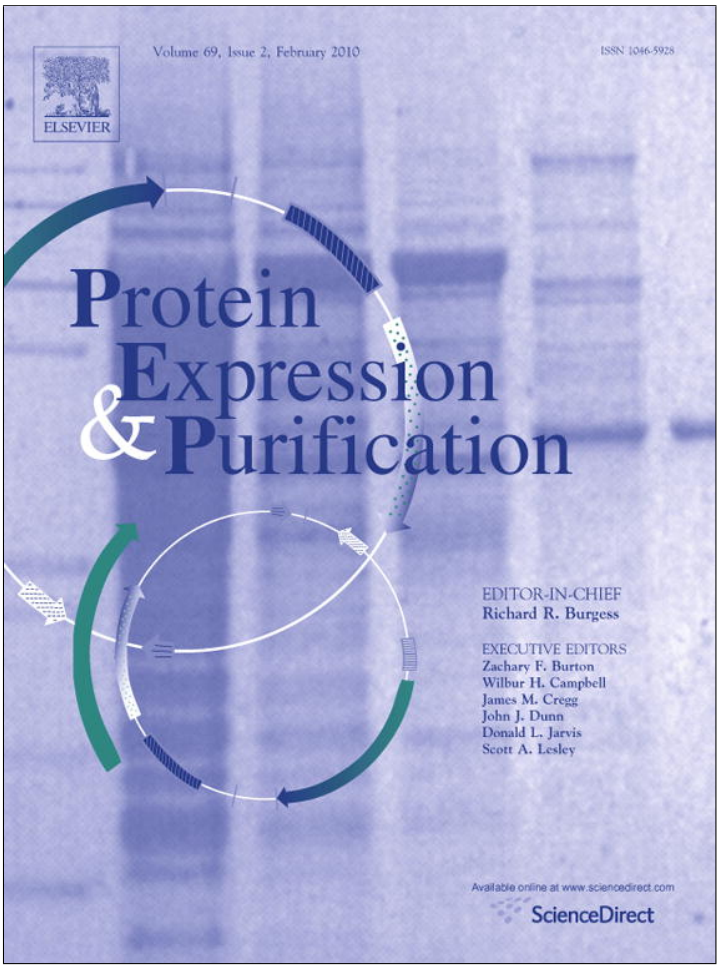

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Overproduction, purification and characterisation of Tbj1, a novel Type III Hsp40 from *Trypanosoma brucei*, the African sleeping sickness parasite

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ABSTRACT

The heat shock protein 40 (Hsp40) family of proteins act as co-chaperones of the heat shock protein 70 (Hsp70) chaperone family, and together they play a vital role in the maintenance of cellular homeostasis. The Type III class of Hsp40s are diverse in terms of both sequence identity and function and have not been extensively characterised. The *Trypanosoma brucei* parasite is the causative agent of Human African Trypanosomiasis, and possesses an unusually large Hsp40 complement, consisting mostly of Type III Hsp40s. A novel *T. brucei* Type III Hsp40, Tbj1, was heterologously expressed, purified, and found to exist as a compact monomer in solution. Using polyclonal antibodies to the full-length recombinant protein, Tbj1 was found by Western analysis to be expressed in the *T. brucei* bloodstream-form. Tbj1 was found to be able to assist two different Hsp70 proteins in the suppression of protein aggregation *in vitro*, despite being unable to stimulate their ATPase activity. This indicated that while Tbj1 did not possess independent chaperone activity, it potentially functioned as a novel co-chaperone of Hsp70 in *T. brucei*.

Introduction

Molecular chaperones are crucial to the maintenance of cellular homeostasis by facilitating the folding of *de novo* synthesised proteins, aiding in protein translocation, and general cytoprotection in response to the effects of a variety of stresses [1,2]. Several of the major classes of molecular chaperones are categorised as heat shock proteins (Hsps) in allusion to their upregulation in response to thermal stress, although not all molecular chaperones are Hsps, or upregulated in response to heat shock [1]. Proteins of the heat shock protein 70 (Hsp70) class comprise the predominant group of Hsps in organisms and have been implicated in a variety of crucial functions that include aiding newly synthesised proteins to fold, the refolding of denatured proteins and degradation of aggregated proteins through the lysosomal pathway [1,3–5]. The ability of Hsp70 proteins to perform their diverse cellular tasks is dependent on their ability to maintain substrate polypeptides in extended conformations, allowing for the stabilisation of exposed hydrophobic regions [6]. The ATPase activity of Hsp70 proteins is regulated by heat shock protein 40 (Hsp40) co-chaperone proteins [1,7,8].

Hsp40 proteins are divided into three distinct classes based on their similarity to the canonical Hsp40, *Escherichia coli* DnaJ, which possesses a highly conserved J-domain that is approximately 70 amino acids in size, a glycine–phenylalanine (G/F)-rich region and a cysteine-rich region containing four motifs (CXXCXGXG) that resemble a zinc-finger domain [9]. Type I Hsp40s are highly conserved with respect to DnaJ and possess all three canonical domains, while Type II Hsp40s possess the J-domain and the G/F-rich region [9]. In addition, Type I and Type II Hsp40s possess a peptide-binding region at their C-termini, which facilitates binding to non-native polypeptides, enabling these Hsp40 proteins to deliver substrates to Hsp70 proteins for folding [8,10,11].

Type III Hsp40s are poorly conserved with respect to *E. coli* DnaJ in that they only contain the highly conserved J-domain, which can occur anywhere on their sequence, unlike the general N-terminal nature of the J-domain in other types of Hsp40s [9,12]. The peptide-binding domain that occurs in Type I and Type II Hsp40s is also absent in Type III Hsp40s [8,10]. The J-domain has been shown to stimulate the ability of partner Hsp70 proteins to hydrolyse ATP and to mediate the interaction between Hsp40 and Hsp70 proteins [2]. Due to their high levels of sequence diversity, the Type III Hsp40s have not been studied extensively, and few Type III Hsp40s have been biochemically characterised [12,13]. It has been proposed that these proteins preside over specialised functions within the cell that could be distinct from the functions of Type I and Type II Hsp40s [12].

Hsp40 proteins are generally produced at low levels *in vivo*, and overexpression of Hsp40s can, in certain cases, lead to toxicity and a decrease in cell viability [14]. The low level of Hsp40 expression in heterologous expression systems can complicate attempts at overproduction and purification [15], and a variety of methods are often examined before successful expression is achieved. The most significant concern when purifying Hsp40 proteins is the

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possibility of Hsp70/DnaK co-purification, and ATP is frequently added to purification buffers to reduce or eliminate such co-purification [15].

Trypanosoma brucei (*T. brucei*) is a unicellular flagellated protozoon parasite that is transmitted by various members of the *Glossina* spp. family (tsetse fly), causing Human African Trypanosomiasis (HAT) in humans and Nagana in cattle [16,17]. HAT is endemic in 36 countries in Sub-Saharan Africa, and is invariably fatal if left untreated [18,19]. The *T. brucei* parasite is characterised by a complex life-cycle involving a transition from its insect vector to its mammalian host and comprises a bloodstream-form (BSF)[1] and a procyclic form (P) [20].

Previous *in silico* studies have indicated that *T. brucei* possesses a large Hsp complement, with 67 Hsp40 proteins [21], of which 47 are Type III Hsp40s, as well as 12 Hsp70 proteins. At present, no *T. brucei* Hsp40 proteins have been expressed and biochemically characterised, although several proteins from the related *Trypanosoma cruzi* (*T. cruzi*) parasite have been studied [22–24]. The cytosolic Hsp70 of *T. cruzi* (TcHsp70) has been recombinantly overproduced and purified and biochemically characterised, paving the way for studies of Hsp40 and Hsp70 interaction within the *T. cruzi* parasite [25]. Tcj1 is a Type III Hsp40 protein from *T. cruzi*. The coding region of Tcj1 has been successfully cloned, and further investigations have shown that Tcj1 is a 35.4 kDa protein that is constitutively expressed and non-heat inducible [22], suggesting a role in cellular homeostasis for this protein. A preliminary analysis of its co-chaperone properties indicated that Tcj1 was unable to stimulate the ATPase activity of TcHsp70, although no investigation of its ability to assist in aggregation suppression has been performed [24]. Tbj1 (Tb11.01.8750), a putative Tcj1 orthologue was recently identified in *T. brucei* [21]. Tbj1 has no known orthologues in humans, making it an attractive protein for further study as it could serve as a novel drug target if found to be essential for *T. brucei* survival in the human host. To our knowledge this is the first report of the expression and purification of this Type III Hsp40 protein from *T. brucei*, in addition to biochemical analyses indicating that Tbj1 presides over a mode of action that is distinct from that of canonical Hsp40 proteins.

Materials and methods

Materials

All reagents were obtained from Sigma Chemicals (St. Louis, MO, USA), Merck Chemicals (Darmstadt, Germany), Roche Molecular Biochemicals (Indianapolis, IN, USA) unless otherwise stated. The pGEM-T Easy® plasmid was purchased from Promega (Madison, WI, USA) while the pQE1 plasmid was purchased from Qiagen (Valencia, CA, USA). Nickel-Chelating Sepharose Fast Flow matrix was purchased from Pharmacia Biotech (Uppsala, Sweden). *Medicago sativa* Hsp70 was purchased from Alfa Biogene International (Germany). *T. brucei TREU927* genomic DNA was a gift from Dr. David Horn (London School of Hygiene and Tropical Medicine, London, UK) while *T. brucei* Pro and BSF lysates were a kind donation from Prof. T. Coetzer (University of KwaZulu Natal, South Africa).

Construction of plasmid expression vectors

pQE1-Tbj1 expression construct

Primers were designed to amplify the coding region of Tbj1 from *T. brucei TREU927* genomic DNA by means of the polymerase chain reaction (PCR): a forward primer (5′- GGT ACC ATG GGG TCA GAT GTG -3′) with an annealing temperature of 58.5 °C and a KpnI restriction site (underlined), and a reverse primer (5′- CAC GTG CTA TCT ACT TCG GTC ATA G -3′) with an annealing temperature of 60.3 °C and a PvuII restriction site (underlined) were utilized. The PCR reaction parameters were standard (Stage 1: 94 °C for 2 min; Stage 2: 25 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min; Stage 3: 1 cycle at 72 °C for 10 min). The PCR-amplified Tbj1 coding region was ligated into pGEM-T Easy® to generate the pGEM-T-Tbj1 construct. The Tbj1 coding region was restricted from pGEM-T-Tbj1 with KpnI and PvuII enzymes and ligated downstream of the coding region for the 6× His tag of the pQE1 expression vector to generate the pQE1-Tbj1 construct. The identity of the construct was confirmed by means of diagnostic restriction analysis and DNA sequencing. The sequence of the Tbj1 coding region amplified was found to possess slight differences with respect to the sequence of Tbj1 lodged in the GeneDB database (www.genedb.org; [26]), and it has been lodged in GenBank [27] with the Accession No. GQ240140.1.

pQE30-TcHsp70 expression construct

Primers were designed to amplify the coding region of TcHsp70 from the coding region inserted into the pET23b-TcHsp70 construct by means of PCR amplification: a forward primer (5′- GGA TCC ACG ATG ACG TAC GAG GGA GC -3′) with an annealing temperature of 65.6 °C and a BamHI restriction site (underlined), and a reverse primer (5′- GTG GAG GAA GTG GAC TGA GAA TTC AAG CTT -3') with an annealing temperature of 63.9 °C and a HindIII restriction site (underlined) were utilized. The PCR reaction parameters were standard (Stage 1: 94 °C for 2 min; Stage 2: 25 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min; Stage 3: 1 cycle at 72 °C for 10 min). The PCR-amplified TcHsp70 coding region was ligated into pGEM-T Easy® to generate the pGEM-T-TcHsp70 construct. The TcHsp70 coding region was restricted from pGEM-T-TcHsp70 with BamHI and HindIII enzymes and ligated downstream of the coding region for the 6× His tag of the pQE30 expression vector to generate the pQE30-TcHsp70 construct. The identity of the construct was confirmed by means of diagnostic restriction analysis and DNA sequencing.

Heterologous protein overproduction and purification

Overproduction and purification of $(His)_6$-Tbj1 and $(His)_6$-TcHsp70

Overnight rich medium broth cultures were prepared (30 ml; containing 100 μg/ml ampicillin), inoculated with a single colony of *E. coli* XLI Blue [pQE1-Tbj1] and grown at 37 °C with shaking. The overnight cultures were diluted into fresh rich medium (225 ml) and grown for 3–4 h at 37 °C with shaking until the cultures reached mid-log phase (A_{600} 0.6–0.7). The expression of $(His)_6$-Tbj1 was initiated by the addition of isopropyl-1-thio-β-galactopyranoside (IPTG) to a final concentration of 1 mM. The broth cultures were allowed to induce for 5 h prior to harvesting of the cells (6000*g*; 20 min) and subsequent resuspension in denaturing lysis buffer (8 M urea, 300 mM NaCl, 10 mM imidazole, 10 mM Tris, 1 mM phenylmethylsulphonyl fluoride (PMSF)). The cells were stored at −20 °C overnight and subsequently thawed resulting in lysis. The cellular debris was harvested by means of centrifugation (12,000*g*; 20 min) and the supernatant was added to nickel-charged Sepharose beads (2 ml; 50% w/v slurry) in lysis buffer. Binding was allowed to occur for 5 h with gentle agitation at 4 °C prior to pelleting of the beads at 500*g* for 3 min. The supernatant (flow through; FT fraction) was removed and the pelleted beads were washed four times with three column volumes of native wash buffer (100 mM Tris–HCl, 300 mM NaCl, 100 mM imidazole; pH 8.0) with added ATP (0.6 mM) to remove endogenous DnaK bound to the $(His)_6$-Tbj1. The $(His)_6$-Tbj1 bound to the nick-

[1] *Abbreviations used:* BSF, bloodstream-form; P, procyclic form; FPLC, fast protein liquid chromatography; MDH, malate dehydrogenase; Hsp40, heat shock protein 40; TbHsp40, *T. brucei* Hsp40.

el-chelating Sepharose was eluted with native elution buffer (100 mM Tris–HCl, 300 mM NaCl, 1 M Imidazole; pH 8.0) in three elution steps, using two column volumes of elution buffer per elution. The purified protein was dialysed against dialysis buffer (50 mM Tris–HCl, 200 mM NaCl; pH 8.0) at 4 °C for a period of 5–6 h to remove imidazole and allow for further refolding of the protein. Where necessary, the protein was concentrated using polyethyleneglycol (PEG) prior to quantification using the method of Bradford [28]. The purity and integrity of the $(His)_6$-Tbj1 protein was analysed using sodium-dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) prior to use for *in vitro* assays. The heterologous production and purification of $(His)_6$-TcHsp70 from *E. coli* XLI Blue [pQE30-TcHsp70] was accomplished in the same way as that of $(His)_6$-Tbj1.

Overproduction and purification of $(His)_6$-Tbj2

The pET28aTcj2 expression construct was transformed into *E. coli* BL21(DE3) and a single colony was inoculated into YT broth containing kanamycin (50 μg/ml) and incubated at 37 °C and shaking (180 rpm) overnight. The overnight culture was diluted 1:10 with fresh 2× YT broth and grown until the cells reached a density of 0.8–1.0 absorbance units (600 nm), prior to the addition of IPTG inducer (1 mM). Overexpression of Tcj2 took place at 30 °C for 6 h with the addition of kanamycin (50 μg/ml) at two hourly intervals to improve the selective pressure on Tcj2-expressing cells. Subsequent to induction, the cells were harvested at 6000*g* for 15 min at 4 °C and the pellets resuspended in native lysis buffer (40 mM Tris–HCl, pH 8.0; 100 mM NaCl; 10 mM imidazole; 0.1% Triton X-100; 1 mg/ml lysozyme; 1 protease inhibitor cocktail pellet, Roche; Germany) prior to aliquotting and freezing overnight at −80 °C. The lysates were thawed at room temperature prior to sonication, and the cellular debris collected by centrifugation at 16,000*g* for 25 min. Purification of the protein from the clarified lysate was achieved using nickel-affinity purification with the His-trap 5 ml nickel-Sepharose columns connected to an FPLC system (Amersham Pharmacia Biotech, USA). The clarified lysate was applied to the column prior to washing with 10–15 column volumes of wash buffer (40 mM Tris–HCl pH 8.0; 100 mM NaCl; 10 mM imidazole). The column was then washed with an increasing gradient of native elution buffer (40 mM Tris–HCl, pH 8.0; 100 mM NaCl; 1 M imidazole) up to 40% native elution buffer and 60% wash buffer over a period of 10 min. Elution of His-Tcj2 was achieved by increasing the percentage of native elution buffer in the mixture to 100% over a period of 6 min again with a flow rate of 1 ml/min. All fractions containing Tcj2 were pooled and dialysed against exchange buffer (100 mM Tris–HCl pH 8.0; 100 mM NaCl). Typical yields of the purified protein using this method were 2–3.5 mg from 2 l of culture.

Antibody production and Western analysis

Purified, dialysed Tbj1 (0.3 mg/ml) was applied to acid treated bacteria prior to injection into a rabbit from which serum was collected at 0, 14, and 28 days [29]. The rabbit polyclonal anti-Tbj1 antibody was tested by Western analysis using *E. coli* [pQE1-Tbj1] lysates and *E. coli* [pQE1] lysates using a concentration range of 1:5000–1:50,000 for the anti-Tbj1 antibody, and 1:5000 for the secondary antibody, horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Amersham, USA). The absence of DnaK contamination in all purified proteins was confirmed by probing with rabbit anti-DnaK antibody and goat anti-rabbit IgG HRP-conjugated secondary antibody. The Western transfer of proteins was performed according to the protocol outlined in [30]. The proteins of interest were resolved on 12% SDS–PAGE gels and transferred to a nitrocellulose membrane (Bio-Rad; USA) that had previously been soaked in Western transfer buffer (25 mM Tris–HCl, 192 mM glycine, 20% v/v methanol). The transfer was performed in a Bio-Rad Western apparatus (Bio-Rad, USA) at a constant voltage of 100 V, and a current of no lower than 300 mA for a period of 60 min. Upon completion of transfer, the blots were stained with Ponceau Red stain (0.5% w/v Ponceau S and 1% glacial acetic acid v/v) to confirm the success of the transfer and placed in block solution (5% non-fat milk powder in Tris-buffered saline with Tween (TBS–Tween; 50 mM Tris–HCl, 150 mM NaCl, 1% Tween; pH 7.5) overnight at 4 °C. The Western blots were developed by incubating with primary antibody for an hour, prior to a wash step with TBS–Tween for an hour and incubation with the secondary antibody for an hour. The blots were washed in TBS–Tween for another hour prior to developing using chemiluminescent reagent (GE Healthcare, UK) and the Chemidoc XRS imaging system (Bio-Rad, USA). The concentration of primary antibody used varied on the antibody titre and the protein being detected.

Gel filtration chromatography

Gel filtration chromatography of $(His)_6$-Tbj1 was performed using an Äkta Basic 10 FPLC instrument and a Superdex™ 20 HR 10/30 size-exclusion column (Amersham Pharmacia Biotech; USA) (10 mm × 30 cm; bed volume 24 ml). The mobile phase comprised a standard FPLC buffer (50 mM Tris, 150 mM NaCl; pH 7.5) that was filtered and degassed for 45 min prior to use. The flow rate was maintained at 0.5 ml/min and elution volumes (1.2 ml) were collected for the duration of the analysis. The presence of protein was monitored by observing the change in absorbance (280 nm). The molecular mass of $(His)_6$-Tbj1 was determined by a comparison with the retention volume of known standards on the column. The standards used were: ferritin (450 kDa), catalase (225 kDa), bovine serum albumen (BSA; 66 kDa), ovalbumin (45 kDa) and lysozyme (14.4 kDa).

Aggregation suppression of malate dehydrogenase (MDH)

The ability of $(His)_6$-Tbj1 to suppress the thermal aggregation of malate dehydrogenase (MDH) was assessed spectrophotometrically. The assay buffer (50 mM Tris–HCl, 100 mM NaCl; pH 7.4) was heated to 48 °C in a Helios Alpha DB Spectrophotometer with a Peltier-controlled cell prior to the addition of MDH (0.72 μM) in order to determine the levels of aggregation of the MDH prior to the addition of the molecular chaperones of interest. The data set obtained for all assays was plotted on an Excel® spreadsheet, and the aggregation of MDH over six independent replicates was taken as the basal aggregation value for all subsequent calculations. BSA (0.72 μM) was added to MDH under the same reaction conditions outlined above as a negative control reaction, while a Type I Hsp40 from *T. cruzi*, $(His)_6$-Tcj2 was used as a positive control. $(His)_6$-Tbj1 (0.72 μM) was added to TcHsp70 (0.36 μM) or *M. sativa* Hsp70 (MsHsp70; 0.36 μM) and MDH (0.72 μM) in MDH assay buffer in order to assess the ability of these proteins to suppress the thermal aggregation of MDH by measuring the light scatter over 30 min at 360 nm. The aggregation of $(His)_6$-Tbj1, TcHsp70 and MsHsp70 was assessed by heating these proteins to 48 °C in the absence of MDH. No increase of turbidity was observed suggesting that these proteins were not aggregation prone at 48 °C (data not shown). The assay was performed in triplicate on three distinct batches of protein.

ATPase stimulation assays

The ability of $(His)_6$-Tbj1 to stimulate the ATPase activity of TcHsp70 and MsHsp70 was determined using a modification of the ammonium molybdate/ascorbic acid assay of Chifflet and col-

leagues [31]. Tbj1 (0.1–0.4 μM) was added to either TcHsp70 or MsHsp70 (0.4 μM) in ATPase buffer (10 mM HEPES, pH 7.4; 10 mM $MgCl_2$, 20 mM KCl and 0.5 mM DTT) at 37 °C. The reaction (1200 μl) was initiated by the addition of ATP (600 μM) and samples (50 μl) were taken in triplicate (0–300 s) and added to SDS (50 μl; 10%) in order to halt the production of phosphate. The SDS-treated samples were developed with ammonium molybdate (1% in 1 N HCl; 50 μl), ascorbic acid (6%; 50 μl) and sodium citrate/acetic acid solution (2%/2%; 125 μl) for 30 min at 37 °C. The absorbance of the reactions was measured at 850 nm in a KC Junior microplate reader (Bio-Tek Instruments, USA). A phosphate standard curve was prepared from KH_2P0_4 with standards ranging from 0 to 10 nmol and a calibration curve was prepared to serve as a basis for calculations of phosphate content in the reactions assayed. The specific ATPase activity was calculated for each reaction and reported as nmol Pi/min/mg protein. The assay was performed in triplicate on three distinct batches of protein. Control reactions containing each protein to be assessed (0.4 μM) without ATP were also set up in tandem with each experiment. A negative control was conducted using TcHsp70 or MsHsp70 that had been inactivated by boiling for 20 min. Both TcHsp70 and MsHsp70 were assayed in the presence and absence of ATP. Tbj1 was assayed in the presence of ATP and the absence of Hsp70 in order to determine if this protein was prone to spontaneous phosphate release or if residual DnaK contamination was present. A further control reaction containing only ATP was used in order to correct for the effect of spontaneous ATP hydrolysis.

Detection of Tbj1 in T. brucei procyclic and bloodstream-form lysates

T. brucei procyclic and bloodstream-form lysates were resuspended in SDS–PAGE loading buffer to a final concentration of 0.4 mg/ml. The lysates (0.010 mg) were resolved on duplicate 12% SDS–PAGE gels prior to Western analysis. The presence of Tbj1 was detected by means of the anti-Tbj1 antibody (1:5000) produced in this study and anti-rabbit IgG conjugated to horseradish peroxidase (HRP) secondary antibody.

Results

The J-domain of Tbj1

The full-length protein sequence of $(His)_6$-Tbj1 was aligned with that of Tbj1 (Tb11.01.8750) and Tcj1 (Tc00.1047053511537.50) from the GeneDB database (www.genedb.org) in order to confirm the identity of the coding region amplified in our study (Fig. 1A). The Tbj1 protein in this study (*T. brucei TREU927* origin) bears 99.4% identity to the Tbj1 protein encoded on the genome generated by the *T. brucei* genomic sequencing project, with three amino acid substitutions, two of which were conservative substitutions (Fig. 1A). Tbj1 was predicted to localize to the *T. brucei* nucleus by the Wolf PSORT localization prediction program (http://wolfpsort.org/ [32]). A comparison with known nuclear localization signals (NLSs) from the literature [33] suggested that Tbj1 possessed an NLS from residues 103–118 (RLFVERKRKEDEEKMR), which supports the *in silico* localization prediction, although this has to be confirmed experimentally.

Tbj1 was found to possess no known orthologues in humans, yeast or prokaryotes, where an orthologue was defined as a protein that had 40% or greater sequence identity to Tbj1. As Tbj1 is a Type III Hsp40 protein with only the J-domain in common with other Hsp40 proteins, the J-domain was examined for conservation of key residues previously implicated as important for Hsp40 function when compared to the J-domains of Hsp40 proteins from *S. cerevisiae*, *E. coli* and *H. sapiens* (Fig. 1B). The Tbj1 J-domain had a greater similarity to the J-domains of eukaryotic origin (Fig. 1B) than those of prokaryotic origin, and differences in residues proposed to be crucial to the interaction between the J-domain and partner Hsp70s were observed (Fig. 1B and C). The most notable amino acid substitution in the Tbj1 J-domain was H_{34}, which is generally a K or R residue at an equivalent position in the J-domains of other Hsp40s, and has been shown to be critical for functional interaction with Hsp70 [34]. The HPD tripeptide which is involved in the ATPase stimulation of a partner Hsp70 protein was fully conserved in Tbj1, suggesting that it could possess the ability to stimulate the ATPase activity of a partner Hsp70 protein ([35,36]; Fig. 1B and C). Helix III and IV of the Tbj1 J-domain were poorly conserved with respect to those of other Hsp40 proteins, with substitutions at positions 51 to yield a VFK instead of a KFK motif, and 66–70 to yield a $T_{66}QREM_{70}$ instead of a QKRAA motif that could have implications for the chaperone function and properties of the protein [36,37]. The sequences of Type III Hsp40 J-domain have previously been shown to be divergent with respect to the J-domains of Type I and Type II Hsp40s [37]. In spite of this, many of the key consensus regions in the J-domain of Type III Hsp40s such as Djla, are well-conserved, indicating that the lack of conservation of these residues in the Tbj1 J-domain could suggest a specialised function for this protein.

Overproduction and purification of Tbj1

To our knowledge, this is the first successful attempt to isolate the coding region of a Type III Hsp40 protein from *T. brucei*, and express the resulting protein in a heterologous *E. coli* system. Previous expression studies have focussed on the Hsp40 complement of *T. cruzi* [22–24] and frequently relied on the use of cDNA libraries. As no cDNA library was available for *T. brucei*, and because of the lack of introns in *T. brucei* genes [38], it was decided to amplify the coding regions of these proteins directly from *T. brucei* genomic DNA. This method has previously been used with great success in biochemical studies on Hsp70 proteins from *T. cruzi* [25]. One major advantage in using the direct amplification from genomic DNA is that trypanosomal parasites often have unusually long leader sequences and poly-A tails [22] which can complicate analysis of novel genes. For the purposes of the present study, the coding regions were not codon harmonised or optimised, as a careful study of the favoured codons in *T. brucei* versus *E. coli* revealed similar preferences, especially in the case of the various TbHsp40 coding regions [39,40].

Tbj1 was successfully overproduced and purified as a recombinant $(His)_6$-tagged protein (Fig. 2). Solubility studies performed on $(His)_6$-Tbj1 revealed that the protein produced in *E. coli* was insoluble (data not shown). To this end, a denaturing nickel-affinity purification method was adopted that incorporated native wash and elution steps in order to facilitate refolding of the protein. *E. coli* XL1 Blue [pQE1-Tbj1] cells overproduced $(His)_6$-Tbj1 when subject to induction with IPTG at 37 °C (Fig. 2B; panel I). A standard purification resulted in an average yield of 6 mg/l of Tbj1 of >80% purity (Fig. 2B; panel I). A minor and a major contaminant was visible after purification of $(His)_6$-Tbj1 (Fig. 2B; panel I), and, as these bands were frequently detected by Western analysis it was proposed that these could correspond to different oligomeric states and/or degradation products of the protein. The identity of the purified Tbj1 was confirmed by detection with antibodies directed against the $(His)_6$-tag (data not shown) and anti-Tbj1 polyclonal antibodies produced during the course of the study (Fig. 2B; panel II). Endogenous DnaK contamination is a major concern when purifying Hsp40 proteins due to the potential ability of this protein to bind to the overexpressed Hsp40 of interest and co-purify with it [15]. In order to reduce the possibility of *E. coli* DnaK contamination of purified Tbj1, we added ATP to the wash buffers used during

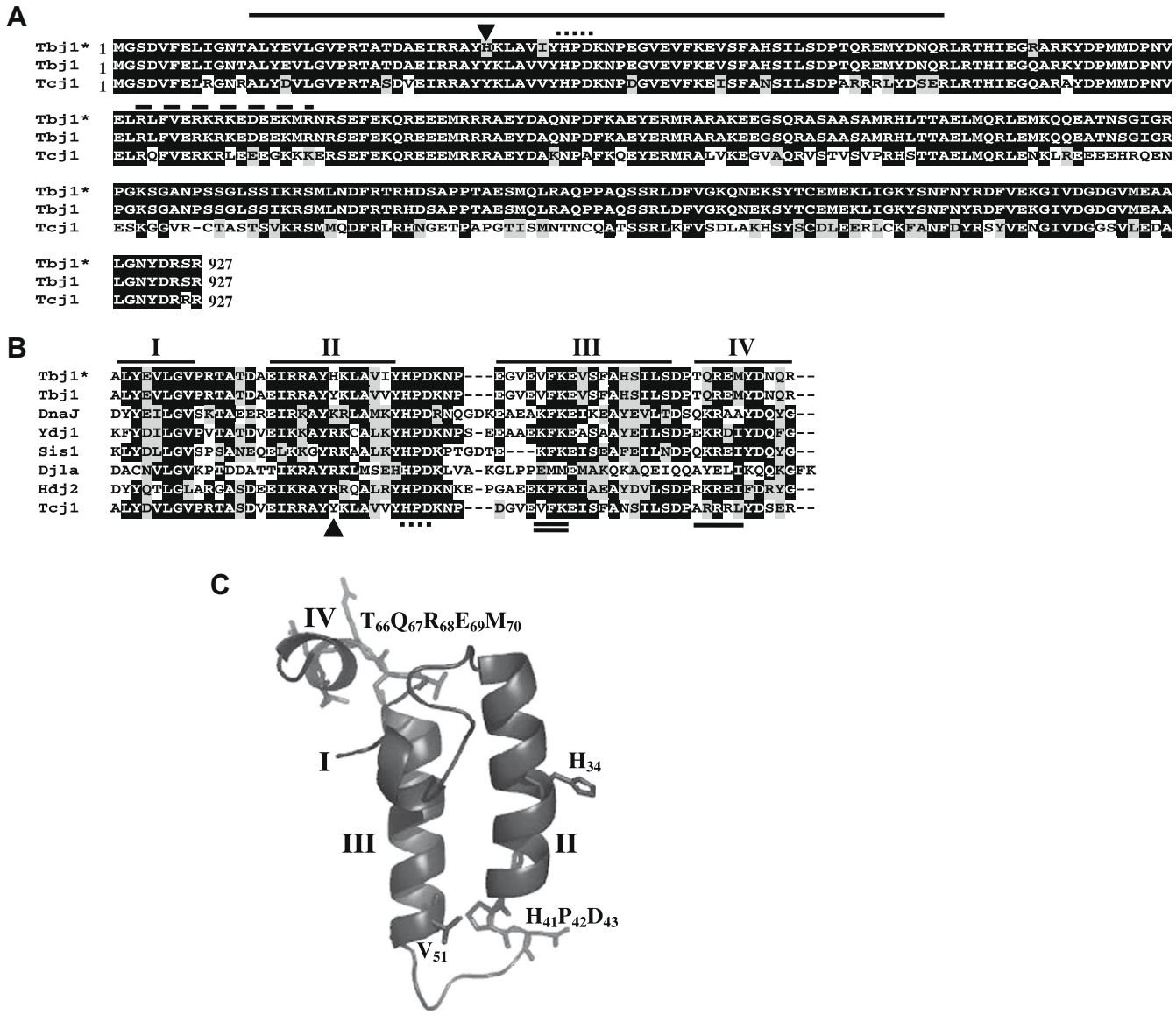

Fig. 1. Sequence analysis of Tbj1. (A) Alignment of the sequence of Tbj1* (this study) with Tbj1 (Tb11.01.8750) and Tcj1 is shown. The J-domain region is indicated by a solid black overline. The Y/H_{34} residue is indicated by a black arrowhead, and the HPD tripeptide motif is highlighted by a dotted overline. The putative NLS is indicated with a dashed overline. The alignment was generated using the ClustalW alignment feature of the BioEdit program [50]. (B) Sequence alignment of the J-domains (GeneBank/GeneDB accession numbers given in brackets) of Tbj1* (this study), Tbj1 (Tb11.01.8750), Tcj1 (Tc00.1047053511537.50), *S. cerivisiae* Ydj1 (CAA95397), Sis1 (CAA95866), Swa2 (QO6677), *E. coli* DnaJ (PO8622), Djla (AP000719) and *H. sapiens* Hdj2 (CAB93418). The helices (I–IV) are annotated on the figure and residues predicted to be important for Hsp40 function and interaction with Hsp70 are annotated as follows: key residue for interaction with Hsp70 (H_{34} in Tbj1; black arrowhead); HPD motif (dotted underline); KFK motif (double solid underline); and QKRAA motif (solid underline). (C) Homology model of the Tbj1* J-domain generated with Modeller software (http://salilab.org/modeller/ [51]) using the *E. coli* J-domain (1 XBL) as a template. The model was rendered using Pymol [52]. The helices are annotated on the figure, and the Tbj1 residues topologically equivalent to those in other Hsp40 proteins predicted to be important for function and interaction with Hsp70 are annotated as follows: key residue for interaction with Hsp70 (H_{34}); $H_{41}PD_{42}$ motif; V_{51} of VFK motif; and the $T_{66}QREM_{70}$ motif.

the first five stages of the purification process. This resulted in the removal of all detectable DnaK from the system (Fig. 2B; panel III).

Detection of Tbj1 in T. brucei bloodstream-form lysates

T. brucei P and BSF lysates were probed with anti-Tbj1 antibody in order to determine at which of these life-cycle-stages of *T. brucei* Tbj1 was expressed (Fig. 3). A single, distinct band of the correct molecular mass (35 kDa) was detected by Western analysis of the lysates of the bloodstream-form and not the procyclic form (Fig. 3). Therefore Tbj1 was shown to be expressed in the BSF, and potentially not in the P form of the parasite. The most important consideration in terms of the detection of Tbj1 in *T. brucei* lysates was the potential detection of a number of Hsp40 proteins because of the conserved nature of the J-domain of Tbj1. A number of TbHsp40s were shown to have sequence similarity with Tbj1, but only over the J-domain region (data not shown). A number of faint bands were observed in the procyclic form of the parasite, and since none of these correspond to the predicted molecular mass of Tbj1, the possibility exists that these protein bands represent cross-reactivity with other TbHsp40 proteins.

Tbj1 occurs as a monomer in solution

The molecular mass and oligomeric state of $(His)_6$-Tbj1 were analysed by means of gel filtration chromatography (Fig. 4). Two peaks were detected; a minor peak that eluted at the void volume of the column, and a major peak corresponding to an elution vol-

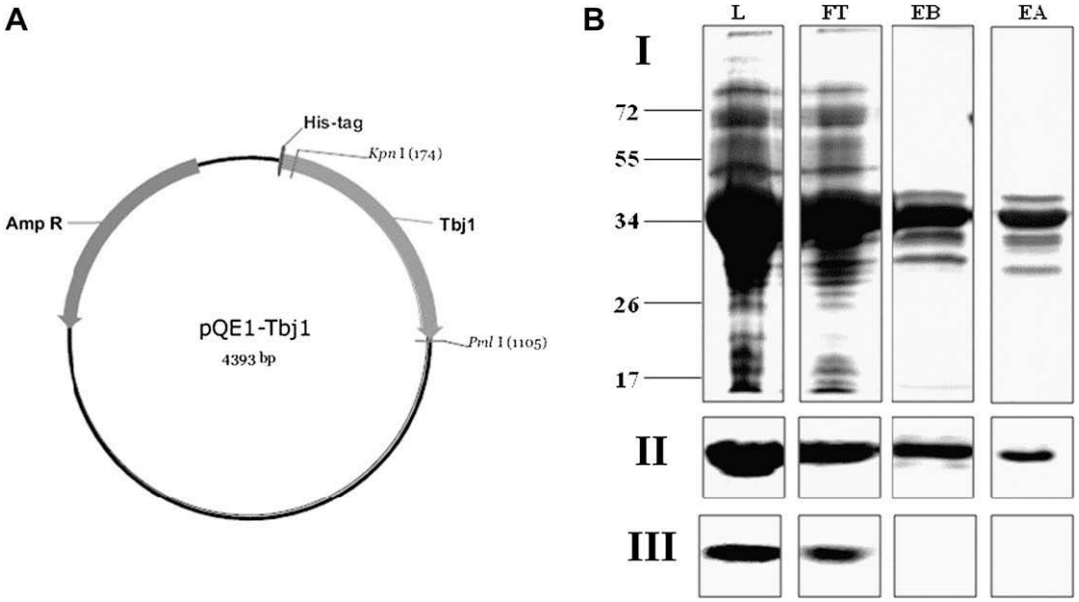

Fig. 2. Tbj1 was successfully overproduced and purified. The map of the pQE1-Tbj1 plasmid generated with Vector NTI software is shown (A) indicating the location of the coding region for Tbj1 and the cleavable $(His)_6$-tag. $(His)_6$-Tbj1 was overproduced and purified (B) using a denaturing nickel-affinity purification with native wash and elution steps. The molecular mass markers (kDa) are shown to the left of the gel. Panel I – Coomassie-stained gel analysis of purified $(His)_6$-Tbj1: [L] – *E. coli* XL1 Blue [pQE1-Tbj1] lysate; [FT] – Flow through after binding to nickel–Sepharose beads; [EB] – Final elution prior to dialysis; [EA] – Final elution subsequent to dialysis and filtering. Panel II – Western analysis of $(His)_6$-Tbj1 purification using anti-Tbj1 antibodies. Panel III – Assessment of endogenous *E. coli* DnaK contamination levels by Western analysis using anti-DnaK antibodies. The lane identity of each figure corresponds to that of figure B.I. The purification data presented in this figure are representative of the samples generated for a typical optimised purification, which was repeated at least three times.

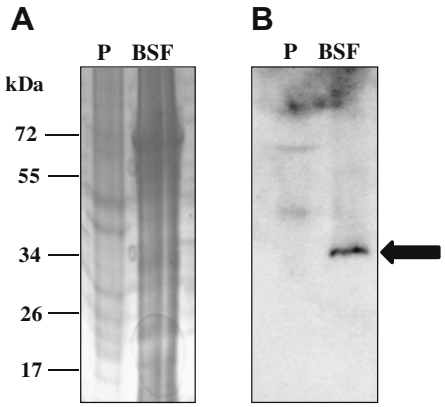

Fig. 3. Detection of Tbj1 in *T. brucei* BSF lysates. *T. brucei* procyclic and bloodstream-form lysates were subjected to Western analysis using anti-Tbj1 antibodies. (A) – Procyclic (P) and bloodstream-form (BSF) *T. brucei* lysates resolved on a 12% SDS–PAGE gel stained with coomassie; (B) – Western analysis of *T. brucei* P and BSF lysates. The black arrow indicates a band detected at 32–35 kDa in the BSF sample lane.

ume of 20–22 ml (Fig. 4). The minor peak was generated by low levels of contaminating aggregated proteins, while the major peak was generated by Tbj1 (confirmed by SDS–PAGE and Western analysis; data not shown). Since the peak corresponding to Tbj1 resolved close to the resolution limit of the column used, an accurate molecular mass could not be determined. However, the Tbj1 elution volume with respect to that of known standards suggested that it existed as a compact monomeric species in solution.

Tbj1 assists Hsp70 in suppression of protein aggregation

The ability of $(His)_6$-Tbj1 to assist in suppressing the thermal aggregation of a model substrate (MDH) was tested in order to assess whether or not $(His)_6$-Tbj1 possessed independent chaperone activity. Incubation of $(His)_6$-Tbj1 in MDH assay buffer at 48 °C in the absence of MDH resulted in no increase in levels of turbidity measured at 360 nm (data not shown) indicating that $(His)_6$-Tbj1 was not aggregation prone. A BSA control was unable to reduce the aggregation of MDH in any significant manner. The addition of $(His)_6$-Tbj1 to MDH (0.72 μM) resulted in an increase in MDH aggregation (to 133%) compared to MDH alone (100%; Fig. 5). A Type I Hsp40 from *T. cruzi* (Tcj2) was used as a control in this reaction and was shown to reduce the aggregation of MDH (43%; Fig. 5), which was anticipated for a Type I Hsp40 protein that possesses a substrate binding domain and independent chaperone activity.

Subsequent to determining that $(His)_6$-Tbj1 alone does not possess the ability to suppress the thermal aggregation of MDH, an attempt was made to determine whether or not $(His)_6$-Tbj1 was able to assist two different Hsp70 proteins in aggregation suppression. The ability of an Hsp70 protein from *T. cruzi* (TcHsp70) and *M. sativa* (MsHsp70) to suppress the aggregation of MDH was analysed at different concentrations. TcHsp70 and MsHsp70 were individually found to greatly reduce the aggregation of MDH (13% and 25% respectively; Fig. 5). The addition of $(His)_6$-Tbj1 (0.72 μM) to TcHsp70 and MsHsp70 resulted in a further reduction in aggregation (10% and 7%, respectively; Fig. 5), although the reduction of aggregation in the case of TcHsp70 and Tbj1 was too slight to be considered significantly different to the reduction by Tchsp70 alone. The Tbj1 ortholog in *T. cruzi*, Tcj1, displayed the same behaviour when added to TcHsp70 and MsHsp70 (data not shown) indicating that the two proteins could possess similar modes of action and chaperoning ability. These data suggest that, while $(His)_6$-Tbj1 does not possess chaperone properties in isolation, it can act as a co-chaperone to Hsp70 proteins, and assist them in aggregation suppression *in vitro*.

Tbj1 is unable to stimulate ATP hydrolysis by Hsp70

The ability of $(His)_6$-Tbj1 to stimulate the ATPase activity of TcHsp70 and MsHsp70 was assessed by means of a modified

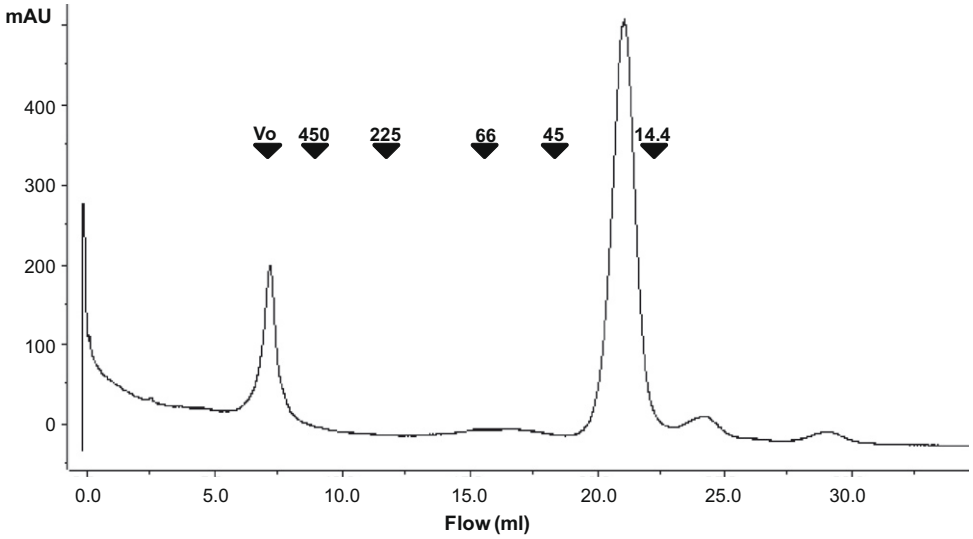

Fig. 4. Tbj1 occurs as a monomer in solution. Gel filtration analysis of $(His)_6$-Tbj1 showing a monomeric species that eluted at 20–22 ml. The elution volumes of the molecular mass standards are indicated with black arrows (molecular masses given in kDa).

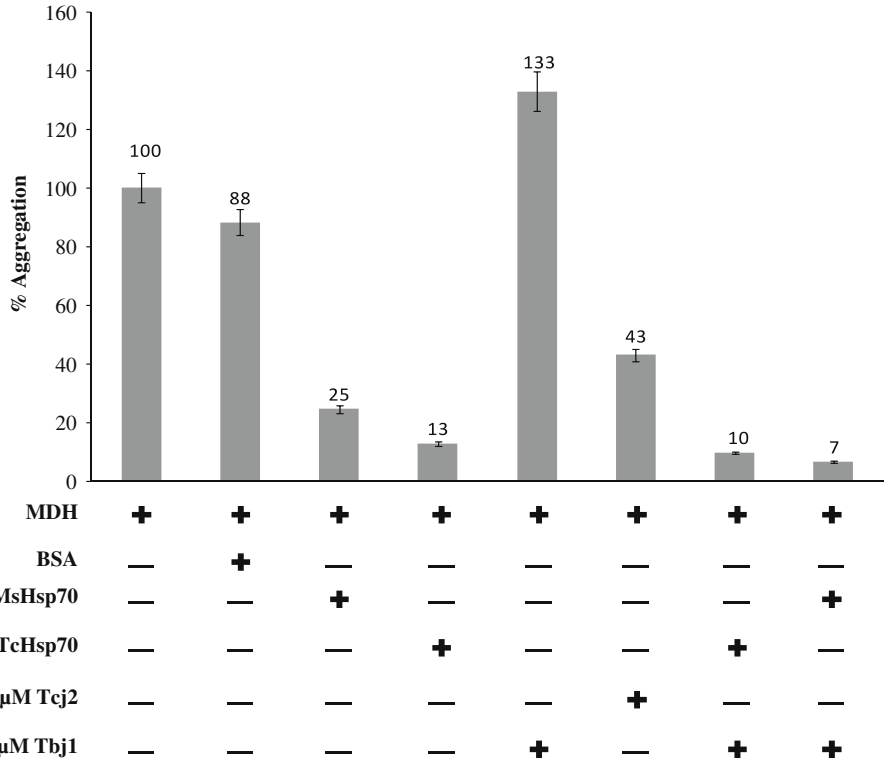

Fig. 5. Tbj1 assists Hsp70 in suppression of protein aggregation. The results shown are obtained for MDH aggregation suppression assays performed in triplicate with three independently purified batches of protein. All samples were assayed at 48 °C over a 30 min period. The '+' symbols indicate constituents present in a given reaction, while the '–' symbols indicate constituents that have been omitted from a given reaction. The concentration of MDH (0.72 μM), BSA (0.72 μM), and the Hsp70s (0.36 μM) were as for the standard assay, and the concentration of the Hsp40s is given. The actual values (% aggregation) are given above each bar of the bar graphs. The error bars in the figure indicate the standard error obtained for each experiment.

ammonium molybdate assay for the determination of free-phosphate production [31]. TcHsp70 was shown to have a basal ATPase activity of 0.56 nmol Pi/min/mg Hsp70, while the addition of an equimolar quantity (0.4 μM) of $(His)_6$-Tbj1 to TcHsp70 resulted in an ATPase activity of 0.22 nmol/min/mg (Fig. 6). This represents a 2.3-fold reduction in ATPase activity from that of the basal rate. The assay of equimolar amounts of $(His)_6$-Tbj1 and TcHsp70 in the absence of ATP resulted in an ATPase activity of 0.018 nmol/min/mg, while $(His)_6$-Tbj1 alone caused a negligible release of phosphate (data not shown). The ATPase activity of TcHsp70 in the presence of increasing concentrations of $(His)_6$-Tbj1 increased (Fig. 6), although these values were never observed to be higher than the basal value for TcHsp70 alone. The same phenomenon was observed when $(His)_6$-Tbj1 was added to MsHsp70, although

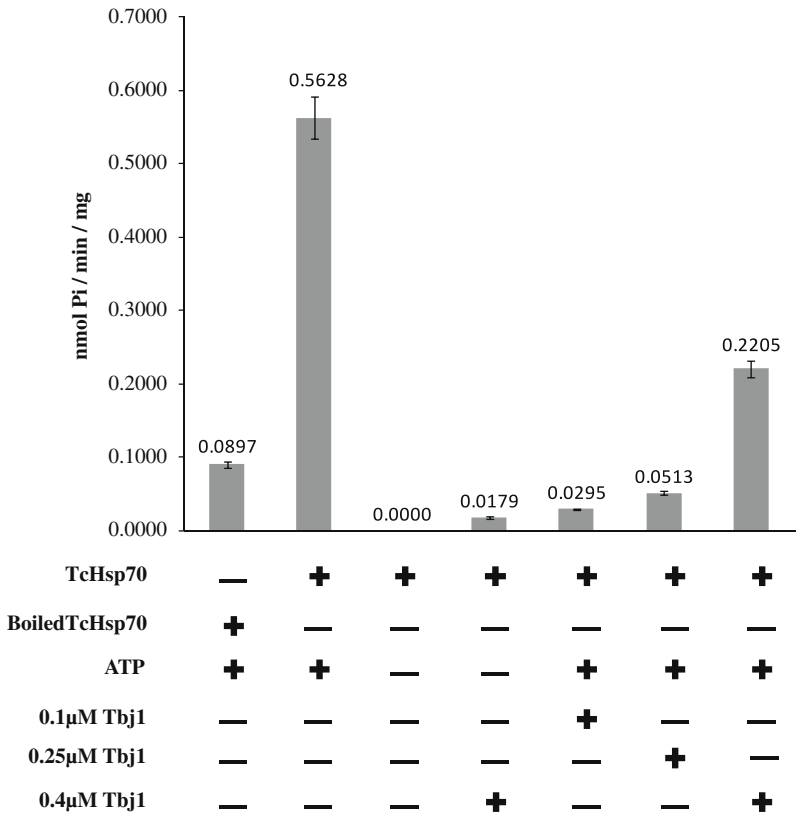

Fig. 6. Tbj1 is unable to stimulate Hsp70 ATP hydrolysis. The results shown are for ATPase assays performed in triplicate with three independently purified batches of protein. The symbols listed below each bar represent the constituents added to each reaction mixture, with (+) indicating the presence of a given reagent and (−) indicating its absence. The concentration of Hsp40 proteins was varied as indicated, while the concentration of TcHsp70 was kept constant (0.4 μM), and samples were assayed after a period of 3 h. The ATPase activity is given in nmol phosphate (Pi) released per minute per mg of TcHsp70 in the reaction and corrected for spontaneous ATP hydrolysis.

the basal activity of MsHsp70 was shown to be significantly higher (38.08 nmol/min/mg) than that of TcHsp70 (data not shown). Tcj1 has previously been shown to be unable to stimulate the ATPase activity of TcHsp70 [24]. Our findings confirm the results of this study, as neither Tbj1 (Fig. 6) nor Tcj1 (data not shown) were able to stimulate the ATPase activity of either TcHsp70 or MsHsp70.

Discussion

We present here the first report to our knowledge of the successful isolation and functional characterisation of a *T. brucei* Hsp40 protein. $(His)_6$-Tbj1 was successfully overproduced in *E. coli* without manipulating its codon bias by means of either optimisation or harmonisation, or the co-expression of tRNA for rare codons. The incorporation of a cleavable hexahistidine tag in the pQE1-Tbj1 construct facilitated purification and detection of the protein, and allows for the removal of the His_6-tag in future experiments in which it is not required. The denaturing purification outlined here was rapid, simple and highly effective for the isolation of an insoluble heterologously expressed protein, and the addition of native wash and elution steps facilitated the production of a functional protein as confirmed by biochemical assays. While a native purification protocol would have been preferred, our attempts at developing such a protocol using solubilising agents such as polyethyleneimine (PEI) and sarkosyl in conjunction with sonication did not yield positive results. It is proposed that the method outlined here is the most rapid and successful method for the purification of the Tbj1 from *E. coli*, but future expression studies in yeast could prove a more viable option.

The isolation of purified Tbj1 facilitated the generation of polyclonal anti-Tbj1 antibodies. These antibodies were successfully used to show that Tbj1 was expressed in the BSF of the *T. brucei* parasite, and potentially not in the P form. This is the first time that Tbj1 has been successfully detected in a *T. brucei* lysate, and merits attention for future studies of the protein, especially in light of the availability of a suitable antibody that appears not to cross react with other proteins in a *T. brucei* BSF lysate. The BSF of the *T. brucei* life-cycle takes place in the warm-blooded mammalian host, and it is proposed that a number of the highly specialised Type III TbHsp40s are predominantly upregulated during this life-cycle stage in order to facilitate the survival of the parasite in the mammalian bloodstream.

The oligomeric state of well characterised Hsp40 proteins such as DnaJ, Djla and Sis1 has been assessed by means of X-ray scattering and size-exclusion chromatography, which indicated that these proteins existed as homodimers [41–44]. The dimerisation potential of Type I and Type II Hsp40s is critical to their function. In this study heterologously expressed Tbj1 was found to exist as a compact monomer in solution. As dimerisation frequently plays a key role in function and mechanism of action, it is unclear what effect this monomeric state has on the role of Tbj1 as a molecular chaperone, but suggests that it behaves differently from more canonical Hsp40s, including the Type III Hsp40 from *E. coli*, Djla [43,44].

The ability of a given Hsp to suppress the aggregation of a thermolabile protein has frequently been used as an indicator of its chaperone activity [45]. Type III Hsp40s are predicted to possess no independent chaperone activity, while certain Type I proteins do, in fact, possess such activity [46]. MDH is an attractive model substrate in thermal aggregation studies due to its thermolabile nature [47], and was well suited to the analysis of the chaperone ability of $(His)_6$-Tbj1 and two potential partner Hsp70s. While $(His)_6$-Tbj1 alone did not aggregate when exposed to increased

temperature, it did enhance the aggregation of MDH. This suggested that either Tbj1 enhanced the aggregation of MDH while remaining unaggregated itself, or that Tbj1, through interaction with itself or MDH, formed aggregates. The enhanced aggregation of a model substrate in the presence of an Hsp40 protein has previously been described for the interaction between the Type II *E. coli* Hsp40, CbpA and rhodanese, where the addition of CbpA to rhodanese resulted in a 20–30% increase in aggregation [48]. Interestingly, the addition of an Hsp70 protein (DnaK) to the reaction resulted in aggregation suppression [48], as was the case in the present study involving Tbj1 and TcHsp70/MsHsp70.

This indicated that Tbj1 did not possess any chaperone activity of its own accord, as would be anticipated for a Type III Hsp40 [36,49], which was in contrast to the ability of a Type I Hsp40 from *T. cruzi*, Tcj2, which was able to suppress the thermal aggregation of MDH in the same system. In spite of its inability to suppress the aggregation of MDH when added on its own, $(His)_6$-Tbj1 was able to assist TcHsp70 and MsHsp70 in the aggregation suppression of MDH when added to the system. While these results suggested that there was possibly a direct interaction between $(His)_6$-Tbj1 and TcHsp70/MsHsp70, such an interaction would have to be confirmed by means of more rigorous analysis involving binding assays (e.g. surface plasmon resonance spectroscopy). In addition, if such an interaction were to occur to facilitate the suppression of thermal aggregation, it is currently not known what the potential mechanism of such an interaction would be.

Tbj1 was found to be unable to stimulate the ATPase activity of either TcHsp70 or MsHsp70, which corresponds to a previous analysis of its ortholog, Tcj1 [24]. While this may be a true *in vitro* situation, it is important to consider that the *in vivo*, native form of the protein may well be able to stimulate the ATPase activity of partner Hsp70 proteins. The addition of lower concentrations of Tbj1 to TcHsp70 resulted in increased apparent inhibition of the ATPase activity of TcHsp70. It is possible that at sub-stoichiometric levels compared to Hsp70, Tbj1 predominantly acts as a negative regulator of Hsp70 through J-domain-based inhibition of the ATPase domain. As the concentration of Tbj1 approaches equimolar levels compared to Hsp70, it may begin to be predominantly recognized as a substrate, and be sequestered to the substrate binding domain of Hsp70, with the resultant stimulation of the ATPase activity of the Hsp70. This indicates that Tbj1 and Tcj1 preside over a different type of interaction in the cell as does the *E. coli* Type III Hsp40, Djla, which was shown to be able to stimulate the ATPase activity of *E. coli* DnaK in a manner indistinguishable from that of DnaJ [36]. What is not known at present, is what mode of interaction Tbj1 presides over, as it appears to assist Hsp70 proteins in their aggregation suppression ability while being unable to stimulate the ATPase activity of these same proteins.

Tbj1, while being an ortholog to Tcj1 and being successfully overexpressed during the course of the present study, leaves many questions as to its biochemical activities and potential functionality *in vivo*. The apparent ability of Tbj1 to assist in the suppression of thermal aggregation, but its inability to stimulate the ATPase activity of two different partner Hsp70 proteins could serve to indicate that this protein possesses a specialised function within the *T. brucei* parasite that differs from the well-conserved function of Type I Hsp40s. At this point it is unclear what this function is, as studies on Type III Hsp40s are not very numerous. Further experimental investigation and biochemical characterisation will hopefully reveal more about the *in vitro* abilities and *in vivo* functions of this protein.

Acknowledgments

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