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Localization of BiP to translating ribosomes increases soluble accumulation of secreted eukaryotic proteins in an *E. coli* cell-free system

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Abstract

The endoplasmic reticulum (ER) resident Hsp70 chaperone, BiP, docks to the Sec translocon and interacts co-translationally with polypeptides entering the ER to encourage proper folding. In order to recreate this interaction in *E. coli* cell-free protein synthesis (CFPS) reactions, a fusion protein was formed between the ribosome binding portion of the *E. coli* protein Trigger Factor (TF) and BiP. The biophysical affinity to ribosomes as well as the characteristic Hsp70 ATPase activity were both verified for the fusion protein. When added to *E. coli*-based CFPS reactions, the TF-BiP fusion chaperone increased soluble yields of several protein fragments that are normally secreted through the ER and have poor solubility in typical CFPS reactions. For comparison, a fusion between TF and the native *E. coli* Hsp70, DnaK, was also constructed. This fusion was also biologically active and increased soluble yields of certain protein targets in CFPS. The TF-BiP fusion described in this study can be seen as a first step in reconstituting and better understanding ER folding pathways in the prokaryotic environment of *E. coli* CFPS.

Keywords

Hsp70 chaperones; protein folding; cell-free protein synthesis; DnaK; BiP; Trigger Factor

Introduction

Understanding protein folding - the assembly of linear polypeptides into stable three-dimensional functional proteins - is a central challenge of biology (Frydman 2001; Hartl and Hayer-Hartl 2002). The endoplasmic reticulum (ER) in eukaryotes provides a particularly complex folding environment. Approximately one-third of all translated proteins, including all membrane and secreted proteins, are translocated through the ER, and molecular chaperones and folding enzymes, which prevent protein aggregation and promote proper folding, may comprise up to 25% of all proteins in the crowded ER environment (van Anken and Braakman 2005). Notably, the Hsp70 chaperone, BiP, is an extremely important ER molecular chaperone that aids in protein folding and assembly, translocation of proteins across the ER membrane, targeting of misfolded proteins for degradation, and maintenance of ER calcium stores (Hendershot 2004).

BiP forms a permeability barrier between the cytoplasm and the ER lumen by docking to the luminal side of the ER membrane. Thus, when nascent polypeptides are translated through

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the translocon, BiP is available to adhere to them co-translationally, detach from its translocon docking site, and direct the folding and ultimate cellular destination of the polypeptide (Hamman et al. 1998). In particular, BiP has been shown to bind members of the immunoglobulin superfamily which includes antibodies (Bole et al. 1986; Haas and Wabl 1983; Knarr et al. 1995) and T-cell receptors (Suzuki et al. 1991), presumably by binding the β -sheets that comprise the barrel structure of immunoglobulin domains, particularly in sections that may be buried in the ultimate structure of the protein (Augustine et al. 2001).

Trigger Factor (TF) is a small ribosome-tethered chaperone in the cytoplasm of *Escherichia coli* that can accept an emerging peptide and be pulled off the ribosome to continue assisting that protein to fold (Kaiser et al. 2006) much as BiP functions in the ER. The first 118 amino acids of TF are independently responsible for its localization near the ribosome exit tunnel (Hestkamp et al. 1997). Henrichs et al. (2005) created a fusion protein between this N-terminal domain of TF and the tobacco etch virus (TEV) NIa protease. They demonstrated that the proteolytic efficiency of the TEV protease was increased by being tethered to the translating ribosomes. Thus, the N-terminal domain of TF was shown to be a viable ribosome localization domain for fusion partners.

E. coli based cell-free protein synthesis (CFPS) has progressed much in recent years from a benchtop research tool to a scalable technology with commercial applications (Swartz 2006; Voloshin and Swartz 2005). The open nature of the CFPS environment provides for facile modification of the chemical environment and has allowed for harnessing of native *E. coli* energy systems (Jewett et al. 2008; Jewett and Swartz 2004), successful production of disulfide bonded proteins at high yield (Goerke et al. 2008; Yin and Swartz 2004), and incorporation of non-natural amino acids that allow for post-translational modifications (Goerke and Swartz 2009). Each of these advances potentially improves the production of valuable mammalian protein drug targets. However, the folding of many complex, secreted mammalian proteins at high yields has remained difficult. The problem of folding larger, multi-domain eukaryotic proteins is a common roadblock for both *in vivo* and *in vitro* *E. coli* systems (Agashe et al. 2004; Baneyx and Mujacic 2004; Netzer and Hartl 1998). Thus, utilizing the ER folding machinery, including BiP, could potentially offer improved folding of complex heterologous targets, and the open nature of the CFPS environment lends itself to facile introduction of these non-native chaperones. However, in order to better harness the chaperone capabilities of BiP for protein folding, it would be useful to localize BiP to the exit tunnel of translating ribosomes to encourage immediate interaction with nascent polypeptides. In order to more fully replicate the immediate availability of BiP to polypeptides emerging into the ER, we fused the localization domain of TF to BiP (Fig. 1). Realizing there was a risk that the domains would interfere with each other's function, we carefully demonstrated the expected ribosome affinity as well as full ATPase activity for the chaperone domain.

In this work, we show that the folding of several secreted mammalian proteins is improved by localizing BiP to translating ribosomes, thereby increasing BiP's local concentration and reproducing its *in vivo* function of co-translational interaction with substrates. To test for Hsp70 chaperone specificities for proteins from different organisms, we compared the effectiveness of BiP to the cytoplasmic Hsp70 chaperone, DnaK, for both eukaryotic and prokaryotic proteins. We also tested a fusion between DnaK and TF to further evaluate the effectiveness of ribosome localization.

Materials and Methods

Cloning, Production, and Purification of Chaperones

Plasmids containing the genes for hamster BiP as well as the human co-chaperones, ERdj3 and BAP (Chung et al. 2002), were kind gifts from Dr. Linda Hendershot, St. Jude Children's Hospital, Memphis, TN. In order to obtain the human BiP sequence, primers to change 6 amino acids in the hamster sequence were used to amplify the gene by PCR in 2 pieces followed by amplification of the full-length, human sequence (Ting and Lee 1988). All genes were cloned without their signal sequences and also encoded a His₆ purification tag at the N-terminus separated from the protein sequence by a G₄S linker. Genes for DnaK and its co-chaperones, DnaJ and GrpE, were obtained through PCR from the *E. coli* genome with the introduction of the coding sequence for a His₆ purification tag at the C-terminus. All genes were cloned into the pY71 vector using NdeI and SalI or AatII restriction enzyme sites at the N- and C-termini, respectively (Table I). pY71 is a reduced size plasmid (2.45 kb) with a pUC19 origin of replication and a kanamycin resistance element under the control of the T7 promoter (Kuchenreuther et al. 2009).

Fusion proteins between the N-terminal TF domain and BiP (TF-BiP) as well as DnaK (TF-DnaK) were designed to include the first 144 amino acids of TF (TF 1-144) followed by the Hsp70 chaperone. The chaperones were separated from TF via a (G₄S)₃ linker and included a His₆ purification tag at the C-terminus (Table I). The genes encoding these fusion proteins were also introduced in the pY71 plasmid.

Plasmids were transformed into *E. coli* BL21(DE3) pLysS cells which were grown in 1 L LB medium supplemented with 40 µg/mL kanamycin and 20 µg/mL chloramphenicol at 37 °C. At an OD of 0.6, cells were induced with 1 mM IPTG and incubated for an additional 3 hours. (Cultures expressing TF-BiP, ERdj3, and TF-DnaK were induced for 5 hours at 30 °C to reduce degradation. For the production of BAP, cells were incubated at 17 °C for 3 hours after induction.) Cells were harvested by centrifugation and resuspended in 15 mL of 50 mM sodium phosphate pH 8, 300 mM sodium chloride, and 10 mM imidazole (Buffer A). A single pass through an Avestin EmulsiFlex C-50 high-pressure homogenizer at 20,000 psig lysed cells. (Protease inhibitor (PMSF) was added before lysis to a concentration of 1.33 mM.) After centrifugation at 30,000 × g, 2,200 U of DNaseI was added to the supernatant to reduce viscosity. The supernatants were loaded on 1 mL nickel chelating columns equilibrated with Buffer A and eluted with Buffer A containing 250 mM imidazole. Pooled fractions were dialyzed into 10 mM potassium phosphate pH 7.2. Sucrose was added to 10% and proteins were stored at -80 °C.

Plasmid Construction for Cell-Free Production of Model Proteins

Cell-free production from plasmid templates was evaluated for the following proteins: the CD-19 extracellular domain (ECD) (Tedder et al. 1994), a single chain T-cell receptor (scTCR), a single-chain antibody (scFv), β-galactosidase, and firefly luciferase. The CD-19 ECD (encoding amino acids 19-287) was constructed without the coding regions for the N-terminal leader sequence or the C-terminal transmembrane and cytoplasmic domains. Additionally, the codons for the first five amino acids of chloramphenicol acetyl transferase (CAT) were added at the N-terminus to improve translation initiation, and six codons for a His₆ tag were added to the C-terminus. The scTCR gene (a kind gift from Ronald Levy, Stanford University) was constructed from sequences obtained from the C6VL cell line, a T-cell tumor of C57BL/Ka origin (Okada et al. 1997). The gene was composed of the variable α- and variable β-domains joined via a (G₄S)₃ linker and followed by the constant β-domain. The model scFv has anti-digoxin affinity (Hunter et al. 1982; Levy et al. 2001) and was composed of the variable light chain region linked to the variable heavy chain region with a

(G₄S)₃ linker. The gene was codon optimized for *E. coli* expression as suggested by DNAworks software (Hoover and Lubkowski 2002). The β -galactosidase gene (Keseler et al. 2005) was cloned from the *E. coli* genome using PCR with NdeI and AatII restriction sites removed from the sequence. The firefly luciferase gene from *Photinus pyralis* (de Wet et al. 1987) was codon optimized for *E. coli* expression as suggested by DNAworks.

Assessing the ATPase Activity of the Fusion Proteins

ATP hydrolysis assays were used to confirm the biological activities of the Hsp70 chaperones and fusion proteins. The reaction buffer consisted of 20 mM HEPES pH 7.2, 50 mM potassium chloride, 5 mM magnesium chloride, 10 mM dithiothreitol, and 250 μ M ATP. Hsp70 chaperones and fusion chaperones were each added at 4 μ M and co-chaperones were each added at 0.25 μ M. Reactions were incubated at 37 °C and ATP concentration measurements were taken every 15 minutes by diluting samples 250-fold into 20 mM HEPES pH 7.2, 50 mM potassium chloride, 5 mM magnesium chloride, and 2 mM dithiothreitol. 100 μ l of these diluted samples was then mixed with 35 μ l of a luciferin mixture consisting of 20 mM HEPES pH 7.2, 5 mM magnesium chloride, 2 mM dithiothreitol, 0.4 mg/mL bovine serum albumin, 0.5 mM luciferin (Invitrogen, Carlsbad, CA), and 0.2 μ g/mL luciferase (Promega, Madison, WI). A luminometer (Mithras LB 940, Berthold Technologies, Oak Ridge, TN) was used to measure ATP concentrations based on luciferase activity in a 96-well plate format. ATP hydrolysis rates were calculated by fitting the linear portion of the ATP concentration versus time curves for each chaperone tested.

Assessing the Ribosome Binding Affinity for the Fusion Chaperones

To test affinities to ribosomes, radioactive (¹⁴C) TF proteins or fusion proteins were added to suspensions of purified ribosomes and adsorbed protein was separated from free protein by ultracentrifugation (Raine et al. 2004). The proteins were expressed in 1 mL PANOX SP CFPS reactions (see below) and radiolabeled with L-[U-¹⁴C]-leucine, loaded onto 1 mL nickel chelating columns in 10 mM imidazole, and eluted at 250 mM imidazole. Ribosomes were purified as follows: 10 g (wet weight) of KC6 *E. coli* cells (Calhoun and Swartz 2006) were resuspended in 10 mL of 20 mM Tris pH 7.2, 100 mM ammonium chloride, 10 mM magnesium chloride, 0.5 mM EDTA, and 6 mM β -mercaptoethanol and were lysed by a single pass through an Avestin EmulsiFlex C-50 high-pressure homogenizer at 20,000 psig. Insoluble cell debris was removed by centrifugation at 30,000 $\times$ g for 15 minutes. Ribosomes were then spun through a 45% sucrose cushion at 200,000 $\times$ g for 20 hours at 4 °C to pellet and separate from native proteins. Ribosomes were resuspended and stored at -80 °C in a final buffer of 10 mM Tris pH 7.5, 60 mM ammonium chloride, 5 mM magnesium chloride, and 0.5 mM EDTA.

Fixed concentrations of TF and TF fusions were mixed for 15 minutes at room temperature with differing concentrations of purified ribosomes in a CFPS mixture (see below) without the addition of extract or template DNA. Mixtures were then spun at 85,000 $\times$ g for 2 hours in a Beckman Airfuge ultracentrifuge (Beckman Coulter, Fullerton, CA) to pellet ribosomes. (The TF and TF fusion concentrations were less than 250 nM for all proteins tested.) Scintillation counting of the resuspended pellet indicated the degree of ribosomal adsorption. Each binding test consisted of 6 different ribosome concentrations and tests were repeated 3 to 4 times for each concentration. After subtracting the scintillation counts in the pellets for the proteins centrifuged without ribosomes, the K_D values were estimated using curve-fitting software in SigmaPlot® (for an example, see Fig. 2) with a one-site saturation ligand binding model. To ensure the validity of the reported K_D values, each binding experiment tested at least 2 ribosome concentrations on either side of the K_D.

Cell-Free Protein Synthesis Reactions

Model proteins were tested for improved folding in the previously described PURE (Shimizu et al. 2001) and PANox SP (Jewett and Swartz 2004) CFPS systems. The PURE system (New England Biolabs, Ipswich, MA) CFPS reactions were conducted as per the recommendations of the manufacturer with several exceptions. An optimized oxidizing environment for the correct formation of disulfide bonds was added for the secreted ER target proteins. The *E. coli* disulfide isomerase, DsbC, was added at a concentration of 75 µg/mL, and an artificial oxidizing buffer of 4 mM oxidized glutathione and 1 mM reduced glutathione was added for the CD-19 ECD and scFv while a 1 mM oxidized glutathione and 4 mM reduced glutathione buffer was used for the scTCR. L-[U-¹⁴C]-leucine was added at 8.4 µM (2.67 µCi) for protein quantification. Reactions were conducted at 15 µl volumes in 1.5 mL Eppendorf tubes for 1 hour at 37 °C as the majority of product accumulated during this time frame.

PANox SP reactions were conducted as previously described (Jewett and Swartz 2004) with 1.2 mM ATP, 0.85 mM each of GMP, UMP and CMP, 33 mM phosphoenol pyruvate (Roche Molecular Biochemicals, Indianapolis, IN), 170 mM potassium glutamate, 10 mM ammonium glutamate, 20 mM magnesium glutamate, 1.5 mM spermidine, 1.0 mM putrescine, 34 µg/mL folinic acid, 171 µg/mL *E. coli* tRNA mixture (Roche Molecular Biochemicals), 13.3 µg/mL plasmid, 300 µg/mL T7 RNA polymerase (prepared by the previously described procedure (Jewett and Swartz 2004)), 2 mM of each of the 20 unlabeled amino acids, 0.33 mM NAD, 0.26 mM Coenzyme A (CoA), 2.7 mM sodium oxalate, and 0.24 volumes of *E. coli* KC6 S30 extract (Calhoun and Swartz 2006). (All reagents were obtained from Sigma–Aldrich, St. Louis, MO unless otherwise noted.) Oxidizing conditions were created for the secreted ER model proteins as described above. L-[U-¹⁴C]-leucine was also added at 8.4 µM (2.67 µCi) for protein quantification. Reactions were conducted at 15 µl volumes in 1.5 mL Eppendorf tubes for 3.5 hours at 37 °C.

Following CFPS reactions, 3-6 µl of reaction volume was spotted on filter paper and allowed to dry. The remaining volume was then spun at 20,800 × g to pellet insoluble protein and the supernatant was also spotted and allowed to dry. A previously described trichloroacetic acid procedure (Calhoun and Swartz 2005) was then used to precipitate protein, and counting of incorporated L-[U-¹⁴C]-leucine in a Beckman LS3801 liquid scintillation counter (Beckman Coulter) was used to quantify protein yields. P-values were calculated using a one-tailed test either for comparison of the reaction yields after chaperone additions versus the no-chaperone control or for fusion chaperone addition versus the native chaperone.

Protein Activity Assays

The activity of firefly luciferase was measured by diluting samples 250-fold in 20 mM HEPES pH 7.2, 50 mM potassium chloride, 5 mM magnesium chloride, 2 mM dithiothreitol, 350 µM ATP. 100 µl of these diluted samples was then mixed with 35 µl of a luciferin mixture consisting of 20 mM HEPES pH 7.2, 5 mM magnesium chloride, 2 mM dithiothreitol, 0.4 mg/mL bovine serum albumin, and 0.5 mM luciferin (Invitrogen). A luminometer (Mithras LB 940, Berthold Technologies) was used to measure activity of the samples in a 96-well plate format.

The activity of β-galactosidase was measured by diluting CFPS reaction samples 100-fold into S30 buffer (10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, and 60 mM potassium acetate). 10 µl of these dilutions was then mixed with 10 µl of 8 mg/mL ONPG and incubated at 37 °C for 15 minutes. The reaction was quenched by adding 80 µl of 0.5 M

sodium carbonate pH 9.6, and absorbance was measured at 413 nm in a 96-well plate format.

Results

Construction of Fusion Chaperones

To mimic the ER function of BiP in CFPS, the chaperone needs to be engineered to: 1) localize to translating ribosomes, 2) detach from ribosomes upon binding with nascent protein substrates, and 3) preserve the native bioactivity of BiP. We chose to create a fusion protein between the TF ribosome localization domain and BiP (or the prokaryotic Hsp70, DnaK) in order to address these goals (Fig. 1). TF is a small prolyl isomerase chaperone in the cytosol of *E. coli* that binds to the L23 ribosomal protein near the ribosome exit tunnel, thereby placing it in an ideal location to interact with emerging polypeptides. TF is an ideal fusion candidate because it cycles on and off of the ribosome with moderate affinity (Kaiser et al. 2006) and has a known ribosome binding domain that has previously been isolated and used in a fusion protein to increase the activity of a protease (Henrichs et al. 2005; Hesterkamp et al. 1997). The N-terminal domain of TF (first 118 amino acids) is responsible for ribosome binding. However, residues 119-144 provide a soluble linker that both increased the solubility of the TF N-terminal domain and provided a convenient linker for TF-Hsp70 fusions, so we used residues 1-144 for our fusion with Hsp70 chaperones. TF was fused to the N-terminus of either BiP or DnaK separated by a 15 residue (G₄S)₃ linker to allow independent functioning of the two portions of the fusion protein (Table I, Fig. 1).

Bioactivity of Fusion Chaperones

To test that the fusion chaperones were active, we measured their ATPase activities. When ATP is bound, the chaperones exhibit a low affinity for substrates, but when ATP is hydrolyzed to ADP, a conformational change in the chaperones leads to much higher affinity for substrates. In the ADP-bound state, the Hsp70 remains bound to substrates until fresh ATP can displace the ADP, releasing the substrates and allowing the cycle to continue (Bukau and Horwich 1998). The cycling on and off of substrates is crucial to Hsp70 function and several co-chaperones facilitate this process. For DnaK, the co-chaperone, DnaJ, speeds the ATP hydrolysis step while the co-chaperone, GrpE, increases the rate at which ADP is exchanged for fresh ATP (Bukau and Horwich 1998; Liberek et al. 1991). In the mammalian ER, the ERdj class of chaperones (ERdj1-6) function similarly to DnaJ to accelerate the ATP hydrolysis step whereas the co-chaperone, BAP, assists in the nucleotide exchange step (Chung et al. 2002; Jin et al. 2009; Shen and Hendershot 2005; Weitzmann et al. 2007).

We tested each Hsp70 chaperone (BiP and DnaK) for its ability to hydrolyze ATP when free or when fused to the TF domain. Co-chaperones at 16:1:1 ratios to the Hsp70 (ERdj3 and BAP for BiP, DnaJ and GrpE for DnaK) were included in each reaction as well as in all subsequent CFPS tests. The rate of ATP hydrolysis was determined throughout the reaction by measuring ATP concentrations using firefly luciferase. Our data show that both of the fusion proteins are bioactive and exhibit ATP hydrolysis rates similar to those measured for the non-tethered chaperones (Table II) and to previously described results (Bonomo et al. 2010; Chung et al. 2002; Liberek et al. 1991; Mayer et al. 2003; Weitzmann et al. 2007).

Ribosome Binding of Fusion Chaperones

To address ribosome localization, we used a previously described ultracentrifugation method (Raine et al. 2004) to separate ribosomes (and adsorbed protein) from unbound protein (Table II, Fig. 2). Proteins were radiolabeled with L-[U-¹⁴C]-leucine for quantification via scintillation counting and K_D values were determined using curve-fitting software from

SigmaPlot®. To confirm the validity of the K_D measurements, the binding curves were extrapolated to higher ribosome concentrations. In all cases the total ligand concentration was correctly indicated.

We first compared the binding affinity of native TF to that of a truncated TF consisting of the first 144 amino acids (TF 1-144). The measured affinity of TF for ribosomes was similar to previous studies at approximately 1 μ M (Mayer et al. 2003; Raine et al. 2004). TF 1-144 had a somewhat higher affinity, K_D equal to 510 nM. The higher affinity possibly results from less steric hindrance for the isolated TF 1-144 domain as it approaches the ribosome as compared to the full-length protein.

We next tested the TF-BiP and TF-DnaK fusions for ribosome binding. TF-BiP had a nearly identical ribosomal affinity as that of TF 1-144 (Table II). Steric hindrances from the BiP domains might possibly be minimized by the (G₄S)₃ linker, thus preserving the higher affinity. TF-DnaK had an affinity between that of native TF and TF 1-144. However, the measured affinity is within experimental error of both that for the native TF as well as for TF-BiP. To confirm that affinity was not due to either BiP or DnaK binding nonspecifically to ribosomes, we also tested the ribosome affinity of these native chaperones. At ribosome concentrations as high as 4 μ M (well above all TF-associated K_D values), only a minor fraction of both proteins was bound to ribosomes indicating that BiP and DnaK alone do not bind to ribosomes at the ribosome concentrations in CFPS reactions (about 2 μ M). Thus, the ribosome affinity of the fusion chaperones is dominated by the TF domain, suggesting proper localization to the L23 protein.

The protein portion of the *E. coli* signal recognition particle (SRP), Ffh, was also tested as a possible fusion partner for BiP and DnaK since it can also bind to the L23 protein, albeit with a somewhat higher affinity than for TF (Raine et al. 2004). The N-terminal domains responsible for ribosome binding (Gu et al. 2003) were tested, but did not display measurable ribosome affinity up to 4 μ M ribosome concentrations (data not shown). Thus, the Ffh protein was therefore abandoned as a potential fusion partner.

CFPS for ER Proteins

Hsp70 chaperones and fusion chaperones were tested for their ability to increase yields of three model secreted, eukaryotic proteins. These proteins, the extracellular domains of CD-19; a single-chain T-cell receptor (scTCR); and a single-chain anti-digoxin antibody (scFv), all contained immunoglobulin domains and had shown poor expression and folding in previous CFPS reactions despite accumulating at the expected size with minimal degradation products. The proteins were tested for increased soluble yields in two different CFPS systems: the PURE translation system (Shimizu et al. 2001) and the PANOx SP system (Jewett and Swartz 2004). The PURE system uses only the individually purified components necessary for transcription and translation in *E. coli*. Thus, the system provides a simpler environment with fewer components to influence protein folding and chaperone function. The lack of native chaperones, including TF, in the PURE system allowed a more direct assessment of the specific effects of chaperone addition and avoided competition for ribosome binding sites. The PANOx SP system is a higher yielding, longer lasting CFPS system that has proven effective for a variety of proteins from multiple hosts (Bundy et al. 2008; Goerke et al. 2008; Goerke and Swartz 2009; Wu and Swartz 2008; Yang et al. 2004). The PANOx SP system uses *E. coli* extracts which have high concentrations of native chaperones including TF and DnaK (both present at approximately 2-3 μ M in CFPS) (Hartl and Hayer-Hartl 2002; Kaiser et al. 2006) and other molecules and cofactors that make the folding environment more crowded than that of the PURE system (albeit still about 20-fold less crowded than the *E. coli* cytoplasm). For all CFPS reactions, Hsp70 chaperones and fusion chaperones were added at a concentration of 8 μ M while co-chaperones were added

at 0.5 μM to approximate *in vivo* ratios (Neidhardt and VanBogelin 1987; Weitzmann et al. 2007). The 8 μM Hsp70 additions increased yields in general and, based on ribosome affinity measurements, would be expected to effectively outcompete native TF for ribosome binding sites in the PANOx SP system.

For the PURE system (Table III, Fig. 3), CFPS soluble yields were higher than the no chaperone control for all Hsp70 chaperones and fusion chaperones and for each model protein tested ($p < 0.05$) except when BiP was tested with the scFv (Fig. 3c, black bars). However, the additions of TF fusions for ribosome localization provided only marginal benefits and were even detrimental in some cases. TF-BiP offered a statistically significant improvement over the non-tethered BiP protein only for the CD-19 ECD protein ($p < 0.01$), whereas TF-DnaK showed no statistical benefit over DnaK.

In contrast, for the PANOx SP system (Table III, Fig. 4), the TF-BiP fusion chaperone offered a much greater benefit (Fig. 4, black bars). TF-BiP gave higher soluble yields than the control for all proteins tested ($p < 0.05$), whereas BiP only improved soluble yields significantly for the scTCR ($p < 0.025$). TF-BiP provided a greater benefit than BiP for all proteins, and this effect was statistically significant for both the CD-19 ECD and scTCR ($p < 0.025$). The much higher percentage of soluble protein (Fig. 4, grey bars) suggests that TF-BiP prevents accumulation of misfolded protein, consistent with a co-translational folding mechanism in the ER in which exposure of hydrophobic residues is minimized during the course of translation. The contribution of the TF 1-144 domain to directly improve protein folding was ruled out as CFPS tests with this domain alone failed to increase yields compared to the no chaperone control (data not shown). TF-DnaK and DnaK, on the other hand, were only beneficial for the scFv ($p < 0.01$). While TF-DnaK gave slightly higher soluble yields than DnaK for all three proteins, none of the improvements were statistically significant. It is possible that the native DnaK concentration and other factors present in the *E. coli* extract are sufficient such that additional DnaK is superfluous even if it is tethered to the ribosome. It is also possible that co-translational interactions for DnaK are less important than for BiP.

Since some of the beneficial effects of tethering BiP were only seen with the PANOx SP system, it appears that in the less complex and less crowded PURE environment, nascent proteins may not need the immediate benefits of protection at the ribosome, and their folding may even be hindered by the presence of the chaperones at the exit tunnel. In contrast, in the more complex environment of the PANOx SP system, the immediate presence of BiP may offer much greater protection from aggregation and misfolding. Alternatively, BiP may need assistance from other chaperones in the PANOx SP cell extract and its benefit might only appear in this context.

CFPS for Cytoplasmic Proteins

Eukaryotic firefly luciferase and prokaryotic β -galactosidase, both cytoplasmic protein targets, were also tested in PANOx SP CFPS reactions for increased soluble yields as well as increased activity (Table IV, Fig. 5). For both proteins, only the DnaK chaperones provided any benefit. In addition, only non-tethered DnaK offered a significant benefit ($p < 0.025$) for soluble yields for the β -galactosidase protein. The lack of improvement from TF fusions indicates that perhaps neither of these proteins requires immediate folding assistance. Furthermore, the detrimental effects seen from the addition of both BiP and TF-BiP suggest that BiP may provide limited assistance for non-native substrates.

Discussion

We have demonstrated the feasibility of localizing molecular chaperones to translating ribosomes in the context of CFPS reactions. Furthermore, we have compared the functions of two different classes of Hsp70 molecular chaperones and offer suggested insights into protein folding in the eukaryotic ER.

In terms of general specificity, the *E. coli* cytoplasmic Hsp70, DnaK, was able to increase soluble yields in CFPS for both prokaryotic and eukaryotic proteins. The eukaryotic ER resident Hsp70, BiP, on the other hand, only provided benefit to secreted eukaryotic proteins. In the prokaryotic cytoplasm, DnaK theoretically could interact with any protein that is expressed, whereas BiP is only exposed to a certain subclass of proteins, namely those that are translocated into the ER. Thus, it is not surprising that DnaK provides a more generally useful chaperone function.

By fusing both DnaK and BiP to the N-terminal domain of TF, we have provided another example of the utility of this TF domain to create fusion proteins where ribosome localization is desired (Eser et al. 2007; Henrichs et al. 2005). More importantly, the fusion chaperones create a method to assess the importance of ribosome localization and co-translational folding assistance *in vitro* for Hsp70 chaperones. For DnaK, the TF fusion was largely ineffective. A previously described mechanism of DnaK function suggests that, while possibly slowing folding through co-translational interaction, DnaK requires multiple post-translational bind-and-release cycles with the substrate to ultimately lead to correct folding (Agashe et al. 2004; Pierpaoli et al. 1997). Thus, it might be expected that DnaK function would not benefit as much from ribosome localization.

In contrast to DnaK, the TF fusion with BiP significantly increased soluble protein yields and the percentage of soluble product for secreted eukaryotic targets in CFPS. The data suggest that BiP localization encourages co-translational folding, supporting previously described mechanisms for eukaryotic protein folding (Christis et al. 2008; Frydman 2001; Netzer and Hartl 1997). For the PANOx SP CFPS, TF-BiP reduced total protein yields suggesting that the presence of TF-BiP at the ribosome may slow the rate of protein elongation or the rate of ribosome recycling.

By localizing BiP to translating ribosomes, we have partially replicated the folding environment of the eukaryotic ER in CFPS leading to increased soluble production of several secreted eukaryotic proteins in CFPS. Production of eukaryotic proteins in heterologous prokaryotic hosts is often difficult and aggregation prone as the secreted proteins are typically larger and include more structural domains (Netzer and Hartl 1998). The open nature of CFPS facilitates reaction system modification, and further additions to the system described here could produce greater increases in eukaryotic protein production. Specifically, addition of the chaperones GRP94 and GRP170 might be beneficial since these have been suggested as recipients of partially folded BiP substrates in a hand-over-hand mechanism (Melnick et al. 1994; Steel et al. 2004) that may be necessary to complete folding. The ER resident protein disulfide isomerase (PDI) might also be more effective than *E. coli* DsbC.

While much progress has been made in the understanding of protein folding in the ER (Jin et al. 2009; Melnick et al. 1994; Meunier et al. 2002; Tatu and Helenius 1997), the difficulty in studying this compartment has prevented a full understanding of how BiP specifically collaborates with the other resident ER chaperones. CFPS may provide a convenient way to study the interaction of various chaperones and folding factors. The TF-BiP fusion system described in this paper demonstrates a first step in reproducing ER folding pathways *in vitro* and, in conjunction with additional ER chaperones, could ultimately provide a more

thorough replication of ER folding, elucidating more fully the folding process in this compartment as well as yielding a platform to produce valuable therapeutic proteins and vaccines in CFPS.

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Figure 1.

A fusion between BiP and the ribosome binding portion of TF. The first 144 amino acids of Trigger Factor (TF 1-144) bind to the L23 protein near the ribosome exit tunnel, thereby localizing the BiP (or DnaK) fusion near the exit tunnel and allowing the chaperone to interact with nascent translating polypeptides.

Figure 2.

Representative ribosome binding curve for radiolabeled TF-DnaK (100nM). Data points represent scintillation counts from the resuspended pellet following centrifugation of L-[U- ^{14}C]-leucine labeled TF-DnaK with the given ribosome concentrations. Each protein binding test was repeated 3-4 times, and K_D values were calculated using SigmaPlot® software.

Figure 3.

Improvements in PURE system CFPS compared to no-chaperone control for model secreted proteins with chaperone additions. Soluble yields (black bars) and percentage soluble protein (grey bars) are displayed for the following proteins: A) CD-19 ECD, B) scTCR, and C) scFv. Each reaction was performed in triplicate, and error bars represent standard deviation.

Figure 4.

Improvements in PANOx SP CFPS compared to no-chaperone control for model secreted proteins with chaperone additions. Soluble yields (black bars) and percentage soluble protein (grey bars) are displayed for the following proteins: A) CD-19 ECD, B) scTCR, and C) scFv. Each reaction was performed in triplicate, and error bars represent standard deviation.

Figure 5. PANOx SP CFPS protein and activity comparison of chaperone additions to the no-chaperone control for A) firefly luciferase and B) β -galactosidase. Soluble yields (black bars), percentage soluble protein (grey bars), and protein activity (white bars) are displayed. Each reaction and activity assay was performed in triplicate, and error bars represent standard deviation.

Table I

Primers used for Hsp70 chaperones and fusion chaperones.

Protein		Primer sequence
BiP	Forward	atatat <u>catatg</u> cat CATCACCATCACCATCAC ggcggcgggagccgaggaggaggacaa
	Reverse	tatatatagtcgacttacaaactcatctttttctgctgatcc
TF-BiP	Forward	tcacta <u>catatg</u> caagtttcagttgaaaccac
	Mid-reverse	GGAACCCCCACCGCCGCTGCCTCCACCACCagagta
	Mid-forward	GGCGGTGGGGGTTCGGGTGGCGGCGGCAGTgaggaggaggacagaag
	Reverse	ttaatagacgcttatta GTGATGGTGTGATGGTGTG Gcaactcatctttttctgctgatcc
DnaK	Forward	tcacta <u>catatg</u> ggtaaaataattggtatcgacctgggtactaccaactc
	Reverse	tcacta <u>tcgactta</u> GTGATGGTGTGATGGTGTG Gccatggtttttgtctttgacttctcaaattcagc
TF-DnaK	Forward	Same as TF-BiP Forward
	Mid-reverse	GGAACCCCCACCGCCGCTGCCTCCACCACCagagtatccagcatgcc
	Mid-forward	GGCGGTGGGGGTTCGGGTGGCGGCGGCAGTatgggtaaaataattggtatcgacc
	Reverse	Same as DnaK reverse

For fusion chaperones, the first two primers used were for the TF portion of the fusion and the final two primers for the Hsp70 portion. Final products resulted from overlapping PCR of the two pieces followed by a final round of amplifications with the outermost primers. Underline- NdeI and SalI or AatII restriction sites for cloning. CAPS- (G4S)₃ linker portions. **BOLD CAPS**- His₆ purification tags.

Table II

Enzymatic ATP hydrolysis rates and ribosome binding affinity of Hsp70 chaperones and fusion chaperones.

Protein	ATPase activity (min^{-1})	Ribosome K_D (nM)
TF	N/D	950 ± 240
TF 1-144	N/D	510 ± 120
BiP	0.37 ± 0.06	N/D
TF-BiP	0.57 ± 0.08	510 ± 160
DnaK	0.64 ± 0.04	N/D
TF-DnaK	0.67 ± 0.04	760 ± 140

TF-BiP and TF-DnaK fusions preserve the ATP hydrolysis activity necessary for chaperone function while also showing characteristic TF affinity to ribosomes (presumably at the exit tunnel). N/D – values were not determined.

Table III

Protein synthesis for model secreted eukaryotic proteins in PURE and PANOx SP CFPS systems without the addition of Hsp70 chaperones.

Cell-free System	Protein	Soluble Yield ($\mu\text{g/mL}$)	% Soluble
PURE	CD-19 ECD	31 ± 1	19 ± 0.3
	scTCR	52 ± 12	28 ± 6
	scFv	44 ± 3	32 ± 2
PANOx SP	CD-19 ECD	57 ± 11	14 ± 6
	scTCR	31 ± 3	8 ± 1
	scFv	65 ± 5	34 ± 8

Percentage soluble represents the fraction of soluble protein to total protein synthesized. Each reaction was performed in triplicate.

Table IV

Primers used for Hsp70 chaperones and fusion chaperones.

Protein	Soluble Yield ($\mu\text{g/mL}$)	% Soluble
Firefly Luciferase	184 ± 42	13 ± 1
β -galactosidase	337 ± 7	41 ± 2

For fusion chaperones, the first two primers used were for the TF portion of the fusion and the final two primers for the Hsp70 portion. Final products resulted from overlapping PCR of the two pieces followed by a final round of amplifications with the outermost primers. Underline- NdeI and SalI or AatII restriction sites for cloning. CAPS- (G4S)₃ linker portions. **BOLD CAPS**- His6 purification tags.