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The molecular chaperone Ssb from *Saccharomyces cerevisiae* is a component of the ribosome–nascent chain complex

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The 70 kDa heat shock proteins (Hsp70s) are a ubiquitous class of molecular chaperones. The Ssbs of *Saccharomyces cerevisiae* are an abundant type of Hsp70 found associated with translating ribosomes. To understand better the function of Ssb in association with ribosomes, the Ssb–ribosome interaction was characterized. Incorporation of the aminoacyl-tRNA analog puromycin by translating ribosomes caused the release of Ssb concomitant with the release of nascent chains. In addition, Ssb could be cross-linked to nascent chains containing a modified lysine residue with a photoactivatable cross-linker. Together, these results suggest an interaction of Ssb with the nascent chain. The interaction of Ssb with the ribosome–nascent chain complex was stable, as demonstrated by resistance to treatment with high salt; however, Ssb interaction with the ribosome in the absence of nascent chain was salt sensitive. We propose that Ssb is a core component of the translating ribosome which interacts with both the nascent polypeptide chain and the ribosome. These interactions allow Ssb to function as a chaperone on the ribosome, preventing the misfolding of newly synthesized proteins.

Keywords: chaperone/protein folding/ribosome–nascent chain complex/ribosomes/Ssb

Introduction

Within the crowded environment of the cell, newly translated proteins must be prevented from folding prematurely or aggregating. Molecular chaperones are thought to prevent these events from occurring by binding reversibly to proteins in their non-native conformations (for reviews, see Craig *et al*., 1993; Morimoto *et al*., 1994; Hartl, 1996). The 70 kDa heat shock proteins (Hsp70s) comprise a highly conserved class of molecular chaperones that are known to bind short, largely hydrophobic amino acid segments likely to be exposed in unfolded or unassembled structures. This binding is thought to prevent the aggregation of non-native proteins (Flynn *et al*., 1989; Blond-Elguindi *et al*., 1993; Gragerov *et al*., 1994). It has been suggested that Hsp70s may interact with nascent polypeptides as they emerge from the ribosome and facilitate the successful folding of newly synthesized proteins (Beckmann *et al*., 1990; Nelson *et al*., 1992; Frydman *et al*., 1994; Kudlicki *et al*., 1995; Eggers *et al*., 1997).

Members of the Hsp70 family share structural features that suggest a common mechanism of action. All Hsp70s have three domains: a highly conserved N-terminal 44 kDa ATPase domain, a less well conserved 18 kDa peptide-binding domain and a C-terminal 10 kDa variable domain (Chappell *et al*., 1987; Milarski and Morimoto, 1989; Flaherty *et al*., 1990; Haus *et al*., 1993; Huang *et al*., 1993; Wang *et al*., 1993; Tsai and Wang, 1994; Zhu *et al*., 1996). Cycles of peptide binding and release are coupled to ATP binding, hydrolysis and nucleotide exchange (Palleros *et al*., 1993; McCarty *et al*., 1995; Banecki and Zylicz, 1996). Current models of Hsp70 action state that the ATP-bound form of Hsp70 binds to substrates with high affinity yet rapidly releases them, and thus is considered the 'fast-binding, fast-release' form. The ADP-bound form of Hsp70 is relatively slow in both the binding and release of substrate, and is considered the 'slow-binding, slow-release form'. Therefore, ATP hydrolysis converts Hsp70 from the 'fast-binding, fast-release' form to the 'slow-binding, slow-release' form, stabilizing the interaction between Hsp70 and its peptide substrate (Palleros *et al*., 1993; Schmid *et al*., 1994; Greene *et al*., 1995; McCarty *et al*., 1995). Evidence also suggests that the DnaJ family of chaperones stimulate ATP hydrolysis and thereby aid in the formation of stable Hsp70–peptide substrate interactions (reviewed by Cyr and Douglas, 1994). Efficient release of substrate then requires the exchange of ADP for ATP.

The cytosol of the budding yeast *Saccharomyces cerevisiae* contains two well-characterized classes of Hsp70s, the Ssas and Ssbs (Craig and Jacobsen, 1985; Craig *et al*., 1995). The Ssas, encoded by the *SSA1–4* genes, comprise an essential subfamily. Ssas are involved in translocation of some precursor proteins into the endoplasmic reticulum (ER) or mitochondria and are critical in regulating the heat-shock response (Craig *et al*., 1995). The Ssbs are encoded by two genes, *SSB1* and *SSB2*, which differ by only four amino acids and are referred to collectively herein as the Ssbs (Boorstein *et al*., 1994). Two phenotypes of strains lacking both Ssbs are: (i) poor growth at all temperatures, but particularly at low temperatures; and (ii) hypersensitivity to certain protein synthesis inhibitors, including aminoglycosides such as hygromycin B (Craig and Jacobsen, 1985; Nelson *et al*., 1992). Analysis of chimeric Ssa–Ssbs demonstrated that rescue of the cold-sensitive phenotype requires the 44 kDa ATPase domain from Ssb. Any two of the three Ssb domains are sufficient for rescue of the antibiotic sensitivity and ribosome association. For example, the

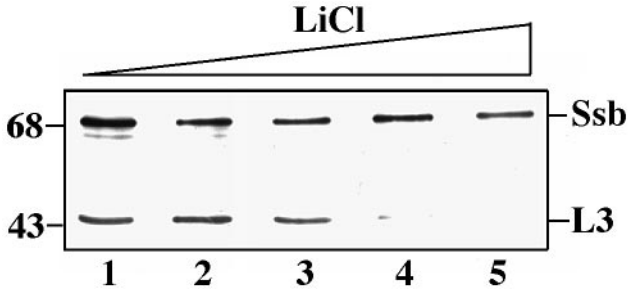

Fig. 1. Effect of increasing concentrations of LiCl on the Ssb–RNC interaction. Cells were pre-treated with cycloheximide and lysates were treated with increasing concentrations of LiCl and centrifuged through sucrose cushions containing the same concentrations of LiCl. Ribosome pellets were subjected to SDS–PAGE and immunoblot analysis using antibodies against Ssb or the ribosomal protein L3. Concentrations of LiCl were as follows: 0 M (1), 0.25 M (2), 0.50 M (3), 1.0 M (4), 1.5 M (5).

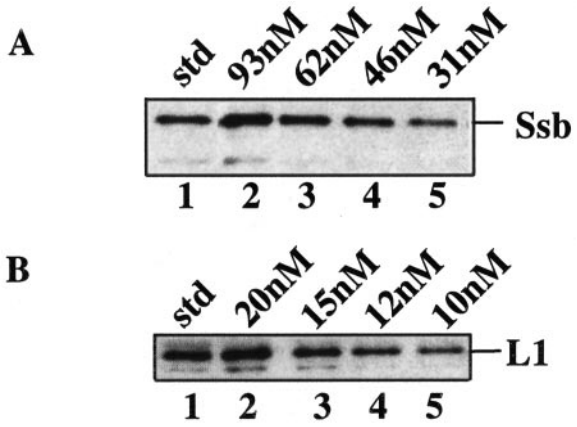

Fig. 2. Determination of the relative levels of Ssb and ribosomes in the cell. Immunoblot band intensities of pre-determined concentrations of Ssb or ribosomal protein L1 were compared with immunoblot band intensities from log-phase whole-cell lysates separated on the same SDS–PAGE gels to determine the molar ratios of Ssb to L1 in the cell. In lane 1 of both (**A**) and (**B**), 1.2 μg (std) of cell extract has ~47 nM of Ssb and 17 nM of L1.

expression of a chimera containing the Ssa1 ATPase domain and the 18 and 10 kDa domains of Ssb1 allows for polysome association as well as growth in the presence of 70 μg/ml hygromycin B, a concentration which inhibits the growth of cells lacking Ssb (James *et al*., 1997).

The sensitivity of *ssb1 ssb2* to protein synthesis inhibitors suggests a role for Ssb in translation. Indeed, Ssb is ribosome-associated (Nelson *et al*., 1992). Here we provide evidence that Ssb interacts both with the ribosome and directly with the nascent chain as it emerges from the ribosome. We propose that Ssb functions as a chaperone for polypeptide chains during translation and is able to do so by its stable interaction with ribosome–nascent chain complexes (RNCs).

Results

Interaction of Ssb with the ribosome–nascent chain complex is highly stable

To assess the stability of the interaction of Ssb with translating ribosomes, we treated RNCs with increasing concentrations of LiCl and determined the amount of Ssb which remained associated with the RNCs. Lysates were isolated from growing cells treated with cycloheximide, which inhibits the translocation step of translation, thereby freezing translating nascent chains on ribosomes and preventing ribosome run-off. RNCs were separated from soluble components by centrifugation through sucrose cushions and resuspended in buffer containing increasing concentrations of LiCl. Salt-treated RNCs were centrifuged through sucrose cushions of the same salt concentration. RNC pellets were resuspended, separated by SDS–PAGE and subjected to immunoblot analysis. As shown in Figure 1, Ssb is stably associated with RNCs even at high concentrations of LiCl. At the same high concentrations of LiCl, the ribosomal protein L3 dissociated from the RNCs, indicating that the interaction of Ssb with RNCs is at least as stable as that of a ribosomal protein. This effect is not exclusive for LiCl as Ssb was also stably associated with RNCs up to 1 M KCl (data not shown).

Ssb is more abundant than ribosomes

An understanding of the interaction of Ssb with ribosomes *in vivo* requires knowledge of the relative abundance of Ssb molecules and ribosomes. Therefore, the relative amount of Ssb and ribosomes was determined using antibodies specific to either Ssb or the ribosomal protein L1. Determination of the total Ssb and L1 in cell extracts was achieved by using serial dilutions of cell extract, purified Ssb and purified ribosomes containing L1 as described in Materials and methods (Figure 2A and B). We compared the signal from cell extracts of log phase cultures with that of determined concentrations of both Ssb or L1. We calculate that there are ~1–5 molecules of Ssb per ribosome in the cell. Thus, sufficient Ssb exists in the cell for each ribosome to be associated with at least one Ssb molecule.

Ssb associates with ribosomes bearing nascent chains

Hsp70s are known to bind short, largely hydrophobic amino acid segments likely to be exposed in unfolded or newly synthesized proteins. Therefore, it was proposed that Ssb may bind to nascent polypeptides emerging from the ribosome (Nelson *et al*., 1992). To assess whether the strong association between Ssb and the translating RNC is dependent on the presence of the nascent polypeptide chain, we examined whether incorporation of the amino-acyl tRNA analog puromycin, which causes the release of nascent polypeptide chains, would also cause release of Ssb from the ribosome. A concentration of puromycin was used which caused 50% release of nascent chains. Growing yeast cells were pulse-labeled with [^{35}S]cysteine/methionine and subsequently treated with cycloheximide. Ribosomes and polysomes were separated from soluble proteins by centrifugation; the ribosomal pellets were resuspended in the presence or absence of puromycin plus high salt, which is necessary for puromycin to work efficiently. Pellets were fractionated in sucrose gradients to separate ribosomal subunits, 80S monosomes and polysomes. Gradient fractions were immunoblotted with antibodies specific for Ssb and for L1. In the absence of puromycin, all of the ^{35}S-labeled nascent chains co-migrated with ribosomes or polysomes as would be expected of label incorporated into nascent chains (Figure

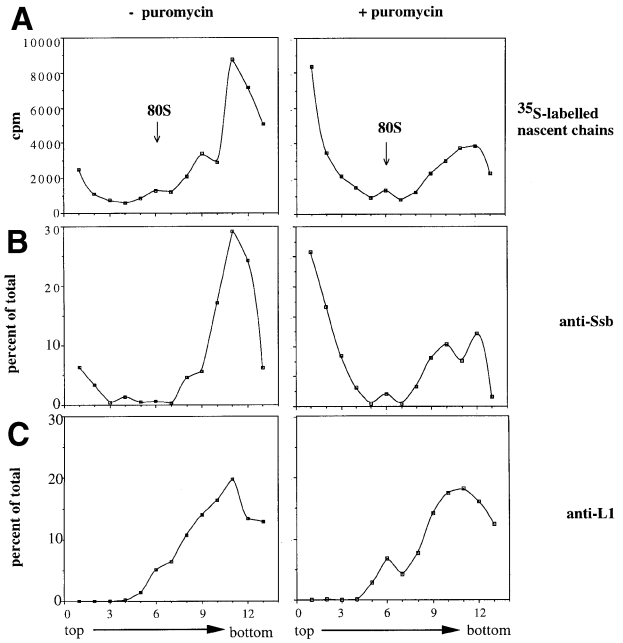

Fig. 3. Concomitant release of both ^{35}S-labeled nascent chains and Ssb from polysomes upon puromycin addition. Cells were pulse-labeled with [^{35}S]methionine prior to lysis and preparation of lysate. Polysomes were recovered and either treated with puromycin or untreated, as described in Materials and methods. Samples were separated on sucrose gradients and individual fractions analyzed for ^{35}S content (**A**). The relative levels of Ssb (**B**) and L1 (**C**) in each fraction were determined. The position of the 80S ribosome in the gradient is indicated by an arrow.

3A). In addition, Ssb co-migrated with ribosomes and polysomes (Figure 3B). Upon addition of puromycin, 50% of ^{35}S-labeled nascent chains were released from translating ribosomes (Figure 3A). This release was mirrored by the release of 50% of the Ssb from the puromycin-treated ribosomes (Figure 3B), while L1 remained in the 80S monosome- and polysome-containing fractions (Figure 3C).

The proportional release of nascent chain and Ssb from puromycin-treated ribosomes suggests that release of the nascent chains causes the release of Ssb in the presence of high salt. This idea is supported by the fact that treatment with a 10-fold higher puromycin concentration (5 mM) for 15 min in the presence of high salt resulted in the release of 98% of the ^{35}S-labeled chains from ribosomes. After this treatment, no detectable Ssb remained in the ribosome fractions (data not shown).

Ssb interacts with ribosome-bound nascent chains

The concomitant release of nascent chain and Ssb upon addition of puromycin in the presence of high salt suggests that Ssb binds directly to the nascent chain; therefore, we tested whether Ssb could be cross-linked to the nascent chain using a photoactivatable cross-linker incorporated into the nascent chain. Based on a protocol previously used to test for nascent chain interactions in wheat germ and rabbit reticulocyte systems (Kurzchalia *et al*., 1986; Wiedmann *et al*., 1987, 1994; Müsch *et al*., 1992; Hendrick *et al*., 1993; Valent *et al*., 1995; Wang *et al*., 1995; Hesterkamp *et al*., 1996), truncated mRNAs were translated *in vitro* in a yeast translation system to generate RNCs in which the translated nascent chains were trapped on the ribosome. *In vitro* translation was performed in the presence of [^{35}S]methionine and lysyl-tRNAs charged with lysines bearing the photoactivatable cross-linker TDBA [4-(3-trifluoromethyl-diazirono)benzoic acid]. Labeled nascent chains bearing this photoactivatable probe can form covalent attachments with interacting proteins. Cross-linked products can be identified by immunoprecipitation using antibodies to proteins of interest.

The N-terminal 72 amino acids of chloramphenicol acetyl transferase (CAT) were synthesized and the translation reactions were subjected to centrifugation to separate pelletable RNCs from non-ribosome-associated proteins. Cross-linked products were visualized by SDS–PAGE and fluorography. Figure 4A shows the RNCs of the samples not exposed to light (lane 1) and the samples irradiated to activate the cross-linker (lane 2). A clear band was observed at M_r ~81 kDa (Figure 4A, lane 2), corresponding to the predicted molecular weight for Ssb (70 kDa) cross-linked to the CAT nascent chain (~11 kDa). The 81 kDa band was present only when the *in vitro* translation reaction was irradiated, thus activating the cross-linker (Figure 4A, lane 2 versus lane 1). A product of the same apparent size was precipitated by antibodies against Ssb (Figure 4A, lane 5) but not by pre-immune sera (con) or anti-Ssa antibodies (Figure 4A, lanes 3 and 4). Cross-linking of Ssb to two other substrates, pre-prolactin (pPL-M) and pCoxIV was also examined. Anti-Ssb antibodies were able to immunoprecipitate labeled 86 amino acid pPL-M and labeled 96 amino acid CoxIV nascent chains cross-linked to Ssb upon irradiation (Figure 4B, lane 5; and 4C, lane 5, respectively). Together, these results demonstrate that Ssb can be cross-linked to a variety of nascent polypeptide chains.

To determine if the Ssb association with nascent chain depends on the nascent chain being ribosome associated, the ability of Ssb to cross-link to released nascent chains was investigated. Puromycin was added to an *in vitro* translation reaction prior to activating the cross-linker, thereby releasing ~70% of the ^{35}S-labeled polypeptides which were then found in the supernatant after centrifugation (Figure 4D). Both supernatants and pellets were immunoprecipitated and no Ssb–nascent chain cross-link product was detected. A band does migrate to the position expected for a Ssb–Cat cross-link product in the supernatant sample (Figure 4D, lane 2), such a band is often seen in the absence of cross-linking (Figure 4C, lane 1). However, this band appears to be a co-migrating band lacking Ssb, as anti-Ssb antibodies were unable to immunoprecipitate this labeled nascent chain. Thus, Ssb does not form a stable interaction with nascent chains removed from the context of the ribosome.

Ssb interaction with ribosome–nascent chain complexes is not affected by ATP

Analyses of unfolded protein–Hsp70s interactions have shown that such interactions are transient in the presence of ATP. ATP was added to the translation at 1.2 mM, a concentration expected to destabilize Hsp70–substrate interactions. However, as discussed above, Ssb can be cross-linked to the nascent chain under such conditions. To ensure that ATP was unable to disrupt the Ssb–RNC such that cross-linking could not occur, the concentration of ATP was raised to 10 mM before cross-linking. Also,

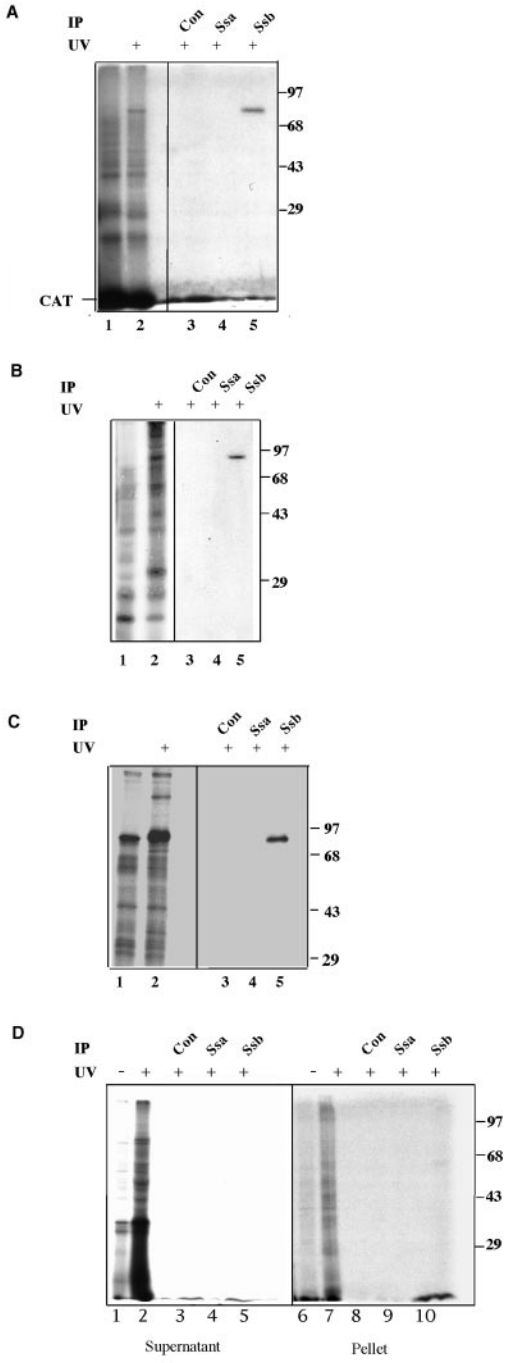

Fig. 4. Photo cross-linking of Ssb to ribosome-associated nascent chains. Truncated mRNAs for 72 amino acid CAT (**A**), 86 amino acid pPL-M (**B**) and 96 amino acid pCOXIV (**C**) were translated in yeast *in vitro* in the presence of TBDA-lysyl-tRNA and [^{35}S]methionine. The resulting RNCs were isolated by centrifugation. Equivalent portions of untreated or irradiated samples were either TCA precipitated or immunoprecipitated using antibodies directed against Ssa, Ssb or pre-immune serum (Con). For puromycin release (**D**), *in vitro* translation reactions were treated with puromycin prior to irradiation. Samples from RNC pellets (lanes 6–10) or non-ribosome-bound fractions (supernatants, lane 1–5) were analyzed. For (A–D), both TCA-precipitated and immunoprecipitated samples were analyzed by SDS–PAGE. Samples from the immunoprecipitations are four times the amount compared with the corresponding TCA samples on the same gel and exposed to film approximately twice as long. The heterogeneity of bands seen in the RNC pellet lanes is due to translation of endogenous mRNAs not completely eliminated by pre-treatment of the lysates with RNase.

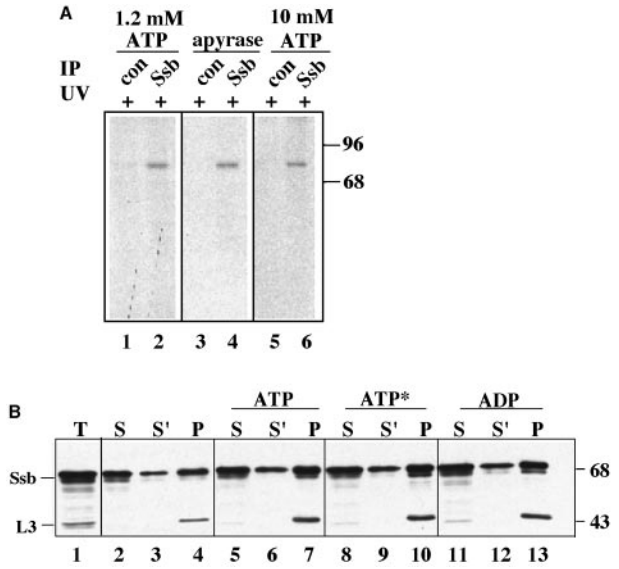

Fig. 5. Effect of increases in ATP levels on the Ssb–RNC interaction. (**A**) Photo cross-linking was performed and products analyzed as described in Figure 4, except that *in vitro* translation reactions were treated with excess ATP or apyrase prior to irradiation. RNC pellets were analyzed by immunoprecipitations with antibodies for Ssb or pre-immune serum (con). (**B**) Cells were pre-treated with cycloheximide and lysates were subsequently treated for 15 min at 30°C with 1 mM ATP (lanes 5–7), 1 mM ATP plus a regenerating system (lanes 8–10, ATP*) or 1 mM ADP (lanes 11–13). Treated or untreated lysates were centrifuged through sucrose cushions. One total lysate is shown in lane 1 (T). Ribosome pellets (P) and the supernatant fractions (S), as well as the sucrose cushion (S') were analyzed by immunoblotting against Ssb or L3. Ssb is detected as a doublet in these samples due to protein degradation which is occasionally observed in these analyses.

apyrase, which can degrade endogenous ATP, was added to a sample prior to cross-linking. Neither increased levels of ATP nor addition of apyrase, which lowers ATP levels, altered the ability of Ssb to be cross-linked to the nascent polypeptide chain (Figure 5A).

Because cross-linking may not distinguish between transient and stable interactions, the stability of the Ssb–RNC interaction in lysates prepared from growing cells was also tested. Yeast lysates were treated with 1 mM ATP, 1 mM ATP plus a regenerating system, or 1 mM ADP. RNCs were then isolated by centrifugation and analyzed by immunoblotting for the presence of Ssb and L3. Surprisingly, addition of 1 mM ATP did not disrupt the association of Ssb with RNCs (Figure 5B), suggesting that the Ssb chaperone, in the ATP-bound form, can remain associated with the RNCs.

Ssb interacts with vacant ribosomes in a salt-sensitive manner

The stability of the Ssb–RNC interaction in the presence of increased ATP suggests that Ssb may bind directly to the ribosome in addition to its interaction with the nascent chain. We predicted that this Ssb–ribosome interaction would be salt-sensitive as suggested by the concomitant release of Ssb from ribosomes treated with puromycin in the presence of high salt. Therefore, we investigated the possible interaction of Ssb with vacant ribosomes (ribosomes lacking nascent chains). Vacant ribosomes were isolated from the yeast strain *prt1-1*, a temperature-sensitive mutant which is unable to initiate translation

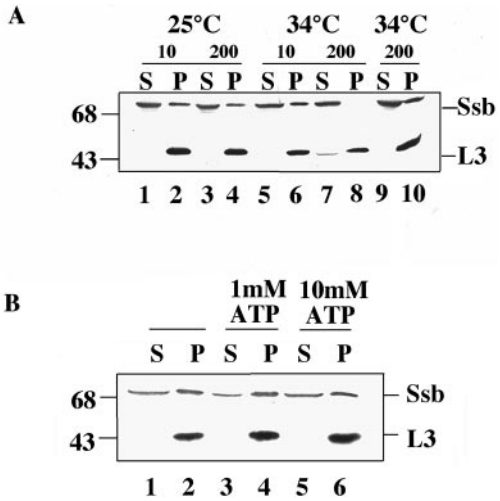

Fig. 6. Analysis of the interaction of Ssb with vacant ribosomes. (**A**) Cultures of *prt1-1* were grown at 25°C and then incubated at 25°C (lanes 1–4) or 34°C (lanes 5–8) for 20 min. Cultures of wild-type cells were grown at 25°C and then incubated at 34°C for 20 min (lanes 9–10). Cells were then treated with cycloheximide and lysates prepared as described. Lysates were centrifuged through sucrose cushions containing 10 (lanes 1–2 and 5–6) or 200 mM KCl (lanes 3–4 and 7–10). (**B**) Cultures of wild-type strains were grown at 30°C. Lysates were prepared as described and treated with no additional ATP (lanes 1–2), 1 mM ATP (lanes 3–4) or 10 mM ATP (lanes 5–6). Ribosome pellets (P) or supernatant fractions (S) were analyzed by immunoblotting against Ssb or the ribosomal protein L3.

after temperature shift (Hartwell and McLaughlin, 1968). *prt1-1* strains were grown at 25°C and shifted to 34°C for 20 min to allow the block of initiation and run-off of those ribosomes which were already translating. The *prt1-1* block in translation is expected to cause the polysomes to collapse into the monosome peak; this collapse was confirmed by centrifugation of lysates through sucrose gradients and monitoring of gradients at A_{260} (data not shown). Lysates were prepared from these cells in the presence of cycloheximide, and the soluble proteins were separated from ribosomes by centrifugation through low or high salt sucrose cushions. As shown in Figure 6A, Ssb associated with the ribosome pellet at both 10 and 200 mM KCl when *prt1-1* was grown at 25°C (Figure 6A, lanes 1–4). However, when cells were shifted to 34°C, Ssb was no longer associated with the ribosome pellet at 200 mM KCl (Figure 6, lanes 7 and 8). This effect was not due to the temperature difference as lysates from wild-type cells shocked at 34°C for 20 min reproducibly showed that Ssb remained associated with ribosome pellets at 200 mM KCl (Figure 6A, lanes 9 and 10). A small amount of L3 appears in the supernatant in lane 7. Varying levels of L3 consistently appeared in the supernatant from *prt1-1* 34°C lysates centrifuged through cushions containing 200 mM KCl; however, we never detected Ssb in these pellets. We think the appearance of L3 in the supernatant reflects a difference in non-translating and translating ribosomes, which in turn affects the stability of L3. These data suggest that Ssb is associated with vacant ribosomes but that this interaction is salt-sensitive in contrast to the salt-resistant interaction of Ssb with translating RNCs.

Two possible explanations for the change in the stability of the Ssb interaction with translating RNCs versus the Ssb interaction with non-translating (vacant) ribosomes are: (i) conformational differences between translating and non-translating ribosomes affect Ssb interaction; and (ii) binding of Ssb to the nascent chain affects Ssb interaction with RNCs. To attempt to differentiate between these possibilities, we examined the salt sensitivity of Ssb with RNCs in the presence of increased ATP. As shown in Figure 6, the Ssb–RNC interaction is resistant to 200 mM KCl, even in the presence of 10 mM ATP. This ATP-independent interaction suggests that although Ssb interacts with the nascent chain, the stability of the Ssb interaction with the ribosome is dependent on the conformation of the ribosome and not its interaction with the nascent chain.

Discussion

Hsp70s may serve as molecular chaperones for newly synthesized proteins by facilitating their proper folding. Based on data presented here, we propose that Ssb is a chaperone on the translating ribosome which interacts with nascent chains as they emerge from the ribosome. If Ssb functions as a chaperone for most translating proteins, the levels of Ssb in the cell would need to be comparable with that of ribosomes. Our results indicate that there are 3 ± 2 molecules of Ssb per ribosome and that Ssb constitutes 1–2% of the total cellular protein, nearly the level of the translation elongation factor, EF-1α, which is estimated at 5% of cell protein (Thiele *et al*., 1985). These data indicate that there is sufficient Ssb in the cell to function as a general chaperone in translation. As a chaperone for newly synthesized proteins, we would predict a direct association between nascent chains and Ssb. Interaction of Ssb with translating nascent chains is supported by the cross-linking of Ssb to nascent chains bearing a photoactivatable cross-linker. This interaction may occur primarily in the context of the ribosome as we did not detect an interaction of Ssb with nascent chains released from the ribosome upon addition of puromycin.

We propose a model in which Ssb associates with both ribosomes and nascent chains during cycles of translation. Accordingly, Ssb interacts in a salt-sensitive manner with the vacant ribosome prior to the initiation of translation; once translation begins, the conformation of the ribosome is altered and Ssb associates in a salt-resistant manner. This salt-resistant interaction indicates that Ssb is one of the core components of the translating ribosome. This stable association of Ssb and the translating ribosome may allow Ssb to interact with the nascent chain as it emerges from the exit channel. Upon completion of translation and the release of nascent chain, interaction with the ribosome is again altered, returning the Ssb association to the salt-sensitive state. Ssb may remain associated with the non-translating ribosome or ribosomal subunit(s) ready for another round of translation. Alternatively, Ssb may be released from the ribosome and be recycled. This model is supported by data which demonstrate that the association of Ssb with vacant ribosomes isolated from *prt1-1* strains at 34°C is sensitive to 200 mM KCl, while the interaction of Ssb with RNCs is extremely stable as shown by the inability to dissociate Ssb from the RNCs even at 1.0 M LiCl, a concentration which has been used to categorize core ribosomal proteins (El-Baradi *et al*., 1984). This

difference in salt sensitivity seems to be due to the conformational differences between translating and non-translating ribosomes and not to Ssb's interaction with the nascent chain, since Ssb is not released from RNCs at 200 mM KCl even in the presence of increased ATP which is expected to disrupt an Hsp70–substrate interaction. These results are reminiscent of the work on two previously characterized nascent chain-binding proteins: signal recognition particle (SRP) and trigger factor (TF). They have also been shown to associate with vacant ribosomes in a salt-sensitive manner and with translating ribosomes in a salt-resistant manner (Hesterkamp *et al*., 1996; Powers and Walter, 1996). Taken together, these data suggest that Ssb becomes a core component of the translational apparatus when it is actively synthesizing proteins.

The data presented here indicate that Ssb interacts with both the ribosome and the nascent chain; however, the nature of these interactions is not understood. The analysis of chimeric Ssa–Ssb proteins, in which the 44 kDa ATPase domain, the 18 kDa peptide-binding domain and the 10 kDa variable domains were exchanged, have provided some clues. James *et al.* (1997) demonstrated that the domains of Ssb necessary for rescue of the hygromycin B sensitivity of an *SSB* deletion strain are the same as those domains needed for association of Ssb with RNCs. Any two of the three domains of Ssb allowed RNC association; however, it did not matter which two of the three domains were present (James *et al*., 1997). This result demonstrates that the peptide-binding domain of Ssb is not absolutely required for RNC association as the chimera containing the ATPase domain of Ssb, the peptide-binding domain of Ssa and the 10 kDa domain of Ssb associates with translating ribosomes. Since the Ssa and Ssb peptide-binding domains are exchangeable, there is not an Ssb-specific interaction with the nascent chain that is necessary for RNC association. The data obtained using the chimeras are consistent with the results presented here, which suggest that Ssb is anchored to the ribosome in a manner independent of nascent chain binding and, therefore, unaffected by ATP. Such an association would allow Ssb to bind both to the ribosome and to the nascent chains with little specificity as they emerge from the ribosome.

Ssb's ability to bind ribosomes may target it efficiently to the exit site where nascent chains emerge from the ribosome (Bernabeu *et al*., 1983; Verschoor *et al*., 1996; Beckmann *et al*., 1997). In *S.cerevisiae*, other Hsp70s have been shown to be targeted to their site of action. For example, Kar2, an Hsp70 of the ER lumen, is associated with the ER membrane by virtue of its interaction with Sec63, a DnaJ-like protein (Sadler *et al*., 1989; Lyman and Schekman, 1995). Ssc1, a mitochondrial Hsp70, associates with the inner membrane of the mitochondria by virtue of its interaction with Tim44 (Blom *et al*., 1993; Schneider *et al*., 1994). Both of these interactions, however, are dependent on ATP, unlike the Ssb–ribosome interaction which appears to be ATP-independent. In this regard, the Ssb–ribosome interaction appears to differ from these previously described interactions of Hsp70s with proteins which target them to their site of action.

The results presented here place Ssb among a limited set of proteins shown to be associated with nascent chains as they emerge from ribosomes. SRP has been shown to interact with nascent chains bearing certain signal sequences, and functions to pause translation and allow for localization of the translating ribosome to the ER membrane (Kurzchalia *et al*., 1986; Ogg and Walter, 1995). Nascent chain-associated complex (NAC), which has also been found to associate with nascent chains emerging from the ribosome, is hypothesized to prevent SRP from binding to proteins lacking signal peptides (Wiedmann *et al*., 1994; Lauring *et al*., 1995; Wang *et al*., 1995). TF, a peptidyl *cis–trans* isomerase and DnaJ, a Hsp40 chaperone, both interact with nascent chains in *Escherichia coli* and are postulated to play roles in early protein folding events (Hendrick *et al*., 1993; Valent *et al*., 1995; Hesterkamp *et al*., 1996).

Other proteins of the Hsp70 class have also been shown to interact with nascent chains but each appears to differ from the Ssb–RNC interaction. Hsc70/Hsp70 from mammalian cells has been shown to interact with nascent chains (Beckmann *et al*., 1990; Frydman *et al*., 1994; Eggers *et al*., 1997), leading to a model in which Hsp70/Hsp40/TRiC interact with ribosome-bound nascent polypeptides in an ordered manner that allows both co-translational folding of protein domains as well as complete folding of the polypeptide once it is released from the ribosome. In *E.coli*, a role is indicated for DnaK, DnaJ, GrpE, GroEL and GroES in folding extended rhodanase while it is still bound as a peptidyl-tRNA to the ribosome. (Kudlicki *et al*., 1995). However, the Hsc70–nascent chain interaction is ATP dependent, unlike the Ssb–RNC interaction (Beckmann *et al*., 1990). Furthermore, there is no evidence of an interaction between mammalian or prokaryotic hsp70 and vacant ribosomes.

Interestingly, the cytosolic Hsp70/Hsc70 of mammalian cells is more closely related to another cytosolic Hsp70 in *S.cerevisiae*, Ssa. The Ssas are neither associated with ribosomes, nor interact with short nascent chains (<86 amino acids) as indicated by the inability to detect a cross-link product of Ssa with CAT, pPL or pCOXIV (Figure 4). However, the Ssas have been shown to interact with polypeptides and function in their translocation across organellar membranes (Chirico *et al*., 1988; Deshaies *et al*., 1988; Murakami *et al*., 1988). Perhaps the Ssas and Ssbs work together. We suggest that the Ssbs may serve a specialized function on the ribosome very early in the folding process, prior to association of the Ssas with the newly synthesized polypeptides. Ssas may predominantly function later in folding or translocation pathways. Ssb may function on the ribosome in a variety of ways to assist in the proper folding of ribosome-bound nascent chains: maintaining nascent chains in an unfolded, yet folding-competent state; preventing the nascent chain from slipping backwards into the ribosome; or facilitating movement of the chain away from the ribosome. Future experiments will be aimed at determining the means by which Ssb can function as a chaperone for the nascent chains as they emerge from the ribosome.

Materials and methods

Yeast strains

The yeast strains used in this study were DS10: *MAT**a**, lys1, lys2, leu2-3,112, ura3-52, his3-11,15, trp1-Δ1*; TZ185: *MAT**a**, trp1-1, ura3-1, leu2-3,112, his3-11,15, ade2-1, can1-100, met2-Δ1, lys2-Δ2*. (T.Ziegelhoffer

and P.James, unpublished); prt1-1: *MAT**a**, prt1-1, ura3-52, leu2-3, 112, ade1* (Hartwell and McLaughlin, 1968). Unless otherwise indicated, yeast were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C.

Preparation and treatment of yeast extracts

Yeast lysates were prepared in CSB buffer as previously described (Nelson *et al*., 1992). For LiCl treatments, 500 µl (20 OD_{260} units) of lysates were centrifuged through 2 ml low salt sucrose cushions (500 mM sucrose in CSB lacking sorbitol) at 50 000 r.p.m. for 3 h at 4°C in a TLA100.3 rotor (Beckman, Fullerton, CA). Pellets were resuspended in 500 µl of CSB containing 0.1 mg/ml cycloheximide (Sigma Chemical Co., St Louis, MO), incubated for 30 min at 4°C in CSB containing the indicated levels of LiCl, followed by centrifugation through 2 ml sucrose cushions containing the appropriate concentrations of LiCl (see above). For analysis of vacant ribosomes, yeast strain *prt1-1* or a wild-type strain were grown at 25°C to an OD_{600} between 0.5 and 0.7. Cultures were then divided in half: one half was incubated at 34°C for 20 min and the other half remained at 25°C for 20 min. Lysates from these cells were prepared as described. Twenty OD units (A_{260}) were run on 10–47% continuous sucrose gradients in CSB lacking sorbitol (54 000 r.p.m., 90 min, 4°C, SW55Ti rotor). Gradients were monitored at A_{260} to detect monosomes and polysomes. Lysates (500 µl) were also centrifuged as described above on 2 ml sucrose cushions containing 10 or 200 mM KCl. For ATP or ADP treatments, ATP or ADP was added to cell lysates at the indicated concentration and incubated for 20 min at 30°C. For the ATP plus the regenerating system, ATP was added to cell lysate, plus 200 mM creatine phosphate and 70 U of creatine kinase. Samples were incubated for 20 min at 30°C and then centrifuged through 2 ml low salt sucrose cushions (see above). For all samples, pellets were resuspended in 100 µl of CSB. The supernatants and cushions were combined and acetone precipitated. Equivalent amounts of supernatant and pellet were mixed with SDS sample buffer and subjected to SDS–PAGE, transferred to nitrocellulose (Hybond-C, Amersham Corp., Arlington Heights, IL) and immunoblotted for Ssb, L1 or L3 using the ECL detection system (Amersham, Arlington Heights, IL).

Quantitation of protein by immunodetection

Whole-cell yeast lysates were prepared from log-phase cultures. Cell pellets were lysed by vortexing for 2 min with glass beads in SDS sample buffer [60 mM Tris–HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue] and boiling for 2 min. Proteins were separated by SDS–PAGE and immunoblotted as described above. Relative protein levels were determined by densitometric analysis of exposed film (BioMax-AR, Eastman Kodak Co., Rochester, NY). Equal loading was confirmed by analyzing each sample in duplicate.

The cellular content of Ssb was quantified using Ssb-specific serum while the total amount of ribosomes in the cell was determined using antibodies against L1. L1 was used as a quantitative marker since it is presumed to be in a 1:1 stoichiometry with the ribosome. Quantification was based on analysis of the cellular content of both L1 and Ssb in cell extracts from wild-type cells. Partially purified preparations of Ssb and ribosomes were used as standards. Ssb and L1 concentrations, in their respective preparations, were determined by assessing the total amount of protein and subtracting from this the percentage of other contaminating proteins as determined by densitometry. Quantification of the total amount of Ssb in the cell extract was achieved by comparing serial dilutions of both cell extracts and Ssb preparations using Ofoto (Light Source Computer Images, Inc.) and Scananalysis (Biosoft) software packages. Quantification of the cellular amount of L1 was achieved using the same procedure.

Pulse labeling of nascent polypeptide chains and puromycin release

Strain TZ185 was grown at 34°C in Met/Cys dropout medium to an OD_{600} of 0.75. The cells were pelleted by centrifugation (5000 r.p.m., 5 min, 4°C, SA600, Dupont, DE) and resuspended in 6 ml of the same medium. Nascent polypeptides were labeled by incubating cells in the presence of 0.63 mCi of Trans^{35}S-label (Dupont, DE) for 1 min. Labeling was terminated by the addition of cycloheximide to a final concentration of 0.1 mg/ml. The culture was chilled rapidly by the addition of an approximately equal volume of crushed ice. Lysates were prepared as described (see Preparation and treatment of yeast extracts). The resulting lysate was loaded on a discontinuous sucrose gradient prepared in CSB lacking sorbitol (1 ml of 2 M, 1 ml of 1 M, 2.2 ml of 0.5 M sucrose) and centrifuged at 45 000 r.p.m., 4°C for 16 h in an SW55 rotor. The polysome pellet was resuspended in 50 µl of dH_2O. For the experimental conditions tested, 20 µl of this polysome solution was added to 20 µl of 2× buffer lacking cycloheximide [final composition: 20 mM HEPES (pH 7.5), 5 mM MgCl, 800 mM KCl]. In the indicated samples, puromycin (Sigma, St. Louis, MO) was added to a final concentration of 0.6 mM. Samples were incubated on ice for 1 h before centrifugation through discontinuous sucrose gradients [1.2 ml each of 20, 30, 40 and 50% sucrose (w/v) in CSB] for 75 min at 54 000 r.p.m. in an SW55 rotor. Then 0.33 ml fractions were removed from the top of the gradient and transferred to 1.5 ml tubes. A portion of each fraction was analyzed by scintillation counting to determine relative levels of ^{35}S. Approximately 20 µg of ovalbumin was added to each fraction to facilitate quantitative precipitation with acetone (0.1 ml of dH_2O and 0.8 ml of acetone per fraction). Precipitates were solubilized in 8 M urea, followed by addition of an equal volume of 2× SDS sample buffer. Samples were subjected to SDS–PAGE and immunoblotted for Ssb and L1 as above.

In vitro transcription, translation and cross-linking

The 72 amino acid CAT, the 86 amino acid pre-prolactin (pPL-M and pPL) and the 96 amino acid pCOXIV templates for *in vitro* transcription are described elsewhere (Wiedmann *et al*., 1994). The positions of the lysine residues for each template are listed as follows: 76 amino acid CAT: 3, 4, 19, 46, 49, 50 and 54; 86 amino acid pPL-M: 4, 9, 72 and 78; 96 amino acid pCOXIV: 12, 28, 32, 52, 75, 85 and 92. For Figure 4B, pPL-M was used. This construct bears a mutated signal sequence and is not able to translocate across microsome membranes. Wild-type pPL was also tested and a cross-link to Ssb was detected (data not shown). *In vitro* transcription by SP6 polymerase (Promega Biotech, Madison, WI) was performed as previously described (Gilmore *et al*., 1991).

In vitro yeast translation was performed as previously described, with some modifications (Waters and Blobel, 1986; Müsch *et al*., 1992). Yeast extracts were incubated for 10 min at 26°C with 1/100 volume of >1500 U/ml S7 nuclease (Boehringer Mannheim, Mannheim, Germany) in 1 mM $CaCl_2$. The reaction was stopped by addition of EGTA to 2 mM. Each 25 µl translation reaction contained: 0.81 µl of a mix of every amino acid at 1 mM except methionine and lysine, 0.32 µl of 0.1 M ATP, 0.11 µl of 0.5 M dithiothreitol (DTT), 0.27 µl of 1 M HEPES–KOH, pH 7.5–7.7, 0.11 µl of 250× protease inhibitor mix (Erickson and Blobel, 1983), 0.32 µl of 0.2 M GTP, 0.43 µl of 0.6 M creatine phosphate, 0.32 µl of 8 mg/ml creatine kinase, 3.5 µl of water, 0.62 µl of 5 M KOAc, 0.55 µl of 0.1 M MgOAc, 0.54 µl of Rnase In (Boehringer Mannheim, Mannheim, Germany), 0.33 µl of TDBA-lysyl-tRNA (Gorlich *et al*., 1991) and [^{35}S]methionine at 15 mCi/ml (ICN, Costa Mesa, CA). This mixture was added to 11 µl of S7 nuclease-treated yeast extract. The reactions were started by addition of 1.5 µl of mRNA (15 mg). Reactions were incubated at 26°C for 20 min. The reactions were stopped by chilling on ice and addition of 1.3 µl of 20 mM cold methionine.

Cross-linking was performed as previously described (Gorlich *et al*., 1991). Briefly, reactions were irradiated for 5 min on a cooled steel block with light of wavelength >330 nm. Following irradiation, KOAc pH 8 was added to a final concentration of 0.5 M. RNCs were recovered by centrifugation (100 000 r.p.m., 1 h, 4°C, TLA 100.4 rotor, Beckman, Fullerton, CA). Supernatants were trichloroacetic acid (TCA) precipitated and resuspended in the original sample volume in SDS sample buffer. RNC pellets were resuspended in the same volume of Tris–HCl pH 8.8 and subsequently treated with RNase A (100 mg/ml) and SDS to 1%. Samples were boiled for 5 min. Irradiated and non-irradiated samples were immunoprecipitated or separated on SDS–PAGE gel and radioactive bands were visualized by fluorography (BioMax-MR, Eastman Kodak Co., Rochester, NY).

Immunoprecipitations

Immunoprecipitation with antibodies against the yeast proteins Ssa, Ssb or the pre-immune serum were performed as follows. Twenty five µl of supernatants and pellets from irradiated and non-irradiated samples (previously treated with 1% SDS and boiled for 5 min) were diluted to 250 µl in buffer A (150 mM NaCl, 50 mM HEPES–KOH, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS). Antisera were diluted 1:10 with buffer, and 50 µl of the appropriate antiserum were added to each diluted sample. Incubation was performed at 4°C overnight. Then 50 µl of 50% protein A–Sepharose (Sigma, St Louis, MO) was added and the mixture was rotated for 1 h at 4°C. The Sepharose beads were washed three times with buffer A, bound material was eluted by boiling for 3 min in SDS sample buffer, and half of the total sample was loaded.

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