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The Hsp70 Ssz1 modulates the function of the ribosome-associated J-protein Zuo1

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J-proteins are obligate partners of Hsp70s, forming a ubiquitous class of molecular chaperone machinery. The ribosome-associated Hsp70 of yeast Ssb binds nascent polypeptides as they exit the ribosome. Here we report that the ribosome-associated J-protein Zuo1 is the partner of Ssb. However, Zuo1 efficiently stimulates the ATPase activity of Ssb only when in complex with another Hsp70, Ssz1. Ssz1 binds ATP, but none of the 11 different amino acid substitutions in the ATP-binding cleft affected Ssz1 function *in vivo*, suggesting that neither nucleotide binding nor hydrolysis is required. We propose that Ssz1's predominant function in the cell is to facilitate Zuo1's ability to function as a J-protein partner of Ssb on the ribosome, serving as an example of an Hsp70 family member that has evolved to carry out functions distinct from that of a chaperone.

Hsp70s are ubiquitous molecular chaperones that function in a wide variety of cellular processes, such as protein folding, translocation and degradation[1,2]. In these diverse cellular roles, Hsp70s act by reversibly binding and releasing short hydrophobic stretches of amino acids in a nucleotide-dependent fashion. Although multigene families of Hsp70s have evolved such that most cellular compartments contain more than one Hsp70, structural features are shared among diverse Hsp70s. Both a highly conserved N-terminal 44-kDa ATPase domain and an adjacent, less conserved, 18-kDa domain, which contains a binding site for short hydrophobic stretches of amino acids, are critical for chaperone activity[3]. Hsp70's interaction with hydrophobic stretches of unfolded polypeptide substrates is regulated by cycles of ATP binding and hydrolysis. Conformational changes generated upon ATP hydrolysis stabilize Hsp70's interaction with its polypeptide substrate, whereas the exchange of ADP for ATP promotes their release.

Hsp70 chaperones do not function alone, but with J-protein (also known as Hsp40) partners. All J-proteins contain a conserved J-domain that is known to transiently interact with the ATPase domain of an Hsp70, stimulating ATP hydrolysis, thus facilitating the chaperone cycle[4]. A His-Pro-Asp (HPD) tripeptide is conserved in all known J-domains[5]. This tripeptide is critical for J-protein function, as alterations, such as a change of the histidine to glutamine, have been shown to disrupt function *in vivo* and *in vitro* in several J-proteins[6–9]. Like Hsp70 genes, J-protein genes have evolved into large multigene families, with each cellular compartment typically containing more than one type of J-protein. For example, the cytosol of the yeast *Saccharomyces cerevisiae* contains 10–12 J-proteins[10]. Defining the partnerships among these multiple J-proteins and Hsp70s is central to understanding molecular chaperone function in the cell.

In *S. cerevisiae*, the Ssb Hsp70s compose a specialized class of Hsp70s that are stoichiometrically associated with ribosomes. The Ssbs, comprising the functionally interchangeable Ssb1 and Ssb2, which differ by only 4 amino acids[11], can be crosslinked to very short nascent chains that extend only 10–15 amino acids beyond the exit tunnel of the ribosome[12–14]. Therefore, Ssbs are thought to play an important role in early folding events as polypeptides exit the ribosome, and may even directly affect the translation process itself[11,15–17].

The identity of the J-protein partner of Ssb is not established. However, the J-protein Zuo1 is considered a promising candidate, as it is also stoichiometrically associated with ribosomes[18]. In addition, strains lacking *SSB* or *ZUO1* have the same phenotypes, including slow growth, particularly at low temperatures, and hypersensitivity to cations, including aminoglycoside antibiotics and NaCl[11,18,19]. This simple scenario is confounded, however, by the fact that Zuo1 is found as a very stable heterodimer, called RAC (ribosome-associated complex), with another Hsp70, Ssz1 (ref. 20). Although Ssz1 is also stoichiometrically associated with ribosomes, this interaction is thought to be indirect as it is not associated with ribosomes in the absence of Zuo1 (ref. 20). Cells lacking Ssz1 have the same phenotype as Δ*ssb* or Δ*zuo1* cells. Combinations of any of these mutations have no additive effects, suggesting that Ssb, Zuo1 and Ssz1 function together in the same pathway[13,21]. Although Ssz1 is categorized as an Hsp70 based on its sequence similarity, the function of Ssz1 is not understood. Unlike Ssb and other Hsp70s[22,23], its entire putative peptide-binding domain can be deleted without affecting its *in vivo* function[13] and an interaction with nascent chains has not been reported.

We set out to test whether Zuo1 is the J-protein partner of Ssb and to better understand the function of Ssz1. We found that, indeed, Zuo1 can stimulate the ATPase activity of Ssb, but only when in complex with Ssz1. This stimulation is dependent on a functional J-domain in Zuo1,

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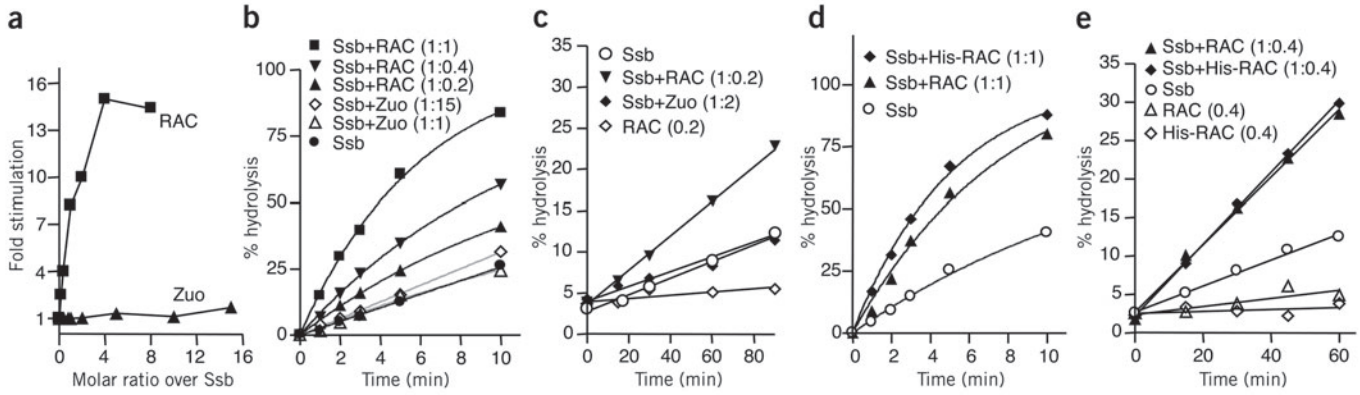

Figure 1 RAC, but not Zuo1, catalytically stimulates Ssb's ATPase activity. (**a**) Single-turnover ATPase assays were done. Preformed Ssb1–[α-^{32}P]ATP complex (~0.5 μM) was isolated and the rate of ATP hydrolysis measured over time at 25 °C in the absence or presence of varying concentrations of Zuo1 or RAC. Fold stimulation under each condition was calculated from the rate of hydrolysis using the curve fitting program, Prism 4.0 (GraphPad Software). (**b**) Single-turnover ATPase assays were carried out as in **a**. The amount of Zuo1 or RAC added is indicated as the molar ratio compared to Ssb. (**c**) Steady-state ATPase assay of Ssb1 (1 μM) in the presence of 50 μM ATP. The molar ratio of Zuo1 or RAC compared to Ssb1 is indicated in parentheses. Assays were carried out at 25 °C. (**d**) Single-turnover ATPase assays were carried out as in **a**. The amount of RAC or His-RAC added is indicated as the molar ratio compared with Ssb. (**e**) Steady-state ATPase assays were carried out as in **c**. The amount of RAC or His-RAC added is indicated as the molar ratio compared with Ssb.

thus we conclude that Zuo1 is the J-protein partner of Ssb. We propose that Ssz1's role is to facilitate Zuo1's ability to function as a J-protein partner of Ssb on the ribosome.

RESULTS

Stimulation of Ssb's ATPase activity by RAC

As a first step toward testing whether Zuo1 is the J-protein partner of Ssb, we asked whether Zuo1 can stimulate Ssb's ATPase activity. A complex between Ssb and radiolabeled ATP was isolated, and the rate of ADP formation in the presence and absence of Zuo1 determined. The rate of hydrolysis, 0.035 ± 0.001 min^{-1}, did not change in the presence of equimolar amounts of purified Zuo1 (**Fig. 1a**,**b**). Even when Zuo1 was in a 15-fold excess over Ssb, only very minimal stimulation was observed in this 'single-turnover' assay. Similarly, no stimulation over Ssb's basal rate was observed in a steady-state ATPase assay when excess Zuo1 was added (**Fig. 1c**, data not shown).

It is possible that Zuo1 fails to stimulate Ssb because it is not the J-protein partner of Ssb, or because Zuo1 is functional only in the presence of Ssz1, with which it forms the RAC heterodimer. To test the second possibility, we measured Ssb's ATPase activity in the presence of RAC. In a single-turnover assay, RAC stimulated Ssb's ATPase activity 8-fold when present at an equimolar concentration and 14-fold when in 4-fold excess (**Fig. 1a**). Stimulation was observed even when RAC was present at substoichiometric levels. For example, ~2.5-fold stimulation was observed at a ratio of Ssb/RAC of 1:0.2 in both steady-state and single-turnover assays (**Fig. 1b**,**c**).

Because of the difficulty of purifying large amounts of RAC by classical purification techniques, we also used a polyhistidine tag at Zuo1's N terminus to assist in purification. Stimulation of the ATPase activity of Ssb by tagged and untagged RAC were indistinguishable in both steady-state and single-turnover assays (**Fig. 1d**,**e**). In addition, the ability of the His-tagged Zuo1 to rescue the phenotypes of the Δ*zuo1* strain (data not shown) indicated that His-tagged Zuo1 forms a functional complex with Ssz1 and that the inability of Zuo1 alone to stimulate Ssb's ATPase activity was not due to the presence of the tag.

Requirement of Zuo's J-domain for RAC stimulation

Because RAC effectively stimulated Ssb's ATPase activity, whereas Zuo1 alone did not, we asked whether Zuo1's J-domain is important for RAC's ability to stimulate Ssb. As with other J-proteins, the J-domain of Zuo1 contains the conserved HPD signature motif. Cells expressing a mutant Zuo1 with histidine modified to glutamine in the HPD motif (Zuo1-H128Q) have growth defects similar to those of Δ*zuo1* cells[13,21]. Therefore, we purified RAC composed of Zuo1-H128Q and wild-type Ssz1 (H128Q-RAC). H128Q-RAC, like wild-type RAC, is a very stable complex, suggesting no marked changes in its conformation[21]. However, H128Q-RAC failed to stimulate Ssb's ATPase activity (**Fig. 2**), indicating that the ATPase stimulation we observed required Zuo1's J-domain.

Specificity of RAC's stimulation of Ssb

To examine the specificity of RAC's stimulation of Ssb's ATPase activity, we asked (i) whether RAC could stimulate another Hsp70 of the cytosol, Ssa1, and (ii) whether Ssb could be stimulated by two other J-proteins of the cytosol, Sis1 and Ydj1. RAC did not increase the ATPase activity of Ssa1 even when present at a 10-fold excess over Ssb (**Fig. 3a**), whereas,

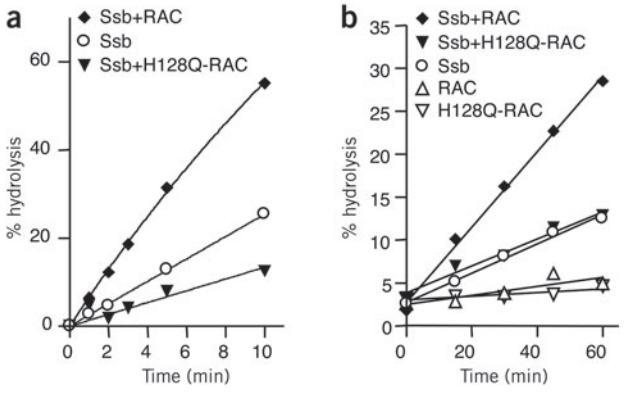

Figure 2 H128Q-RAC fails to stimulate the ATPase activity of Ssb. (**a**) Single-turnover ATPase activity of Ssb1 (~0.3 μM) was measured at 25 °C in the absence or presence of 0.24 μM RAC or H128Q-RAC. (**b**) Steady-state ATPase activity of Ssb1 (1 μM) with 50 μM ATP was measured at 25 °C in the absence or presence of 0.4 μM H128Q-RAC or RAC.

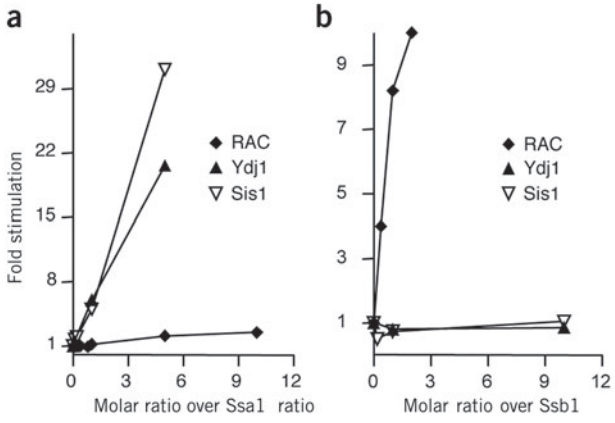

Figure 3 RAC does not stimulate Ssa1; Ssb1 is not stimulated by Ydj1 and Sis1. (**a**,**b**) Single-turnover ATPase activity of ~0.3 μM Ssa1 (**a**) or ~0.5 μM Ssb1 (**b**) was measured at 25 °C in the absence or presence of RAC, Ydj1 or Sis1. The concentration of the added J-protein (Ydj1, Sis1 or RAC) is indicated as the molar ratio of J-protein/Hsp70.

as expected from previous results[24], Ydj1 and Sisl, two well-established J-protein partners of Ssa1, stimulated the activity >15-fold. On the other hand, neither Ydj1 nor Sis1 stimulated Ssb's ATPase activity (**Fig. 3b**), even when in a ten-fold excess. These results suggest that the Ssb-RAC interaction is specific.

Suppression of *zuo1-H128Q* by mutations in *SSB*

The biochemical data described thus far suggest that Zuo1 is the J-protein partner of Ssb, because (i) when in complex with Ssz1, it specifically stimulates Ssb's ATPase activity, and (ii) Zuo1's J-domain is important for this interaction. To test this hypothesis *in vivo,* we asked whether mutations in *SSB* could be found that overcome the *in vivo* defect of the *zuo1-H128Q* mutation. Using a library of *SSB* genes containing mutations in the DNA segment encoding the ATPase domain, we selected for cells that could grow at 18 °C, thus suppressing the cold-sensitivity of the *zuo1-H128Q* strain (**Fig. 4a**,**b**). Seven mutations in *SSB1* that partially suppressed the cold-sensitive phenotype of *zuo1-H128Q* were isolated (**Fig. 4c**). All were found to partially suppress the cation-sensitivity of *zuo1-H128Q* as well (data not shown).

The suppressor mutations cause single-residue substitutions of six different residues. Alteration of Ile174 was isolated twice, to threonine and valine in suppressors 3 and 7, respectively (**Fig. 4c**). Among the six residues, three are hydrophobic (Ile174, Ile200 and Phe208), one is positively charged (Arg79), and two are polar uncharged (Gln119 and Asn176). The amino acids altered in the suppressors are all surface-exposed when placed on the modeled structure of the Ssb ATPase domain (**Fig. 4d**)[22]. Furthermore, they cluster in one area on Ssb's ATPase domain, suggesting that this surface of Ssb1 may be involved in the interaction with Zuo1's J-domain. Consistent with this idea, residues in the same region of the ATPase domain were previously found to be important for inter-

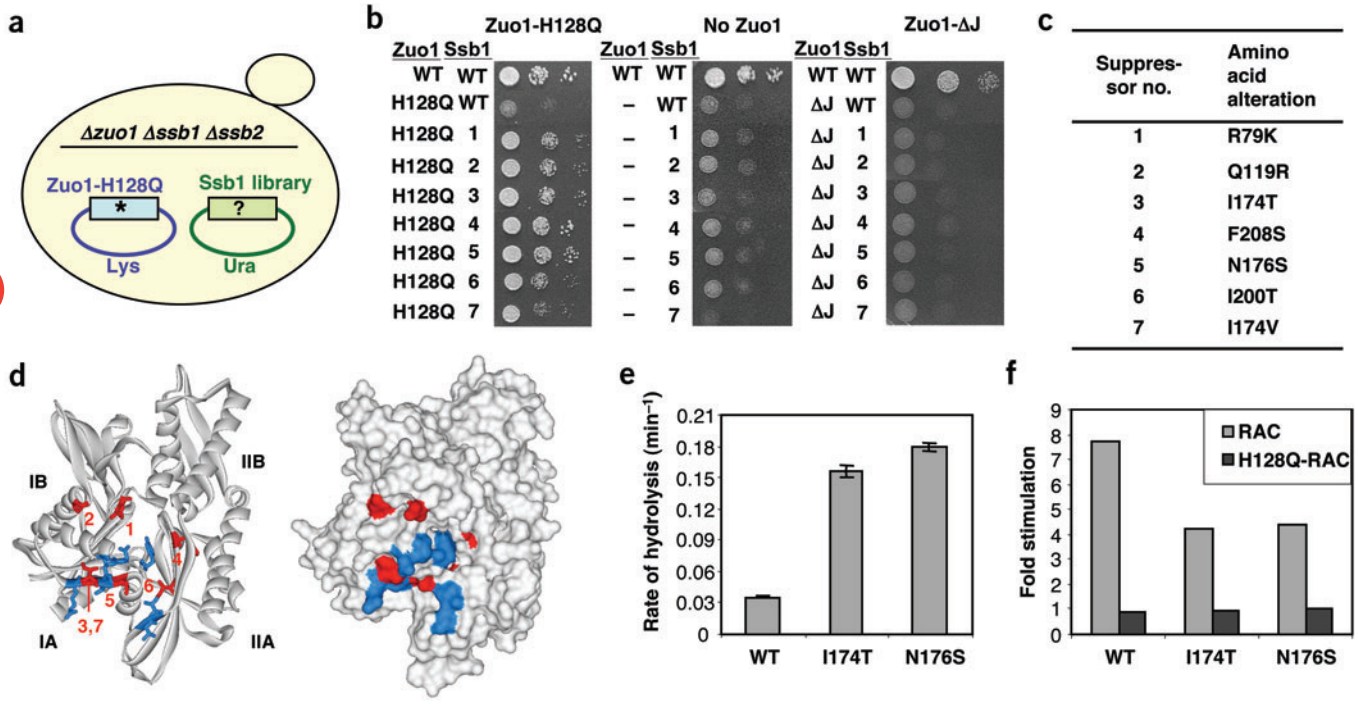

Figure 4 *SSB1* ATPase domain mutants rescue the phenotype of *zuo1-H128Q*. (**a**) Schematic of the genetic selection used in this study. Δ*ssb1*Δ*ssb2*Δ*zuo1* cells containing two centromeric plasmids, one carrying *zuo1-H128Q* and the other carrying a *SSB1*-ATPase domain mutant were grown on selective media at 18 °C. Fast growers were selected. (**b**) Serial dilutions of PH16 (Δ*ssb1*Δ*ssb2*Δ*zuo1*) cells expressing Ssb suppressors in combination with Zuo1-H128Q (left), an empty vector (middle), or Zuo1-ΔJ (right) were grown on selective media at 18 °C for 5 d. (**c**) The amino acid alterations carried by the isolated *SSB1* suppressor genes are listed. WT, wild type. (**d**) Ribbon diagram (left) and solvent-accessible surface (right) of modeled Ssb1 ATPase domain with the residues altered in the suppressors highlighted in red. These residues are labeled with the suppressor numbers according to **c**. The analogous residues shown to be important for DnaK-DnaJ interaction are blue[25,26]. The structures were visualized and generated using DS ViewerPro 5.0 (Accelrys). (**e**) Intrinsic ATPase activities of 0.3 μM wild-type Ssb1 or suppressors (I174T and N176S) as determined by single-turnover ATPase assays at 25 °C. (**f**) The ability of 0.3 μM RAC or H128Q-RAC to stimulate the ATPase activity of 0.3 μM Ssb1 or suppressors, I174T and N176S, as determined by single-turnover ATPase assays at 25 °C.

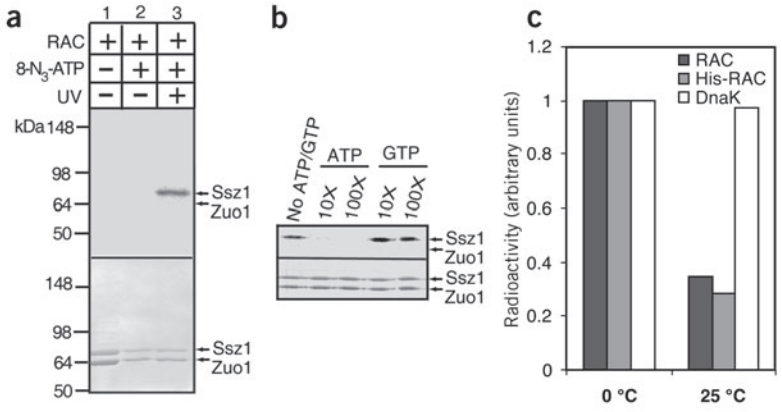

Figure 5 Ssz1 binds ATP, but the interaction is unstable. (**a**) UV photocrosslinking of radiolabeled 8-N_3-ATP to Ssz1. [α-^{32}P]8-N_3-ATP was incubated with RAC. After separation from unbound 8-N_3-ATP, RAC was subjected to UV irradiation (lane 3) or not (lane 2). Samples were subjected to SDS-PAGE, stained with Coomassie blue (bottom) and exposed to phosphorimager screen (top). An equivalent amount of RAC was not subjected to the chromatography step, served as a control for protein recovery (lane 1). (**b**) Competition by ATP. Experiment was conducted as in **a**, except a 10- or 100-fold excess of ATP or GTP compared with radiolabeled 8-N_3-ATP was added to the reaction. The autoradiograph (top) and Coomassie blue staining (bottom) of the same gel were aligned for comparison. (**c**) Stability of ATP binding. RAC, His-RAC and DnaK were incubated with [α-^{32}P]8-N_3-ATP and separated from free nucleotide as described in **a**. Radioactivity of crosslinked adducts kept on ice for 30 s (0 °C) or incubated at 25 °C for 5 min (25 °C) was quantified and normalized to protein recovered. Complex present at 0 °C was set at 1.

Ssz1 binds ATP

The biochemical and genetic results presented above indicate that Zuo1 is the J-protein partner of Ssb only when bound to Ssz1, but they do not address how Ssz1 might function. Our previous work demonstrated that the N-terminal 407 amino acids of Ssz1, the putative ATPase domain, are sufficient for rescue of both the slow-growth phenotype and cation-sensitivity of Δ*ssz1* cells[13]. Therefore, to address Ssz1's function in stimulating Ssb's ATPase activity, we first asked whether Ssz1 could bind ATP.

RAC was incubated with the radiolabeled photocrosslinkable nucleotide analog, 8-N_3-ATP, and the RAC–ATP complex isolated by gel filtration. The complex was subjected to UV irradiation to induce formation of a covalent bond with nucleotide, and components of the mixture were separated by electrophoresis. Radioactivity was detected at the position of Ssz1 but not Zuo1 (**Fig. 5a**). No radioactivity was detected in the absence of UV irradiation, indicating that crosslinking was required for a stable interaction. To test the specificity of binding of 8-N_3-ATP to Ssz1, we added 10- or 100-fold excess of ATP or GTP during the incubation of the analog with RAC. In the presence of ATP, crosslinking of 8-N_3-ATP was decreased, whereas the addition of GTP had no effect on crosslinking (**Fig. 5b**). Thus Ssz1 binds ATP.

To test the stability of the nucleotide-Ssz1 interaction, we compared complex that had been maintained on ice after isolation, and complex that was incubated at 25 °C for 5 min, before crosslinking. DnaK was used as a control. Consistent with DnaK's reportedly stable interaction with nucleotide[27], only a 3% reduction in complex was seen upon incubation at 25 °C (**Fig. 5c**). However, in the case of RAC, such incubation resulted in a 65% reduction in the amount of RAC–ATP complex detected, whether untagged or His-tagged RAC was tested (**Fig. 5c**). Thus, the interaction between Ssz1 and ATP is unstable.

Because RAC binds ATP, we also tested whether our RAC preparations had ATPase activity. We measured a low ATPase activity of the preparation of untagged RAC purified from ribosomal salt wash in both steady-state (**Fig. 1c,e**) and in single-turnover ATPase assays (data not shown). However, the His-tagged RAC, which stimulated Ssb1 as well as untagged

action between the *Escherichia coli* Hsp70, DnaK and the J-protein DnaJ (**Fig. 4d**)[25,26]. Collectively, these residues form a surface having a groove near Ile200 and Phe208. This groove is very close to the nucleotide-binding site. Thus it is easy to envision binding of Zuo1's J-domain stimulating ATP hydrolysis.

To understand the functional basis of the suppression, we purified two suppressor mutant proteins, Ssb1-I174T and Ssb1-N176S. The basal ATPase activity of both Ssb1-I174T and Ssb1-N176S were approximately five-fold higher than that of wild-type Ssb1 (**Fig. 4e**). Like wild-type, neither was stimulated by mutant RAC, but both were stimulated by wild-type RAC (**Fig. 4f**). These results suggest that the increased basal ATPase activity of the mutant proteins is important for their ability to suppress the *in vivo* defects caused by the altered J domain of Zuo1.

Because the basal ATPase activity of both the *SSB1* mutant proteins was increased, but not further enhanced by H128Q-RAC (Zuo1-H128Q–Ssz1, we tested whether the *SSB1* genes having the suppressor mutations were able to function in the absence of Zuo1. None rescued the cold- or cation-sensitivity of a Δ*ssb* Δ*zuo1* strain (**Fig. 4b**, middle panel; data not shown), indicating that the need for Zuo1 was not bypassed. To determine whether suppression specifically required the J-domain, a variant lacking the J-domain, Zuo1-ΔJ, which is expressed at normal levels but nonfunctional[18], was used. Δ*ssb* Δ*zuo1* cells expressing the mutant Ssb1 proteins were transformed with a plasmid expressing Zuo1-ΔJ. No improvement in growth was observed (**Fig. 4b**, data not shown). These results indicate that the suppressor mutations allow Ssb to function with a defective J-domain, but do not bypass the requirement for a J-domain. Perhaps the interaction of the Zuo1 J-domain with Ssb1 has a function in addition to stimulation of ATPase activity.

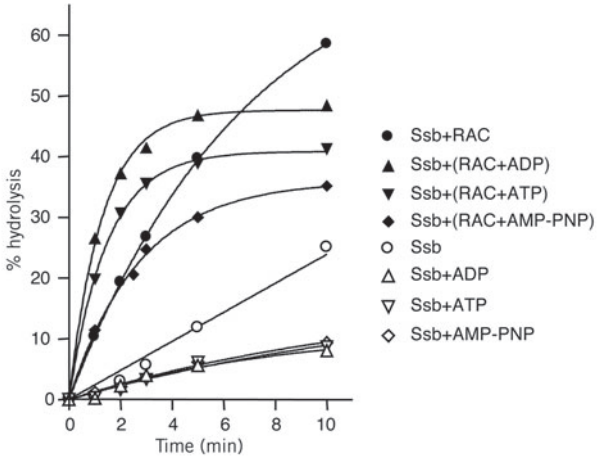

Figure 6 Effect of Ssz1's nucleotide-binding state on RAC's stimulation of Ssb. RAC (0.3 μM) preincubated with 1 mM ADP, AMP-PNP, or ATP at 25 °C before mixing with an equal amount of preformed Ssb1-[α-^{32}P]ATP complex (~0.3 μM). Single-turnover ATPase activity of Ssb1 in the absence or presence of RAC preincubated with different nucleotides was then measured at 25 °C.

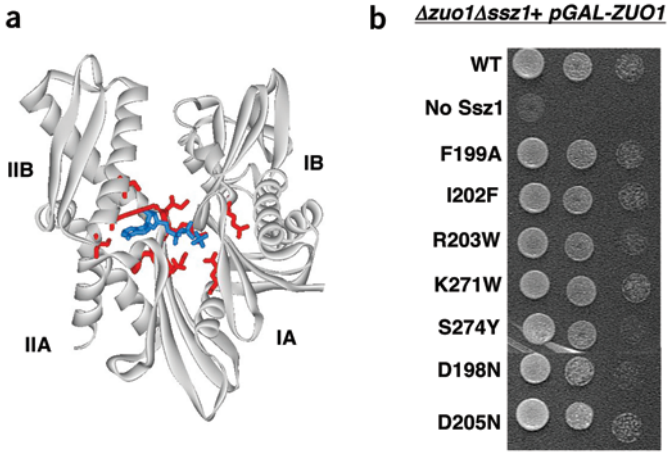

Figure 7 Alteration of ATP-binding cleft of Ssz1 has no obvious effect on growth. (**a**) Ribbon diagram of the modeled ATPase domain structure of Ssz1. ADP, P_i (blue) and Mg^{2+} (green) are placed in the nucleotide-binding cleft of Ssz1 by superimposition of the modeled Ssz1 structure and the crystal structure of Hsc70's ATPase domain with bound ADP and P_i[38]. Substituted residues (**Table 1**) are red. Coordinates of Ssz1 model were generated by SWISSMODE[39] based on a multiple sequence alignment of Hsp70s across species. The structure was visualized and generated using the program DS ViewerPro 5.0 (Accelrys). (**b**) Serial dilution of HE12 (Δ*zuo1* Δ*ssz1*) cells expressing a low level of Zuo1 and wild-type Ssz1 or Ssz1 mutant proteins with alterations in their ATP-binding cleft were grown on selective medium in the presence of 500 μg ml^{-1} paromomycin at 30 °C for 2 d.

RAC (**Fig. 1d**,**e**), had no detectable ATPase activity in either assay, even though it bound ATP as well as untagged RAC (data not shown, **Fig. 5c**). It is possible that the presence of the His-tag on Zuo1 in His-RAC is sufficient to inactivate Ssz1's ATPase activity. Alternatively, the low ATPase activity could be due to a contaminant in the untagged RAC preparation. Regardless, both RAC and His-tagged RAC stimulated the ATPase activity of Ssb with similar efficiencies (**Fig. 1d**,**e**), indicating that Ssz1's ATPase activity is not critical for RAC's ability to stimulate Ssb's ATPase activity.

Minimal effect of nucleotide binding by Ssz1

As outlined above, RAC lacking bound nucleotide stimulates the ATPase activity of Ssb. Because Ssz1 binds nucleotide, we asked whether binding had either a substantial positive or negative effect on RAC's ability to stimulate Ssb's ATPase activity. The instability of the interaction between Ssz1 and nucleotide presented technical challenges to this analysis. We decided to use the single-turnover ATPase assay described above. RAC was preincubated with an excess of nonradioactive ADP, ATP or AMP-PNP, a nonhydrolyzable ATP analog. The mixture was then added to a preformed Ssb1–[^{32}P]ATP complex before ATP hydrolysis was measured.

As a control, nonradioactive nucleotide was added to the Ssb1–[^{32}P]ATP complex in the absence of RAC. Hydrolysis of radioactive ATP was reduced in the presence of unlabeled nucleotides, reaching a plateau of ~10% (**Fig. 6**, data not shown). This inhibition is presumably due to the release of [^{32}P]ATP from Ssb1, which in the absence of an excess of unlabeled nucleotide rebinds to Ssb1 and is ultimately hydrolyzed.

RAC preincubated with 1 mM ADP, ATP or AMP-PNP and added at a molar ratio of 1:1 with Ssb resulted in stimulation of ATPase activity. Consistently, stimulation was greatest in the presence of ADP, but at most two-fold higher than in the presence of AMP-PNP. The effect of ATP binding was less than that of ADP, but more than for AMP-PNP. This difference could be due to hydrolysis of ATP during the incubation, or because AMP-PNP does not precisely mimic the effects of ATP on the conformation of Ssz1. In all cases hydrolysis plateaued at the 50% level or below, owing to the presence of the added nonradioactive nucleotide. Therefore, it was not possible to quantitatively determine the rates of hydrolysis in the presence and absence of nucleotide. Regardless, these results indicate that the nature of the nucleotide bound does not have a marked effect on RAC's ability to stimulate ATP hydrolysis.

Alterations in Ssz1's ATP-binding cleft

The experiments described above suggest that nucleotide binding of Ssz1 does not markedly affect RAC's ability to facilitate stimulation of Ssb1's ATPase activity. To test whether nucleotide binding is important for Ssz1 function *in vivo*, we constructed and tested *SSZ1* alleles encoding alterations in the predicted Ssz1 nucleotide-binding site. Residues in Hsp70's ATPase domain that are important for ATP hydrolysis have been identified for several Hsp70s based on mutagenesis and biochemical studies[28]. Alteration of these residues in other Hsp70s resulted in reduced Hsp70 activity and caused any obvious phenotypic effects *in vivo*. The N-terminal domain of Ssz1 shares 30–35% sequence identity with the ATPase domain of Hsp70s across species, allowing us to model the N-terminal domain of Ssz1 based on the known structures of other Hsp70s (**Fig. 7a**). Overall the modeled ATPase domain of Ssz1 is similar to that of well-defined Hsp70s. However, the modeled N-terminal domain of Ssz1 has a slightly wider cleft for nucleotide binding, perhaps explaining the instability of Ssz1–ATP complex we observed. In addition, the predicted nucleotide-binding cleft of Ssz1 contains several notable differences compared with other Hsp70s, including Arg71, Phe199, Ile202 and Arg203 (see **Table 1** for analogous residues in bovine Hsc70), raising the possibility that Ssz1 may interact with nucleotides differently from other Hsp70s.

Based on this model and the effect of alterations in the nucleotide-binding cleft of other Hsp70s, we created mutations encoding 13 single-residue substitutions in Ssz1 predicted to disrupt either ATP binding or hydrolysis (**Table 1** and **Fig. 7a**). All mutants rescued the growth defects of a Δ*ssz1* strain as well as wild-type Ssz1 under all the conditions that Δ*ssz1* cells are known to show phenotypic effects, including low temperatures and the presence of cations (data not shown). In addition, we constructed four double mutants (**Table 1**). None showed any phenotypic effects. These results support the idea that neither ATP binding nor hydrolysis is critical for Ssz1's ability to rescue an Ssz1 deletion strain.

It has been shown that 2% of the normal levels of Ssz1 and Zuo1 is sufficient for wild-type growth rates and normal resistance to cations[13]. To test whether the effects of these mutations might be masked by the level of expression, we placed seven mutant *SSZ1* genes (D198N, F199A, I202F, R203W, D205N, K271W and S274Y) under the control of the *GAL1* promoter. In the presence of glucose, these Ssz1 mutant genes were expressed at 2–5% of wild-type levels. All mutants tested were able to fully rescue the phenotypes of Δ*ssz1* strains. To test under even more stringent conditions, the mutant Ssz1 proteins were expressed at 2–5% of the normal levels in the presence of 2% of the normal levels of Zuo1. Again, no growth defects were observed (**Fig. 7b**, data not shown).

DISCUSSION

Based on the biochemical and genetic results presented here, we conclude that Zuo1 is the J-protein partner of the ribosome-associated Hsp70 Ssb. Zuo1, when in complex with Ssz1, stimulates the ATPase activity of Ssb1. A single-residue alteration in the J-domain (H128Q) of Zuo1, which renders Zuo1 nonfunctional *in vivo*, eliminates stimulation by the Zuo1–Ssz1 complex. However, the most convincing demonstration of Ssb and Zuo1's *in vivo* partnership is our successful isolation of *SSB* mutants that can overcome the growth defects incurred by this Zuo1

Table 1 Ssz1 ATPase domain substitutions

Ssz1 alteration	Analogous Hsc70 residue
R71A	K71
E174D	E175
D198N	D199
F199A	L200
G200D	G201
I202F	G203
R203W	T204
D205N	D206
S267A	E268
K271A	R272
K271W	R272
S274A	S275
S274Y	S275
R71A, D198N	K71, D199
R71A, D205N	K71, D206
K271A, G200D	R272, G201
S267A, K271A	E268, R272

alteration in the J-domain. All these alterations mapped to the face of the ATPase domain, previously shown to be important for stimulation of an Hsp70's ATPase activity by a J-domain[25,26].

The fact that Zuo1 stimulates Ssb1's ATPase activity efficiently only when in complex with another Hsp70, Ssz1, is highly unusual. However, this observation is consistent with the genetic result that the phenotypes of a Δ*ssb*, Δ*zuo1* or Δ*ssz1* strain are the same, and identical to those of a strain lacking all of these genes. This identity in phenotype would be expected if an Hsp70 and J-protein formed an exclusive pair, because Hsp70s are known to absolutely require a J-protein for function. Identical phenotypes might well not be observed if an Hsp70 functions with more than one J-protein or a J-protein functions with more than one Hsp70. Similarly, if a second factor were required for that J-protein to carry out its role, then the lack of this factor (for example, Ssz1) would have the same effect as lack of the J-protein itself. This idea is also consistent with the previous result that crosslinking of Ssb to the nascent chain was not observed when Zuo1 but not Ssz1 was present[21].

Under some conditions Zuo1 can function *in vivo* in the absence of Ssz1. For example, an increase in expression of Zuo1 can partially suppress the growth of Δ*ssz1* cells[21]. Notably, when Zuo1 was added at high concentrations, at a 15-fold molar excess over Ssb, slight stimulation was observed even though no Ssz1 was present. In light of the fact that Zuo1 and Ssz1 need to be present at only 2% of their normal levels to allow wild-type growth *in vivo*[13], any expression over the normal level of Zuo1 is substantial compared with what is actually required. Therefore, Zuo1 by itself probably has residual ability to function as a J-protein on its own.

Although the importance of Ssz1 in the function of Zuo1 as a J-protein is demonstrated by the experiments reported here, the mechanistic role of Ssz1 is less clear. Two distinct possibilities can be envisioned. Ssz1 could play a structural role, and thus be necessary for maintaining a conformation of Zuo1 that is able to function efficiently as a J-protein. Alternatively, it could play a regulatory role, modulating the ability of Zuo1 to function as a J-protein in response to some signal. If Ssz1 were playing a regulatory role, we would hypothesize that it could regulate Zuo1 either through binding of substrates in its peptide-binding cleft or through nucleotide binding or hydrolysis. However, Ssz1 is functional in the absence of its C-terminal 14-kDa region, the putative peptide-binding domain[13]. In addition, many alterations in the ATP-binding cleft of Ssz1 have no effect on Ssz1's ability to function. Together, our results suggest that Ssz1 primarily plays a structural role, functioning to maintain Zuo1 in an active conformation. Any regulatory role may either be quite subtle, or occur under physiological conditions that we have not tested.

To our knowledge, a J-protein requiring a second factor for activity has not been reported previously, and is thus of interest on its own right. However, a particularly notable aspect of RAC is that the second factor is an Hsp70 family member. The *SSZ1* gene has evolved to carry out a function unlike that of other known family members—interacting with a J-protein to allow it to function as the partner of another Hsp70. Whether Ssz1 can, in fact, act as a chaperone that transiently binds short stretches of amino acid sequences, regulated by ATP binding and hydrolysis, is not resolved. Arguing against possible chaperone activity is the fact that neither of the two known *in vivo* functions of Ssz1 requires its C-terminal putative peptide-binding domain. As discussed above, the ATPase domain of Ssz1 alone is sufficient for rescue of a Δ*ssz1* strain. In addition, overexpression of Ssz1 has been shown to increase pleiotropic drug resistance (PDR) of yeast cells, through regulation of the activity of a transcription factor, Pdr1, which in turns regulates the expression of several ATP-binding cassette transporters[29]. Although the mechanism of action of Ssz1 in this activity is not known, the C-terminal domain is not required, as the ATPase domain has been shown to be sufficient[30]. Unfortunately, owing to the instability of the mutant Ssz1 proteins when overexpressed, we could not test their ability to function in PDR.

Although Ssz1 is the first Hsp70 family member found to facilitate a J-protein's function, it is not the only Hsp70 that has been found to play an atypical role. Two Hsp70s of the endoplasmic reticulum, Kar2 and Lhs1, have been found to associate, stimulating the other's ATPase activity, in part by Lhs1 serving as a nucleotide exchange factor for Kar2 (ref. 31). When the 14 Hsp70s of *S. cerevisiae* are compared, Ssz1 and Lhs1, along with the closely related Sse1 and Sse2, are outliers compared with the majority of Hsp70s. These Hsp70s fall into the divergent Hsp110 subclass[32]. Notably, the Sse Hsp70s have been shown to function through a distinctive mechanism as their ATPase domains and peptide-binding domains can function together *in vivo* without being tethered to each other[33]. All of them may serve as examples of how some members of the Hsp70 class of proteins have evolved to carry out very different functions. Whether all of them have maintained their ability to act as chaperones and whether such atypical Hsp70s exist in higher organisms to play regulatory roles remain to be determined.

METHODS

Yeast strains and media. All yeast strains used in this study are isogenic with DS10 (ref. 22) and contain the following mutations: *his3-11,15 leu2-3,112 lys1 lys2 Δtrp1 ura3-52*, in addition to mutations described below. The strains used and their additional mutations were PH16, *ssb1:TRP1 ssb2:LEU2 Δzuo:HIS3*; HE12, *Δzuo1:HIS3 Δssz:*LYS2 (ref. 30); HH4, *Δzuo1:HIS3 Δssz:LYS2 Δpep4:TRP1*, which was derived from HE12.

All yeast cultures were grown in either YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) or minimal media (0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose, supplemented with all amino acids except those needed for selection). Paromomycin (Sigma Chemical) was added at 500 μg ml^{-1} to selective minimal medium.

Protein purification. His-tagged Ssb1p and Ssa1p were purified from yeast cells using a Ni^{2+} chelating column followed by an ATP agarose column, as described[22]. Purified Ssb has a basal ATPase activity similar to that of untagged Ssb reported previously[24]. This construct has been shown to be fully functional *in vivo*[22] (data not shown). RAC and H128Q-RAC were purified from yeast ribosomal salt wash fractions using two anion-exchange columns and one gel-filtration column as described[20,21]. For purification of Zuo1, we used

N-terminal His-tagged Zuo1 that has been shown to be functional *in vivo* as cells expressing this construct are phenotypically equal to wild type (data not shown). To overexpress His-tagged Zuo1 or His-tagged RAC (His-Zuo1–Ssz1) for purification, the plasmid pYW45 (pRS416-GPD-His-Zuo1) was transformed into HH4 strain in the absence or presence of pHE31 (pRS415-GPD-Ssz1) and transformants were used in the following purification procedures. Cells (6 l) were grown overnight at 30 °C to an A_{600} of ~3. Pelleted cells were resuspended in binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 8.0, 10% (v/v) glycerol, 1 mM PMSF, protease inhibitor cocktail tablet (Roche)) to complete the composition of the buffer and disrupted by French press. Cleared and filtered lysate was loaded onto a 1 ml column of His-Bind^R resin (Novagen). After extensive washing with binding buffer, bound proteins were eluted with a 40 ml imidazole gradient from 5 mM to 200 mM. The peak fractions were then subjected to a Superdex 200 (Pharmacia) column and eluted with buffer A (20 mM HEPES, 100 mM KCl, 11 mM $Mg(OAc)_2$, 10% (v/v) glycerol). Ydj1p and Sis1p were purified as described[34,35]. To purify the Ssb1 suppressors, Ssb1-I174T and Ssb1-N176S, the suppressor genes were subcloned into the pRS416-*TEF-HIS-SSB1* plasmid[22] as BlpI-MunI fragments to encode a His_6-tag on their N terminus and to allow overexpression under the *TEF* promoter. Purification was carried out as for wild-type protein[22].

ATPase assays. Single-turnover ATPase assays of Ssb were carried out as described[24] with some modification. Ssb1-[α-^{32}P]ATP complex was formed by incubating 25 µg of His-Ssb1p with 100 µCi [α-^{32}P]ATP (PerkinElmer Life Sciences, 3,000 Ci mmol^{-1}) in buffer A containing 6 µM ATP in a 100 µl final volume at 25 °C for 5 min. The reaction mix was immediately chilled on ice and Ssb1–ATP complex was purified away from free ATP by fractionation over a NICK (Sephadex G50) gel filtration column (Amersham Pharmacia Biotech) at 4 °C with buffer A. For single-turnover reaction, the isolated [α-^{32}P]ATP–Ssb1 complex was incubated with different proteins (Zuo1, RAC and H128Q-RAC) at various concentrations at 25 °C or 30 °C in buffer A. At the indicated time, aliquots were removed and mixed with the stop solution (36 mM ATP, 2 M LiCl, 1 M formic acid) to terminate the reaction. The aliquots were then applied to polyethylenimine TLC plates and developed as described[36]. The fraction of ATP hydrolyzed to ADP at each time point was quantified by phosphorimager analysis (Molecular Dynamics) and the rate of ATP hydrolysis was calculated by fitting the data using Prism 4.0 (GraphPad Software). Steady-state ATPase assays were carried out in the presence of 50 µM ATP at 25 °C in a similar way but the Ssb1–ATP complex formation and isolation steps were excluded. AMP-PNP used in the RAC preincubation experiment was purified to >99% pure using a Resource Q column and a HPLC system. In the RAC pre-incubation experiments, RAC was preincubated with 1mM ADP or AMP-PNP for 30 min at 25 °C. For ATP, the preincubation time was 1 min at 25 °C to minimize any possible ATP hydrolysis.

Genetic selection for Zuo-H128Q suppressors. To generate the *SSB1* ATPase domain library, the ATPase domain of *SSB1* was mutagenized using error-prone PCR with Taq polymerase[37]. The PCR products were cloned into the pRS316k-SSB1 (ref. 22) using the engineered KpnI site (at +30 base pairs) and the natural MunI site. *Escherichia coli* transformants (7,200) were collected to generate the library. To select for Zuo-H128Q suppressors, PH16 yeast cells were transformed with pRS317-Zuo1-H128Q and pRS316k-*SSB1*-ATPase domain library. Suppressors were selected as fast growers on Ura, Lys omission medium at 18 °C for 5 d. *SSB1* plasmids were then isolated from the faster-growing transformants and sequenced to identify the mutations. All the suppressor plasmids were subcloned into pRS316k-*SSB1* as KpnI-MunI fragments and retransformed into PH16 with pRS317-Zuo1-H128Q to confirm the suppression phenotype.

Photocrosslinking of 8-N_3-ATP to Ssz1/RAC. Purified RAC (2 µM) was incubated with 4 µM [α-^{32}P]8-N_3-ATP (Affinity Labeling Technologies) in buffer A in a total volume of 20 µl for 5 min at 25 °C. The reaction mixtures were then applied to gel filtration spin columns (Micro Bio-Spin P30 chromatography columns, Biorad) pre-equilibrated in buffer A. After centrifugation at 1,000*g* for 4 min at 4 °C, the eluates containing 8-N_3-ATP bound RAC were then irradiated for 2 min using a hand-held UV lamp at 254 nm. Immediately after UV irradiation, 3 µl of 100 mM DTT was added to quench the crosslinking. Reaction mixtures were then separated on an 8.5% (v/v) polyacrylamide gel, stained with Coomassie blue, dried and exposed to phosphorimager screen. In the case of the nucleotide competition experiments, ATP or GTP was added at a concentration of 40 or 400 µM, a 10- or 100-fold excess over 8-N_3-ATP. In the DnaK control experiments, 2 µM of purified DnaK was incubated with 2.2 µM [α-^{32}P]8-N_3-ATP in a 20 µl reaction.

Ssz1 mutants carrying alterations in the ATP-binding cleft. Mutations in Ssz1's ATP-binding cleft were created using the PCR QuikChange method (Stratagene). PCR was used to introduce a 5′ BamHI site and a 3′ SalI site of *SSZ1* containing different mutations. The PCR products were digested with BamHI and SalI, and fragments were cloned into BamHI-XhoI sites of pYES2 (Invitrogen). The entire *SSZ1* coding region of these plasmids was sequenced to confirm that no other mutations were present. The expression level was examined by immunoblot analysis using antibody against the C-terminal region of Ssz1. All were expressed between 2% and 5% of the wild-type level in the presence of glucose. The pYES2 based *GAL-Zuo1* plasmid[13] was used for expressing 2% of Zuo1 in combination with these Ssz1 mutant plasmids.

Accession codes. BIND identifiers (http://bind.ca): 262695, 262696, 262697, 262698.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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