Eukaryotic ribosomes carry a stable chaperone complex termed ribosome-associated complex consisting of the J-domain protein Zuo1 and the Hsp70 Ssz1. Zuo1 and Ssz1 together with the Hsp70 homolog Ssb1/2 form a functional triad involved in translation and early polypeptide folding processes. Strains lacking one of these components display slow growth, cold sensitivity, and defects in translational fidelity. Ssz1 diverges from canonical Hsp70s insofar that neither the ability to hydrolyze ATP nor binding to peptide substrates is essential in vivo. The exact role within the chaperone triad and whether or not Ssz1 can hydrolyze ATP has remained unclear. We now find that Ssz1 is not an ATPase in vitro, and even its ability to bind ATP is dispensable in vivo. Furthermore, Ssz1 function was independent of ribosome-associated complex formation, indicating that Ssz1 is not merely a structural scaffold for Zuo1. Finally, Ssz1 function in vivo was inactivated when both nucleotide binding domain with hydrophobic patches of polypeptide substrates. Substrate binding and release are regulated via the C-terminal domain via the C-terminal domain were disrupted in the same mutant. The two domains of this protein thus cooperate in a way that allows for severe interference in either but not in both of them.

Chaperones of the Hsp70 family usually consist of an ~45-kDa N-terminal ATPase, an ~15-kDa peptide binding, and an ~10-kDa variable C-terminal domain (1). The function of classical Hsp70 homologs is based on the interaction of the peptide binding domain with hydrophobic patches of polypeptide substrates. Substrate binding and release are regulated via the ATPase cycle of the N-terminal domain. In the case of classical Hsp70s, ATPase activity can be regulated by a diverse set of substrates. Substrate binding and release are regulated via the C-terminal domain. In the case of classical Hsp70/J-domain partners, the role of Ssz1 in the system has remained obscure. At first, Ssz1 contains an unusually short peptide binding domain and lacks the variable C-terminal domain (8). In fact, the function of Ssz1 does not strictly depend on substrate binding as a truncated version lacking nearly the entire peptide binding domain was reported to support growth as efficiently as the wild type protein (7). Moreover, Ssz1 also contains a particular ATPase domain. Within the nucleotide binding pocket of wild type Ssz1, a number of important residues diverge from canonical Hsp70s (10). Whether Ssz1 can hydrolyze ATP at all has not been conclusively decided. Preparations of authentic RAC displayed ATPase activity, but interfering contaminations could not be excluded. On the other hand, RAC purified via a His tag on Zuo1 did not hydrolyze ATP but might have been functionally disturbed (10–12). The difficulty in tackling this basic question

3 Ssb1/2 represents Ssb1 and Ssb2, two 99% identical proteins with identical function and similar expression levels (39).

4 The abbreviations used are: RAC, ribosome-associated complex consisting of Ssz1 and Zuo1; BN-PAGE, blue native PAGE; Tricine, N-[2-hydroxy-1,1-bis(3-hydroxymethyl)ethyl] glycine.
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is due to the fact that Hsp70s in general display low ATP turnover rates, which are experimentally difficult to distinguish even from marginal contaminations with active ATPases (1). One possibility to avoid this problem is to analyze the effect of mutations that have been shown to eliminate ATP hydrolysis in other Hsp70s. For instance, mutations in the ATPase domain of Ssz1 were found to fully complement the phenotype of a Δssz1 strain, indicating that ATP hydrolysis was not an essential function of Ssz1 in vivo (10). However, whether or not an Hsp70 can hydrolyze ATP cannot be decided solely based on in vivo studies. On this note, it recently became evident that the ATPase activity of some Hsp70s is dispensable in vivo. For example, yeast Sse1 is capable of ATP hydrolysis in vitro, but its function does not depend on this activity in vivo (13–15).

Here we set out to better understand the role of the non-canonical Hsp70 homolog Ssz1. To this end, we have generated a set of mutations in Ssz1 that enabled us to comparatively analyze in vivo and in vitro the roles of ATP binding, ATP hydrolysis, and the peptide binding domain. Furthermore, we have analyzed how Ssb1 acts as a multicopy suppressor in a Δssz1 strain with respect to these properties (6). The combined data suggest that the role of Ssz1 in the chaperone triad is robust. Loss of the Ssz1 peptide binding domain, stable interaction with its partner subunit Zuo1, and ATP binding were well tolerated, displaying only minor reduction of its in vivo activity. Our data also resolve the issue of the ATPase activity and demonstrate that Ssz1 contained in purified RAC does not hydrolyze ATP. Only when Ssz1 lacks its peptide binding domain and at the same time is deficient in nucleotide binding does it appear completely non-functional. We find that Ssz1 is involved in two separable processes, one related to translational fidelity and the other to general growth defects. Ssb1 substrates for the role in translational fidelity by a mechanism that does not require ATP hydrolysis. However, with respect to slow growth and cold sensitivity, Ssb1 is unable to replace Ssz1.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Culture Conditions—Standard yeast genetic techniques were applied (16). MH272-3F a/α (ura3/ura3, leu2/leu2, his3/his3, trp1/trp1, ade2/ade2) is the parental wild type strain of all haploid derivatives used in this study (17). Deletion strains lacking SSZ1 (Ida2: Δssz1), ZUO1 (Ida1: Δzuo1), or both genes (Ida12: Δzuo1Δssz1) have been previously described (8). To generate the Δssz1Δssb2 strain, the ClaI/AgeI 1.4-kb fragment contained with the coding region of SSB1 as well as SSB2 were replaced by the ADE2 marker. Single disruptions were generated in the haploid a and α strains. The resulting strain ssb1::ADE2 was transformed with a plasmid containing the URA3 marker, and the ssb2::ADE2 strain was transformed with a plasmid containing the TRP1 marker; after mating, a diploid ssb1::ADE2 ssb2::ADE2 strain that had lost the plasmids was selected and subsequently sporulated. ssb1::ADE2 ssb2::ADE2 haploid strains (Ida56E) were generated after dissection of the spores by tetrad analysis. Deletion of both copies of Ssb was also confirmed by Western blotting (data not shown).

Strains were grown to log phase on 1% yeast extract, 2% peptone, 2% dextrose (YPD) or in glucose-containing minimal medium. Growth defects were analyzed by spotting 10-fold serial dilutions containing the same number of cells to YPD plates. Plates were supplemented with paromomycin at the concentration specified in the legends of Figs. 3–5. Plates were incubated at 30 °C to analyze the slow growth phenotype and at 20 °C to determine cold sensitivity.

Mutant Versions of Ssz1 for Expression in Yeast and Escherichia coli—For a summary of the mutants used in this study, compare Fig. 1. For expression in yeast, mutant versions of SSZ1, SSBI, and chimera were generated by PCR and were transferred into the centromeric yeast expression vector pYCPlac33 (URA3) (6, 18). Mutant Ssz1-R71A has been described (10). In Ssz1-LKA, three consecutive amino acids, G201I, I202K, and R203A, were exchanged. Ssz1-ΔC is a C-terminally truncated version of Ssz1 (1–394). Ssz1-LKA-ΔC corresponds to Ssz1-ΔC, but in addition, contains G201I, I202K, and R203A. Ssb1-ΔC is a C-terminally truncated version of Ssb1 (1–391). The Ssb1-Ssz1 chimera was generated by fusing the ATPase domain of Ssb1 (1–391) in-frame to the peptide binding domain of Ssz1 (396–538). Ssb1*-Ssz1 contains K73A within the ATPase domain of Ssb1, which abolishes ATPase activity (19). Ssz1-ΔC and Ssb1-ΔC correspond to the ATPase domain fragment of Hsc70 (amino acids 1–386), of which the structure was solved by x-ray crystallography (20). All mutant versions of Ssz1 and Ssb1 were verified by sequencing. Expression levels were analyzed by immunoblotting (supplemental Fig. 1). To ensure comparable cellular concentrations of the RAC subunits, we have overexpressed Zuo1 in Δssz1 + Ssb1-Δssz1 and Δssz1 + Ssb1*-Ssz1 strains from the 2μ, ADE2 marker plasmid pYEpLac555-zuo1. To purify RAC and RAC-LKA for in vitro experiments, Ssz1 or Ssz1-LKA and Zuo1 were co-expressed in E. coli. With the exception of the LKA mutation, Ssz1 was identical to the authentic yeast protein, and Zuo1 contained a single amino acid exchange (F2H). Neither Ssz1 nor Zuo1 contained tags. Ssz1 and Ssb1-LKA were expressed from pET28N, a derivative of pET28a (Novagen), and Zuo1 was expressed from pETcoco-2 (Novagen).

Analysis of Protein Complexes and Ribosome Association—For the preparation of total cell extracts, 50-mL mid-log cultures grown on YPD were harvested, washed with 10 mL of double distilled H2O, and subsequently treated with 1 volume of acid-washed glass beads and 2 volumes of lysis buffer (20 mM HEPES-KOH, pH 7.4, 5 mM magnesium acetate, 120 mM potassium acetate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mix (1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastatin, 5 μg/ml pepstatin A dissolved in Me2SO)). Cells were disrupted by six 1-min cycles of vortexing followed by cooling on ice and cleared by centrifugation for 10 min at 20,000 × g. Aliquots of the resulting extracts were analyzed either by BN-PAGE using 6–16.5% gradient gels at 4 °C as described previously (9) or by immunoprecipitations under native conditions using α-Zuo1- or α-FLAG (Sigma)-coated agarose beads as indicated in the legends for Figs. 4 and 5 (5, 21). An aliquot corresponding to the material applied to each immunoprecipitation reaction was precipitated with a final concentration 5% trichloroacetic acid and was analyzed in parallel. Preparation of cytosol and ribosome binding assays were performed as described previously (8).
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| Ssz1       | Ssz1-R71A | Ssz1-LKA | Ssz1-ΔC |
|------------|-----------|----------|---------|
|            | R71A      | G201L,D03K,R203A | (1-394) |
| N-terminal |            |          |         |
| C-terminal |           |          | 538     |

**Untagged RAC Purified from E. coli Does Not Hydrolyze ATP**

In most cases, objective evidence for ATP hydrolysis by an Hsp70 is based on the ability of specific co-chaperones to stimulate this reaction that occurs at an intrinsically low rate. If such a co-chaperone has not been identified, analysis depends on highly purified protein preparations. To obtain highly purified, authentic RAC, we have co-expressed untagged versions of Ssz1 and Zuol (Fig. 1) in E. coli and have purified the resulting complex via a series of conventional column chromatographic steps (Fig. 2A).

**RESULTS**

1. **Protein Purification**—E. coli cells were disrupted by sonication in buffer A (40 mM HEPES-KOH, pH 7.4, 240 mM potassium acetate, 0.014% (v/v) β-mercaptoethanol, 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride). Crude extracts were cleared by three successive centrifugation steps for 30 min at 12,000 × g, 20 min at 30,000 × g, and 10 min at 36,000 × g. The resulting supernatant was loaded onto a 16-ml ResourceQ column (GE Healthcare) (250–800 mM, 60-mL linear potassium acetate gradient in 40 mM HEPES-KOH, pH 7.4). RAC eluted at a concentration of 440–540 mM potassium acetate. RAC-containing fractions were pooled, adjusted to a concentration of 200 mM potassium acetate, and loaded onto a MonoQ HR10/10 (GE Healthcare) column (200–1000 mM, 50-mL linear potassium acetate acetate gradient in 40 mM HEPES-KOH, pH 7.4). RAC-containing fractions (750–840 mM potassium acetate) were adjusted to a final concentration of 100 mM potassium acetate and were loaded onto a MonoS HR5/5 (GE Healthcare) column (100–1000 mM, 18-mL linear potassium acetate gradient in 40 mM HEPES-KOH, pH 7.4). During this final purification procedure, RAC eluted at a potassium acetate concentration of 240–400 mM. RAC-R71A and RAC-QPD were purified from yeast as described previously (8). Purified RAC preparations were concentrated to ~2 mg/ml, supplemented with 10% glycerol, and stored at −80 °C. DnaK and DnaJ were purified according to established protocols (22, 23).

2. **ATPase Assays**—ATPase activities of RAC and DnaK were determined in single turnover as well as steady-state experiments. ATP hydrolysis was monitored by thin layer chromatography. Single-turnover assays were performed basically as described (24). A 50-μl reaction contained 8.2 μM DnaK or RAC, 25 μM ATP, 0.82 MBq of [α-32P]ATP in buffer B (20 mM HEPES-KOH, pH 7.4, 120 mM potassium acetate, 10 mM magnesium acetate). Protein-ATP complexes were rapidly separated from free nucleotide by gel filtration on a nickel column (GE Healthcare) equilibrated in buffer B. Single-drop fractions were collected, and the radioactivity was monitored with a Geiger–Müller counter (Mini Instruments Ltd., Mini 900). The first four fractions containing [α-32P]ATP were pooled, and aliquots of 19 μl were frozen in liquid nitrogen and stored at −80 °C. For the assay, an aliquot was quickly thawed at 30 °C, 1 μl was spotted onto a polyethyleneimine-celluloseF TLC plate (Merck) for the zero value, and 32 μl of prewarmed buffer B (containing 1 μM DnaK when indicated) was added. At the indicated times, 2-μl aliquots of the reaction mix were spotted onto TLC plates, which were subsequently developed in 3.6% (v/v) acetic acid, 400 mM LiCl. Quantification of the amounts of ADP and ATP was performed using the AIDA software (raytest), and the data were fitted to a single-exponential decay function (Kaleidagraph, Synergy Software). Steady-state ATPase assays were carried out at 25 °C in a volume of 50 μl. The reactions contained 50 μM ATP, 0.037 MBq of [α-32P]ATP, 20 mM HEPES-KOH, pH 7.4, 120 mM potassium acetate, 5 mM magnesium acetate. The rate of ATP hydrolysis at steady-state was calculated by linear fitting of the data.

3. **Photocross-linking of [α-32P]8-N3-ATP to Ssz1**—Photocross-linking experiments were performed as described with minor modifications (10). Twenty-μl reactions containing 1 μM of the purified RAC preparations were incubated in buffer C (20 mM HEPES-KOH, pH 7.4, 120 mM potassium acetate, 5 mM magnesium acetate) supplemented with 4 μM [α-32P]8-N3-ATP (MP Biomedicals) and incubated for 5 min at 25 °C. For details, compare “Experimental Procedures” and “Results.”

**RESULTS**

**Untagged RAC Purified from E. coli Does Not Hydrolyze ATP**

**Miscellaneous**—Total yeast protein for immunoblot analysis was prepared by the method of Yaffe and Schatz (25). Protein concentrations were determined by the BCA assay according to the instructions of the manufacturer (Sigma). Immunoblots were developed using ECL or 125I-labeled protein A (26). Quantification of immunoblots was performed with AIDA Image Analyzer software (raytest).
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First, we analyzed the ATPase activity of RAC under steady-state conditions using E. coli DnaK as a positive control. Consistent with published data (28), DnaK hydrolyzed ATP at a rate of $1 \times 10^{-3}$ s$^{-1}$. This rate was stimulated 7-fold in the presence of DnaJ. In contrast, the determined rate of RAC ($7.5 \times 10^{-5}$ s$^{-1}$) was in the same order of magnitude as that of purified DnaK, which served as a non-ATPase control (Fig. 2B). It is therefore most likely that the measured ATPase activity of RAC originated from small contaminations of co-purified ATPases from the E. coli cytosol. In steady-state assays, even small contaminations with high ATPase rates can contribute significantly to the observed hydrolysis rate since the observed velocity of the hydrolysis reaction is proportional to the product of $k_{cat}$ and concentration of the contaminating ATPase. To circumvent this problem, we performed single-turnover ATPase experiments with isolated RAC:ATP complexes. Under these conditions, any contaminating ATPase would contribute to the observed rate and amplitude of ATP hydrolysis according to its relative amount, which is negligible when highly purified proteins are used (Fig. 2A). RAC formed stable [$\alpha$-32P]ATP complexes, which could be isolated via rapid gel filtration (data not shown). However, upon incubation at 25 °C, bound ATP was not hydrolyzed (Fig. 2C).

**ATP Binding Has No Effect on the in Vivo Function of Ssz1**—Previous analysis of a large set of mutants suggested that neither nucleotide binding nor hydrolysis was critical for the function of Ssz1 in vivo (10). However, the extent to which ATP binding was affected in mutant Ssz1 versions had not been assessed directly. To interfere with ATP binding, we have introduced three consecutive mutations (G201L, I202K, and R203A, Fig. 1) within the nucleotide binding pocket of Ssz1. The residues are located within a region homologous to a loop of Hsc70 required for the accommodation of the $\beta$- and $\gamma$-phosphate groups of bound nucleotides (20). The resulting mutant, named Ssz1-LKA, was co-expressed with Zuo1 in E. coli, and the mutant complex (RAC-LKA) was purified (Fig. 3A, upper panel). ATP binding to wild-type RAC and RAC-LKA was determined by UV-induced cross-linking of the $\alpha$-32P-labeled ATP analog 8-azido-ATP (10). As a control, we also tested 8-azido-ATP binding to RAC-R71A containing Ssz1-R71A (Fig. 1), a mutant designed to eliminate any potential ATPase activity of Ssz1 (10), and to RAC-QPD containing Zuo1-QPD (Fig. 1), which contains an inactivating mutation within Zuo1 with respect to Ssb1 stimulation (10). When compared with normalized wild-type RAC, the 8-azido-ATP cross-link to RAC-LKA was reduced to less than 2%, indicating that ATP binding was significantly disturbed. Wild-type RAC and RAC-QPD were cross-linked to 8-azido-ATP with similar efficiency, whereas cross-linking efficiency to RAC-R71A was reproducibly increased by about 50% (Fig. 3A, lower panel, and 3B). When the ATP non-binding mutant Ssz1-LKA was expressed in a Δssz1 background, no significant growth defect was observed (Fig. 3C). The lack of a phenotype strongly suggests that ATP binding is not an essential property of Ssz1 (compare also below).
The N-terminal Domain of Ssz1 Does Not Stably Interact with Zuo1—To explore the role of the peptide binding domain of Ssz1, we have generated a C-terminally truncated version (Ssz1-ΔC, Fig. 1). This mutant corresponds precisely to the 44-kDa ATPase fragment of bovine Hsc70 as solved by x-ray crystallography (20). We were unable to isolate a complex between Ssz1-ΔC and Zuo1 when the two proteins were co-expressed in E. coli (data not shown). To test whether complex formation occurred in yeast, Ssz1-ΔC was expressed in a Δssz1 strain. We have previously observed that Zuo1 is destabilized in the absence of its partner subunit Ssz1 (8). A comparison of the Zuo1 levels in wild type, Δssz1, and Δssz1 + Ssz1-ΔC revealed that expression of Ssz1-ΔC did not stabilize Zuo1, suggesting that the two proteins failed to stably interact (Fig. 4A). Consistently, Ssz1-ΔC was not detected in a complex with Zuo1 on BN-PAGE (Fig. 4B). Moreover, Ssz1-ΔC was not anchored to ribosomes via Zuo1 (Fig. 4C), whereas this is the case for wild type Ssz1 (8). We conclude that the peptide binding domain of Ssz1 is required to form stable heterodimeric RAC. Phenotypic analysis of a Δssz1 strain expressing Ssz1-ΔC revealed that the strain grew like wild type at 30 °C as well as at 20 °C (Fig. 4D) (7). Only at high concentrations of paromomycin did Ssz1-ΔC not fully restore growth of Δssz1 (Fig. 4D). With respect to the phenotype, it is important to note that the paromomycin concentrations required to inhibit growth of a Δssz1 strain were ~2 orders of magnitude lower (Ref. 29 and data not shown). This rather mild phenotype of Ssz1-ΔC suggests that tight interaction between Zuo1 and Ssz1 is not strictly required for the chaperone triad to function.

We finally generated Ssz1-LKA-ΔC (Fig. 1), which is the N-terminal domain of Ssz1 containing the triple mutation that inactivates ATP binding (Fig. 3A). Ssz1-LKA-ΔC was stably expressed in a Δssz1 background (supplemental Fig. 1); however, it failed to complement growth defects of Δssz1 at 30 or 20 °C or in the presence of even low concentrations of paromomycin (Fig. 4E and data not shown). It appears that nucleotide binding becomes an essential feature of Ssz1 when its peptide binding domain is truncated, and consequently, Zuo1 no longer stably interacts.

The C-terminal Domain of Ssz1 Does Not Stably Interact with Zuo1—To test whether the peptide binding domain of Ssz1 by itself was sufficient to force a structurally similar domain to interact with Zuo1, we constructed a chimera consisting of the ATPase domain of Ssb1 fused to the peptide binding domain of Ssz1 (Ssb1-Ssz1, Fig. 1). To exclude that ATP hydrolysis by Ssb1-Ssz1 would influence the interaction with Zuo1, a second chimera was constructed in which the ATPase of Ssb1 was inactivated by an exchange of lysine at position 73 for an alanine (Ssb1*-Ssz1, Fig. 1). Both chimeras were expressed in a Δssz1 background and tested for their ability to form a complex with Zuo1. Ssb1-Ssz1 and Ssb1*-Ssz1 were not co-isolated together with Zuo1 in native immunoprecipitation reactions using a Zuo1-specific antisera (α-Zuo1) (Fig. 5A), nor was a complex detected on BN-PAGE between the chimera and Zuo1 (Fig. 5B). We conclude that neither Ssb1-Ssz1 nor Ssb1*-Ssz1 formed a stable complex with Zuo1. The combined data indicate that neither the ATPase domain nor the peptide binding domain of Ssz1 are sufficient to mediate complex formation with Zuo1.

Seemingly, within RAC, the two subunits share essential protein-protein interfaces that involve both domains of Ssz1 (Fig. 6). Alternatively, only one of the two Ssz1 domains may directly interact. In this case, conformational changes in the remaining domain of Ssz1 may impair stable interaction with Zuo1.

Ssb1 Can Substitute for the Function of Ssz1 in Translational Fidelity—It was previously observed that growth defects of a Δssz1 strain can be partly complemented by high expression levels of Ssb1 (6). From our results, two scenarios may explain how Ssb1 substitutes for the function of Ssz1 in translational
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To distinguish between the two possibilities, we have generated Ssb1*, a mutant unable to hydrolyze ATP, and Ssb1-ΔC, lacking the peptide binding domain (Fig. 1). Neither Ssb1* nor Ssb1-ΔC was able to complement growth defects of a Δssz1 strain, confirming that the function of Ssb1 required the ability to hydrolyze ATP as well as its peptide binding domain (data not shown). We have also expressed Ssb1-Ssz1 in a Δssb1Δssz2 strain (Fig. 5C). As a result, the peptide binding domain of Ssz1 cannot functionally replace the peptide binding domain of Ssb1. Next, we have expressed the different mutants and chimera from low copy number plasmids in a haploid yeast strain, confirming that the function of Ssb1 depends on its ATPase activity and peptide binding domain, the data strongly suggest that Ssb1 can directly replace Ssz1 with respect to its role in translational fidelity.

**DISCUSSION**

**Ssz1 Cannot Hydrolyze ATP and Binding of ATP Is Not Strictly Required in Vivo**—Until recently, it was presumed that Hsp70 function in general depends on ATP hydrolysis. However, it is now recognized that the major function of some non-canonical Hsp70s does not depend on their ATPase activity. For example, yeast Sse1 hydrolyzes ATP in vitro, but cell growth does not rely on this activity (11, 13). Similarly, mutational analysis revealed that Ssz1 function is independent of ATP hydrolysis (10). We now find that Ssz1 contained in the highly purified RAC complex is not an ATPase. Formally, it...
cannot be ruled out that ATP hydrolysis by Ssz1 depends on a yet unidentified co-chaperone, occurs only when Ssz1 is bound to the ribosome, or depends on a functionally important modification missing due to its heterologous expression. However, all currently available data indicate that Ssz1 function is independent of ATP hydrolysis and that Ssz1 does not hydrolyze ATP. Moreover, the function of Ssz1 also does not strictly depend on ATP binding. Whether this degree of degeneration is common for Hsp70s in RAC-type complexes is currently not clear. Hsp70L1, the mammalian counterpart, contains an ATPase domain more closely related to canonical Hsp70s when compared with that of Ssz1. The biochemical properties of Hsp70L1 are currently unknown. Some evidence suggests that there might be differences between RAC complexes in yeast and higher eukaryotes. For example, MPP11, the J-domain partner of Hsp70L1, contains two C-terminal Myb domains, characteristic for animals and plants, which are absent in Zuo1. Phylogenetic analysis revealed that fungal homologs, such as Zuo1, have developed from MPP11-like proteins by the complete loss of the Myb domain (30), suggesting that some aspects of RAC function might have become dispensable in fungi during evolution. By the same token, Ssz1 might have become more degenerated when compared with its mammalian counterparts.

Tight Interaction between Ssz1 and Zuo1 is Not Strictly Required in Vivo—RAC is a very stable complex, and Ssz1 and Zuo1 can be separated only upon denaturation (data not shown, Fig. 6). Conservation of this unusual type of ribosome-bound chaperone complex in mammalian cells (9) argues for its functional significance. A variety of methods now revealed that Ssz1-ΔC did not form a complex with Zuo1 but that the consequences in vivo were minor and only detectable in the presence of high concentrations of error-inducing drugs. This observation disfavors the idea that the effect of Ssz1 is mainly mediated via direct structural effects on Zuo1 (Fig. 6). In an earlier study, a C-terminally truncated version, only 13 amino acids longer than Ssz1-ΔC, was reported to fully complement in a Δssz1 background (7). Although strain-specific differences may account for the variation, it is also plausible that the longer version of Ssz1 displayed higher affinity for Zuo1. Growth defects in the presence of Ssz1-ΔC, however, may not be confined to the loss of Zuo1 or ribosome interaction but may originate from an yet unknown function of the C-terminal domain. The latter is suggested by the finding that Ssb1-ΔC does not complement any of the growth defects displayed by Δssz1. However, Ssb1-Ssz1, which is a fusion of Ssb1-ΔC to the C-terminal domain of Ssz1, restores translational fidelity of Δssz1. As the Ssb1-Ssz1 chimera does not form a stable complex with Zuo1, the peptide binding domain of Ssz1 likely contributes to complementation by different means.

Slow Growth at Different Temperatures and Sensitivity toward Aminoglycosides Relate to Separable Functions of Ssz1—Complementation of Δssz1-related growth defects by Ssb1, Ssb1-SSz1, or Ssb1*-Ssz1 was confined to aminoglycoside sensitivity. Slow growth and cold sensitivity were only insignificantly affected by expression of Ssb1 or of the chimera, suggesting at least two separable cellular roles for Ssz1. Similar phenotypic separation was previously reported for Ssb. In this case, genetic and biochemical analysis of chimeras built from Ssb and Ssa revealed that tolerance to aminoglycosides correlated with ribosome association and could be separated from cold sensitivity (31). Interestingly, in the case of Ssz1-ΔC, aminoglycoside sensitivity was also associated with loss of ribosome binding. Recently, it was found that expression of the prokaryotic chaperone trigger factor in yeast partly rescued aminoglycoside sensitivity of a yeast strain lacking Ssb1/2, Ssz1, and Zuo1, whereas cold sensitivity remained by and large unaffected (32). How exactly ribosome-bound chaperones mediate their different roles is ill-defined. However, there is accumulating evidence that aminoglycoside sensitivity of mutants within the chaperone triad is directly connected to defects in the translation process, specifically in aspects related to translational fidelity. Stop codon readthrough and incorporation of the wrong amino acids is increased in the absence of functional RAC or Ssb1/2. The effect is significantly enhanced in the presence of aminoglycosides (29). In addition, lack of either RAC or Ssb1/2 results in specific inhibition of programmed −1 ribosomal frame shifting and causes defects in Killer virus maintenance (33). Finally, Ssb1 was recently found in an unbiased screen for factors that increase translation termination when overexpressed (34). It has been speculated that ribosomal proteins of the exit tunnel and chaperones that bind to this area of the ribosome may couple to the ribosomal decoding center via the nascent polypeptide. In this model, the absence of the chaperones would lead to a feedback of the nascent polypeptide from the tunnel exit to the decoding center (33, 35–37). Consistent with this hypothesis, simple binding of different chaperones without the requirement of ATP hydrolysis, for instance, of mutant Ssb1* or trigger factor, may partially prevent this effect of the nascent polypeptide.

Ssz1 Requires Either Nucleotide Binding or Its C-terminal Domain—It is difficult to compromise the two domains of Ssz1 more severely than by eliminating the ability of the N-terminal domain to bind to ATP or to completely delete the C-terminal domain. However, only a combination of both resulted in a loss of function phenotype in vivo. It is challenging to define the role of an Hsp70 that does not hydrolyze ATP, is only marginally affected when ATP binding is abolished, and depends neither on substrate binding nor on stable interaction with its protein complex partner. In light of the recently emerging hypothesis that Hsp70s can act on each other as nucleotide exchange factors, it is tempting to speculate that Ssz1 may not exclusively bind Zuo1 but also potentially interact with Ssb1 to facilitate nucleotide exchange. In this case, the rather weak phenotypes observed for most Ssz1 mutant versions may be due to the presence of other nucleotide exchange factors, such as Sse1 or Fes1 (14, 15, 38), which could substitute for this specific role of Ssz1. Experiments to test for such an additional function of Ssz1 are on their way.

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