Molecular chaperones such as Hsp70 use ATP binding and hydrolysis to prevent aggregation and ensure the efficient folding of newly translated and stress-denatured polypeptides. Eukaryotic cells contain several cytosolic Hsp70 subfamilies. In yeast, these include the Hsp70s SSB and SSA as well as the Hsp110-like Sse1p. The cellular functions and interplay between these different Hsp70 systems remain ill-defined. Here we show that the different cytosolic Hsp70 systems functionally interact with Hsp110 to form a chaperone network that interacts with newly translated polypeptides during their biogenesis. Both SSB and SSA Hsp70s form stable complexes with the Hsp110 Sse1p. Pulse-chase analysis indicates that these Hsp70/Hsp110 teams, SSB/SSA and SSA/SSA, transiently associate with newly synthesized polypeptides with different kinetics. SSA Hsp70s bind cotranslationally to a large fraction of nascent chains, suggesting an early role in the stabilization of nascent chains. SSA Hsp70s bind mostly post-translationally to a more restricted subset of newly translated polypeptides, suggesting a downstream function in the folding pathway. Notably, loss of SSB dramatically enhances the cotranslational association of SSA with nascent chains, suggesting SSA can partially fulfill an SSB-like function. On the other hand, the absence of Sse1p enhances polypeptide binding to both SSB and SSA and impairs cell growth. It, thus, appears that Hsp110 is an important regulator of Hsp70-substrate interactions. Based on our data, we propose that Hsp110 cooperates with the SSB and SSA Hsp70 subfamilies, which act sequentially during de novo folding.

Newly synthesized proteins are faced with the difficult task of transforming their linear amino acid sequence into a three-dimensional folded structure within the complex and crowded environment of the cell (1–3). During translation, molecular chaperones, such as Hsp70, use ATP binding and hydrolysis to protect the emerging polypeptide from aggregation and facilitate its correct folding (1, 4, 5). A unique subclass of Hsp70 homologues called Hsp110s, found only in eukaryotic cells, is distinguished by an insertion of flexible loops after the polypeptide binding domain and an extended tail at the C terminus (8, 9). The function of these additional domains in Hsp110 remains unclear.

Binding and release of an unfolded polypeptide by all Hsp70s rely on the modulation of the intrinsic peptide affinity of Hsp70 by cycles of ATP binding and hydrolysis (2, 6, 7). Cycling of Hsp70s between the different nucleotide states is regulated by an expanding list of cofactors (1, 7, 10), including J-domain proteins that stimulate the ATPase activity of Hsp70 and several structurally distinct nucleotide exchange factors (6, 11, 12).

An intriguing aspect of the eukaryotic cytosol is that it contains multiple Hsp70 subfamilies (5). For instance, the cytosol of the yeast Saccharomyces cerevisiae contains four functionally redundant Hsp70 homologues called Ssa1–4p, sharing more than 85% identity (herein SSA) and three ribosome-associated Hsp70s, Ssz1p, and the functionally redundant Ssb1p and Ssb2p, sharing more than 99% identity (herein SSB). In addition, yeast has two Hsp110-like homologues called Sse1p and Sse2p, sharing 76% identity (5, 9, 13). It is unclear why eukaryotic cells have so many Hsp70 homologues. Although they appear to have distinct cellular functions, as only SSA is essential for viability and cannot be replaced by SSB (5, 14), the precise role of the different Hsp70s in cellular folding remains undefined. Genetic and biochemical studies indicate that SSA is required for the folding of some newly synthesized proteins, including TRP3 (tryptophan-requiring protein 3) and the heterologous proteins ornithine transcarbamoylase and the von Hippel-Lindau tumor suppressor protein (15–17). SSA also facilitates in import of precursor proteins into both the endoplasmic reticulum and mitochondria (18, 19) and is important for rescue of stress-denatured proteins (5). On the other hand, in vitro experiments showed that the ribosome-bound SSB can be cross-linked to nascent chains in close proximity to the polypeptide exit channel (20, 21). However, association of SSB with nascent chains has not yet been observed in vivo. Although not essential, deletion of SSB results in a slow growth phenotype (14). It has recently been suggested that SSA function at the ribosome is modulated by another Hsp70, Ssz1p, in a complex with the J-domain protein Zuo1p (21–23). It is further proposed that the Ssz1p–Zuo1p complex is essential for SSB interaction with nascent chains (21).

The cellular function of Hsp110s is also unclear. Hsp110s have been shown to prevent aggregation of model substrates in vitro (24) and have been implicated in thermotolerance (25–27). In S. cerevisiae, cells lacking Sse1p are slow growing and temperature-sensitive (13), and genetic studies also implicate Sse1p in Hsp90-dependent processes (24, 28). Interestingly, biochemical studies suggest that Hsp110 chaperones may be involved in the regulation of Hsp70 ATP hydrolysis and that Hsp70 may have some stimulatory activity on Hsp110 ATPase as well (29, 30).
However, the ATPase activity of Sse1p appears to be dispensable for its function (31).

To obtain insights into the function of the different cytosolic Hsp70s in de novo folding, we searched for cellular binding partners of the Hsp70s SSB and SSA and examined their interactions with nascent chains. We find that the major cytosolic Hsp70s, SSA and SSB, both associate independently with the cytosolic Hsp110 homologue Sse1p.

To evaluate the interplay and relative contribution of each Hsp70 subfamily to de novo protein folding, we examined the flux of newly synthesized proteins through the different Hsp70 chaperones. Our data suggest that the SSB-Sse1p complex plays an early role in cotranslational nascent chain stabilization, whereas SSA-Sse1p acts mostly post-translationally to facilitate the folding of a more restricted protein subset.

Analysis of Hsp70 interactions in Δsse1 cells further indicates that Hsp110 is an important regulator for both SSB- and SSA-substrate interactions. Our analysis indicates that a network of different cytosolic Hsp70s cooperates in de novo folding.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—SSA1, SSB2, and SSE1 were N-terminal HA-tagged (or 3HA-tagged for SSE1) and expressed in the copper-inducible vector pCu426 (noted as pCu) (32). Importantly, all the tagged chaperones fully rescued the corresponding null strain (not shown). A chromosomal copy of SSE1 was also HA-tagged at the C terminus via the integration of a kanamycin cassette (33). Except where indicated, the Hsp70s were expressed in the absence of copper. Expression resulting from leakiness of the promoter closely approached physiological expression levels, as determined by immunoblot analysis with the corresponding antibodies (data not shown). In Fig. 2B, overexpression was achieved by the addition of 200 μM CuSO4. All experiments were done in the BY4743 background (MAT a/α his3Δ1/Δ1 leu2Δ0/Δ0 met15Δ0/Δ0 ura3Δ0/Δ0) and expressed as a -fold difference over the wild type and mutant cells were expressed as a -fold difference over the wild type.

Gel Filtration Analysis—Wild type cells with chromosomally tagged SSE1-3HA were grown in rich media, harvested in log phase, and lysed at 4 °C in buffer A (50 mM Tris, pH 7.5, 10 mM HEPES, pH 7.5, 100 mM KCl, 5 mM MgCl2, 1% Triton X-100) (34). The ribosomal pellets were resuspended, and the Hsp70-bound polypeptides in the supernatant were quantitated with ImageQuant software on a Typhoon PhosphorImager (Amersham Biosciences, Version 5.2) by measuring the radioactivity in each lane in gel exposures that were within the linear range of the instrument. All quantifications were normalized over the total amount of labeled proteins (i.e. the total radioactivity in the ribosomal fraction for Fig. 4 or total amount of labeled protein for Figs. 5 and 6). For pulse-chase samples, the label corresponding to newly synthesized Hsp70 and Hsp110 bands was subtracted from the total radioactivity in each lane. Immunoprecipitations were adjusted for equivalent protein amounts in each time point. To compare the effect of chaperone deletions on substrate binding to Hsp70s, the amount of Hsp70-bound nascent chains within paired experiments in wild type and mutant cells were expressed as a -fold difference over the Hsp70-bound nascent chains in wild type cells.

RESULTS

The Hsp70-like Sse1p Associates with the Cytosolic Hsp70s SSB and SSA—To better understand the function of Hsp70s in de novo folding, we analyzed the interactions of the ribosome-bound Hsp70 SSB. Upon immunoprecipitation of Ssb2p, a very prominent associated protein of higher molecular weight was readily observable (Fig. 1A, lane 1). Mass spectrometry analysis indicated that the SSB-associated protein was the Hsp110 homologue Sse1p. In like fashion, immunoprecipitation of Sse1p revealed association with two different polypeptides of ~70 kDa (Fig. 1B, lane 1). Mass spectrometry analysis revealed that one of the bands corresponded to Ssb1/2p, and the other band contained the cytosolic Hsp70s Ssa1/2p. The relative amounts of SSB and SSA in the Sse1p immunoprecipitation were comparable, suggesting that both Hsp70s associate to a similar extent with Sse1p (Fig. 1B, lane 1; see also Fig. 1C, lane 2). The interaction between Sse1p and Ssa1/2p was also observed in Δssb1/2 cells, indicating that the complexes can form independently of each other (Fig. 1B, lane 2). Immunoprecipitation of SSA and analysis of its associated proteins confirmed that SSA interacts with Sse1p, but it indicated that only a small fraction of SSA is in a complex with Sse1p (Fig. 1C, lane 1; see Fig. 2). The association between these Hsp70s was unaffected by the presence or absence of Mg2+-ATP in the lysis buffer (not shown) and was independent of the levels of expression of either Hsp70, as was observed using either plasmid-borne or chromosomally encoded chaperones (e.g. compare Fig. 1, B (lane 1) and C (lane 2), for plasmid-borne or chromosomally tagged Ssa1p, respectively). Of note,
Hsp110 Cooperates with Cytosolic Hsp70s

FIGURE 1. Interaction of Sse1p with the Hsp70 proteins Ssa1/2p and Ssb1/2p. A, SSB associates with Sse1p but not with SSA-Hsp70s. Wild type cells expressing HA-Ssb2p were lysed and immunoprecipitated with anti-HA antibodies as described under "Experimental Procedures." As a control a parallel analysis was carried out on cells transformed with the backbone vector alone (pCu). Ssb2p binding partners were identified by mass spectrometry. MW, molecular weight. B, Sse1p interacts directly with both Hsp70s Ssa1/2p, and Ssb1/2p and can associate with Ssa1/2p independently of Ssb1/2p. Wild type (WT) or ssb1/2 cells expressing 3HA-SSE1 from the pCu plasmid were analyzed for Sse1p-interacting proteins as in A. C, HA-Ssa1p associates with Sse1p but not with SSB. Wild type cells carrying pCu SSA1 or the pCu vector alone (lanes 1 and 3) were analyzed for Ssa1p-interacting proteins as in A. In parallel, Sse1p-interacting proteins were also immunoprecipitated from cells carrying a chromosomally tagged copy of SSE1–3HA (lane 2). All immunoprecipitations were separated by SDS-PAGE and either Coomassie-stained (A and B) or silver-stained (C). Positions of tagged and untagged Hsp70 chaperones are indicated as are the positions of the heavy chain (HC), light chain (LC), and bovine serum albumin (asterisk) that is added to the immunoprecipitation.

these immunoprecipitation experiments did not detect a direct interaction between SSA and SSB, suggesting that Sse1p forms two distinct complexes with SSA and SSB (Fig. 1, A and C). On the other hand, we did not observe the other Hsp110 homologue, Sse2p, in any of our immunoprecipitations. Experiments using tagged Sse2p indicated an association with SSA Hsp70s but failed to detect association with SSB (not shown). Because the stress-inducible Sse2p is expressed at much lower levels than Sse1p (13, 36, 37), it is possible that its function is less relevant than Sse1p under normal conditions. Alternatively, the two homologues may have distinct cellular functions whereby only Sse1p can associate with SSB Hsp70s. Notably, immunoprecipitation of HA-Ssa1p using antibodies against the epitope tag also isolated untagged Ssa1/2p, suggesting that this Hsp70 self-associates in vivo, as suggested by in vitro experiments using purified Hsp70 (38).

The Hsp110 Sse1p Forms Distinct Complexes with the Hsp70s SSA and SSB—We next examined the distribution of Hsp70-containing complexes in cell extracts using size exclusion chromatography (Fig. 2). Lysates obtained from wild type cells carrying a chromosomally integrated 3HA tag at the C terminus of SSE1 were fractionated on a Superdex S200 column, and the elution profiles of the Hsp70s SSA, SSB, and Sse1p were determined by immunoblot analysis (Fig. 2A). As previously described for mammalian Hsp70 (39), SSA associated with numerous cellular complexes and migrated very broadly in the separation, ranging in size from monomeric forms to high molecular weight complexes eluting in the void of the column (Fig. 2A). In contrast, SSB complexes were found primarily in two major peaks. One SSB complex of very high molecular weight eluted in the void together with the ribosomes (Fig. 2A) and probably corresponds to ribosome-bound SSB (20). The other broad peak displayed a molecular mass range between 60 and 200 kDa and probably corresponds to monomeric SSB and soluble complexes containing SSB. This is consistent with previous observations that SSB partitions between ribosome-bound and ribosome-free forms (20). The elution profile of Sse1p was more restricted than that of SSA or SSB (Fig. 2A). Very little Sse1p eluted at its expected monomeric molecular mass; instead, Sse1p was primarily found in a peak centered around a molecular mass of ~180 kDa, with a minor shoulder at ~210 kDa. Because the major Sse1p fraction corresponds in approximate size to a heterodimeric complex between Hsp110 and Hsp70 and the immunoprecipitation analysis suggests that most Sse1p is associated with either SSA or SSB (Fig. 1B), it appears that Sse1p is predominantly in complex with Hsp70. Additionally, a small fraction eluted together with the ribosomal peak.

To further examine the Sse1p-containing complexes, we immunoprecipitated individual column fractions for the endogenously tagged Sse1–3HAp followed by immunoblot analysis of SSA and SSB (Fig. 2B). Sse1p and SSA associated in a major complex of ~180 kDa. SSB also coimmunoprecipitated with Sse1p in defined complexes. One complex coeluted with the ribosomal fraction (lane 18), whereas the other complex peaked at ~180 kDa, with an elution profile almost identical to that of Sse1p (Fig. 2A). A minor peak at ~210 kDa, also observed in this analysis of Sse1p complexes with both Hsp70s, may correspond to Sse1p-Hsp70 complexes with either additional cofactors or substrates. The gel filtration analysis of these Hsp70s supports the idea that not all the SSA or SSB Hsp70s are in a complex with Sse1p, but most Sse1p forms complexes with either SSA or SSB. Because we did not detect a ternary SSA-SSE1-SSB complex (Fig. 1), it is likely that SSA and SSB complexes with Sse1p are mutually exclusive.

Genetic Interaction between Cytosolic Hsp70s—Given the high degree of homology between Sse1p and Sse2p (76% identity), the differences between their association with SSA and SSB prompted us to examine the genetic interactions between the two Hsp110 homologues (Fig. 3A). Deletion of SSE1, although viable, results in a very severe growth phe-
observed a less severe phenotype for the Δsse1 cells and a synthetic lethal effect for Δsse1Δsse2 (40). In any case, the genetic analysis demonstrates that SSE function plays a fundamental role in normal cell growth. Furthermore, in our genetic background, Sse1p is the major source of Hsp110 activity, in agreement with estimates that Sse1p is >10-fold more abundant than Sse2p (37).

The severity of the Δsse1 phenotype together with the observation that most Sse1p is associated with either SSB or SSA raised the possibility that Sse1p plays an important role in the function of these Hsp70s. Indeed, analysis of the consequences of Ssa1p or Sse1p overexpression in Δssb1/2 cells indicated that the interplay between these cytosolic Hsp70 systems is important for cellular function (Fig. 3B). Loss of SSB function results in a slow growth phenotype. Although low levels of expression of Ssa1p or Sse1p did not affect viability of either wild type or Δssb1/2 cells (Fig. 3B, −copper), overexpression of either Ssa1p or Sse1p in the Δssb1/2 background has a severe toxic effect on cell growth (Fig. 3B, +copper). Thus, there is an optimum balance between the functions of these cytosolic Hsp70s that has to be maintained for cell viability.

The SSB and SSA Hsp70s Associate with Distinct Subsets of Nascent Chains—Previous studies in mammalian systems found that Hsp70 assists de novo folding by interacting co- and post-translationally with newly made proteins (4, 35). To better understand the functional interplay between SSB, SSA, and Sse1p, we next examined their interaction with newly translated polypeptides. The SSB known ribosome association together with Sse1p migration in ribosome-containing fractions led us to initially assess Hsp70 substrate interactions that may occur either during or soon after polypeptide synthesis. Accordingly, we initially tested chaperone binding to both ribosome-associated nascent chains as well as completed full-length polypeptides already released from the ribosome (Fig. 4). Newly translated polypeptides were specifically labeled by a very short pulse with radiolabeled methionine to preferentially label polypeptides that are still bound to the ribosome (Fig. 4B, lane 3), although some proteins are completed and released during this pulse (Fig. 4B, lane 1). Cells were then depleted of ATP to stabilize Hsp70-substrate interactions and lysed in the presence of cycloheximide to stabilize ribosome-nascent chain complexes. To distinguish between co- and post-translational interactions, we separated the ribosome-bound nascent chains (R) from the released polypeptides in the soluble fraction (S) by ultracentrifugation through dense sucrose cushions (C) (Fig. 4, B–F, see Totals in lanes 1–6). After separation, ribosomal (R), sucrose (C), and supernatant (S) fractions were either directly analyzed by SDS-PAGE (Fig. 4, B–F, lanes 1–6) or subjected to immunoprecipitation to assess nascent chain interactions with the indicated chaperones (Fig. 4, B–F, lanes 7–15). Newly synthesized polypeptides were then detected by autoradiography.

We first examined nascent chain binding to SSB in wild type cells (Fig. 4B). We found that SSB associates mostly to ribosome-bound nascent chains found in the ribosomal fractions (Fig. 4B, lane 9), consistent with the results that SSB binds ribosomes in close proximity of the exit site (20). Notably, although many completed 35S-labeled proteins were already released into the soluble fraction (Fig. 4B, lane 1), only newly made SSB, Sse1p, and an additional polypeptide were observed in the SSB immunoprecipitation of the supernatant of the centrifugation (Fig. 4B, lane 7), suggesting that SSB associates preferentially with ribosome-bound nascent chains but less effectively with released full-length polypeptides. Because in vitro experiments suggested that another cytosolic Hsp70, Ssz1p, is required for nascent chain binding to SSB (21), we next examined SSB nascent chain binding in a Δssz1 strain. Notably, loss of Ssz1p did not diminish the proportion of SSB-bound polypeptides (Fig. 4B, compare lanes 9 and 12; Fig. 4G), indicating that this Hsp70 is
FIGURE 4. Nascent chain interaction with SSA, SSB, and Sse1p.

A, experimental design. Wild type (WT) or mutant cells were pulse-labeled for 1 min with [35S]methionine to preferentially label nascent chains. Ribosome-bound (R) and soluble (S) nascent chains were separated by ultracentrifugation through a sucrose cushion (C). Chaperone-substrate interactions were determined after immunoprecipitation (IP) of the various fractions with anti-HA antibodies, as described under “Experimental Procedures.”

B and C, binding of Ssb2p to nascent chains was assessed in wild type versus Δssz1 cells (B) and wild type versus Δsse1 cells (C). D and E, binding of Ssa1p to nascent chains was assessed in wild type versus Δssb1/2 cells (D) and wild type versus Δsse1 cells (E). F, binding of Sse1p to nascent chains was assessed in wild type and Δssb1/2 cells. Paired experiments for each panel were done
not required for association of SSB with nascent chains in vivo. However, this does not exclude that SSE1 may regulate other aspects of SSB function, such as polypeptide release or ATPase activity (22).

Because a substantial proportion of SSB was found in a complex with SSE1, we also considered that SSE1 may regulate polypeptide binding to SSB. Interestingly, we observed a marked increase in the proportion of SSB-bound nascent chains in Δsse1 cells over that observed in wild type cells (Fig. 4, C (compare lanes 9 and 12) and G). This suggests that SSE1 is not required for SSB binding to newly synthesized proteins but instead may assist in SSB-mediated processing of unfolded polypeptides.

We next examined the contribution of SSB to nascent chain binding in wild type and chaperone-deleted strains. To facilitate comparison between experiments, all Hsp70s contained the same epitope tag; accordingly, all chaperone immunoprecipitations were carried out using the same antibody on cell lysates containing comparable proportions of 35S-labeled proteins. In wild type cells immunoprecipitation of SSA in the ribosomal fractions revealed an interaction with nascent chains (Fig. 4D, lane 9), albeit at a much lower level than observed for SSB. In further contrast to SSB, the immunoprecipitation of SSA in the soluble fraction (S) revealed an association of this Hsp70 with many full-length polypeptides released from the ribosome (Fig. 4D, lane 7). This analysis, thus, suggests that SSA does not bind as efficiently as SSB to ribosome-bound chains but does associate post-translationally with newly made proteins. Strikingly, in Δssb1/2 cells the association of SSA with nascent chains increased about 10-fold (Fig. 4, C (compare lanes 9 and 12) and G). Therefore, it does appear that SSA can also interact cotranslationally with a broad spectrum of nascent chains when SSB is absent. Accordingly, the ribosome-bound SSB may effectively prevent SSA from early cotranslational binding. These results suggest that SSB is specifically recruited to the ribosome, whereas SSA is not and instead interacts post-translationally with newly made proteins.

We next tested whether SSA polypeptide association was affected in Δsse1 cells. Unlike what was observed for SSB, the level of SSA nascent chain binding was not substantially altered in Δsse1 cells relative to wild type cells (Fig. 4, E (compare lanes 9 and 12) and G). Thus, although SSE1 is not required for association of newly made polypeptides to SSA, this experiment does not reveal a significant role of SSE1 in the early interaction of SSA with ribosome-bound nascent chains.

Finally, we examined the interactions of SSE1 with newly synthesized proteins. Analysis in wild type cells revealed an association of SSE1 with both ribosome-bound nascent chains (Fig. 4F, lane 9) and released full-length proteins (Fig. 4F, lane 7), consistent with the idea that SSE1 interacts with SSB. However, the extent of interaction between newly made polypeptides and SSE1 was decreased relative to that of SSB and SSA. This could be caused by a higher substrate dissociation rate from this chaperone or could indicate that the association of SSE1 with substrate is indirect, possibly mediated by SSA or SSB. A parallel analysis of SSE1 interactions in Δssb1/2 cells indicated that SSE1 is not exclusively recruited to translating polypeptides by SSB. Thus, SSE1 is still associated with nascent chains and full-length proteins in Δssb1/2 cells (Fig. 4F). This association is probably mediated through a complex with SSA, since SSA takes over most nascent chain binding in the absence of SSE1 (Fig. 4D).

The Hsp70 Chaperones SSB, SSA, and SSE1 Interact Transiently with Newly Translated Polypeptides during Their Biogenesis—Molecular chaperones have been shown to facilitate de novo folding by transiently associating with newly synthesized proteins until they reach the native state (2, 35, 41, 42). To examine the relative contributions of SSB, SSA, and SSE1 to de novo folding, we analyzed the flux of newly translated polypeptides through these Hsp70 proteins (Fig. 5). Newly translated proteins were 35S-labeled with a short pulse, and chaperone interactions during folding were followed by a chase in unlabeled media. The kinetics of transit through different Hsp70 systems was examined by immunoprecipitation of HA-tagged chaperones.

Immunoprecipitation of SSB-associated polypeptides revealed SSB binding to a broad spectrum of newly made polypeptides at the earliest time point (Fig. 5B, lane 1). However, these rapidly dissociated from SSB after only 1 min of chase (Fig. 5B, lanes 1 and 2 and E; see also Fig. 6A). The diffuse nature of the immunoprecipitated polypeptides in the earliest time point together with the rapid dissociation kinetics is consistent with our observation that SSB interacts with a large proportion of ribosome-bound polypeptides (Fig. 4). Only few full-length proteins remained associated to SSB during the later chase times and dissociated with slower kinetics (Fig. 5B, lanes 2–7). Together these results indicate that SSB associates with nascent chains and that few proteins stay bound to SSB after completion of synthesis.

SSA also associated transiently with newly made polypeptides (Fig. 5, C and E). However, the spectrum of SSA-bound polypeptides and the kinetics of interaction differed from those observed for SSB (Fig. 5C, lanes 1–7; see also Fig. 6B). Polypeptides dissociated more slowly from SSA than from SSB, with many proteins still bound after 6 min of chase (Fig. 5, C (lane 5) and E). In addition, most SSA-bound proteins were larger than 36 kDa. Given that a single folding domain is ~25–30 kDa, this suggests most SSA-bound polypeptides are slow folding multidomain proteins. Interestingly, similar results were obtained upon analysis of protein flux through mammalian Hsp70 (35). A similar pulse-chase analysis measuring the flux of newly synthesized proteins through SSE1 indicated that SSE1 also associates transiently with newly synthesized proteins (Fig. 5D, lanes 1–6). SSE1 bound a diffuse smear of proteins ranging from ~20–200 kDa early in the time course (Fig. 5D, lanes 1 and 2). At later chase times SSE1 interacted with full-length proteins with dissociation kinetics similar to those observed for SSA (Fig. 5, D and E).

SSE1 Modulates the Association of SSB and SSA with Newly Synthesized Polypeptides—Our finding that loss of SSE1 altered the interaction between SSB and ribosome-bound nascent chains (Fig. 4) led us to examine the effect of SSE1 on the flux of nascent chains through SSB and SSA. Initially, we examined the transit of newly translated polypeptides through SSB in both wild type and Δsse1 cells (Fig. 6A). Although SSB still interacted transiently with newly made proteins in Δsse1 cells, loss of SSE1 caused more than a 4-fold increase in the fraction of SSB-bound polypeptides over that bound in wild type cells (Fig. 6A). This analysis indicates that SSE1 is not required for polypeptide binding to SSB nor is it essential for polypeptide release. However, SSE1 modulates the SSB-substrate interaction either by regulating its affinity or the rate of release during folding of newly synthesized proteins.
We next examined the effect of \( \text{SSE1} \) on SSA-substrate interactions. We reasoned that the very short pulse used in Fig. 4, which primarily labeled partially synthesized ribosome-bound polypeptides, may not have revealed the effects of \( \text{SSE1} \) on SSA, which appears to bind primarily to full-length polypeptides. Instead, the pulse-chase analysis used to assess the flux of newly made polypeptides through Hsp70 uses a longer pulse (2.5 min) that already labels full-length polypeptides and allows us to observe later effects, including changes in the rates of polypeptide release. Strikingly, when we compared polypeptide transit through SSA in wild type and \( \Delta \text{sse1} \) cells, we observed that SSA associated transiently with a similar spectrum of proteins in wild type and \( \Delta \text{sse1} \) cells, but the level of association was 3–4-fold higher in cells lacking Sse1p (Fig. 6B). Thus, the absence of Sse1p increased the proportion of newly made polypeptides associated to SSA, as observed for SSB. These experiments indicate that Sse1p enhances substrate binding to both SSB and SSA by comparable levels without noticeably affecting the specificity of the Hsp70s for their client proteins. These findings are consistent with our observations that Sse1p is not required to recruit these Hsp70s to bind polypeptide substrates (Fig. 4, C and E) and indicate that Sse1p modulates the activity and substrate interactions of both Hsp70s SSA and SSB by a similar mechanism of action.

**FIGURE 5.** Newly synthesized proteins transit with different kinetics through the cytosolic Hsp70s SSB, SSA, and Sse1p. A, experimental design. Cells expressing HA-tagged chaperones were pulse-labeled with \( \text{[35S]} \)methionine, chased with cold methionine for the indicated time points, and analyzed as described under “Experimental Procedures.” Chaperone-substrate interactions at the indicated chase times were determined after immunoprecipitation with anti-HA antibodies. Control immunoprecipitations were carried out with a nonimmune antibody. B–D, the flux of newly translated proteins through SSB (B), SSA (C), and Sse1p (D) was assessed in wild type cells. NI, nonimmune antibody. The total fraction (Tot, lane 8) represents 5% of the total labeled sample at 0 min of chase and highlights the reproducibility of \( \text{[35S]} \) labeling between experiments. E, quantification of the flux of newly translated proteins through the different Hsp70s. Results are representative of at least three independent experiments.
In this study we characterize the interplay of the major cytosolic Hsp70 systems during de novo folding. We find that most of the cytosolic pool of the yeast Hsp110 homologue Sse1p is found in complexes with either of the two major cytosolic Hsp70s, SSB and SSA (Figs. 1 and 2). Consistent with these findings, non-denaturing PAGE analysis by Shaner et al. (Ref. 49, the companion study) also indicates that cytosolic Sse1p is found exclusively in complexes with either SSA or SSB. It, thus, appears that the cellular role of Sse1p is functionally linked to these Hsp70s. The severe growth defect of Δsse1 cells indicates that the Hsp110 Sse1p plays an important role regulating the function of both Hsp70s (Fig. 3A). The idea of a functional interplay between cytosolic Hsp110s and the Hsp70s SSB and SSA is further supported by our findings that Sse1p or Ssa1p overexpression is toxic in Δsse1/Δssb1/2 cells (Fig. 3B). All three Hsp70s, SSB, SSA, and Sse1p, transiently associate with newly synthesized polypeptides (Fig. 5), which suggests a role in de novo folding. SSA appears to interact primarily with ribosome-bound nascent chains, although SSA instead associates mostly post-translationally with newly made proteins (Fig. 4). Although Sse1p is not essential for polypeptide binding by either SSB or SSA (Figs. 4 and 6), we find that polypeptide association to the Hsp70s SSA and SSB is considerably enhanced in Δsse1 cells. Accordingly, we propose that Sse1p functions to modulate the activities of SSA and SSB during folding of newly made proteins.

Distinct Roles of Cytosolic Hsp70s in de Novo Folding—We find that the three cytosolic Hsp70s, SSA, SSB, and Sse1p, transiently associate with newly synthesized polypeptides (Fig. 5). Notably, the distinct spectrum and kinetics of polypeptide flux through these Hsp70s suggest distinct functions in de novo folding. The predominant association of SSB with ribosome-bound nascent chains (Fig. 4B) together with the rapid kinetics of polypeptide dissociation from SSB (Fig. 5B) suggest that the primary function of this Hsp70 occurs early during synthesis and is probably to protect emerging ribosome-bound nascent chains (Fig. 6). However, SSB function can be partially replaced by SSA (Fig. 4D) or by other chaperone systems (43). This may explain why full-length proteins such as ornithine transcarbamoylase or von Hippel-Lindau tumor suppressor protein are observed to need SSA for folding (16, 17), whereas, no proteins have been shown to require SSB. On the other hand, SSA associates with few ribosome-bound nascent chains and binds predominantly polypeptides released from the ribosome (Fig. 4D). Notably, SSA can switch to a cotranslational mode of interaction when SSA is absent, suggesting this chaperone may block access of SSA to the ribosome exit site (Fig. 4D). In pulse-chase analysis the spectrum of SSA-bound polypeptides consists mostly of full-length proteins of greater than 40 kDa; these polypeptides dissociate from SSA with significantly slower kinetics than for SSB (Fig. 5E). Sse1p also associates with both ribosome-bound nascent chains and with full-length

FIGURE 6. Sse1p modulates the flux of newly synthesized proteins through SSA and SSB. To determine the effect of SSE1 on the association of newly synthesized polypeptides with SSB and SSA, wild type (WT) and Δsse1 cells expressing HA-SSB2 (A) or HA-SSA1 (B) were pulse-labeled with [35S]methionine, and chaperone-substrate interactions were analyzed and quantified as in Fig. 5. NI, control immunoprecipitations (IP) with a nonimmune antibody. Chaperone-bound polypeptides were quantified as described under "Experimental Procedures" and expressed as a-fold difference over SSB- or SSA-bound polypeptides at 0 min in wild type cells.
polypeptides (Figs. 4F and 5E). As discussed below, it is unclear at this point whether the interaction is direct or only mediated by its association with either SSB or SSA.

Our data argue for a sequential pathway of interactions of different Hsp70s with newly made polypeptides. It is likely that the early interaction of SSB with most nascent chains is mediated by its direct affinity for the ribosome (20). This interaction may suffice to fold smaller proteins, whereas larger multidomain proteins may require SSA to prevent folding intermediates from engaging in inter-molecular "domain swapping" that may lead to aggregation. Although early interaction with SSB is clearly optimal for folding, as evidenced by the slow growth phenotype of Δssb1/2 cells, the sequential pathway is not obligate because SSA can interact with nascent chains when SSB is deleted.

The Hsp110 Sse1 Regulates Hsp70-Substrate Interactions—We find that Sse1p binds to two different cytosolic Hsp70s and regulates their interactions with substrates. Our finding that both SSB and SSA have an enhanced association with newly made polypeptides in the absence of Sse1p raises several possible mechanisms for Hsp110-mediated regulation of Hsp70s. One option is that the Sse1p/Hsp70 heterodimer has reduced affinity or a slower binding kinetics for polypeptide binding than Hsp70 homodimer complexes. However, this scenario is unlikely given the large excess of SSB and SSA over Sse1p in the cytosol (4- and 9-fold, respectively (37)). Because even in wild type cells most SSB and/or SSA are not in a heterodimer with Sse1p and would be available to bind polypeptides, a lower substrate affinity or slower on-rate Sse1p-containing complex would be unable to compete effectively for polypeptide binding. More plausibly, Sse1p may modulate the Hsp70-substrate interaction once it is established. For instance, it may cooperate with SSA and SSB to promote folding such that folded polypeptides would not return to Hsp70s for binding. Alternatively, Sse1p may act catalytically to regulate the release of substrate from SSB and SSA, based on the reported effects of Hsp110s on Hsp70 ATPase activity. These possibilities are not mutually exclusive, and it is conceivable that more than one describes the function of Hsp110s in the cell.

Because ATP binding and hydrolysis are essential to regulate Hsp70 substrate binding and release, our findings on the role of Sse1p resonate with studies suggesting that Hsp110s can regulate Hsp70 ATPase activity. For instance, in the endoplasmic reticulum, a distant Hsp110 homologue, Lhs1p, stimulates the ATPase activity of the endoplasmic reticulum Hsp70 Kar2p by acting as a nucleotide exchange factor, whereas Kar2p coordinately regulates Lhs1p activity by stimulating its ATPase (30). Interestingly, mammalian Hsp110 has been reported to inhibit the ATPase activity of nucleotide-loaded Hsp70 (29), which could be consistent with an Hsp110 nucleotide exchange activity. In addition, Shaner et al. (49) report that Sse1p can stimulate the ATPase of SSA in conjunction with its J-protein Ydj1. Because nucleotide exchange by Hsp70 promotes polypeptide release, the suggestion that Sse1p may act as a nucleotide exchange factor is consistent with our functional analysis. Indeed, the enhanced association of newly made proteins with SSA and SSB in ∆sse1 cells (Fig. 6) is consistent with the idea that Sse1p could function as a nucleotide exchange factor that causes substrate release.

A Pathway of Hsp70 Interactions during de Novo Folding—Previous studies showing that different chaperone systems act sequentially to promote folding in the cell focused on the cooperation of Hsp70 with other classes of chaperones such as chaperonins, Hsp90 or Hsp104 (4, 17, 44, 45). Remarkably, our data suggest sequential cooperation between different types of Hsp70s whereby SSB interacts first with nascent chains as they emerge from the ribosome, whereas SSA interacts later with a more restricted subset of larger proteins (Fig. 7). What may be the function of such a pathway? Coupling of SSB binding to transla-

\textbf{FIGURE 7. A model for sequential chaperone interactions during de novo folding.} SSA and SSA form complexes with Sse1p, where Sse1p may have a role in modulating Hsp70 activity. These chaperone systems together can create a network for the folding of newly synthesized proteins. Ribosome-associated SSB Hsp70 has first contact with the emerging nascent chain. Restricted subsets of slow folding polypeptides may then interact co- or post-translationally with downstream chaperones such as the Hsp70-SSA or the TRIC/CCT (chaperonin tailless complex polypeptide 1 (TCP1) ring complex/chaperonin containing TCP1).

Notably, our findings of sequential cooperation between SSB and SSA bear striking parallels to the Escherichia coli chaperones trigger factor and the Hsp70 DnaK (41, 42). In E. coli, the trigger factor binds directly to the ribosome where it recognizes shorter nascent chains, whereas the Hsp70 DnaK binds mostly post-translationally to larger proteins. Furthermore, the substrates of DnaK are also predominantly large proteins that probably have more than one folding domain (41, 42), similar to what is observed for SSA-Hsp70 (Fig. 5) and for mammalian Hsp70 (35). Therefore, two structurally different chaperones, trigger factor and DnaK, stabilize and fold newly synthesized proteins in E. coli. In contrast, in the eukaryotic cytosol these functions appear to involve the cooperation of different Hsp70s. This feature of eukaryotes may afford the possibility of regulated binding and release, as Hsp70s are influenced by various cofactors, such as J-domain and nucleotide exchange factors (2, 7, 10, 46). Thus, substrate binding to SSA is regulated by the J-domain proteins Ydj1p and Sis1p, whereas substrate binding to SSB is thought to be regulated by the J-domain protein Zuo1p. Our observation that substrate interactions with both SSB and SSA is in turn regulated by another Hsp70-like protein, the Hsp110 Sse1p, may add another layer of complexity and regulation to de novo folding in the eukaryotic cytosol. Interestingly, there are no identified homologues of SSB in mammalian cells. Instead, Hsp70 binds to polypeptides both co- and post-translationally (4), while probably also interacting with Hsp110 (39). Addi-
tional regulatory features are also conserved in mammalian cells in light of the recent characterization of the mammalian Zuo1p homologue (47, 48). Perhaps the functions of SSB and SSA are encompassed by the mammalian Hsp/Hsc70 homologues, raising the possibility of a handover between ribosome-bound Hsp70 and soluble Hsp70.

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