The Refolding Activity of the Yeast Heat Shock Proteins Ssa1 and Ssa2 Defines Their Role in Protein Translocation

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Abstract. Ssa1/2p, members of one of the yeast cytosolic hsp70 subfamilies, have been implicated in the translocation of secretory proteins into the lumen of the ER. The involvement of these hsp70s in translocation was tested directly by examining the effect of immunodepleting Ssa1/2p from yeast cytosol and subsequently testing the cytosol for its ability to support co- and posttranslational translocation of prepro-α-factor. Depletion of Ssa1/2p had no effect on the efficiency of translocation in this in vitro assay. The system was used to examine the effect of the absence of Ssa1/2p on two other putative hsp70 functions: cotranslational folding of nascent luciferase and refolding of denatured luciferase. Depletion of Ssa1/2p had no effect on the ability of the yeast lysate to synthesize enzymatically active luciferase, but had a dramatic effect on the ability of the lysate to refold chemically denatured luciferase. These results demonstrate, for the first time, the refolding activity of Ssa1/2p in the context of the yeast cytosol, and define refolding activity as a chaperone function specific to Ssa1/2p, apart from other cytosolic hsp70s. They also suggest that Ssa1/2p do not play a significant role in chaperoning the folding of nascent polypeptides. The implications of these findings for Ssa1/2p activity on their proposed role in the process of translocation are discussed.

THE translocation of nascent secretory and membrane proteins into the endoplasmic reticulum (ER) represents their entry into the secretory pathway and the first step in targeting to a number of subcellular organelles (36). Although models of translocation have stressed the cotranslational, signal recognition particle (SRP)[1]-dependent nature of the process, the yeast *Saccharomyces cerevisiae* has developed an alternative, SRP-independent means of translocation that is sufficient to maintain cell viability in the absence of SRP (25). The severity of the translocation defect caused by the absence of SRP varies with different proteins, leading to the suggestion that some proteins preferentially use an SRP-independent pathway, while others can adapt to the absence of SRP (3, 25). Two scenarios have been suggested to account for SRP-independent translocation. Elongation of constitutively SRP-independent proteins may be slow enough to allow the ribosome to interact with the membrane, allowing cotranslational translocation independent of SRP and its receptor to ensue. Alternatively, the secretory protein may be released from the ribosome into the cytosol and subsequently translocated across the ER membrane in a posttranslational manner (48).

Posttranslational translocation has been shown to occur in yeast systems both in vivo and in vitro (24, 26, 27, 39, 41, 47, 49). An important prerequisite for posttranslational translocation must be the maintenance of the preprotein in a translocation-competent conformation before the initiation of translocation. Cytosolic members of the hsp70 family of heat shock proteins have been postulated to serve as molecular chaperones for nascent polypeptides in the process of protein translocation into the ER (5, 18, 28, 29, 38, 52). In yeast, two particular hsp70s, Ssa1p and Ssa2p, have been implicated in this process (11, 17). Ssa1p and Ssa2p are constitutively expressed and nearly indistinguishable biochemically, while Ssa3p and Ssa4p are not expressed under normal growth conditions (12). The results of two investigations support the proposal that Ssa1/2p are involved in protein translocation. First, posttranslational translocation into yeast microsomes of prepro-α-factor that was synthesized in a wheat germ lysate, and thus incapable of being translocated, was stimulated by the addition of Ssa1/2p in combination with other cytosolic factors (11, 17). Second, prepro-α-factor, as well as the precursor of the β-subunit of mitochondrial F_1ATPase, accumulated in the cytosol in vivo when the plasmid-based expression of Ssa1p was repressed in a strain containing chromosomal deletions in the *SSA1*, *2*, and *4* genes (17). From these studies, it was concluded that these Ssa proteins are involved in posttranslational translocation in yeast.

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; G-6-PD, glucose-6-phosphate dehydrogenase; SRP, signal recognition particle.

As molecular chaperones, hsp70s are involved in diverse cell processes. In various systems, hsp70s have been shown to interact with nascent chains in the process of translation, assisting in both co- and posttranslational protein folding, as well as playing roles in targeting proteins for degradation, heat shock response, and vesicle trafficking (12). In *S. cerevisiae*, there are currently ten identified hsp70 genes, six of which are constitutively expressed and have protein products localized to the cytosol (4, 33, 46). Of the cytosolic hsp70s, the *SSA* subfamily is the only essential subfamily (4). The *Ssb* subfamily, although not essential, is also of interest in this context. Since Ssb1/2 have been shown to be associated with ribosomes through an interaction with the nascent chain, they are therefore thought to be involved in translation (34). The specific tasks of the different hsp70s have yet to be delineated.

In addition to their presumptive role in protein translocation, Ssa1/2p have been shown by in vitro biochemical assays to participate in the prevention of protein aggregation and in refolding of denatured proteins (16, 31). Furthermore, Ssa2p has the highest clathrin uncoating activity measured for yeast hsp70s in vitro (22). Other potential functions for Ssa1/2p have yet to be identified and it is unknown which activity or activities of Ssa1/2p are essential to the cell. The requirement of Ssa1/2p for cell viability, however, raises the possibility that the translocation defect observed as a function of in vivo depletion of Ssa1p is a result of decreased cell viability rather than a direct result of the absence of Ssa1/2p on translocation. Posttranslational translocation has been effectively characterized by in vitro studies (23, 24, 26, 27, 37, 39, 41, 47, 49, 50). To assess the role of Ssa proteins in translocation, independent of other cellular processes, an in vitro approach was taken in which Ssa1/2p was immunochemically depleted from a yeast cellular extract which was then assayed for its ability to support posttranslational translocation.

Materials and Methods

Antibodies and Reagents

Rabbit polyclonal antibodies were generated against full-length Ssa1p, purified from *E. coli* using a six-histidine tag placed at the COOH terminus of the coding region. The COOH-terminal antibody was raised against a bacterially synthesized peptide containing the COOH-terminal 126 amino acids of Ssa1p tagged with six-histidines on the COOH terminus and four-histidines on the NH_2 terminus. Ssb antibodies were generously given by Dr. M. Werner-Washburne (University of New Mexico, Albuquerque, NM) and Dr. E. Craig (University of Wisconsin, Madison, WI). Ydj1p antibodies were a gift from Dr. M. Douglas (University of North Carolina, Chapel Hill, NC). Antibodies against glucose-6-phosphate dehydrogenase (G-6-PD) and luciferase were purchased commercially (Sigma Chem. Co., St. Louis, MO; Promega, Madison, WI, respectively). Luciferase was purchased from Sigma Chem. Co. and the luciferase assay system was purchased from Promega.

Cell Culture Conditions

S. cerevisiae strain ABYS1 (MATa *pra1 prb1 prc1 cps1 ade*) (1) was grown to early log phase and used to prepare cytosolic extract as previously described (40). To induce heat shock proteins seen in Fig. 1 *B*, ABYS1 cells were incubated for 2 h at 39°C. Lysate proteins were separated using 10% SDS-PAGE or two-dimensional gel electrophoresis (2D) with 7.5% SDS-PAGE.

Immunodepletion

Lysate was recirculated over a protein A–agarose (Schleicher and Schuell, Keene, NH) column containing affinity-purified rabbit antibodies to either the COOH-terminal 126 amino acids of Ssa1p or aprotinin for 45 min. The lysates (flowthroughs) were collected, the columns were washed with lysate buffer (100 mM potassium acetate, 2 mM magnesium acetate, 20% glycerol, 20 mM Hepes, pH 7.4, 2 mM DTT), and the bound fraction was eluted with 100 mM glycine, pH 2.5.

Immunoblotting

The immunoblot was visualized using chemiluminescent substrate (Renaissance, New England Nuclear, Wilmington, DE). For quantitation of depletion, serial dilutions of depleted and mock-depleted lysate were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antisera directed against Ssa1p and G-6-PD. The filter-bound antibodies were decorated with ^{35}S-Protein A (Amersham Life Sciences, Arlington Heights, IL) and quantified using phosphorimaging (Molecular Dynamics, Sunnyvale, CA). The G-6-PD present was used as a control for the amount of lysate proteins loaded in each lane; the amount of Ssa1p was normalized using G-6-PD. In four separate column runs, the amount of Ssa1/2p present in the depleted lysate ranged from 4% to <1% of that remaining in the mock-depleted lysate (data not shown).

Translocation

A modified prepro-α-factor construct (43) was translated in yeast lysate, as previously described (7) using a 100-μl reaction mixture, either in the presence (cotranslational) or absence (posttranslational) of membranes for 45 min. The cotranslational translocation reactions were processed for immunoprecipitation immediately after the translation reaction. The posttranslational translocation reactions were treated with cycloheximide (0.1 μg/ml, final concentration) for 5 min at 25°C to halt translation, and supplemented with an energy regeneration system. Membranes were added and the translocation was allowed to proceed for 45 min at 25°C. Membranes were prepared as previously described (53) with the final pellet being resuspended in 0.25 M sucrose, 50 mM potassium acetate, 20 mM Hepes, and 1 mM DTT. Antisera directed against prepro-α-factor (40) was used for immunoprecipitation. Immunoprecipitates were separated by 16% SDS-PAGE. Bands were quantified using phosphorimaging.

Luciferase Synthesis

Transcription of Luciferase. Luciferase mRNA was transcribed from pH LucS4 (received as a gift from Dr. J. McCarthy, National Biotechnology Research Center, Braunschweig, Germany) as follows. The plasmid was linearized with NsiI (Boehringer Mannheim, Indianapolis, IN) and the digestion reaction was added to a T7 transcription reaction (Promega) following manufacturer's directions, except that after incubation at 40° for 10 min, the reaction mixture was supplemented with 1 μl of 40 mM GTP. Incubation was continued for an additional 30 min at 40°C. The transcription reaction was stored as aliquots at −80°C.

Luciferase synthesis. The translation reaction contained ~7.5 A_{280} U/ml of yeast lysate in lysate buffer. The 100-μl translation reaction, containing 4 μl of the transcription reaction, was essentially as described previously (7), except the concentration of potassium acetate was adjusted to 160 mM, and the concentration of magnesium acetate was adjusted to 4.8 mM. Translation was carried out at 25°C. At 30-min intervals, 10 μl of the translation reaction was assayed in 100 μl luciferase assay buffer for 20 s in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). An additional 20 μl of the synthesis reaction was removed, diluted in 40 ml solubilization buffer (55 mM Tris, pH 7.5, 7.5 mM EDTA, 1.14% SDS, 15 mM DTT, and 1.5 mg/ml PMSF) to stop protein synthesis and stored on ice for subsequent immunoprecipitation. Luciferase was immunoprecipitated from the reaction using a commercially available polyclonal antibody. The products were assayed by SDS-PAGE and quantified using phosphorimaging. Specific activity values were calculated as a ratio of enzyme activity: amount of full-length protein.

Renaturation of Luciferase

Luciferase (12 μM) was denatured in buffer A (25 mM Hepes-KOH, pH 7.5, 50 mM potassium acetate, 5 mM magnesium acetate, 5 mM DTT), plus 6 M guanidine HCl. The solution was incubated at 25° for 1 h. Reaction mixtures in a final volume of 48 μl, containing yeast lysate (protein concentration ~8.2 A_{280} U/ml), lysate buffer, supplemented with 2 μl 25 mM ATP and 2 μl buffer A or 2 μl Ssa1p in lysate buffer (where indicated), were prepared on ice. The denatured luciferase was diluted 1:40 in buffer

A. Diluted luciferase (2 μl) was added to the reaction mixture and the reaction was incubated at 25°. At 15-min intervals, 2 μl were withdrawn from the reaction and the activity of luciferase was assayed using 50 μl of a luciferase assay system for 10 s. Duplicate readings were made at each time point and the average value used for analysis. For the Western blot, 2 μl of the reaction mix were removed at the end of the renaturation assay and separated by 10% SDS-PAGE.

Purification of Ssa1p. Purification of Ssa1p was essentially as described (13) using yeast strain MW332 (a gift from Dr. M. Werner-Washburne, University of New Mexico, Albuquerque, NM), which carries disrupted genomic copies of SSA1, SSA2, SSA3, and SSA4, with plasmid-based expression of SSA1 under galactose control. Ssa1p was eluted with 3 mM ATP and dialyzed against yeast lysate buffer.

Results

Immunodepletion of Ssa1/2p

The success of the immunodepletion approach depended upon obtaining antibodies capable of discriminating Ssa1/2p from other cytosolic hsp70s. Rabbit polyclonal antibodies were generated against full-length Ssa1p (anti-Ssa1p) and against the COOH-terminal 126 amino acids of Ssa1p (anti–C-Ssa1p). Both antisera recognized Ssa1p and Ssa2p, but not Ssb1/2p on two-dimensional immunoblots (Fig. 1 *A*), although anti-Ssb weakly recognizes both Ssa proteins. Both anti-Ssa1p antibodies also recognized Ssa3p and Ssa4p when these proteins were induced by heat shock (Fig. 1 *B*). As previously reported (51), neither Ssa3p nor Ssa4p are expressed under normal growth conditions. Therefore depletion of Ssa1p and Ssa2p from lysates prepared from cells grown under normal growth conditions constitutes the removal of all cellular Ssa subfamily proteins.

Affinity columns containing an anti–C-Ssa1p antibody proved to be quite effective in specifically depleting the yeast cytosolic fraction (lysate) of Ssa1/2p. Columns composed of affinity-purified polyclonal antibodies raised against aprotinin were run in parallel to generate the mock-depleted lysate that was used as the control for all assays. Aliquots of lysate were recirculated over antibody columns for 45 min. The column flowthroughs were collected and used for subsequent assays. The antibody columns were washed and proteins bound to the columns were eluted with 100 mM glycine, pH 2.5. Immunoblotting and two-dimensional gel electrophoresis revealed the level of Ssa1/2p remaining in depleted and mock-depleted lysates (Fig. 2). Only Ssa1/2p were retained by the antibody column and there was no nonspecific protein binding detected. The level of depletion was consistently >96% compared to mock-depleted controls. The process of immunodepletion of Ssa1/2p had relatively little effect on the lysate levels of Ssb1/2p or Ydj1p (Fig. 3, compare depleted and mock-depleted lanes).

Effect of Ssa1/2p Depletion on Translocation

To assess the role of Ssa1/2p in translocation, the efficacy of the depleted lysate in supporting the co- and posttranslational translocation of prepro-α-factor was compared to that of the mock-depleted as well as the untreated lysate. Prepro-α-factor mRNA, transcribed from a construct modified to remove the three glycosylation sites (43), was translated in lysates that were supplemented with yeast microsomes either co- or posttranslationally.

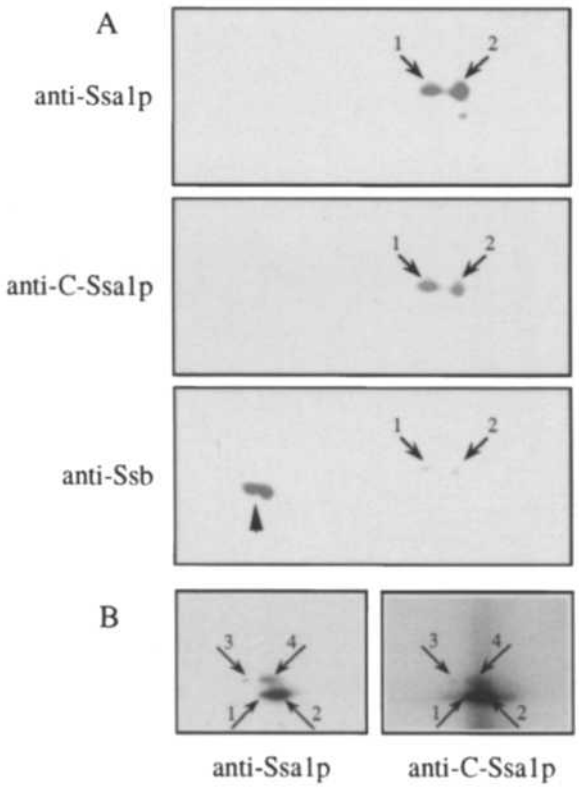

Figure 1. Anti-Ssa1p and anti–C-Ssa1p recognize Ssa proteins but not Ssb proteins. (*A*) Lysate prepared from cells grown under normal growth conditions was separated by 2D gel electrophoresis, transferred to a nitrocellulose membrane, and sequentially immunoblotted with anti-Ssa1p, anti–C-Ssa1p, and anti-Ssb. The three panels are pictures of the same blot, stripped, and checked for residual activity before staining with the next antibody. The difference in staining of Ssa1p and Ssa2p between the two top panels reflect differences in antibody affinity. The arrows indicate Ssa1p (*1*) and Ssa2p (*2*); the arrowhead in the third panel indicates Ssb1/2p. (*B*) Lysate prepared from cells exposed to heat shock conditions (39°) was separated by 2D gel electrophoresis and transferred to nitrocellulose. Duplicate blots were stained with anti-Ssa1p and anti–C-Ssa1p. Ssa1-4p are indicated by numbered arrows.

Translocation was measured by the appearance of the signal sequence–cleaved form of pro-α-factor and verified by protease protection (data not shown). The level of translation was the same in the three lysates (Fig. 4 *A*). Depletion of Ssa1/2p had no detectable effect on the amount of co- or posttranslational translocation (Fig. 4, *A* and *B*) nor on the rate of posttranslational translocation per se (Fig. 4 *C*). Since it is conceivable that the yeast microsomes added to the translocation reaction served as a sufficiently large source of Ssa1/2p to functionally compensate for the depletion, the Ssa1/2p content of the entire translocation reaction was tested by immunoblotting (Fig. 5). Additional Ssa1/2p was not detected in reactions containing membranes. Thus, despite the correlation between loss of Ssa1p and accumulation of prepro-α-factor observed in vivo (17), depletion of Ssa1/2p in vitro had no discernible effect on either co-translational or posttranslational translocation activity.

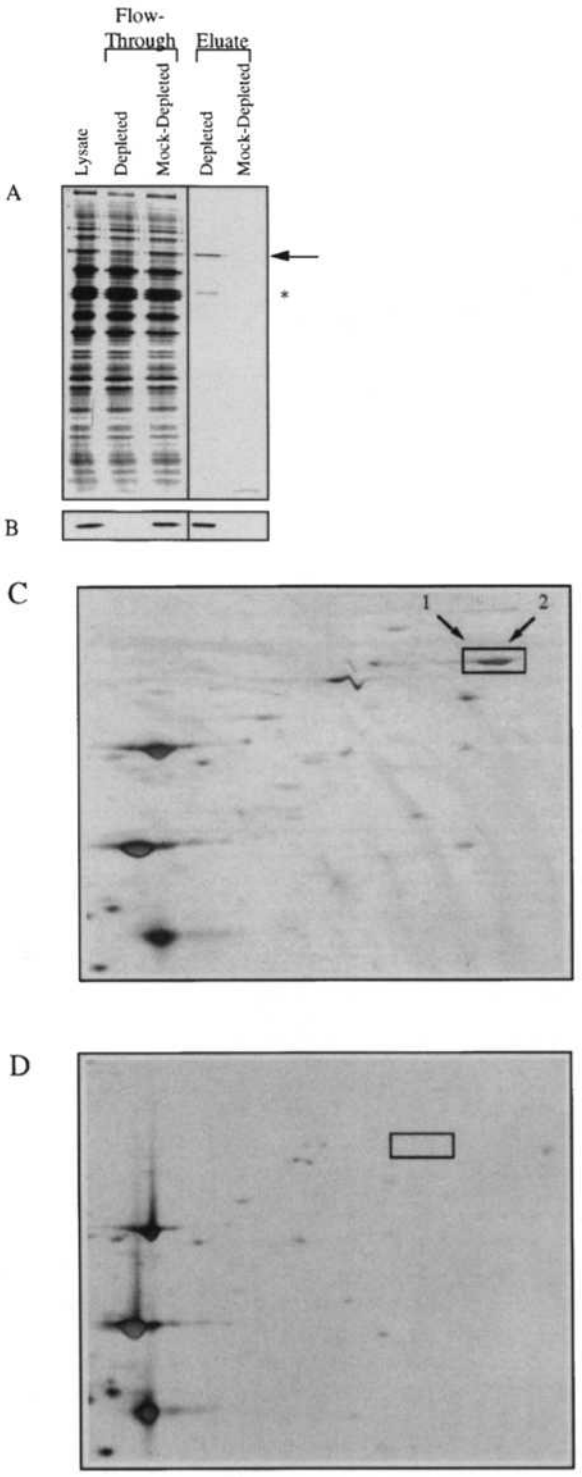

Figure 2. Anti–C-Ssa1p antibody affinity chromatography efficiently depletes Ssa proteins from yeast lysate. Yeast lysate was recirculated over an antibody column containing affinity-purified antibodies to either Ssa1p (*Depleted*) or aprotinin (*Mock-Depleted*). After washing, the columns were eluted with 100 mM glycine, pH 2.5. The starting material (*Lysate*), the column flowthroughs, and the eluates were separated by SDS-PAGE and visualized by (*A*) silver staining or (*B*) by immunoblotting with anti-Ssa1p. The arrow indicates full-length Ssa1p and the asterisk marks a band consistent with a reported degradation product (20). The (*C*) mock-depleted and (*D*) depleted column flowthroughs were also separated by 2D gel electrophoresis and visualized by silver staining. The numbered arrows in *C* indicate Ssa1p and Ssa2p.

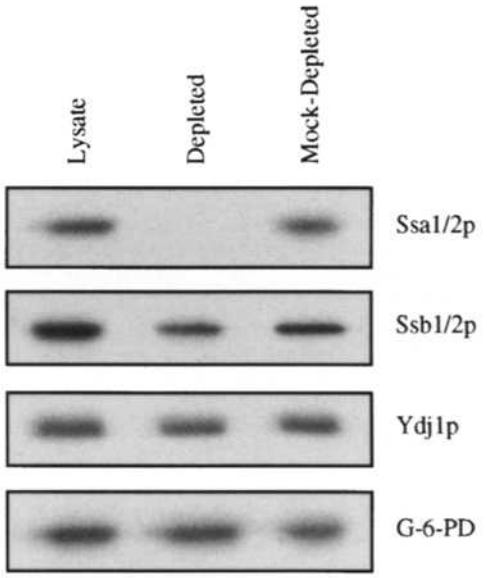

Figure 3. Depletion is specific for Ssa1/2p. Starting lysate, depleted lysate, and mock-depleted lysate were separated by SDS-PAGE. Duplicate lanes were decorated with antibodies against full-length Ssa1p, Ssb, or Ydj1p. Depletion of Ssa1/2p had little effect on the levels of Ssb1/2p or Ydj1p. The presence of G-6-PD, measured in the same blot as Ssa1/2p, indicates the relative amounts of protein loaded in each lane.

Effect of Ssa1/2p Depletion on Cotranslational Protein Folding

To show that depletion of more than 96% of Ssa1/2p was capable of affecting at least one postulated function, nascent chain folding and protein refolding activities were compared in depleted and mock-depleted lysates. Hsp70 has been shown to interact with nascent polypeptides (2, 42) and has been proposed to mediate protein folding and to maintain nascent polypeptides in an unfolded state (28). Moreover, firefly luciferase has been shown to fold cotranslationally (19, 30, 32) and to require the cotranslational presence of hsp70 for the acquisition of activity (21). In reticulocyte lysate, even the partial depletion of hsp70 resulted in a significant decrease in luciferase specific activity, reported as an amount of enzyme activity per unit of luciferase. Thus, the reduction in hsp70 in the reticulocyte lysate resulted in the impaired folding, but not the synthesis of luciferase.

The function of Ssa1/2p in protein folding was assessed by examining the effect of their depletion on luciferase synthesis and acquisition of enzymatic activity. A plasmid construct containing the coding region of firefly luciferase was modified in the 5′-untranslated region to permit translation in yeast lysate. Because previous studies were conducted in wheat germ lysate and reticulocyte lysate, it was necessary to confirm that luciferase translated in yeast lysate also folded cotranslationally. The relationship between luciferase synthesis and enzyme activity was linear over a period of 90 min (Fig. 6, *A–C*). The increase in luciferase activity ceased with the termination of protein synthesis, consistent with previously published results establishing the cotranslational nature of luciferase folding (Fig 6 *D*). Luciferase mRNA was translated in Ssa1/2p-

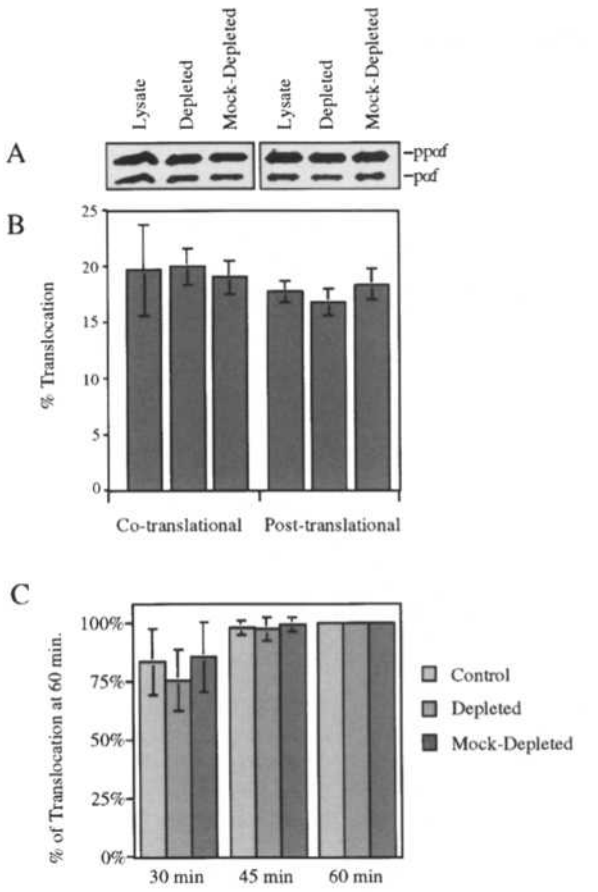

Figure 4. Depletion of Ssa1/2p from lysates has no effect on the translocation of prepro-α-factor. Lysates were used to synthesize radiolabeled prepro-α-factor either in the presence (Co-translational) or absence (Post-translational) of membranes. In the post-translational translocation assay, the translation reaction was treated with cycloheximide before the addition of membranes. Prepro-α-factor (ppαf) and pro-α-factor (pαf) were immunoprecipitated from the translocation reaction and separated by 16% SDS-PAGE. Translocation was determined by the appearance of the signal sequence-cleaved pro-α-factor form. Background levels of translocation in lysates not supplemented with membranes was minimal and did not differ between the three lysates. (*A*) Representative gel from one experiment. (*B*) Average of the percent translocation from six experiments. (*C*) Prepro-α-factor was translated in vitro for 45 min in each of the three lysates. Translation was terminated by the addition of cycloheximide. The translation reactions were supplemented with membranes and translocation was allowed to proceed. At the times indicated an aliquot of the translocation reactions were withdrawn and subjected to immunoprecipitation. The immunoprecipitated products were separated by SDS-PAGE and the percent translocation was determined by phosphorimaging analysis. In the histogram the amount of translocation at each time point is reported as a fraction of the translocation obtained at 60 min for each lysate. The histogram values are the average of two experiments.

depleted and mock-depleted lysates and aliquots were removed at 30-min intervals to determine the levels of luciferase synthesis and enzyme activity. The data in Fig. 6 documents the fact that comparable rates and levels of synthesis, (Fig. 7 *A*) as well as enzyme activity (Fig. 7 *B*), were obtained in the two lysates. From these data, it would

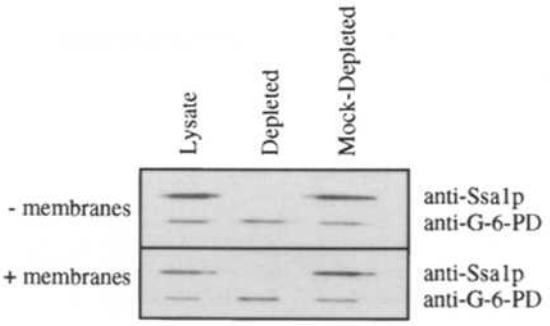

Figure 5. Microsomal membranes did not increase the level of Ssa1/2p in the translocation reaction. Mock translocation reactions, with and without membranes, lacking only radiolabel and transcript, were separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted to determine the amount of Ssa1/2p added to the reactions by the membranes. The same immunoblot was stained with anti-G-6-PD in order to assess the relative amounts of lysate proteins loaded in each lane.

appear that Ssa1/2p do not function identically to the hsp70s that were removed from reticulocyte lysate, i.e., Ssa1/2p are not involved in the folding of nascent luciferase.

Effect of Ssa1/2p Depletion on Protein Refolding

Hsp70 chaperones have also been implicated in the refolding of denatured proteins (44). In assays using various purified protein substrates, Ssa1/2p have been shown to prevent polypeptide aggregation and to assist in the refolding of proteins, including luciferase (16, 31). Additionally, the refolding of denatured luciferase in reticulocyte lysate has been shown to be ATP-dependent and to cofractionate with hsp70 (35, 45). Accordingly, yeast lysates lacking Ssa1/2p were assayed for their ability to refold denatured luciferase. In striking contrast to the results obtained in the translocation and luciferase synthesis assays, depletion of Ssa1/2p from yeast lysates severely compromised the ability of the lysate to promote a recovery of luciferase activity that had been lost through chemical denaturation.

Luciferase incubated in 6 M guanidine hydrochloride for 1 h retained <1% of its original activity (data not shown). The addition of the denatured enzyme to depleted lysates resulted in levels of enzyme activity only 25–30% of that observed in mock-depleted controls (Fig. 8, *A* and *B*). Addition of purified Ssa1p (Fig. 8 *C*) at wild-type levels (Fig. 8 *D*) to depleted lysates resulted in the restoration of refolding activity (Fig. 8, *A* and *B*, *circles*), indicating that the depletion of Ssa1/2p was specifically responsible for the loss of activity. Furthermore, the addition of Ssa1p restored the refolding capability of the depleted lysate in a dose-dependent manner (data not shown) suggesting that the amount of Ssa1/2p in the lysate was directly correlated with the refolding activity observed. This result clearly demonstrates the involvement of Ssa1/2 proteins in renaturation activity in yeast lysate, and supports the validity of the depletion approach to testing Ssa1/2p function in vitro.

Taken together, the results from Ssa1/2p depletion on de novo luciferase folding and luciferase refolding suggest that the trivial explanation, i.e., that the remaining 1–4% Ssa1/2p is responsible for the lack of effect in translocation, is not likely. The level of depletion was sufficient to

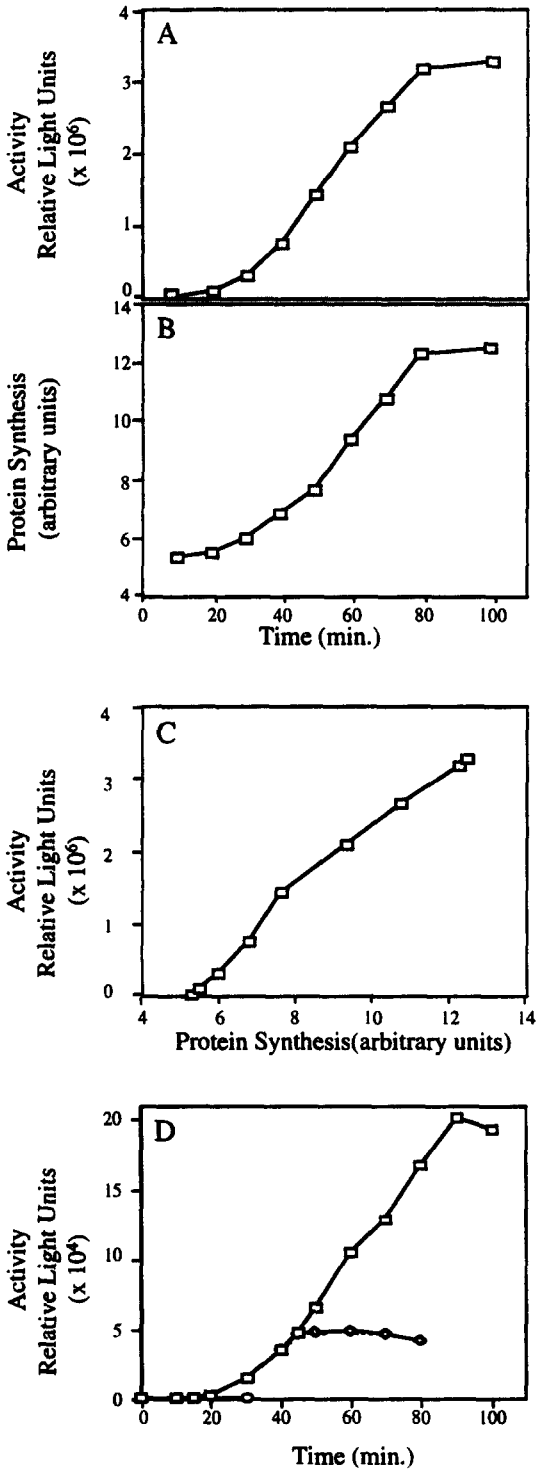

Figure 6. Firefly luciferase enzyme activity and synthesis in yeast lysate is consistent with established activity in reticulocyte and wheat germ systems. Radiolabeled luciferase was translated in yeast lysate. At indicated time points one aliquot was removed and assayed for (*A*) enzyme activity and a second aliquot was removed for immunoprecipitation and quantitation of (*B*) protein synthesis. Synthesis was measured by separating the immunoprecipitated material by 10% SDS-PAGE and quantifying the amount of radioactivity incorporated into luciferase using phosphorimaging. (*C*) The increase in enzyme activity was linear with respect to the increase in protein accumulation over 90 min. (*D*) The effect of the blocking protein synthesis of the increase in luciferase activity was measured by the addition of cycloheximide (0.1 mg/ml, final concentration) to the translation reaction. Three translation reactions were started simultaneously; one was untreated (*squares*), one received cycloheximide after 15 min (*circles*), and one was treated with cycloheximide after 45 min (*diamond*). Addition of cycloheximide brought about an immediate cessation in the increase in enzyme activity.

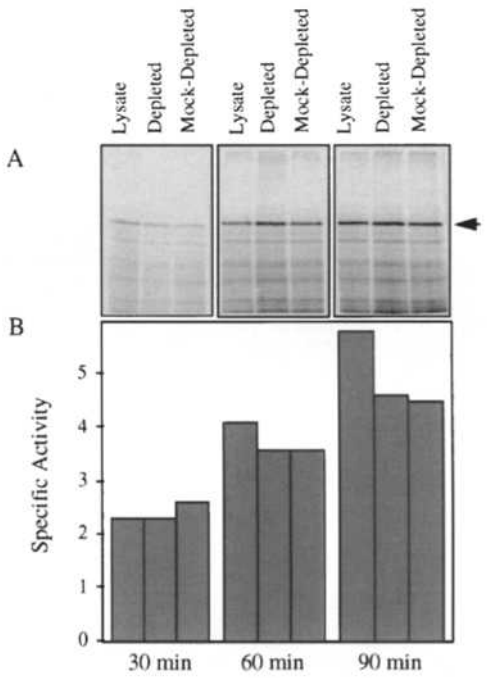

Figure 7. Lysate depleted of Ssa1/2p synthesizes active luciferase. Luciferase was synthesized in Ssa1/2p-depleted and mock-depleted lysates. At 30-min intervals an aliquot of the translation reaction was withdrawn and assayed for protein synthesis by immunoprecipitation and luciferase activity. Protein synthesis was assayed as in Fig. 5. (*A*) Luciferase synthesis demonstrated by separating the immunoprecipitates on SDS-PAGE (arrow indicates full-length luciferase). (*B*) Specific activity (the ratio of enzyme activity to amount of protein synthesized) from a representative experiment.

obtain a dramatic effect on the capability of the lysate to refold denatured luciferase, but had no effect on the folding of nascent luciferase. As both the folding and the refolding assays contained comparable amounts of luciferase activity, the different effects of Ssa1/2p depletion observed in these two assays cannot be explained by the different amounts of luciferase. In reticulocyte lysate, a similar experiment showed that removal of only 70% of the cytosolic hsp70s was sufficient to affect folding of nascent luciferase (21). These results suggest that the lack of effect on protein folding and, by extension, protein translocation, is not a consequence of inadequate depletion but rather an indication that the depletion of Ssa1/2p does not have significant impact on these processes.

Discussion

The data presented here define, for the first time in the context of yeast cytosol, a specific and essential role for

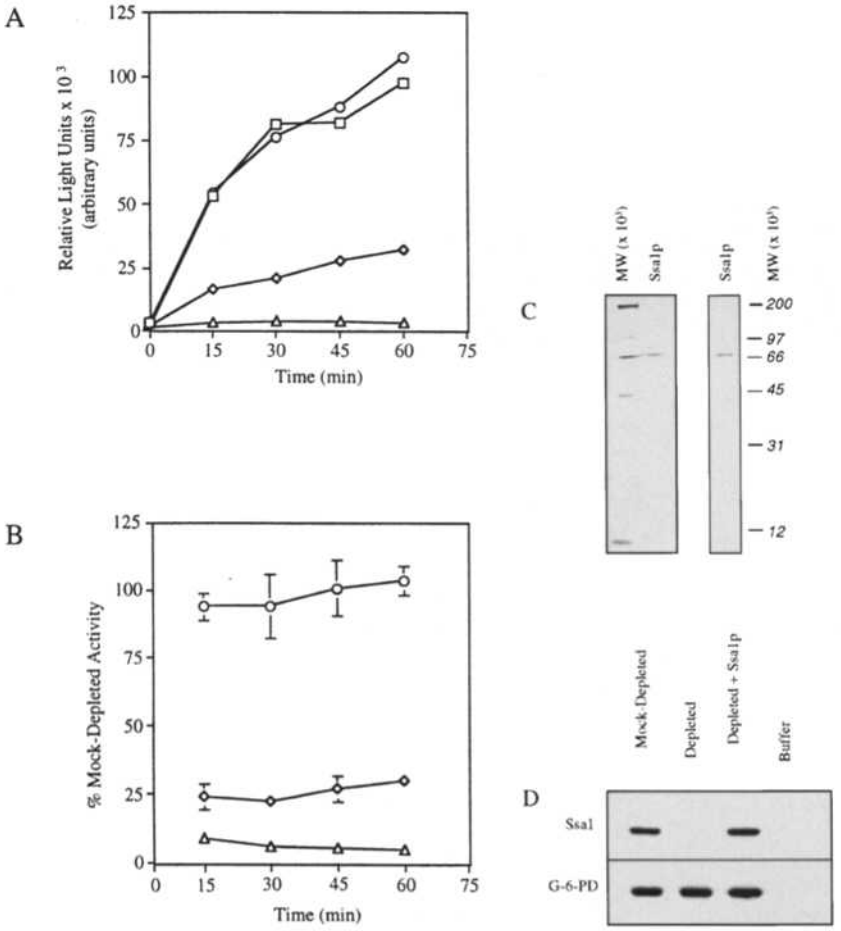

Figure 8. Lysate depleted of Ssa1/2p does not support efficient refolding of luciferase. Luciferase was denatured in 6 M guanidine hydrochloride and diluted into either mock-depleted lysate (*squares*), depleted lysate (*diamonds*), depleted lysate with purified Ssa1p added exogenously (*circles*), or buffered alone (*triangles*). Aliquots of the reaction were removed every 15 min and assayed for luciferase activity. (*A*) Time course of renaturation in one experiment. (*B*) Time course with luciferase renaturation represented as a percent of the mock-depleted lysate activity, average of four experiments with error bars. (*C*) SDS-PAGE of purified Ssa1p used for renaturation. The left panel is visualized by silver stain and the right panel is an immunoblot decorated with anti-Ssa1p. (*D*) Immunoblot of renaturation assays, decorated with antibodies against Ssa1p and G-6-PD as a control for amount of lysate proteins.

Ssa1/2p as molecular chaperones involved in protein refolding. This conclusion is consistent with results from biochemical studies using purified Ssa proteins and purified substrates (31). Our results also distinguish Ssa1/2p functionally from the other major cytosolic hsp70 subfamily, Ssb, since Ssb1/2p, though still present in the lysate (Fig. 3), did not compensate for the loss of the Ssa proteins. Ssa1/2p therefore represent essential chaperones for refolding activity. It is this refolding activity that we argue is crucial for posttranslational translocation.

The activity of Ssa1/2p is further characterized by the observation that the absence of these chaperones had no effect on the folding of nascent luciferase. This result is in contrast to that obtained in the renaturation experiments, as well as to those of Frydman et al. (21), who showed that luciferase required the cotranslational presence of hsp70 for activity in reticulocyte lysate (21). In yeast lysate, the depletion of Ssa1/2p removed only a subset of the cytosolic hsp70s. Since the depletion of Ssa1/2p had no effect on nascent luciferase activity, Ssa1/2p either do not act as chaperones for folding nascent polypeptides, or, the folding function unlike refolding, is redundant with the activity of other chaperones.

The finding that the in vitro depletion of Ssa1/2p had no effect on posttranslational translocation was surprising in light of the published data that implicates these particular hsp70s in the translocation process. Deshaies et al. (17) demonstrated that an accumulation of prepro-α-factor was correlated with the in vivo decrease of Ssa1p. Furthermore, both Chirico et al. (11) and Deshaies et al. (17) observed that Ssa1p was necessary to promote the posttranslational translocation of prepro-α-factor synthesized in wheat germ lysate. This requirement for Ssa1p was particularly interesting since prepro-α-factor physically interacts with the endogenous hsp70s in wheat germ cytosol (10). This suggests that the need for hsp70 in posttranslational translocation reflects a requirement for a specific function beyond a generic requirement for a molecular chaperone.

We maintain that this function is the specific refolding activity we have found for Ssa1/2p in our assays. Based on our findings, we propose the existence of two SRP-independent pathways that support translocation across the ER membrane, only one of which involves Ssa proteins (Fig. 9). In the first, the Ssa1/2p-dependent pathway, preproteins are released from the ribosome and/or nascent chain chaperones and assume a translocation-incompetent conformation in the cytosol (11, 17, 48). Translocation competence is restored through the refolding action of Ssa1/2p and translocation ensues. A loss of the "renaturation" capacity through, for example, the in vivo depletion of Ssa proteins (17) would result in the accumulation of a certain subset of preproteins in the cytosol. The published data suggest that this group includes prepro-α-factor and the β subunit of F_1ATPase, but not prepro-carboxypeptidase Y (prepro-CPY). This pathway would explain the need for Ssa1/2p to enable the translocation of prepro-

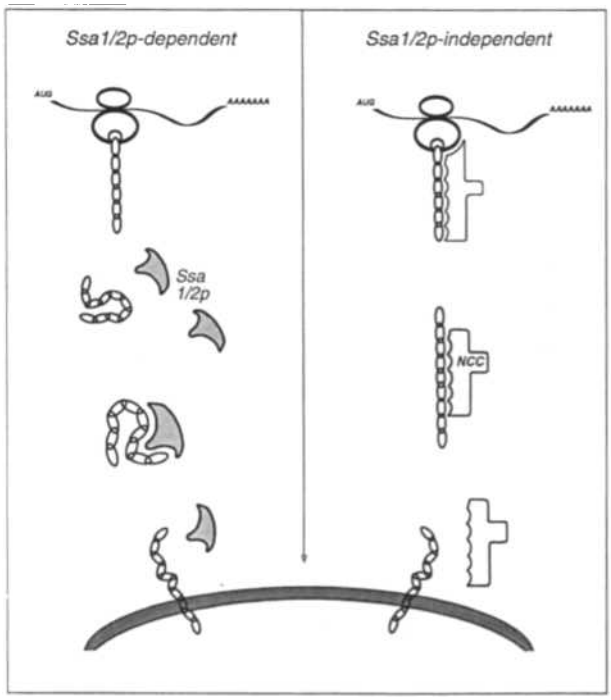

Figure 9. Model for SRP-independent translocation. In the Ssa1/2p-dependent pathway the preprotein, upon release from the ribosome and/or any nascent chain chaperones, folds into a translocation-incompetent conformation in the cytosol. Ssa1/2p refold the preprotein into a translocation-competent conformation, maintaining that conformation until translocation occurs. This pathway is necessarily posttranslational. In contrast, the Ssa1/2p-independent pathway can occur cotranslationally or posttranslationally (as depicted here). In this alternative, the preprotein interacts with a nascent chain chaperone (NCC) upon emerging from the ribosome. The NCC maintains the preprotein in a translocation-competent conformation until translocation occurs. This same NCC would also be responsible for the cotranslational folding of nascent polypeptides.

α-factor synthesized in wheat germ lysate. In these cases, the association of prepro-α-factor with wheat germ hsp70 was unproductive, leading to a conformation that was inconsistent with translocation across yeast microsomes. Ssa1/2p was required to displace wheat germ hsp70 (10) and to refold prepro-α-factor to a translocation-competent conformation.

The existence of such an SRP-independent, Ssa1/2p-dependent pathway is amply supported in the literature. In addition to the work previously cited, the involvement of Ssa1/2p in translocation is corroborated by studies on Ydj1p, one of the yeast DnaJ homologues (8). Temperature-sensitive mutants in Ydj1p give the same translocation phenotype as seen in the in vivo depletion of Ssa1p (9). The relationship between Ydj1p and Ssa1/2p has been demonstrated by biochemical studies showing that Ydj1p affects both the ATPase activity and the peptide-binding activity of Ssa1/2p (14, 15, 54). The action of Ydj1p appears to be specific for Ssa1/2p; there is no corresponding stimulation of Ssb1/2p. Although these studies do not address the mechanism of Ssa involvement, they clearly provide further support for its role in the translocation process.

The second pathway, which is Ssa1/2p-independent, is consistent with our finding that in vitro Ssa1/2p depletion had no effect on translocation. We propose that the chaperone(s) involved in nascent polypeptide folding also functions in translocation. This idea is supported by the finding that depletion of Ssa1/2p had no effect on the folding of nascent luciferase, suggesting that these particular hsp70s do not function as nascent polypeptide chaperones. There are, in fact, other cytosolic hsp70s that may perform this function. For example, Ssb1/2p are excellent candidates, as they have been shown to have a nascent chain-dependent association with ribosomes (34). Nascent chain chaperones (Ssb1/2p or otherwise) may have catalyzed the folding, but not the refolding, of luciferase in our assays. We suggest that they could also mediate co- and posttranslational translocation in vitro, thus serving as important participants in an SRP-independent translocation mechanism. This pathway would also provide a mechanism for the cotranslational, SRP-independent pathway that has been proposed as a formal hypothesis by others (48).

We propose that the postulated nascent chain chaperone mediates the translocation of prepro-α-factor in vitro. How then can one explain the in vivo result showing an accumulation of cytoplasmic prepro-α-factor upon the reduction of Ssa1p expression (17)? The simplest explanation reflects inherent differences in protein synthesis between in vitro and in vivo systems. In vitro translation proceeds at a markedly slower rate than in vivo, and the system is translating a single mRNA. In this case all of the lysate's potential chaperone activity would be available for functional interaction with a small complement of translation product. In contrast, in vivo translation rates are significantly higher, and all cellular proteins are being synthesized with their attendant demands on chaperone activity. In vivo, prepro-α-factor that fails to translocate cotranslationally or posttranslationally with the assistance of other chaperones (such as SRP or the postulated nascent chain chaperone), is released to the cytosol and becomes dependent on the refolding activity of Ssa1/2p to resume translocation competence. In the case of reduced *SSA1* expression, the diminishing pool of available refolding chaperone causes an accumulation of prepro-α-factor.

A translocation mechanism that is both SRP- and Ssa1/2p-independent provides an explanation for observations regarding the translocation of prepro-CPY, a preprotein whose translocation does not fit the current models of translocation. Prepro-CPY, of all preproteins examined thus far, appears most independent of SRP (6, 25). One would predict, therefore, that the translocation of prepro-CPY would be severely affected by the loss of Ssa1/2p activity. Yet, prepro-CPY translocation was affected, albeit modestly, by the in vivo depletion of Ssa1p (17) and not affected at all by the conditionally lethal YDJ1 (9). In vitro, prepro-CPY was found either unable (41), or only minimally capable (27), of crossing yeast microsomes posttranslationally. We suggest that the translocation of prepro-CPY may use this postulated nascent chain chaperone activity. The proposal of an SRP- and Ssa-independent mechanism, capable of accommodating both co- and posttranslational translocation, is therefore most appropriate.

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