Control of folding and membrane translocation by binding of the chaperone DnaJ to nascent polypeptides

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ABSTRACT   Recent evidence supports the view that cellular protein folding may be mediated by molecular chaperones. A fundamental question concerns the stage in its biogenesis at which the folding protein makes first contact with these components. We show here by crosslinking that the chaperone DnaJ binds nascent ribosome-bound polypeptide chains as short as 55 residues. Cotranslational binding of DnaJ to firefly luciferase and chloramphenicol acetyltransferase resulted in an arrest of folding as long as the functional partners of DnaJ in *Escherichia coli*, DnaK and GrpE, were missing. Protein uptake into microsomes and mitochondria was also interrupted by DnaJ. Both folding and post-translational translocation recommenced upon addition of DnaK and GrpE. We propose that DnaJ protects nascent polypeptide chains against aggregation and, in cooperation with Hsp70, controls their productive folding once a complete polypeptide or a polypeptide domain has been synthesized.

Homologs of the bacterial chaperone protein DnaJ in the cytosol (1–3) and the endoplasmic reticulum (4) of yeast cells are involved in the membrane translocation of newly synthesized proteins. Recently, homologs of DnaJ have been detected in the cytosol of mammalian cells (5). DnaJ itself prevents the aggregation of unfolded protein and cooperates with DnaK [a member of the 70-kDa heat shock protein (Hsp70) family] and its nucleotide exchange factor GrpE in protein folding *in vitro* (6). DnaJ homologs generally act in conjunction with Hsp70 (7, 8). Hsp70s have been copurified with ribosomes and nascent chains (9, 10) and are believed to prevent premature folding during translation (11–13), although as yet no direct evidence for this has been presented. Newly synthesized precursor proteins in the cytosol are kept competent for translocation into organelles by Hsp70s acting together with other factors (14, 15). Reasoning from the ability of DnaJ to prevent protein aggregation (6), DnaJ and DnaJ homologs might have the potential to bind to nascent polypeptides and prevent incorrect folding. We analyzed the effects of DnaJ and the Hsp70 DnaK on the folding of proteins synthesized *in vitro* in translation extracts derived from eukaryotic cells. We reasoned that the prokaryotic chaperones, added at levels comparable to their intracellular abundance, would not interact efficiently with the eukaryotic chaperones which are present in the translation extract at a fraction of their cellular concentration. Our results suggest that DnaJ has a general ability to bind to nascent polypeptide chains. In contrast, we did not observe a stable interaction of DnaK with nascent polypeptides under the ATP-rich conditions used in the translation extract.

MATERIALS AND METHODS

Isolation of Chaperones. Isolation of DnaK, DnaJ, and GrpE proteins was as previously described (6).

In Vitro **Translation of Chloramphenicol Acetyltransferase (CAT) and Firefly Luciferase (FFLuc) in the Presence of Chaperones.** FFLuc mRNA was transcribed from plasmid pGEM-luc (Promega) and translated (30 min, 30°C, 40% lysate) in a reticulocyte cell-free translation system (Promega), using the supplier's recommendations, or in wheat germ translation system prepared as described (16, 17). CAT mRNA for *in vitro* translation was transcribed by using SP6 RNA polymerase (Promega) and plasmid pCAT (kindly provided by M. Strauss, Berlin). Protein was translated at 26°C for 45 min, then cycloheximide was added to 2 mM. Where indicated, translation was synchronized by the addition of 8 mM 7-methylguanosine 5'-monophosphate (Sigma) after 2 min at 26°C. DnaJ protein was added from a 100 μM stock in 40 mM sodium phosphate buffer, pH 6.8/100 mM KCl/5 mM dithiothreitol/0.05% Brij 58/10% (vol/vol) glycerol. Translations without DnaJ received this buffer alone.

FFLuc and CAT activities were determined at 20°C and 37°C, respectively, as published (18, 19). Specific activities were calculated from quantitation of the storage phosphor image (Molecular Dynamics PhosphorImager) of ^{35}S-labeled protein separated by SDS/PAGE.

Post-Translational Folding of FFLuc. Translation was halted after 45 min by addition of cycloheximide, and the reaction mixture was made 1 mM in ATP, 6 mM in creatine phosphate, and 8 μg/ml in creatine kinase. DnaK (10 μM) and GrpE (15 μM) were added as indicated, and incubation was continued at 30°C for another 30 min. At the indicated times during this incubation FFLuc activity was determined (18). The duration of the enzyme assay from dilution of sample into assay mix until reading of the result was 2 min.

Protease Digestion of FFLuc. Translated samples were placed on ice after 50 min and treated with proteinase K (Sigma; 10 μg/ml, 20 min, 4°C) in the presence of bovine serum albumin carrier at 5 mg/ml. Digestion was stopped by addition of phenylmethylsulfonyl fluoride to 1 mM and protein was precipitated by addition of trichloroacetic acid to 10%, dissolved in Laemmli sample buffer, and separated by SDS/PAGE. Extent of digestion was determined by PhosphorImager.

Photocrosslinking. FFLuc 77-mer mRNA was produced by transcription of pGEM-luc linearized by restriction with *Hin*fI and used to program synthesis in a wheat germ cell-free translation system in the presence of 4-(3-trifluoromethyldiazirino)benzoic acid (TDBA)-modified lysyl-tRNA and ^{35}S-labeled methionine (17). To enrich for nascent chains and to reduce background, polyribosomes were isolated prior to crosslinking by sedimentation (Beckmann TLA-120.1 rotor, 20 min, 120,000 rpm; 4°C) through a sucrose cushion [0.5 M sucrose/0.5 M KOAc/20 mM Hepes/KOH, pH 7.5/5 mM Mg(OAc)$_2$/1 mM dithiothreitol/0.5 mM cycloheximide/500

Abbreviations: TDBA, 4-(3-trifluoromethyldiazirino)benzoic acid; CAT, chloramphenicol acetyltransferase; FFLuc, firefly luciferase; pPL, preprolactin; PL, processed prolactin; ppαF, yeast prepro-α-factor; pF$_1\beta$, pre-F$_1$ ATPase β subunit; F$_1\beta$, processed pF$_1\beta$; 3GαF, processed and triply glycosylated ppαF; SRP, signal recognition particle.

units of RNasin (Promega) per ml] and resuspension in translation blank buffer [50 mM Hepes/KOH, pH 7.5/100 mM KOAc/5 mM Mg(OAc)$_2$/1 mM dithiothreitol/500 units of RNasin per ml/0.5 mM cycloheximide/1.2 mM ATP/0.2 mM GTP/10 mM creatine phosphate/100 μg of creatine kinase per ml/0.1 μg of Trasylol per ml] (16). DnaJ was added to 15 μM and allowed to bind for 2 min at 26°C, then crosslinking was initiated and crosslinked samples were analyzed by SDS/PAGE as described (16, 17). Alternatively, samples were denatured in 1% SDS, diluted 1:40 into 50 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM Mg(OAc)$_2$/0.5 mM EDTA/1% Triton X-100 and immunoprecipitated with rabbit antibodies to DnaJ or preimmune antibodies crosslinked to staphylococcal protein-A-Sepharose (Pharmacia) (20) prior to SDS/PAGE and fluorography. Transcription/translation of bovine preprolactin (pPL) 55-mer and incorporation of TDBA-lysine were as published previously (16, 17).

Protein Translocation into Pancreatic Microsomes, Yeast Microsomes, and Mitochondria. Bovine pPL mRNA was translated for 45 min at 30°C in rabbit reticulocyte lysate supplemented with dog pancreas microsomes depleted of signal recognition particle (SRP) by EDTA and high-salt washing (21). Samples of this reaction mixture were analyzed by SDS/PAGE and fluorography. Synchronized translation of bovine pPL in wheat germ lysate, purification of SRP and SRP-depleted dog pancreas microsomes, and translocation were as described.

Wheat germ translation system programmed with pPL mRNA was supplemented with DnaJ and SRP as indicated. Translation was initiated for 2 min at 26°C, further initiation was inhibited by addition of 7-methylguanosine 5'-monophosphate (8 mM), and translation was continued for 10 min prior to addition of membranes or buffer. Translocation was allowed to proceed for 5 min. Trichloroacetic acid precipitates of all reaction mixtures were analyzed by SDS/PAGE and fluorography. Extent of translocation or translation (in the absence of microsomes) was determined by densitometric analysis of pPL and processed prolactin (PL) bands on fluorographs.

Yeast prepro-α-factor (ppαF) and pre-F$_1$ ATPase β subunit (pF$_1\beta$) were translated in rabbit reticulocyte lysate (30 min, 30°C, 40% lysate), translation was halted by addition of 2 mM cycloheximide, and DnaJ (plus DnaK and GrpE where indicated) was added. A 15,000 $\times$ g membrane fraction of yeast (containing a mixture of mitochondria and microsomes) was added (final protein concentration 0.1 mg/ml), and incubation was continued for another 30 min at 30°C. An aliquot of each reaction mixture was subjected to SDS/PAGE and fluorography to detect proteolytically processed pre-F$_1\beta$ (F$_1\beta$) and triply glycosylated ppαF (3GαF).

RESULTS

Addition of micromolar concentrations of DnaJ to a translation reaction mixture prevented the folding of newly synthesized full-length FFLuc, lowering the specific activity of this monomeric 60-kDa enzyme by up to 60% (Fig. 1A). Higher concentrations of DnaJ, while further reducing the specific activity of FFLuc, also markedly reduced the efficiency of translation (data not shown). DnaJ had a more pronounced inhibitory effect on the folding and assembly of CAT, a 25-kDa protein that is active as a trimer. Without significantly inhibiting translation, 3 μM DnaJ prevented the formation of active CAT almost completely (Fig. 1A). Surprisingly, addition of the Hsp70 homolog DnaK had little effect on folding of either FFLuc or CAT (Fig. 1B). Cotranslational addition of DnaK did not reverse the antifolding effect of DnaJ (data not shown). However, post-translational addition of DnaK in the presence of *Escherichia coli* GrpE did reverse the effect of DnaJ, as will be shown in Fig. 4.

The inhibitory effect of DnaJ was strictly cotranslational. FFLuc protein that was synthesized under these conditions was highly sensitive to proteinase K, while native FFLuc is folded into a protease-resistant form that is unaffected by post-translationally added DnaJ (Fig. 1C).

To determine at which stage during synthesis the interaction of DnaJ with nascent polypeptide chains occurs, we synthesized a nascent FFLuc polypeptide carrying a photoactivatable diazirino crosslinker (16) that can be incorporated at positions 5, 8, 9, 28, 31, and 67 of the FFLuc sequence. The FFLuc transcript was truncated by using an appropriate restriction site to give rise to a nascent chain consisting of the 77 amino-terminal residues of FFLuc. Translation was performed in a wheat germ extract in the presence of a TDBA-modified lysyl-tRNA. The effect of added DnaJ on FFLuc folding in the wheat germ translation system is identical to that found in reticulocyte lysate (data not shown). When isolated ribosomes containing the nascent chain were incubated with DnaJ, irradiation resulted in the formation of a radiolabeled crosslink product of approximately 47 kDa corresponding to the combined size of the 77-mer of FFLuc and the 41-kDa DnaJ (Fig. 1D). On highly resolving polyacrylamide gels the DnaJ crosslink behaved as a doublet of bands, reflecting the presence of two closely migrating forms of DnaJ in our purified preparation. These crosslink products, which were specifically immunoprecipitated with DnaJ antibodies (Fig. 1D), were also observed when DnaJ was added to ribosome-bound chains in the complete wheat germ extract (data not shown). These results indicate that DnaJ is able to recognize peptide segments in unfolded proteins very early in translation. We could not detect a crosslink product between DnaK and nascent FFLuc under similar conditions.

The binding of DnaJ to short nascent chains is reminiscent of the interaction between the SRP and the amino-terminal signal sequence of ribosome-bound secretory precursors (21). SRP ensures the cotranslational translocation of proteins into the endoplasmic reticulum (22) and has been extensively characterized using the secretory precursor protein pPL both by functional analysis (21) and by specific crosslinking of nascent pPL to the 54-kDa subunit of SRP protein (SRP54) (23, 24), here repeated in Fig. 2C. Functional interaction of SRP with the nascent chain leads to translocation of pPL into microsomes; in the absence of a target membrane, SRP binding causes translation arrest (21). Surprisingly, both the cotranslational targeting of pPL to microsomes (Fig. 2A) and the translation arrest were blocked by DnaJ (Fig. 2B). Similar observations were made with the precursors to human placental lactogen, ppαF, and β-lactamase (not shown). Purified SRP added at 0.08 μM during translation almost fully reversed the block in translocation imposed by 8 μM DnaJ and reestablished efficient translation arrest in the absence of microsomes (Fig. 2B). These results suggested that, like SRP, DnaJ was also able to interact with a short nascent pPL peptide. To test this directly, isolated ribosomes carrying a radiolabeled 55 amino acid nascent pPL containing photoactivatible crosslinker at lysines 4 and 9 of the signal peptide were incubated with DnaJ. Upon irradiation, a crosslinked product of approximately 45 kDa appeared (Fig. 2C, lanes 2–4). This product could be immunoprecipitated under denaturing conditions with anti-DnaJ antibodies and with PL antibodies (data not shown). Significantly, addition of DnaJ diminished the crosslinking of nascent pPL to SRP54 (Fig. 2C, lane 5). This suggests that both components may interact with the same or with overlapping sequences of pPL exposed on the ribosome. Our translocation experiments indicate that the affinity of SRP for the pPL nascent chain is about 100-fold higher than that of DnaJ. The interaction of secretory precursors with SRP present at about 10 nM in the cytosol (25) would be favored even if the cellular
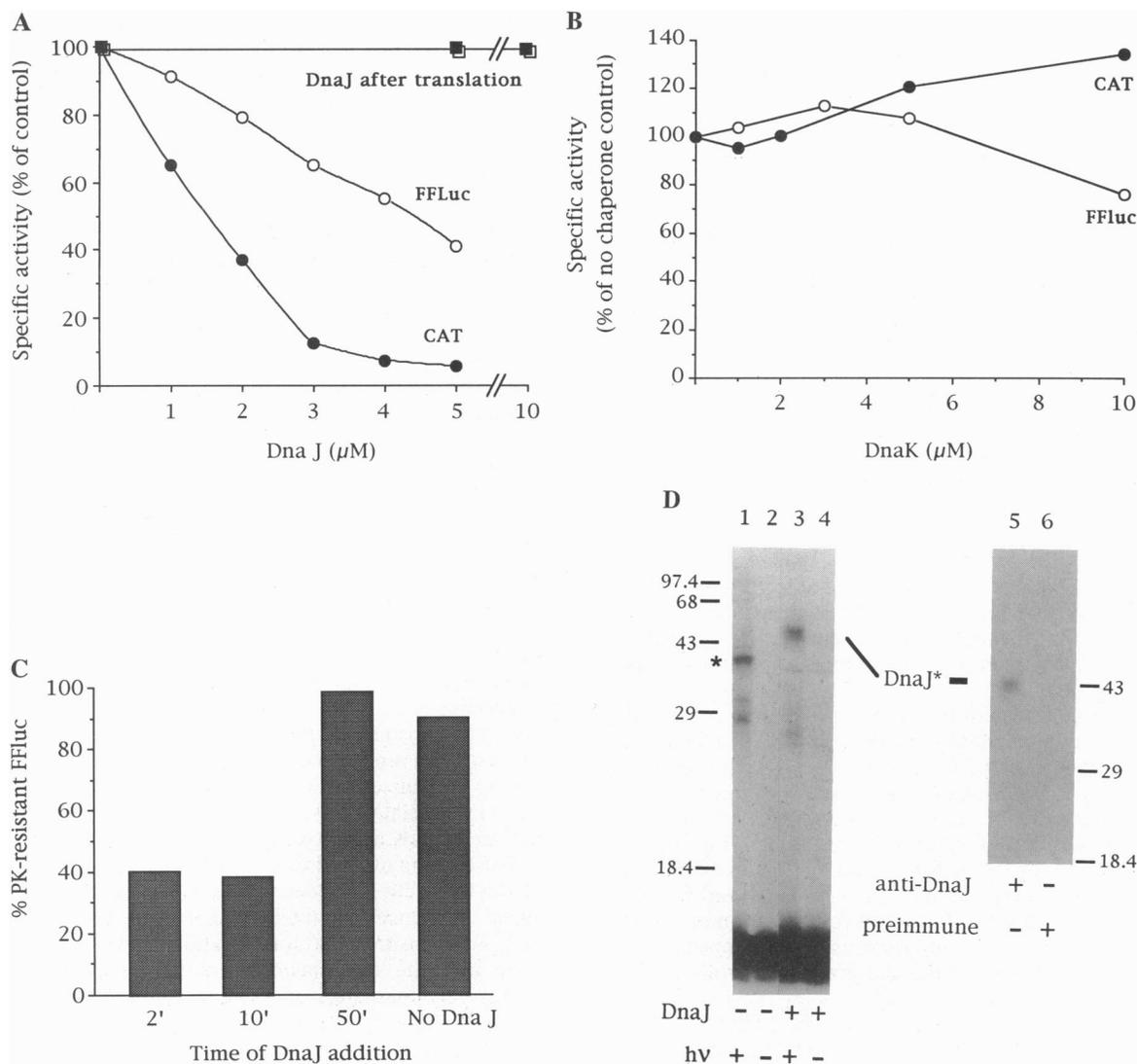
FIG. 1.   Prevention of protein folding by cotranslational binding of DnaJ. (*A*) Synthesis of inactive protein in the presence of DnaJ. Specific activity of FFLuc (○, □) and CAT (●, ■) was determined as a function of DnaJ added during (○, ●) or after (□, ■) translation. Specific enzyme activities measured in the absence of added DnaJ are set to 100%. (*B*) Synthesis of active protein in the presence of DnaK. Specific activity of FFLuc (○) and CAT (●) was determined as a function of DnaK added during translation. Specific enzyme activities measured in the absence of added DnaK are set to 100%. (*C*) Protease sensitivity of FFLuc synthesized in the presence of DnaJ added 2, 10, or 50 min after initiation of translation. Translation was inhibited by cycloheximide at 45 min. Amounts of proteinase K (PK)-resistant protein is expressed in percentage of total full-length FFLuc synthesized. (*D*) Crosslinking of nascent FFLuc peptide to DnaJ. Lanes 1–4, analysis of crosslink products by SDS/PAGE. Lanes 5 and 6, immunoprecipitation of crosslinked product with anti-DnaJ antibody and preimmune control serum. Numbers on left and right are masses of marker proteins in kDa; *hv* indicates crosslinking by light. Sample loaded in lanes 1–4 corresponds to 3 times that in lanes 5 and 6. DnaJ*, crosslinked product. * marks a crosslink product with an unidentified protein in wheat germ lysate that competes with DnaJ. The collapse of the crosslinked product from a doublet in lane 3 to a single band in lane 5 reflects band compression due to immunoglobulin heavy chains.

concentration of eukaryotic DnaJ homologs is, like that of DnaJ in *E. coli*, in the micromolar range (26).

DnaJ also inhibited the post-translational translocation of yeast ppαF into microsomes (measured as the formation of 3GαF) and the post-translational import into mitochondria (measured as proteolytic processing) of several precursor proteins, including pF₁β (Fig. 3), the Rieske Fe-S protein, and cytochrome oxidase subunit IV (data not shown). In contrast to the effects of DnaJ on FFLuc and CAT folding, inhibition of post-translational translocation was observed whether DnaJ was added during or after translation (data not shown). This, together with the previous observation that DnaJ binds proteins refolding from denaturant, leads us to conclude that DnaJ has the general ability to recognize nascent or newly synthesized polypeptides that are not yet folded.

If these interactions reflect the normal function of DnaJ during protein biosynthesis, they must be reversible in the presence of its functional partners DnaK and GrpE. Membrane translocation of DnaJ-bound precursor was restored by DnaK and GrpE but was unaffected by either protein alone (Fig. 4*A*). In a similar experiment FFLuc was translated in the presence of 4 μM DnaJ, further translation was halted with cycloheximide, and DnaK, GrpE, or both DnaK and GrpE were added. A 70–80% increase in FFLuc activity and a corresponding increase of protease-resistant FFLuc was observed when both DnaK and GrpE were present, while either DnaK or GrpE alone was without effect (Fig. 4*B*). The final specific activity of FFLuc reached that of a parallel synthesis from which DnaJ had been omitted. Reversibility of the DnaJ inhibition was also seen for the folding and assembly of CAT. When instead of DnaJ, DnaK and GrpE were added
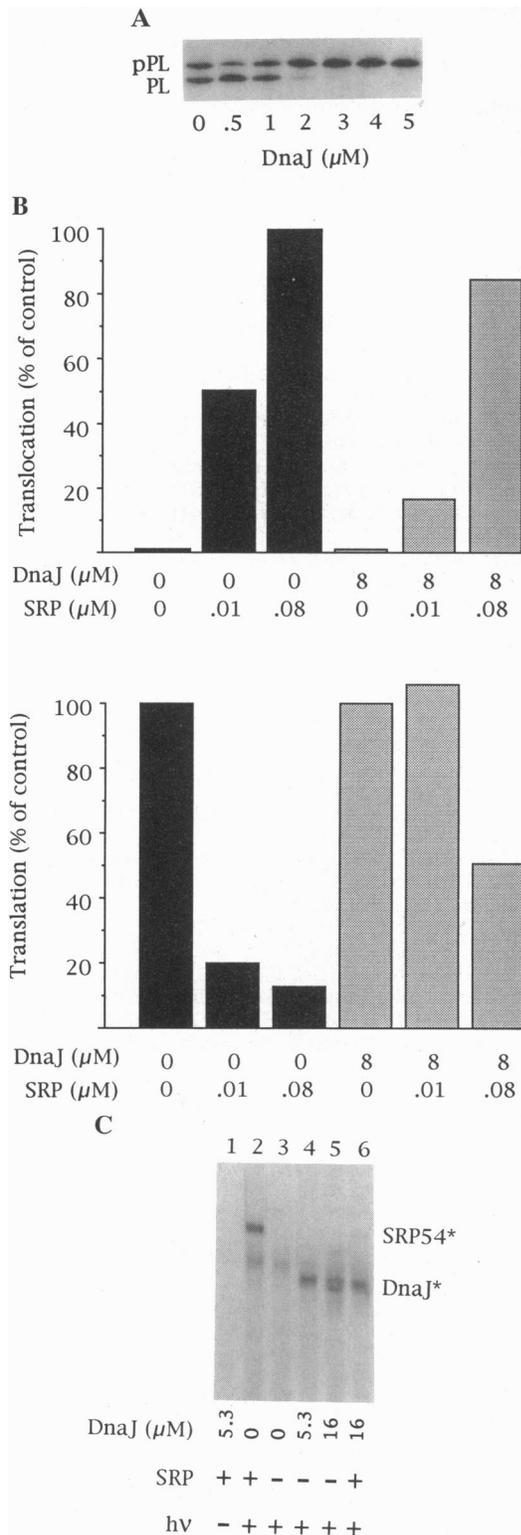
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A

pPL
PL

0 .5 1 2 3 4 5

DnaJ (µM)

B

FIG. 3. Inhibition of post-translational import of ppαF into yeast microsomes and pF₁β into yeast mitochondria. In each case, the processed species can be isolated with the organelle fraction after treatment with proteinase K, indicating that processing is a reliable indicator of protein import (not shown). The intermediate-size F₁β species in lanes 5–8 was not characterized, but it may reflect cytosolic degradation of pF₁β.

post-translationally, no increases in specific enzyme activities were observed (data not shown). Thus the interaction of DnaJ-like factors with proteins folding in the cytosol may normally be controlled by cooperating proteins so as to be transient and largely limited to the nascent chain.

DISCUSSION

Current concepts of protein folding suggest that a ribosome-bound polypeptide would be unable to reach a stably folded structure until at least a complete protein domain (100–200 amino acid residues) has been synthesized (12, 27–29). We propose that the Hsp70/DnaJ chaperone machinery has its vital role in the maintenance of such growing polypeptide chains in a folding-competent state. This view is supported here by the observation that DnaJ binds a nascent chain very early in translation and that this interaction is productive for folding to the native state upon addition of DnaK (Hsp70) and GrpE. The broad meaning of this finding is that chaperones can become involved in controlling folding before any unproductive interactions within or between folding polypeptide chains have occurred.

In light of the known functional interaction between Hsp70 and DnaJ (8), our results raise the formal possibility that the previously described binding of Hsp70 to nascent polypeptide chains (9, 10) is mediated solely by DnaJ homologs. There is evidence, however, that Hsp70s bind extended peptides in the absence of DnaJ (6, 30, 31). Mitochondrial (32) and microsomal (33) Hsp70s have been crosslinked to proteins partially translocated across their respective membranes, circumstances resembling a nascent polypeptide emerging from the ribosome. Binding of Hsp70 to protein and peptide substrates is weakened in the presence of bound ATP (6, 30, 31). A transient interaction of DnaK with nascent chains in the presence of ATP would not be expected to perturb protein folding in the translation extract, and it might not be long-lived enough for efficient crosslinking. We propose that the association of DnaJ and Hsp70 with nascent polypeptides is normally a cooperative event and that the sequence by which they bind may depend on the structural properties of the

FIG. 2. Competition of DnaJ and SRP for binding to nascent pPL. (*A*) Inhibition of pPL processing by DnaJ. pPL was synthesized in reticulocyte lysate in the presence of dog pancreas microsomes. PL is produced from pPL by cleavage of the signal sequence. In control experiments (data not shown) the processed PL was resistant to digestion by proteinase K at 150 µg/ml, indicating that processing reflects translocation of pPL into the microsomal lumen. (*B*) Effects of SRP on pPL translocation into microsomes (*Upper*) and translation in the absence of microsomes (*Lower*). pPL was synthesized in wheat germ lysate, which has low levels of endogenous SRP. Black bars, translocation or translation without added DnaJ; grey bars, with added DnaJ. Translocation (pPL processing to PL) in the
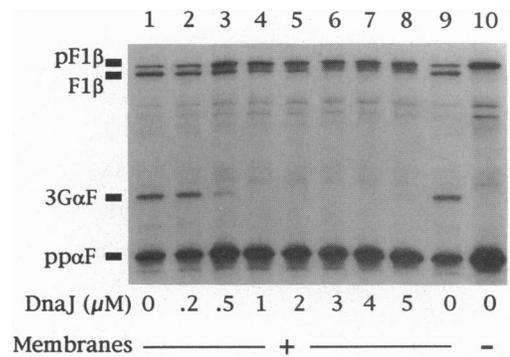
presence of 0.08 µM SRP is set to 100% (*Upper*). Translation in the absence of SRP is set to 100% (*Lower*). (*C*) Crosslinking of DnaJ or SRP to nascent 55-mer pPL fragment. SRP54*, crosslink product immunoprecipitable by anti-SRP54 antiserum. DnaJ*, crosslink product immunoprecipitable with anti-DnaJ antiserum. When indicated, SRP was present at 0.08 µM. Endogenous wheat germ protein crosslinked to pPL 55-mer is visible between the DnaJ* and SRP54* products in lanes 2 and 3.
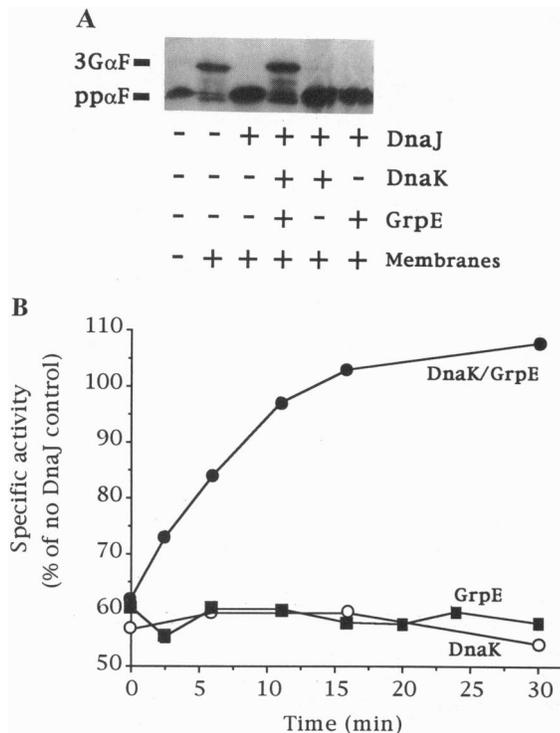
FIG. 4. Restoration of post-translational translocation and folding by DnaK and GrpE. (*A*) Reversal of DnaJ-dependent inhibition of ppαF translocation. (*B*) Specific activity of FFLuc after post-translational addition of DnaK (o), GrpE (■), or both (●) to FFLuc synthesized in the presence of 4 μM DnaJ. The specific activity of FFLuc synthesized in the absence of DnaJ was set to 100%.

polypeptide emerging from the ribosome. The binding specificity of DnaJ for polypeptide segments has not yet been analyzed. Our findings suggest the hydrophobic domain of the pPL signal peptide as a potential binding element. The amino-terminal 50 residues of FFLuc, which were crosslinked to DnaJ, do not contain regions of comparable hydrophobicity (34). Two uncharged segments of about 10 residues that contain prolines and glycines are present, however.

The complete course of protein folding in the cell-free translation system is not known. In our model, the DnaJ/Hsp70 system would deliver a protein in the form of a collapsed folding intermediate to a further chaperone component, for example GroEL/ES, that mediates its folding to the native state (6). Transfer could occur before completion of synthesis as soon as structural elements recognized by the downstream chaperone become available. GrpE, or a eukaryotic GrpE homolog, would promote release of bound Hsp70/DnaJ, allowing the polypeptide segment to fold productively or be withdrawn by binding to another chaperone. This is supported by our recent observations with such a transfer reaction reconstituted *in vitro* (6). The TCP-1 ring complex (TRiC), a possible GroEL analogue in the eukaryotic cytosol, may promote the folding of FFLuc upon release from DnaJ. Purified TRiC is known to mediate the folding of denatured FFLuc *in vitro* (18).

A complete Hsp70/DnaJ/GrpE system could also stabilize a precursor protein in an unfolded state for post-translational translocation. In this case, membrane binding would be critical in removing the protein from its chaperones because the cytosolic precursor would be unable to fold efficiently to the native state. The yeast DnaJ homolog Ydj1p is required for protein import into the endoplasmic reticulum and into mitochondria in addition to Hsp70 (1, 2, 35). This, and the finding that Ydj1p can directly interact with Hsp70 (7),

suggests that a mechanism similar to that described here for the DnaK/DnaJ/GrpE chaperones is indeed operative in the eukaryotic cytosol.

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