

## Mitochondrial GrpE Is Present in a Complex with hsp70 and Preproteins in Transit across Membranes

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**We characterized a 24-kDa protein associated with matrix hsp70 (mt-hsp70) of *Neurospora crassa* and *Saccharomyces cerevisiae* mitochondria. By using specific antibodies, the protein was identified as MGE, a mitochondrial homolog of the prokaryotic heat shock protein GrpE. MGE extracted from mitochondria was quantitatively bound to hsp70. It was efficiently released from hsp70 by the addition of Mg-ATP but not by nonhydrolyzable ATP analogs or high salt. A mutant mt-hsp70, which was impaired in release of bound precursor proteins, released MGE in an ATP-dependent manner, indicating that precursor proteins and MGE bind to different sites of hsp70. A preprotein accumulated in transit across the mitochondrial membranes was specifically coprecipitated by either antibodies directed against MGE or antibodies directed against mt-hsp70. The preprotein accumulated at the outer membrane was not coprecipitated by either antibody preparation. After being imported into the matrix, the preprotein could be coprecipitated only by antibodies against mt-hsp70. We propose that mt-hsp70 and MGE cooperate in membrane translocation of preproteins.**

Heat shock proteins with molecular masses of 70 kDa (hsp70s) form a major class of molecular chaperones present in prokaryotes and nearly every compartment of eukaryotic cells. DnaK, an hsp70 of *Escherichia coli*, interacts with two other heat shock proteins, a 44-kDa protein (DnaJ) and a 24-kDa protein (GrpE). Together, these three hsp70s function in a variety of cellular processes, including regulation of the heat shock response and bacteriophage  $\lambda$  replication (for reviews, see references 1, 11, 12, and 39). Langer et al. (20) showed that the folding of a denatured protein involves the successive action of DnaK, DnaJ, and GroEL/GroES (hsp60/cpn10). GrpE assists in the release of nucleotides from DnaK (21) and is required for the transfer of polypeptide chains from DnaK/DnaJ to GroEL/GroES.

It has been proposed that the function of hsp70s in eukaryotes also involves cooperation of DnaJ and GrpE homologs (12). Several homologs of DnaJ have been identified in eukaryotic cells, and current evidence suggests that at least some of them functionally interact with hsp70s (3, 4, 6, 37). An interaction between eukaryotic hsp70s and a GrpE homolog has not been reported so far. Of all eukaryotic hsp70s, the hsp70 of the matrix of mitochondria (mt-hsp70) shows the highest degree of homology to DnaK (5). We thus reasoned that it might provide the best opportunity to uncover interactions between an hsp70 and a eukaryotic GrpE homolog.

mt-hsp70 (Ssc1p) is essential for import of preproteins across the mitochondrial membranes (9, 17, 30, 38). In addition, it appears to be involved in protein folding in the matrix, in a way that is comparable to the function of DnaK (17). Here, we report the isolation of a 24-kDa protein from *Neurospora crassa* and *Saccharomyces cerevisiae* mitochondria, which is associated with mt-hsp70 under nondenaturing conditions. We identify this 24-kDa protein as a mitochondrial GrpE homolog,

MGE. MGE is associated with a preprotein in transit across the mitochondrial membranes and cooperates with hsp70 in protein translocation across membranes.

### MATERIALS AND METHODS

**Growth of cells and isolation of mitochondria.** *N. crassa* wild-type 74A cells were grown and harvested as described elsewhere (31, 32). When indicated, the cells were grown in the presence of [<sup>35</sup>S]sulfate. Mitochondria were isolated by differential centrifugation (27). *S. cerevisiae* cells were grown, and mitochondria were isolated as described elsewhere (7, 13). The following *S. cerevisiae* strains were used: PK82 (*MAT $\alpha$  his4-713 lys2 ura3-52  $\Delta$ trp1 leu2-3,112*; referred to as wild-type), PK81 [*MAT $\alpha$  ade2-101 lys2 ura3-52  $\Delta$ trp1 leu2-3,112 ssc1-2(LEU2)*; referred to as *ssc1-2*], and PK83 [*MAT $\alpha$  ade2-101 lys2 ura3-52  $\Delta$ trp1 leu2-3,112 ssc1-3(LEU2)*; referred to as *ssc1-3*] (9, 17).

**Immunolabeling under renaturing conditions.** Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was incubated three times in renaturation buffer (6 M urea, 10 mM Tris-HCl [pH 7], 50 mM NaCl, 20 mM EDTA, 0.1 mM dithiothreitol) for 90 min at room temperature with gentle agitation. The electrophoretic transfer of proteins to nitrocellulose membranes submerged in blot buffer (25 mM Tris-base, 192 mM glycine) was performed at 400 mA on ice overnight. The wet nitrocellulose membrane was stained with Ponceau S (Serva), blocked in incubation buffer (0.5% [vol/vol] Tween 20, 50 mM Tris-base, 150 mM NaCl), immunolabeled for 1 h with diluted antiserum in incubation buffer, washed three times for 5 min in washing buffer (50 mM Tris-base, 150 mM NaCl), and incubated for 30 min with peroxidase coupled to anti-rabbit antibodies (Sigma) diluted in incubation buffer. After washing, signals were detected using the ECL detection system (Amersham).

**Covalent coupling of antibodies to protein A-Sepharose.** Antiserum (1 ml) was added to 300  $\mu$ l (wet volume) of protein A-Sepharose (Pharmacia), and the mixture was suspended in 2

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ml of 100 mM potassium phosphate buffer (pH 7.5) and gently shaken for 1 h at room temperature. After washing twice with 0.1 M sodium borate buffer (pH 9), the protein A-Sepharose was resuspended in 7 ml of sodium borate buffer, and 35 mg of solid dimethylpimelimidate was added. After mixing, the protein A-Sepharose was again incubated for 30 min at room temperature. The coupling reaction was quenched by washing and incubation for 2 h in 1 M Tris-HCl (pH 7.5) at room temperature. The Sepharose matrix with coupled antibodies was stored suspended in TBS (10 mM Tris-HCl [pH 7.5], 0.9% [wt/vol] NaCl) at 4°C.

**Isolation of mt-hsp70-associated proteins.** <sup>35</sup>S-labeled mitochondria (20 µg of mitochondrial protein) were pelleted by centrifugation at 14,000 × *g* and were lysed in 100 µl of ice-cold digitonin buffer (0.5% [wt/vol] digitonin, 30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.3 mM phenylmethylsulfonyl fluoride) after addition of 1 µl of 1% (wt/vol) bovine serum albumin (BSA) and 1 µl of 100× protease-inhibitor mix (125 µg of leupeptin, 50 µg of pepstatin A, 100 µg of antipain, 25 µg of chymostatin, 500 µg of aprotinin per ml) by vigorous shaking at 4°C. The mitochondrial lysate was centrifuged for 5 min at 14,000 × *g* to remove insoluble aggregates. The supernatant was gently agitated with 50 µl (wet volume) of antibodies specifically directed against *N. crassa* mt-hsp70 (covalently coupled to protein A-Sepharose) for 1 h at 4°C in 1-ml spin columns (Mobitec) (the antibodies are monospecific for mt-hsp70 under denaturing conditions). The Sepharose matrix was washed four times with 150 µl of digitonin buffer. Flowthrough and wash fractions were collected by centrifugation for 30 s at 300 × *g* in a microcentrifuge. Bound proteins were eluted by a short incubation of the Sepharose matrix in 150 µl of 100 mM glycine, pH 2.5, and centrifugation as above. Collected fractions were treated with 10% (wt/vol) trichloroacetic acid (TCA) and were separated by SDS-PAGE. Standard procedures were used for analysis by SDS-PAGE, transfer to nitrocellulose, immunodecoration (summarized in reference 36), detection by the ECL system (Amersham Buchler), and densitometry, unless otherwise indicated. The antibodies against yeast MGE1 were raised against a glutathione *S*-transferase fusion protein containing amino acid residues 91 to 228 of MGE1 (19). Radioactive samples were analyzed by the phosphor storage technology (Molecular Dynamics).

For isolation in chemical amounts, isolated mitochondria from *S. cerevisiae* (250 µg of mitochondrial protein) were pelleted, lysed in digitonin buffer, and incubated with immobilized antibodies against Ssc1p as described above, except that three times more buffer volume was used for incubations and washing steps (the antibodies are monospecific for mt-hsp70 [Ssc1p] under denaturing conditions [9, 17]). After elution of bound protein complexes at pH 2.5, the proteins were precipitated by TCA, separated on SDS-polyacrylamide gels, and analyzed by Western blotting (immunoblotting) with anti-MGE1 serum.

**Import of preproteins into isolated mitochondria and coimmunoprecipitation with mt-hsp70.** Two hybrid precursor proteins were used for *in vitro* import assays: a fusion protein between the amino-terminal 69 amino acids of the subunit 9 of the mitochondrial *F*<sub>0</sub>-ATPase and the entire mouse dihydrofolate reductase sequence (Su9-DHFR) (28) and a fusion protein between the amino-terminal 167 amino acids of cytochrome *b*<sub>2</sub> (with a deletion in the intermembrane space targeting sequence [amino acids 47 to 65]) and the entire DHFR (*b*<sub>2</sub>-DHFR) (18). Precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine. Import into isolated mitochondria was performed in the pres-

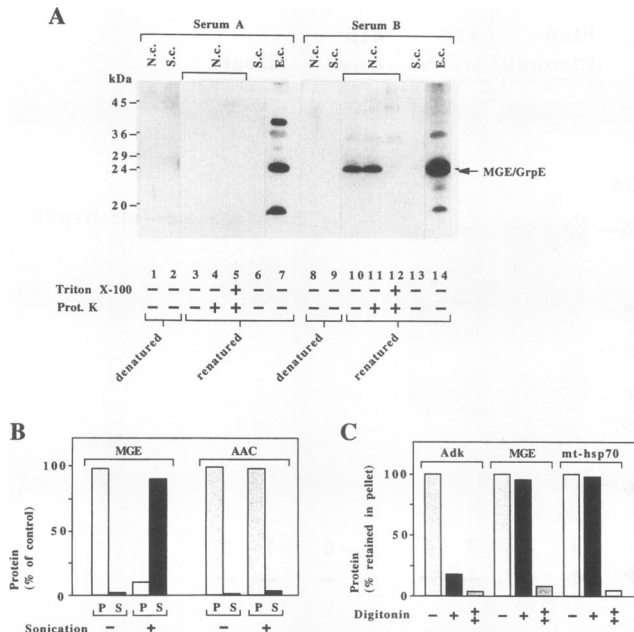
ence of 2 mM ATP and 4 mM NADH for 5 min at 25°C in BSA buffer (3% [wt/vol] BSA, 250 mM sucrose, 10 mM morpholinepropanesulfonic acid [MOPS]-KOH, [pH 7.2], 80 mM KCl, 5 mM MgCl<sub>2</sub>) in a final volume of 100 µl (import assay). When indicated, the samples were treated with proteinase K (100 to 150 µg/ml). The mitochondria were reisolated and analyzed by SDS-PAGE and autoradiography (13, 17, 26). For accumulation of membrane-spanning intermediates, the lysate containing the precursor of *b*<sub>2</sub>-DHFR was preincubated for 15 min in the presence of 1 µM methotrexate and 5 mM dithiothreitol at 0°C (on ice) before mitochondria were added to start the import reaction (8, 29). For binding of precursors to the outer membrane, *b*<sub>2</sub>-DHFR and mitochondria were incubated in the presence of 0.5 µM valinomycin, 8 µM antimycin A, and 20 µM oligomycin to dissipate the membrane potential across the inner membrane. For coimmunoprecipitations, the mitochondria were lysed after the import reaction in the digitonin buffer described above and, after a clarifying spin (5 min, 23,000 × *g*), were incubated with antisera directed against DHFR, preimmune serum, or antiserum directed against Ssc1p or MGE1. The immunoprecipitates were washed three times in digitonin buffer and once in 10 mM Tris (pH 7.5) and were analyzed by SDS-PAGE as described.

## RESULTS

**Identification of an association between mt-hsp70 and MGE in *N. crassa* and *S. cerevisiae*.** To identify a homolog of GrpE in eukaryotic cells, two different antisera directed against *E. coli* GrpE were tested for cross-reaction with total cellular or mitochondrial protein extracts of *N. crassa* and *S. cerevisiae*. No significant reaction was found when the assay was performed under standard Western blotting conditions, i.e., when SDS-denatured proteins were transferred to nitrocellulose (Fig. 1A, lanes 1, 2, 8, and 9). We then treated the gels with 6 M urea to allow renaturation of proteins before the transfer to nitrocellulose. Following this treatment, a band of about 24 kDa from *N. crassa* mitochondria was recognized by one of the anti-GrpE sera (Fig. 1A, lane 10). As described in this report, the 24-kDa protein represents a mitochondrial GrpE and is thus termed MGE. No reaction was found with the other anti-GrpE serum (Fig. 1A, lane 3) or with yeast mitochondria (Fig. 1A, lanes 6 and 13). As expected, GrpE of *E. coli* was recognized by each antiserum also after renaturation (Fig. 1A, lanes 7 and 14). The fact that renaturation was required to observe reactivity raised the possibility that a nonlinear epitope of the putative *N. crassa* GrpE homolog was recognized by anti-*E. coli*-GrpE antibodies.

The *N. crassa* MGE was resistant to protease treatment of intact mitochondria but was digested by protease after lysis of the membranes by detergent (Fig. 1A, lanes 11 and 12). MGE was released into the supernatant following sonication of mitochondria, while the membrane protein ADP-ATP carrier remained in the membrane fraction (Fig. 1B). The intramitochondrial location of the protein was determined by analyzing the solubility of MGE upon successive opening of the outer membrane and inner membrane by digitonin (Fig. 1C). MGE was released into the supernatant and became accessible to protease only after opening of the matrix space by treatment with a high concentration of digitonin (Fig. 1C) (adenylate kinase was a marker for the intermembrane space and mt-hsp70 was a marker for the matrix). MGE thus behaves as a soluble protein of the mitochondrial matrix.

We then tested if MGE was associated with the hsp70 of the matrix. After lysis of *N. crassa* mitochondria with digitonin, mt-hsp70 was affinity purified under nondenaturing conditions



**FIG. 1.** Identification of *N. crassa* MGE and localization to the mitochondrial matrix. (A) Immunological cross-reaction after renaturation. Mitochondria were isolated from *N. crassa* (lanes 1, 3 to 5, 8, and 10 to 12) or *S. cerevisiae* (lanes 2, 6, 9, and 13). Mitochondrial proteins (75  $\mu$ g) were separated by SDS-PAGE and were transferred to nitrocellulose. Prior to electrophoresis, samples with intact (lanes 4 and 11) or solubilized (lanes 5 and 12) mitochondria were treated with 100  $\mu$ g of proteinase K (Prot. K) per ml. Solubilization was performed by resuspending in 1% (vol/vol) Triton X-100–300 mM NaCl–10 mM Tris-HCl (pH 7.4) and by 5 min of vigorous shaking at 4°C. Proteins of Triton X-100-treated mitochondria were precipitated with TCA. For samples 7 and 14, lysed *E. coli* cells were applied (50  $\mu$ g of total cellular protein). When indicated, the gel was renatured prior to electrophoretic transfer of proteins to nitrocellulose as described in Materials and Methods. Nitrocellulose membranes were decorated with antisera against *E. coli* GrpE. (B) MGE is a soluble protein. Isolated *N. crassa* mitochondria were sonified three times for 30 s and separated in soluble (S) and insoluble (P) fractions by centrifugation as described elsewhere (2). After precipitation of soluble proteins with TCA, the samples were analyzed by SDS-PAGE and Western blotting (renaturation of the gel for MGE). The total amount of MGE or ADP/ATP carrier (AAC) in untreated mitochondria was set to 100%. (C) MGE is located in the matrix. Isolated *N. crassa* mitochondria were treated with 0% (–), 0.1% (+), or 0.5% (wt/vol) (++) digitonin and then with proteinase K (30  $\mu$ g/ml) (14). After centrifugation for 15 min at 22,000  $\times$  g, the enzymatic activity of adenylate kinase (Adk) and the amounts of mt-hsp70 and MGE were determined for the pellet and supernatant. The total amount of each protein in pellet plus supernatant in the absence of digitonin was set to 100%.

using a column with covalently bound antibodies directed against *N. crassa* mt-hsp70. Binding of mt-hsp70 and several other proteins to the column was independent of the presence of ATP in the loading buffer (Fig. 2A, lanes 1 and 2). However, a 24-kDa protein was bound to the affinity column only in the absence of added Mg-ATP (and presence of EDTA) (Fig. 2A, lane 2). This protein was identified as MGE by the use of anti-GrpE antibodies (Fig. 2A, lane 3). While this work was in progress, a yeast gene located upstream of the *KIN4* gene (16) was sequenced and found to be significantly similar to *E. coli* *grpE*. The essential gene, termed *MGE1* or *YGE1*, encodes a 24-kDa protein located in mitochondria (apparent molecular mass of about 22 kDa on SDS-PAGE). *MGE1/YGE1* is able to

functionally replace *E. coli* *grpE* (15, 19, 37). Antibodies which were directed against yeast *MGE1* (19) selectively recognized the 24-kDa protein of *N. crassa* mitochondria that we found associated with mt-hsp70 (Fig. 2A, lane 5), confirming its identity as MGE. We conclude that MGE of *N. crassa* mitochondria is associated with the hsp70 in the matrix.

In analogy to the procedure described for *N. crassa* mitochondria, digitonin-lysed yeast mitochondria were passed over a column containing covalently bound antibodies directed against yeast mt-hsp70. After washing of the column, bound proteins were eluted from the antibodies at pH 2.5 (Fig. 2B, lanes 7 and 8). In the presence of Mg-ATP, MGE was found in the flowthrough fraction (Fig. 2B, lane 9). As with *N. crassa* mitochondria, MGE was efficiently bound to mt-hsp70 when no ATP was added to the extract (Fig. 2B, lanes 8 and 16). To exclude the possibility that binding of MGE to mt-hsp70 occurred after the lysis of mitochondria,  $^{35}$ S-labeled yeast mitochondria were lysed in the presence of a fivefold excess of unlabeled mitochondrial matrix extract. This did not decrease the amount of labeled MGE bound to labeled mt-hsp70 (data not shown), indicating that the MGE was already bound to mt-hsp70 in intact mitochondria.

We conclude that MGE in the matrices of *N. crassa* and *S. cerevisiae* mitochondria is associated with hsp70 and that ATP is required for its dissociation. Since in the absence of ATP practically all of the MGE is retained on the column, as is all of the hsp70, the molar ratio of the two proteins in mitochondria can be determined. By comparison of the intensity of staining by Coomassie blue, the molar ratio of MGE to mt-hsp70 was found to be approximately 1:3.

**Characteristics of interaction of MGE with mt-hsp70.** As described above, the MGE-hsp70 complex is disrupted by the addition of Mg-ATP (Fig. 3A, column 2). The addition of  $Mg^{2+}$  ions alone did not release MGE from mt-hsp70, since MGE was still bound to the anti-hsp70 column (Fig. 3A, column 3). When the mitochondrial extract was incubated with EDTA to lower the concentration of free  $Mg^{2+}$ , the addition of ATP resulted in the release of about 80% of the MGE (Fig. 3A, column 4). Calculations according to Perrin (25) indicate that under these conditions the concentration of available magnesium ions is below the micromolar range (35), suggesting that very low  $Mg^{2+}$  concentrations are sufficient for the ATP-dependent release. Only about 25% of the MGE was released from mt-hsp70 by the addition of the nonhydrolyzable analog adenosine-5'-O-3-thiotriphosphate (ATP $\gamma$ S) or 5'-adenylylimido diphosphate (AMP-PNP) to the extracts (Fig. 3A, columns 5 and 6). In addition, the MGE-hsp70 complex was largely resistant to high salt concentrations. KCl at concentrations of up to 1.5 M only slightly reduced the amount of bound MGE (Fig. 3B, lanes 9 and 11, and data not shown). Even at high salt concentrations, the addition of Mg-ATP led to a quantitative release of MGE (Fig. 3B, lanes 4 and 6), demonstrating the specificity of the interaction and the ATP-dependent dissociation of the complex.

To compare the interaction between mt-hsp70 and precursor polypeptides with the interaction with MGE, we made use of a temperature-sensitive *S. cerevisiae* mutant of mt-hsp70, termed Ssc1-2p (17). Ssc1-2p has a single amino acid change in the putative peptide-binding domain of hsp70. An import defect can be induced by preincubating isolated *ssc1-2* mitochondria at 37°C. Ssc1-2p binds preproteins but is strongly impaired in releasing them (9), while wild-type mt-hsp70 transiently interacts with preproteins (22, 24).

A fusion protein of the presequence of  $F_0$ -ATPase subunit 9 and DHFR (Su9-DHFR) was synthesized in rabbit reticulocyte lysate in the presence of [ $^{35}$ S]methionine. After denaturation

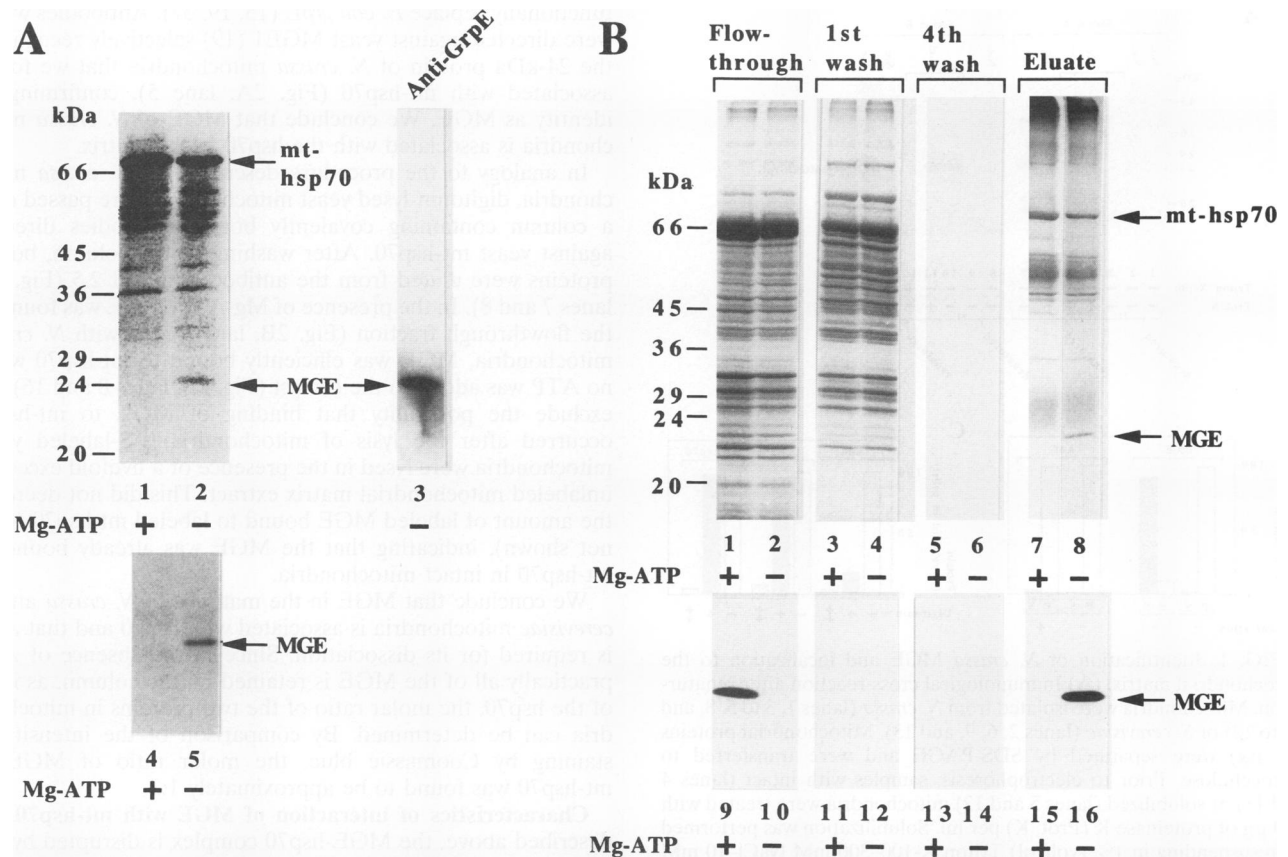


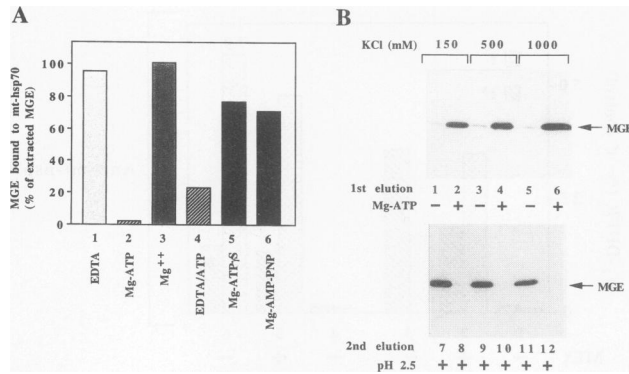
FIG. 2. Association of MGE with mt-hsp70 from *N. crassa* and *S. cerevisiae*. (A) *N. crassa* MGE. For lanes 1 and 2, [<sup>35</sup>S]sulfate-labeled mitochondria (20  $\mu$ g of mitochondrial protein) were isolated from *N. crassa*, lysed in digitonin buffer in the presence of either 5 mM ATP and 10 mM magnesium acetate (+) or 10 mM EDTA (-). mt-hsp70 and associated proteins were affinity purified with a column containing covalently coupled anti-mt-hsp70 antibodies as described in Materials and Methods. The proteins were analyzed by SDS-PAGE. For lane 3, 250  $\mu$ g of mitochondrial protein was used to isolate proteins associated with mt-hsp70 in the presence of 10 mM EDTA as described for lane 2. After TCA precipitation, eluted proteins were separated by SDS-PAGE, renatured, and transferred to nitrocellulose. Immunodecoration was performed with antiserum against *E. coli* GrpE. For lanes 4 and 5, proteins bound to mt-hsp70 were isolated as described for lanes 1 and 2, separated by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with antiserum against yeast MGE1. (B) *S. cerevisiae* MGE. Mitochondria were isolated from *S. cerevisiae* (500  $\mu$ g of mitochondrial protein) and lysed in the presence (+) or absence (-) of Mg-ATP. Proteins bound to mt-hsp70 were isolated by affinity chromatography as described in Material and Methods. After TCA precipitations of the flowthrough, the wash fractions, and the eluate fraction, proteins were separated by SDS-PAGE. One-half of the samples was analyzed by staining with Coomassie blue R250 (lanes 1 to 8). The other half of the samples was transferred to nitrocellulose, and the amount of MGE was determined by immunodecoration with antiserum against yeast MGE1 (lanes 9 to 16). The prominent band of about 66 kDa seen in lanes 1 to 4 is BSA that was present in the lysis buffer.

in 8 M urea, the preprotein was imported into *ssc1-2* mitochondria. The mitochondria were lysed with detergent, and coimmunoprecipitations with anti-mt-hsp70 antibodies were performed. Su9-DHFR associated with Ssc1-2p (Fig. 4, column 3) was released only to a small degree by the addition of Mg-ATP (Fig. 4, column 6). In wild-type mitochondria, the preprotein transiently associated with mt-hsp70 was efficiently released by the addition of Mg-ATP (Fig. 4, columns 2 and 5).

By coimmunoprecipitation with anti-mt-hsp70 antibodies and Western blotting with anti-MGE antibodies, we probed whether MGE was associated with Ssc1-2p in isolated mitochondria (in the absence of preproteins). In the absence of added ATP, the large majority of MGE was associated with Ssc1-2p (Fig. 4, lane 3). It was efficiently released by the addition of Mg-ATP (Fig. 4, lane 6). Thus, the mutation in the peptide-binding region of Ssc1-2p impairs the release of precursor polypeptide chains but not the release of MGE, demonstrating that the interaction of hsp70 with polypeptides is distinct from the interaction of hsp70 with MGE.

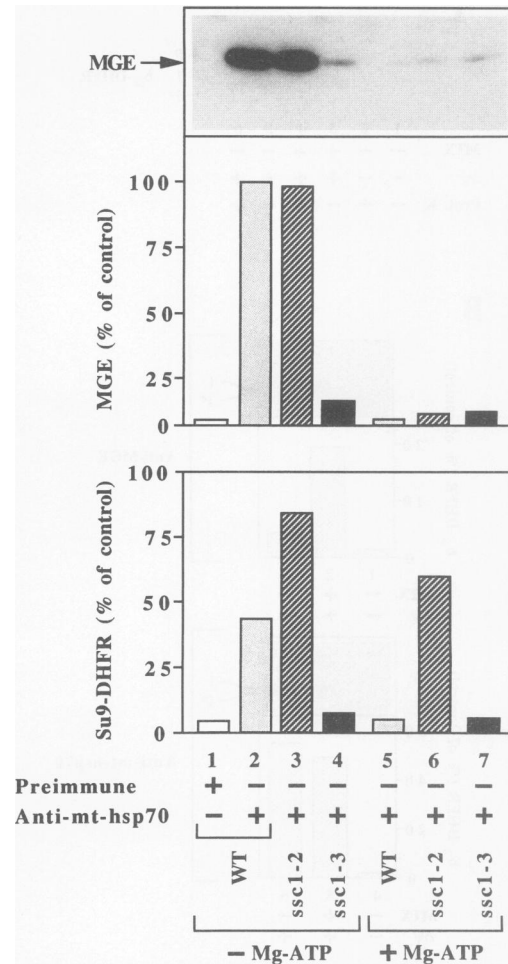
Another yeast mutant of mt-hsp70, termed Ssc1-3p, carries a point mutation in the ATPase domain. *ssc1-3* mitochondria partially import some preproteins such as Su9-DHFR, but no significant interaction between Ssc1-3p and preproteins is detectable (9) (Fig. 4, columns 4 and 7). Similarly, we did not detect a significant interaction of MGE with Ssc1-3p (Fig. 4, lanes 4 and 7), indicating that a functional ATPase domain of hsp70 is needed for both binding of polypeptide chains and binding of MGE.

**Association of MGE with a preprotein in transit across the mitochondrial membranes.** We attempted to more closely define the interaction of MGE with mt-hsp70 in relation to the sequence of events of the import process. To this end, we analyzed the translocation of a matrix-targeted precursor protein that could be accumulated at distinct stages, a fusion protein between an amino-terminal portion of the precursor of cytochrome *b<sub>2</sub>* and DHFR (29). This protein can be accumulated (i) at the mitochondrial surface in the absence of a membrane potential (Fig. 5A, lane 1); (ii) in a membrane-



**FIG. 3.** Characterization of interaction of mt-hsp70 and MGE. (A) Association of MGE and mt-hsp70 is nucleotide dependent. Proteins bound to mt-hsp70 were isolated from mitochondrial lysates (200  $\mu$ g of protein per sample) of *S. cerevisiae* as described in Materials and Methods and in the legend to Fig. 2. The amount of bound MGE (MGE found in the eluate fraction) was analyzed by SDS-PAGE and immunodecoration. To determine the nucleotide dependence of the interaction between mt-hsp70 and MGE, lysis and isolation by affinity chromatography were performed in the presence of the following additions (as indicated): 10 mM EDTA (EDTA), 10 mM magnesium acetate and 5 mM ATP (Mg-ATP), 10 mM magnesium acetate (Mg<sup>2+</sup>), 10 mM magnesium acetate and 5 mM ATP $\gamma$ S, and 10 mM magnesium acetate and 5 mM AMP-PNP. The total amount of MGE extracted from mitochondria was set to 100%. (B) Binding of MGE to mt-hsp70 is resistant to ionic strength. Proteins associated with mt-hsp70 were isolated from yeast mitochondrial lysates and analyzed as described in Material and Methods, except that lysis and isolation were performed in the presence of KCl. When indicated, MGE was eluted in the presence of 10 mM magnesium acetate and 5 mM ATP (Mg-ATP) in the third washing buffer (1st elution). Afterward, all samples were treated with 100 mM glycine, pH 2.5, to elute all residual protein (2nd elution). After TCA precipitation, the amounts of MGE were analyzed by Western blotting.

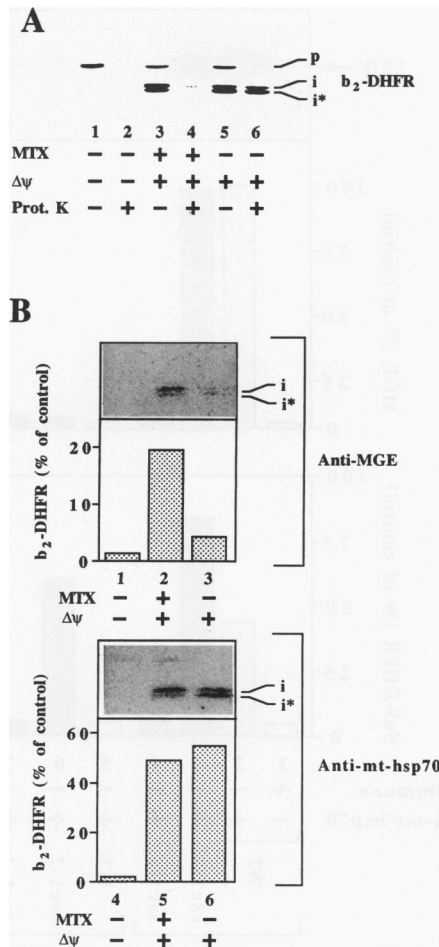
spanning form with the amino-terminal portion of the fusion spanning across both membranes and the DHFR moiety on the cytosolic surface by performing the import reaction in the presence of methotrexate, which stabilizes DHFR in an import-competent conformation (Fig. 5A, lane 3); and (iii) in an imported cleaved form in the matrix by performing the import reaction in the absence of methotrexate (Fig. 5A, lane 6). As expected, the preprotein accumulated on the surface was not coimmunoprecipitated by either anti-MGE or anti-mt-hsp70 antibodies (Fig. 5B, lanes 1 and 4). When methotrexate was added to the import reaction mixtures using energized mitochondria, the presequence was cleaved as expected (Fig. 5A, lane 3) and the DHFR domain on the cytosolic side remained accessible to added protease (Fig. 5A, lane 4) (29, 38). This membrane-spanning intermediate was coprecipitated by both anti-MGE antibodies and anti-mt-hsp70 antibodies (Fig. 5B, lanes 2 and 5), while no coimmunoprecipitation was observed with preimmune antibodies (data not shown). The efficiency of coimmunoprecipitation of *b*<sub>2</sub>-DHFR by anti-mt-hsp70 antibodies is about twofold higher than that by anti-MGE antibodies. Considering what is known about GrpE of *E. coli*, it is likely that the interaction of MGE with the preprotein is not a direct one but rather is mediated by mt-hsp70. Thus, all three proteins have to be present in a ternary complex to observe a coimmunoprecipitation by anti-MGE antibodies, since this ternary complex may be partially dissociated during immunoprecipitation. The preprotein that was completely imported into the matrix of energized mitochondria in the



**FIG. 4.** Comparison of the interaction of MGE and preproteins with mt-hsp70. Proteins associated with mt-hsp70 were isolated from mitochondria of wild-type yeast (WT) and the temperature-sensitive mutant strains *ssc1-2* and *ssc1-3* (9) in the presence of 10 mM EDTA (-Mg-ATP) or 10 mM magnesium acetate and 5 mM ATP (+Mg-ATP) as described in Material and Methods. Prior to lysis, the mitochondria were incubated for 15 min at 37°C to induce the mutant phenotype. The amount of bound MGE was determined as described in the legend to Fig. 3A. The total amount of extracted MGE was set to 100%. The fusion protein Su9-DHFR was synthesized in rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine and was imported into isolated yeast mitochondria of the wild type and the temperature-sensitive mutant strains *ssc1-2* and *ssc1-3* under nonpermissive conditions as described in Material and Methods. After treatment with proteinase K (100  $\mu$ g/ml), the mitochondria were lysed, and immunoprecipitations with antisera against mt-hsp70 or DHFR or preimmune serum were performed in digitonin buffer as described above. When indicated, the digitonin buffer contained 10 mM magnesium acetate and 5 mM ATP or 10 mM EDTA. The amount of immunoprecipitated Su9-DHFR obtained with antiserum against DHFR was set to 100%.

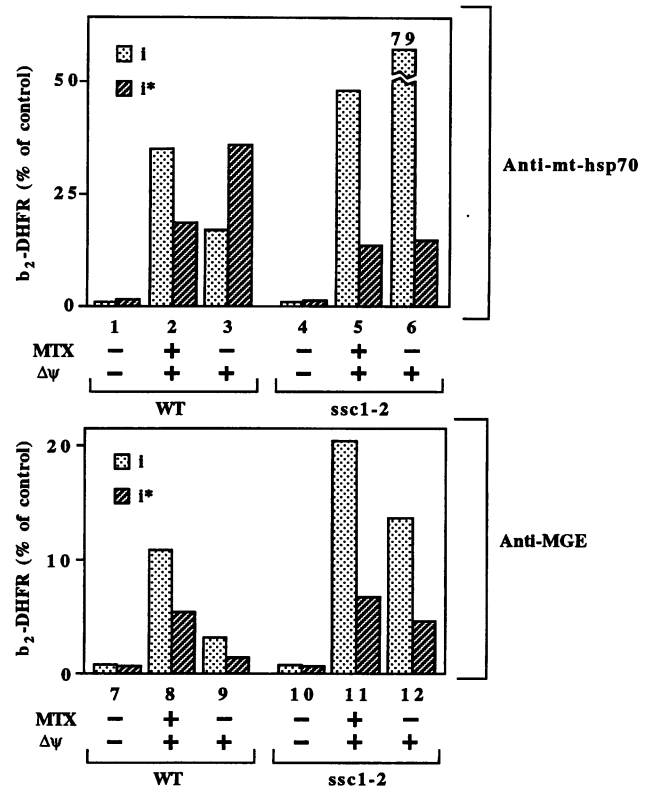
absence of methotrexate (Fig. 5A, lane 6) was found to be associated with mt-hsp70 (Fig. 5B, lane 6), but only to a small degree with MGE (Fig. 5B, lane 3).

Some matrix-targeted *b*<sub>2</sub>-DHFR fusion proteins, such as the one used here, yield two processing products with slightly different molecular weights (33). When import reactions are carried out in the presence of methotrexate, we observed a processing product, i, preferentially as the the membrane-



**FIG. 5.** MGE interacts with a preprotein in transit across the mitochondrial membranes. (A) Import steps of  $b_2$ -DHFR. The matrix-targeted precursor protein  $b_2$ -DHFR (containing the amino-terminal region of the precursor of cytochrome  $b_2$  with a deletion of the intermembrane space targeting signal) was synthesized in rabbit reticulocyte lysate in the presence of [ $^{35}$ S]methionine and incubated with isolated yeast mitochondria in the presence (+) or absence (-) of a membrane potential ( $\Delta\psi$ ) as described in Materials and Methods. For samples 3 and 4, the preprotein was preincubated with methotrexate (MTX). When indicated, the samples were treated with 150  $\mu$ g of proteinase K (Prot. K) per ml after the import incubation. Analysis was by SDS-PAGE and autoradiography. (B) Association of  $b_2$ -DHFR with MGE and mt-hsp70 during translocation. The precursor protein  $b_2$ -DHFR was incubated with isolated mitochondria as indicated. In the samples for lanes 3 and 6, the mitochondria were treated with proteinase K (150  $\mu$ g/ml) after the import reaction. The samples were split into thirds. The reisolated mitochondria were lysed in digitonin buffer, and immunoprecipitations under nonreducing conditions with antisera directed against MGE, mt-hsp70, or DHFR were performed. The amount of  $b_2$ -DHFR immunoprecipitated with antiserum against DHFR was set to 100%. p, precursor form; i and  $i^*$ , processed forms of  $b_2$ -DHFR.

spanning intermediate (Fig. 5A, lane 3), while in the absence of methotrexate most of the  $b_2$ -DHFR was in the form of the slightly smaller product,  $i^*$  (Fig. 5A, lane 6). Whereas the  $i$  form that accumulated in the presence of methotrexate was coimmunoprecipitated by both anti-mt-hsp70 and anti-MGE antibodies (Fig. 6, columns 2 and 8), the matrix-imported  $i^*$  form found in import reaction mixtures lacking methotrexate



**FIG. 6.** MGE shows a prolonged interaction with precursor proteins in *ssc1-2* mutant mitochondria. In vitro import reactions using isolated mitochondria of wild-type and mutant strain *ssc1-2* and the precursor protein  $b_2$ -DHFR were performed as described in the legend to Fig. 5B. Prior to the import reaction, the mitochondria were incubated for 15 min at 37°C in BSA buffer to induce the mutant phenotype. Samples 3, 6, 9, and 12 were treated with proteinase K (150  $\mu$ g/ml) after the import reaction. After reisolation of the mitochondria, immunoprecipitations using antisera against mt-hsp70, MGE, and DHFR were performed under native conditions. The amount of  $b_2$ -DHFR immunoprecipitated with antiserum directed against DHFR was set to 100%.  $i$  and  $i^*$ , processed forms of  $b_2$ -DHFR.

was rather efficiently coimmunoprecipitated with anti-mt-hsp70 antibodies (Fig. 6, columns 3) but not with anti-MGE antibodies (Fig. 6, columns 9). The distinct processing pattern thus seems to serve as a convenient tool to monitor the stage specificity of import and coprecipitation analysis, since the  $i$  form represents an earlier import step than the  $i^*$  form. This is supported by the observation that in *ssc1-2* mitochondria, in which the release of preproteins from mt-hsp70 is impaired, the amount of the  $i$  form is increased (Fig. 6, columns 5 and 11). In *ssc1-2* mitochondria, even the preproteins imported into the mitochondrial matrix and associated with mt-hsp70 were mainly of the  $i$  form (Fig. 6, columns 6), indicating that the defect in *Ssc1-2p* retarded the import and maturation pathway of the preprotein at the level of the  $i$  form. Interestingly, in *ssc1-2* mitochondria, MGE was also found to interact with preproteins imported into the matrix (Fig. 6, columns 12). The presence of MGE in a ternary complex with preprotein and mt-hsp70 is thus prolonged by the *ssc1-2* mutation, whereas, as shown in Fig. 4, the interaction between MGE and mt-hsp70 in the absence of preproteins is not influenced by the *ssc1-2* mutation.

We conclude that MGE and mt-hsp70 are associated with a

membrane-spanning translocation intermediate. This association suggests a role for a GrpE homolog in the import of precursor proteins.

### DISCUSSION

We have identified a GrpE homolog (MGE) in the matrix of *N. crassa* mitochondria by cross-reaction with an antiserum directed against *E. coli* GrpE. The cross-reaction was possible only when the 24-kDa mitochondrial protein was renatured prior to immunodetection, suggesting the conservation of a nonlinear epitope between *N. crassa* and *E. coli* GrpE. MGE is associated with mt-hsp70 and is dissociated by Mg-ATP. MGE is thus comparable to *E. coli* GrpE in size and in association with hsp70 (DnaK) (40). We detected a similar complex between mt-hsp70 and a 24-kDa protein of yeast mitochondria, the very recently identified yeast GrpE (termed MGE1 or YGE1). Yeast MGE1/YGE1, identified by its sequence homology to *E. coli* GrpE, is essential for the viability of yeast cells and can functionally substitute for *E. coli* GrpE (15, 19, 37).

MGE extracted from mitochondria by detergent is largely bound to mt-hsp70. The interaction is not destroyed by high concentrations of salt, pointing to a role for nonionic binding forces. MGE is completely released from hsp70 by the addition of Mg-ATP but not by the nonhydrolyzable analogs ATP $\gamma$ S and AMP-PNP. Very low concentrations of magnesium ions are sufficient for the release of MGE from mt-hsp70 by ATP. Similar dependencies on nucleotides and ions were found for the interaction of *E. coli* GrpE and DnaK (10, 34, 35, 40). The nucleotide-dependent interaction of GrpE/MGE and DnaK/hsp70 has thus been found in prokaryotes and the evolutionarily distant eukaryotes *N. crassa* and *S. cerevisiae*, indicating its high conservation.

Since the characteristics of interaction between MGE and mt-hsp70 are very similar to those between GrpE and DnaK, it is possible that the mitochondrial proteins use mechanisms for fulfilling their chaperone functions that are analogous to those of the corresponding bacterial proteins. In addition, however, mt-hsp70 has a function that is not performed by DnaK. Namely, it binds to precursor polypeptide chains during translocation across the mitochondrial membranes and is thus essential for the import of proteins and not only for the folding of imported proteins (9, 17, 38). Therefore, we investigated if MGE participated in the membrane translocation of preproteins and indeed found that MGE as well as mt-hsp70 was associated with a preprotein spanning across the mitochondrial membranes. Since MGE extracted from mitochondria was nearly quantitatively bound to mt-hsp70, we conclude that a complex containing mt-hsp70 and MGE interacts with a membrane-spanning preprotein. The specificity of the coimmunoprecipitation approach was demonstrated by the lack of association of MGE and mt-hsp70 with a preprotein accumulated at the mitochondrial outer membrane. This indicates that MGE is involved in membrane translocation of mitochondrial preproteins. In support of this, yeast cells with reduced expression of MGE accumulate the precursor forms of mitochondrial preproteins (19).

Osipiuk et al. (23) showed that *E. coli* GrpE stimulated the binding of DnaK to a substrate protein (in this case, the bacteriophage  $\lambda$ P protein that binds to the origin of DNA replication), provided that DnaJ was present even in low amounts. It is thus conceivable that a putative mitochondrial DnaJ homolog participates in the interaction of mt-hsp70 and MGE with a precursor protein in transit. It cannot be excluded that one of the additional protein bands that bound to mt-

hsp70 under nondenaturing conditions represents a mitochondrial DnaJ homolog. The recently identified mitochondrial homolog of DnaJ (MDJ1), however, is not required for membrane translocation of preproteins. It is involved in refolding of imported proteins (37). Future studies will have to address if mitochondria may contain more than one DnaJ homolog.

Interestingly, a preprotein that was further imported into the matrix was found still to be associated with mt-hsp70 but no longer with MGE. mt-hsp70 is about threefold more abundant than MGE. MGE-free mt-hsp70 molecules apparently interact with imported preproteins that are in an unfolded conformation (17, 22) to initiate the further folding steps. In analogy to the situation in *E. coli*, it is speculated that this occurs in cooperation with a mitochondrial DnaJ homolog such as MDJ1 (12, 37). An MGE would then be needed again to mediate the transfer of the polypeptide chains to the hsp60-cpn10 system. As with *E. coli* GrpE, this role of MGE may be a very transient one, explaining the lack of detection of a significant association of MGE and precursor proteins imported into the matrix.

A yeast mutant of mt-hsp70 with an altered ATPase domain (Ssc1-3p) is unable to bind MGE. Nor does Ssc1-3p bind preproteins in transit across the membranes, raising the possibility that the lack of interaction with MGE causes the inability to bind preproteins. Another yeast mutant of mt-hsp70 with a mutation in the putative peptide-binding region, termed Ssc1-2p, still binds precursor proteins; however, most of the bound protein is not released in the presence of Mg-ATP, unlike wild-type mt-hsp70. In contrast, MGE bound to Ssc1-2p in the absence of preproteins is fully released by the addition of Mg-ATP. We conclude that a functional ATPase domain of mt-hsp70 is required for association with MGE and that the alteration of the peptide-binding region in Ssc1-2p does not affect the interaction between mt-hsp70 and MGE when preproteins are not bound. These results suggest that precursor proteins and MGE are bound to different sites of mt-hsp70. However, the *ssc1-2* mutation enhanced and prolonged the association of MGE with preproteins, suggesting that mt-hsp70 is structurally and functionally involved in the interaction between MGE and preproteins. We speculate that mt-hsp70 is the core component of a ternary complex including MGE and preproteins. MGE and preproteins may not interact with each other directly, but rather via mt-hsp70. The mode of interaction of one partner (MGE or preprotein) with mt-hsp70 seems to influence the binding of the other partner.

In conclusion, we describe a role for mitochondrial GrpE, i.e., the cooperation with hsp70 to drive preprotein translocation at the *trans* side of a membrane. In the sequence of events of mitochondrial protein import, this interaction of preprotein, mt-hsp70, and MGE occurs before the steps that lead to folding of imported proteins. It is likely that the processes leading to protein folding, which involve mt-hsp70, a mitochondrial DnaJ, MGE, hsp60, and cpn10, then occur by mechanisms comparable to those established with the homologous *E. coli* components. While a situation analogous to import of preproteins does not exist in prokaryotes, such a situation may exist in the endoplasmic reticulum and in chloroplasts. We speculate that the hsp70 (Kar2p, BiP) in the lumen of the endoplasmic reticulum cooperates with a hypothetical endoplasmic reticulum GrpE (EGE) in driving the import of preproteins.

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