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70-kD Heat Shock–related Protein Is One of at Least Two Distinct Cytosolic Factors Stimulating Protein Import into Mitochondria

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Abstract. We have developed an in vitro system in which the posttranslational import of Put2 (delta[1]-pyrroline-5-carboxylate dehydrogenase), into yeast mitochondria is dependent on the addition of yeast postribosomal supernatant (PRS). When mRNA for a nuclear-encoded yeast mitochondrial matrix protein, Put2, was translated in a wheat germ cell-free system, import into posttranslationally added yeast mitochondria was negligible. However, when a yeast PRS was added, significant import was observed. The import stimulating activity of the yeast PRS was shown to consist of at least two distinct factors. One of these is the recently purified 70-kD heat shock–related protein Ssalp/Ssa2p, two proteins that are 98% homologous. The other factor is an *N*-ethylmaleimide-sensitive protein(s). Both factors act synergistically.

SEVERAL laboratories have reported that in vitro import of proteins into mitochondria is stimulated by cytosolic factors (Argan et al., 1983; Miura et al., 1983; Firgaira et al., 1984; Ohta and Schatz, 1984; Argan and Shore, 1985; Pfanner and Neupert, 1987; Ono and Tuboi, 1988). So far, however, these cytosolic factors have not been purified to homogeneity. In fact, proteins of rather different molecular mass of 40–50 (Ohta and Schatz, 1984; Argan and Shore, 1985) or 200 kD (Ono and Tuboi, 1988) or a ribonucleoprotein of 400 kD (Firgaira et al., 1984) have been reported to be the active component in partially purified fractions from rabbit reticulocyte lysate or yeast cytosol.

Recently, a yeast cytosolic protein, Ssalp, related to the 70-kD heat shock protein (hsp70), has been suggested to function in mitochondrial protein import. In a yeast strain where the chromosomal copies of three hsp70-related genes (*SSA1*, *SSA2*, and *SSA4*) were deleted and the production of plasmid-borne Ssalp was turned off, accumulation of precursors of a nuclear-encoded mitochondrial protein was observed (Deshaies et al., 1988).

To investigate the requirement for cytosolic factors in the in vitro import of proteins into mitochondria, we took advantage of the wheat germ cell-free translation system. Unlike the rabbit reticulocyte lysate translation system that is conventionally used for the synthesis of nuclear-encoded mitochondrial proteins and subsequent import into mitochondria (Maccecchini et al., 1979; Gasser, 1983; Argan et al., 1983; Chen and Douglas, 1987; Pfanner and Neupert, 1987), the wheat germ–based translation system has been shown to lack (or to contain either an inactive form or a negligible amount of) all those cytosolic factors that have so far been shown to be involved in protein translocation across microsomal membranes (Walter and Blobel, 1980; Waters et al., 1986; Fecycz and Blobel, 1987; Chirico et al., 1988). In fact, we show here that nuclear-encoded mitochondrial proteins, when synthesized in a wheat germ system, are not imported into yeast mitochondria to a significant extent unless a yeast postribosomal supernatant (PRS)[1] is added to the import reaction. We found that the yeast PRS contains at least two distinct import stimulating activities. One of these is the recently purified Ssalp/Ssa2p (Chirico et al., 1988), a mixture of two hsp70-related proteins of 98% homology. The other one is an *N*-ethylmaleimide (NEM)-sensitive protein(s) that remains to be purified. The implications of these findings for protein import into mitochondria are discussed.

Materials and Methods

Materials

The source of most materials was as described (Waters and Blobel, 1986). 7-methyldiguanosine triphosphate was from Pharmacia Inc. (Piscataway, NJ). NADH, calf liver tRNA, and proteinase K were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Restriction enzymes were from New England Biolabs (Beverly, MA). Sodium deoxycholate was from Schwarz/Mann Biotec (Orangeburg, NY) and was four times recrystallized from 90% acetone before use. Oligomycin, antimycin A, and carbonyl cyanide *m*-chlorophenylhydrazone were from Sigma Chemical Co. (St. Louis, MO).

SP6 In Vitro Transcription

The *PUT2* gene (pKB8) (Brandriss, 1983; Krzywicki and Brandriss, 1984)

1. *Abbreviations used in this paper*: NEM, *N*-ethylmaleimide; PRS, postribosomal supernatant; Put2, delta[1]-pyrroline-5-carboxylate dehydrogenase.

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was kindly provided by Dr. M. C. Brandriss (New Jersey Medical School, Newark, NJ). The *PvuII–SacI* fragment of this gene was inserted between the Sma I and Sac I sites of pSP64 (Promega Biotec, WI). This construction was carried out by Dr. J. Kaput (University of Illinois, Urbana, IL).

Before transcription, the plasmid was linearized downstream from the gene with Dra I. SP6 transcription was carried out in 40 mM Tris-HCl, pH 7.5, 6 mM magnesium chloride, 4 mM spermidine, 10 mM DTT, 0.25 mM each of ATP, GTP, CTP, UTP, and cap-analog, 7-methyldiguanosine triphosphate, 1 U/μl of RNase inhibitor (Promega Biotec, Madison, WI) and 0.05 mg/ml of the linearized DNA, at 40°C for 1 h. After transcription, mRNA was extracted by phenol/chloroform and collected by ethanol and lithium chloride precipitation.

Wheat Germ Cell-free Translation System

Translation in the wheat germ cell-free system was carried out essentially as described (Erickson and Blobel, 1983; Pain and Blobel, 1987). The translation mixture was also supplemented with 0.1 mg/ml of calf liver tRNA, 1 U/μl of RNase inhibitor, 10 U/ml of Trasylol, and 0.1 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin. After incubation, the ribosomes were removed by centrifugation for 22 min at 4°C in an airfuge at 30 psi (140,000 *g*; Beckman Instruments, Inc., Palo Alto, CA) and PRS, which contained in vitro–synthesized pre-delta^1-pyrroline-5-carboxylate dehydrogenase, (pre-Put2), was used for the import assay.

Preparation of Yeast PRS

Yeast PRS was prepared from *Saccharomyces cerevisiae* strain SKQ2N as described (Waters et al., 1986; Chirico et al., 1988). PRS was dialyzed against buffer A (20 mM Hepes-KOH, pH 7.4, 2 mM magnesium acetate, 2 mM DTT) containing 100 mM KOAc, 1 mM Mg-ATP, and 0.5 mM PMSF for 4 h at 4°C. To inactivate the metal requiring mitochondrial signal peptidase which might have leaked from mitochondria broken during the homogenization, PRS was incubated with 5 mM EDTA for 10 min on ice (McAda and Douglas, 1982) and then magnesium acetate was added to 5 mM to compensate for EDTA just before the import reaction.

Preparation of Ssalp/Ssa2p

Yeast Ssalp/Ssa2p was purified as described (Chirico et al., 1988) with slight modifications; yeast PRS was applied to a DEAE-cellulose column without prior salt exchanging on Sephadex G-25. The active fractions eluted with buffer A containing 300 mM KOAc and 1 mM Mg-ATP were pooled, dialyzed against buffer A containing 10 mM KOAc, and applied to an ATP-agarose column. The fractions containing Ssalp/Ssa2p were eluted as described (Chirico et al., 1988), concentrated by Amicon ultrafiltration with a PM30 membrane (Amicon Corp., Danvers, MA) and three volumes of buffer A containing 100 mM KOAc and 1 mM Mg-ATP were added. After concentration, this step was repeated once more. Finally the Ssalp/Ssa2p preparation was concentrated by Centricon 10 (Amicon Corp.), frozen in aliquots in liquid nitrogen, and stored at −80°C.

Isolation of Yeast Mitochondria

Mitochondria were isolated from *S. cerevisiae* wild-type strain D273-10B (ATCC25657) by the procedure described by Gasser (1983) with modifications. Yeast cells were cultured in 12 liters of 2% lactate medium and harvested as described (Gasser, 1983). After incubation with 0.1 M Tris-SO_4, pH 9.4 and 10 mM DTT, cells were incubated with 0.8 mg of Zymolyase-100T per gram of cells in the spheroplast buffer (20 mM potassium phosphate, pH 7.4, 1.2 M sorbitol) at 30°C for 60 min. The spheroplasts were washed twice with the spheroplast buffer, suspended in ice-cold homogenization buffer (20 mM Hepes-KOH, pH 7.4, 0.6 M sorbitol, 0.1% BSA, 1 mM EDTA, 1 mM PMSF, and 10 U/ml of Trasylol) to a concentration of 0.8 g of cells per milliliter, and then homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ).

The homogenate (45 ml) was diluted with 1 vol of the homogenization buffer and centrifuged at 1,464 *g* for 5 min at 2°C. The supernatant was saved and the pellet was homogenized in 40 ml of homogenization buffer, and the homogenate was centrifuged as above. Both supernatants were combined and centrifuged again as before to remove residual cell debris. Mitochondria were collected from the supernatant by centrifugation (10 min, 9,681 *g*, 2°C), resuspended in a small volume (1–2 ml) of homogenization buffer, and further purified by Percoll (Pharmacia Inc.) step-density gradient centrifugation; the mitochondrial fraction was loaded onto 25 ml of 20% Percoll and 7 ml of 40% Percoll in the homogenization buffer, and was centrifuged in an SW28 rotor (25 min, 70,000 *g*, 4°C; Beckman Instruments, Inc.). Mitochondria were collected from the interphase between 20 and 40% Percoll, diluted with 10 vol of buffer M (the homogenization buffer without EDTA) and centrifuged at 9,681 *g* for 10 min at 2°C. Finally mitochondria were resuspended in buffer M.

The protein concentration of the purified mitochondria was calculated by measuring the absorbance at 280 nm as described by Gasser (1983), and was adjusted to 30 mg/ml. For storage, 1 vol of an ice-cold solution of 20% DMSO, 20 mg/ml BSA, 20 mM Hepes-KOH, pH 7.4, 0.6 M sorbitol, 40 U/ml Trasylol, and 5 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin was added dropwise. Aliquots containing 1 mg of mitochondria per 66.7 μl were immediately frozen in liquid nitrogen and stored at −80°C. For all experiments aliquots of mitochondria were thawed only once.

Posttranslational Import into Mitochondria

Just before the import assay, an aliquot of frozen mitochondria was thawed at room temperature, immediately diluted with 1 ml of ice-cold buffer D (20 mM Hepes-KOH, pH 7.4, 0.6 M sorbitol, 0.1% BSA, 0.5 mM magnesium acetate and, 20 U/ml Trasylol) and mitochondria were sedimented in an Eppendorf centrifuge (16,000 *g*; Brinkmann Instruments, Inc., Westbury, NY) for 2 min at 4°C. The mitochondrial pellet was gently resuspended in 0.1 ml of ice-cold buffer S (20 mM Hepes-KOH, pH 7.4, 0.6 M sorbitol, 0.5% BSA, 50 U/ml Trasylol, and 1.25 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin).

The import assay mixture (40 μl) contained 2 μl of wheat germ PRS containing newly synthesized pre-Put2, 28 mM Hepes-KOH, pH 7.4, 0.6 M sorbitol, 62 mM KOAc, 13 mM magnesium acetate, 1 mM methionine, 2.4 mM ATP, 5 mM phosphoenolpyruvate, 2 mM GTP, 20 U/ml pyruvate kinase, 10 mM NADH, 1.8 mM DTT, 10 U/ml Trasylol, and 1.25 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin. NADH was included because it was found to stimulate Put2 import. Mitochondria were added to a concentration of 1 mg/ml, which was found to be the saturating amount for this import system. The import reaction was carried out at 30°C for 20 min. After incubation, each import reaction mixture was chilled on ice and then precipitated by adding an equal volume of 20% ice-cold TCA. The precipitates were resuspended and analyzed by SDS-PAGE in 6.5% separating gels, and 4% stacking gels, and autoradiography as described by Waters and Blobel (1986).

Protease Protection

After import, one of two aliquots received sodium deoxycholate at a concentration of 0.5%, then both aliquots were chilled on ice for 5 min. Proteinase K was added to a concentration of 120 μg/ml and the samples were incubated for 40 min on ice. The digestion was stopped by adding an equal volume of ice-cold 2 mM PMSF, 0.25 mg/ml of hemoglobin (Sigma Chemical Co.), 20 mM Hepes-KOH, pH 7.4, and 0.6 M sorbitol. After incubation for 5 min on ice, the samples were precipitated by TCA, and prepared for SDS-PAGE as described above.

Treatment of Yeast PRS or Ssalp/Ssa2p with NEM, Trypsin, or Heat

Aliquots of yeast PRS or purified Ssalp/Ssa2p were incubated with a final concentration of 10 mM NEM or 10 mM NEM and 20 mM DTT at 25°C for 15 min. The reactions were chilled on ice and a final concentration of 20 mM DTT was used to inactivate unreacted NEM in the NEM-treated samples.

Aliquots of yeast PRS were incubated with 40 μg/ml of trypsin at 30°C for 30 min in the presence or absence of a protease inhibitor mixture (final concentrations were 0.4 mg/ml of trypsin inhibitor (Sigma Chemical Co.), 40 U/ml of Trasylol and 1 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin). The reactions were chilled on ice and the protease inhibitor mixture was added to the aliquot that had not received it previously to inactivate trypsin.

An aliquot of yeast PRS was treated at 100°C for 2 min, chilled on ice, centrifuged for 2 min in an Eppendorf centrifuge (Brinkmann Instruments, Inc.), and the supernatant was collected for the import assay.

Determination of Percent Import

Dried SDS–polyacrylamide gels were directly scanned by a Beta Scanning System (Automated Microbiology Systems, Inc., San Diego, CA). Based on the data of the amino acid sequence of Put2 (Krzywicki and Brandriss,

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1984) and relative molecular mass of the precursor (64 kD) and mature forms (61 kD), we assumed that one of twelve methionine residues was lost during the processing. Accounting for this, the percent import was calculated as $100 \times m/(m + p \times 11/12)$, where m and p are radioactivities of mature and precursor forms of Put2, respectively. The percent conversion of the precursor form to the mature form that was not related to import was determined by carrying out corresponding mock import reactions in the absence of mitochondria. These values, ranging from 12.1% in the absence of yeast PRS to 20.2% in the presence of 4 mg/ml yeast PRS, were subtracted from the values obtained from the corresponding import reactions carried out in the presence of mitochondria.

Miscellaneous

Proteins were measured by the BCA protein assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard.

Results

Protein Import into Mitochondria Is Stimulated by Yeast PRS

A control wheat germ cell-free translation without any added mRNA did not show incorporation of [^{35}S]methionine into any protein (data not shown). However, when mRNA for Put2, a nuclear encoded mitochondrial matrix protein, was translated in a wheat germ cell-free system two translation products were detected in the PRS prepared posttranslationally (Fig. 1 *A*, lane *1*). The major one was the full-length, 64-kD precursor of Put2 (*p*). The other one (58 kD) (*asterisk*) presumably represented a truncated form of Put2, due to initiation from a downstream methionine codon at position 52 of the amino acid sequence (Krzywicki and Brandriss, 1984), and therefore lacked the amino-terminal signal sequence. After posttranslational incubation of the wheat germ PRS containing pre-Put2 with yeast mitochondria (Fig. 1 *A*, lane *4*), there was no significant import as there was neither significant conversion of the precursor to the mature form (*m*) by the matrix-localized signal peptidase, nor protection of the precursor from externally added proteinase K (data not shown). However, with increasing amounts of yeast PRS added to the import reaction, there was increasing conversion of the precursor to the mature form (Fig. 1 *A*, lanes *5–9* and quantitative data in *B*). Most of this processing was inhibited by adding inhibitors of the mitochondrial energy-generating systems (Fig. 1, lane *10*), suggesting that it was due to energy-dependent import. Moreover, equivalently high concentrations of hemoglobin or wheat germ PRS had no significant stimulatory effect on the processing in the absence of yeast PRS (data not shown), suggesting that the stimulation is due to some specific component(s) in the yeast PRS and not due to a nonspecific effect of the elevated protein concentration. During incubation without mitochondria and yeast PRS (Fig. 1 *A*, lane *2*), the precursor was neither processed to mature form nor degraded. The processing that was observed during the incubation with the maximum amount (4.0 mg/ml) of yeast PRS in the absence of mitochondria (Fig. 1 *A*, lane *3*), was likely due to the mitochondrial signal peptidase that had leaked from the mitochondrial matrix during homogenization of cells and subsequent preparation of PRS and that was not completely inactivated by the EDTA treatment of the yeast PRS (see Materials and Methods).

Furthermore, much of the mature form generated in the presence of PRS was protected from proteinase K digestion

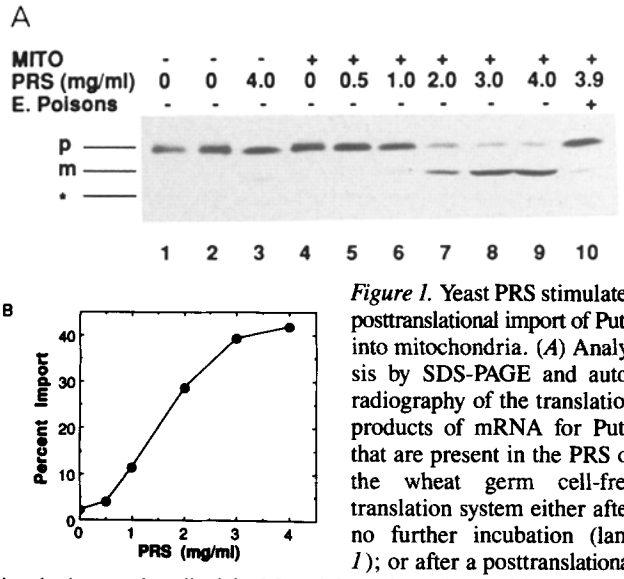

Figure 1. Yeast PRS stimulates posttranslational import of Put2 into mitochondria. (*A*) Analysis by SDS-PAGE and autoradiography of the translation products of mRNA for Put2 that are present in the PRS of the wheat germ cell-free translation system either after no further incubation (lane *1*); or after a posttranslational incubation as described in Materials and Methods either without supplements (lane *2*); or in the presence of 4 mg/ml yeast PRS (lane *3*); or 1 mg/ml of yeast mitochondria (lane *4*); or yeast mitochondria (1 mg/ml) plus 0, 0.5, 1.0, 2.0, 3.0, or 4.0 mg/ml of yeast PRS (lanes *5–9*, respectively); or 3.9 mg/ml of yeast PRS, yeast mitochondria (1 mg/ml), plus a cocktail of energy poisons (2.5 mM KCN, 2.5 mM carbonyl cyanide m-chlorophenylhydrazone, 10 μM oligomycin, and 5 μM antimycin A) (lane *10*). *MITO*, mitochondria; *E. Poisons*, cocktail of energy poisons; *p*, precursor form of Put2; *m*, mature form of Put2; *asterisk*, truncated form of Put2 presumably lacking 51-amino-terminal residues of the primary translation product of Put2. (*B*) Quantitative analysis of the data in *A* (see Materials and Methods).

(Fig. 2, lane *3*), whereas the precursor form was degraded. Protection was abolished when protease digestion was carried out in the presence of deoxycholate (Fig. 2, lane *4*). The mature form also sedimented with mitochondria (data not shown), suggesting that it was associated with them.

We conclude from these data that yeast PRS stimulates posttranslational import into yeast mitochondria when the nuclear-encoded mitochondrial protein, pre-Put2, was synthesized in a wheat germ cell-free system.

Import Stimulating Activity Is Sensitive to NEM

When yeast PRS was preincubated with 10 mM NEM and then added to the import reaction, its import-stimulating activity was abolished (Fig. 3, compare lanes *2* and *3*). Inactivation was prevented when preincubation of PRS was carried out with both 10 mM NEM and 20 mM DTT (Fig. 3, lane *4*). These data indicate that the import-stimulating factor contains a sulfhydryl group that is required for its activity.

Ssa1p/Ssa2p Stimulates Import Only in the Presence of Yeast PRS

Turning off the plasmid borne production of the hsp70-related gene product, Ssa1p, in a yeast strain that had its chromosomal copies of three hsp70-related genes deleted (*SSA1*, *SSA2*, and *SSA4*) has been shown to result in the in vivo accumulation of precursors of both an imported mitochondrial protein as well as secretory proteins (Deshaies et al., 1988).

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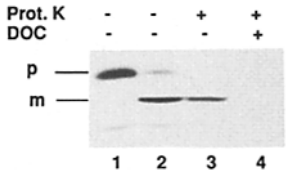

Figure 2. Imported Put2 is protected from proteinase K digestion. Aliquots of a wheat germ PRS containing the translation products of *PUT2* mRNA were analyzed (see Fig. 1) after either no further incubation (lane *1*); or after posttranslational incubation with mitochondria plus 3 mg/ml of yeast PRS (lane *2*) and a subsequent incubation with either proteinase K alone (lane *3*) or with proteinase K plus deoxycholate (lane *4*) as described in Materials and Methods. *Prot. K*, proteinase K. *DOC*, deoxycholate. *p* and *m*, as in Fig. 1.

To determine whether Ssalp, or the closely related Ssa2p that is copurified with Ssalp (Chirico et al., 1988), is also required for mitochondrial import in vitro, we added increasing amounts of Ssalp/Ssa2p (hsp70) purified from yeast PRS to the import reaction. Increasing amounts of Ssalp/Ssa2p stimulated import only slightly (Fig. 4, *A*, lanes *1–5* and *B*). However, when a "limiting" amount of yeast PRS (1.0 mg/ml) was added together with increasing amounts of Ssalp/Ssa2p, increased processing of the precursor to the mature form was observed (Fig. 4, *A*, lanes *6–10* and *B*). The mature form was protected from proteinase K digestion and also sedimented with mitochondria (data not shown). Moreover, this Ssalp/Ssa2p-dependent processing was sensitive to the inhibitors of the mitochondrial energy generating systems (data not shown). Thus, as is the case for translocation of secretory proteins across microsomal membranes (Deshaies et al., 1988; Waters, M. G., and G. Blobel, manuscript in preparation), Ssalp/Ssa2p by itself had no significant stimulatory effect on protein import into mitochondria, but stimulation was observed in the presence of yeast PRS. The stimulatory effect of Ssalp/Ssa2p in the presence of 1 mg/ml yeast PRS was saturated at a concentration of 0.2 mg/ml (Fig. 4 *B*). Therefore, the amount of Ssalp/Ssa2p present in the added yeast PRS was a limiting factor for protein import under these experimental conditions. These results suggested that, in addition to Ssalp/Ssa2p, another factor(s) was required for protein import into mitochondria.

Non-Ssalp/Ssa2p Activity of Yeast PRS Is Inactivated by Heat or Trypsin Treatment

When an import reaction was carried out in the presence of an excess of added Ssalp/Ssa2p (0.36 mg/ml) and of yeast PRS that had been preincubated at 100°C (Fig. 5, lane *5*) or

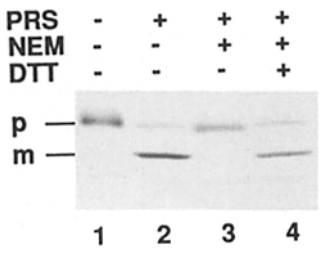

Figure 3. NEM inhibits the import-stimulating activity of yeast PRS. Aliquots of wheat germ PRS containing the translation products of *PUT2* mRNA were analyzed (see Fig. 1) after either no further incubation (lane *1*); or after posttranslational incubation with mitochondria plus either 3.7 mg/ml of untreated yeast PRS (lane *2*), or 3.7 mg/ml of yeast PRS pretreated with NEM alone (lane *3*), or with NEM plus DTT simultaneously (lane *4*) as described in Materials and Methods. *p* and *m*, as in Fig. 1.

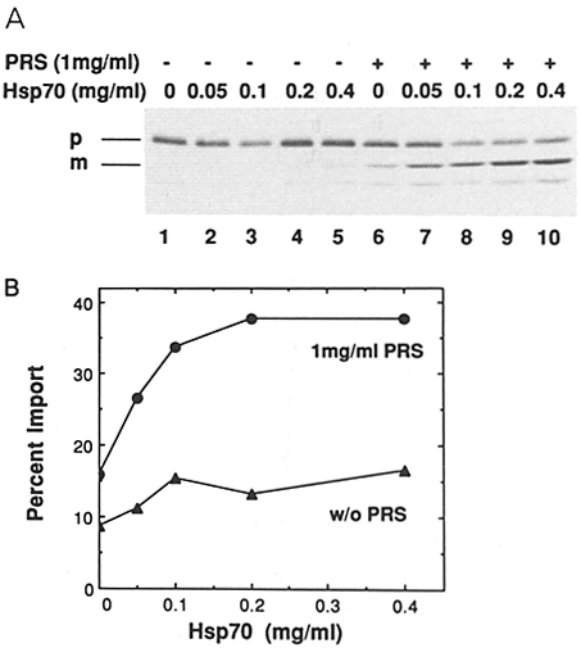

Figure 4. Purified yeast Ssalp/Ssa2p (hsp70) stimulates Put2 import in the presence of yeast PRS. (*A*) Aliquots of wheat germ PRS containing the translation products of *PUT2* mRNA were incubated with mitochondria and the indicated amount of purified yeast Ssalp/Ssa2p (hsp70) either in the absence (lanes *1–5*) or the presence of 1 mg/ml of yeast PRS (lanes *6–10*) and analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. *Hsp70*, purified yeast Ssalp/Ssa2p. *p* and *m*, as in Fig. 1. (*B*) Quantitative analysis of the data in *A* as described in Materials and Methods. (●) Percent import calculated from the data of lanes *6–10* in *A* (import reactions in the presence of 1 mg/ml yeast PRS). (▲) Percent import calculated from the data of lanes *1–5* in *A* (import reactions in the absence of yeast PRS).

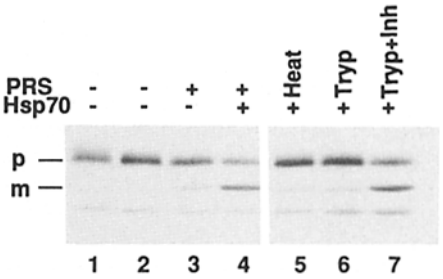

Figure 5. The import-stimulating activity in yeast PRS is inactivated by heat and trypsin treatment. Aliquots of wheat germ PRS containing the translation products of *PUT2* mRNA were analyzed as in Fig. 1 after either no further incubation (lane *1*); or after posttranslational incubation with mitochondria alone (lane *2*); or mitochondria plus 1 mg/ml of untreated yeast PRS (lane *3*); or mitochondria plus saturating amount (0.36 mg/ml, see Fig. 4 *B*) of Ssalp/Ssa2p (hsp70) plus, either 1 mg/ml of untreated PRS (lane *4*), or the corresponding amount of heat-pretreated PRS (lane *5*) or 1 mg/ml of yeast PRS pretreated with trypsin (lane *6*), or trypsin in the presence of protease inhibitor mixture (lane *7*) as described in Materials and Methods. *Heat*, heat-pretreated yeast PRS. *Tryp*, yeast PRS pretreated with trypsin. *Tryp+Inh*, yeast PRS pretreated with trypsin in the presence of protease inhibitor mixture. *Hsp70*, purified yeast Ssalp/Ssa2p. *p* and *m*, as in Fig. 1.

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with 40 μg/ml of trypsin (Fig. 5, lane *6*), import was abolished (compare with lane *4* in Fig. 5). Import was not affected when the trypsin pretreatment of yeast PRS was in the presence of protease inhibitors (Fig. 5, lane *7*). Thus, the non-Ssalp/Ssa2p activity of yeast PRS is a heat- and trypsin-sensitive protein.

Non-Ssalp/Ssa2p Activity of Yeast PRS Is NEM Sensitive

As Ssalp/Ssa2p is NEM insensitive (Chirico et al., 1988) (at least its activity stimulating secretory protein translocation across microsomal membranes) and as the import-stimulating activity of yeast PRS was inactivated by NEM (see Fig. 3), it is likely that the non-Ssalp/Ssa2p activity is NEM sensitive.

To determine whether the import stimulating activity of purified Ssalp/Ssa2p is indeed resistant to NEM treatment, an import reaction containing a limiting amount of yeast PRS (1.0 mg/ml) was incubated with a saturating amount of Ssalp/Ssa2p (hsp70) (0.37 mg/ml), either untreated (Fig. 6, *A* and *B*, lanes *4*) or preincubated with either 10 mM NEM alone (lanes *5*) or 10 mM NEM together with 20 mM DTT (lanes *6*). The data indicated that most of the import-stimulating activity of Ssalp/Ssa2p was not inactivated by NEM. Therefore the import-stimulating activity of yeast PRS, which is NEM sensitive (see Fig. 3), is unlikely to be due to that amount of Ssalp/Ssa2p that was introduced into the import reaction as part of the added yeast PRS.

To demonstrate that the NEM-sensitive activity is due to another factor present in yeast PRS, an excess amount of Ssalp/Ssa2p (0.37 mg/ml) was added to an import reaction together with a limiting amount of yeast PRS (1.0 mg/ml) that had been preincubated with either 10 mM NEM alone or with 10 mM NEM plus 20 mM DTT. Preincubation of yeast PRS with NEM abolished its import-stimulating activity even in the presence of an excess amount of Ssalp/Ssa2p (Fig. 6, *A* and *B*, lanes *7*), whereas simultaneous preincubation with NEM and DTT protected the activity (Fig. 6, *A* and *B*, lanes *8*). Therefore the non-Ssalp/Ssa2p activity in the yeast PRS was NEM sensitive and is most likely identical to the heat- and trypsin-sensitive factor(s) (Fig. 5).

Synergism between Ssalp/Ssa2p and NEM-sensitive Activity of Yeast PRS

An import reaction that contained increasing amounts of yeast PRS (containing both Ssalp/Ssa2p and the NEM-sensitive activity) yielded increasing import (Fig. 7, *A*, lanes *1–6*, and *B*). When excess Ssalp/Ssa2p (hsp70) (0.45 mg/ml) was added together with each of the amounts of PRS tested,

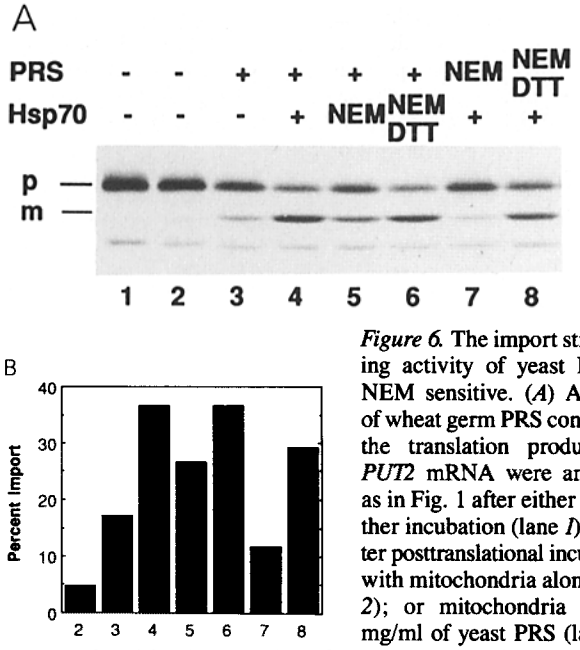

Figure 6. The import stimulating activity of yeast PRS is NEM sensitive. (*A*) Aliquots of wheat germ PRS containing the translation products of *PUT2* mRNA were analyzed as in Fig. 1 after either no further incubation (lane *1*); or after posttranslational incubation with mitochondria alone (lane *2*); or mitochondria plus 1 mg/ml of yeast PRS (lane *3*); or mitochondria plus 1 mg/ml of yeast PRS plus either saturating amount (0.37 mg/ml, see Fig. 4 *B*) of untreated Ssalp/Ssa2p (hsp70) (lane *4*); or 0.37 mg/ml of Ssalp/Ssa2p pretreated with NEM alone (lane *5*); or pretreated with NEM and DTT (lane *6*); or mitochondria plus 0.37 mg/ml of untreated Ssalp/Ssa2p plus 1 mg/ml of yeast PRS either pretreated with NEM alone (lane *7*); or pretreated with NEM and DTT (lane *8*) as described in Materials and Methods. *NEM*, NEM-treated PRS or hsp70 (Ssalp/Ssa2p). *NEM-DTT*, PRS or hsp70 (Ssalp/Ssa2p) treated with NEM in the presence of DTT. *Hsp70*, purified yeast Ssalp/Ssa2p. *p* and *m*, as in Fig. 1. (*B*) Quantitative analysis of the data in *A* as described in Materials and Methods. Numbers below the bars correspond to the lane numbers in *A*.

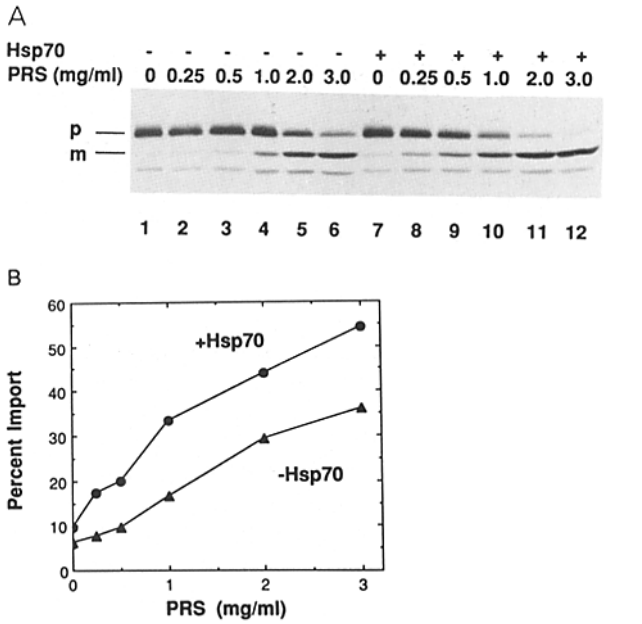

Figure 7. Increasing amounts of yeast PRS yield increasing stimulation of import in the presence of saturating amount of Ssalp/Ssa2p (hsp70). (*A*) Aliquots of wheat germ PRS containing the translation products of *PUT2* mRNA were incubated with mitochondria plus increasing amounts of yeast PRS as indicated, either in the absence (lanes *1–6*) or the presence (lanes *7–12*) of a saturating amount (0.45 mg/ml) of yeast Ssalp/Ssa2p (hsp70) and analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. *Hsp70*, purified yeast Ssalp/Ssa2p. *p* and *m*, as in Fig. 1. (*B*) Quantitative analysis of the data in Fig. 7 *A* as described in Materials and Methods. (●) Percent import calculated from the data of lanes *7–12* in *A* (import reactions in the presence of a saturating amount of Ssalp/Ssa2p [hsp70]). (▲) Percent import calculated from the data of lanes *1–6* in *A* (import reactions without adding Ssalp/Ssa2p [hsp70]).

additional stimulation of import was observed (Fig. 7, *A*, lanes *7–12* and *B*). Since Ssalp/Ssa2p alone has little effect (Fig. 4, *A* and *B*), these data suggest that Ssalp/Ssa2p and the NEM-sensitive activities in the PRS act synergistically. Moreover, under the condition where a saturating amount of Ssalp/Ssa2p was added to the import reaction, the NEM-sensitive factor(s) is limiting, that is, the import was dependent on the amount of NEM-sensitive factor(s). Therefore, this system provides a functional assay for the purification of the NEM-sensitive factor(s), which is presently in progress.

Discussion

We have developed an in vitro system where protein import into yeast mitochondria is stimulated by added yeast PRS. We have shown here that yeast PRS contains at least two distinct stimulatory activities.

One of these activities is represented by the recently purified Ssalp/Ssa2p (Chirico et al., 1988), two hsp70-related proteins that are 98% homologous (Craig, E., personal communication), and that are therefore not readily separable from each other. Because of the near identity of these two proteins and the phenotypes of mutants in either one or both genes (Craig and Jacobsen, 1984), we assume that they can substitute for each other. Consistent with this assumption are the findings that only the triple deletion of the chromosomal genes for Ssalp, Ssa2p, and Ssa4p is lethal (Werner-Washburne et al., 1987) and that introduction of the *SSA1* gene on a plasmid (under the control of a galactose inducible promoter) restores viability (Deshaies et al., 1988). When galactose was replaced by glucose in the growth medium, Ssalp was no longer produced and eventually diluted to a point where precursors for both mitochondrial and secretory proteins accumulated before the cells ceased to be viable (Deshaies et al., 1988). Our data here show that a function for Ssalp/Ssa2p in mitochondrial protein import can be demonstrated also in a cell-free in vitro system. This system should permit us to define more precisely how, and at what, step Ssalp/Ssa2p functions in the protein import reactions. Based on the proposal by Pelham (1986) it is possible that Ssalp/Ssa2p interacts directly with pre-Put2 to keep it in or to transform it to a translocation-competent state. In addition, or alternatively, Ssalp/Ssa2p could protect sites (Chirico et al., 1988) of the soluble or membrane-bound signal recognition factors (see below).

Besides Ssalp/Ssa2p we have identified a second cytosolic activity that stimulates protein import into mitochondria. This activity is proteinaceous as it is inactivated by trypsin or heat and, unlike Ssalp/Ssa2p, it is NEM sensitive. Both Ssalp/Ssa2p and the NEM-sensitive protein act synergistically. By itself Ssalp/Ssa2p does not stimulate import significantly (Fig. 4). It does so only in conjunction with the NEM-sensitive protein.

Our finding here that the import reaction can be carried out in the presence of an excess amount of purified Ssalp/Ssa2p and, under these conditions, is solely dependent on the addition of the NEM-sensitive proteinaceous factor(s), has provided an assay for the purification of the NEM-sensitive proteinaceous factor(s).

By analogy to protein translocation across microsomal membranes we presume that the NEM-sensitive protein may function like the signal recognition particle (SRP), namely to recognize and to bind to the signal sequence of proteins targeted for translocation from the cytosol to the mitochondrial matrix. By further analogy, interaction of this soluble signal recognition factor with its cognate receptor on the mitochondrial membrane may cause dissociation of the soluble signal recognition factor from the signal sequence and thereby free the signal sequence to interact with a second membrane-bound signal recognition system located in contact zones between inner and outer mitochondrial membrane (Pain, D., H. Murakami, and G. Blobel, manuscript in preparation).

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