The EMBO Journal vol.15 no.2 pp.399–407, 1996

Cytoplasmic chaperones determine the targeting pathway of precursor proteins to mitochondria

Tohru Komiya, Masao Sakaguchi and Katsuyoshi Mihara[1]

Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812, Japan

[1]Corresponding author

Two ATP-dependent cytosolic chaperones, mitochondrial import stimulation factor (MSF) and hsp70, are known to be involved in the import of precursor proteins into mitochondria. Hsp70 generally recognizes unfolded proteins, while MSF specifically recognizes mitochondrial precursor proteins and targets them to mitochondria in a NEM-sensitive manner. Here we analyzed the relative contribution of these chaperones in the import process and confirmed that the precursor proteins are targeted to mitochondria via two distinct pathways: one requiring MSF and the other requiring hsp70. Both pathways depend on distinct proteinaceous components of the outer mitochondrial membrane. The MSF-dependent pathway is NEM-sensitive and requires the hydrolysis of extra-mitochondrial ATP for the release of MSF from the mitochondrial import receptor, whereas the hsp70-dependent pathway is NEM-insensitive and does not require extra-mitochondrial ATP. The NEM-insensitive, hsp70-dependent import became NEM-sensitive depending on the amount of MSF added. The relative importance of the two pathways appears to be determined by the affinities of MSF and hsp70 for the precursor proteins.

Keywords: ATPase/chaperones/mitochondria/precursor protein/protein import

Introduction

Most mitochondrial proteins are synthesized in the cytosol as precursors, directed to the outer surface of the mitochondria and then translocated either into or across the membranes to their correct intra-mitochondrial location. This import process requires ATP in both the cytosol and matrix as well as the electrochemical potential across the inner membrane (Attardi and Schatz, 1988; Pfanner and Neupert, 1990; Glick *et al.*, 1992; Stuart *et al.*, 1994; Glick, 1995). Mitochondrial protein import has been intensively studied and several components of the import machinery of the outer and inner membranes have been characterized (Söllner *et al.*, 1989; Baker *et al.*, 1990; Hines *et al.*, 1990; Kiebler *et al.*, 1990; Ono and Tuboi, 1990; Söllner *et al.*, 1990; Baker and Schatz, 1991; Pfanner *et al.*, 1991; Hines and Schatz, 1993; Kiebler *et al.*, 1993; Nakai *et al.*, 1993; Ramage *et al.*, 1993; Lithgow *et al.*, 1994; Pfanner *et al.*, 1994; Nakai and Endo, 1995). Nevertheless, the events that occur in the cytoplasm are still poorly understood. It has been shown that hsp70 in the cytosol is involved in the import of precursor proteins into mitochondria. Deshaies *et al.* (1988) speculated that hsp70 maintains the import-competent, unfolded conformation of the precursors in an ATP-dependent manner (Neupert *et al.*, 1990; Sheffield *et al.*, 1990; Craig, 1993). Another cytosolic factor sensitive to N-ethylmaleimide (NEM) has been reported to be involved in yeast mitochondrial protein import, a role for it in precursor recognition and mitochondria targeting has been suggested (Murakami *et al.*, 1988). In this respect, a cytoplasmic factor that stimulates precursor import into mitochondria *in vitro* has been purified from rat liver and termed mitochondrial import stimulation factor (MSF) (Hachiya *et al.*, 1993). MSF is a heterodimer of a 32 kDa and a 30 kDa subunit; it unfolds aggregated precursors in a reaction requiring ATP hydrolysis and also targets precursors to mitochondria in a NEM-sensitive manner (Hachiya *et al.*, 1993; Lithgow *et al.*, 1993; Hachiya *et al.*, 1994; Lithgow *et al.*, 1995).

Although these cytosolic chaperone proteins are clearly involved in the import of precursor proteins into mitochondria, the molecular mechanisms of their actions are still poorly understood. Hsp70 generally recognizes unfolded proteins while MSF specifically recognizes mitochondrial precursor proteins and targets them to mitochondria. In addition, the mechanism requiring cytoplasmic ATP in the process of mitochondrial protein import and the way in which the newly synthesized precursor proteins are transferred to the 'import receptors' on the mitochondrial surface are also unknown. Two such receptor complexes of yeast mitochondria are known: Mas70p/Mas37p and Mas22p/Mas20p (Gratzer *et al.*; 1995; Lithgow *et al.*, 1995). The *Neurospora crassa* proteins corresponding to Mas70p, Mas22p and Mas20p are termed MOM72, MOM22 and MOM19 respectively (Söllner *et al.*, 1989, 1990; Hines *et al.*, 1990; Kiebler *et al.*, 1993; Ramage *et al.*, 1993; Lithgow *et al.*, 1994; Hönlinger *et al.*, 1995).

Here we analyze the chaperone-mediated import of the precursor proteins into mitochondria by means of an *in vitro* protein import system using the chemically pure precursor proteins, preadrenodoxin (pAd) and porin and the purified chaperones hsp70 and MSF.

We show that the precursor proteins can be imported into mitochondria by both a NEM-sensitive, MSF-dependent pathway and a NEM-insensitive, hsp70-dependent pathway. In addition, hsp70-dependent precursor targeting occurs through a direct interaction of the precursors with the membrane receptor. MSF-dependent import of the precursor proteins requires extra-mitochondrial ATP for release of MSF. During hsp70-dependent import, hsp70 spontaneously dissociates from the precursor without requiring extra-mitochondrial ATP.

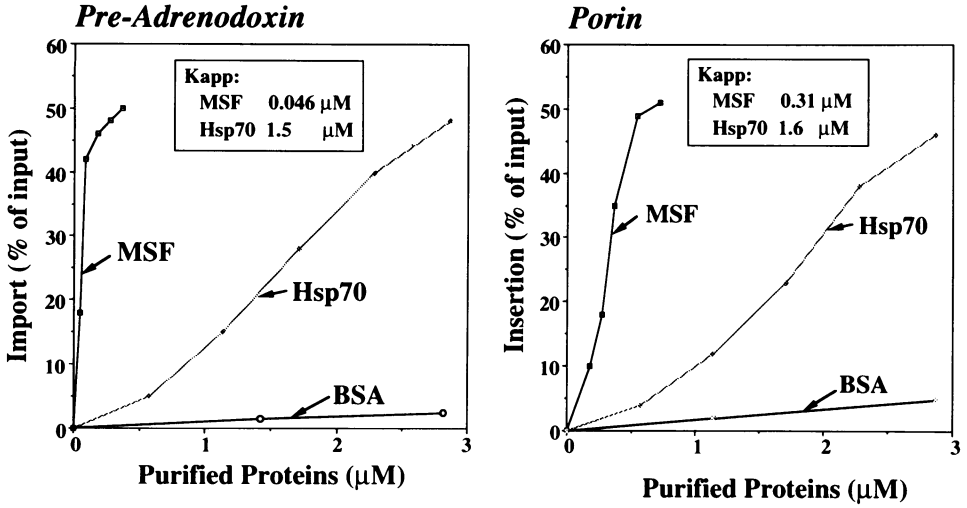

Fig. 1. MSF and hsp70 maintain the import-competence of pAd and porin. ^{125}I-labeled pAd or ^{125}I-labeled porin in 7 M urea was diluted 40-fold into the 'import buffer' containing the indicated amounts of MSF, hsp70 or bovine serum albumin (BSA). After incubation at 0°C for 30 min, the reaction mixtures were assayed for mitochondrial import of pAd or insertion of porin into mitochondria as described in Materials and methods. The concentrations MSF and hsp70 were calculated assuming the molecular masses of MSF and hsp70 to be 57 kDa and 70 kDa, respectively. K_{app}: the concentrations of MSF and hsp70 giving a half maximum import of the precursor proteins.

Results

MSF and hsp70 maintain the import-competence of mitochondrial precursor proteins

MSF stimulated the import of precursor proteins synthesized in a wheat germ system; there was no additional stimulation by hsp70 (Hachiya *et al.*, 1993). Since it has been reported that hsp70 and an NEM-sensitive fraction synergistically stimulate mitochondrial protein import in yeast (Murakami *et al.*, 1988), we examined the effect of hsp70 and MSF on mitochondrial import of two chemically pure, urea-unfolded mitochondrial precursor proteins: pAd and porin.

Since hsp70 binds to the hydrophobic stretches on unfolded protein molecules (Flynn *et al.*, 1989, 1991; Blond-Elguindi *et al.*, 1993; Fourie *et al.*, 1994; Gragerov *et al.*, 1994; Hartl *et al.*, 1994), we first determined whether hsp70 recognized unfolded mitochondrial precursor proteins and stabilized their import-competent states in the same manner as that previously reported for MSF (Hachiya *et al.*, 1994). pAd and porin purified in the presence of 7 M urea rapidly lost their import-competence after a 40- to 50-fold dilution into the import buffer (Hachiya *et al.*, 1994). As shown in Figure 1, the presence of both hsp70 and MSF in the incubation mixture prevented pAd (left panel) and porin (right panel) from losing their import-competence dose-dependently. The concentrations (K_{app}) of hsp70 and MSF providing half the maximum import-competence were: 1.5 and 0.046 μM for pAd and 1.6 and 0.31 μM for porin respectively. These results suggest that the affinity of MSF differs significantly among precursor proteins (see also Figure 3). It should be noted that porin is the major mitochondrial outer membrane protein that lacks a cleavable signal sequence. Thus, MSF recognized mitochondrial precursor proteins irrespective of the presence of the cleavable signal sequence, confirming the previous results that pAd and porin induced significant MSF ATPase activity (Hachiya *et al.*, 1994). The element in the structure of porin that is recognized by MSF remains to be identified. In addition, BSA did not maintain the import-competence of pAd and porin.

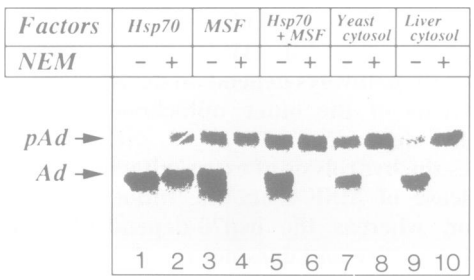

Fig. 2. MSF-dependent import of pAd but not that of hsp70, is NEM sensitive. 3.58 μM hsp70, 0.438 μM MSF, 3.58 μM hsp70 plus 0.438 μM MSF, 1.25 mg/ml of yeast cytosol or 5 mg/ml rat liver cytosol treated in 40 μl of the import buffer with 1.25 mM NEM at 30°C for 10 min, then the unreacted NEM was inactivated with 1.25 mM DTT (NEM+). As the control, NEM-treatment was performed in the presence of DTT (NEM–). ^{125}I-labeled pAd was diluted into the mixtures at 0.00625 μM, incubated at 0°C for 30 min and followed by the import reaction in 50 μl as described in Materials and methods. Positions of pAd and mature adrenodoxin Ad are indicated in the figure.

Two distinct precursor-targeting pathways

These results indicated that saturating amounts of MSF and hsp70 can independently support the import of urea-denatured pAd to a similar extent. Since MSF has a precursor-targeting function which is inhibited by NEM (Hachiya *et al.*, 1993, 1994), we examined the NEM-sensitivity of hsp70-dependent import. Whereas MSF-supported import was impaired by NEM, hsp70-supported import was NEM-insensitive (Figure 2, lanes 1–4). In addition, in the presence of saturating levels of MSF and hsp70, import of pAd mainly occurred via the MSF-mediated pathway (lanes 5 and 6). Hence the *in vitro* assays of MSF-mediated pathway were performed in the presence of a saturating amount of hsp70 (Figures 4A, 8 and 9). Yeast cytosol- and liver cytosol-supported pAd

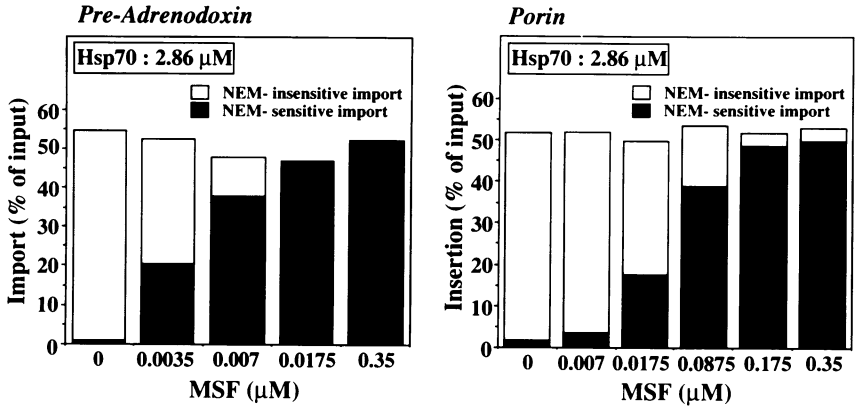

Fig. 3. Hsp70-dependent, NEM-insensitive import becomes NEM-sensitive depending upon the amount of MSF added. 2.86 μM hsp70 plus the indicated amounts of MSF in the import buffer were treated or mock-treated with NEM as described in the legend to Figure 2. ^{125}I-labeled pAd (0.00625 μM) or ^{125}I-labeled porin (0.00625 μM) was added to each reaction mixture and incubated at 0°C for 30 min, followed by the import reaction as described in Materials and methods.

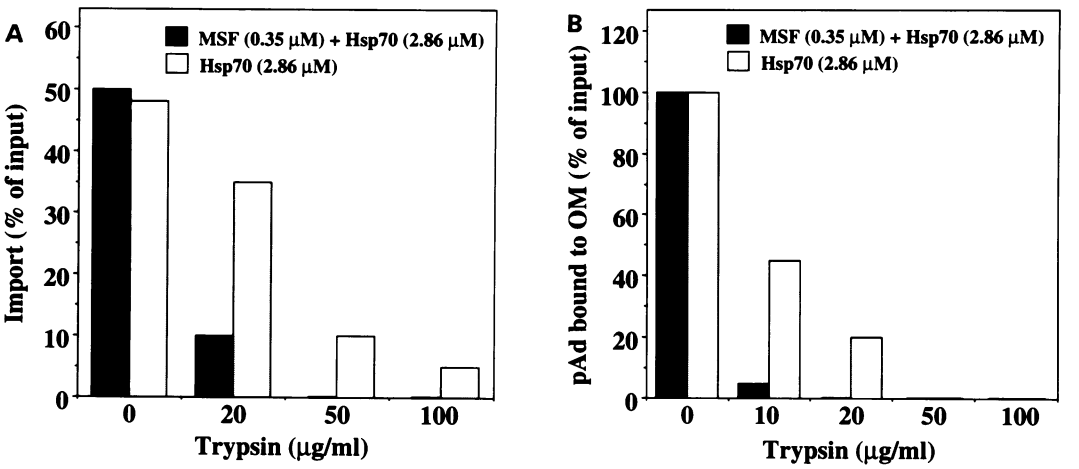

Fig. 4. (**A**) MSF- and hsp70-dependent import of pAd depend on distinct proteinaceous components of OM. ^{125}I-labeled pAd in 7 M urea was diluted into the import buffer containing the indicated amounts of hsp70 or MSF plus hsp70 and incubated at 0°C for 30 min. Trypsin-treated mitochondria were then added to the mixture and the import reactions were performed as described in Materials and methods. (**B**) Trypsin susceptibility of MSF- and hsp70-dependent binding of pAd to OM. [^{125}I]pAd in 7 M urea was diluted into the import buffer containing the indicated amounts of [^{125}I]hsp70 or [^{125}I]MSF plus [^{125}I]hsp70 and incubated at 0°C for 30 min to form complexes. The complexes were incubated with 0–100 μg/ml trypsin-treated OM preparations at 30°C for 30 min. The reaction mixtures were mixed with TKM and ultracentrifuged to separate the supernatant and OM fractions. Both fractions were resolved by SDS–PAGE.

import was NEM-sensitive (lanes 7–10). We found further that the NEM-insensitive import became NEM-sensitive in the presence of MSF (Figure 3, left panel). The import rate did not significantly change at any ratios of hsp70:MSF which suggests that the precursor-targeting step was not rate-limiting in this assay. At 0.0175 μM MSF, which is the concentration corresponding to that in the cytosol, pAd import into the mitochondria was completely NEM-sensitive. Essentially the same results were also obtained for the mitochondrial insertion of porin except that an ~10-fold higher concentration of MSF was required to reach the plateau of NEM-sensitive import (Figure 3 right panel), suggesting that the affinity of MSF differs between different precursor proteins.

We then addressed the issue of whether different receptors on the outer membrane are utilized in the MSF- and hsp70-supported import of pAd into mitochondria. Rat liver mitochondria were incubated with various concentrations of trypsin and their capacity for MSF- and hsp70-dependent pAd import was then assayed (Figure 4A). Both MSF- and hsp70-dependent import was significantly impaired by trypsin although the former was the more sensitive. Similar results were obtained for MSF- and hsp70-dependent binding of pAd to the isolated outer mitochondrial membrane (OM) (Figure 4B). These results suggested that the MSF- and hsp70-dependent import of precursor proteins occurs via distinct import receptors on the OM.

To determine whether or not these two receptor types function independently, we examined the competition between pAd and porin for binding to OM. Figure 5A shows that the hsp70–pAd complex but not the MSF–pAd complex, significantly inhibited the hsp70-supported binding of porin (lanes 3–6). Conversely, the MSF–pAd complex but not the hsp70–pAd complex, interfered with the MSF-supported binding of porin (lanes 9–12), thus indicating that the two receptors function independently.

In a previous report we showed that MSF binds to OM only in the presence of pAd (Hachiya *et al.*, 1994). To check whether binding of the complex to mitochondria is

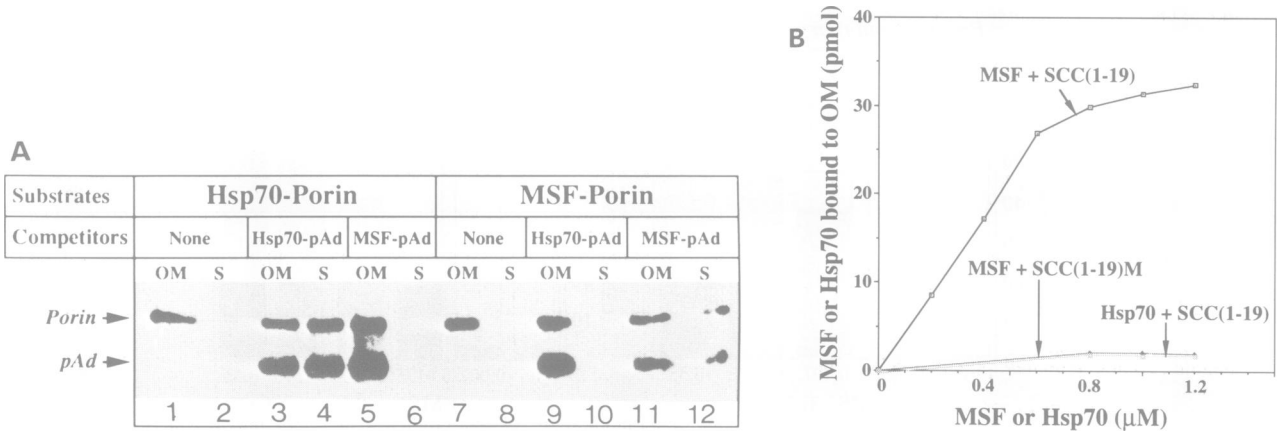

Fig. 5. (**A**) Two membrane receptors function independently. The competition between ^{125}I-labeled porin (defined as substrate) and ^{125}I-labeled pAd (defined as competitor) for binding to OM was examined in the presence of hsp70, MSF or both as described in Materials and methods. The reaction mixtures were ultracentrifuged to separate the supernatant (S) and OM fractions and both fractions were resolved by SDS–PAGE. (**B**) A functional signal peptide induces the binding of MSF to OM. The binding of ^{125}I-labeled MSF and ^{125}I-labeled hsp70 to OM was examined in the presence of a functional signal peptide, SCC(1-19) or a non-functional mutant peptide, SCC(1-19)M as described in Materials and methods.

mediated by the precursor's signal peptide, we replaced the precursor with synthetic signal peptides (Figure 5B). MSF bound to OM in the presence of a functional signal peptide SCC(1-19) but not in the presence of the corresponding mutant peptide, SCC(1-19)M, in which three basic amino acids of SCC(1-19) had been changed to serine or threonine (Komiya *et al.*, 1994). No significant binding of hsp70 was observed even in the presence of the functional signal peptide. These findings suggest that OM contains a receptor for MSF and that MSF binding to the receptor is regulated by the signal peptide of the precursor. From Figure 5B, the putative MSF receptor was calculated to be present at a concentration of ~7.5–15 pmol/mg mitochondrial protein, assuming that the OM comprises ~5–10% (w/w) of total mitochondrial protein. For comparison, MOM72 and MOM19 of the mitochondrial OM of *N.crassa* are present at ~2–5 and 2–4 pmol/mg mitochondrial protein, respectively (Söllner *et al.*, 1989, 1990).

MSF- and hsp70-dependent binding of pAd to the outer mitochondrial membrane

We first examined whether urea-unfolded pAd can form complexes with hsp70 or hsp70 plus MSF. The complex formation was examined by co-immunoprecipitation with IgG raised against mature adrenodoxin (Ad) or the MSF large subunit (MSFL) or with pre-immune IgG (Figure 6). When urea-unfolded [^{125}I]pAd was mixed with [^{125}I]hsp70 at a molar ratio of 1:5, pAd and hsp70 were both immunoprecipitated with anti-Ad IgGs (lanes 3 and 4). Similarly, when [^{125} I]Ad was mixed with [^{125}I]MSF and [^{125}I]hsp70 at a molar ratio of 1:1.4:5, all of the components were immunoprecipitated either with anti-Ad IgGs (lanes 11 and 12) or with anti-MSFL IgGs (lanes 19 and 20). These results indicate that hsp70 or hsp70 plus MSF formed a binary or ternary complex with pAd. These complexes dissociated in the presence of ATP (lanes 5, 6 and 13–16) and this dissociation was inhibited by an excess of AMP-PNP, a non-hydrolyzable ATP analogue (lanes 7, 8, 17 and 18). Dissociation of the complexes thus required ATP hydrolysis. No interaction between MSF and hsp70 was observed (data not shown). We then

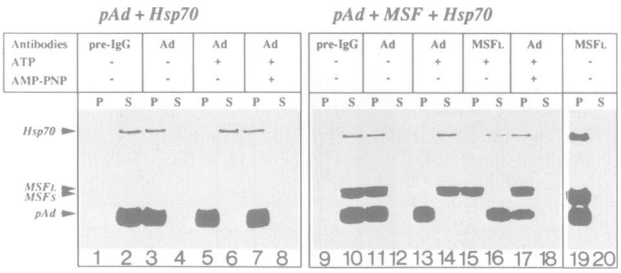

Fig. 6. Unfolded pAd forms a complex with hsp70 or MSF and hsp70. ^{125}I-labeled pAd was diluted into TKM containing ^{125}I-labeled hsp70 or ^{125}I-labeled hsp70 plus ^{125}I-labeled MSF and incubated at 30°C for 30 min (the final concentrations of pAd, hsp70 and MSF were 0.005, 0.025 and 0.007 μM, respectively). The reaction mixtures were incubated with or without ATP or with ATP plus excess AMP-PNP, for another 30 min. The complex formation was examined by co-immunoprecipitation using IgGs raised against mature Ad or the large subunit of MSF (MSFL) or pre-immune IgG (Pre-IgG). The immunprecipitate (P) and the supernatant (S) fractions were resolved by SDS–PAGE. Lanes 19 and 20 show the results of another experiment.

examined the binding of these complexes to OM (Figure 7). When the pAd–hsp70 complex was incubated with OM in the absence of ATP, only pAd was recovered from the membrane while, unexpectedly, hsp70 was recovered from the supernatant (lanes 1 and 2). In marked contrast to the data shown in Figure 6, neither treatment with apyrase nor AMP-PNP prevented dissociation of hsp70 from the complex (lanes 3–6). In addition, the pAd–hsp70 complex did not bind to trypsin-treated OM (t-OM; lanes 7 and 8). These results further indicate that pAd interacts directly with an import receptor on OM in the hsp70-dependent targeting. When the pAd–MSF–hsp70 complex was incubated with OM, hsp70 dissociated from the complex and MSF and pAd were bound to the OM (lanes 9 and 10). Again, neither apyrase (lanes 11 and 12) nor AMP-PNP (data not shown) prevented dissociation of hsp70 from the complex. Furthermore, ATP did not cause dissociation of MSF from the complex (lanes 13 and 14), which seems to be consistent with the data that OM inhibited the ATPase activity of MSF (Hachiya *et al.*,

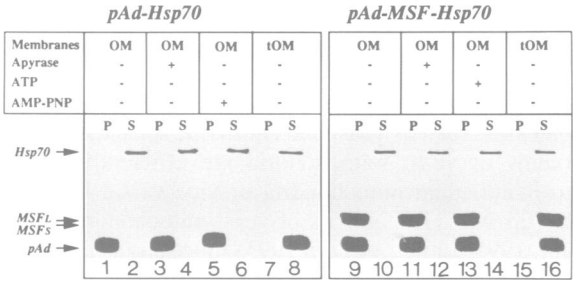

Fig. 7. Interaction of the binary or ternary targeting complexes with OM induces the release of hsp70 in the absence of ATP hydrolysis. pAd–hsp70 or pAd–MSF–hsp70 complexes prepared as described in the legend to Figure 6 were incubated at 30°C for 30 min, each with 5 μg of either OM or trypsin-treated OM (tOM) under the indicated conditions. The reaction mixtures (50 μl) were mixed with 300 μl of TKM and ultracentrifuged to separate supernatant (S) and OM (P). Both fractions were resolved by SDS–PAGE. Other conditions are described in Materials and methods.

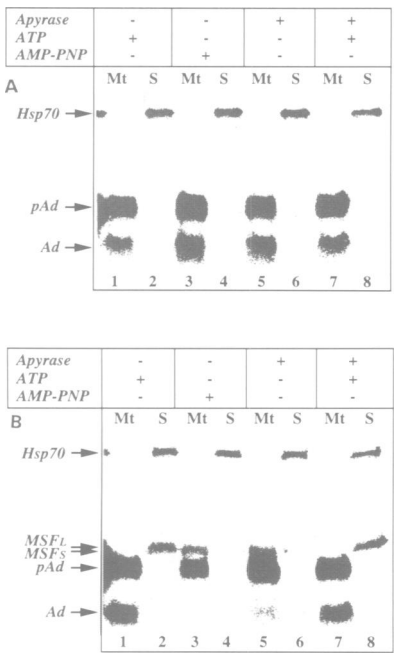

Fig. 8. (**A**) Hsp70-dependent import of pAd does not require extra-mitochondrial ATP. ^{125}I-labeled pAd and ^{125}I-labeled hsp70 were mixed in TKM and incubated at 30°C for 30 min. This complex was subjected to the import assay. Where indicated, 1 mM ATP (ATP+) or 1 mM ATP plus 5 mM AMP-PNP (AMP-PNP+) was added to the reaction mixture or apyrase-treated mitochondria (Apyrase+) was used in lieu of the intact mitochondria. After the import reaction mitochondria (Mt) and supernatant (S) fractions were separated by centrifugation and were subjected to SDS–PAGE. Percent import in lanes 1, 3, 5 and 7 were 45%, 58%, 56% and 48%, respectively. (**B**) MSF-dependent import of pAd requires extra-mitochondrial ATP. The ternary complex was prepared from ^{125}I-labeled preparations of pAd, MSF and hsp70 and subjected to the import assay under the indicated conditions. Other conditions are described in the legend to Figure 8A and in Materials and methods. Percent import in lanes 1 and 7 as calculated from c.p.m. of Ad/c.p.m. of (pAd + Ad)×100 were 55% and 52%, respectively.

1994). Apparently some components required for the release of MSF were lost or inactivated in the isolated OM (for comparison, see Figure 8B).

These results suggest that the interaction of pAd–hsp70 or pAd–MSF–hsp70 complexes with the receptors on OM causes conformational changes in the precursor and thereby induces the dissociation of hsp70 from the complex even in the absence of ATP. These experiments, in conjunction with the data that NEM inhibits the precursor targeting function of MSF, further suggest that pAd binds to the membrane via MSF and its membrane receptor in MSF-dependent targeting, whereas pAd interacts directly with the membrane receptor in the hsp70-dependent precursor targeting.

Hsp70- and MSF-dependent import of pAd into mitochondria

We then examined the fate of hsp70 and MSF during the mitochondrial import of pAd in pAd–hsp70 and pAd–MSF–hsp70 targeting complexes (Figure 8A and B). When the pAd–hsp70 complex was analyzed by the standard import assay, pAd was imported and both pAd and mature Ad were recovered from the mitochondria, while hsp70 was released into the supernatant (Figure 8A, lanes 1 and 2). Import of pAd into the mitochondria and release of hsp70 into the supernatant occurred even in the absence of extra-mitochondrial ATP (apyrase treatment in the presence of carboxyatractyloside, an inhibitor of the ATP–ADP translocator of the mitochondrial inner membrane; lanes 5 and 6) or in the presence of AMP-PNP (lanes 3 and 4). However, when the pAd–MSF–hsp70 complex was subjected to the standard import assay, pAd was imported into mitochondria while both MSF and hsp70 were recovered from the supernatant fraction (Figure 8B, lanes 1 and 2). When AMP-PNP was present (lanes 3 and 4) or when extra-mitochondrial ATP was depleted with apyrase in the presence of carboxyatractyloside (lanes 5 and 6), hsp70 was released into the supernatant but pAd import was completely arrested and MSF remained bound to the mitochodria. When the apyrase-treated reaction mixture was replenished with ATP, the mitochondrial import of pAd was restored, concomitant with the release of MSF into the supernatant (lanes 7 and 8). These results indicate that the MSF-dependent import pathway requires extra-mitochondrial ATP to release MSF from its receptor on the OM whereas the hsp70-dependent pathway does not.

Characterization of the receptors of MSF- and hsp70-dependent import pathways

In order to characterize the putative import receptors in the two targeting pathways, we examined the effect of IgGs against OM proteins on MSF- and hsp70-dependent import of pAd (Figure 9). IgG against a 37 kDa OM protein (OM37) inhibited MSF-dependent binding and import of pAd to mitochondria (lanes 5 and 6) but it did not inhibit hsp70-dependent import of pAd (lanes 7 and 8). In contrast, IgG against a 14 kDa OM protein (OM14) which recognized a rat homolog of Mas20p (J.Iwahashi, S.Yamazaki, T.Komiya, H.Matsuo, N.Nomura and K.Mihara, unpublished data) inhibited the binding as well as the import of pAd in the hsp70-dependent pathway (lanes 11 and 12). On the other hand, it did not inhibit MSF-dependent binding of pAd to mitochondria but inhibited pAd's import therein (lanes 9 and 10). Pre-immune IgG did not inhibit MSF-dependent import of pAd (lanes 3 and 4). These results indicate that the pAd–MSF–hsp70 complex is first targeted to OM37 in the

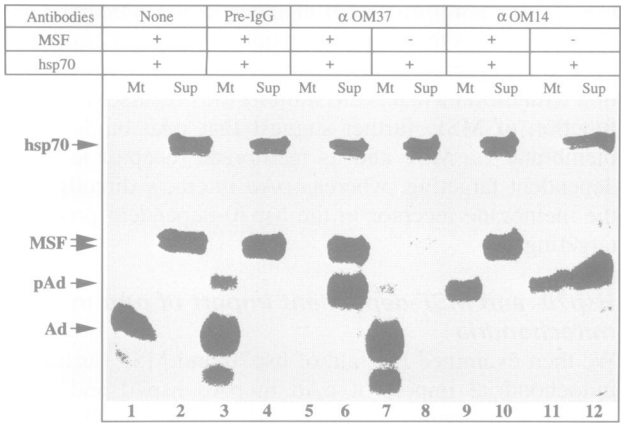

Fig. 9. IgGs raised against two OM proteins discriminate between MSF- and hsp70-dependent binding of pAd to mitochondria. [^{125}I]pAd in 7 M urea was diluted into 50 μl of the import buffer containing [^{125}I]MSF plus [^{125}I]hsp70 (MSF+, hsp70+) or [^{125}I]hsp70 (MSF–, hsp70+) and incubated at 0°C for 30 min to form complexes under the conditions described in the legend to Figure 8. The complexes were subjected to the import assay in 100 μl in the presence of mitochondria which had been mock-treated (none) or treated with IgGs raised against OM14 (αOM14), OM37 (αOM37) or pre-immune IgG (Pre-IgG) as described in Materials and methods. After the import reaction, mitochondria (Mt) and supernatant (sup) fractions were separated by centrifugation and were subjected to SDS–PAGE. The positions of hsp70, MSF large and small subunits, pAd and Ad are indicated in the figure.

MSF-dependent pathway. The precursor is then imported into mitochondria via rat Mas20p. In contrast, pAd bypasses OM37, binds to rat Mas20p and is then imported into mitochondria in the hsp70-dependent pathway.

Discussion

We showed that pAd can be imported into rat liver mitochondria via MSF-dependent, NEM-sensitive, as well as hsp70-dependent, NEM-insensitive targeting pathways. Both pathways depend upon the distinct proteinaceous receptors on OM. This study indicated that pAd in the targeting complexes docks onto the membrane receptor mediated by MSF in the MSF-dependent pathway (referred to as the 'MSF receptor'), whereas in the hsp70-dependent pathway, precursor proteins interact directly with the membrane receptor (referred to as the 'precursor receptor'). The receptor for the former pathway is more susceptible to trypsin digestion than that for the latter pathway. The protease-sensitivity is reminiscent of the two 'import receptors' of yeast and *N.crassa* mitochondria, Mas70p/MOM72 for the former and Mas20p/MOM19 respectively. Mas70p/MOM72 is completedy digested with trypsin at concentrations as low as 25–50 μg/ml, whereas Mas20p/MOM19 requires 100–150 μg/ml trypsin to be completely digested (Hines *et al.*, 1990; Kiebler *et al.*, 1990, 1993). In support of this conjecture, IgGs raised against OM14, which reacted with a rat homolog of Mas20p, inhibited hsp70-dependent binding of pAd to mitochondria, indicating that rat Mas20p is a component of the 'precursor-receptor'. In marked contrast, IgGs raised against OM37 inhibited MSF-dependent but not hsp70-dependent binding of pAd to mitochondria. Thus, distinct roles of the membrane receptors in the MSF- and hsp70-dependent precursor targeting pathways could be demonstrated, although the MSF receptor of rat liver mitochondria still remains to be characterized.

The protein import system of mammalian mitochondria would be expected to be basically similar to that of other organisms, such as yeast and *N.crassa*, since the precursor proteins of yeast mitochondria are efficiently imported into mammalian mitochondria or vice versa. Indeed, the Mas70p–Mas37p complex of yeast mitochondria (Gratzer *et al.*, 1995; Lithgow *et al.*, 1995) functions as the receptor for MSF–precursor complexes (Hachiya *et al.*, 1995). Furthermore, Mas20p/MOM19 has been reported to function as the receptor for a chemically pure, urea-denatured precursor (Becker *et al.*, 1992) interacting with the mitochondrial targeting signal (Haucke *et al.*, 1995).

The relative contribution of MSF-dependent and hsp70-dependent pathways in the precursor import depended on the affinity of the precursors for the two cytoplasmic chaperones. The apparent affinity of MSF for porin is ~10-fold lower than that for pAd. In physiological concentrations of MSF and hsp70 (0.0175 and 2.86 μM, respectively), pAd would be imported almost exclusively via the MSF-dependent pathway, whereas ~70% of porin would be imported via the hsp70-pathway with the rest imported via the MSF-dependent pathway. Pfanner *et al.* (1988) have reported that integration of the reticulocyte lysate-synthesized porin into the OM requires extra-mitochondrial ATP. However, the reported result cannot be compared directly with the present data since the estimation of the amounts of MSF and hsp70 that had been carried over from the reticulocyte lysate in the import system is difficult. We speculate that the relative importance of the targeting pathway is determined by the affinity of the precursor proteins for the two chaperones as well as by the relative abundance of the chaperones in the cytosol.

Cytoplasmic ATP is required for the mitochondrial import of a class of precursor proteins and it seems to promote the release of the precursor proteins from cytoplasmic chaperones (Wachter *et al.*, 1994; Lithgow *et al.*, 1995). However, no direct interaction has yet been demonstrated between the cytoplasmic chaperones and the precursor proteins during mitochondrial protein import. We have found that the MSF- and hsp70-dependent import pathways differ markedly in their requirement for the extra-mitochondrial ATP, even though the same precursor was used as the import substrate; the former required cytoplasmic ATP while the latter did not. In this context we found that hsp70, be it in the form of pAd–hsp70 complex or pAd–MSF–hsp70 complex, dissociated from the complexes in the absence of ATP when incubated with either OM or intact mitochondria. The binding of the targeting complexes to the membrane receptors would probably cause a conformational change of the precursor, thus inducing the dissociation of hsp70 from the complexes. Hence, hsp70-dependent precursor import into mitochondria does not require extra-mitochondrial ATP. On the other hand, MSF and pAd in the ternary targeting complex remained bound to the mitochondria when the complex was incubated with mitochondria in the absence of ATP; the addition of ATP induced the release of MSF from the membrane, thus triggering the import of pAd into mitochondria. The ATP-dependent release of MSF from mitochondria occurred in the absence of either the

electrochemical potential across the inner membrane or the matrix ATP (T.Komiya and K.Mihara, unpublished data). These results indicate that the MSF-dependent import requires extra-mitochondrial ATP for the release of MSF from the receptor. The mechanism of releasing MSF from the membrane remains to be clarified.

In the MSF-dependent targeting pathway, MSF probably binds to the mitochondrial targeting signals of the nascent chains as soon as they emerge from the ribosomes. This in turn increases the affinity of MSF for the mitochondrial OM receptor, thus accomplishing the docking of the targeting complex to the membrane. On the other hand, hsp70 is considered to bind to the mature portions of the precursors and co-operate with MSF to keep the precursor's unfolded conformation, since both chaperones exhibit different recognition specificity (Flynn *et al.*, 1989; 1991; Blond-Elguindi *et al.*, 1993; Fourie *et al.*, 1994; Gragerov *et al.*, 1994; Hachiya *et al.*, 1994; Hartl *et al.*, 1994; Komiya *et al.*, 1994). These processes require a mechanism that stabilizes the ternary targeting complex against the dissociating action of ATP in the cytosol, since MSF exhibits higher ATPase activity *in vitro* in the presence of the precursors (Hachiya *et al.*, 1994). Such a stabilization was indeed found, since urea-unfolded pAd diluted into rat liver cytosol was present in a stable complex with hsp70 and MSF even in the presence of ATP (T.Komiya and K.Mihara, unpublished results).

Materials and methods

Materials

MSF and hsp70 were purified from rat liver cytosol according to the procedures of Hachiya *et al.* (1993) and Chirico *et al.* (1988) respectively. pAd and porin were expressed in *Escherichia coli* and purified in 7 M urea solution as described previously (Iwahashi *et al.*, 1992). Outer mitochondrial membrane was prepared from rat liver mitochondria as described by Hachiya *et al.* (1994).

Import of precursor proteins into mitochondria

Mitochondrial import of *E.coli*-expressed precursor proteins was assayed as described previously (Hachiya *et al.*, 1994) with a slight modification. The substrate mixture containing ^{125}I-labeled pAd or ^{125}I-labeled porin was incubated with rat liver mitochondria (1 μg/μl) in the 'import buffer' [10 mM HEPES–KOH buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT, 0.22 M mannitol and 0.07 M sucrose] containing 5 mM NADH, 20 mM sodium succinate and the 'ATP-generation system' (0.5 mM ATP, 0.1 mM GTP, 1.5 mM creatine phosphate and 15 μg/ml creatine phosphokinase) at 30°C for 50 min. The reaction mixtures were then centrifuged to separate mitochondria and the supernatant. Both fractions were resolved by SDS–PAGE.

^{125}I-labeling of pAd, porin, MSF and hsp70

Iodination was performed using IODO–BEADS (Pierce) according to the manufacturer's protocol. Briefly, 50 μg each of pAd, porin, hsp70 or MSF were incubated at room temperature for 20 min in a 100 μl of the reaction mixture containing 500 mM sodium phosphate buffer (pH 7.0), 500 μCi Na[^{125}I] and three grains of IODO–BEADS. The reaction was terminated by 20 mM HEPES–KOH buffer (pH 7.4) containing 10 mg/ml tyrosine, 0.1% glycerol and 0.1% xylene cyanol, followed by gel-filtration through a Sephadex G-25 column.

Analysis of SDS–PAGE gels

Unless otherwise stated, SDS–PAGE gels were analyzed with a Fuji Bioimage Analyzer BAS2000.

Assay for the maintenance of the import-competence of pAd and porin by hsp70 and MSF

^{125}I-labeled pAd or ^{125}I-labled porin in 7 M urea was diluted 40-fold into the import buffer either with or without the indicated amounts of hsp70 or MSF. The final concentration of ^{125}I-labeled pAd or ^{125}I-labeled porin was 0.005 μM. After incubation at 0°C for 30 min, the reaction mixtures were assayed for mitochondrial import in the presence of an ATP-generation system and respiratory substrates as described above. The reaction mixtures were then treated with 0.2 mg/ml trypsin at 0°C for 30 min to measure the integration of porin into mitochondria by the criterion of resistance to trypsin digestion (Mihara *et al.*, 1982).

Trypsin treatment of mitochondria and outer mitochondrial membrane

Mitochondria (0.5 mg/ml) were incubated with the indicated amounts of trypsin at 0°C for 30 min, then a 10-fold excess amount of soybean trypsin inhibitor was added and the mixture was incubated at 0°C for 20 min. Mitochondria were recovered by centrifugation, washed once with the 'import buffer' and re-suspended in the same buffer. Trypsin digestion of mitochondrial OM was performed as follows: 0.1 mg/ml of OM was incubated with 0–100 μg/ml of trypsin at 0°C for 30 min. Then, 500 μg/ml soybean trypsin inhibitor was added to the reaction mixture and incubated at 0°C for 20 min.

Complex formation of unfolded pAd with hsp70 or MSF plus hsp70

^{125}I-labeled pAd was diluted in 50 μl of 'TKM' [20 mM Tris–HCl buffer (pH 7.5) containing 150 mM potassium acetate and 2 mM magnesium acetate] containing ^{125}I-labeled hsp70 or ^{125}I-labeled hsp70 plus ^{125}I-labeled MSF (the final concentrations of pAd, MSF and hsp70 were 0.005, 0.007 and 0.025 μM, respectively) and incubated at 30°C for 30 min to form the binary or ternary complex. The complexes were incubated with or without 1 mM ATP or 1 mM ATP plus 5 mM AMP-PNP for another 30 min. Twenty-five micrograms each of pre-immune IgG, anti-adrenodoxin IgG (Hachiya *et al.*, 1994) or anti-MSF large subunit IgG (Alam *et al.*, 1994) were added to the mixtures and incubated overnight at 4°C. The reaction mixtures were incubated with protein A–Sepharose at 4°C for 120 min and centrifuged to separate the supernatant and protein A–Sepharose fractions. The supernatant was TCA precipitated and the protein A–Sepharose was washed twice with PBS containing 0.05% Tween 20 and suspended in gel-loading buffer. Both fractions were resolved and analyzed by SDS–PAGE.

Interaction of pAd–hsp70 and pAd–MSF–hsp70 complexes with mitochondrial OM

pAd–hsp70 and pAd–MSF–hsp70 complexes were prepared from ^{125}I-labeled components as described above and incubated at 30°C for 30 min with 5 μg each of OM or trypsin-treated OM (tOM) in the presence or absence of 1 mM ATP, 1 mM ATP plus 5 mM AMP-PNP or 20 U/ml apyrase in a final volume of 50 μl. The reaction mixtures were diluted with 300 μl of TKM, layered over a cushion of 100 μl 0.54 M sucrose and then centrifuged at 100 000 *g* for 90 min to separate OM and supernatant fractions and both fractions were subjected to SDS–PAGE.

Competition between pAd and porin for the binding to OM

^{125}I-labeled porin (0.01 μM, defined as substrate) and ^{125}I-labeled pAd (0.03 μM, defined as competitor) were incubated separately at 30°C for 30 min in 25 μl of TKM with 5.72 μM hsp70 (unlabeled) or 0.7 μM MSF (unlabeled). The substrate mixture was incubated with 2 μg of OM in the presence or absence of the competitor at 30°C for 30 min in a final volume of 50 μl. The reaction mixtures were ultracentrifuged to separate OM and the supernatant fractions and both fractions were resolved by SDS–PAGE.

Mitochondrial import of pAd in pAd–hsp70 and pAd–MSF–hsp70 complexes

^{125}I-labeled pAd (0.01 μM) and ^{125}I-labeled hsp70 (0.05 μM) were mixed in the absence or presence of ^{125}I-labeled MSF (0.014 μM) in 50 μl of TKM buffer and incubated at 30°C for 30 min. The binary and ternary complexes thus obtained were incubated at 30°C for 50 min in 100 μl of 10 mM HEPES–KOH buffer (pH 7.4) containing 0.25 M sucrose, 1 mM DTT, 1 mM EDTA, 20 mM sodium succinate, 5 mM NADH and 100 μg of mitochondria. Where indicated, 1 mM ATP or 1 mM ATP plus 5 mM AMP-PNP was added to the reaction mixture, or apyrase-treated mitochondria were used. The reaction mixtures were then centrifuged to separate the mitochondria and supernatant fractions; both fractions were resolved by SDS–PAGE.

Apyrase treatment of mitochondria

Mitochondria (100 μg) were incubated with 20 U/ml apyrase in the import buffer (50 μl) containing 200 μg/ml carboxyatractyloside at 30°C for 30 min. Mitochondria were then isolated by centrifugation, washed

once with the import buffer, suspended into the same buffer and used in the import assay.

Treatment of mitochondria with antibodies

Mitochondria (100 μg) were incubated with 10 μg each of IgGs raised against OM14 or OM37 or pre-immune IgG in 50 μl of the import buffer at 0°C for 60 min, then sedimented by centrifugation. The mitochondria were washed once with the import buffer by centrifugation, resuspended into the same buffer and used for the import assay.

Preparation of the antibodies against OM37 and OM14

Purified rat liver outer mitochondrial membranes (OM) were resolved by SDS–PAGE and the proteins with the apparent molecular weight of 37 kDa (OM37) and 14 kDa (OM14) were electro-eluted. The antibodies against both proteins were raised separately in rabbits using Ribi Adjuvant system (RIBI Immunochem Research, Inc.) according to the manufacturer's protocol.

Signal peptide-induced binding of MSF to OM

The indicated amounts of ^{125}I-labeled MSF or ^{125}I-labeled hsp70 were incubated with 3 μM synthetic peptides, SCC(1-19) or SCC(1-19)M in 50 μl of TKM at 30°C for 30 min. Two micrograms each of OM were added to the mixtures and incubated at 30°C for 30 min. The mixtures were centrifuged at 100 000 *g* for 90 min to separate the supernatant and OM fractions and both fractions were resolved by SDS–PAGE.

Acknowledgements

We would like to thank Drs T.Omura and G.Schatz for their helpful discussions and comments. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

Alam,R., Hachiya,N., Sakaguchi,M., Kawabata,S., Iwanaga,S., Kitajima,M., Mihara,K. and Omura,T. (1994) cDNA cloning and characterization of mitochondrial import stimulation factor (MSF) purified from rat liver cytosol. *J. Biochem.*, **116**, 416–425.

Attardi,G. and Schatz,G. (1988) Biogenesis of mitochondria. *Annu. Rev. Cell Biol.*, **4**, 289–333.

Baker,K.P. and Schatz,G. (1991) Mitochondrial proteins essential for viability mediate protein import into yeast mitochondria. *Nature*, **349**, 205–208.

Baker,K.P., Schaniel,A., Vestweber,D. and Schatz,G. (1990) A yeast mitochondrial outer membrane protein essential for protein import and cell viability. *Nature*, **348**, 605–609.

Becker,K., Guiard,B., Rassow,J., Söllner,T. and Pfanner,N. (1992) Targeting of a chemically pure preprotein to mitochondria does not require the addition of a cytosolic signal recognition factor. *J. Biol. Chem.*, **267**, 5637–5643.

Blond-Elguindi,S., Cwiria,S.E., Dower,W.J., Lipshutz,R.J., Sprang,S.R., Sambrook,J.F. and Gething,M.-J.H. (1993) Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of Bip. *Cell*, **75**, 717–728.

Chirico,W.J., Waters,M.G. and Blobel,G. (1988) 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature*, **332**, 805–810.

Craig,E.A. (1993) Chaperones: helpers along the pathways to protein folding. *Science*, **260**, 1902–1903.

Deshaies,R.J., Koch,B.D., Werner-Washburne,M., Craig,E.A. and Schekman,R. (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature*, **332**, 800–805.

Flynn,G.C., Chappell,T.G. and Rothman,J.E. (1989) Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science*, **245**, 385–390.

Flynn,G.C., Pohl,J., Flocco,M.T. and Rothman,J.E. (1991) Peptide-binding specificity of the molecular chaperone BiP. *Nature*, **353**, 726–730.

Fourie,A.M., Sambrook,J.F. and Gething,M.-J.H. (1994) Common and divergent peptide binding specificities of hsp70 molecular chaperone. *J. Biol. Chem.*, **269**, 30470–30478.

Glick,B.S. (1995) Can hsp70 proteins act as force-generating motors? *Cell*, **80**, 11–14.

Glick,B.S., Beasley,E.M. and Schatz,G. (1992) Protein sorting in mitochondria. *Trends Biochem. Sci.*, **17**, 453–459.

Gragerov,A., Zeng,L., Zhao,X., Burkholder,W. and Gottesman,M.E. (1994) Specificity of DnaK-peptide binding. *J. Mol. Biol.*, **235**, 848–854.

Gratzer,S., Lithgow,T., Bauer,R.E., Lamping,E., Paltauf,F., Kohlwein,S.D., Haucke,V., Schatz,G. and Horst,M. (1995) Mas37p, a novel receptor subunit for protein import into mitochondria. *J. Cell Biol.*, **129**, 25–34.

Hachiya,N., Alam,R., Sakasegawa,Y., Sakaguchi,M., Mihara,K. and Omura,T. (1993) A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent conformational modulator for precursor proteins. *EMBO J.*, **12**, 1579–1586.

Hachiya,N., Komiya,T., Alam,R., Iwahashi,J., Sakaguchi,M., Omura,T. and Mihara,K. (1994) MSF, a novel cytoplasmic chaperone which functions in precursor targeting to mitochondria. *EMBO J.*, **13**, 5146–5154.

Hachiya,N., Mihara,K., Suda,K., Horst,M., Schatz,G. and Lithgow,M. (1995) Reconstitution of the initial steps of mitochondrial protein import. *Nature*, **376**, 705–709.

Hartl,F.-U., Hlodan,R. and Langer,T. (1994) Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem. Sci.*, **19**, 20–25.

Haucke,V., Lithgow,T., Rospert,S., Hahne,K. and Schatz,G. (1995) The yeast mitochondrial protein import receptor Mas20p binds precursor proteins through electrostatic interaction with the positively charged presequence. *J. Biol. Chem.*, **270**, 5565–5570.

Hines,V. and Schatz,G. (1993) Precursor binding to yeast mitochondria. A general role for the outer membrane protein Mas70p. *J. Biol. Chem.*, **268**, 449–454.

Hines,V., Brandt,A., Griffiths,G., Horstmann,H., Brutsh,H. and Schatz,G. (1990) Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70. *EMBO J.*, **9**, 3191–3200.

Hönlinger,A. *et al.* (1995) The mitochondrial receptor complex: MOM22 is essential for cell viability and directly interacts with preproteins. *Mol. Cell. Biol.*, **15**, 3382–3389.

Iwahashi,J., Furuya,S., Mihara,K. and Omura,T. (1992) Characterization of adrenodoxin precursor expressed in *Escherichia coli*. *J. Biochem. (Tokyo)*, **111**, 451–455.

Kiebler,M., Pfaller,R., Söllner,T., Griffiths,G., Horstmann,H., Pfanner,N. and Neupert,W. (1990) Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. *Nature*, **348**, 610–616.

Kiebler,M., Keil,P., Schneider,H., van der Klei,I.J.P., Pfanner,N. and Neupert,W. (1993) The mitochondrial receptor complex: a central role of MOM22 in mediating preprotein transfer from receptors to the general insertion pore. *Cell*, **74**, 483–492.

Komiya,T., Hachiya,N., Sakaguchi,M., Omura,T. and Mihara,K. (1994) Recognition of mitochondria-targeting signals by a cytosolic import stimulation factor, MSF. *J. Biol. Chem.*, **269**, 30893–30897.

Lithgow,T., Hoj,P.B. and Hoogenraad,N.J. (1993) Do cytosolic factors prevent promiscuity at the membrane surface? *FEBS Lett.*, **329**, 1–4.

Lithgow,T., Junne,T., Suda,K., Gratzer,S. and Schatz,G. (1994) The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast. *Proc. Natl Acad. Sci. USA*, **91**, 11973–11977.

Lithgow,T., Glick,B.S. and Schatz, G. (1995) The protein import receptor of mitochondria. *Trends Biochem. Sci.*, **20**, 98–101.

Mihara,K., Blobel,G. and Sato,R. (1982) *In vitro* synthesis and integration into mitochondria of porin, a major protein of the outer mitochondrial membrane of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **79**, 7102–7106.

Murakami,H., Pain,D. and Blobel,G. (1988) 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J. Cell Biol.*, **107**, 2051–2057.

Nakai,M. and Endo,T. (1995) Identification of yeast MAS17 encoding the functional counterpart of the mitochondrial receptor complex protein MOM22 of *Neurospora crassa*. *FEBS Lett.*, **357**, 202–206.

Nakai,M., Endo,T., Hase,T. and Matsubara,H. (1993) Intramitochondrial protein sorting. isolation and characterization of the yeast MSP1 gene which belongs to a novel family of putative ATPases. *J. Biol. Chem.*, **268**, 24262–24269.

Neupert,W., Hartl,F.-U., Craig,E.A. and Pfanner,N. (1990) How do polypeptides cross the mitochondrial membranes? *Cell*, **63**, 447–450.

Ono,H. and Tuboi,S. (1990) Purification of the putative import-receptor for the precursor of the mitochondrial protein. *J. Biochem.*, **107**, 840–845.

Pfanner,N. and Neupert,W. (1990) The mitochondrial protein import apparatus. *Annu. Rev. Biochem.*, **59**, 331–353.

Pfanner,N., Pfaller,R., Kleene,R., Ito,M., Tropschung,M. and Neupert,W. (1988) Role of ATP in mitochondrial protein import. Conformational alteration of a precursor protein can substitute for ATP requirement. *J. Biol. Chem.*, **263**, 4049–4051.

Pfanner,N., Söllner,T. and Neupert,W. (1991) Mitochondrial import receptors for precursor proteins. *Trends Biochem. Sci.*, **16**, 63–67.

Pfanner,N., Craig,E.A. and Meijer,M. (1994) The protein import machinery of the mitochondrial inner membrane. *Trends Biochem. Sci.*, **19**, 368–372.

Ramage,L., Junne,T., Hahne,K., Lithgow,T. and Schatz,G. (1993) Functional cooperation of mitochondrial protein import receptors in yeast. *EMBO J.*, **12**, 4115–4123.

Sheffield,W.P., Shore,G.C. and Randall,S.K. (1990) Mitochondrial precursor protein. Effects of 70-kilodalton heat shock protein on polypeptide folding, aggregation, import competence. *J. Biol. Chem.*, **265**, 11069–11076.

Söllner,T., Griffiths,G., Pfaller,R., Pfanner,N. and Neupert,W. (1989) MOM19, an import receptor for mitochondrial precursor proteins. *Cell*, **59**, 1061–1070.

Söllner,T., Pfaller, R., Griffiths,G., Pfanner,N. and Neupert,W. (1990) A mitochondrial import receptor for the ADP/ATP carrier. *Cell*, **62**, 107–115.

Stuart,R.A., Cyr,D.M., Craig,E.A. and Neupert,W. (1994) Mitochondrial molecular chaperones: their role in protein translocation. *Trends Biochem. Sci.*, **19**, 87–92.

Wachter,C., Schatz,G. and Glick,B.S. (1994) Protein import into mitochondria: the requirement for external ATP is precursor-specific whereas intramitochondrial ATP is universally needed for translocation into the matrix. *Mol. Biol. Cell*, **5**, 465–474.

Received on July 18, 1995; revised on September 25, 1995