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Cytosolic factors in mitochondrial protein import

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Abstract. In vitro import studies have confirmed the participation of cytosolic protein factors in the import of various precursor proteins into mitochondria. The requirement for extramitochondrial adenosine triphosphate for the import of a group of precursor proteins seems to be correlated with the chaperone activity of the cytosolic protein factors. One of the cytosolic protein factors is hsp70, which generally recognizes and binds unfolded proteins in the cytoplasm. Hsp70 keeps the newly synthesized mitochondrial precursor proteins in import-competent unfolded conformations. Another cytosolic protein factor that has been characterized is mitochondrial import stimulation factor (MSF), which seems to be specific to mitochondrial precursor proteins. MSF recognizes the mitochondrial precursor proteins, forms a complex with them and targets them to the receptors on the outer surface of mitochondria.

Key words. Mitochondrial protein import; cytosolic factors; hsp70; targeting factor; presequence-binding factor (PBF); mitochondrial import stimulation factor (MSF); MSF-dependent import.

Involvement of cytosolic protein factor(s) in protein import into mitochondria was first suggested by the stimulative effect of reticulocyte lysate on the in vitro import of a precursor protein into liver mitochondria [1]. The in vitro import of a purified precursor protein into yeast mitochondria also showed a requirement for a cytosolic protein fraction [2]. Since then, in vitro import studies have confirmed the need for cytosolic protein factor(s) for efficient import of various precursor proteins into the mitochondria isolated from animal tissues [3–7], yeast [8] and *Neurospora* [9], although some precursor proteins [10] and small peptides [11, 12] did not show a requirement for cytosolic factors.

Various lines of evidence suggest that the newly synthesized precursor proteins released from the ribosomes must be kept in an unfolded conformation for efficient import into mitochondria across the outer and inner membranes [13, 14], and it has been shown with yeast that a cytosolic stress protein, hsp70, is needed to keep the conformations of the precursor proteins importable, although the molecular chaperone function of hsp70 does not seem to be specific to mitochondrial precursor proteins [8, 15]. The role of hsp70 or 70-kDa heat shock 'cognate' (hsc70) in the import of precursor proteins into animal mitochondria has also been studied [7, 16, 17].

The role of the cytosolic protein factors in the targeting of precursor proteins to their receptors on the outer surface of mitochondria has been studied. Such cytosolic factors with a targeting function are expected to recognize the mitochondria-targeting signal in the presequence of the precursor proteins, and three cytosolic protein factors of mammalian origin have so far been reported. They are targeting factor [3] and presequence-binding factor (PBF) [5], both purified from rabbit reticulocyte lysate, and mitochondrial import stimulation factor (MSF) [7], which we purified from rat liver cytosol.

In this paper, we briefly review the studies on the roles of hsp70, targeting factor and PBF in mitochondrial protein import, and then describe our studies on MSF in more detail.

Participation of a 70-kDa stress protein, hsp70, in mitochondrial protein import

Members of the 70-kDa stress protein, hsp70, family are widespread among various organisms and perform adenosine triphosphate (ATP)-dependent chaperone functions. The first evidence for the involvement of cytosolic hsp70 in mitochondrial protein import was obtained by Deshaires et al. [15] by the in vivo depletion of a subset of cytosolic hsp70 proteins, Ssa1p and Ssa2p, which are constitutively expressed in *Saccharomyces cerevisiae* cells. The hsp70 depletion resulted in accumulation of the precursor form of the β subunit of mitochondrial F/1-ATPase in the cytosol, indicating the necessity of cytosolic hsp70 for the import of the precursor protein into mitochondria. The precursor protein did not accumulate in the cytosol when one of the hsp70 proteins, Ssa1p, was expressed.

The necessity of hsp70 for mitochondrial protein import in yeast cells was further confirmed by an in vitro study by Murakami et al. [8]. They found that the precursor of Put2 protein was not imported into yeast mitochondria in vitro when it was translated from the mRNA in a wheat germ lysate cell-free system, but the addition of

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a yeast postribosomal supernatant to the import reaction mixture resulted in an efficient import of the precursor protein. The yeast postribosomal supernatant contained at least two distinct protein factors which were both needed for efficient import and which acted synergistically. One of the cytosolic factors was identified as hsp70 (Ssa1p/Ssa2p), whereas another protein factor, whose import-stimulating activity was sensitive to *N*-ethylmaleimide (NEM), was not characterized. However, the chaperone function of hsp70 family proteins was not specific to mitochondrial precursor proteins. They were also needed for the post-translational import of precursor polypeptides into the lumen of the endoplasmic reticulum [15, 18]. It is generally believed that the peptide-binding site of hsp70s selects for hydrophobic amino acid residues, which are usually hidden in the hydrophobic interior of folded globular proteins and exposed upon the unfolding of the native protein molecules [19]. However, a recent paper by Endo et al. [17] reports that a yeast cytosolic hsp70, Ssa1p, shows high affinity for amphiphilic peptides such as the presequences of mitochondrial precursor proteins, although it interacts with a broad range of amino acid sequences.

The role of cytosolic hsp70 in mitochondrial protein import was also examined with mammalian mitochondria. It was shown by Sheffield et al. [6] that hsp70 retards the folding of the precursor polypeptides and prevents their aggregation in an ATP-dependent manner. Terada et al. [16] examined the possible role of hsp70 in the stimulation of in vitro protein import into rat liver mitochondria by reticulocyte lysate, which contained hsc70 as the major form of hsp70 family proteins, and concluded that hsc70 must be present in the translation mixture when a precursor protein, ornithine transcarbamylase precursor (pOTC), is synthesized in vitro. Hsc70 binds to pOTC and maintains it in an import-competent form, but once the import competence of pOTC was lost, hsc70 could not restore the import-competent conformation of the precursor protein even in the presence of ATP.

Targeting factor and presequence-binding factor purified from reticulocyte lysate

Miura et al. noticed a stimulative effect of reticulocyte lysate on the in vitro import of pOTC into rat liver mitochondria [1]. They further examined the in vitro import of pure recombinant pOTC into mitochondria, and concluded that a protein factor present in the reticulocyte lysate interacts with pOTC and holds it in an import-competent form [20]. A protein factor that binds to pOTC but not to mature OTC was purified from rabbit reticulocyte lysate and named presequence-binding factor (PBF) [5]. The purified PBF markedly stimulated the mitochondrial import of purified recombinant pOTC or in vitro synthesized pOTC, and the PBF-stimulated import was further enhanced by hsp70. The urea-denatured recombinant pOTC aggregates upon dilution and loses import competence, whereas its complex with PBF does not aggregate and remains importable in diluted solutions. Since the formation of pOTC-PBF complex was inhibited by low concentrations of the synthetic presequence of pOTC and also those of other mitochondrial precursor proteins, PBF seemed to bind to the presequence portion of the precursor proteins and prevent their aggregation.

It was also found that PBF was required for the import of various mitochondrial precursor proteins which have the cleavable presequence at the aminoterminus, whereas rat 3-oxoacyl CoA thiolase, which has no cleavable presequence, was efficiently imported into mitochondria in vitro in the absence of PBF [21], suggesting the existence of both PBF-dependent and -independent pathways of mitochondrial protein import. However, the molecular characterization of PBF has not yet been reported.

Ono and Tuboi [3] examined the import of a synthetic peptide of the presequence of ornithine aminotransferase into isolated rat liver mitochondria, and found the import was stimulated by addition of rabbit reticulocyte lysate. The import stimulation factor in the reticulocyte lysate was needed for the import of various precursor proteins into mitochondria, and the binding of the precursor proteins to mitochondria was found to be increased by addition of increasing amounts of the factor to the import reaction mixture, suggesting that the cytosolic factor participates in the recognition step in the import process. The factor was purified and named 'targeting factor' for import of mitochondrial proteins [4, 22]. The targeting factor lost its import-stimulating activity during purification, but the identity of the purified targeting factor with the import stimulation factor in the reticulocyte lysate was confirmed by the use of the antibody prepared against the purified protein. The molecular weight of the targeting factor was 28 kDa on SDS polyacrylamide gel electrophoresis (PAGE), but its detailed molecular properties have not yet been reported.

Mitochondrial import stimulation factor, a conformational modulator of mitochondrial precursor proteins

It is known that mitochondrial precursor proteins synthesized in reticulocyte lysate are efficiently imported in vitro into mitochondria, whereas those synthesized in wheat germ lysate are only poorly imported, suggesting that the wheat germ lysate lacks the cytosolic factor(s) required for mitochondrial protein import [5]. We observed that the wheat germ-synthesized adrenodoxin precursor (pAd) was not imported into isolated liver mitochondria unless the import reaction mixture was

supplemented with reticulocyte lysate or liver cytosol. Taking advantage of this system and using COX IV presequence affinity chromatography, an import-stimulating factor was purified from rat liver cytosol [7]. The purified protein factor, which was termed mitochondrial import stimulation factor, or MSF, was a heterodimer of a large (32 kDa) and a small (30 kDa) subunit. MSF stimulated mitochondrial import of several precursor proteins such as porin, pAd, presuperoxide dismutase or a fusion protein of yeast COX IV presequence and porin, which were all synthesized in the wheat germ lysate translation system, suggesting that MSF generally recognizes mitochondrial precursor proteins irrespective of the presence or absence of the cleavable presequence and stimulates their import into mitochondria.

MSF catalysed depolymerization of the wheat germ lysate-synthesized, aggregated precursor proteins, depending on ATP hydrolysis. The depolymerization of the in vitro synthesized precursor proteins seemed to be accompanied by their conformational change, since the aggregated pAd was almost completely degraded by treatment with a low concentration of trypsin in the presence of MSF and ATP, while it was more resistant to the proteolysis in the presence of MSF and β,γ-imidoadenosine 5′-triphosphate (AMP-PNP) [7]. In addition, MSF was found to form a stable complex with urea-denatured precursor proteins and prevent them from losing their import competence [23]. The MSF-precursor protein complex dissociated in the presence of ATP, and once dissociated, the precursor refolded and formed large aggregates. The import stimulation activity of MSF was inhibited by treatment with NEM, whereas the ATP-dependent chaperone activity of MSF was NEM-insensitive, which suggested an additional function of MSF: involvement in precursor-targeting to mitochondria [7, 23].

Precursor-induced ATPase activity of MSF

Mitochondrial precursor proteins induced significant ATPase activity of MSF, irrespective of the presence or absence of the cleavable mitochondria-targeting sequence and submitochondrial localization [23]. However, nonmitochondrial proteins or mature forms of mitochondrial proteins did not induce the ATPase activity, suggesting that MSF ATPase is induced by the mitochondria-targeting sequence. In fact, the chemically synthesized peptides of 8–21mer, corresponding to the mitochondrial-targeting sequences, induced the ATPase activity of MSF [24]. Basic amino acid residues in the mitochondria-targeting sequence seem to be critical for recognition by MSF. In addition, the ATPase activity of MSF was dependent on the folded state of the precursors; the import-competent, unfolded pAd induced a low ATPase activity, whereas the aggregated, import-incompetent pAd induced a higher activity [7, 23]. MSF seems to recognize several features of the precursor proteins, but the primary target of recognition is apparently the mitochondria-targeting sequence.

MSF thus shows properties similar to those of the molecular chaperones of the hsp70 family in the following respects. (1) MSF and hsp70 family proteins recognize aggregated proteins and unfold them in an ATP-dependent manner, (2) they complex with the unfolded proteins to stabilize their unfolded conformations, and (3) they exhibit ATPase activity depending on the presence of peptides or aggregated proteins. There are, however, some conspicuous differences between them: (1) MSF recognizes mitochondrial precursor proteins, while hsp70 family proteins recognize the unfolded proteins in general, and (2) MSF binds the mitochondria-targeting sequence, whereas hsp70 family proteins bind peptides enriched in hydrophobic amino acid residues.

MSF- and hsp70-dependent mitochondria-targeting pathways

Hsp70 has also been shown to be involved in the import of precursor proteins into mitochondria by maintaining the import-competent, unfolded conformation of the precursors in an ATP-dependent manner [8, 15]. How are the two cytoplasmic chaperones, hsp70 and MSF, involved in the mitochondrial import of precursor proteins? The in vitro import studies with chemically pure precursor proteins, pAd and porin, and purified MSF and hsp70, have shown that both MSF and hsp70, at their saturated concentrations, can independently support the mitochondrial import of pAd to a similar extent, although the apparent affinity of MSF for pAd is about 30-fold higher than that of hsp70 [25]. MSF- and hsp70-dependent imports could be distinguished from one another by NEM sensitivity: the former was inhibited by NEM, while the latter was not. NEM-insensitive, hsp70-dependent import became NEM-sensitive on addition of MSF [25]. The apparent affinity of MSF for pAd was about 10-fold higher than that for porin, suggesting that the affinity of MSF differs significantly between the precursor proteins.

The MSF- and hsp70-supported imports seem to be dependent on different receptors on the mitochondrial outer membrane (OM), since both exhibited distinct trypsin sensitivity. Coimmunoprecipitation experiments with the antibodies against adrenodoxin and MSF subunits showed that urea-unfolded pAd formed binary or ternary complexes with hsp70 or hsp70 and MSF [25]. When hsp70-pAd complex was incubated with OM, hsp70 dissociated from the complex in the absence of ATP, and pAd was recovered from the OM fraction. On the other hand, when MSF-hsp70-pAd complex was incubated with OM in the absence of ATP, hsp70 was released from the complex, whereas both MSF and pAd

were recovered from the OM fraction [25]. These results, in conjunction with the evidence that NEM treatment abolished the precursor targeting function of MSF [7, 23], indicate that pAd directly interacts with the receptor on OM in hsp70-dependent precursor targeting, whereas pAd binds indirectly to OM via MSF and its membrane receptor in MSF-dependent targeting.

ATP requirement of MSF- and hsp70-dependent mitochondrial protein import

Cytoplasmic ATP is required for the mitochondrial import of a class of precursor proteins [26], and the ATP appears to be involved in conferring import competence to the precursor proteins through the action of cytoplasmic chaperone proteins. In this respect, hsp70 and MSF are the candidates for the ATP-dependent cytoplasmic chaperones involved in this process. We therefore examined the ATP-requirement for the mitochondrial import of pAd in the hsp70-dependent and MSF-dependent imports.

When hsp70-pAd complex was incubated with isolated mitochondria in the absence of extramitochondrial ATP, hsp70 was released to the supernatant and pAd was imported into the mitochondria. AMP-PNP, which interferes with the ATP-dependent dissociation of the binary complex, did not inhibit the import [25]. On the other hand, when MSF-hsp70-pAd complex was incubated with mitochondria in the absence of extramitochondrial ATP, pAd was not imported. Hsp70 was released from the complex into the supernatant, and MSF and pAd were recovered from the mitochondria. The import arrest was relieved by the addition of ATP, which released MSF to the supernatant [25]. These results indicate that the MSF-dependent pathway requires extramitochondrial ATP to release MSF from its receptor on OM, whereas the hsp70-dependent pathway does not [25]. The binding of hsp70-pAd or MSF-hsp70-pAd to the membrane receptor seems to cause a conformational change of the precursor protein and induces dissociation of hsp70 from the complex in the absence of ATP, which fits well with a recent report by Meyer et al. [27] that binding of the presequence at the trans side of OM induces unfolding of the precursor protein.

The membrane receptor for MSF-precursor protein complex

Sucrose density gradient centrifugation showed that MSF formed a complex with urea-unfolded pAd, and the complex was bound to isolated OM [23]. No binding of MSF-pAd complex to OM was observed when either trypsin-treated OM or NEM-treated MSF was used. MSF by itself did not bind to OM, which indicated that the binding of MSF to its receptor required the presence of a mitochondrial precursor protein [30]. In this respect, a chemically synthesized functional mitochondria-targeting sequence, but not its mutated version, induced the binding of MSF to OM [25]. It remains to be clarified whether MSF interacts directly with the receptor or whether it interacts with the receptor via the precursor protein. However, the fact that NEM blocked the precursor-targeting activity of MSF but not its chaperone activity seems to support the former possibility.

Interestingly, the pAd-induced ATPase activity of MSF was inhibited by OM, and the inhibition correlated well with the binding of MSF-pAd complex to OM; inhibition was not observed when either trypsin-treated OM or NEM-treated MSF was used for the ATPase assay [23]. This assay provided a way of identifying the OM components that interact with the MSF-precursor complex. In the yeast *S. cerevisiae*, four OM proteins (Tom20, Tom22, Tom70 and Tom37) have been shown to be the components of the protein import receptor complex, and these OM proteins are present as the subcomplexes of Tom20/Tom22 and Tom37/Tom70 [28]. Hachiya et al. [29] have shown that yeast OM inhibited MSF-ATPase, and pretreatment of OM with the IgGs against Tom37 or Tom70 suppressed the inhibition, whereas no suppression was observed with the IgGs against Tom20 or Tom22. They have also shown by coimmunoprecipitation that MSF-pAd complex physically interacts with the Tom37/Tom70 subcomplex, indicating that a subcomplex of the mitochondrial protein import receptor Tom37/Tom70, but not Tom20/Tom22, functions as the receptor for MSF-precursor protein complexes [29].

Early steps in mitochondrial protein import

The role of Tom37/Tom70 and Tom20/Tom22 in the import of pAd in the absence or presence of MSF has been examined. The binding of the urea-denatured precursor was found to be completely blocked by the IgGs against Tom20 and Tom22, while the binding was unaffected by the IgGs against Tom37 and Tom70 [29]. In contrast, the binding of MSF-pAd complex to mitochondria was completely inhibited by IgGs against Tom37 and Tom70, but was only slightly affected by the IgGs against Tom20 and Tom22. The import of pAd in the MSF-pAd complex, which had been prebound to mitochondria, was inhibited by the IgGs against Tom20 and Tom22. These results indicate that the MSF-pAd complex docks on the Tom37/Tom70 subcomplex. MSF is then released, and thereafter the precursor protein is transferred across OM via the Tom20/Tom22 subcomplex [29]. Similar results were also obtained with rat liver mitochondria; the IgGs against a 37-kDa OM protein (OM37) inhibited MSF-dependent binding of pAd. In contrast, the IgGs against a rat homologue of Tom20 did not inhibit the binding

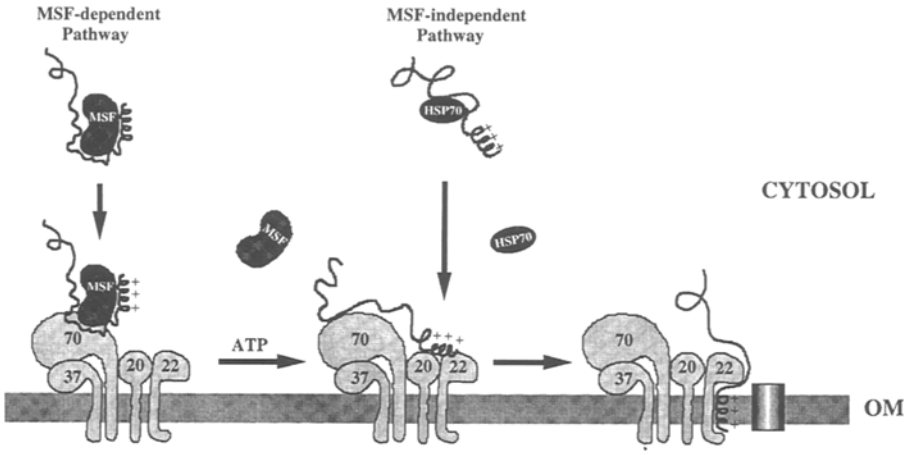

Figure 1. A model for precursor protein targeting to mitochondria. MSF and its receptor-dependent and hsp70-dependent precursor protein-targeting pathways are outlined.

of MSF-pAd complex to OM, but the antibodies completely inhibited the subsequent import of pAd as well as the hsp70-dependent binding of pAd [25, 30]. The relation between Tom37 and OM37 remains to be clarified.

In summary, all the available evidence obtained by in vitro import experiments indicates that the precursor proteins can be imported into mitochondria through an NEM-sensitive, MSF and Tom37/Tom70-dependent pathway and also through an NEM-insensitive, MSF-independent pathway (fig. 1). In the former pathway, the MSF-precursor protein complex first docks onto Tom37/Tom70. MSF is then released from the receptor, depending on ATP hydrolysis, and the precursor protein is transferred to Tom20/Tom22 and imported into mitochondria. On the other hand, the precursor proteins which are not recognized by MSF but are able to maintain the unfolded conformation either by themselves or by the interaction with hsp70 are directly targeted to Tom20/Tom22, and then transported across OM. The latter pathway does not require extramitochondrial ATP, since the precursor proteins do not require hsp70 to keep the import-competent unfolded conformation, or if they do, hsp70 spontaneously dissociates from the precursor proteins upon interaction of the hsp70-precursor protein complex with Tom20/Tom22.

Import studies with yeast mitochondria have shown that the precursor proteins to be imported into mitochondria can be classified into two groups [26]. The first group consists of hsp60, COX IV, dihydrofolate reductase (DHFR) fusion proteins and cytochrome c heme lyase, which remain loosely folded outside the mitochondria and do not require extramitochondrial ATP for their import. The second group includes β subunit of F/1-ATPase, α subunit of mitochondrial processing peptidase, ADH III, cytochrome c1 and adenine nucleotide translocator. The import of the precursor proteins belonging to the second group requires extramitochondrial ATP. The role of cytoplasmic molecular chaperones including hsp70 and MSF in the import of various precursor proteins into mitochondria in animal and microbial cells must be further clarified by future studies.

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