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Minireview

The First Steps of Protein Import into Mitochondria

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Protein import into mitochondria is initiated by the recognition and binding of precursor proteins by import components in the cytosol, on the mitochondrial surface, and in the mitochondrial outer membrane. Following their synthesis on cytoplasmic ribosomes, some precursor proteins interact with molecular chaperones in the cytosol which function in maintaining the precursor protein in an import-competent state and may also aid in the delivery of the precursor to the mitochondria. A multisubunit protein import receptor then recognises and binds precursor proteins before feeding them into the outer membrane import site. Some proteins are sorted from the import site into the outer membrane, but most precursor proteins travel through the outer membrane import site into the mitochondria, where the later steps of protein import take place.

KEY WORDS: Protein targeting; protein import; mitochondria; molecular chaperones.

INTRODUCTION

Mitochondria are essential organelles and fulfill a variety of metabolic functions (Attardi and Schatz, 1988). The mitochondrial subcompartments—the outer membrane, the intermembrane space, the inner membrane, and the matrix—each contain a specific set of proteins, totalling around a thousand for the entire organelle. Since more than 90% of all mitochondrial proteins are translated in the cytosol, they have to be imported into the mitochondria.

The most common targeting signal to direct a newly-made protein from the cytosol to the mitochondria is a basic, amphipathic helix situated at its amino terminus (Roise *et al.*, 1986; von Heijne, 1986). As this signal emerges from the ribosome, it becomes available to interact with components of the targeting machinery in the cytosol, on the mitochondrial surface, and situated within the mitochondrial outer membrane (Fig. 1).

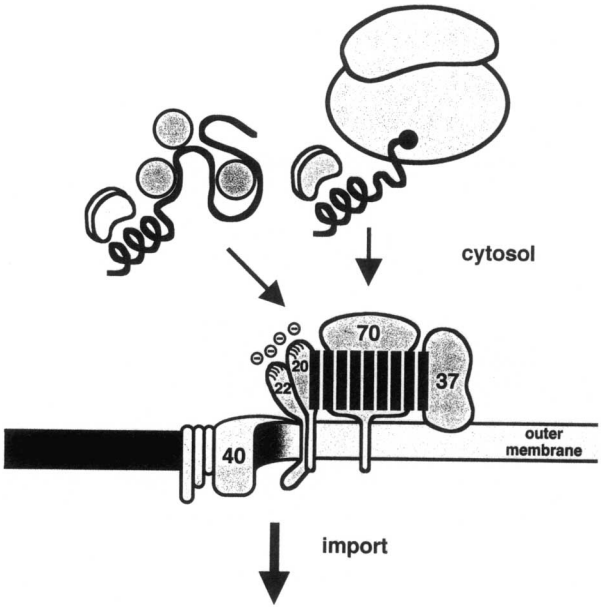

Fig. 1. Precursor proteins are made on ribosomes in the cytosol. In some cases, import might be initiated before translation is complete, but there appears to be no mechanism to ensure that this be the case. Instead, molecular chaperones in the cytosol will bind to the precursor to keep it "import-competent" until it finds its way to the receptor on the mitochondrial surface, and enters the import site in the outer membrane.

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THE RIBOSOMES

Ribosomes in the cytosol translate proteins bound for all subcellular compartments, including the mitochondria. There is evidence that some mitochondrial precursor proteins can initiate import before translation is complete (reviewed by Verner, 1993), and that 80S ribosomes attached to the mitochondrial outer membrane may translate precursors that are being imported (Kellems *et al.*, 1975; Pon *et al.*, 1989). While co-translational import can occur, there is no evidence that it is the requisite form of protein import into mitochondria *in vivo*. Indeed, a number of cytosolic factors have been identified on the basis of assisting the transport of fully-translated precursors through the cytosol to the mitochondrial surface.

THE MOLECULAR CHAPERONES

Molecular chaperones, including isoforms of the protein families hsp70,[3] DnaJ, and 14-3-3, have been shown to bind mitochondrial precursors post-translationally and maintain them in an "import-competent" state (Cyr *et al.*, 1994; Mihara and Omura, 1996). Import competent means: (a) that the protein should not fold prematurely in the cytosol, since fully-folded proteins tend not to cross the mitochondrial membranes (Eilers and Schatz, 1986), and (b) that the protein should not misfold, since it would then be subject to proteolysis in the cytosol (Jentsch, 1992; Craig *et al.*, 1994). Thus, molecular chaperones increase the lifetime of the precursor protein while it searches for the mitochondrial surface.

In one case, a chaperone has been shown to play a proactive role in directing the precursor to the mitochondrial surface. The molecular chaperone MSF selectively binds mitochondrial precursor proteins, and the bound precursor elicits ATPase activity from MSF (Hachiya *et al.*, 1993). The isolated MSF-precursor is able to dock with the outer membrane (Hachiya *et al.*, 1994). Using the well-defined yeast system, it has been shown that the MSF-precursor complex binds stably to the Tom70 and Tom37 subunits of the import receptor (see below) in the mitochondrial outer membrane (Hachiya *et al.*, 1995). An ATP-dependent release of MSF follows, returning the chaperone to the cytosol and leaving the precursor bound to the import receptor, ready for import.

One question that cannot yet be answered is whether MSF is the only chaperone able to specifically deliver precursors to the mitochondrial surface. Other molecular chaperones have been implicated in the import of mitochondrial precursor proteins (reviewed in Cyr *et al.*, 1994), and hsp70 also promotes the productive binding of preadrenodoxin to the mitochondrial surface (Komiya *et al.*, 1996). Indeed, the selective binding of hsp70 to the mitochondrial (but not the cytosolic) isoform of aspartate aminotransferase prevents folding of the precursor, and thereby promotes import into the mitochondria (Lain *et al.*, 1995). Does the mitochondrial surface carry a specific binding site for each of these chaperones? Or does the import receptor simply accept an import-competent precursor protein from any source: as an MSF-complex, as a hsp70-complex, or as a "naked" precursor?

THE PROTEIN IMPORT RECEPTOR

The receptor on the mitochondrial surface recognizes and binds precursor proteins, to initiate their translocation into and across the outer membrane (Lithgow *et al.*, 1995). Early studies had identified two proteins, now called Tom70 and Tom20, which were postulated to function independently as separate receptors for different subclasses of precursor proteins (Söllner *et al.*, 1989, 1990; Hines *et al.*, 1990; Steger *et al.*, 1990). However, it has become clear that no mitochondrial precursor is strictly dependent on either Tom70 or Tom20, since either subunit can be deleted without catastrophe, and yeast cells deleted for their chromosomal copy of the *TOM20* gene or the *TOM70* gene grow as fast as wild-type cells (Lithgow *et al.*, 1994; Moczko *et al.*, 1994). Recent studies undertaken with both yeast and *Neurospora* make clear that at least four outer membrane proteins, Tom70, Tom20, Tom37, and Tom22, contribute to form a single receptor, whose function is to recognize and bind the great variety of mitochondrial precursor proteins (Lithgow *et al.*, 1995; the only known exception being apocytochrome *c*, whose peculiar import pathway has been reviewed by Stuart and Neupert, 1990). The need for multiple subunits in this receptor is dictated by the

[3] Abbreviations: MSF, mitochondrial import stimulating factor; SRP, signal recognition particle; hsp70, 70-kDa heat shock protein; Tom*X*, *X*-kDa translocation component in the outer membrane; TPR, tetratricopeptide repeat.

different properties of the various precursors that must be imported by mitochondria.

Another Subunit of the Import Receptor: Tom72

An exciting observation has been made in the course of the *Saccharomyces cerevisiae* genome sequencing project (Johnston *et al.*, 1994). A gene (YHR117W) encoding a second isoform of Tom70 has been uncovered; since the predicted molecular weight of this novel protein is 72 kDa, it has been christened Tom72 (Bömer *et al.*, 1996). Tom70 and Tom72 are highly homologous, and disruption of either gene results in equivalent mild growth defects in haploid yeast cells. At the protein level, the only real difference in Tom70 and Tom72 are two short segments of amino acid sequence (Fig. 2). Given the conservation in sequence throughout the rest of Tom70 and Tom72, it is tempting to speculate that these segments hold important clues for dissecting import receptor function, and more work is clearly required to define the mechanisms by which these receptor subunits release precursor proteins from molecular chaperones, and themselves bind the partly-folded precursor proteins.

While the Tom37 and Tom70 subunits play a role in the binding of precursor proteins normally delivered by the cytosolic ATP-dependent chaperone MSF, the other two subunits of the import receptor, Tom20 and Tom22, provide acidic domains responsible for recognition and binding of the basic, amphipathic targeting sequence of a mitochondrial precursor protein.

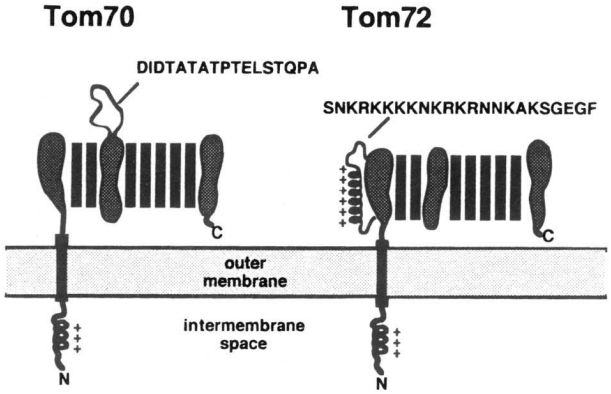

Fig. 2. The receptor subunits Tom70 and Tom72. Each protein has a single, amino-terminal transmembrane domain and a large soluble domain. The soluble domains consist largely of the seven repeats of TPR helices (black), but each protein also carries a unique stretch of amino acids. The amino acid sequences are shown. In Tom70, this "TATA loop" is predicted to be largely unstructured, and is situated between the second and third TPR helix. In Tom72 a very basic stretch, predicted to form a long helix, sits between the transmembrane domain and the soluble domain.

THE "ACID BRISTLE" DOMAIN AND RECOGNITION OF MITOCHONDRIAL TARGETING SEQUENCES

By analogy to the "methionine bristles" by which the signal recognition particle recognizes hydrophobic secretory targeting sequences (Rothman, 1989), it has been proposed that "acid bristles" formed by Tom22 and Tom20 might enable the import receptor to recognize the general features of positively-charged, amphipathic mitochondrial targeting sequences (Lithgow *et al.*, 1994). Precursor proteins can bind to the import receptor through electrostatic interactions involving the presequence (Haucke *et al.*, 1995), and the flexible side chains of the aspartic acid and glutamic acid residues in the putative acid bristle domain formed by Tom20 and Tom22 would deform sufficiently to accommodate the very different primary structures found in mitochondrial targeting sequences.

To test this model, point mutations in the acid bristle region of Tom20 and Tom22 were made, and shown to diminish the ability of precursors to bind to the mitochondrial surface (Bolliger *et al.*, 1995). Both receptor subunits are involved in the productive binding of precursor proteins at the mitochondrial surface: bound precursors can be cross-linked through their targeting sequence to either Tom20 (Haucke *et al.*, 1995) or Tom22 (Hönlinger *et al.*, 1995). The acid-bristle subunits Tom20 and Tom22 can be cross-linked to each other, confirming their status as neighboring subunits of the import receptor (Mayer *et al.*, 1995a).

Figure 3 presents a model that summarizes the roles of the Tom proteins in the early steps of protein import. Any given precursor is able to interact with each of the receptor's subunits, but might be imported at near normal rates in the absence of one of these subunits. For example, pre-alcohol dehydrogenase III interacts with Tom20, Tom37, and Tom70, but can be imported at near normal rates into mitochondria from Δ*tom37* (Gratzer *et al.*, 1995) or Δ*tom70* (Hines and Schatz, 1993) yeast cells. COXVa (subunit Va of cytochrome oxidase) interacts with Tom20 (Lithgow and Schatz, 1995), but can be imported at near normal rates into Δ*tom20* mitochondria (Gärtner *et al.*, 1995).

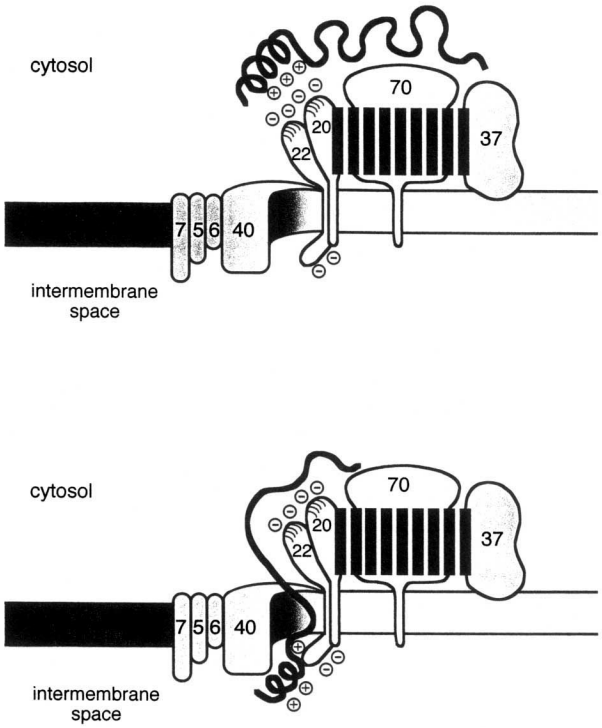

Fig. 3. The Toms. The import receptor consists of the four subunits Tom22, Tom20, Tom70, and Tom37. TPR helices (black) are proposed to tie the receptor subunits together. Tom37 and Tom70 bind predominantly to the mature region of precursor proteins and effect the release of cytosolic chaperones from the precursor. Tom22 and Tom20 together form an acid bristle domain (shaded) that recognizes the targeting sequence of a bound precursor. Tom40, and the tiny Toms Tom5, Tom6, and Tom7, contribute to the import site at which the precursor crosses the outer membrane.

With this multi-functional receptor, mitochondria have a comprehensive means to recognize and bind any of the very different proteins which may need to cross their membranes.

WHAT HOLDS THE IMPORT RECEPTOR TOGETHER?

Under most solubilization conditions, the import receptor dissociates into two subcomplexes, a Tom37/Tom70 dimer, and a Tom20/Tom22 complex (Gratzer *et al.*, 1995; Mayer *et al.*, 1995a; Haucke *et al.*, 1996). We believe, however, that in the outer membrane the import receptor exists as a single functional entity. While a complete characterization of the import receptor has not yet been reported, a sequence motif has been noted in three of the subunits which might provide the basis for holding the receptor together. Tom70 contains seven copies of a 34-amino acid helical repeat (Hines *et al.*, 1990) known as a tetratricopeptide repeat (TPR). These TPR motifs are perfectly maintained in the isoform Tom72 (Fig. 2). TPR motifs have been proposed to mediate protein–protein interactions (Goebl and Yanagida, 1991). Tom20 and Tom37 each contain a single TPR motif (Fig. 3). Indeed, mutations strategically placed in the TPR helix of Tom20 virtually abolish the association of Tom20 and Tom70 and specifically slow the import of precursor proteins that are first bound to the partner subunits Tom37 and Tom70 (Haucke *et al.*, 1996).

THE IMPORT SITE

The import site, functionally defined as the site penetrated by a precursor in transit across the outer membrane, incorporates the protein Tom40 to which transiting precursors can be cross-linked (Vestweber *et al.*, 1989). Most of Tom40 is probably buried within the outer membrane, but at least one small domain is exposed in the intermembrane space (Kiebler *et al.*, 1993; Lithgow and Schatz, 1995). Tom40 can be immunoprecipitated from detergent-solubilized mitochondria in a complex that contains a number of the Tom proteins (Kiebler *et al.*, 1990; Söllner *et al.*, 1992).

THE TINY TOMS

A set of small proteins have been identified in association with the import site in the outer membrane. The proteins Tom5 and Tom7 were identified as part of the complex purified from detergent-solubilized outer membranes (Söllner *et al.*, 1992), and the gene encoding Tom6 was cloned as a multicopy suppressor of a defect in the import site component Tom40 (Kassenbrock *et al.*, 1993). Tom6 is a proteolipid that probably sits within the outer membrane, and may facilitate the interaction of the receptor with Tom40 (Alconada *et al.*, 1995). The function of Tom7 has recently been characterized. The *TOM7* gene encodes a small integral protein of the outer membrane import site. The loss of Tom7 stabilizes the interaction between the receptor subunits and Tom40, resulting in impaired lateral movement of proteins to be inserted into the lipid phase of the outer membrane (Hönlinger *et al.*, 1996). The gene encoding Tom5 has not yet been cloned.

THE TRANS DOMAIN OF THE IMPORT SITE

Precursor proteins cross the outer membrane by entering the import site, and are held in place by a site binding to the precursor's targeting sequence on the *trans* side of the outer membrane (Mayer *et al.*, 1995b). The cryptic nature of this *trans* domain has made an unequivocal identification of the proteins contributing to it difficult, but two good candidates have been put forward.

Mitochondria isolated from a yeast strain lacking the intermembrane space domain of Tom22 have a greatly reduced ability to hold a precursor in the outer membrane import site, and the domain of Tom22 normally situated in the intermembrane space has been purified and shown to recognize and bind targeting sequences (Bolliger *et al.*, 1995). This is strong evidence that Tom22 forms at least part of the *trans* domain. Nonetheless, since a yeast mutant lacking this domain of Tom22 is viable (Bolliger *et al.*, 1995; Nakai *et al.*, 1995), additional components are likely to contribute to the *trans* domain. Like the import receptor on the mitochondrial surface, the *trans* binding domain might be comprised of several subunits. One further candidate is Tom40 (Mayer *et al.*, 1995b), since the precursor in transit is in contact with Tom40 (Vestweber *et al.*, 1989) and part of Tom40 is exposed in the intermembrane space (Kiebler *et al.*, 1993).

SORTING EVENTS WITHIN THE IMPORT SITE

The protein translocation machinery in the outer mitochondrial membrane not only participates in protein import to the inner mitochondrial compartments, but is also involved in sorting of proteins to the outer membrane (Fig. 4). The outer membrane protein Tom70 has a signal-anchor sequence at its extreme amino terminus, consisting of a matrix-targeting signal followed by a transmembrane domain. *In toto,* this signal-anchor sequence initiates entry into the import site, and then abbrogates further translocation such that Tom70 exits laterally into the plane of the outer membrane (Hase *et al.*, 1984; Nakai *et al.*, 1989; McBride *et al.*, 1992)

An interesting example of sorting from the outer membrane import site is the mitochondrial NADH-cytochrome b_5 reductase (Mcr1). The Mcr1 precursor is inserted into the outer membrane import site. Some

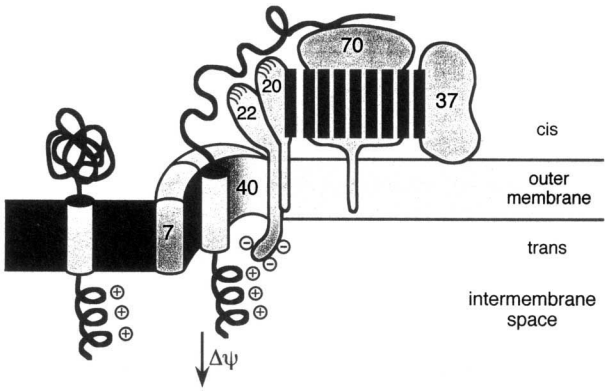

Fig. 4. Sorting of precursors from the import site in the outer membrane. Precursor proteins entering the import site can be held in place by a *trans* domain situated in the intermembrane space. Sorting sequences, held in the import site, would determine if the protein exits the import site into the plane of the outer membrane, or is translocated into the inner mitochondrial compartments. Tom7 might be one of the components that mediates this sorting process.

of the imported molecules exit the import site to become firmly inserted into the outer membrane. The remaining molecules are, however, translocated through the import site to the inner membrane, cleaved by inner membrane protease 1, and released into the intermembrane space (Hahne *et al.*, 1994). How this "leaky stop-transfer" in the outer membrane is accomplished remains unsolved. Two novel candidates which may play a role in this process are Msp1 and Tom7. *MSP1* was isolated as a gene whose overexpression causes the mislocalization of a $Tom70_{1-61}$–cytochrome c_1 fusion protein from the outer membrane to the intermembrane space (Nakai *et al.*, 1993). The *MSP1* gene encodes a 40-kDa outer membrane protein, with a large cytosolic domain. Msp1 has sequence similarity to Sec18, Cdc48, and bacterial FtsH, a prokaryotic ATP-dependent protease participating in the degradation of cytoplasmic proteins (Herman *et al.*, 1995). While Msp1 might be directly involved in protein sorting, it is also possible that Msp1 has a proteolytic activity that leads indirectly to the mislocalization of the fusion protein. A second component, Tom7, has been suggested as a gate in the import site, which would allow the escape of proteins destined to remain in the outer membrane (Hönlinger *et al.*, 1996). Lastly, the *trans* domain of the import site might also be involved in registering signal-anchor sequences (Fig. 4). It will be interesting to see whether any of these components are involved in maintaining the delicate balance between the insertion of Mcr1p into the outer mem-

brane and its further transport to the intermembrane space.

THE LATER STEPS OF PROTEIN IMPORT

Although some proteins are sorted from the import site into the outer membrane, most precursor proteins are destined to travel through the outer membrane import site and into the mitochondria. Since binding of a targeting sequence to the *trans* domain is stable (Mayer *et al.*, 1995b; Bolliger *et al.*, 1995), a switch is required to release the targeting sequence so that import can be completed. Local pH gradients close to the surface of the mitochondrial inner membrane may play a role in loosening the grip of the *trans* domain on the precursor's targeting sequence, or a component in the intermembrane space might promote release. Given the tools currently available, it should not be long until we understand how precursor proteins are transferred from the outer membrane import site into the mitochondria, where the later steps of protein import take place.

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