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Minireview

Targeting of proteins to mitochondria

Trevor Lithgow*

Russell Grimwade School of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Vic. 3010, Australia

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Abstract A clear picture has emerged over the past years on how a 'classic' mitochondrial protein, like subunit IV of cytochrome *c* oxidase, might be targeted to mitochondria. The targeting and subsequent import process involves the commitment of the TOM (translocase in the outer mitochondrial membrane) receptor complex on the mitochondrial surface, a TIM (translocase in the inner mitochondrial membrane) translocation complex in the mitochondrial inner membrane, and assorted chaperones and processing enzymes within the organelle. Recent work suggests that while very many mitochondrial precursor proteins might follow this basic targeting pathway, a large number have further requirements if they are to be successfully imported. These include ribosome-associated factors and soluble factors in the cytosol, soluble factors in the mitochondrial intermembrane space, an additional TIM translocase in the inner membrane and a range of narrow specificity assembly factors in the inner membrane. This review is focused on the targeting of proteins up to the stage at which they enter the TOM complex in the outer membrane.

Key words: Mitochondrion; Ribosome; Protein targeting; Membrane; Protein translocation

1. Introduction

In the yeast *Saccharomyces cerevisiae*, two-dimensional gels resolve around 200–300 individual proteins (Lithgow, unpublished data). Given that there will be some proteins present at levels too low to be detected after electrophoresis, there might be as many as 400–500 different polypeptides targeted to mitochondria in this organism whose entire genome encodes only 6000 or so proteins. Amongst the mitochondrial proteins, eight are known to be encoded and synthesised within the mitochondria itself, the rest are encoded on nuclear genes and synthesised on ribosomes in the cytosol. Each one of these polypeptides has its own biochemical identity and each will require different degrees of assistance from the cellular components that mediate protein targeting to mitochondria.

Using a few model substrates, a very clear picture of the basic mitochondrial protein import pathway has been put together (Fig. 1). For a 'classic' mitochondrial protein, like subunit number IV of cytochrome *c* oxidase, a short presequence at the amino terminal end of the newly synthesised polypeptide contains the information required to commit the protein to mitochondria. The presequences need only be around 20 amino acids in length and able to form a basic, amphipathic helix in order to fulfil this function [1–3]. After synthesis is complete, the precursor protein can be bound by receptor subunits of the TOM complex (translocase in the outer mitochondrial membrane) on the surface of the mitochondria, translocated through the outer membrane and sorted, folded, processed and assembled into the correct submitochondrial compartment [4–6].

2. To fold or not to fold: the dilemma of every new polypeptide

2.1. Characteristics of some polypeptides targeted to mitochondria

Since the presequence of CoxIV is clearly necessary and sufficient for targeting, this and similar presequences have been appended to various reporter proteins providing information on the mechanics of the import pathway. The cytosolic enzyme dihydrofolate reductase (DHFR) has been a common workhorse, since the tightly folded conformation of DHFR can be stabilised with the substrate analogue methotrexate. Since the addition of methotrexate to yeast cells transformed to express mitochondrial-targeted DHFR fusions prevents import, most and perhaps all of the molecules of DHFR fusions must be imported after translation is completed in the cytosol [7].

However, it was always clear that some mitochondrial precursor proteins, while following the same pathway to the mitochondria, would have more difficulty making the journey than a DHFR fusion protein. Mitochondrial precursors tend, by and large, to be more hydrophobic than polypeptides that remain in the cytosol [8]. Many of them are integral membrane proteins, or internal parts of multisubunit complexes, that could not be expected to remain soluble. The in vitro assays used to study protein import have been used to demonstrate these features of mitochondrial proteins: presequence DHFR can be synthesised and urea-denatured and still be imported by isolated mitochondria [9], but the precursor form of the hydrophobic ATPase subunit 9 is imported poorly if at all after urea-denaturation of cytosolic extract [10]. The precursor form of mammalian ornithine-transcarbamylase folds slowly [11] and import of the loosely folded precursor into the mitochondrial matrix is rapid [12,13]. The opposite is true of the precursor form of fumarase: domains of this precursor fold rapidly and prevent import into isolated mitochondria [14].

2.2. Molecular chaperones can assist import post-translationally

For those mitochondrial precursors prone to misfolding or

*Fax: (61)-3-9348 2251.
E-mail: t.lithgow@biochemistry.unimelb.edu.au

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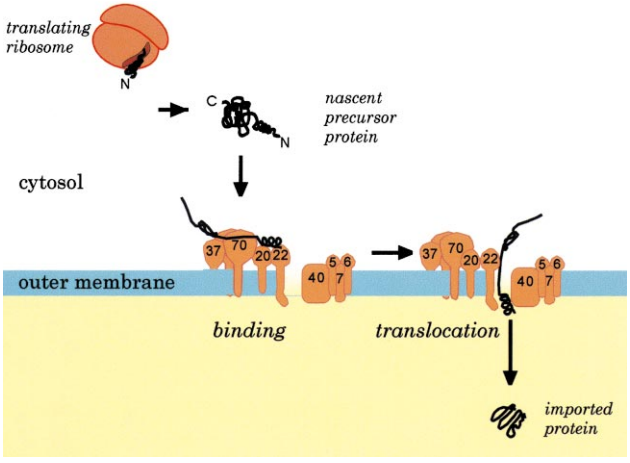

Fig. 1. Fundamental steps of protein import into mitochondria. Most mitochondrial proteins are synthesised on cytosolic ribosomes, many have an amino-terminal targeting sequence. After release from the ribosome, the precursor protein can bind to the receptor subunits (37, 70, 20 and 22 represent the apparent sizes in kDa of the Tom proteins) of the TOM complex. Multiple interactions of the precursor with the channel subunits (Tom5, Tom22 and Tom40) initiates translocation of the precursor protein across the outer membrane [4,40,52]. The final stage of translocation, and subsequent processing and assembly of the imported protein, relies on components of the TIM complexes and various soluble factors not shown in this diagram. See the accompanying review by Koehler [6] for details.

aggregation, cytosolic chaperones are available to prolong their 'import competence' [15]. Cytosolic HSP70s interact with a broad range, perhaps all, nascent polypeptides transiently [16]. Interaction with polypeptides bound for mitochondria might be exaggerated by the increased hydrophobicity of the precursor polypeptides and the presence of the mitochondrial presequence: addition of purified HSP70 is sufficient to stimulate the import of several precursor proteins in vitro [17–20], and the depletion of HSP70 in vivo leads to the accumulation of the precursor form of an ATPase subunit [21]. In the case of aspartate amino-transferase, the presence of a presequence influences the folding of precursor proteins to enable HSP70 to recognise them as substrates [18].

Other chaperones can also promote post-translational import by presenting a precursor in a conformation suitable for binding to the TOM complex (an 'import competent' state). The best characterised of these is MSF, a 14-3-3 protein, that readily binds several mitochondrial precursor proteins, can prevent and even reverse their aggregation, and directly transfer precursors to the Tom70 and Tom37 subunits of the TOM complex [22].

3. Mitochondrial protein import can start during translation

3.1. Kinetics of translation and translocation

Since on average there are only one or two molecules of mRNA encoding a given mitochondrial precursor protein per yeast cell [23], the rate and localisation of protein synthesis has to be considered to understand all of the components that can influence the protein import pathway. After export into the cytoplasm, a mRNA will become translationally active [24]. If the mRNA encodes a mitochondrial precursor protein, that probably means that early passes of ribosomes will generate complete precursors that will encounter the mitochondrial surface post-translationally and might require assistance from molecular chaperones in the cytosol. Given the dynamic nature of the cytoplasm (including the mitochondria themselves), a stable mRNA–polysome complex will eventually come into close proximity to the mitochondria, and once one precursor has engaged the TOM complex on the mitochondrial surface, all subsequent precursors are likely to be imported co-translationally (Fig. 2).

There is no evidence to support distinct co-translational and post-translational protein import mechanisms. Rather, the model presented in Fig. 2 suggests that the relative kinetics of translation and import, as well as the stability of particular mRNAs, might effect the requirement for cytosolic factors that assist either the folding or import of newly-translated precursor polypeptides to mitochondria post-translationally. Furthermore, it supports recent evidence that factors associated with active polysome complexes can assist protein import co-translationally.

3.2. A co-translational phase can assist the import of some precursor proteins

Using electron microscopy, Kellems et al. [25] demonstrated that ribosomes accumulate on the surface of yeast mitochondria. These ribosomes are translationally active and are programmed by mRNA encoding mitochondrial precursor proteins [26,27]. According to the model presented in Fig. 2, these represent polysome complexes in the later phase of translation.

Using a modified precursor protein that is imported post-translationally with reduced efficiency, Ellis and Reid [28] screened for mitochondrial targeting suppressors. The gene

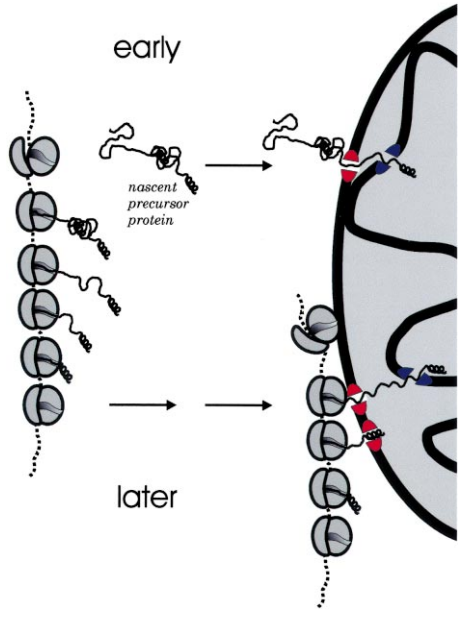

Fig. 2. Kinetic model linking translation and translocation of mitochondrial precursor proteins. A molecule of mRNA encoding any given precursor protein is likely to initiate translation distant from the mitochondrial surface, and in the early phase of translation precursor proteins will be complete before they encounter the TOM complex on the mitochondrial surface (shown in red). Eventually, short nascent chains are likely to encounter the mitochondrial surface, and be recognised by the TOM complex. During the later phase of translation, the precursor proteins synthesised would have the opportunity to be imported co-translationally.

mutated in the *mts1* cells they recovered encodes Npl3, an mRNA-binding protein which shuttles mRNA out into the cytoplasm [28–31]. The *mts1* mutation was mapped to the RNA-binding domain of Npl3 and found to result in an increased residency of the mutant Npl3 protein throughout the cytoplasm, increasing the stability of some mRNAs including those encoding mitochondrial precursor proteins [32].

Using a multi-copy suppressor approach to cure a defect based on minimal import of an exceptionally hydrophobic precursor, the karyopherins Pse1p/Kap121p and Kap123p, factors mediating the nucleo-cytoplasmic shuttling of proteins like Npl3, were found to suppress mitochondrial protein import defects [33]. One explanation for this exciting data is that the karyopherins control levels of factors like Npl3 in the cytoplasm, thereby stabilising some mRNAs and promote co-translational protein import into mitochondria. This would explain the increased import efficiency of the hydrophobic precursors: even for model precursors like DHFR fusion proteins co-translational import can be an order of magnitude more efficient than post-translational import [34,35].

The precursor form of the Krebs cycle enzyme fumarase provides another example of a precursor protein which can be assisted into mitochondria co-translationally. In yeast, fumarase is found in both the cytosol and the mitochondrial matrix. The structure of fumarase has been solved [36] and the amino-terminal domain of fumarase appears to fold tightly as molecules of the precursor leave the ribosome. Those molecules interacting with the TOM complex post-translationally cannot be successfully imported: after translocation of only a short segment of the precursor polypeptide, the presequence is cleaved and the folded polypeptide falls back out of the translocases and remains enzymatically active in the cytosol. Only when import of the fumarase precursor is assayed co-translationally is the polypeptide fully translocated into the mitochondrial matrix [14].

One prediction that can be made from the kinetic model in Fig. 2 is that components assisting in the co-translational phase of protein import should be important in maintaining the level of mitochondrial fumarase. The nascent polypeptide-associated complex (NAC) is one such component. NAC was originally identified in mammalian cell extracts as a ribosome-associated factor that interacts with nascent polypeptides [37]. In yeast, disruption of either of the genes encoding the subunits of the NAC heterodimer leads to defects in protein targeting to the mitochondria. In particular, the steady-state level of fumarase is reduced at least three-fold in Δ*egd1*, Δ*egd2* cells [38; George and Lithgow, in preparation].

A direct involvement of NAC in the co-translational import of another mitochondrial precursor, malate dehydrogenase, has been measured in an elegant in vitro assay [39]. The precursor was presented to isolated mitochondria as nascent chain–ribosome complexes and efficient import depended entirely on the presence of NAC on the ribosomal surface: salt-washing the nascent chain–ribosome complexes removes NAC and prevents import of the precursor, re-addition of purified NAC restores import. NAC could act on the nascent precursor to ensure its presentation in an 'import-competent' conformation, but NAC might also assist the docking of ribosomes on the mitochondrial surface (Fig. 3).

4. The TOM complex: recognition, binding and translocation

4.1. Presequence recognition by the TOM complex

Whether the precursor protein has been released from the ribosome or remains in the process of translation, an amino-terminal targeting sequence will be able to interact with the

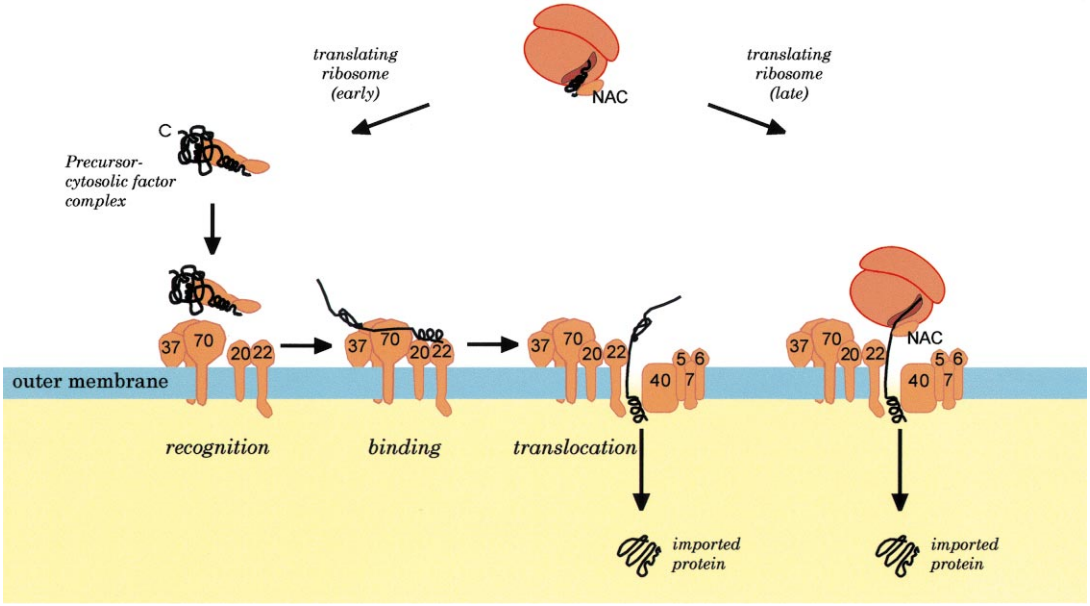

Fig. 3. Protein targeting and import into mitochondria. Most mitochondrial proteins are synthesised on cytosolic ribosomes and a large proportion of the molecules synthesised from each mRNA will be completely translated before encountering a mitochondrion. Many of these polypeptides have chemical properties that promote binding of cytosolic factors, assisting their import by preventing aggregation, misfolding or proteolysis. These import competent precursor molecules are recognised by receptor subunits of the TOM complex: these might always be part of the translocase 'holo-complex', or dock to the 'core-complex' as depicted here [43,46]. Multiple interactions of the precursor with the channel subunits (Tom5, Tom22 and Tom40) initiate translocation of the precursor protein across the outer membrane [4,40,52]. The need for cytosolic factors is obviated in the later stages of translation where the polysome complex can be close to the mitochondrial surface and precursor proteins could be imported co-translationally. Factors such as NAC promote the import of ribosome-associated nascent chains [38,39].

acidic receptor components of the TOM complex to initiate translocation across the mitochondrial outer membrane [40]. There are six subunits in the TOM complex purified from *Neurospora crassa*, eight in *S. cerevisiae* and seven in *Solanum tuberosum* [41–43]. The acidic receptor subunits Tom20 and Tom22 are highly conserved in fungi and animals and, though they are specifically modified in plants, they have probably retained the function of presequence-binding throughout evolution [44].

Tom20 and Tom22 bind exclusively to the amino-terminal presequence of the 'classic' CoxIV precursor [45]. For precursor proteins with 'internal' targeting sequences, like the ADP/ATP carrier, the acidic receptors bind to short segments dispersed within the precursor [45] and it is likely that they also bind the unusual carboxy-terminal signals in tail-anchored proteins targeted to mitochondria. The additional receptor subunits Tom37 and Tom70 interact with other parts of the precursor, perhaps reflecting an ability to take up unfolded polypeptides from cytosolic chaperones like MSF [22].

4.2. Roving receptors, dynamic interactions and docking to the core translocase complex

Interaction of the receptor subunits Tom20, Tom37 and Tom70 with the rest of the TOM complex is somewhat labile and can be disrupted during detergent solubilisation of the complex from the outer mitochondrial membrane. Ahting et al. [46] used this property of the complex to deliberately solubilise a 'core complex', consisting of only Tom22, Tom40, Tom6 and Tom7 from *N. crassa* mitochondria. The core complex represents the translocation channel for protein import.

To be imported into mitochondria, nascent polypeptides are threaded through a 20 Å opening in the outer membrane translocase [47], and electron microscopy of the detergent-solubilised core complex shows a ~20 Å feature that accumulates stain and is very likely to be the channel [43,46]. Since the purified Tom40 subunit reconstituted into artificial bilayers also displays electrophysiological properties of a ~20 Å channel, it is reasonable to conclude that Tom40 forms the channel of the TOM complex through which a nascent polypeptide is threaded across the mitochondrial outer membrane [48]. Indeed, Tom40 was first identified through cross-linking to a CoxIV–DHFR fusion protein deliberately arrested to span the outer membrane translocation site [49].

While the receptor subunits of the TOM complex can be readily isolated from the core complex, there is good evidence to suggest that in the intact membrane they interact with the core complex and with each other. For example, Tom20 and Tom22 work together to recognise and bind presequences [50] and probably act together to recognise distinct features of the presequence [45]. One possibility, represented in Fig. 3, is that the Tom22 'receptor' is only ever found in the core complex with Tom40 and the tiny Toms, and represents the docking point for the roving Tom20 receptor subunit. Also, Tom20 and Tom70 interact via their tetratricopeptide repeat domains and precursors bound to Tom70 are imported poorly if at all in yeast with a point mutation in the tetratricopeptide repeat domain of Tom20, suggesting that Tom70 docks with Tom20 to feed precursors into the translocase channel [51].

5. Concluding remarks and future directions

We have come a long way towards understanding how proteins are imported into mitochondria, but many aspects of the process remain to be studied. The dynamic interactions between subunits of the TOM complex are likely to explain *how* precursor molecules are translocated, but as yet no energy input has been defined for this process. High resolution structures will become available in the next years, but these represent the complex frozen in one conformation or another: how does the complex move in order to achieve precursor recognition, precursor binding, and then translocation? How does the TOM complex recognise both the 'classic' basic, amphipathic presequence and 'atypical' targeting sequences like those found on proteins tip- or tail-anchored in the outer membrane, the tiny TIMs (translocase in the inner mitochondrial membrane) in the intermembrane space and the various inner membrane proteins that don't have presequences? To what extent do ribosome-associated factors like NAC, and cytosolic chaperones like mitochondrial stimulating factor (MSF), set-up and dictate the efficiency of the overall import process? Does a ribosome ever make intimate contact with the TOM complex, or does each machine work oblivious to the presence of the other? The answers to these questions promise a comprehensive understanding of a most complicated biological process.

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