Intracellular Sorting of Macromolecules

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Targeting proteins to mitochondria: is there a role for mRNA localization?
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Introduction
Mitochondria contain more than 1000 different gene products. Although the organelle contains its own genome, mitochondrial DNA encodes only a minor subset of mitochondrial proteins. The vast majority of proteins are encoded by the nucleus and synthesized on cytoplasmic poly-somes. How are polypeptides sorted to the mitochondrion and how are they imported? These fascinating questions have occupied numerous eminent research groups for the past 20 years, and advances in our knowledge concerning the molecular mechanisms underlying these processes have been the subject of several excellent reviews [1–4]. The most detailed knowledge of these processes has been elucidated in lower eukaryotes, in particular in the fungi Saccharomyces cerevisiae and Neurospora crassa, driven by the powerful partnership of molecular genetics and protein biochemistry. It is already clear, however, that much of this information will prove directly relevant to the higher eukaryote system.

The role of the presequence in sorting and import
Most mitochondrially destined polypeptides are synthesized as precursor proteins with short (10–80 amino acids) N-terminal extensions. The importance of this presequence extension in mitochondrial protein import was originally demonstrated by showing that import of a non-mitochondrial protein such as dihydrofolate reductase could only be accomplished by fusing a presequence on to its N-terminus [5]. Although there is little primary sequence similarity evident, these presequences exhibit several similarities. There is a virtual absence of negatively charged residues, a preponderance of hydroxylated and basic amino acids, and the sequence is often predicted to fold as an amphipathic α-helix with hydrophobic and positively charged faces [6]. Once internalized, the presequence is removed by one or two proteolytic steps. The major protease is a heterodimeric metalloendopeptidase found in the matrix [7], whilst for sorting of some proteins to the intracristal space, a second processing event is performed by an inner-membrane-bound peptidase [8].

Is this N-terminal extension required for mitochondrial localization, for import or for both functions? Synthetic presequences have been shown to translocate across lipid bilayers in a membrane-potential-dependent manner in the absence of any other proteins, suggesting that specificity of import may be entirely due to the membrane-permeant cationic nature of the presequence [9]. In contrast, protease shaving of isolated mitochondria severely inhibits import of...
numerous mitochondrial preproteins, consistent with the existence of specific outer-membrane presequence or preprotein receptors [10]. Indeed, recent in vitro and in vivo studies have revealed two outer-membrane receptor complexes (reviewed in [11]). Homologues of the protein components of these receptors have been identified in both Neurospora and Saccharomyces and bear different names: in this article, I will utilize the nomenclature of Schatz and colleagues [11]. By the nature of their acidic cytosolic domains, the MAS20–MAS22 outer-membrane subcomplex can bind the presequence directly, whilst a second complex MAS37–MAS70 has been shown to interact with precursors bound to cytosolic chaperones such as heat shock protein 70 (hsp70) or the mitochondrial import stimulating factor (MSF) [12].

Another intriguing possibility is that the presequence aids in maintaining an import-competent conformation of the precursor and helps to prevent its aggregation. This may be achieved by maintaining the preprotein in an unfolded state, as a lack of stable tertiary structure is a prerequisite for import [5,13]. It is apparent that the presequence plays a fundamental role in initiating import of preproteins into mitochondria. Its role in targeting per se, however, remains to be clarified.

**Translocation of precursors into the mitochondrial matrix**

The vast majority of mitochondrial proteins are located either within the inner membrane or in the mitochondrial matrix. Once localized to the outer membrane, how are the precursor proteins directed across two hydrophobic barriers to find their correct destination? Again, almost all our current understanding of these processes concerns import into fungal mitochondria. Precursors generally become associated with a receptor subcomplex, promoting an interaction with a multipeptide pore (general insertion pore) which functions as the outer-membrane translocation channel [14]. At least nine outer-membrane proteins are involved in this process (reviewed in [4]). Until recently, it was believed that this translocation channel spanned both inner and outer membrane at areas of membrane juxtaposition, referred to as contact sites [15,16]. Contrary to this expectation, numerous experiments have now shown that the outer- and inner-membrane transfer systems function independently, and that the translocation pore is not static but consists of mobile components in the two membranes which come together only on protein import (detailed in [3]).

Although the inner-membrane import machinery is less well documented, at least three protein components are required for precursor binding and translocation [17]. For successful transfer of precursor proteins across the inner membrane, a membrane potential, matrix ATP and mitochondrial hsp70 are required. Mitochondrial hsp70 plays a dual role in protein import: it maintains the imported precursor in an unfolded state and fuels the translocation process in an ATP-dependent manner. A putative mechanism for describing this ATP-driven process, referred to as the molecular ratchet mechanism, has recently been reported [18]. The mitochondrial hsp70 has been shown to interact directly with GrpE (another mitochondrial chaperone) in the matrix and Isp45, the protein that lines the inner-membrane transport channel, an interaction that is lost on hydrolysis of ATP [19].

**Folding of the mature protein**

How do the imported proteins adopt their native conformations? The processed peptide is transferred in its unfolded configuration from hsp70 to a multimeric complex of the mitochondrial chaperonin system first described in Escherichia coli [21,22]). The mechanisms underlying multi-subunit complex assembly in the mitochondrion and the role these chaperones play in this process are currently not known.

**Submitochondrial sorting of peptides**

In addition to localizing those proteins that are destined to the mitochondrial matrix, mechanisms must also exist for faithfully subcompartmentalizing peptides to the outer membrane, the intracristal (inter-membrane) space or the inner membrane (for review see [4]). It is generally accepted that outer-membrane proteins do not require redirection from the matrix, as they do not need to be proteolytically processed. These proteins interact either directly with the membrane or via surface receptors [23]. There are several import routes for intracristal-space proteins. Apocytochrome c has a natural membrane translocating activity, and becomes trapped at its
destination by the covalent addition of a haem moiety [24]. Other intracristal-space proteins first enter the matrix by utilizing the standard import machinery, where they are cleaved, translocated back through the inner membrane and fully matured by the inner-membrane peptidase. Alternatively they can be passed directly into the intracristal space via the outer-membrane translocation site, bypassing the inner-membrane site completely (i.e. cytochrome c haem lyase [25]). Most inner-membrane proteins appear to be fully imported into the matrix, where they interact with the mitochondrial chaperones, are proteolytically processed and enter the membrane, where a hydrophobic domain functions as a stop-transfer sequence, anchoring the peptide in the membrane.

**Co-translational versus post-translational translocation of mitochondrial proteins**

Are mitochondria-destined peptides synthesized in the cytosol before being imported, or, as is the case with proteins that are transported across the endoplasmic reticular membranes, are they translated and imported simultaneously? Initial experiments performed in the mid-1970s by Butow and colleagues demonstrated that isolated mitochondria from yeast treated with cytosolic protein synthesis inhibitors were coated with polysomes [26]. These polysomes were tightly bound and could only be released by incubation with puromycin [27]. Furthermore these polysomes were enriched for transcripts encoding mitochondrially destined polypeptides and were clustered around contact sites between inner and outer mitochondrial membranes [28]. Alternatively, however, post-translational translocation has clearly been demonstrated both in vivo and in vitro. For example, when cells are grown in the presence of mitochondrial-membrane-potential dissipaters, precursor peptides accumulate within the cell. On removal of the dissipater, these precursors are successfully imported and matured [29,30]. Fujiki and Verner [31] recently repeated these studies, but in addition were unable to see any discernible pool of precursor under normal physiological conditions. Furthermore compelling evidence was presented for tight coupling between protein synthesis and mitochondrial import. The authors concluded that, although precursors such as the β-subunit of the F,F₁-ATPase could be imported post-translationally, this was not its normal manner of import.

Cytosolic chaperones such as hsp70 and MSF (see above) have been implicated in maintaining the import competence of newly synthesized precursor proteins, a function that would seem to be redundant under conditions of co-translational translocation. A unifying hypothesis would therefore be that co-translational translocation is a 'leaky' process, with complete synthesis of precursors routinely occurring before interaction with the outer-membrane receptors. The latter proteins would be inhibited from aggregating by interacting with the cytosolic chaperones until docking to the receptor was completed.

**Could co-translational translocation be facilitated by mRNA localization?**

An intriguing adjunct to the question of whether translocation occurs during or after peptide synthesis is that for some proteins the initial sorting mechanism may occur before synthesis, at the level of mRNA location. Many transcripts localize to specific intracellular compartments, and it has recently been demonstrated that this process is often mediated by cis-acting elements within the 3'-untranslated region of these transcripts (reviewed in [32–34]). Mitochondria are known to be dynamically associated with microtubules [35], cytoskeletal elements that have also been implicated in transporting certain mRNA species to their subcellular locale [36]. It is therefore feasible that the initial selection event for a subset of mitochondrial proteins may be the localization of their transcripts to the mitochondrial periphery. Although no formal proof for this hypothesis exists, it is interesting to note that an RNA-binding protein that interacts with nuclear-derived transcripts encoding several mitochondrial proteins has been reported to be located within the mitochondrial matrix and has since been shown to be mammalian glutamate dehydrogenase [37,38]. Scatchard analysis and surface plasmon resonance techniques have shown that only a subset of glutamate dehydrogenase is able to bind RNA (T. Preiss, E. A. Sang and R. N. Lightowlers, unpublished work). This subset is phosphorylated at a residue(s) that is not tyrosine, serine or threonine. Binding is also sensitive to varying concentrations of nucleotides. Preliminary data are consistent with the RNA-binding domain being accessible to the cytosol and thus may function as a 'receptor' for
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It is well documented that there are two submitochondrial locations of glutamate dehydrogenase: matrix soluble and inner-mitochondrial-membrane-associated [40,41]. It is possible that the RNA-binding domain of glutamate dehydrogenase may span both membranes at contact sites, and functions to localize several species of cytosolic mRNA at the mitochondrial periphery (Figure 1). This mechanism is appealing in that it would facilitate co-translational translocation by maintaining the transcript in close proximity to the mitochondrion and infers a communication mechanism between the mitochondrial matrix and the cytosol. By interacting with the cytosolic mRNA, the glutamate dehydrogenase subset would effectively anchor the translation complex to the mitochondrial surface. This could be of particular relevance for short peptides (Figure 1B) such as are commonly found in the respiratory chain complexes.

**Conclusion**

In summary, it is clear from the enormous body of elegant experiments performed on fungal mitochondria that the presequence facilitates precursor protein import both in vivo and in vitro. It is also clear that targeting of preproteins can also be facilitated by the presequence interacting with the receptor subcomplexes on the outer membrane. The possibility that for at least a subset of mitochondrially destined proteins targeting may be initiated by mRNA localization to the mitochondrial periphery is, however, intriguing. Although there are still no compelling data to support this possibility we are currently investigating the physiological role of the RNA-binding activity of mammalian glutamate dehydrogenase.

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Localization signals in proteins internalized from the cell surface

G. Banting

The mechanism by which certain specific integral membrane proteins (both those with single membrane-spanning domains and those with multiple membrane-spanning domains) are internalized from the cell surface and either recycled to the plasma membrane or delivered to specific intracellular organelles has been an area of intense study for several years. Clathrin and associated adaptor proteins have been shown to play vital roles in receptor-mediated endocytosis [1], whilst defined sequences within the cytosolic domains of many integral membrane proteins have been shown to be required for their efficient endocytosis [2]. The structure of such sequence motifs is clearly important in terms of their interaction with cognate molecules. However, until recently, only one class of such motifs has been subjected to any direct structural analysis.

A number of integral membrane proteins