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review

Finding the right organelle

Targeting signals in mitochondrial outer-membrane proteins

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The mitochondrial outer membrane contains a diverse set of proteins that includes enzymes, components of the preprotein translocation machinery, pore-forming proteins, regulators of programmed cell death, and those that control the morphology of the organelle. All these proteins, like the vast majority of mitochondrial proteins, are encoded in the nucleus, so they are synthesized in the cytosol and contain signals that are essential for their subsequent import into mitochondria. This review summarizes our current knowledge of the signals that target mitochondrial outer-membrane proteins to their correct intracellular location. In addition, the mechanisms by which these signals are decoded by the mitochondria are discussed.

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INTRODUCTION

The mitochondrial outer membrane mediates numerous interactions between the mitochondrial metabolic and genetic systems and the rest of the eukaryotic cell. Proteins that reside in the outer membrane include a diverse set of enzymes, components of the translocase of the outer membrane of mitochondria (TOM) complex and pore-forming components (porins). In addition, the outer membrane contains proteins that control the inheritance and morphology of the organelle, as well as members of the pro- and anti-apoptotic Bcl-2 family.

All these proteins, like the vast majority of mitochondrial proteins, are encoded by nuclear DNA and are synthesized on cytosolic ribosomes. How do outer-membrane proteins reach their correct intracellular address? Two elements are required to target a protein to the mitochondria and place it within the correct sub-mitochondrial compartment: first, the mitochondrial preprotein must contain a mitochondria-targeting signal; second, the cell must have machinery that decodes this information and delivers the protein to its correct subcellular location. The targeting of most mitochondrial preproteins is mediated by a cleavable amino-terminal extension of about 20–50 amino-acid residues (called the presequence or matrix-targeting signal), which is necessary and sufficient to direct proteins into the mitochondria (Neupert, 1997). The import process of presequence-containing preproteins has been extensively studied and is summarized in several recent review articles (for example, see Pfanner & Geissler, 2001; Jensen & Dunn, 2002).

In contrast to presequence-containing preproteins, all proteins that reside in the outer membrane contain non-cleavable targeting and sorting signals within the protein sequence itself. This review summarizes the data that are emerging about the nature of these internal targeting signals and discusses how they are deciphered at the outer membrane.

Topologies of outer-membrane proteins

Proteins of the outer membrane span the membrane once or twice or are polytopic proteins (Fig. 1). One class of these proteins, which include Tom20 and Tom70, contain a single transmembrane segment at their N terminus. These proteins are orientated in the outer membrane such that the bulk of the polypeptide is exposed to the cytosol and only a small N-terminal segment crosses the outer membrane. Tail-anchored proteins, such as Tom5 and Bcl-2, form another class of outer-membrane proteins that have a single membrane-insertion sequence at their carboxyl terminus, and their large N-terminal region is exposed to the cytosol (Wattenberg & Lithgow, 2001). The third type of protein span the outer membrane twice, so that they have a small loop in the intermembrane space. Fzo1 is the only known member of this group so far (Fritz *et al*., 2001). Finally, porins and Tom40 are predicted to traverse the outer membrane as a series of anti-parallel β-strands that form a β-barrel structure (Mannella *et al*., 1996).

The TOM complex as an insertion site

The TOM complex has been found to facilitate the import of almost all mitochondrial proteins analysed so far. Hence, it is an obvious candidate for the machinery that decodes targeting signals in outer-membrane proteins and mediates their insertion into the outer membrane. Indeed, the endogenous TOM complex has been found to mediate the insertion of newly synthesized Tom components and porin (Schleiff *et al*., 1999; Krimmer *et al*., 2001; Rapaport *et al*., 2001; Model *et al*., 2001).

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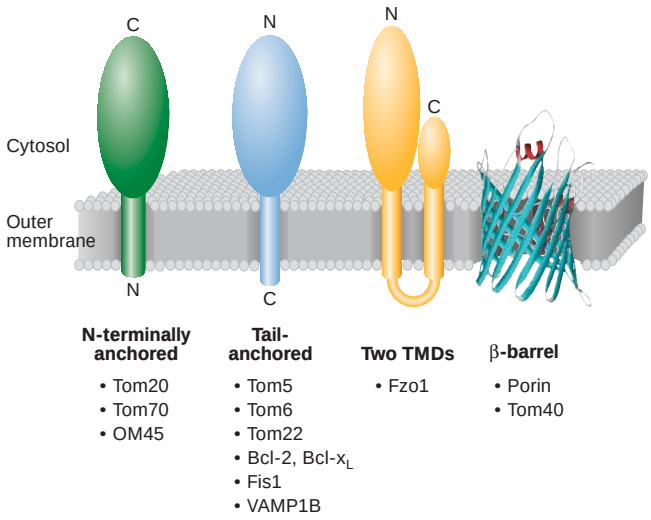

Fig. 1 | Topologies of proteins that reside in the outer membrane of mitochondria. OM45, outer-membrane protein of 45 kDa; TMD, transmembrane domain; Tom, translocase of the outer membrane of mitochondria; VAMP1B, vesicle-associated membrane protein/synaptobrevin 1B.

Within the TOM complex, components with domains that are exposed to the cytosol function as preprotein receptors. Tom20 and Tom22 are involved in the translocation of most protein precursors, in particular those with N-terminal targeting signals (Harkness *et al*., 1994; Lithgow *et al*., 1995). Another receptor, Tom70, forms a binding site for a more restricted set of preproteins, most notably the mitochondrial carrier family that is responsible for metabolite transport across the inner membrane (Schlossmann *et al*., 1994; Brix *et al*., 1999; Wiedemann *et al*., 2001). The subunits Tom40, Tom22, Tom7, Tom6 and Tom5 are embedded in the outer membrane and form the TOM core complex, which is also called the general insertion pore (Dekker *et al*., 1998; Künkele *et al*., 1998; Ahting *et al*., 1999). Tom40 interacts with polypeptide chains in transit and is the main component of the protein-conducting pore (Rapaport *et al*., 1997; Hill *et al*., 1998; Stan *et al*., 2000; Ahting *et al*., 2001).

Amino-terminally anchored proteins

Amino-terminally anchored proteins are also known as 'signal-anchored' proteins because their transmembrane domain (TMD) and its flanking regions function both as an intracellular sorting signal and as an anchor to the membrane (Shore *et al*., 1995). However, these proteins do not share any sequence similarity in their signal–anchor domain (Fig. 2) and therefore the targeting information is probably encoded in structural elements rather than in a specific primary sequence. What structural features make such a domain competent for mitochondrial targeting?

In vitro import experiments have revealed that the targeting information in yeast Tom70 resides in a linear sequence that includes the predicted TMD (residues 11–29), as well as amino-acid residues 1–10, which comprise a hydrophilic, positively charged segment (Fig. 2; Shore *et al*., 1995). The TMD is required for both mitochondrial targeting and membrane anchoring (a signal–anchor function), whereas the positively charged residues cooperate with the TMD to increase the overall rate of import (McBride *et al*., 1992).

A more detailed picture emerged when the structural features of the signal–anchor domains of mammalian Tom20 and Tom70 were characterized. Residues were deleted or mutated in either the TMD or its flanking regions and these proteins were fused to green fluorescent protein (GFP). The intracellular localizations of the resulting proteins were determined by fluorescence microscopy and cell fractionation. Moderate TMD hydrophobicity and a net positive charge within five residues of the C-terminal flanking region were found to be crucial for the mitochondrial localization of mammalian Tom20 (Fig. 2; Kanaji *et al*., 2000). Similarly, at least three basic residues in the C-terminal flanking region of the TMD of Tom70 were necessary for mitochondrial targeting (Suzuki *et al*., 2002). The importance of a net positive charge at the C-terminal flanking region of the TMD is emphasized by the fact that it is also present in fungal proteins (Fig. 2).

The mechanism by which this signal is deciphered at the outer membrane is only partially understood. The targeting of Tom20 and Tom70 to mitochondria was found to be independent of protease-accessible surface receptors (Schneider *et al*., 1991; Schlossmann & Neupert, 1995; Suzuki *et al*., 2002). In addition, antibodies against the pore protein Tom40 inhibited the membrane integration of newly synthesized Tom20, whereas those against the receptors Tom20 and Tom70 did not (Schneider *et al*., 1991). This suggests that both Tom20 and Tom70 are either recognized directly by Tom40, or are recognized by the trypsin-resistant Tom5 before being transferred to Tom40. Two scenarios can be proposed for the steps that take place after the initial recognition. In the first, the signal–anchor domain is inserted into the lumen of the translocation pore formed by Tom40, after which the β-barrel structure opens and the protein is laterally released into the lipid core of the membrane. Such an opening of a β-barrel structure, however, is thermodynamically unfavourable (Gabriel *et al*., 2001). A more likely proposal is that the precursor protein is inserted at the interface area between the TOM core complex and the lipid phase of the membrane. A recent report describing a Tom40 mutant that is ineffective in the transfer of presequence-containing preproteins, but can support the normal insertion of outer-membrane proteins, is consistent with the latter proposal (Gabriel *et al*., 2003).

Can this working model be applied to other N-terminally anchored proteins? Tom20 and Tom70, the only two such proteins

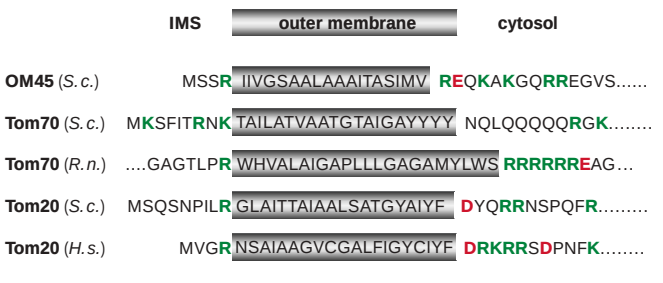

Fig. 2 | Sequences of the transmembrane domains and their flanking regions of amino-terminally anchored outer-membrane proteins. Positively charged amino-acid residues are shown in green and negatively charged residues are shown in red. H.s., *Homo sapiens*; IMS, intermembrane space; OM45, outer membrane protein of 45 kDa; R.n., *Rattus norvegicus*; S.c., *Saccharomyces cerevisiae*; Tom, translocase of the outer membrane of mitochondria.

characterized so far, are components of the TOM complex, and therefore may follow a unique pathway. Studies with other model proteins are therefore required to verify whether the TOM complex is generally involved in the import of N-terminally anchored proteins and, if so, by which mechanism.

Tail-anchored proteins

Tail-anchored proteins in the outer membrane include Fis1, a protein involved in fission of mitochondria (Mozdy *et al*., 2000); the four TOM complex subunits (Tom5, Tom6, Tom7 and Tom22) that interact with Tom40 to form the TOM core complex (Allen *et al*., 2002; Beilharz *et al*., 2003); regulators of apoptosis belonging to the Bcl-2 family (Cory & Adams, 2002); the mitochondrial form of cytochrome *b*5 (Cyt*b*5; D'Arrigo *et al*., 1993); and an alternatively spliced isoform of vesicle-associated membrane protein (VAMP)/synaptobrevin-1B; Isenmann *et al*., 1998; Fig. 3). For some of these proteins, it has been shown that their signal–anchor domain is necessary and sufficient for targeting to mitochondria (Nguyen *et al*., 1993; Egan *et al*., 1999; Dembowski *et al*., 2001; Beilharz *et al*., 2003).

Like the N-terminally anchored proteins, those that are tail-anchored do not share any sequence conservation in their tail region, and the mitochondrial-targeting information is encoded instead in the structural features of this region (Fig. 3). The TMD of these proteins is moderately hydrophobic, relatively short, and has positive charges at its flanking regions. The relative contribution of each of these structural features seems to vary from protein to protein.

The importance of positive charges on either side of the TMD is emphasized by studies of the intracellular distribution of proteins belonging to the Bcl-2 family. These key regulators of apoptosis are divided into two groups: anti-apoptotic members such as Bcl-2 and Bcl-x_L that protect cells from death, and pro-apoptotic members such as Bax and Bak that trigger, or sensitize cells to, apoptosis (Cory & Adams, 2002). Bcl-x_L localizes exclusively to mitochondria (Gonzalez-Garcia *et al*., 1994; Kaufmann *et al*., 2003), whereas Bcl-2 can also be found in the nuclear envelope and the endoplasmic reticulum (ER) membrane (Janiak *et al*., 1994; Kaufmann *et al*., 2003). The TMDs of Bcl-2 and Bcl-x_L have similar hydrophobicities and lengths. However, two positively charged amino-acid residues at either side of the TMD of Bcl-x_L have a crucial role in targeting this protein exclusively to mitochondria (Fig. 3; Kaufmann *et al*., 2003). Bcl-2 lacks this signal as it contains only one basic residue on either side of its TMD, and it therefore stably inserts into several intracellular membranes in a relatively nonspecific manner.

Another example of the importance of positive charges in the tail segment is provided by the subcellular distribution of Cyt*b*5 and VAMP1. Both of these proteins exist in two homologous isoforms that are localized specifically either to the mitochondrial outer membrane or to the ER (Fig. 3). It has been shown that the mitochondrial localization is conferred by a short TMD within the tail region in conjunction with a lack of a net negative charge at its C-terminal flanking region (Isenmann *et al*., 1998; Kuroda *et al*., 1998; Borgese *et al*., 2003).

With the exception of Tom40, all components of the TOM core complex (Tom5, Tom6, Tom7 and Tom22) are tail-anchored proteins. In yeast, all of these Tom components have a proline residue within their transmembrane segment, and this has been shown to be required for the efficient targeting of Tom7 (Allen *et al*., 2002). It is possible that this proline residue, a known α-helix destabilizer, introduces some flexibility into the TMD, which is required for the membrane integration of these proteins.

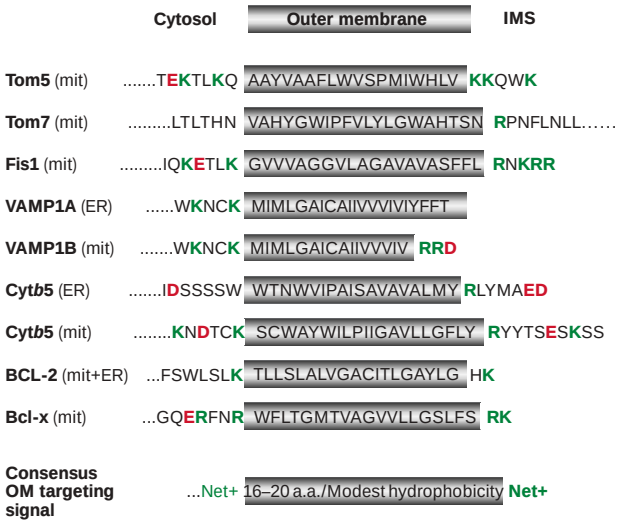

Fig. 3 | Sequences of tail–anchor domains of mitochondrial and endoplasmic reticulum proteins. Positively charged amino-acid residues are shown in green and negatively charged residues are shown in red. Cyt*b*5, cytochrome *b*5; ER, endoplasmic reticulum; IMS, intermembrane space; mit, mitochondrial; Net+, net positive charge; OM, outer membrane; Tom, translocase of the outer membrane of mitochondria; VAMP, vesicle-associated membrane protein/synaptobrevin.

The targeting signal of yeast Tom5 has been studied in more detail (Horie *et al*., 2002). Mutated versions of the protein were fused to GFP, the hybrid proteins were transfected into COS7 cells and their intracellular distribution was examined by fluorescence microscopy. Importantly, yeast Tom5 was targeted to the mammalian mitochondria but did not assemble into the TOM complex. Hence, in this study, Tom5 represented a protein that is uniformly dispersed in the outer membrane. The localization of the different mutants implies that it is the length of the TMD that is important for mitochondrial targeting rather than its hydrophobicity. In addition, positive charges within a segment of five residues C-terminal to the TMD, and the distance between the TMD and this segment, were found to be crucial for mitochondrial targeting. Surprisingly, these positively charged amino-acid residues are not required for the import of yeast Tom5 into mitochondria (Horie *et al*., 2002). Thus, it is possible that the structural requirements for a mitochondrial-targeting signal in yeast cells are not as stringent as in the mammalian system.

Whereas the targeting signals of tail-anchored proteins are quite well characterized, the mechanisms by which these signals are recognized at the mitochondrial surface and how these proteins are inserted into the membrane are not completely understood. One reason for this is the difficulty in differentiating between the nonspecific binding of precursor proteins and physiological membrane integration in an *in vitro* import system (Borgese *et al*., 2003). This difficulty might be the source of conflicting reports regarding the requirements for surface receptors,

external energy and cytosolic chaperones in the import process of tail-anchored proteins. For example, whereas the targeting of VAMP1B to mitochondria was found to be dependent on surface receptors (Lan *et al*., 2000), the membrane targeting of Bcl-2 and Cyt*b*5 has been suggested to be independent of trypsin-sensitive components of the recipient membranes (Janiak *et al*., 1994). A recent study supports the need for receptors, as it reports that the import of the Bcl-2 precursor into yeast mitochondria that lack Tom20 is reduced compared with its import into wild-type mitochondria (Motz *et al*., 2002). This study further suggests that neither the other receptor, Tom70, nor components of the TOM core complex are involved in the import of Bcl-2.

With regard to the tail-anchored TOM components, membrane integration of full-length Tom22 has been found to be dependent on the receptor proteins Tom20 and Tom70 (Keil & Pfanner, 1993). Similarly, the insertion of Tom6 and Tom7 into the outer membrane of isolated mitochondria was inhibited by the proteolytic removal of the cytosolic domains of surface receptors (Dembowski *et al*., 2001). Thus, these proteins are first recognized by import receptors before being assembled into the TOM core complex.

β-barrel proteins

Both porins and Tom40 have been proposed to form β-barrel pore structures that allow the passage of small solute molecules and precursor proteins, respectively, across the membrane. Several attempts have been made to characterize possible targeting signals in these proteins. Truncated versions of *Neurospora crassa* Tom40, in which either the N- or C-terminal segment was removed, were still targeted to the outer membrane, which suggests that it is not these domains exclusively that contain targeting information (Rapaport & Neupert, 1999). The targeting information within porin is located in several regions of the protein, including its extreme C terminus (Hamajima *et al*., 1988; Court *et al*., 1996). These studies suggest that, in contrast to proteins that span the outer membrane once, the targeting information in β-barrel proteins is encoded in a structural element that involves different regions rather than a linear sequence.

How are β-barrel proteins recognized at and inserted into the mitochondrial outer membrane? The initial targeting of the Tom40 precursor to mitochondria is mediated by the import receptors Tom20 and Tom70. The subsequent assembly of the protein into functional complexes involves various high-molecular-weight intermediates that are in equilibrium with the fully assembled TOM complex (Rapaport & Neupert, 1999; Model *et al*., 2001; Rapaport *et al*., 2001; for a review, see Rapaport, 2002). Similarly, the biogenesis of porin is known to involve the import receptor Tom20 and components of the TOM core complex, including Tom40. The cytosolic domain of human Tom20 can bind to the porin precursor and mediate the insertion of the latter into lipid vesicles (Schleiff *et al*., 1999). The subsequent involvement of Tom core components in the import of porin has been shown in various studies. For example, the import of precursors destined for various mitochondrial compartments was inhibited by an excess of water-soluble porin at a stage in the import pathway after that involving protease-accessible binding sites (Pfaller *et al*., 1988). Similarly, excess amounts of matrix-destined preproteins can compete with the import of porin (Krimmer *et al*., 2001). Furthermore, studies using mutant alleles of the essential pore protein Tom40 or yeast strains that lack either Tom5 or Tom22 have shown that the import of porin requires functional Tom5, Tom22 and Tom40 (Krimmer *et al*., 2001).

Perspectives

Our understanding of the biogenesis of mitochondrial outer-membrane proteins has progressed significantly during recent years. For proteins that traverse the outer membrane once, it is now clear that the targeting signal is contained within the single TMD and its flanking regions. To target proteins to mitochondria, this signal must contain a relatively short membrane-spanning segment that has moderate hydrophobicity and is flanked by positively charged residues. These structural characteristics apply regardless of whether the protein is tail-anchored or N-terminally anchored. A challenge for the future is to similarly identify the structural features that comprise the targeting information in β-barrel proteins.

However, the mechanisms by which these signals are deciphered at the outer membrane are not completely understood. Whereas it is clear that the TOM complex is involved in the import of some precursor proteins, whether this involvement can be generalized to all outer membrane proteins is still an open question. To obtain a more comprehensive picture of the biogenesis of outer-membrane proteins, the following questions still need to be addressed: are some precursors of outer-membrane proteins inserted 'spontaneously' into the outer membrane? Do cytosolic factors contribute to the mitochondrial targeting of outer membrane proteins? Does the specific lipid composition of the mitochondrial outer membrane have a role in targeting? Finally, does the same mechanism apply to proteins irrespective of whether the signals are contained within the C- or N-terminal regions? The development of reliable cell-free experimental assays might help us to obtain answers to these questions in the near future.

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Doron Rapaport