Review

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Unlocking the presequence import pathway

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Translocation of presequence-containing precursor proteins into the inner mitochondrial membrane and matrix is an essential process that is facilitated by the translocase of the outer membrane (TOM) together with the presequence translocase of the inner membrane (TIM23). After initial recognition by receptors of the TOM complex followed by transport across the outer membrane, the precursor emerges into the intermembrane space (IMS). Recognition of the presequence by Tim50 triggers rearrangements of the presequence translocase, priming it for inner membrane translocation. Subsequently, the precursor can be released into the membrane or translocated into the mitochondrial matrix aided by the import motor. This heat-shock protein 70 (Hsp70)-based motor drives precursor unfolding and translocation and is subject to dynamic remodelling. Here, we review recent advances in understanding of the mechanisms underlying protein transport along the presequence pathway.

Most mitochondrial proteins are transported along the presequence pathway

Organelles are a hallmark of eukaryotic cells. Among these, mitochondria play key roles in cellular regulation processes and energy metabolism. Although mitochondria have retained their genome, they rely on the import of nuclearly encoded proteins synthesised by cytoplasmic ribosomes. Based on the signal hypothesis, proteins destined for an organellar compartment carry a signal that determines their destination [1]. While this concept still provides the conceptual framework for studies of protein translocation, the number of known mitochondrial targeting signals has increased during recent years (for reviews see [2,3]).

The initial delivery of precursor proteins to mitochondria was long thought to occur post-translationally and in many cases to be chaperone assisted [4,5]. However, a recent study found a wide variety of nascent chains in close vicinity to mitochondria, indicating that a significant fraction of precursor proteins could be transported cotranslationally [6,7]. In line with this, it was shown that *in vivo* import is enhanced by signals in the 3′ untranslated region (UTR) of the precursor mRNAs, targeting them to the mitochondrial surface [8–11]. Interestingly, integral membrane proteins appear to have a uracil bias in their mRNA [12]. Whether this provides a mechanism to target mRNAs to the various cellular organelles remains to be investigated.

On the protein level, the most common mitochondrial targeting signals are N-terminal presequences [13]. Presequences are approximately 15–50 residues long and form net positively charged amphipathic helices [2]. On the surface of mitochondria these signals are recognised by TOM receptors, translocated across the outer membrane, and handed over to TIM23. The TIM23 complex facilitates the membrane potential-driven transport of the presequence across the inner membrane and inserts into the inner membrane those precursors that contain a stop-transfer signal. Alternatively, in the absence of such a signal, the presequence translocase-associated motor (PAM) is recruited and drives full matrix translocation at the expense of ATP.

After the identification of several subunits of these translocases in past decades, research has focussed on the mechanisms of translocation, especially by the TIM23 complex. Here we address current ideas on the mechanism of precursor handover between TOM and TIM23 and how targeting signals trigger rearrangements in the presequence translocase.

Passage across the outer membrane

The general entry gate for protein import into mitochondria is the TOM complex, through which almost all precursor classes pass. The TOM complex comprises the pore forming β-barrel protein Tom40, receptor subunits (Tom20, Tom22, and Tom70), and small subunits important for assembly and stabilisation (Tom5, Tom6, and Tom7) (Figure 1). Electron microscopy revealed that the complex contains two or three pores with a diameter of 21 Å. A combination of Tom22 and Tom40 form the core of the complex, with the transmembrane segment (TMS) of Tom22 contacting at least two Tom40 molecules [14]. Further emphasising the central role of Tom22 is its requirement for the docking of Tom20 and Tom70 to the complex. Interestingly, recent analyses show that most TOM subunits are subject to regulation by phosphorylation, which modulates TOM biogenesis and transport capacity (Box 1).

Transport of a presequence-containing precursor is initiated by recognition of the targeting signal via the interaction of Tom20 with the hydrophobic face of the presequence (Figure 2A). Subsequently, the hydrophilic

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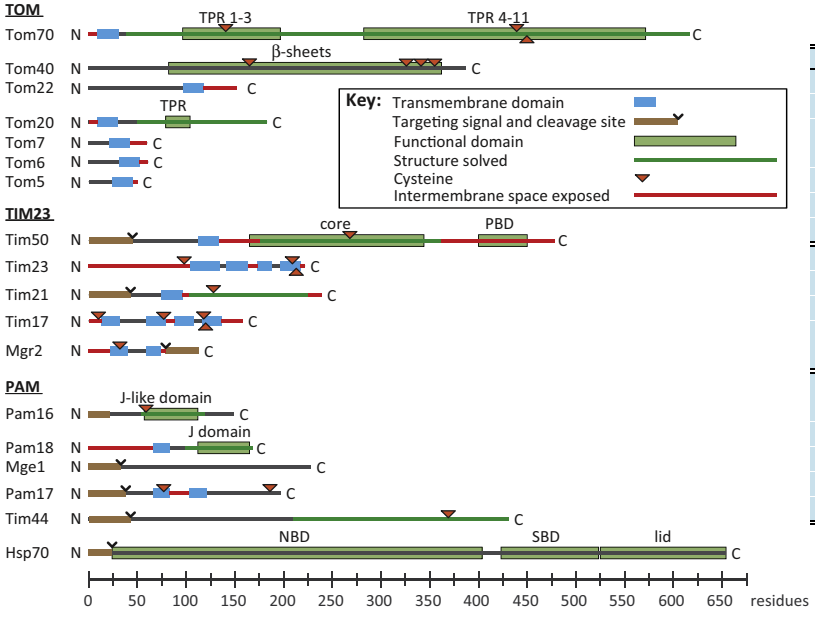

Subunit	Function
Tom70	Receptor (carrier substrates)
Tom40	Channel, binds presequence
Tom22	Presequence receptor (*cis*, *trans*), central TOM organiser
Tom20	Presequence receptor (*cis*)
Tom7	Assembly of TOM, presequence binding (*trans*)
Tom6	Assembly and stability of TOM
Tom5	Presequence receptor (*cis*)
Tim50	Presequence receptor, channel closure at resting state
Tim23	Channel, binds presequence
Tim21	Binds to $Tom22^{IMS}$ and Qcr6 (bc_1 complex)
Tim17	Part of the native channel, gating, sorting
Mgr2	Lateral gate keeper, modulates Pam18 cycle
Pam16	Recruits and inhibits Pam18
Pam18	J-protein, activates ATPase activity of Hsp70
Mge1	Nucelotide exchange factor of Hsp70
Pam17	Initial precursor transport across IM, PAM assembly
Tim44	Binds presequence, tethers Hsp70 to exit site
Hsp70	ATP dependent precursor binding, driving force

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Figure 1. Subunits of the translocase of the outer membrane (TOM) presequence, translocase of the inner membrane (TIM23), and presequence translocase-associated motor (PAM) complex. Domain structure, topology, and targeting signals of the TOM, TIM23, and PAM subunits. The nomenclature of the indicated segments is described in the legend. Cysteine residues, frequently used in crosslinking studies, are highlighted. The functions described on the right present an overview. Abbreviations: TPR, tetratricopeptide repeat; PBD, presequence-binding domain; NBD, nucleotide-binding domain; SBD, substrate-binding domain; N, amino terminus; C, carboxy terminus.

side of the presequence is recognised by Tom22 [15,16]. In contrast to a sequential mode of recognition, a parallel mode of action, in which Tom20 and Tom22 recognise the presequence simultaneously, can also be envisioned [14]. A model in which Tom22 and Tom20 act together as a composite receptor is supported by the close proximity of the subunits in the resting state, as well as the fusion of Tom20 and the N-terminal acidic domain of Tom22 in *Saccharomyces castellii* [14,17,18].

Following initial recognition, the presequence of the precursor is handed over to Tom40 with the help of Tom5 (Figure 2B) [19]. Rather than simply acting as a passive translocation pore, Tom40 also binds the targeting signal and maintains the precursor in an unfolded state (Figure 2C) [20–22]. On emergence of the presequence on the *trans* side of the TOM complex, it can be bound by Tom40, Tom7, and the IMS domain of Tom22 ($Tom22^{IMS}$), from where it is subsequently transferred to the TIM23 complex (Figure 2D) [20,23–26].

Despite our detailed knowledge of the TOM subunits, the driving forces of precursor translocation across the outer membrane remain enigmatic. A relay of hydrophobic and ionic interactions between the presequence and receptors, as well as an increase in affinity toward the *trans* side, has been suggested to drive the transport process [23,27].

While the TOM complex represents the general entry gate for all types of imported mitochondrial protein (except Mim1-dependent α-helical outer membrane proteins), a recent study indicated that the TOM complexes used by presequence-containing precursors and those used by cysteine motif-containing substrates, targeted to the IMS, are not identical [28].

Organisation of the presequence translocase

During protein translocation and the sorting of presequence-containing precursors across and into the inner mitochondrial membrane, the TIM23 complex has to prevent wasteful proton leakage across the inner membrane. Although the stoichiometry of the native channel remains unknown, it is clear that Tim23, as well as the related Tim17, switches between a sealed and a membrane potential-dependent gating state. Another subunit that was suggested to contribute to the protein-conducting channel unit is the small hydrophobic Mgr2. Apart from these mostly membrane-embedded subunits, the TIM23 complex contains Tim50, a receptor subunit, as well as Tim21, which couples the translocase to the respiratory chain (Figure 1).

Detailed analysis of the contacts of these subunits within the presequence translocase revealed an intricate interaction network, which is outlined below. The central subunit, Tim23, is sufficient to form a hydrophilic channel, which opens in a polarised membrane in the presence of presequences [29–32]. The IMS domain of Tim23 is naturally unstructured and interacts with several partners in the IMS: $Tom22^{IMS}$, presequences, $Tim50^{core}$, and the IMS domain of other Tim23 molecules [33–41]. The membrane potential-triggered dimerisation of Tim23 is thought to be mediated by a putative leucine zipper and dissociated in the presence of presequence peptides [33,36]. The first TMS of Tim23 is in close proximity to Tim50 and one or two molecules of Tim17 (presumably contacting TMS4) [36]. While the interaction with Tim17 is altered in the presence of a precursor or the absence of a membrane potential, the interaction with Tim50 is enhanced on membrane depolarisation [36]. The first matrix-exposed loop of