Minireview

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Protein transport along the presequence pathway

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Abstract: Most mitochondrial proteins are nuclear-encoded and imported by the protein import machinery based on specific targeting signals. The proteins that carry an amino-terminal targeting signal (presequence) are imported via the presequence import pathway that involves the translocases of the outer and inner membranes – TOM and TIM23 complexes. In this article, we discuss how mitochondrial matrix and inner membrane precursor proteins are imported along the presequence pathway in *Saccharomyces cerevisiae* with a focus on the dynamics of the TIM23 complex, and further update with some of the key findings that advanced the field in the last few years.

Keywords: mitochondria; PAM; presequence translocase; protein translocation; TIM23 complex; TOM complex.

1 Introduction

Mitochondria contribute to essential cellular processes such as energy metabolism, iron-sulfur cluster biosynthesis, lipid and amino acid metabolism, and control of apoptosis. Most mitochondrial proteins are nuclear-encoded, synthesized on cytosolic ribosomes, and targeted to the mitochondrial surface by specific targeting signals in a process aided by cytosolic chaperones. Further, protein import into the organelle is mediated by multi-subunit complexes, out of which, presequence-carrying proteins are imported via the TOM-TIM23 pathway, or also called the presequence pathway (Busch et al. 2023; Schulz et al. 2015). While most presequence-carrying proteins localize to the mitochondrial matrix, some of them either anchor to the inner membrane or localize in the intermembrane space (IMS). Most of our knowledge on the presequence pathway is based on the studies conducted in baker's yeast, *Saccharomyces cerevisiae*.

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2 Presequences

Mitochondrial targeting signals can be classified into 2 types: cleavable N-terminal presequence and non-cleavable internal targeting signal (ITS) (Pfanner et al. 2019). Around 70 % or more of the mitochondrial proteins have a presequence (Vögtle et al. 2009). The presequence is an amphipathic alpha helix in the range of 10–50 amino acid residues with a positive net charge. Upon import, the presequences are proteolytically removed by the mitochondrial processing peptidase (MPP) in the matrix (Figure 1). Some inner membrane proteins have an additional non-cleavable hydrophobic sorting or stop-transfer signal adjacent to the presequence that allows their sorting into the inner membrane (Glick et al. 1992; Ieva et al. 2014; Laan et al. 2007). The hydrophobic sorting signal functions as a transmembrane domain in the mature protein. Presequence-carrying IMS proteins also follow the same mechanism as aforementioned inner membrane proteins. However, the stop-transfer signal is removed by the inner membrane peptidase and the proteins are released into the IMS (Glick et al. 1992; Ieva et al. 2014; Schendzielorz et al. 2018). Interestingly, some mitochondrial precursor proteins contain internal matrix targeting sequence-like signal(s) (iMTS-L) that are processed by the MPP to give rise to two or more functional proteins (Backes et al. 2018). Certain matrix proteins contain neither a predictable presequence nor any known ITS, suggesting that their import into mitochondria is presequence-independent and unconventional (Bykov et al. 2022; Rada et al. 2015). Most likely, these proteins contain internal targeting information that is non-cleavable.

3 Translocase of the outer membrane (TOM complex)

Most proteins enter into mitochondria through the TOM complex. The highly conserved β-barrel protein Tom40

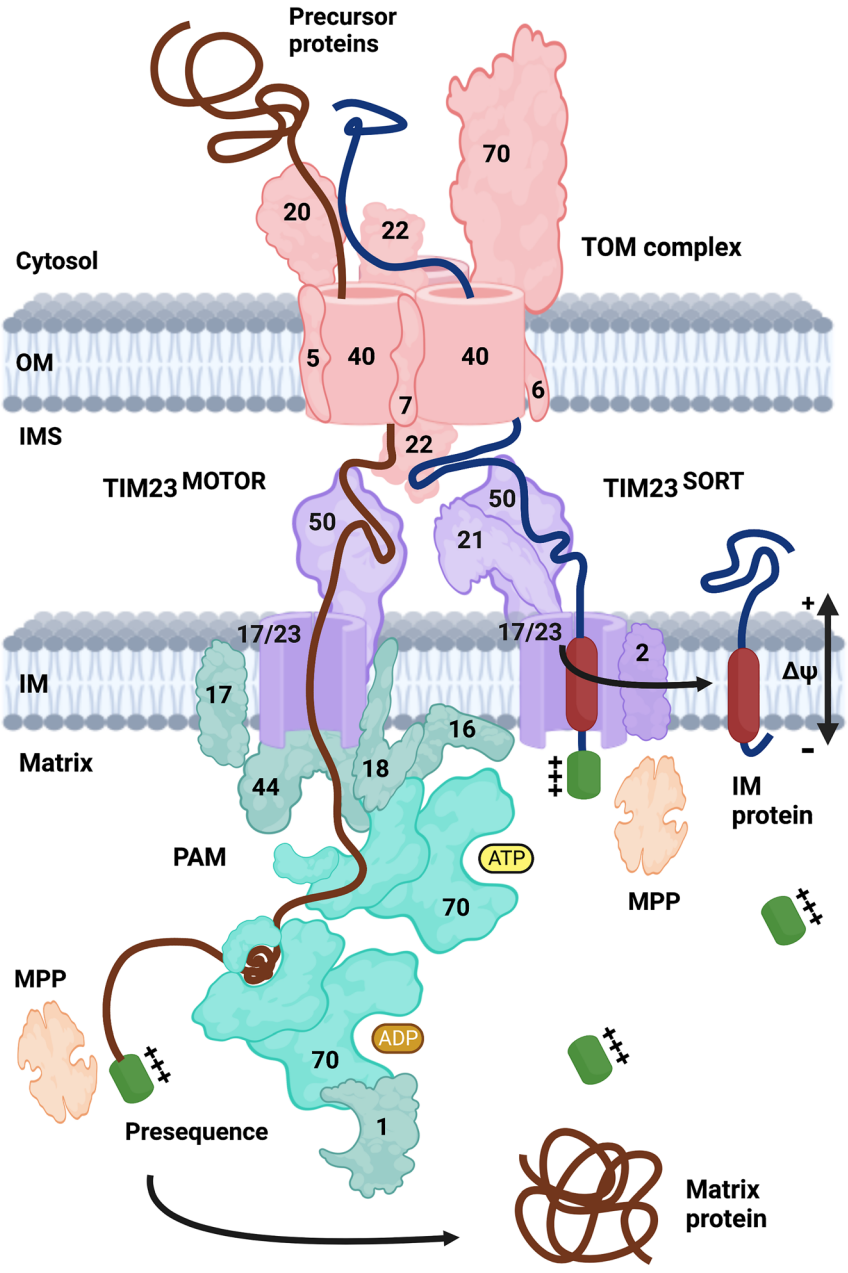

Figure 1: Presequence pathway for matrix and inner membrane proteins in *Saccharomyces cerevisiae* mitochondria. Translocase of the outer membrane (TOM complex) (pale pink), translocase of the inner membrane (TIM23 complex – $TIM23^{SORT}$ and $TIM23^{MOTOR}$) (light purple), presequence translocase-associated motor (PAM) (cyan), mitochondrial processing peptidase (MPP) (light orange), matrix protein (brown), inner membrane (IM) protein (navy blue), presequence (green) and hydrophobic sorting signal (red). Tom5, Tom6, Tom7, Tom20, Tom22, Tom40 and Tom70 are subunits of the TOM complex. Tim17, Tim23, Tim50, Tim44, mtHsp70, Pam16, Pam17, Pam18 and Mge1 form the $TIM23^{MOTOR}$ module, while Tim17, Tim23, Tim50, Tim21 and Mgr2 form the $TIM23^{SORT}$ module. Presequence-carrying matrix proteins are imported by the TOM and $TIM23^{MOTOR}$ complexes, whereas inner membrane proteins with a presequence and a hydrophobic sorting signal are imported via TOM and $TIM23^{SORT}$ complexes. MPP cleaves the presequence from the precursor proteins. Membrane potential (Δ ψ) across the inner membrane and ATP hydrolysis drive protein translocation by the TIM23 complex.

forms the protein-conducting channel across the outer membrane, and exists as a dimer (twin-pore) or a trimer (triplet-pore) (Figure 1) (Araiso et al. 2019; Hill et al. 1998; Makki et al. 2019; Model et al. 2008; Shiota et al. 2015). Besides Tom40, the TOM complex consists of six α-helical membrane proteins – Tom5, Tom6, Tom7, Tom20, Tom22 and Tom70 that are anchored to the outer membrane via a single transmembrane domain (Figure 1). Tom20, Tom22 and Tom70 function as receptors (Brix et al. 1997). Tom20 and Tom22 have negatively charged patches, and are primary receptors for canonical presequence-carrying proteins (Abe et al. 2000; Brix et al. 1997). Although Tom70 is mostly involved in the import of hydrophobic metabolite carrier proteins with internal targeting signals, it also assists in the import of certain hydrophobic proteins with a presequence and polypeptides containing iMTS-L (Backes et al. 2018; Melin et al. 2015). The roles of Tom5, Tom6 and Tom7 are less clear but, seem to have supportive roles in TOM biogenesis and substrate transfer (Araiso et al. 2019; Dietmeier et al. 1997).

4 Presequence translocase of the inner membrane (TIM23 complex)

The TIM23 complex is highly dynamic, and exists either in a motor-associated or motor-free state (Figure 1) (Chacinska et al. 2005; Laan et al. 2007). The core of the TIM23 complex is formed by three proteins: Tim17, Tim23 and Tim50 (Figure 1) (Dekker et al. 1997; Geissler et al. 2002; Mokranjac et al. 2003; Yamamoto et al. 2002). Tim17 and Tim23 are α-helical proteins embedded in the inner membrane via four transmembrane domains. The N-terminal soluble part of Tim23 localizes to the IMS while the C-terminal membrane segment is a part of the protein conducting module (Gomkale et al. 2021; Lytovchenko et al. 2013; Tamura et al. 2009; Truscott et al. 2001). Although, the exact stoichiometry of the subunits of the TIM23 complex is not clear, it is now known that more than one copy of Tim17, Tim23 and Tim50 are present in a single complex (Dekker et al. 1997; Gomkale et al. 2021; Lytovchenko et al. 2013). The presequence translocase receptor Tim50 is anchored to the inner membrane at the N-terminus, and has a large IMS domain (Geissler et al. 2002; Mokranjac et al. 2003; Schulz et al. 2011; Yamamoto et al. 2002). $Tim50_{IMS}$ has two presequence-binding domains, one that is located at the C-terminus and a second that forms a negatively-charged groove located in between the former and the transmembrane domain (Lytovchenko et al. 2013; Schulz et al. 2011; Tamura et al. 2009).

5 $TIM23^{SORT}$

The TIM23 complex exists in 2 states: $TIM23^{SORT}$ that consists of two more proteins, Tim21 and Mgr2, or $TIM23^{MOTOR}$ in which the TIM23 complex lacks Tim21 and Mgr2 but associates to the presequence translocase-associated motor (PAM) (Figure 1) (Chacinska et al. 2005; Gebert et al. 2012; Laan et al. 2007). A constitutive association between the TIM23 complex and PAM decreases the import efficiency of presequence-carrying proteins into the inner membrane (Schendzielorz et al. 2018). Tim21 anchors to the inner membrane via a single transmembrane domain and its C-terminal IMS domain interacts with both $Tim50_{IMS}$ and $Tom22_{IMS}$ to form a dynamic TOM-TIM23 supercomplex (Chacinska et al. 2005; Gomkale et al. 2021; Lytovchenko et al. 2013). Tim21 links the TIM23 complex to respiratory chain complexes III and IV. This coupling promotes the import and inner membrane sorting of precursor proteins (Laan et al. 2006). Mgr2 functions as a coupling factor between $TIM23^{CORE}$ and Tim21, and also as a lateral gatekeeper of the presequence translocase (Gebert et al. 2012; Ieva et al. 2014). Mgr2 is thought to exert a quality-controlled release of protein into the lipid phase.

6 $TIM23^{MOTOR}$

PAM in yeast mitochondria consists of Tim44, mtHsp70, Pam16, Pam17, Pam18 and Mge1 (Figure 1). The central subunit is the ATP-driven chaperone mtHsp70. The TIM23 complex positions mtHsp70 via Tim44 at the matrix side of the inner membrane (Figure 1) (Kang et al. 1990; Schulz and Rehling 2014). MtHsp70 contains an N-terminal nucleotide-binding domain and a C-terminal peptide-binding domain. Tim44 has a lipid-binding segment that tethers it to the matrix-facing side of the inner membrane to facilitate its interaction with the TIM23 complex. The interaction between Tim44 and mtHsp70 depends on whether mtHsp70 is bound to ATP or ADP, and the presence of a translocating protein (D'Silva et al. 2004; Mapa et al. 2010; Slutsky-Leiderman et al. 2007). Mge1 is a nucleotide-exchange factor that triggers ADP to ATP exchange on mtHsp70 (Figure 1) (Bolliger et al. 1994). In addition, the J-domain containing co-chaperones, Pam18 and Pam16 regulate mtHsp70 – Pam18 stimulates ATPase activity while, Pam16 antagonizes this activity by regulating Pam18 (Figure 1) (D'Silva et al. 2003; Frazier et al. 2004; Li et al. 2004). Pam17, an inner membrane protein specific to yeast, promotes the translocation of matrix proteins that are hypersensitive to the membrane potential (Figure 1) (Caumont-Sarcos et al. 2020; Schendzielorz et al. 2017).

7 Precursor transport from TOM to TIM23

When the cytosolic chaperones present the newly synthesized precursor proteins at the mitochondrial surface, Tom20 interacts with the presequence via hydrophobic interaction and simultaneously, the acidic cytosolic domain of Tom22 binds to the positively charged side of the presequence (Brix et al. 1997; Yamano et al. 2008). iMTS-L containing polypeptides are recognized and stabilized by Tom70 that engages with the cytosolic chaperones at the outer membrane (Backes et al. 2018). Tom22 in cooperation with Tom5 transfers the precursor protein to the Tom40 pore (Dietmeier et al. 1997). Within the Tom40 channel, presequence-carrying proteins take an acidic path and exit at the presequence-binding site of the Tom40 pore in the IMS (Araiso et al. 2019; Shiota et al. 2015). Here, the C-terminal

segment of Tom40, $Tom22_{IMS}$ and $Tom7_{IMS}$ receive the exiting polypeptide and hand it over to $Tim50_{IMS}$ and $Tim23_{IMS}$ (Araiso et al. 2019; Gomkale et al. 2021). Thus, a tight cooperation facilitates the handover of presequence-carrying proteins from the TOM to the TIM23 complex (Araiso et al. 2019; Callegari et al. 2020; Chacinska et al. 2003; Gomkale et al. 2021; Lytovchenko et al. 2013; Shiota et al. 2015). The interaction between the incoming polypeptide and $Tim50_{IMS}$-$Tim23_{IMS}$ activates the Tim17-Tim23 module for protein translocation and enables the polypeptide translocation across the inner membrane. Most likely, similar to its passage through Tom40, the polypeptide might follow a path along Tim17-Tim23 that is relatively rich in acidic or hydrophilic residues. The membrane potential across the inner membrane plays a key role in driving protein translocation. The negative charges on the matrix side are thought to act through an electrophoretic effect on the positively charged presequences.

Matrix proteins exiting the TIM23 complex are received by Tim44 on the matrix side and transferred to mtHsp70 (Figure 2A) (Gomkale et al. 2021; Kang et al. 1990). ATP-bound mtHsp70, is proximal to the presequence translocase and in an open conformation primed to bind to the substrate (Figure 2A). Upon mtHsp70 binding to the client protein, ATP is hydrolyzed, stimulated by Pam18, triggering a conformational change in mtHsp70 from open to closed (Figures 2A and B) (D'Silva et al. 2004; Mapa et al. 2010). Dissociation of substrate bound mtHsp70-ADP from Tim44 prevents back sliding of the translocating polypeptide and enables binding of a new ATP-bound mtHsp70 to the polypeptide chain at the translocase (Callegari et al. 2020; D'Silva et al. 2004; Mapa et al. 2010; Schulz and Rehling 2014). Mge1 triggers ADP to ATP exchange on mtHsp70. The concomitant conformational change allows for polypeptide release (Figures 2B and C). The ATPase activity of mtHsp70 is highly critical for the maintenance of a TOM-TIM23 supercomplex (Dekker et al. 1997; Schulz and Rehling 2014). Taken together, the import of presequence-carrying matrix proteins requires both membrane potential across the inner membrane and ATP hydrolysis by the import motor complex.

Presequence-carrying proteins of the inner membrane and IMS follow a similar mechanism as matrix proteins until the point where the presequence exits the TIM23 complex. However, the TOM-tethering function of Tim21 in the sorting module significantly increases the efficiency of handover of substrates from TOM to TIM23 complex (Figure 1) (Callegari

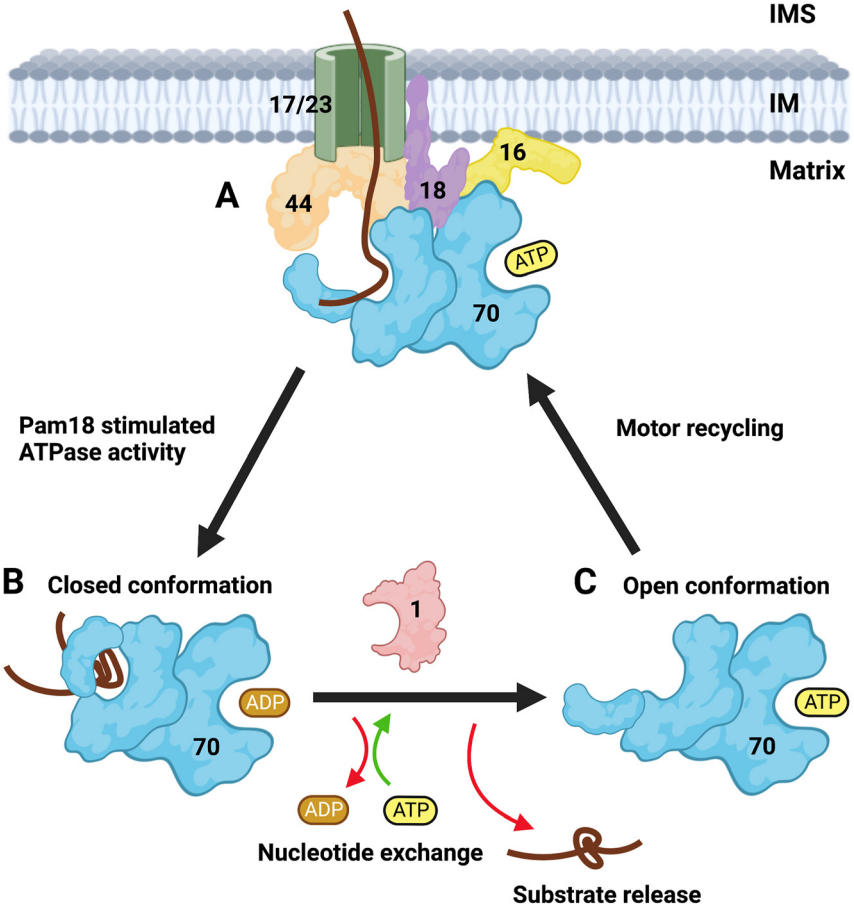

Figure 2: ATP/ADP cycle of mitochondrial Hsp70. Tim17/Tim23 (green), Tim44 (light orange), mtHsp70 (light blue), Pam18 (light purple), Pam16 (yellow) and Mge1 (pale pink). (A) The translocating polypeptide (brown) in the presequence translocase is received by mtHsp70-ATP in an open conformation. Tim44 docks mtHsp70-ATP to the presequence translocase. (B) J-protein Pam18 stimulates the ATPase activity of mtHsp70, causing a switch in the mtHsp70 conformation from open to closed, and the polypeptide is tightly bound by mtHsp70-ADP. (C) Mge1 removes ADP from mtHsp70, allowing mtHsp70 to bind to ATP, and this triggers a change in the mtHsp70 conformation from closed to open facilitating the release of the substrate. Now, mtHsp70-ATP is ready to interact with Tim44 at the exit of the presequence translocase and start a new cycle.

et al. 2020; Chacinska et al. 2005). Also, in the presence of the translocating precursor, the $Tim21_{IMS}$ seems to interact with the extreme C-terminus of $Tim50_{IMS}$ suggesting that Tim50 and Tim21 cooperate during the handover step from TOM to TIM23 complex (Gomkale et al. 2021). When the hydrophobic sorting signal is positioned within the presequence translocase, translocation is stalled. At that stage, Mgr2 modulates the lateral release of the polypeptide into the inner membrane (Figure 1) (Ieva et al. 2014). In the case of presequence-carrying IMS proteins, after their release from the presequence translocase, the stop-transfer signal is removed by the inner membrane peptidase, and the mature proteins are released to the IMS (Glick et al. 1992; Ieva et al. 2014; Schendzielorz et al. 2018). Unlike matrix proteins, the precursor proteins of the inner membrane and IMS require only a membrane potential for the transport across the inner membrane and removal of the presequence.

8 Future perspectives

Our understanding on how the presequence translocase works has advanced considerably in the last two decades. However, several important questions have remained unanswered. Although it is known that the presequence translocase exists either as sort or motor form in mitochondria, whether these are constitutively present at distinct positions, or if the translocase constantly switches between these forms depending on the import load is not clear. If a precursor-dependent switching mechanism exists, it remains open which factors control such a process. Furthermore, the recruitment and recycling of the motor at the presequence translocase remains understudied.

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