Cooperation of translocase complexes in mitochondrial protein import

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Most mitochondrial proteins are synthesized in the cytosol and imported into one of the four mitochondrial compartments: outer membrane, intermembrane space, inner membrane, and matrix. Each compartment contains protein complexes that interact with precursor proteins and promote their transport. These translocase complexes do not act as independent units but cooperate with each other and further membrane complexes in a dynamic manner. We propose that a regulated coupling of translocases is important for the coordination of preprotein translocation and efficient sorting to intramitochondrial compartments.

Introduction

Mitochondria synthesize only a small number of proteins in their matrix. 99% of the ~1,000 different mitochondrial proteins are produced on cytosolic ribosomes and are imported into the organelle (Sickmann et al., 2003; Prokisch et al., 2004; Dolezal et al., 2006; van der Laan et al., 2006a). The classic pathway of protein import into mitochondria involves N-terminal presequences on the precursor proteins (Fig. 1). The presequences target the proteins to receptors of the translocase of outer mitochondrial membrane (TOM) complex. After translocation through the TOM channel, the preproteins are directed to the TIM23 (presequence translocase of inner mitochondrial membrane) complex. The presequence translocase-associated motor (PAM) completes preprotein translocation into the matrix. Here, the mitochondrial processing peptidase removes the presequences, and the proteins are folded to their mature forms.

However, many mitochondrial precursor proteins are not synthesized with cleavable presequences but possess internal targeting signals. Although the TOM complex functions as the central import site for most precursors, the subsequent transport of proteins to the four mitochondrial compartments is mediated by different machineries. Three main import pathways for noncleavable precursor proteins have been defined (Fig. 1). (1) The precursors of outer membrane β-barrel proteins are transferred by the Tim9–Tim10 chaperone complex to the sorting and assembly machinery (SAM) complex of the outer membrane (Wiedemann et al., 2003, 2004; Hoppins and Nargang, 2004). (2) Multispanning proteins of the inner membrane like the metabolite carriers also use the Tim9–Tim10 chaperone complex to traverse the intermembrane space and are inserted into the inner membrane by the TIM22 (carrier translocase of inner mitochondrial membrane) complex (Curran et al., 2002; Vial et al., 2002; Rehling et al., 2003). (3) Many proteins of the intermembrane space contain cysteine motifs and are imported and oxidized by the mitochondrial intermembrane space assembly system (Chacinska et al., 2004; Naoé et al., 2004; Allen et al., 2005; Mesecke et al., 2005).

The protein translocases in the four mitochondrial compartments do not function as independent complexes but cooperate in a dynamic manner. This includes transient contacts between translocases located in different compartments and the involvement of protein complexes that have previously been thought not to be related to protein biogenesis, such as the respiratory chain and mitochondrial morphology components.

Transient connection of outer and inner membrane translocases

Cleavable preproteins are guided into mitochondria by a chain of sequential binding sites for presequences, including the receptor domains of Tom20 and Tom22, the channel formed by Tom40, and the intermembrane space tail of Tom22 (Fig. 2; Komiya et al., 1998; Abe et al., 2000; Chacinska et al., 2005). Tom40 is not simply a passive channel but recognizes the presequences and participates in the selection of precursors for the subsequent sorting pathways (Gabriel et al., 2003). It has been a long-standing question of how preproteins are transferred from the TOM complex to the TIM23 complex of the inner membrane. Models ranged from a permanent TOM–TIM connection to two fully independent translocase complexes.

The identification of Tim50 and Tim21 as new subunits of the TIM23 complex revealed a dynamic mechanism of TOM–TIM cooperation (Geissler et al., 2002; Yamamoto et al., 2002;

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Abbreviations used in this paper: Hsp, heat-shock protein; MDM, mitochondrial distribution and morphology; mtHsp70, mitochondrial Hsp70; PAM, presequence translocase-associated motor; SAM, sorting and assembly machinery; TOM, translocase of outer mitochondrial membrane.

The Journal of Cell Biology, Vol. 179, No. 4, November 19, 2007 585–591
http://www.jcb.org/cgi/doi/10.1083/jcb.200708199

Chacinska et al., 2005). In the absence of preproteins, Tim50 keeps the inner membrane channel formed by Tim23 in a closed state (Meinecke et al., 2006). As soon as preproteins emerge from the Tom40 channel, Tim50 binds to them and stimulates interaction of the presequence with the intermembrane space tail of Tom22. Thus, remarkably, a Tim protein helps a preprotein in transit to make contact with the trans-side of the TOM machinery. Subsequently, Tim21 binds to Tom22, representing a direct but transient interaction between TOM and TIM during protein import (Fig. 2, stage 2). Presequences and Tim21 compete for binding to the intermembrane space tail of Tom22. Thereby, Tim21 induces a release of the presequence from Tom22 and promotes transfer of the preprotein to the next stage, insertion into the inner membrane (Chacinska et al., 2005; Albrecht et al., 2006). Presequence binding to Tim23 completes the chain of binding sites for preproteins on the way from TOM to TIM (Truscott et al., 2001).

Sorting switch of the presequence translocase: alternative binding of respiratory chain and import motor

Transport of presequences through the TIM23 complex is driven by the inner membrane potential $\Delta\psi$, which performs a dual role. It activates the channel protein Tim23 and drives translocation of the positively charged presequences by an electrophoretic mechanism (Truscott et al., 2001). Now, a decision has to be made about the further pathway of the preprotein: either lateral sorting into the inner membrane or complete transport into the matrix. Recent studies showed that two different modular forms of the presequence translocase exist (Chacinska et al., 2005; van der Laan et al., 2006b). The core of both forms consists of Tim50, Tim23, and Tim17, whereas the presence of additional subunits or partners depends on the import route of the preprotein in transit (Chacinska et al., 2005). The sorting form of the TIM23 complex, which is responsible for lateral release of proteins into the inner membrane, contains Tim21 but not the import motor PAM (Fig. 2, stage 3a), whereas the matrix transport form of TIM23 lacks Tim21 but is associated with the multicomponent PAM machinery (Fig. 2, stage 3b).

Several preproteins carry a hydrophobic segment behind the presequence (Fig. 2, preprotein type a). This sorting signal stops translocation across the inner membrane, and the protein is released into the lipid phase by the motor-free TIM23 complex (Fig. 2, stages 3a and 4a; Chacinska et al., 2005). Surprisingly, Tim21 was found to recruit a supercomplex of the mitochondrial respiratory chain consisting of the bc_1 complex and cytochrome *c* oxidase (Fig. 2, stage 3a; van der Laan et al., 2006b). What could be the function of a direct association between TIM23 and the respiratory chain? Lateral sorting into the inner

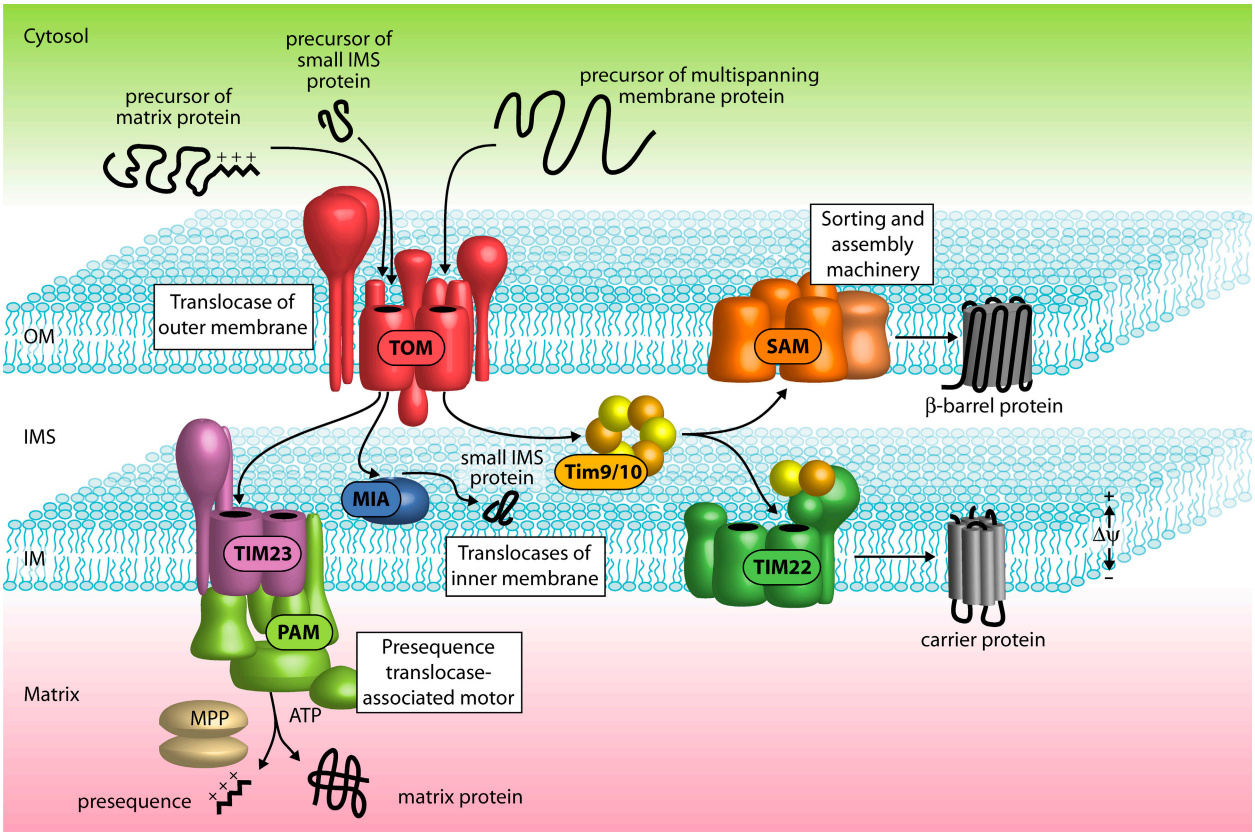

Figure 1. **Mitochondrial import pathways for precursor proteins.** Nuclear-encoded mitochondrial proteins are imported by the TOM complex. Subsequently, they follow different pathways. Presequence-carrying proteins are transported by the TIM23 complex and the motor PAM into the matrix, where mitochondrial processing peptidase (MPP) cleaves off the presequences. Small proteins of the intermembrane space (IMS) are imported via the mitochondrial intermembrane space assembly machinery (MIA). β-barrel precursors of the outer membrane (OM) are transferred by the Tim9–Tim10 chaperone complex from TOM to SAM. Precursors of inner membrane (IM) carriers use Tim9–Tim10 for transfer to the TIM22 complex that drives insertion into the inner membrane.

membrane can be driven by the electrochemical potential as the only external energy source without a requirement for the ATP-dependent motor PAM. Indeed, upon lowering of the overall electrochemical potential of the inner membrane, TIM23 complexes that are in the direct vicinity of the respiratory chain are still competent in preprotein insertion, whereas other transport processes across the inner membrane are diminished (van der Laan et al., 2006b). We envisage two possibilities. The proton motive force may be higher in close proximity to a proton-pumping complex, or protons may be directly translocated to the TIM23 complex and facilitate preprotein transport.

However, the majority of presequence-carrying preproteins are completely translocated into the matrix. To perform this task, the TIM23 complex associates with PAM, which consists of several modules (Fig. 2, stage 3b; Chacinska et al., 2005; Mokranjac et al., 2007). Mitochondrial heat-shock protein 70 (Hsp70 [mtHsp70]) is the central component of PAM. This molecular chaperone binds unfolded preproteins in an ATP-regulated manner. Four membrane-bound cochaperones, Tim44, Pam18, Pam17, and Pam16, interact with the TIM23 complex and coordinate the function of mtHsp70 directly at the TIM channel. Tim44 provides a binding site for mtHsp70, whereas the J protein Pam18 (Tim14) stimulates the ATPase activity of mtHsp70. Pam16 (Tim16) regulates the activity of Pam18, and Pam17 is required to organize the Pam18–Pam16 module (D'Silva et al., 2005; van der Laan et al., 2005; Mokranjac et al., 2007). Finally, mitochondrial GrpE (Mge1) promotes release of the nucleotides from mtHsp70, completing the motor reaction cycle. Thus, PAM is a multistep motor that involves a coordinated action of membrane-bound and soluble proteins to promote the unfolding of preproteins and drive them into the matrix (van der Laan et al., 2005; Wilcox et al., 2005).

Collectively, the TIM23 complex functions at a junction of protein import. Three partner complexes interact with TIM23 in an alternating manner: the TOM complex in early transfer from outer membrane to inner membrane, the respiratory chain for promoting sorting into the inner membrane, and PAM for translocation into the matrix. Tim21 alternates between binding to TOM and the respiratory chain (van der Laan et al., 2006b), whereas Tim17 is involved in the switch between inner membrane sorting and PAM binding (Chacinska et al., 2005). We propose that cooperation of the TIM23 complex with its partner complexes involves more than one interaction site in each case. For the TOM–TIM connection, Tim50 was found to cooperate

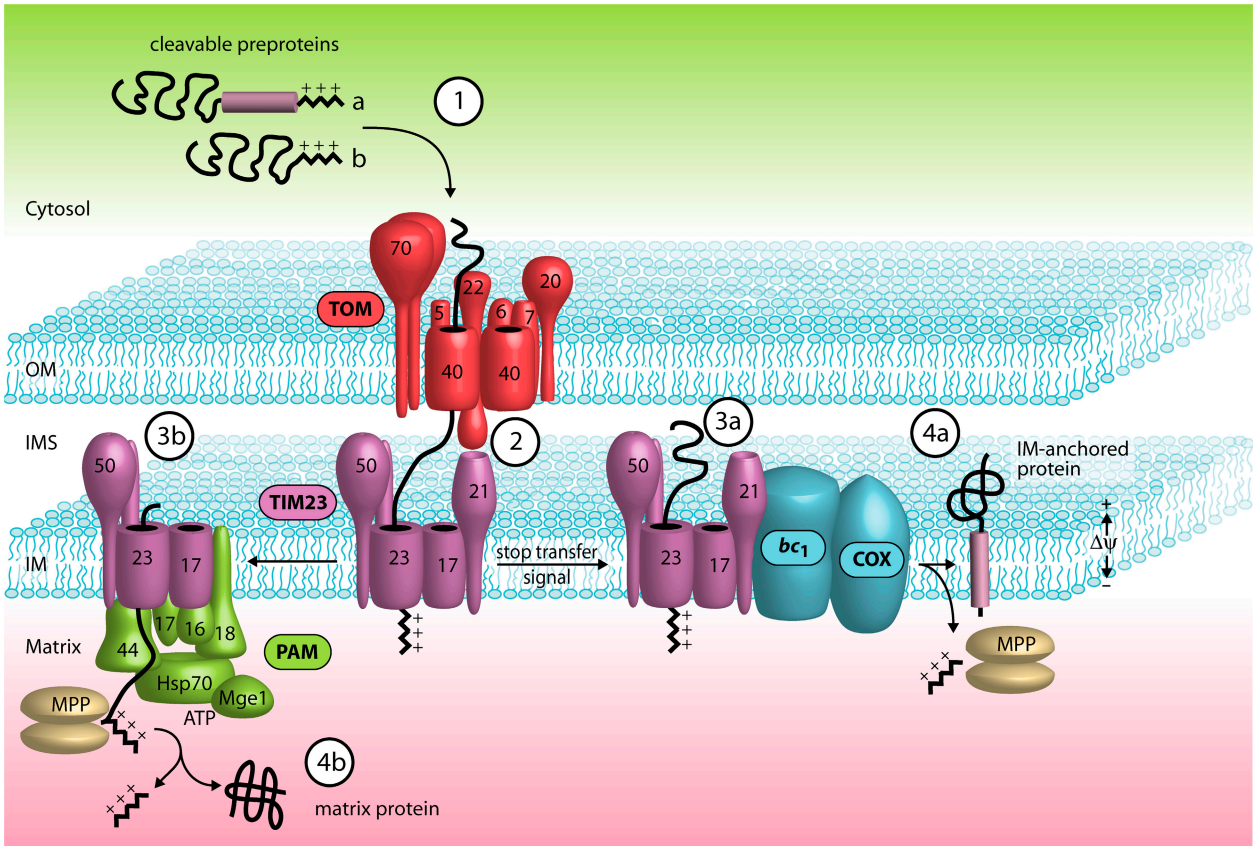

Figure 2. **The presequence translocase of the inner membrane cooperates with different partner complexes.** Preproteins with cleavable presequences are targeted to the TOM complex (stage 1). Upon translocation through the Tom40 channel, Tim50 interacts with the preprotein, and the presequence binds to the intermembrane space (IMS) tail of Tom22. Subsequently, Tim21 binds to Tom22 and promotes release of the presequence (stage 2). The presequence inserts into the Tim23 channel. Then, two pathways are possible. Pathway a: preproteins with a hydrophobic sorting (stop transfer) signal are laterally released into the inner membrane (IM) by a Tim21-containing motor-free TIM23 complex; interaction of the TIM23 complex with the bc_1 complex and cytochrome *c* oxidase (COX) of the respiratory chain stimulates the membrane potential–driven sorting step. Pathway b: preproteins that only carry a matrix-targeting signal are transported into the matrix by a TIM23 complex that associates with the motor PAM. OM, outer membrane; MPP, mitochondrial processing peptidase.

with Tim21 (Chacinska et al., 2005), whereas for the coupling to respiratory chain and PAM, further interacting partners have to be defined in future studies. As the TIM23–respiratory chain interaction is impaired but not blocked by the deletion of Tim21 (van der Laan et al., 2006b), the existence of at least one more interaction site is apparent.

Cooperation of chaperones and translocases in the import of multispanning membrane proteins

Most mitochondrial membrane proteins with several transmembrane segments (multispanning proteins) are synthesized without cleavable presequences. Two major classes are the β-barrel proteins of the outer membrane and the metabolite carriers of the inner membrane (Fig. 3). To prevent aggregation of the hydrophobic precursors, chaperones operate at several stages of the biogenesis pathway. For transfer from cytosolic ribosomes to the Tom receptors, chaperones of the Hsp90 and Hsp70 classes bind to the precursors (Fig. 3, stage 1; Young et al., 2003; Humphries et al., 2005). The receptor Tom70 possesses a specific binding site for the chaperones, and, thus, the precursor–chaperone complex docks onto Tom70 and delivers the substrate. Tom70 oligomerizes in the presence of substrate such that several Tom70 molecules bind to one precursor polypeptide and prevent aggregation during transfer to the Tom40 channel (Wiedemann et al., 2001; Esaki et al., 2003).

The intermembrane space is an aqueous compartment, and hydrophobic proteins would aggregate here. Therefore, the intermembrane space contains a soluble translocase, the Tim9–Tim10 complex, which binds to the precursors of carrier proteins as soon as part of the polypeptide chain has traversed the Tom40 channel (Wiedemann et al., 2001). Tim9–Tim10 forms a hexameric TIM chaperone complex that protects the hydrophobic segments of precursors from aggregation (Curran et al., 2002; Vial et al., 2002; Webb et al., 2006). The carrier precursors do not cross the outer membrane as linear polypeptide chain–like cleavable preproteins but are translocated through Tom40 in a loop formation (Fig. 3, stage 2). Precursor release from TOM requires an active TIM chaperone complex, indicating a close cooperation of both translocases (Wiedemann et al., 2001; Zara et al., 2001; Truscott et al., 2002). The intermembrane space contains a second TIM chaperone complex, the Tim8–Tim13 complex, which is homologous to the Tim9–Tim10 complex and interacts with a subset of hydrophobic precursor proteins (Hoppins and Nargang, 2004; Davis et al., 2007).

The Tim9–Tim10 chaperone delivers the carrier precursors to the TIM22 complex. This involves a rearrangement of the chaperone at the surface of the inner membrane. Tim12, a small Tim protein peripherally bound to the TIM22 complex, associates with Tim9 and Tim10 in a ternary complex, and so these small Tim proteins become membrane bound (Fig. 3, stage 3b; Murphy et al., 2001; Gentle et al., 2007). The TIM22 complex contains three integral membrane proteins: Tim54, Tim22, and Tim18. Tim22 is the channel-forming protein and mediates protein insertion into the inner membrane in a membrane potential–driven manner (Rehling et al., 2003). It is not known which of the three integral subunits binds the small Tim proteins. We speculate that Tim54, with its large domain in the intermembrane space, functions as a docking site for small Tim proteins at the carrier translocase.

The TIM chaperone complexes cooperate with a third membrane translocase, the SAM complex of the outer membrane (Wiedemann et al., 2003). Upon translocation via the Tom40 channel, the precursors of β-barrel proteins bind to Tim9–Tim10 or Tim8–Tim13 and are transferred to SAM (Fig. 3, stage 3a; Hoppins and Nargang, 2004; Wiedemann et al., 2004). Precursor insertion into the outer membrane is initiated by Sam50 (Omp85/Tob55), the central component of the SAM complex (Kozjak et al., 2003; Gentle et al., 2004; Habib et al., 2007). It is not yet known whether Sam50 provides a direct interaction site for the TIM chaperones.

In summary, the soluble TIM chaperone complexes, Tim9–Tim10 and Tim8–Tim13, provide a shuttle system between TOM and the membrane insertases TIM22 and SAM and, thus, ensure that precursors are kept in a translocation-competent conformation.

Linking mitochondrial morphology to outer membrane protein assembly

The outer membrane SAM complex contains three core components: the channel-forming protein Sam50, Sam37, and Sam35 (Wiedemann et al., 2003; Gentle et al., 2004; Ishikawa et al., 2004; Milenkovic et al., 2004; Habib et al., 2007). Sam50 is a β-barrel protein itself. The lateral opening of a β-barrel protein is energetically unfavorable, as many hydrogen bonds would have to be broken. We envisage that the β-barrel precursors, which are delivered by the TIM chaperones, may insert between several Sam50 molecules and, thus, have access to the lipid phase (Fig. 3, stage 3a). The exact function of Sam35 and Sam37 is not yet known. They likely participate in the insertion and lateral release of precursor proteins.

Sam50 is homologous to Omp85/YaeT of Gram-negative bacteria, implying a conserved mechanism of β-barrel insertion in mitochondria and bacteria (Voulhoux et al., 2003; Wu et al., 2005; Dolezal et al., 2006; Bredemeier et al., 2007). However, the partner proteins of Sam50 and Omp85/YaeT are not homologous to each other. In addition, as the lipid composition of bacterial and mitochondrial outer membranes differs considerably, it is likely that the mitochondrial assembly machinery was originally derived from the bacterial one but underwent substantial changes during evolution.

Further characterization of the SAM pathway revealed an unexpected connection to the machinery that maintains mitochondrial morphology. A fourth subunit found in a fraction of SAM complexes turned out to be the morphology protein Mdm10 (Meisinger et al., 2004). Mdm10 is required to assemble β-barrel precursors, in particular the precursor of Tom40, into functional complexes. Mdm10 not only associates with the SAM complex but also with two further morphology proteins, Mdm12 and Mmm1, to form a different complex (Boldogh et al., 2003; Meisinger et al., 2007). Remarkably, this mitochondrial distribution and morphology (MDM) complex is also required for the β-barrel assembly pathway of the mitochondrial outer membrane at a stage after the SAM core components (Fig. 3, stages 3a to 4a).

The MDM complex possibly mediates the cooperation of both mitochondrial membranes because MDM proteins are enriched in punctate structures near contact sites of outer and inner membranes (Aiken Hobbs et al., 2001; Boldogh et al., 2003, Kondo-Okamoto et al., 2003, Jensen, 2005). It should be emphasized that the majority of proteins that were reported to function in the maintenance of mitochondrial morphology are not involved in the assembly of β-barrel proteins (Meisinger et al., 2007). Only a subset of morphology proteins associating with the SAM complex or the MDM complex perform a primary function in protein assembly. As their function involves the biogenesis of the TOM complex (i.e., assembly of the main entry gate of mitochondria), a defect of these morphology proteins leads to a defect of the TOM complex and, consequently, to a defect in the import of genuine morphology components.

We are just beginning to understand how the interplay between TOM, SAM, and MDM complexes is organized. Tom7, a small subunit of the TOM complex, plays a second role outside the mature TOM complex. Tom7 regulates the association of Mdm10 with the SAM complex in an antagonistic manner. Upon deletion of Tom7, the amount of Mdm10 at the SAM complex is increased, and the assembly of Tom40 is accelerated (Meisinger et al., 2006). Thus, Tom7 has two functions. It is a subunit of the mature TOM complex and acts as a negative regulator of the assembly pathway of Tom40. We suggest that the biogenesis of outer membrane β-barrel proteins involves a dynamic cooperation of TOM, TIM chaperones, SAM, and MDM to ensure an efficient and regulated transfer of precursor proteins.

Conclusions and perspectives

We suggest a new level of organization of the mitochondrial protein import machinery. Although the initial characterization of protein transport led to the identification of numerous components and their presence in stable translocase complexes, we have reviewed here that the translocases are highly dynamic machineries. Depending on the sorting signals present in precursor proteins, the translocases undergo modular rearrangements and transiently interact with each other. Importantly, this involves a dynamic interaction between transport complexes located in different mitochondrial compartments, such as the TOM–TIM23 connection, the TIM23–PAM interaction, and the cooperation of TIM chaperones of the intermembrane space with translocases of both outer and inner membranes. The cooperation not only involves the known translocases but also complexes that have not been related to protein import so far, such as the respiratory chain and the MDM complex.

The dynamic nature of the protein import machinery is also reflected in increasing evidence that transport components perform two or more functions or interact with alternating partners. We outlined the examples of Tom22, Tim50, Tim21, and Tim17 in the TIM23 reaction cycle, the cooperation of Tim9–Tim10 with three different translocases, and the dual role of Tom7 as TOM subunit and regulator of Mdm10. Seeing this growing list, we speculate that import components that play

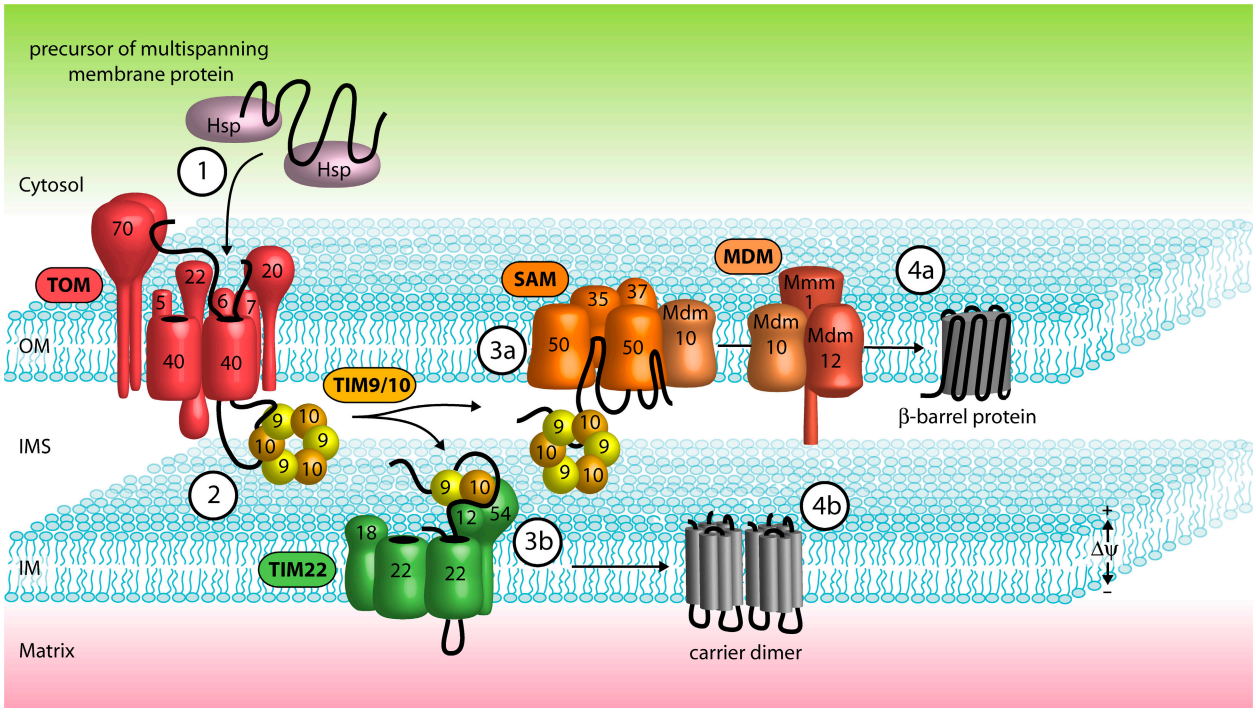

Figure 3. **The Tim9–Tim10 chaperone complex cooperates with translocases of both mitochondrial membranes.** The import pathways of two classes of multispanning membrane proteins are shown: β-barrel proteins of the outer membrane (OM; Pathway a) and carrier proteins of the inner membrane (IM; Pathway b). The precursors are delivered to the TOM complex with the help of cytosolic chaperones (stage 1). The Tim9–Tim10 complex binds to precursors emerging on the intermembrane space (IMS) side of Tom40 (stage 2). β-barrel precursors are transferred to the SAM complex (stage 3a) that cooperates with the MDM complex for membrane insertion and protein assembly (stage 4a). Carrier precursors dock onto the TIM22 complex via the Tim9–Tim10–Tim12 module. Tim22 inserts the precursors into the inner membrane in a membrane potential ($\Delta\psi$)–driven process.

more than one function are much more common than anticipated. Multifunctionality of an import component may be the rule, not the exception.

The rapid increase in knowledge of the cooperation of preprotein translocases suggests that future studies will reveal more dynamic interactions between translocases, be it for preprotein transfer or for regulatory purposes. For example, the three stages defined for the TIM23 reaction cycle likely represent only snapshots that are accessible to our current experimental tools. It is conceivable that the switch between inner membrane sorting and motor binding occurs in several intermediate steps (e.g., for preproteins, which possess a sorting signal but also contain folded domains that require the unfolding power of PAM). We speculate that TOM, SAM, and MDM may be organized in transient, larger assemblies. Moreover, the inner membrane contains machineries for the export of mitochondrially encoded proteins from the matrix (Frazier et al., 2006; Ott et al., 2006; Jia et al., 2007). It will be interesting to see whether these export machineries cooperate with the TIM import machineries.

A cooperation of machineries and components located in different compartments of mitochondria is not only important for protein biogenesis but also for tethering mitochondria to the cytoskeleton, for fusion and fission of the mitochondrial membranes, and for apoptotic processes (Jensen, 2005; Meeusen and Nunnari, 2005; Okamoto and Shaw, 2005; Perfettini et al., 2005). Thus, a characterization of the mechanisms, which coordinate and regulate the activities of both mitochondrial membranes and the two aqueous compartments, will be a major challenge toward a molecular understanding of this highly dynamic cell organelle.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereiche, the Gottfried Wilhelm Leibniz Program, the Max Planck Research Award, Bundesministerium für Bildung und Forschung, Fonds der Chemischen Industrie, and a Boehringer Ingelheim Fonds predoctoral fellowship (to S. Kutik).

Submitted: 29 August 2007
Accepted: 22 October 2007

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