Functional cooperation and separation of translocators in protein import into mitochondria, the double-membrane bounded organelles

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Summary

Nearly all mitochondrial proteins are synthesized in the cytosol and subsequently imported into mitochondria with the aid of translocators: the TOM complex in the outer membrane, and the TIM23 and TIM22 complexes in the inner membrane. The TOM complex and the TIM complexes cooperate to achieve efficient transport of proteins to the matrix or into the inner membrane and several components, including Tom22, Tim23, Tim50 and small Tim proteins, mediate functional coupling of the two translocator systems. The TOM complex can be disconnected from the TIM systems and their energy sources (ATP and $\Delta\Psi$), however, using alternative mechanisms to achieve vectorial protein translocation across the outer membrane

Key words: Mitochondria, Protein import, Translocator, TOM, TIM

Introduction

Mitochondria are essential organelles in eukaryotic cells and involved in many cellular processes ranging from energy production to apoptosis. They are bounded by two membranes, the outer and inner membranes, that divide two aqueous compartments – the intermembrane space (IMS) and matrix. Mitochondria consist of ~1000 different polypeptides, most of which are encoded by the nuclear genome, synthesized on the cytoplasmic ribosomes and imported into mitochondria.

Information for targeting to mitochondria is contained in the mitochondrial proteins themselves (Schatz and Dobberstein, 1996). Typical mitochondrial targeting signals are encoded in the N-terminal presequences, which have the potential to form positively charged amphiphilic helices and are removed upon import of the protein into mitochondria (Roise and Schatz, 1988) (Fig. 1). Some other mitochondrial proteins are synthesized without cleavable presequences and contain targeting signals within the mature protein (Fig. 1).

Import into mitochondria is mediated by translocators (also termed translocases or translocons) in the mitochondrial membranes (Neupert, 1997; Pfanner and Geissler, 2001). The translocator is an assembly of multiple membrane-protein subunits and performs multiple functions. First, it functions as a receptor for recognition of the targeting and/or intramitochondrial sorting signals. Second, it provides a protein-conducting channel through which precursor proteins cross the membrane in an unfolded state. Third, it provides the driving force for vectorial movement of the translocating polypeptide chain.

In *Saccharomyces cerevisiae*, three mitochondrial translocators have been identified: the TOM (the translocase of the outer mitochondrial membrane) complex in the outer membrane, and the TIM23 (TIM, the translocase of the inner mitochondrial membrane) and TIM22 complexes in the inner membrane (Fig. 2) (Lill and Neupert, 1996; Neupert, 1997; Koehler et al., 1999; Bauer et al., 2000; Pfanner and Geissler, 2001; Endo and Kohda, 2002; Jensen and Dunn, 2002). These translocators mediate protein translocation across or into the mitochondrial membranes and guide the proteins to their destinations within mitochondria.

We review recent progress in our understanding of mitochondrial protein import. In particular, we focus on the functional cooperation and separation of the translocators in the two membranes.

The translocators

The TOM complex

The TOM complex consists of a core assembly of Tom40, Tom22, Tom5, Tom6 and Tom7, and the peripheral components Tom20 and Tom70 in yeast (Lill and Neupert, 1996; Neupert, 1997; Pfanner and Geissler, 2001). Tom20 and Tom70 function as receptors; Tom20 is the general receptor protein for presequences and Tom70 probably recognizes targeting signals present in the mature protein and/or those in presequences (Endo and Kohda, 2002). The NMR structure of a complex of the cytosolic core domain of rat Tom20 and a peptide derived from the presequence of rat aldehyde dehydrogenase revealed that the presequence peptide forms an amphiphilic helix when bound to Tom20 and that Tom20 recognizes the hydrophobic side of this helix (Abe et al., 2000). The cytosolic domain of Tom22, which is rich in acidic residues, may well complement the role of Tom20 as a receptor by recognizing the basic residues of the presequences (Brix et al., 1997).

The TOM complex purified from *Neurospora crassa* is a

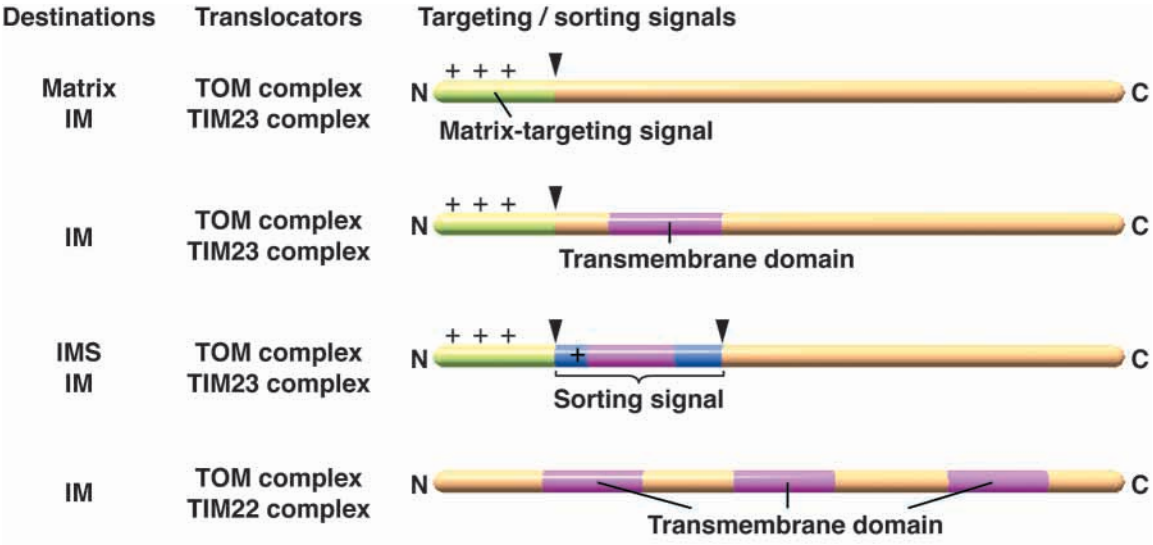

Fig. 1. Targeting and sorting signals of mitochondrial proteins. N-terminal cleavable presequences are shown in light green, transmembrane segments in purple. Sorting signals to the IMS (the intermembrane space) of IM (inner membrane) consist of a hydrophobic segment (purple) flanked by hydrophilic segments (dark blue). Cleavage sites are indicated by arrowheads. From the top to the bottom, precursor proteins with a presequence that are targeted to the matrix or inserted into the IM from the matrix side, IM proteins with a presequence that are anchored to the IM by a single transmembrane segment with the N_{in}-C_{out} orientation (e.g. D-lactate dehydrogenase and subunit Va of cytochrome c oxidase), precursor proteins with a bipartite presequence containing a sorting signal to the IMS (e.g. cytochrome b_2 and cytochrome c_1), and polytopic IM proteins without a presequence (e.g. AAC) are shown. + indicates positively charged regions.

cation-selective high-conductance channel (Künkele et al., 1998). Electron microscopic analyses revealed that the holo TOM complex contains two or three pores, each of which has a diameter of ~20 Å (Ahting et al., 1999). Tom40 is the main component of this channel and purified Tom40 alone can function as a cation-sensitive channel when reconstituted into liposomes (Hill et al., 1998). Tom22 and Tom5 may functionally link the receptors to the Tom40 channel (Kiebler et al., 1993; Dietmeier et al., 1997; van Wilpe et al., 1999).

The TIM23 complex

The TIM23 complex mediates the translocation of presequence-containing proteins across the inner membrane. It consists of two integral membrane proteins – Tim23 and Tim17 – and a peripheral membrane protein, Tim44, and is functionally assisted by soluble matrix proteins Ssc1p and Yge1p (Mge1p). Tim17 and Tim23 could form a protein-conducting channel. Tim23 forms a dimer through its N-terminal domain (residues 51-101) (Bauer et al., 1996). Dimerization depends on the membrane potential across the inner membrane ($\Delta\Psi$), and dissociation of the dimer depends on the presence of presequences (Bauer et al., 1996). Purified recombinant Tim23, when integrated into liposomes, functions as a voltage-activated cation-selective channel that is inhibited by presequence peptides but is activated by both presequence peptides and $\Delta\Psi$ (Truscott et al., 2001). Therefore, in the presence of $\Delta\Psi$, presequences trigger dissociation of the Tim23 dimer, probably leading to the opening of the TIM23 channel. Although a channel-forming activity of Tim17 has not been directly demonstrated, the sequence similarity shared by Tim17 and Tim23 suggests that Tim17 could also constitute a pore, perhaps with Tim23.

Tim44, Ssc1p and Yge1p (Mge1p) form a motor that drives translocation across the inner membrane (Stuart et al., 1994; Rassow et al., 1995). Ssc1p is a mitochondrial Hsp70 (mHsp70) and cycles between high-affinity and low-affinity states for unfolded polypeptide segments at the expense of ATP hydrolysis (Schneider et al., 1994). Yge1p is a nucleotide-exchange factor for Ssc1p. Tim44 is a peripheral inner membrane protein, and Ssc1p binds to it in a nucleotide-dependent manner. The current model proposes that two molecules of Ssc1p, tethered to the TIM23 complex through Tim44, bind in a hand-over-hand manner to the mitochondrial precursor protein, which emerges through the exit of the TIM channel in an unfolded state (Moro et al., 1999). How Ssc1p facilitates the movement of the precursor through the TIM channel has been a matter of debate: two conceptually distinct models, the Brownian ratchet and power stroke models, have been proposed (Glick, 1995; Neupert and Brunner, 2002). In the power stroke model, Ssc1p undergoes a conformational change to generate a pulling force on the precursor protein, which drives unfolding of a folded domain outside the mitochondria. The Brownian ratchet model suggests the transient local unfolding of the precursor protein, which allows translocation of the unfolded segments through the import channel. Multiple rounds of binding of Ssc1p to the translocated precursor segments in the matrix would thus result in unidirectional movement and global unfolding of the precursor protein.

Tim50: a new component of the TIM23 complex

Although several reports have described possible additional components of the TIM23 complex, it was only in the last year that Tim50, a new component of the TIM23 complex, was

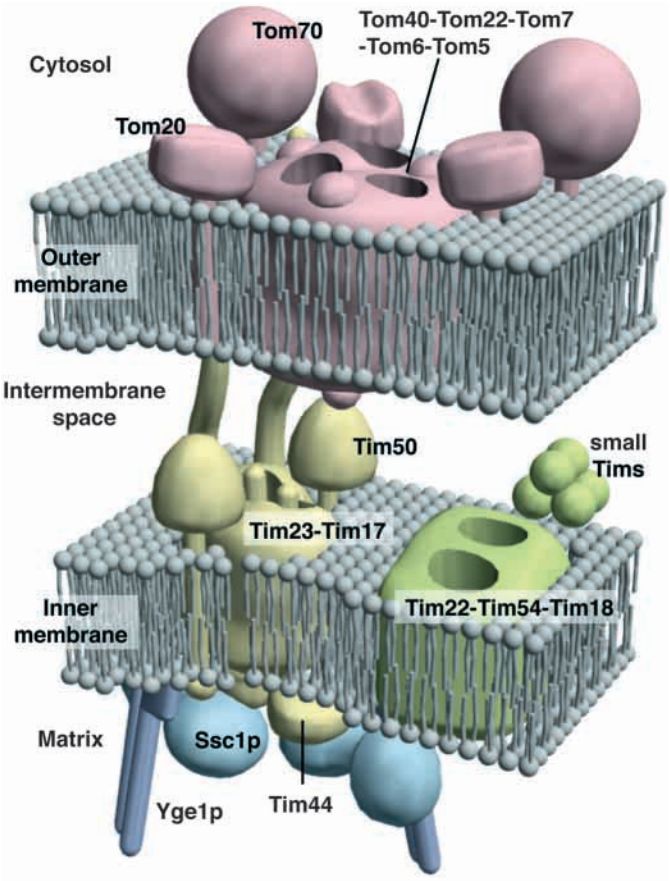

Fig. 2. Translocator complexes in mitochondria. The TOM complex (pink) in the outer membrane, the TIM23 complex (yellow) and the TIM22 complex (light green) in the inner membrane are shown. Ssc1p and Yge1p (Mge1p), peripheral components assisting the function of TIM23 complex (blue) in the matrix, are also included.

identified. Geissler et al. (Geissler et al., 2002) isolated the TIM23 complex from yeast cells and identified Tim50 as an additional protein co-purifying with the complex. Yamamoto et al. (Yamamoto et al., 2002) identified it by characterizing a protein previously shown to be crosslinked to a translocation intermediate. Tim50 was also identified in *Neurospora crassa* by co-isolation with the TIM23 complex (Mokranjac et al., 2003).

Tim50 is essential for yeast growth. When one selectively depletes it, mitochondria lose the ability to import presequence-containing precursor proteins but not the presequence-less inner membrane protein ADP-ATP carrier (AAC) (Geissler et al., 2002; Yamamoto et al., 2002). Anti-Tim50 antibodies block the import of presequence-containing proteins, but not of presequence-less inner membrane proteins into mitochondria when the outer membrane is broken open to allow the access of the antibodies to Tim50 (Yamamoto et al., 2002). Therefore, Tim50 is essential for the translocation of presequence-containing mitochondrial precursor proteins across the inner membrane. Interestingly, the effects of Tim50 depletion on import by the TIM23 complex is somehow suppressed when the positively charged matrix-targeting signal in the presequence is followed by a hydrophobic sorting signal/transmembrane segment (Fig. 1) that arrests translocation across the inner membrane (Geissler et al., 2002; Mokranjac et al., 2003).

The TIM22 complex

The TIM22 complex facilitates insertion of presequence-less polytopic membrane proteins, including members of the metabolite carrier protein family (e.g. AAC) and some Tim proteins (Sirrenberg et al., 1996; Koehler et al., 1999; Bauer et al., 2000; Pfanner and Geissler, 2001; Jensen and Dunn, 2002). The TIM22 complex functions in cooperation with a family of homologous proteins in the IMS called small Tim proteins: Tim8, Tim9, Tim10, Tim12 and Tim13. Three hetero-oligomeric complexes of small Tim proteins have been found: the Tim9-Tim10 complex and the Tim8-Tim13 complex in the IMS, and the Tim9-Tim10-Tim12 complex associated with the TIM22 complex in the inner membrane. The TIM22 complex consists of at least three integral membrane proteins: Tim18, Tim22 and Tim54. Electron microscopic analyses revealed that the reconstituted TIM22 complex forms pores that have a diameter of ~16Å (Rehling et al., 2003). Although precise roles of each component of the TIM22 complex in insertion of proteins into the inner membrane are not known, purified Tim22, whose sequence shows similarity to that of Tim23, can form a hydrophilic channel when reconstituted into liposomes (Kovermann et al., 2002). Tim18 and Tim54 could stabilize the oligomeric structure of the TIM22 complex.

Cooperation of the translocators

The contact site

The outer and inner membranes of mitochondria are physically separated from each other by the aqueous IMS. This poses a problem for proteins destined for the inner membrane and matrix. Does translocation across the outer and inner membranes involve independent steps or a concerted process?

Electron microscopic analyses have shown that mitochondria contain sites where the outer and inner membranes are closely apposed (18-20 nm apart) (Reichert and Neupert, 2002). These sites are called ‘contact sites’ or ‘membrane adhesion sites’. Accumulated evidence suggests that translocation mediated by the TOM and TIM complexes is coupled at such contact sites. For example, when post-translational translocation of the C-terminal domain of the presequence-containing precursor protein is inhibited in vitro, it forms a translocation intermediate that spans the two membranes, which suggests that the translocation takes place at contact sites (Schleyer and Neupert, 1985; Rassow et al., 1990: Jascur et al., 1992; Kanamori et al., 1997).

From TOM to TIM23

The translocation of precursor proteins through the TIM23 complex is tightly linked to their translocation through the TOM complex, because no soluble intermediates in the IMS can be observed. Nevertheless, the TOM and TIM23 complexes are not permanently linked; they can interact transiently but only in the presence of a translocating precursor protein (Berthold et al., 1995; Horst et al., 1995; Dekker et al., 1997). Therefore, a precursor protein translocating through the

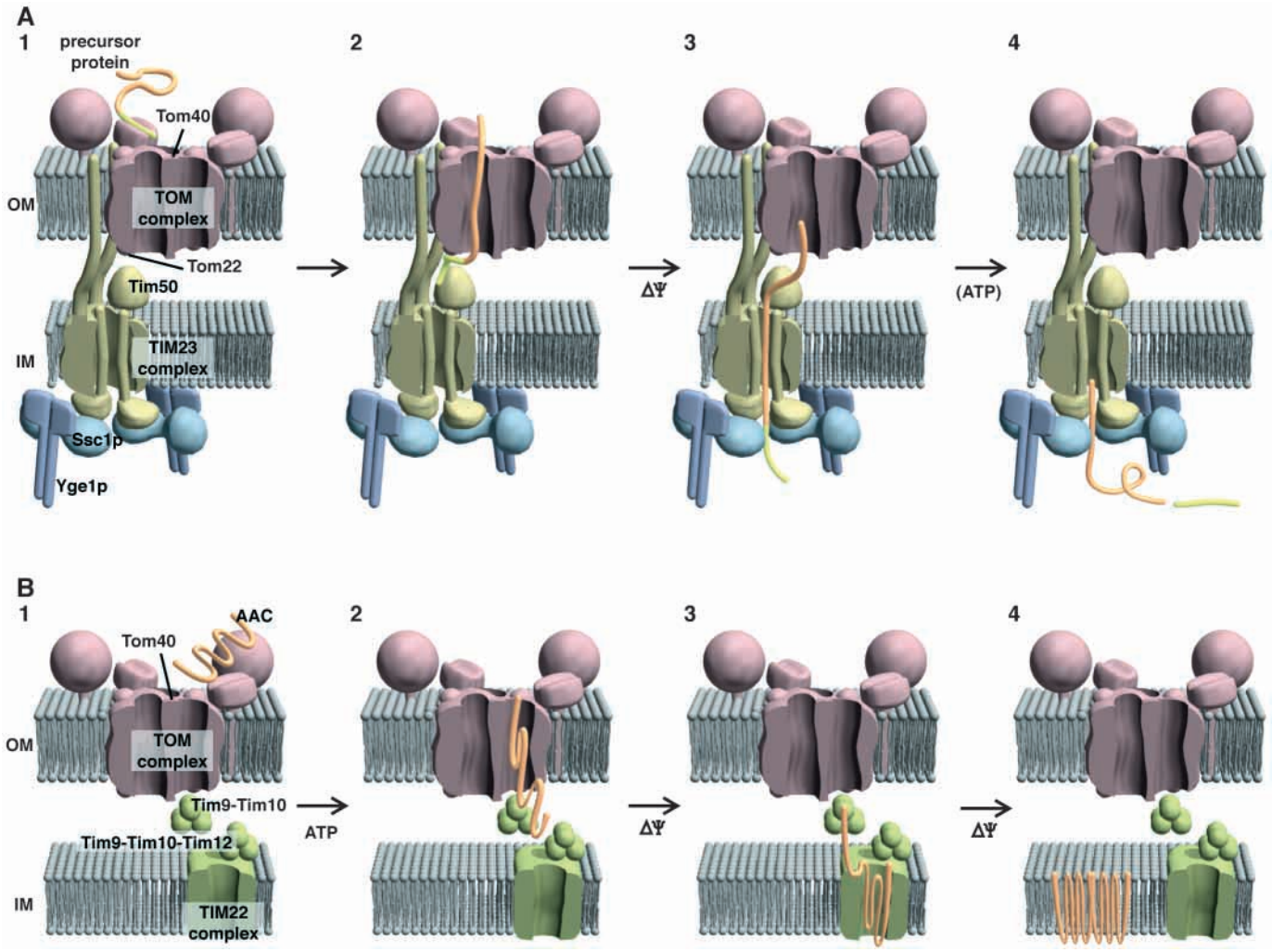

Fig. 3. Functional cooperation of the TOM and TIM complexes. (A) A presequence-containing precursor protein is transferred from the TOM complex to the TIM23 complex. (1) The N-terminal domain of Tim23 tethers the TIM23 complex to the outer membrane. (2) The presequence of the precursor protein reaches the presequence-binding site on the IMS side of the TOM complex (trans site) and is close to Tim50 of the TIM23 complex. (3) $\Delta\Psi$ facilitates transfer of the presequence from the TOM complex to the TIM23 complex via Tim50 and translocation of the presequence across the inner membrane. (4) Translocation of the entire precursor proteins through the TIM23 complex is facilitated by mHsp70 (Ssc1p), in most cases, at the expense of matrix ATP. (B) Polytopic inner membrane proteins including AAC are transferred from the TOM complex to the TIM22 complex. (1) AAC enters the import pathway via Tom70. (2) ATP drives translocation of AAC through the TOM channel probably in a loop conformation to bind to the Tim9-Tim10 complex. (3) AAC is transferred to the TIM22 complex via the Tim9/10/12 complex with the aid of $\Delta\Psi$. (4) AAC is inserted into the inner membrane by the TIM22 complex and $\Delta\Psi$, and forms a dimer. OM, the outer membrane; IM, the inner membrane.

TOM complex needs to engage with the TIM23 complex in the inner membrane to trigger formation of the ternary supracomplex (Glick et al., 1991: Pfanner et al., 1992).

The TOM complex is distributed throughout the outer membrane. Tim23, however, is thought to exhibit an unusual transmembrane topology in which the C-terminal domain (residues 101-222) is integrated into the inner membrane, whereas the N-terminal 50 residues are inserted into the outer membrane, leaving residues 51-100 exposed to the IMS (Donzeau et al., 2000). The double-membrane spanning topology of Tim23 suggests its enrichment around contact sites (Fig. 3A).

Studies by several groups showed that the IMS domain of Tom22 (Court et al., 1996; Moczko et al., 1997; Kanamori et al., 1999) and the N-terminal 50-residue segment of Tim23 (Donzeau et al., 2000) might facilitate the transfer of precursor proteins from the TOM complex to the TIM23 complex. The coupling of translocation across the outer and inner membranes can be assessed by two-step import experiments in vitro. Briefly, when incubated with mitochondria in the absence of $\Delta\Psi$, which is essential for presequence translocation through the TIM23 channel, a precursor protein destined for the matrix stays in the TOM channel. In this intermediate, the presequence reaches the presequence-binding site on the IMS side of the TOM complex (the 'trans site'), which involves Tom40 (Fig. 3A) (Rapaport et al., 1997; Kanamori et al., 1999). This surface-bound intermediate can be chased into the matrix by replenishment of $\Delta\Psi$. Mitochondria containing a mutant

Tom22 that lacks its IMS domain exhibit no defect in the accumulation of the translocation intermediate in the absence of $\Delta\Psi$, but this impairs the passage of the intermediate into the matrix after regeneration of $\Delta\Psi$. Therefore the IMS domain of Tom22 mediates the transfer of the presequence from the TOM complex to the TIM23 complex.

Mitochondria containing mutant Tim23 that lacks the N-terminal 50 residues have defects in protein import, but mitoplasts in which the outer membrane is selectively ruptured do not (Donzeau et al., 2000). This suggests that the N-terminal domain of Tim23 also facilitates the transfer of precursor proteins from the TOM complex to the TIM23 complex. Tethering of the TIM23 complex to the outer membrane by the N-terminal domain of Tim23 may be important for efficient recruitment of the presequence of precursor proteins associated with the TOM channel.

Tim50 also plays a role in the transfer of precursor proteins between the TOM and TIM23 complexes. Although the presequence of the surface-bound translocation intermediates generated in the absence of $\Delta\Psi$ reaches the trans site of the TOM complex, early attempts to detect a direct interaction of this intermediate with a component of the TIM23 complex were unsuccessful. However, Yamamoto et al. (Yamamoto et al., 2002) have been able to crosslink the surface-bound intermediate and Tim50. Therefore, the translocation intermediate lodged in the TOM channel interacts with Tim50 in the TIM23 complex, which suggests that Tim50 links translocation through the TOM channel and that through the TIM23 channel. It is tempting to suggest that the IMS domain of Tim50 is a receptor for the presequence. Regardless, it interacts directly with the IMS domain of Tim23 (Geissler et al., 2002; Yamamoto et al., 2002). Through this interaction, Tim50 stabilizes the form of Tim23 that has the N-terminal segment inserted into the outer membrane, and probably thereby increases the efficiency of the transfer of a precursor protein from the TOM complex to the TIM23 complex (Fig. 3A) (Yamamoto et al., 2002).

From TOM to TIM22

Substrate proteins for TIM22-mediated translocation contain multiple hydrophobic segments to be inserted into the inner membrane. How can such highly hydrophobic proteins cross the aqueous IMS to reach the TIM22 complex?

Import of AAC comprises several distinct steps, and the intermediates at each have been characterized. AAC travels from the cytosol to the inner membrane through a series of Tom and Tim protein complexes: first the TOM complex, then the Tim9-Tim10 complex, the Tim9-Tim10-Tim12 complex and finally the TIM22 complex (Fig. 3B) (Koehler et al., 1998; Sirrenberg et al., 1998; Ryan et al., 1999; Truscott et al., 2002). In the absence of $\Delta\Psi$, AAC accumulates at the point at which it interacts with both Tom40 and Tim10 of the Tim9-Tim10 complex. Replenishment of $\Delta\Psi$ allows the translocation intermediate to interacts with Tim12 of the Tim9-Tim10-Tim12 complex, which is associated with the TIM22 complex. This requires functional Tim10. As the purified Tim9-Tim10 complex binds specifically to the transmembrane segments of AAC, Curran et al. (Curran et al., 2002) have suggested that the Tim9-Tim10 complex, which is partially soluble in the IMS, has a chaperone-like action on unfolded hydrophobic AAC in the aqueous IMS. However, there is no direct evidence for a soluble AAC intermediate chaperoned by Tim9-Tim10. Instead, AAC can be directly pass from the TOM complex to the TIM22 complex with the aid of the Tim9-Tim10 and Tim9-Tim10-Tim12 complexes, minimizing its exposure to the soluble compartment (Endres et al., 1999). As in the case of the TIM23 complex, there is no stable interaction between the TOM complex and the Tim9-Tim10, Tim9-Tim10-Tim12 or the TIM22 complex in the absence of translocating AAC.

Functional separation of the translocators

The driving force for translocation

Translocators are fuelled by different forms of energy to drive unidirectional translocation of polypeptide segments across membranes (Herrmann and Neupert, 2000). The TIM23 complex drives the passage of the N-terminal presequence of a precursor protein across the inner membrane with the aid of $\Delta\Psi$ across the inner membrane, and mHsp70 (Ssc1p) on the matrix side of the inner membrane 'pulls' the rest of the protein across the membrane at the expense of ATP. The TIM22 complex requires $\Delta\Psi$ for facilitating insertion of the transmembrane segments of presequence-less proteins into the inner membrane. Both TIM23 and TIM22 complexes can import precursor proteins directly and independently of the TOM complex in mitoplast preparations (Hwang et al., 1989; Káldi et al., 1998; Yamamoto et al., 2002).

The TOM complex, however, cannot use $\Delta\Psi$ or ATP because there is no $\Delta\Psi$ across the outer membrane and no ATP-dependent chaperone in the IMS. When translocation by the TOM complex is coupled with that by the TIM23 or TIM22 complex, the TOM channel can operate as a passive pore to allow passage of the polypeptide segment, which is 'pulled' by the TIM complex with the aid of $\Delta\Psi$ and/or ATP. However, when translocation is uncoupled from the inner-membrane translocators, the TOM complex has to use different energy sources to ensure vectorial movement of the polypeptide chain to the IMS.

TOM-mediated translocation unplugged

There are several cases in which translocation across the outer membrane is uncoupled from that across the inner membrane. Indeed, under normal circumstances, the TOM complex has to take up precursor proteins destined for the matrix or inner membrane and drives their transmembrane movement until they can engage with the translocator systems in the inner membrane. Reflecting this ability, TOM complexes in outer membrane vesicles or purified and reconstituted into liposomes can transfer the N-terminal presequence of a precursor protein to the trans side of the membrane, although the rest of the polypeptide remains outside the vesicles (Mayer et al., 1995a; Stan et al., 2000). This transfer of the presequence across the membrane is mediated by an array of presequence-binding sites on the TOM complex that are spatially arranged in order of affinity for the presequence (Schatz, 1997; Komiya et al., 1998). Indeed, the TOM complex contains presequence-binding sites on both sides of the membrane, and presequences can spontaneously go through the TOM channel to reach the

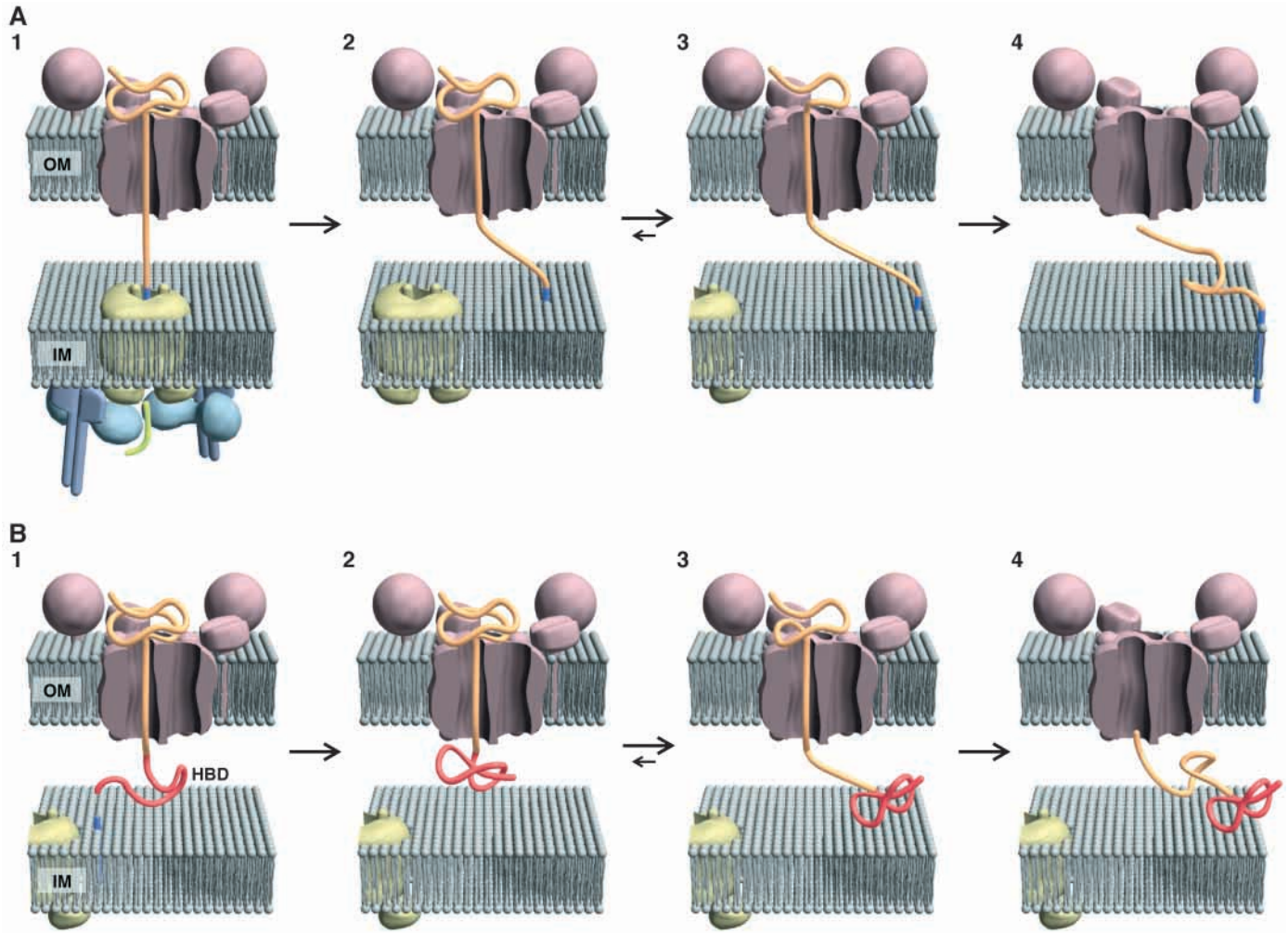

Fig. 4. Translocation across the outer membrane mediated by the TOM complex in the absence of apparent energy. (A) The anchor diffusion mechanism. (B) The folding-driven Brownian ratchet mechanism. OM, the outer membrane; IM, the inner membrane.

trans site on the IMS side (Rapaport et al., 1997; Rapaport et al., 1998; Kanamori et al., 1999).

Does the TOM complex possess a trans site for presequence-less inner membrane proteins? AAC goes through the TOM channel not as a linear chain but in a loop conformation (Endres et al., 1999; Wiedemann et al., 2001). The AAC segments exposed to the IMS can bind to the Tim9-Tim10 complex in the IMS at the exit of the TOM channel, which suggests that small Tim proteins provide the high-affinity trans binding site for presequence-less proteins (Endres et al., 1999; Curran et al., 2002). In both presequence-containing and presequence-less proteins, the precursor proteins should be removed from the high-affinity binding site at the end of the sequential binding array by an exergonic reaction. The bound segments are indeed cleared from the trans site of the TOM complex or small Tim proteins by the TIM23 or TIM22 complex, respectively, and this requires $\Delta\Psi$.

The TOM complex has at least two other mechanisms to drive translocation of proteins across the outer membrane. These mechanisms are exemplified by the proteins targeted to the inner membrane or IMS, including cytochrome b_2, cytochrome c_1 and cytochrome c. Precursors of cytochrome b_2 and cytochrome c_1 have IMS sorting signals near the N termini (Fig. 1) and follow the stop-transfer pathway. This process consists of two steps, the first of which requires $\Delta\Psi$ and an ATP-dependent chaperone, mHsp70, but the second of which is independent of $\Delta\Psi$ and mHsp70 (Glick et al., 2993; Gärtner et al., 1995). The TOM complex drives the second step to transfer polypeptide domains downstream of the sorting signals to the IMS without the aid of the TIM systems. In the ‘anchor diffusion’ mechanism (Glick et al., 1991; Glick et al., 1993; Esaki et al., 1999) (Fig. 4A), the N-terminal segment in the presequence that precedes the sorting signal of a precursor protein crosses both the outer and inner membranes with the aid of $\Delta\Psi$ and an ATP-dependent chaperone, mHsp70 in the matrix (the first step). However, when its sorting signal contacts the TIM23 complex, translocation through the TIM23 channel is arrested and the hydrophobic part of the sorting signal is anchored to the inner membrane. The rest of the polypeptide, which is no longer exposed to the matrix, becomes disconnected from mHsp70. The sorting signal

anchored in the inner membrane then laterally diffuses away from the site of close contact with the TOM complex, thereby pulling the rest of the polypeptide through the TOM channel into the IMS (the second step). Cytochrome b_2 and cytochrome c_1 probably use this mechanism to transfer their mature domains to the IMS when processing of the sorting signals in the presequences is retarded (Wachter et al., 1992; Glick et al., 1993; Arnold et al., 1998; Esaki et al., 1999). The inner membrane proteins, including D-lactate dehydrogenase, that are N-terminally anchored to the inner membrane and face the IMS (Fig. 1) may well use this mechanism to move across the outer membrane as well (Rojo et al., 1998).

A second mechanism involves folding of an N-terminal domain that has already crossed the outer membrane and can function as a trap in the IMS to drive translocation of the C-terminal part of the protein by a Brownian ratchet mechanism (Fig. 4B). In the case of cytochrome b_2 fusion proteins, the mature part of cytochrome b_2 contains a heme-binding domain (HBD) in the N-terminal region, which can fold independently of the rest of the molecule. As the HBD is just downstream of the sorting signal in the presequence, it moves across the outer membrane with the aid of coupled translocation of the presequence through the TIM23 complex and $\Delta\Psi$ and mHsp70 (Glick et al., 1993) (the first step). When the processing of the sorting signal of the presequence takes place faster than translocation of the rest of the molecule across the outer membrane, the anchor diffusion mechanism cannot complete translocation, because the N terminus is free in the IMS. Instead, the tightly folded HBD in the IMS prevents Brownian backsliding, but not forward movement, of the reminder of the molecule in the TOM channel, which results in the forward displacement of the C-terminal part of the protein across the outer membrane (Esaki et al., 1999) (the second step). Cytochrome *c*, a soluble protein in the IMS, also uses folding in the IMS induced by the attachment of heme as a driving force for translocation across the outer membrane (Mayer et al., 1995b). Many small and single-domain proteins, including small Tim proteins in the IMS, may also employ this mechanism to cross the outer membrane without engagement with the TIM23 or TIM22 complex, because they often bind to co-factors or metals in the IMS, which promote tight folding of the proteins (Segui-Real et al., 1993; Steiner et al., 1995; Sirrenberg et al., 1998).

Conclusions and perspective

In spite of the rapid progress in our understanding of the mechanisms of protein import into mitochondria, many questions still remain to be answered. For example, general import pathways have been revealed on the basis of the detailed analyses of the import of various proteins that depend on one or two of the translocators, the TOM, TIM23 and TIM22 complexes. However, more specific pathways have also been unraveled for several mitochondrial proteins, including those synthesized inside mitochondria (Stuart and Neupert, 1996). A proteome-wide approach should be applied to analyses of the intramitochondrial sorting pathways to reveal the entire picture of the import flux of mitochondrial proteins. Like chloroplast thylakoid membranes, which consist of grana and stroma thylakoids, the mitochondrial inner membrane is not homogeneous, but instead consists of several distinct regions, such as the inner boundary membrane, contact sites, and cristae membrane (Reichert and Neupert, 2002). However, the molecular mechanism for the delivery of specific proteins to each of these distinct membrane regions is not known. In contrast to the retrograde polypeptide translocation in the endoplasmic reticulum (ER) membrane, which uses the same Sec61p translocator complex for forward translocation (Brodsky and McCracken, 1997), mitochondria contain an export system for IMS proteins, including cytochrome *c*, that is distinct from the TOM complex and operates when cells need to trigger apoptosis (Vander Heiden and Thompson, 1999). The molecular mechanisms, as well as the components involved in this process are elusive at the moment. These issues need to be tackled in the next decade, and the answers will certainly surprise and excite us.

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