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Transport of the ADP/ATP carrier of mitochondria from the TOM complex to the TIM22·54 complex

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Members of the mitochondrial carrier family such as the ADP/ATP carrier (AAC) are composed of three structurally related modules. Here we show that each of the modules contains a mitochondrial import signal recognized by Tim10 and Tim12 in the intermembrane space. The first and the second module are translocated across the outer membrane independently of the membrane potential, Δψ, but they are not inserted into the inner membrane. The third module interacts tightly with the TOM complex and thereby prevents complete translocation of the precursor across the outer membrane. At this stage, binding of a TIM9·10 complex confers a topology to the translocation intermediate which reflects the modular structure of the AAC. The precursor is then transferred to the TIM9·10·12 complex, still interacting with the TOM complex. Release of the precursor from the TOM complex and insertion into the inner membrane by the TIM22·54 complex requires a Δψ-responsive signal in the third module.

Keywords: ADP/ATP carrier/mitochondria/TOM complex/TIM22·54 complex

Introduction

Nuclear-encoded mitochondrial precursor proteins are imported and sorted to mitochondrial subcompartments by preprotein translocases which are located in the outer and inner membranes of the organelle. The preprotein translocase of the outer membrane, the TOM complex, is composed of preprotein receptor molecules, which are exposed on the surface of the outer membrane, and a protein-conducting channel across the outer membrane which is used by all preproteins (Ryan and Jensen, 1995; Schatz, 1996; Neupert, 1997; Pfanner and Meijer, 1997). The inner membrane contains two preprotein translocases, the TIM17·23 complex and the TIM22·54 complex (Emtage and Jensen, 1993; Ryan *et al*., 1994; Sirrenberg *et al*., 1996, 1998; Kerscher *et al*., 1997; Koehler *et al*., 1998a,b). Both TIM complexes co-operate with the TOM complex in the outer membrane but they differ in their specificity for preproteins. Precursors which contain a positively charged N-terminal matrix targeting signal are imported by the TIM17·23 complex (Bauer *et al*., 1996; Sirrenberg *et al*., 1996). The import of such precursors into the matrix requires the membrane potential Δψ across the inner membrane and ATP in the matrix (Ryan and Jensen, 1995; Schatz, 1996; Neupert, 1997; Pfanner and Meijer, 1997). The Δψ is required for translocation of the presequence across a channel that contains Tim17 and Tim23 (Bauer *et al*., 1996). The peripheral membrane protein Tim44 is associated with the outlet of this channel (Schneider *et al*., 1994; Horst *et al*., 1995, 1997). It recruits the mitochondrial chaperone mt-Hsp70 to the sites of protein import, which drives further translocation with ATP as an energy source (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994, 1996; Azem *et al*., 1997; Bomer *et al*., 1998).

Many integral inner membrane proteins, such as the ADP/ATP carrier (AAC) and other members of the mitochondrial carrier family, are synthesized without a classical N-terminal matrix targeting signal (Smagula and Douglas, 1988; Kübrich *et al*., 1998; Nelson *et al*., 1998; Palmisano *et al*., 1998). Such precursors are imported via the TIM22·54 complex (Sirrenberg *et al*., 1996, 1998; Kerscher *et al*., 1997; Koehler *et al*., 1998a,b). Import of the AAC has been dissected into distinct stages (Pfanner and Neupert, 1987; Wachter *et al*., 1992; Sirrenberg *et al*., 1996; Pfanner *et al*., 1997; Kübrich *et al*., 1998; Palmisano *et al*., 1998). The cytosolic precursor of the AAC (stage I) is recognized by specialized receptors of the TOM complex (stage II) (Pfanner and Neupert, 1987; Dietmeier *et al*., 1997). It is partially translocated across the TOM channel and interacts with the TIM9·10 complex in the intermembrane space (stage IIIa) (Koehler *et al*., 1998a,b; Sirrenberg *et al*., 1998; Adam *et al*., 1999). The precursor is then transferred to the TIM9·10·12 complex (stage IIIb) which is associated with Tim22 at the outer face of the inner membrane (Sirrenberg *et al*., 1996, 1998; Koehler *et al*., 1998a,b; Adam *et al*., 1999). In the presence of a membrane potential, Δψ, Tim22 mediates the insertion of the AAC precursor into the inner membrane (stage IV) (Sirrenberg *et al*., 1996). When Δψ is dissipated, the AAC precursor accumulates as an intermediate (stage III) that is partially translocated across the outer membrane, but not yet inserted into the inner membrane (Pfanner and Neupert, 1987; Kübrich *et al*., 1998). This reaction requires Tim10 but not Tim12 or Tim22 (Koehler *et al*., 1998a; Sirrenberg *et al*., 1998).

Members of the mitochondrial carrier family are composed of three structurally related modules, each consisting of a pair of membrane-spanning helices connected by a hydrophilic loop segment of ~50 amino acid residues which, in the assembled protein, is exposed into the matrix (Saraste and Walker, 1982; Palmieri, 1994; Nelson *et al*., 1998). Carriers are synthesized without a cleavable presequence and contain internal signals for mitochondrial targeting and import (Pfanner and Neupert, 1987; Pfanner *et al*., 1987; Smagula and Douglas, 1988; Palmisano *et al*., 1998).

To identify mitochondrial import signals in carrier proteins, we constructed truncated precursors of the AAC, consisting of single modules and C-terminal deletions lacking one or two transmembrane segments. Precursors consisting of the first and the second module are translocated across the outer membrane in a $\Delta\psi$-independent manner, but not inserted into the inner membrane. The third module contains a signal for $\Delta\psi$-dependent import and insertion into the inner membrane. In the absence of a $\Delta\psi$, the third module accumulates like full-size AAC as a translocation intermediate, stage III, which is partially translocated across the outer membrane. When AAC was accumulated at stage III, each of the three modules was in contact with Tim10 in the intermembrane space, while distinct regions of the precursor were exposed on the surface of the mitochondria. Stage III intermediates were also in contact with Tim12 at the outer face of the inner membrane. Our data suggest that stage III intermediates of the AAC accumulate at contact sites between TOM and TIM; they are structured and appear to span the TOM complex several times. Import of AAC occurs at contact sites and does not involve soluble intermediates in the intermembrane space.

Results

Three internal mitochondrial targeting signals in the ADP/ATP carrier

To identify mitochondrial import signals of carrier proteins, we constructed truncated versions of the AAC of *Neurospora crassa*. TM1-L5, a C-terminally truncated AAC precursor, consists of amino acid residues 1–277 of the AAC but lacks the sixth transmembrane segment (Figure 1A). Furthermore, we constructed TM1-2 which corresponds to the first module (residues 1–105) of the AAC and contains the first and the second membrane-spanning segment, TM3-4 which corresponds to the second module (residues 106–209) containing the third and fourth transmembrane segment, and TM1-4 a fusion of TM1-2 with TM3-4 (Materials and methods). In addition, we constructed TM5-6 which corresponds to the third module (residues 210–313) and contains the fifth and sixth transmembrane segment.

These precursors were synthesized in reticulocyte lysate containing [^{35}S]methionine. The radiolabelled precursors were then incubated with energized yeast mitochondria (Figure 1B, left panels) or with mitochondria whose membrane potential, $\Delta\psi$, had been dissipated with valinomycin (Figure 1B, right panels). Translocation of precursors across the outer membrane was measured by their resistance to degradation by externally added proteinase K (PK). To assess insertion of the precursors into the inner membrane, mitoplasts were generated and treated with PK. In the presence of $\Delta\psi$, full-size AAC was resistant to treatment of mitochondria with PK, indicating that the precursor was translocated across the outer membrane (Figure 1B). When mitoplasts were generated and treated with PK, AAC was clipped and a protease-resistant fragment was generated, which is indicative of its insertion into the inner membrane, stage IV along the import pathway (Pfanner and Neupert, 1987; Sirrenberg *et al*., 1996; Kübrich *et al*., 1998). In the absence of $\Delta\psi$, the AAC precursor was partially resistant to treatment of

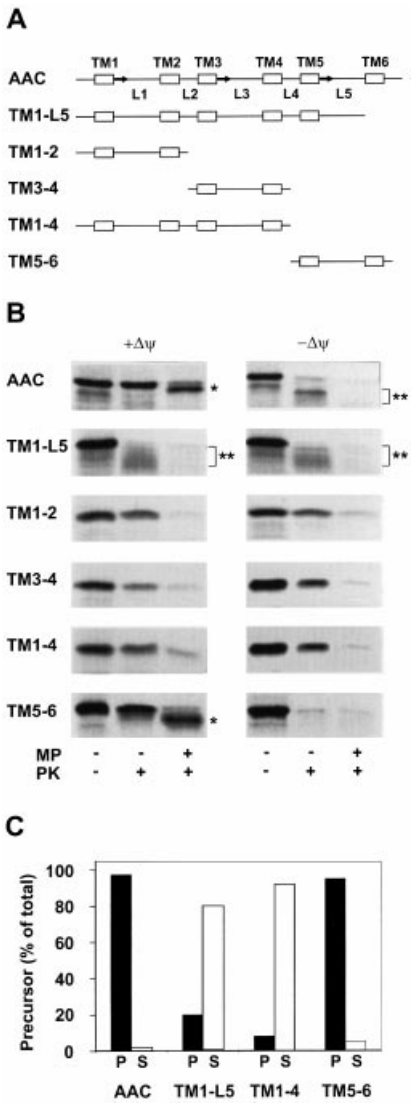

Fig. 1. Internal import signals in the three modules of the AAC precursors. (**A**) Structure of AAC and derived precursors. Precursors are outlined schematically. Transmembrane segments (TM) and connecting loop regions (L) are indicated. The conserved carrier signatures are indicated by arrows. (**B**) Import into mitochondria and membrane insertion of precursors. Radiolabelled precursors were imported into isolated yeast mitochondria in the presence (left panels) and absence (right panels) of a membrane potential, $\Delta\psi$. Samples were diluted 10-fold in 20 mM HEPES–KOH, pH 7.2, 0.6 M sorbitol and treated with PK (30 μg/ml) to measure translocation of the precursors across the outer membrane, or samples were diluted 10-fold in 20 mM HEPES–KOH, pH 7.2 to generate mitoplasts (MP) and treated with PK to determine insertion into the inner membrane. A concentration of 200 μg/ml was used to measure import and insertion of AAC and TM5-6 in the presence of $\Delta\psi$. AAC-specific fragments generated by PK treatment of mitochondria (**) and mitoplasts (*) are indicated. (**C**) Carbonate extraction of imported precursors. Radiolabelled precursors were imported into energized wild-type mitochondria and treated with PK (200 μg/ml) to remove non-imported material (TM1-4 and TM1-L5) or mitochondria were subjected to hypotonic swelling and treated with PK (200 μg/ml) to remove non-inserted precursors (AAC and TM5-6). The samples were then extracted with 100 mM Na_2CO_3, pH 11 and separated into pellet and supernatant. Samples were analysed by SDS–PAGE and autoradiography, and quantified with a phosphorimaging system. The signal in supernatant (S) plus the signal in the membrane pellet (P) was set equal to 100%.

mitochondria with PK, resulting in the generation of fragments which are ~4–6 kDa smaller than the full-size precursor. When the mitochondria were converted to mitoplasts, the AAC precursor was degraded by the protease. This indicates that the precursor accumulated at stage III, a distinct intermediate which is partially translocated across the outer membrane (Pfanner and Neupert, 1987; Sirrenberg *et al*., 1996; Koehler *et al*., 1998a; Kübrich *et al*., 1998).

TM1-L5 which lacks transmembrane segment 6, was imported into the intermembrane space in the presence and absence of Δψ (Figure 1B). When mitochondria were treated with PK, TM1-L5 was clipped and fragments were generated which were of similar size to those obtained with full-size AAC precursor. When mitoplasts were treated with PK, TM1-L5 was degraded, indicating that it was not inserted into the inner membrane.

TM1-2 and TM3-4 were both imported in the presence and absence of Δψ but not inserted into the inner membrane (Figure 1B). The same was found for TM1-4 (Figure 1B). Thus, TM1-2 and TM3-4 contain internal targeting signals that mediate translocation across the outer membrane in a Δψ-independent fashion but contain no signals for Δψ-dependent insertion into the inner membrane.

TM5-6, after import in the presence of Δψ, was protected from degradation by added PK (Figure 1B). When mitoplasts were generated and treated with PK, a protease-resistant fragment was generated, indicating that TM5-6 was inserted into the inner membrane. In the absence of Δψ, TM5-6 associated with the mitochondria. However, it was degraded by added PK, indicating that it was not fully translocated across the outer membrane. Thus, TM5-6 contains mitochondrial targeting information that mediates import and insertion into the inner membrane in a strictly Δψ-dependent manner.

AAC-derived precursors were imported into energized mitochondria, and membrane insertion was measured by extraction of the mitochondrial membranes with alkaline carbonate (Figure 1C). Full-size AAC was inserted into the inner membrane and not extractable by carbonate. TM1-L5 and T1-4 were extracted readily into the supernatant fraction, indicating that the precursors were not inserted into the inner membrane. In contrast, TM5-6 became inserted into the inner membrane as it was recovered in the pellet fraction.

In summary, each of the three structurally related modules of the AAC contains an internal signal for mitochondrial targeting and, accordingly, the full-size AAC precursor contains three regions with independent internal targeting information. The targeting signals in the first and second module direct translocation of the precursor across the outer membrane in a Δψ-independent fashion. The third module, TM5-6, contains a signal for Δψ-dependent import and insertion into the inner membrane. In the absence of Δψ, the precursor accumulates in a membrane-spanning fashion presumably in association with the TOM complex. The TM5-L5 region is required for interaction of AAC with the TOM complex in the absence of Δψ (stage III), while insertion into the inner membrane in the presence of Δψ requires the entire third module.

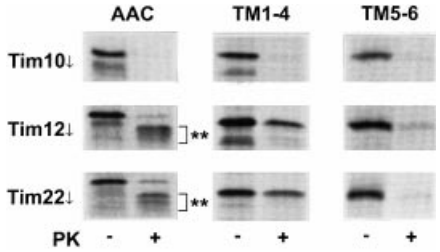

Fig. 2. Interaction of AAC-derived precursors with components of the Tim22-dependent import pathway. Radiolabelled precursors, AAC, TM1-4 and TM5-6, were imported into energized mitochondria (10 min, 25°C) which were depleted of Tim22 (Tim22↓), Tim12 (Tim12↓) and Tim10 (Tim10↓). The samples were split into two; one half was kept on ice (–PK) and the other half was treated with 30 μg/ml PK (+PK). Samples were subjected to SDS–PAGE and analysed by autoradiography. **: stage III–specific fragments.

Interaction of Tim components with stage III translocation intermediates

Translocation of AAC across the outer membrane and its subsequent insertion into the inner membrane requires the successive action of the TIM9·10 complex, the TIM9·10·12 complex and the Tim22·54 complex (Sirrenberg *et al*., 1996, 1998; Kerscher *et al*., 1997; Koehler *et al*., 1998a,b; Adam *et al*., 1999). To determine whether these components interact with AAC-derived precursors, we used yeast cells which express the *TIM* genes under control of the *gal* promoter. These cells were shifted to galactose-free medium and mitochondria were prepared which contained reduced amounts of the respective Tim protein (↓) (Sirrenberg *et al*., 1996, 1998). AAC precursor was incubated with Tim10↓ mitochondria in the presence of Δψ and then treated with PK. The precursor was degraded, demonstrating its presence on the surface of the mitochondria, i.e it did not reach stage III (Figure 2). In Tim12↓ mitochondria and in Tim22↓ mitochondria, the AAC precursor reached stage III but was not inserted into the inner membrane. TM1-4 was not translocated across the outer membrane in Tim10↓ mitochondria but was imported into the intermembrane space in Tim22↓ mitochondria and in Tim12↓ mitochondria. Thus, Tim10, but not Tim12 or Tim22, is required for stage III import of AAC and for the import of TM1-4 into the intermembrane space. When TM5-6 was imported into energized mitochondria, it was accessible to added PK in intact Tim10↓ mitochondria, in Tim12↓ mitochondria and in Tim22↓ mitochondria (Figure 2). Apparently, Tim10, Tim12 and Tim22, i.e. a functional Tim22·54 complex, are required for the Δψ-dependent release of TM5-6 from the outer membrane and its insertion into the inner membrane.

We used chemical cross-linking to analyse the interaction of TIM components with translocation intermediates of AAC and AAC-derived precursors. Radiolabelled AAC precursor was accumulated at stage III by import into mitochondria in the absence of Δψ. Amongst a variety of different cross-linking reagents, we found that *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) efficiently detected interactions between stage III intermediates of AAC and Tim10 (Sirrenberg *et al*., 1998). The major Tim10-specific cross-link with AAC was 42 kDa, which corresponds to an adduct of AAC with one Tim10 molecule (Figure 3A). A second Tim10-containing adduct was 52 kDa and may represent a cross-link of AAC with two

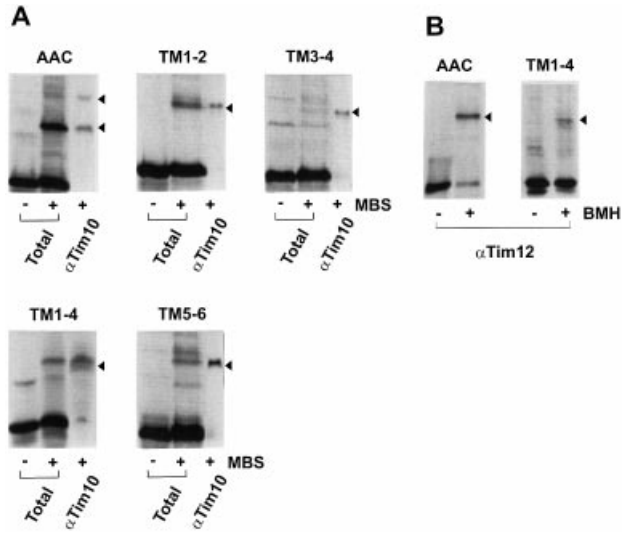

Fig. 3. Stage 3 translocation intermediates in contact with Tim10 and Tim12. (**A**) Radiolabelled precursors were imported into de-energized mitochondria (20 min, 25°C) and then cross-linked with 1 mM MBS on ice (Total). Mitochondria were lysed and subjected to immunoprecipitation with affinity-purified antibodies against Tim10 (α-Tim10). Samples were analysed by SDS–PAGE and autoradiography. Tim10-specific adducts with the radiolabelled precursors are indicated by arrowheads. (**B**) Interaction of Tim12 with stage 3 intermediates. AAC and TM1-4 were imported into de-energized mitochondria (20 min, 25°C) in the presence or absence of 1 mM BMH. Immunoprecipitation with affinity-purified Tim12 antibodies was performed as described above. Tim12-specific adducts are indicated by arrowheads.

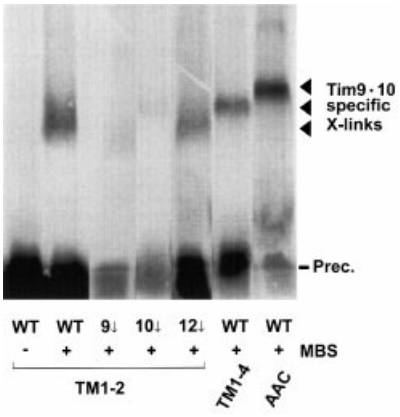

Fig. 4. Interaction of precursors with the TIM9·10. Precursors were imported into de-energized mitochondria. Mitochondria were treated with 50 μg/ml trypsin to remove surface-bound precursors, re-isolated and incubated with 1 mM MBS (20 min, 4°C). Following re-isolation, the mitochondria were lysed with digitonin and analysed by blue native electrophoresis (Schägger and Jagow, 1991) and autoradiography. Arrowheads indicate Tim10-specific adducts with the radiolabelled precursors.

Tim10 molecules. TM1-2 accumulated in mitochondria under the same conditions was cross-linked efficiently to Tim10, yielding an adduct of 22 kDa (Figure 3A). TM3-4, TM1-4 and TM5-6 were cross-linked with Tim10 in a similar manner (Figure 3A). Thus, at stage III, each of the three modules of the AAC are exposed to the intermembrane space and interact with Tim10.

Tim12 is ~10-fold less abundant than Tim10 (Adam *et al*., 1999). Interaction of Tim12 with stage III intermediates of AAC and AAC-derived precursors was detected by cross-linking with 1,6-bismaleimidohexane (BMH) and subsequent immunoprecipitation of the radiolabelled adduct with antibodies against Tim12 (Figure 3B). When AAC was incubated with mitochondria in the absence of $\Delta\psi$ and cross-linking was performed, a 44 kDa adduct was precipitated with antibodies against Tim12, indicating that the stage III intermediate reached Tim12 at the outer face of the inner membrane. TM1-4 was also cross-linked to Tim12, while a cross-link of TM5-6 with Tim12 was not detected (data not shown). Thus, accumulated intermediates of the AAC and TM1-4 interact with Tim12 on the outer face of the inner membrane, while stage III intermediates of TM5-6 may not. Cross-linking of the various precursors with Tim9 was very inefficient using several cross-linkers (not shown).

To characterize translocation intermediates further, we used blue native gel electrophoresis (Schägger and Jagow, 1991). TM1-2, TM1-4 and full-size AAC precursor were imported into mitochondria whose membrane potential previously was dissipated. The mitochondria were treated with a low concentration of trypsin to remove surface-bound precursors. The precursors were then cross-linked with MBS. Under these conditions, the precursors were cross-linked specifically to Tim10, as shown by immunoprecipitation (see Figure 3A). Subsequently, the mitochondria were lysed and subjected to blue native electrophoresis (Figure 4). Without cross-linking, TM1-2 migrated in a low molecular weight form. The TM1-2·Tim10 adduct obtained by cross-linking with MBS migrated with an apparent native mol. wt of ~75 kDa. The 75 kDa species was reduced significantly in Tim9↓ and Tim10↓ mitochondria but not in Tim12↓ mitochondria, which contain approximately normal levels of the TIM9·10 complex. This indicates that TM1-2 was cross-linked preferentially to a Tim10 molecule which was part of the ~70 kDa TIM9·10 complex (Koehler *et al*., 1998a,b; Sirrenberg *et al*., 1998; Adam *et al*., 1999). The native Tim10-specific adducts with TM1-4 migrated as 85 kDa species. Tim10-specific adducts with full-size AAC were formed efficiently and migrated in a 95 kDa complex. These findings suggest that one AAC precursor interacts with one TIM9·10 complex which contains three molecules of Tim10 and three molecules of Tim9 (Koehler *et al*., 1998b; Adam *et al*., 1999). This interaction does not lead to a dissociation of the hexameric TIM9·10 complex. As Tim9 is abundant and occurs in a complex with Tim10 and in a complex with Tim12 (Koehler *et al*., 1998b; Adam *et al*., 1999), the lack of efficient cross-linking to Tim9 may indicate that it does not interact directly with the AAC precursor.

Accumulation of AAC at translocation contact sites

To characterize further the intermediates in contact with Tim10, radiolabelled AAC-derived precursors were accumulated at stage III and cross-linked using MBS (Figure 5A). When mitochondria were then treated with low concentrations of PK, the Tim10-specific adduct with full-size AAC was clipped and smaller fragments were generated, indicating that the translocation intermediate in contact with Tim10 was spanning the outer membrane. In contrast, the cross-linked adduct of TM1-4 with Tim10

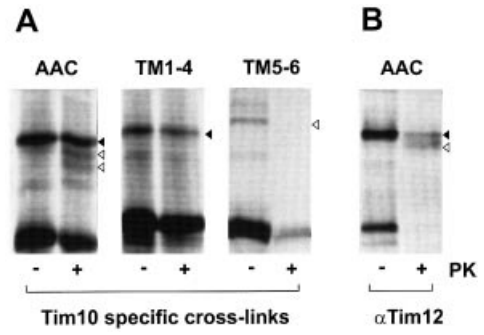

Fig. 5. Stage 3 translocation intermediates spanning the outer membrane. (**A**) Precursors were accumulated at stage 3 (20 min, 25°C, –Δψ). The mitochondria were re-isolated and incubated with 1 mM MBS on ice. Aliquots were kept on ice (–PK) or treated with 50 μg/ml PK (+PK). Total cross-links were analysed by SDS–PAGE and autoradiography. Tim10-specific adducts and proteolytic breakdown products are indicated by arrowheads. (**B**) Precursors were incubated with de-energized mitochondria in the presence of 1 mM BMH (20 min, 25°C, –Δψ). Where indicated, samples were treated with 200 μg/ml PK (+PK). Mitochondria were lysed and subjected to immunoprecipitation with Tim12 antibodies. Tim12-specific adducts with the radiolabelled AAC precursor are indicated by arrowheads.

was not degraded by externally added PK, confirming the earlier conclusion that it was completely translocated across the outer membrane. The adduct of TM5-6 with Tim10 was accessible to PK. Thus, TM5-6 in contact with Tim10 in the intermembrane space accumulated in a membrane-spanning fashion in the absence of Δψ.

Full-size AAC was arrested at stage III and cross-linking with BMH was performed. The resulting 44 kDa radiolabelled adduct of AAC with Tim12 was accessible to externally added PK, indicating that stage III intermediates of AAC accumulated at translocation contact sites (Figure 5B).

Topology of translocation intermediates of AAC

Stage III intermediates of AAC were defined previously by their partial resistance to PK (Pfanner and Neupert, 1987; Sirrenberg *et al*., 1996; Koehler *et al*., 1998a; Kübrich *et al*., 1998). At stage III, the accumulated precursor was resistant to low concentrations of PK; at high concentrations of PK, the precursor was clipped to a series of fragments 4–6 kDa smaller than full-size AAC.

To characterize the topology of stage III intermediates, radiolabelled AAC precursor was incubated with mitochondria whose membrane potential had been dissipated. The mitochondria were then treated with a number of proteases that differ in their substrate specificity: PK which cleaves preferentially after non-polar amino acid residues, trypsin, cleaving after basic amino acid residues, and chymotrypsin, cleaving preferentially after aromatic amino acid residues. Despite differences in their substrate specificity, the three proteases generated remarkably similar patterns of fragments, indicating that only limited regions of the precursor were accessible to proteolysis when accumulated at stage III (Figure 6A, left panel). Either protease generated multiple fragments in the size range of 27 kDa (III_1), 18 kDa (III_2) and 9 kDa (III_3).

For comparison, carrier was imported into energized mitochondria. Then mitoplasts were generated and treated with PK, trypsin and chymotrypsin. The fragments produced from the inserted AAC (Figure 6A, right panel)

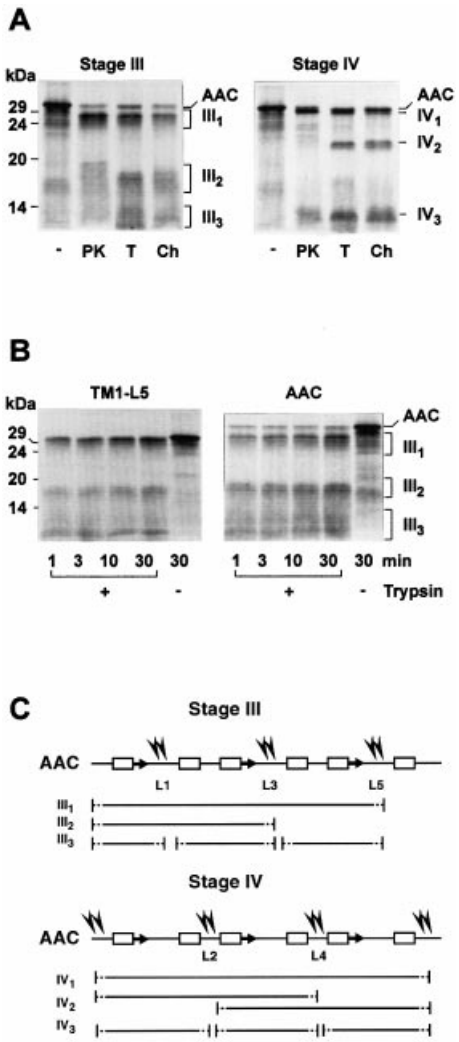

Fig. 6. Topology of stage III intermediates. (**A**) AAC was imported for 10 min into mitochondria in the absence or presence (stage IV) of Δψ. The samples were split into four aliquots. Stage III: import into de-energized mitochondria (–Δψ) was followed by incubation on ice without added protease (–), with 200 μg/ml PK (PK), 100 μg/ml trypsin (T) and 100 μg/ml chymotrypsin (Ch). Stage IV: following import of AAC in the presence of Δψ, mitochondria were converted to mitoplasts (MP) by incubation on ice in 20 mM HEPES–KOH, pH 7.2 in the absence or presence of proteases. Stage III-specific fragments III_1, III_2 and III_3 and stage IV-specific fragments IV_1, IV_2 and IV_3 are indicated. (**B**) TM1-L5 (left) and AAC (right) were incubated with de-energized mitochondria for the indicated times. Samples were then treated with 100 μg/ml trypsin on ice. (**C**) Stage III- and stage IV-specific proteolysis of AAC. The AAC precursor is outlined schematically and trypsin cleavage sites are indicated by double arrowheads. Putative proteolytic fragments specific to stage III and stage IV are shown. The dotted regions indicate heterogeneity of trypsin cleavage.

differed significantly from those obtained from stage III intermediates. Each protease generated fragments of ~30 kDa (IV_1), 22 kDa (IV_2) and 12 kDa (IV_3). This pattern is compatible with cleavage of the inserted carrier at sites exposed to the intermembrane space, i.e. at the N- and C-termini and in the loop segments L2 and L4 (Figure 6C). When import of the precursor of the phosphate carrier was analysed, similar differences in fragmentation patterns between stage III and stage IV were observed (not shown).

Next, TM1-L5 and AAC were allowed to accumulate

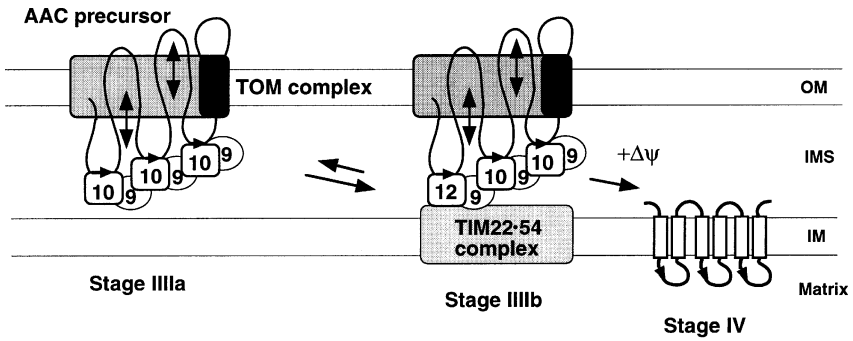

Fig. 7. Model for the transfer of the AAC precursor across the intermembrane space at contact sites. Translocation of the AAC precursor across the TOM complex is facilitated by the TIM9·10 complex in the intermembrane space which binds to conserved carrier signature motifs (small arrow heads). The third module of the AAC precursor interacts tightly with a site in the TOM complex (dark grey area) and prevents complete translocation of the precursor across the outer membrane (stage IIIa). Segments between the carrier signatures reversibly flip through the TOM channel (double-headed arrows) and become exposed on the surface of the mitochondria. The stalled precursor is transferred from the TIM9·10 complex to the TIM9·10·12 complex (stage IIIb) on the outer face of the inner membrane but remains bound to the TOM complex via module 3. This transfer could involve the exchange of components of the TIM9·10 complex and the TIM9·10·12 complex, which is associated with the TIM22·54 complex. In the presence of a membrane potential, Δψ, TIM22·54 triggers the release of the stalled precursor from the TOM complex and mediates its insertion into the inner membrane (stage IV).

at stage III for different times before trypsin was added (Figure 6B). The amount of stage III intermediates increased with time; the ratio of fragments III_1, III_2 and III_3, however, did not change between 1 and 30 min. This suggests that the fragments were generated by proteolysis of intermediates at alternate cleavage sites and not from different sequential intermediates. The same fragments were generated from TM1-L5 and full-size AAC, suggesting that they may represent N-terminal fragments. As the stage III-specific fragments were ~5 kDa smaller than the corresponding stage IV-specific fragments (IV_1>III_1>IV_2>III_2>IV_3), fragments III_1 and III_2 are probably generated by cleavage of AAC in the L3 and L5 regions (Figure 6C). The fragments in the 9 kDa range (III_3) may result from cleavage of AAC in L1, L2 and L3. Fragments III_1, III_2 and III_3 were not kinetic intermediates of proteolysis as they were generated by low (10 μg/ml) and high (100 μg/ml) concentrations of trypsin (not shown). No such fragments were generated when carrier was treated with trypsin in the absence of mitochondria or when it was incubated with Tim10↓ mitochondria and treated with protease (not shown). This demonstrates that the fragments were stage III specific. Apparently, stage III intermediates of the AAC are not stalled randomly in the TOM complex but rather adopt a topology which reflects the modular structure of carriers.

Discussion

Members of the mitochondrial carrier family, such as the AAC, are synthesized without an N-terminal presequence. They are composed of three related modules, each consisting of a pair of membrane-spanning segments connected by a loop which is exposed to the matrix in the assembled protein. Here we show that the AAC precursor contains multiple distinct signals for interaction with the TOM complex, for translocation across the outer membrane and for insertion into the inner membrane.

Each of the three modules of the AAC contains information for mitochondrial targeting. The first and second module contain import signals that direct translocation across the outer membrane independently of the membrane potential Δψ. These targeting signals, however, are not sufficient to drive the insertion of AAC-derived precursors into the inner membrane. The third module of the AAC contains signals which direct import across the outer membrane and insertion into the inner membrane in a strictly Δψ-dependent manner. Thereby, this module not only mediates its own insertion but, in addition, drives membrane insertion of modules 1 and 2, which by themselves cannot insert. In the absence of Δψ, translocation of module 3 stalls and it becomes arrested in the TOM complex. In full-size AAC, the interaction of the third module with the TOM complex prevents complete translocation of the precursor across the outer membrane and leads to the accumulation of the stage III intermediate which spans the TOM complex. TM1-L5, lacking the second transmembrane domain of module 3, accumulated in the TOM complex like full-size AAC, while TM1-4, consisting of the first two modules, was completely translocated across the outer membrane. Thus, a signal for interaction with the TOM complex resides in a segment of 67 amino acid residues (residues 210–277 of AAC) which comprises the C-terminal half of L4 plus TM5 and L5. In contrast to full-size AAC, however, TM1-L5 was neither released from the TOM complex nor inserted into the inner membrane in the presence of Δψ. Accordingly, the Δψ-dependent import and insertion signal appears to be distinct from the TOM-binding site and is comprised of a sequence located C-terminal to L5.

Import of full-size carrier and of single carrier-derived modules required the TIM9·10 complex, and each of the three modules was in close proximity to Tim10 in the intermembrane space during import. This indicates that the carrier contains three related signals that are recognized by Tim10. Full-size AAC was interacting with one TIM9·10 complex; it was in contact with at least two, but presumably all three, Tim10 molecules in this complex. An interaction of the stage III intermediate with the three Tim9 molecules was not observed. At the same time, the loop regions L5, L3 and L1 were accessible from the outside of the mitochondria. AAC at stage III was also in contact with Tim12 which is tightly associated with the outer face of the inner membrane (Jarosch *et al*., 1996,

1997; Koehler *et al*., 1998a; Adam *et al*., 1999). Intermediates in contact with Tim12 were also spanning the TOM complex and, thus, accumulated at translocation contact sites where TOM and TIM are closely apposed. AAC may exhibit similar affinities for Tim10 and Tim12 such that precursors may equilibrate between the TIM9·10 complex (stage IIIa) and the less abundant TIM9·10·12 complex (stage IIIb). As recently suggested, the Zn^{2+} fingers of Tim10 and of Tim12, but not that of Tim9, contain a pattern of charged and hydrophobic amino acid residues which may interact with the carrier signatures (Sirrenberg *et al*., 1998; Adam *et al*., 1999), conserved sequence motifs which are repeated three times in all carrier proteins immediately after transmembrane segments 1, 3 and 5 (Saraste and Walker, 1982; Palmieri, 1994; Nelson *et al*., 1998).

The following working hypothesis of the import pathway for carrier proteins integrates the results from this and previous studies. Initially, the precursor is targeted to receptors on the surface of the mitochondria. The third module of the AAC binds tightly to the TOM complex (Figure 7). When segments of the carrier are translocated across the TOM complex, the carrier signatures in modules 1, 2 and 3 are sequestered by a Tim9·10 complex in the intermembrane space. At this stage (stage IIIa), the interaction of three Tim10 molecules of one TIM9·10 complex with the three carrier signatures in modules 1, 2 and 3 may confer a topology to the translocation intermediate which reflects the structure of the membrane-inserted AAC. As a consequence, the long segments between the signatures may, one at a time, flip back and forth through the pore of the TOM complex (Hill *et al*., 1998; Künkele *et al*., 1998) which is ~20 Å in diameter. In this way, the regions L1, L3 and L5 become accessible to proteases from the outside of the mitochondria. Subsequently, the three segments containing carrier signatures are transferred to the Tim9·10·12 complex at the inner membrane which contains two molecules of Tim10 and one molecule of Tim12 (Adam *et al*., 1999) for interaction with the carrier. The third module of the AAC remains, however, tightly bound to the TOM complex, and segments of the precursor are still exposed on the mitochondrial surface (stage IIIb). Release of the AAC from the TOM complex requires both a $\Delta\psi$ across the inner membrane and recruitment of a functional TIM22·54 complex to the TOM complex. Thus, translocation across the outer membrane and insertion into the inner membrane of the carrier occurs at translocation contact sites and does not involve a soluble intermediate in the intermembrane space. The role of the signal in the third module, according to our hypothesis, is to arrest translocation of the AAC precursor in the TOM complex until a functional TIM22·54 complex is available to take over the precursor directly and mediate its $\Delta\psi$-dependent insertion.

The molecular basis for the interaction of module 3 with the TOM complex and the signalling between TIM and TOM machinery to mediate the $\Delta\psi$-dependent release remains to be studied. Modules 1 and 2 may have similar characteristics to module 3 but differ in their affinity for the TOM complex and/or in their responsiveness to $\Delta\psi$. For successful import and membrane insertion of carriers, it may be sufficient to have one module which interacts with high affinity with the TOM complex and which responds to $\Delta\psi$ when a TIM22·54 complex is recruited. In different carrier proteins, other modules may harbour these features.

The precursor forms of carriers are hydrophobic and shielded by chaperones and factors in the cytosol. As these components are released when a carrier is translocated across the outer membrane, the translocation arrest at stage III may prevent misfolding and aggregation of an unshielded precursor in the intermembrane space. The TOM complex together with the TIM9·10 and TIM9·10·12 complexes in the intermembrane space would substitute for cytosolic chaperones and confer a conformation to the precursors that renders them competent for insertion.

Materials and methods

Construction of AAC-derived precursors

Carrier-derived PCR fragments were amplified with specific oligonucleotide primers from pGEM4-AAC which carried an *Eco*RI–*Hin*dIII fragment encoding the *AAC2* gene of *N.crassa*. All 5′-oligonucleotide primers contained a 5′-CCCCGAATTCGTCGACAAAATG extension and the 3′-oligonucleotides contained a 5′-CCCCAAGCTTAGGATC-CCTCGAG extension. The PCR products were digested with *Eco*RI and *Hin*dIII and cloned into pGEM4. PCR fragments TM1-2, TM3-4, TM5-6 and TM1-L5 encode amino acid residues 1–105, 106–209, 210–313 and 1–277, respectively, of AAC. For the construction of TM1-4, the TM3-4 PCR fragment was digested with *Sal*I and *Hin*dIII and cloned into pGEM4-TM1-2 using the *Xho*I and *Hin*dIII sites. Thereby the amino acid residues L, D, K and M were introduced between residues 105 and 106 of the AAC.

In vitro synthesis of precursor proteins and import into mitochondria

Precursor proteins were synthesized by coupled transcription–translation in reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Adam *et al*., 1999). Mitochondria were isolated as described (Sirrenberg *et al*., 1996). Import reactions were carried out for the indicated times at 25°C in 100 µl of import buffer (600 mM sorbitol, 0.1 mg/ml bovine serum albumin, 80 mM KCl, 10 mM Mg_2Ac, 2.5 mM EDTA, 2 mM KH_2PO_4, 5 mM NADH, 50 mM HEPES–KOH, pH 7.2). An assay contained 25 µg of mitochondria and 3% reticulocyte lysate with the radiolabelled precursor protein. Membrane potential was dissipated by omission of NADH and pre-incubation for 5 min at 25°C with 1 µM valinomycin. Protease treatment of mitochondria and mitoplasts was carried out on ice for 20 min.

Cross-linking

Import reactions were performed in import buffer using 5% reticulocyte lysate. A 50 µg aliquot of mitochondria was used for total cross-links, and 100 µg for cross-linking and subsequent immunoprecipitation.

Immunoprecipitation

MBS cross-linking. Mitochondria were lysed on ice for 15 min in 15 mM Tris pH 7.4, 150 mM NaCl, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Triton X-100, 0.05% SDS. *BMH cross-linking*. Mitochondria were lysed on ice for 15 min in 10 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.5% Triton X-100. After a clarifying spin, the supernatant was subjected to immunoprecipitation with affinity-purified anti-Tim10 or anti-Tim12 IgG (0.5 µg) or pre-immune IgG for 1 h at 4°C.

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References

Adam,A., Endres,M., Sirrenberg,C., Lottspeich,F., Neupert,W. and Brunner,M. (1999) Tim9, a new component of the TIM22·54 translocase in mitochondria. *EMBO J.*, **18**, 313–319.

Azem,A., Oppliger,W., Lustig,A., Jeno,P., Feifel,B., Schatz,G. and Horst,M. (1997) The mitochondrial hsp70 chaperone system. Effect of adenine nucleotides, peptide substrate and mGrpE on the oligomeric state of mhsp70. *J. Biol. Chem.*, **33**, 20901–20906.

Bauer,M.F., Sirrenberg,C., Neupert,W. and Brunner,M. (1996) Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell*, **87**, 33–41.

Bomer,U., Maarse,A.C., Martin,F., Geissler,A., Merlin,A., Schonfisch,B., Meijer,M., Pfanner,N. and Rassow,J. (1998) Separation of structural and dynamic functions of the mitochondrial translocase: Tim44 is crucial for the inner membrane import sites in translocation of tightly folded domains, but not of loosely folded preproteins. *EMBO J.*, **17**, 4226–4237.

Dietmeier,K., Hönlinger,A., Bömer,U., Dekker,P.J.T. Eckerskorn,C., Lottspeich,F., Kübrich,M. and Pfanner,N. (1997) Tom5 functionally links mitochondrial preprotein receptors to the general import pore. *Nature*, **388**, 195–200.

Emtage,J.L.T. and Jensen,R.E. (1993) MAS6 encodes an essential inner membrane component of the yeast mitochondrial import pathway. *J. Cell Biol.*, **122**, 1003–1012.

Hill,K., Model,K., Ryan,M.T., Dietmeier,K., Martin,F., Wagner,R. and Pfanner,N. (1998) Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature*, **395**, 516–521.

Horst,M., Hilfiker-Rothenfluh,S., Oppliger,W. and Schatz,G. (1995) Dynamic interaction of the protein translocation systems in the inner and outer membranes of yeast mitochondria. *EMBO J.*, **14**, 2293–2297.

Horst,M., Oppliger,W., Rospert,S., Schonfeld,H.J., Schatz G. and Azem,A. (1997) Sequential action of two hsp70 complexes during protein import into mitochondria. *EMBO J.*, **16**, 1842–1849.

Jarosch,E., Tuller,G., Daum,G., Waldherr,M., Voskova,A. and Schweyen,R.J. (1996) Mrs5p, an essential protein of the mitochondrial intermembrane space, affects protein import into yeast mitochondria. *J. Biol. Chem.*, **271**, 17219–17225.

Jarosch,E., Rödel,G. and Schweyen,R.J. (1997) A soluble 12-kDa protein of the mitochondrial intermembrane space, Mrs11p, is essential for mitochondrial biogenesis. *Mol. Gen. Genet.*, **255**, 157–165.

Kerscher,O., Holder,J., Srinivasan,M., Leung,R.S. and Jensen,R.E. (1997) The Tim54p–Tim22p complex mediates insertion of proteins into the mitochondrial inner membrane. *J. Cell Biol.*, **139**, 1663–1675.

Koehler,C.M., Jarosch,E., Tokatlidis,K., Schmid,K., Schweyen,R.J. and Schatz,G. (1998a) Import of mitochondrial carrier proteins mediated by essential proteins of the intermembrane space. *Science*, **279**, 369–373.

Koehler,C.M., Merchant,S., Oppliger,W., Schmid,K., Jarosch,E., Dolfini,L., Junne,T., Schatz,G. and Tokatlidis,K. (1998b) Tim9p, an essential partner subunit of Tim10p for the import of mitochondrial carrier proteins. *EMBO J.*, **17**, 6477–6486.

Kronidou,N.G., Oppliger,W., Bolliger,L., Hannavy,K., Glick,B.S., Schatz,G. and Horst,M. (1994) Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of yeast mitochondrial inner membrane. *Proc. Natl Acad. Sci. USA*, **91**, 12818–12822.

Kübrich,M., Rassow,J., Voos,W., Pfanner,N. and Hönlinger,A. (1998) The import route of ADP/ATP carrier into mitochondria separates from the general import pathway of cleavable preproteins at the *trans* site of the outer membrane. *J. Biol. Chem.*, **273**, 16374–16381.

Künkele,K. *et al.* (1998) The preprotein translocation channel of the outer membrane of mitochondria. *Cell*, **93**, 1009–1019.

Nelson,D.R., Cherise,M.F. and Swanson,J.M. (1998) Highly conserved charge-pair networks in the mitochondrial carrier family. *J. Mol. Biol.*, **277**, 285–308.

Neupert,W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.*, **66**, 863–917.

Palmieri,F. (1994) Mitochondrial carrier proteins. *FEBS Lett.*, **346**, 48–54.

Palmisano,A., Zara,V., Honlinger,A., Vozza,A., Dekker,P.J., Pfanner,N. and Palmieri,F. (1998) Targeting and assembly of the oxoglutarate carrier: general principles for biogenesis of carrier proteins of the mitochondrial inner membrane. *Biochem. J.*, **333**, 151–158.

Pfanner,N. and Meijer,M. (1997) The Tom and Tim machine. *Curr. Biol.*, **7**, 100–103.

Pfanner,N. and Neupert,W. (1987) Distinct steps in the import of ADP/ATP carrier into mitochondria. *J. Biol. Chem.*, **262**, 7528–7536.

Pfanner,N., Hoeben,P., Tropschug,M. and Neupert,W. (1987) The carboxyl-terminal two thirds of the ADP/ATP carrier polypeptide contains sufficient information to direct translocation into mitochondria. *J. Biol. Chem.*, **262**, 14851–14854.

Rassow,J., Maarse,A.C., Krainer,E., Kübrich,M., Müller,H., Meijer,M., Craig,E.A. and Pfanner,N. (1994) Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *J. Cell Biol.*, **127**, 1547–1556.

Ryan,K.R. and Jensen,R.E. (1995) Protein translocation across mitochondrial membranes: what a long strange trip it is. *Cell*, **83**, 517–519.

Ryan,K.R., Menold,M.M., Garrett,S. and Jensen,R.E. (1994) SMS1, a high-copy suppressor of the yeast mas6 mutant, encodes an essential inner membrane protein required for mitochondrial protein import. *Mol. Biol. Cell*, **5**, 529–538.

Saraste,M. and Walker,J.E. (1982) Internal sequence repeats and the path of polypeptide in mitochondrial ADP/ATP translocase. *FEBS Lett.*, **144**, 250–254.

Schägger,H. and von Jagow,G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.*, **199**, 223–231.

Schatz,G. (1996) The protein import system of mitochondria. *J. Biol. Chem.*, **271**, 31763–31766.

Schneider,H.-C., Berthold,J., Bauer,M.F., Dietmeier,K., Guiard,B., Brunner,M. and Neupert,W. (1994) Mitochondrial Hsp70/MIM44 complex facilitates import. *Nature*, **371**, 768–774.

Schneider,H.-C., Westermann,B., Neupert,W. and Brunner,M. (1996) The nucleotide exchange factor MGE exerts a key function in the ATP-dependent cycle of mt-Hsp70–Tim44 interaction driving mitochondrial protein import. *EMBO J.*, **15**, 5796–5803.

Sirrenberg,C., Bauer,M.F., Guiard,B., Neupert,W. and Brunner,M. (1996) Import of carrier proteins into the mitochondrial inner membrane mediated by Tim22. *Nature*, **384**, 582–585.

Sirrenberg,C., Endres,M., Fölsch,H., Stuart,R.A., Neupert,W. and Brunner,M. (1998) Carrier protein import into mitochondria mediated by the intermembrane proteins Tim10/Mrs11 and Tim12/Mrs5. *Nature*, **391**, 912–915.

Smagula,C.S. and Douglas,M.G. (1988) Mitochondrial import of the ADP/ATP carrier protein in *Saccharomyces cerevisiae*. Sequences required for receptor binding and membrane translocation. *J. Biol. Chem.*, **263**, 6783–6790.

Wachter,C., Schatz,G. and Glick,B.S. (1992) Role of ATP in the intramitochondrial sorting of cytochrome c_1 and the adenine nucleotide translocator. *EMBO J.*, **11**, 4787–4794.

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