Different import pathways through the mitochondrial intermembrane space for inner membrane proteins

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Earlier work on the protein import system of yeast mitochondria has identified two soluble 70 kDa protein complexes in the intermembrane space. One complex contains the essential proteins Tim9p and Tim10p and mediates transport of cytosolically-made metabolite carrier proteins from the outer to the inner membrane. The other complex contains the non-essential proteins Tim8p and Tim13p as well as loosely associated Tim9p; its function was unclear, but it interacted structurally or functionally with the Tim9p–Tim10p complex. We now show that the two 70 kDa complexes each mediate the import of a different subset of integral inner membrane proteins and that they can transfer these proteins to one of three different membrane insertion sites: the TIM22 complex, the TIM23 complex or an as yet uncharacterized insertion site. Yeast mitochondria thus use multiple pathways for escorting hydrophobic inner membrane proteins across the aqueous intermembrane space.

Keywords: ADP/ATP carrier/polytopic membrane protein/protein import/*Saccharomyces cerevisiae*/TIM complex

Introduction

The majority of mitochondrial proteins are synthesized in the cytosol as precursors with a cleavable N-terminal presequence and are imported into mitochondria by the general protein import pathway. This pathway includes cytosolic chaperones, a hetero-oligomeric translocase of the outer mitochondrial membrane (TOM) complex in the mitochondrial outer membrane, a complex comprised of Tim17p and Tim23p (termed TIM23 complex) in the inner membrane, an ATP-driven import motor associated with the inner face of the TIM23 complex and soluble matrix proteins mediating proteolytic maturation and folding of the imported proteins (Schatz and Dobberstein, 1996; Neupert, 1997; Pfanner *et al.*, 1997).

Work during the past 2 years has revealed that multispanning proteins of the mitochondrial inner membrane are imported by another pathway. Many of these proteins

function as metabolite transporters and are synthesized without a cleavable N-terminal targeting sequence. Studies on the ADP/ATP carrier (AAC) have suggested that the targeting information of this carrier protein is present in discrete regions of the mature protein (Pfanner and Neupert, 1987; Pfanner *et al.*, 1987; Smagula and Douglas, 1988a,b; Davis *et al.*, 1998). Import of mitochondrial metabolite carriers initially follows the general import pathway but diverges from it after the TOM channel. As the carrier's N-terminal region becomes exposed to the soluble intermembrane space, the carrier is pulled across the TOM channel by binding to a soluble 70 kDa complex composed of Tim9p and Tim10p (Koehler *et al.*, 1998a,b; Sirrenberg *et al.*, 1998; Adam *et al.*, 1999). It is then delivered to an inner membrane insertion complex that contains the integral proteins Tim22p and Tim54p, the peripheral membrane protein Tim12p and a small fraction of Tim9p and Tim10p (termed TIM22 complex; Sirrenberg *et al.*, 1996, 1998; Kerscher *et al.*, 1997; Koehler *et al.*, 1998a,b; Adam *et al.*, 1999). Aided by the electric potential across the inner membrane, the TIM22 complex catalyzes the correct insertion of the multispanning carrier into the inner membrane.

Tim9p, Tim10p, Tim12p, Tim22p and Tim54p are all essential for viability of yeast. Recently we have identified two additional yeast proteins, Tim8p and Tim13p, and two human proteins, DDP1 and DDP2 (Deafness Dystonia Polypeptide), which are closely related to Tim9p, Tim10p and Tim12p (Koehler *et al.*, 1999). Loss of DDP1 causes a recessive, X-linked neurodegenerative disorder characterized by deafness, cortical blindness, dystonia, dysphagia and paranoia (Tranebjaerg *et al.*, 1995; Jin *et al.*, 1996). DDP1 and DDP2 are most similar to Tim8p; and DDP1, like Tim8p, is a mitochondrial protein (Koehler *et al.*, 1999).

The function of Tim8p and Tim13p has remained uncertain. Both proteins exist in the intermembrane space as a soluble 70 kDa complex together with a subfraction of Tim9p. Deletion of this complex was not lethal and did not severely affect import of several metabolite carriers into mitochondria, but was synthetically lethal with a conditional mutation in Tim10p (Koehler *et al.*, 1999). This last result suggested that the two 70 kDa Tim complexes in the intermembrane space interact with one another.

The study reported here was prompted by the hypothesis that the Tim8p–Tim9p–Tim13p complex mediates the import of a specific subset of integral inner membrane proteins. To test this hypothesis, we studied the role of the two 70 kDa complexes in the import of several integral inner membrane proteins that do not transport metabolites but perform other functions. We now report that the Tim8p–Tim9p–Tim13p complex functions preferentially in the import of Tim23p, whereas the previously described

Tim9p–Tim10p complex functions in the import of Tim17p, Tim22p, AAC, Coq2p and Tim54p. Tim11p utilizes either complex to cross the intermembrane space. In general, both 70 kDa complexes transfer their cargo to the TIM22 complex. However, some proteins are delivered to the TIM23 complex or to an as yet uncharacterized insertion site. Transport of hydrophobic inner membrane proteins from the cytosol into the inner membrane thus involves multiple pathways across the aqueous intermembrane space.

Results

Tim8p and Tim13p mediate import of Tim23p

Deletion of either Tim8p or Tim13p does not significantly affect import of AAC or the phosphate carrier into isolated mitochondria (Koehler *et al.*, 1999). We decided to re-investigate the role of Tim8p and Tim13p in mitochondrial protein import with a larger spectrum of imported inner membrane proteins, most of them lacking a cleaved targeting signal. To this end, we tested the import of radiolabeled Tim23p, Tim17p, Tim54p, Tim11p and Coq2p into wild-type or mutant mitochondria that either contained temperature-sensitive Tim10p (*tim10-1* mitochondria; Koehler *et al.*, 1998a) or lacked Tim8p (Δ *tim8* mitochondria; Koehler *et al.*, 1999). As deletion of Tim8p also causes loss of Tim13p, Δ *tim8* mitochondria lack the entire Tim8p–Tim9p–Tim13p complex. As controls, we included the import of the metabolite carrier AAC and SU9–DHFR, an artificial precursor targeted to the matrix through the general protein import pathway.

In agreement with our earlier findings, mitochondria lacking the Tim8p–Tim9p–Tim13p complex imported AAC and the phosphate carrier at half the rate of wild-type mitochondria, in contrast to the reduced import of 95% in *tim10-1* mutant mitochondria (Figure 1F; unpublished data). This was also true for the matrix-targeted precursor SU9–DHFR (Figure 1G). Deletion of the Tim8p–Tim9p–Tim13p complex therefore does not inflict a general damage on the mitochondria. However, deletion of the Tim8p–Tim9p–Tim13p complex strongly inhibited import of Tim23p (Figure 1A). In contrast, inactivation of the Tim9p–Tim10p complex retarded import of Tim17p, the partner subunit of Tim23p (Figure 1B), as well as import of Tim54p, a partner subunit of Tim22p (Figure 1C). Both 70 kDa complexes were required for efficient import of Tim11p (Figure 1D; Tokatlidis *et al.*, 1996). Coq2p (polyprenyl diphosphate:4-HB transferase) was used as an additional model protein because it is an integral inner membrane protein with six putative transmembrane regions, yet has an N-terminal targeting presequence (Ashby *et al.*, 1992; Clarke, 1999). Inactivation of the Tim9p–Tim10p complex inhibited import of Coq2p. We checked for insertion of all radiolabeled membrane proteins into the inner membrane by verifying their resistance to alkali extraction. None of the radiolabeled membrane proteins became alkali-resistant if the import was performed with uncoupled mitochondria (unpublished results).

When radiolabeled AAC is imported into isolated uncoupled mitochondria, it accumulates in the intermembrane space in association with the Tim9p–Tim10p complex and can be cross-linked to Tim9p and Tim10p

with a bifunctional cross-linking reagent (Koehler *et al.*, 1998a,b; Sirrenberg *et al.*, 1998). We used this approach to check whether the radiolabeled precursors shown in Figure 1 bind to the Tim8p–Tim9p–Tim13p complex or the Tim9p–Tim10p complex during import. We imported the precursors into uncoupled wild-type mitochondria, treated the mitochondria with the cross-linkers *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester (MBS) or di-succinimidyl suberate (DSS), solubilized the mitochondria with SDS, subjected the extract to immunoprecipitation with antibodies against Tim8p, Tim9p, Tim10p or Tim13p and analyzed the immunoprecipitates by SDS–PAGE and fluorography. With Tim23p as the radiolabeled precursor, antibodies against Tim8p and Tim13p specifically precipitated radiolabeled cross-linked products whose electrophoretic mobilities corresponded to the combined masses of either Tim23p and Tim8p, or Tim23p and Tim13p. Antibodies against Tim9p and Tim10p precipitated little, if any, radiolabeled cross-linked products (Figure 2A). This specificity is remarkable as Tim9p and Tim10p are at least ten-fold more abundant than either Tim8p or Tim13p (unpublished data). The opposite result was obtained with radiolabeled AAC: this precursor formed cross-links with Tim9p and Tim10p, but not with Tim8p or Tim13p. (Figure 2B; unpublished data; Koehler *et al.*, 1998a,b). Using different cross-linkers did not alter this result (unpublished data). The Tim22p precursor was mainly cross-linked to Tim10p (Figure 2C), but also yielded minor cross-links to Tim8p and Tim9p. Finally, the Tim17p precursor was cross-linked to Tim9p and Tim10p, but not to Tim8p or Tim13p, giving a similar result to mitochondrial carrier proteins (Figure 2D). Cross-linking of the Tim54p translocation intermediate mostly yielded radioactive products with molecular masses >100 kDa, none of which contained Tim8p, Tim9p, Tim10p or Tim13p (unpublished data). Cross-linked Coq2p products were immunoprecipitated by antibodies against Tim9p and Tim10p, but barely entered the gel (unpublished data).

To summarize, *in vitro* import assays and cross-linking of translocation intermediates both indicate that the Tim8p–Tim9p–Tim13p complex mediates import of Tim23p, whereas the Tim9p–Tim10p complex mediates import of the mitochondrial carrier proteins Tim17p, Tim22p, Tim54p and Coq2p. Tim11p import requires either complex. Thus, the two soluble 70 kDa complexes mediate the import of different subsets of inner membrane proteins.

Both 70 kDa Tim complexes of the intermembrane space can interact with either the TIM22 complex or the TIM23 complex in the inner membrane

Do the different intermembrane space pathways described in the preceding section all converge at the 300 kDa TIM22 complex in the inner membrane, or can they also deliver precursors to the TIM23 complex of the general import pathway? We approached this question with the aid of yeast mutants containing temperature-sensitive Tim22p or Tim23p. A temperature-sensitive *tim23* mutant had already been isolated (Dekker *et al.*, 1997) and was kindly made available to us. As a temperature-sensitive Tim22p mutant had not been described, we constructed several such mutants by *in vitro* mutagenesis of the cloned

Fig. 1. Tim8p and Tim13p are required for efficient import of Tim23p and Tim11p, but not for that of Tim17p, Tim54p, Coq2p, AAC or Su9-DHFR. (A) Radiolabeled Tim23p was synthesized *in vitro* and incubated for 1, 2 and 4 min at 25°C in the presence or absence of a membrane potential ($\Delta\Psi$) with wild-type mitochondria (WT), *tim10-1* mitochondria (Koehler *et al.*, 1998a) or *Δtim8* mitochondria. Samples were treated with trypsin to remove non-imported precursor, followed by addition of soybean trypsin inhibitor. All samples were extracted with 0.1 M Na_2CO_3 for 30 min on ice, followed by centrifugation at 100 000 g for 15 min and pellets were analyzed by SDS-PAGE and fluorography. Standard (STD) refers to 10% of the radioactive precursor added to each assay. (B) Tim17p was imported as in (A) into WT, *Δtim8* or *tim10-1* mitochondria. (C) Import of Tim54p was as in (B) except that the time points were 10, 20 and 30 min. (D) Tim11p was imported as in (A) and the pellet (P) was examined after alkali extraction. The * denotes a second translation product that probably results from initiation at an internal methionine located after the transmembrane domain. (E) Coq2p was imported as in (A). Each sample was processed further by Na_2CO_3 extraction and the pellet was analyzed. Precursor, p, and mature, m, (i.e. cleaved) forms of protein are shown. (F) The ADP/ATP carrier (AAC) was imported for 1, 2 and 4 min into WT as in (A). Extraction with Na_2CO_3 was performed after import in the presence or absence of a membrane potential. (G) The artificial precursor Su9-DHFR was imported for 0.5, 1 and 2 min into WT or *Δtim8* mitochondria as in (A), except that protease addition was omitted. Translocation of the fusion protein (p) is assessed by the $\Delta\Psi$ -dependent generation of the presequence-free (i.e. mature) form of the fusion protein (m). Import was analyzed by precipitation with trichloroacetic acid, SDS-PAGE and fluorography. Percentage import, fluorographs of import reactions, was quantified by scanning-laser densitometry, and 100% was set as the amount of mature protein that was imported into WT at the longest time point.

TIM22 gene. Haploid yeast cells expressing temperature-sensitive Tim22p grew slightly slower at 25°C than the parental strain, but arrested after 4–6 h at 37°C on fermentable or nonfermentable carbon sources (unpublished data). Several *tim22* mutants had strongly pleiotropic defects, such as low levels of Tim23p that affected the general import pathway into the matrix (unpublished data). After extensive screening we selected the *tim22-4* mutant for further analysis because its pleiotropic defects were less severe than those of the other *tim22* mutants.

When the *tim22-4* mutant was grown at the permissive temperature (25°C), its mitochondria contained two-fold less Tim8p, Tim10p and Tim13p, three-fold less Tim23p and significantly less Tim12p than wild-type mitochondria. Tim22p was undetectable with our antibodies, whereas Tim54p was present at near wild-type levels (Figure 3A; unpublished data). To check whether the *tim22-4* mutation affected the TIM22 complex in the inner membrane (Sirrenberg *et al.*, 1996; Koehler *et al.*, 1998b), we solubilized mitochondria with non-ionic detergent and subjected the extracts to two-dimensional electrophoresis (non-denaturing blue native gel followed by SDS-PAGE; Schägger and von Jagow, 1991; Schägger *et al.*, 1994). The mobilities of Tim10p, Tim12p, Tim22p and Tim54p were determined by immunoblotting (Figure 3B). With

wild-type mitochondria, all of Tim12p, Tim22p and Tim54p co-migrated as a 300 kDa complex and most of Tim10p migrated as a 70 kDa complex in agreement with earlier results (Koehler *et al.*, 1998b). Analysis of the mutant mitochondria failed to reveal any Tim22p, as expected. The residual Tim12p migrated as a 70 kDa complex, whereas Tim54p migrated as a 150 kDa complex (Figure 3B). Thus, inactivation of Tim22p either greatly destabilizes or causes loss of the TIM22 complex.

We tested the import of several inner membrane proteins for its dependence on functional Tim22p. Mutants containing temperature-sensitive Tim22p or temperature-sensitive Tim23p were grown at the permissive temperature. Their mitochondria were isolated, incubated for 30 min at 25 or 37°C in the presence of an energy-regenerating system and tested for their ability to import radiolabeled AAC, Tim23p, Tim22p, Tim17p, Tim54p and Tim11p (Figure 4; Dekker *et al.*, 1997). Import of AAC, Tim22p and Tim23p was inhibited by heat-inactivation of Tim22p, but was essentially unaffected by heat-inactivation of Tim23p (Figure 4A and C). Import of Coq2p and Su9-DHFR was essentially unaffected by heat-inactivation of Tim22p but was inhibited by inactivation of Tim23p (Figure 4A–C). Inactivation of Tim23p also inhibits import of Tim54p (Kurz *et al.*, 1999).

Fig. 2. Tim8p and Tim13p can be cross-linked to a translocation intermediate of Tim23p. (A) Radiolabeled Tim23p was synthesized *in vitro* and imported into uncoupled wild-type mitochondria. A fraction of the import reaction was removed as an untreated control (–MBS) and the remainder was subjected to cross-linking with 0.1 mM MBS for 30 min on ice. After quenching, an aliquot was removed for direct analysis (+MBS). The remainder was denatured with SDS and equal aliquots were incubated with protein A–Sepharose beads containing immobilized rabbit IgGs monospecific for Tim8p (α8), Tim9p (α9), Tim10p (α10) or Tim13p (α13). After centrifugation, bound proteins were eluted with SDS-containing sample buffer and analyzed by SDS–PAGE and fluorography. The arrow marked ‘Tim23p’ denotes the position of monomeric Tim23p. The position of size markers (with masses given in kDa) is indicated on the left. Standard (STD) refers to 10% of the added radiolabeled Tim23p corresponding to each aliquot analyzed. (B) Essentially as (A) except that the cross-linker was 1 mM DSS and immunoprecipitations were performed with IgGs against Tim9p (α9) and Tim8p (α8). The arrow marked ‘AAC’ denotes the position of monomeric AAC. (C) Essentially as (A) except that the radiolabeled precursor was Tim22p. The arrow marked ‘Tim22p’ denotes the position of monomeric Tim22p. (D) As (A) and (C) except that the radiolabeled precursor was Tim17p.

In contrast, import of Tim17p, Tim11p and Tim54p was insensitive to inactivation of Tim22p (Figure 4C and D; unpublished data). Import of Tim11p and Tim17p was also insensitive to heat-inactivation of Tim23p. In similar experiments, Neupert and colleagues have found that the efficiency of Tim17p import is decreased by 50% in mitochondria that have been depleted of Tim23p or Tim22p (Káldi *et al.*, 1998). Insertion of Tim11p and Tim17p into the inner membrane may therefore be mediated by an as yet undefined insertion machinery. Because import of Coq2p, Tim54p and Tim17p is mediated by the Tim9p–Tim10p complex (see above), this complex can deliver a bound precursor either to the TIM23 complex or to an as yet uncharacterized insertion machinery.

In summary, the two 70 kDa Tim complexes of the soluble intermembrane space define two branches of the pathway that delivers cytoplasmically-made inner membrane proteins from the TOM channel in the outer membrane to a TIM system in the inner membrane. These two branches can deliver a hydrophobic inner membrane protein not only to the TIM22 complex, but also to the Tim23 complex and apparently to still other as yet undefined insertion systems.

Discussion

The results of this study show that the Tim8p–Tim9p–Tim13p complex of the yeast mitochondrial intermem-

Fig. 3. Depletion of functional Tim22p causes Tim54p to accumulate as a 150 kDa complex. (A) The intramitochondrial levels of Tim22p and Tim12p are strongly decreased in the temperature-sensitive *tim22-4* mutant grown at the permissive temperature. The parental WT strain and the *tim22-4* mutant were grown in lactate medium for 16 h at 25°C. The mitochondria were isolated and aliquots (50, 100 and 150 µg from left to right) were analyzed by SDS–PAGE and immunoblotting with rabbit antisera monospecific against the proteins indicated on the left. Blots were treated with [¹²⁵I]protein A and subjected to autoradiography. Autoradiographs were quantified by scanning-laser densitometry, and 100% was set as the amount of protein present in the lane for WT mitochondria containing 150 µg of protein. (B) Tim54p accumulates as a 150 kDa subcomplex in mitochondria from the *tim22-4* mutant grown at the permissive temperature. Mitochondria from the *tim22-4* mutant were solubilized in 0.16% *n*-dodecylmaltoside and subjected first to blue native gel electrophoresis (6–16% acrylamide) and then to Tricine-SDS–PAGE (10% acrylamide; Schägger *et al.*, 1994; Dekker *et al.*, 1996). Tim10p, Tim12p, Tim22p and Tim54p were detected by immunoblotting and decoration with [¹²⁵I]protein A. Note the virtual absence of Tim22p. The arrow on the gel denotes the small amount of Tim12p migrating as a 70 kDa complex. A separate gel (lowest horizontal panel) was probed only with antibody against Tim12p and exposed for several days to verify the specific location of Tim12p. T, a sample of wild-type mitochondria was included to show the position of Tim22p in this gel system.

brane space is part of the import machinery for integral inner membrane proteins. Such a function had already been suggested by our earlier observation that deletion of either Tim8p or Tim13p was synthetically lethal with a temperature-sensitive mutation of Tim10p (Koehler *et al.*, 1999), but the present evidence is now compelling. First, Tim8p and Tim13p can be cross-linked to the translocation intermediates of two polytopic inner membrane proteins. Secondly, deletion of the Tim8p–Tim9p–Tim13p complex inhibits the mitochondrial import of Tim23p and Tim11p, but not that of AAC, Tim17p, Tim22p, Tim54p, Coq2p or SU9–DHFR. We conclude that the Tim8p–Tim9p–Tim13p complex mediates the import of a subset of inner membrane proteins, including those with only a single transmembrane span such as Tim11p (Tokatlidis *et al.*, 1996). The path of integral inner membrane proteins across the intermembrane space therefore has at least two branches: one via the Tim9p–Tim10p complex and the other via the Tim8p–Tim9p–Tim13p complex (Figure 5A and B). It is not clear why Tim9p and Tim10p are essential for viability, whereas Tim8p or Tim13p are not. Either

Fig. 4. The temperature-sensitive *tim22-4* mutation inhibits import of Tim23p, AAC and Tim22p, but not of Su9-DHFR into isolated mitochondria. (A) Radiolabeled AAC, Tim23p and Su9-DHFR were synthesized *in vitro* and incubated in the presence or absence of a membrane potential ($\Delta\psi$) at 25°C with mitochondria from the WT, the *tim22-4* mutant or the *tim23-2* mutant (Dekker *et al.*, 1997). Before adding the precursors, mitochondria were incubated for 30 min at 25 or 37°C (as indicated) in the presence of 10 mM succinate, 0.6 M creatine phosphate and 8 mg/ml creatine kinase to inactivate temperature-sensitive Tim22p or Tim23p. After the import reaction samples were treated with proteinase K to remove non-imported precursor, adjusted to 1 mM PMSF, precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE and fluorography. (B) Mitochondria were pre-incubated at 37°C and Tim22p was imported as in (A). Standard (STD) refers to 10% of the radioactive precursor present in each assay. (C) Mitochondria were pre-incubated at 37°C and radiolabeled Coq2p was imported as in (A) for 1, 2 and 4 min. An additional aliquot isolated at the 4 min time point was extracted with 0.1 M Na_2CO_3 for 30 min on ice followed by centrifugation at 100 000 *g* for 15 min to separate pellet (P) and supernatant (S). (D) Radiolabeled Tim17p was imported as in (C) for 1, 2 and 4 min. (E) Radiolabeled Tim11p was imported as in (D). Samples were analyzed by SDS-PAGE and fluorography. Standard (STD) refers to 10% of the radioactive precursor added to each assay. Percentage import, fluorographs of import reactions, was quantified by scanning-laser densitometry, and 100% was set as the amount of mature protein that was imported into WT at 37°C (for temperature shifts) or the longest time point (for time courses).

the escorting function of the Tim9p–Tim10p complex is more important, or Tim9p and Tim10p are required for the function of the membrane-associated insertion complex that also contains Tim12p, Tim22p and Tim54p. The existence of several ‘escorting systems’ for inner membrane proteins in the intermembrane space may reflect the diversity of these hydrophobic proteins.

We have recently reported that the small protein DDP, whose mutation causes an X-linked deafness/dystonia syndrome in humans (Tranebjaerg *et al.*, 1995; Jin *et al.*, 1996) is located in mitochondria and is closely related to yeast Tim8p (Koehler *et al.*, 1999). The present results reinforce our earlier suggestion that this syndrome is the first known human disease caused by a defect in the mitochondrial protein import system.

Since the Tim9p–Tim10p complex had originally been shown to transfer its cargo to the TIM22 complex (Koehler *et al.*, 1998a,b; Sirrenberg *et al.*, 1998), we had assumed that this would also be true for the Tim8p–Tim9p–Tim13p complex. When we tested this hypothesis with a yeast mutant expressing a temperature-sensitive Tim22p, it proved to be generally correct for the mitochondrial metabolite carriers as well as for Tim22p and Tim23p (Figure 5, denoted A and B). However, we found notable exceptions: import of the inner membrane proteins Tim17p, Tim54p, Tim11p and Coq2p does not require

Tim22p even though the journey of these proteins across the intermembrane space is mediated by the Tim9p–Tim10p complex. Pfanner and colleagues recently found that import of Tim54p is inhibited in the *tim23* mutant used in our present work (Kurz *et al.*, 1999). Their result with Tim54p, and ours reported here, suggests that Tim54p and Coq2p are delivered from the Tim9p–Tim10p complex to the TIM23 complex of the general import pathway, and that their import pathways are thus different from those of the other polytopic inner membrane proteins tested so far (Figure 5, denoted C). Because import of Tim17p and Tim11p does not require Tim23p, there may be as yet undiscovered insertion systems (denoted by a question mark in Figure 5).

The operation of yet more pathways for transporting inner membrane proteins across the intermembrane space is also suggested by our unpublished observations that neither the temperature-sensitive *tim10-1* mutation nor the deletion of Tim8p decreased the steady-state concentration of Tim23p in mitochondria. These results contrast with the fact that the Tim23p translocation intermediate can be cross-linked to Tim8p. Alternatively, this discrepancy may merely reflect the frequently observed fact that a subtle import defect *in vivo* is accentuated when isolated mitochondria are analyzed (Yaffe *et al.*, 1985).

It is conceivable that all imported mitochondrial proteins

Fig. 5. Different import pathways for inner membrane proteins across the intermembrane space. Outer membrane (OM) proteins 5, 6, 7, 20, 22, 37, 40 and 70 are subunits of the TOM system that transports proteins across the OM. Proteins 9 and 10 are subunits of a 70 kDa soluble complex in the intermembrane space (IMS) that escorts mitochondrial metabolite carriers Tim17p and Tim22p across the IMS (A). Proteins 8, 9 and 13 are subunits of a separate 70 kDa soluble complex in the IMS that escorts Tim23p across the IMS (B). Pathways A and B can deliver precursors to the TIM22 complex consisting of the inner membrane (IM) proteins 9, 10, 12, 22 and 54. In addition, the Tim9p–Tim10p complex delivers some precursors such as Coq2p and Tim54p to the TIM23 complex (comprised of proteins 17 and 23) of the general import pathway (C). The TIM23 complex is closely associated with Tim11p and Tim44p and mediates transport of precursors carrying a cleaved presequence into the matrix (D). IM proteins such as Tim11p and Tim17p may use still other import pathways (?).

that accumulate in the intermembrane space before passing through or into the inner membrane require escorting by the two 70 kDa Tim complexes. These complexes have a broad substrate specificity and can guide precursors to both known Tim complexes in the inner membrane. Our present work suggests that the insertion routes of proteins into the mitochondrial inner membrane may be as varied as those found in the thylakoid membrane of chloroplasts (Dalbey and Robinson, 1999).

Materials and methods

Plasmids and strains

For *in vitro* transcription/translation, the DNA fragments encoding Tim23p (Haucke and Schatz, 1997) and Tim54p were subcloned into pGEM3Z (Promega), the fragment encoding Tim22p was subcloned into pSP64 (Promega; Koehler *et al.*, 1998a) and the fragments encoding Coq2p and Tim11p were subcloned into pSP65. The plasmid carrying the *TIM17* gene was kindly provided by Dr Nikolaus Pfanner (University of Freiburg, Germany).

Standard techniques were used for growth, manipulation and transformation of yeast strains (Gietz and Sugino, 1988; Guthrie and Fink, 1991). The yeast strains deleted for *TIM8* ($\Delta tim8$) or carrying a temperature-sensitive Tim10p (*tim10-1*) have been described (Koehler *et al.*, 1998a, 1999). The temperature-sensitive *tim23-2* strain (Dekker *et al.*, 1997) was kindly provided by Dr Michiel Meijer (University of Amsterdam, The Netherlands) and by Dr Nikolaus Pfanner. The temperature-sensitive *tim22* strains were constructed using a low-fidelity PCR followed by *in vivo* recombination (Muhlrad *et al.*, 1992; Staples and Dieckmann, 1993; Koehler *et al.*, 1998a). *TIM22* (including 5' and 3' flanking regions) was subcloned into pRS314 (*CEN, TRP1*) and amplified by PCR in the presence of 0.6–1.0 mM MgCl₂ and 0.1–

0.2 mM MnCl₂; the primers were complementary to regions 200 nucleotides away from either side of the multiple cloning region. The amplified fragments were purified and co-transformed with linearized pRS314 into the yeast strain $\Delta tim22$ (pTIM22) [genotype $\Delta tim22::HIS3$ with the plasmid (pTIM22::URA3 *CEN*)]. Trp⁺ transformants were selected at 25°C and screened for growth at 25 and 37°C on minimal glucose media containing 5-fluoro-orotic acid and appropriate growth supplements.

Import of radiolabeled proteins into isolated mitochondria

Mitochondria were purified from lactate-grown yeast cells (Glick and Pon, 1995) and assayed for protein import as described (Hines *et al.*, 1990; Glick *et al.*, 1992; Rospert and Schatz, 1998). Proteins were synthesized in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine after *in vitro* transcription of the corresponding gene by SP6 or T7 polymerase. The reticulocyte lysate containing radiolabeled precursor was incubated at 25°C with isolated mitochondria in import buffer (1 mg/ml bovine serum albumin, 0.6 M sorbitol, 150 mM KCl, 10 mM MgCl₂, 2.5 mM EDTA, 2 mM ATP, 2 mM NADH and 20 mM HEPES–KOH pH 7.4). Mitochondria from the temperature-sensitive *tim22-4* and *tim23-2* mutants were purified from cells grown at 25°C and incubated at 25 or 37°C immediately before import in import buffer at a concentration of 10 mg/ml with an energy-regenerating system (10 mM succinate, 0.6 M creatine phosphate and 8 mg/ml creatine kinase; Wachter *et al.*, 1994). Where indicated, the potential across the mitochondrial inner membrane was dissipated with 1 μ M valinomycin and 25 μ M carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine (FCCP). Non-imported radiolabeled proteins were removed by treatment with 100 μ g/ml trypsin or 50 μ g/ml proteinase K for 15–30 min on ice; trypsin was inhibited with 200 μ g/ml soybean trypsin inhibitor and proteinase K with 1 mM phenylmethylsulfonyl fluoride (PMSF).

For alkali extraction, the mitochondria of an import reaction were sedimented by centrifugation, suspended to 0.1 mg/ml in 100 mM Na₂CO₃ and incubated for 30 min on ice (Fujiki *et al.*, 1982). Supernatant and pellet were separated by centrifugation at 100 000 g for 15 min. The translocation intermediates of Tim23p, Tim17p and Tim22p were cross-linked to adjacent proteins with 0.1 mM MBS. For the translocation intermediate of AAC, cross-linking was performed with 1 mM DSS. The cross-linking protocol was as described (Koehler *et al.*, 1998a,b) with the following modifications: instead of adding protease after generation of the translocation intermediate, mitochondria were washed, suspended at 1 mg/ml in import buffer and incubated with the cross-linker on ice for 30 min followed by a quench with 100 mM Tris–HCl pH 7.5 (for DSS, MBS) or 1 mM 2-mercaptoethanol (for MBS). For immunoprecipitation, solubilized mitochondria were incubated with the corresponding monospecific rabbit IgGs coupled to protein A–Sepharose (Rospert *et al.*, 1994).

Blue native gel analysis

Mitochondria (2.5 mg/ml) were solubilized in 20 mM HEPES–KOH pH 7.4, 50 mM NaCl, 10% glycerol, 2.5 mM MgCl₂, 1 mM EDTA and 1.6 mg/ml *n*-dodecylmaltoside (Boehringer Mannheim) for 30 min on ice. Insoluble material was removed by centrifugation at 100 000 g for 10 min. Solubilized proteins were analyzed by blue native gel electrophoresis on a 6–16% linear polyacrylamide gel (Schägger and von Jagow, 1991; Schägger *et al.*, 1994; Dekker *et al.*, 1996; Koehler *et al.*, 1998b). The separated proteins were detected by immunoblotting using nitrocellulose or polyvinylidene fluoride (PVDF) membranes followed by visualization of the immune complexes with [¹²⁵I]protein A and autoradiography.

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