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Biogenesis of Tim23 and Tim17, integral components of the TIM machinery for matrix-targeted preproteins

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We analysed the import pathway of Tim23 and of Tim17, components of the mitochondrial import machinery for matrix-targeted preproteins. Tim23 contains two independent import signals. One is located within the first 62 amino acid residues of the hydrophilic domain that, in the assembled protein, is exposed to the intermembrane space. This signal mediates translocation of Tim23 across the outer membrane independently of the membrane potential, $\Delta\Psi$. A second import signal is located in the C-terminal membrane-integrated portion of Tim23. It mediates translocation across the outer membrane and insertion into the inner membrane in a strictly $\Delta\Psi$-dependent fashion. Structurally, Tim17 is related to Tim23 but lacks a hydrophilic domain. It contains an import signal in the C-terminal half and its import requires $\Delta\Psi$. The $\Delta\Psi$-dependent import signals of Tim23 and Tim17 are located at corresponding sites in these two homologous proteins. They exhibit features reminiscent of the positively charged N-terminal presequences of matrix-targeted precursors. Import of Tim23 and its insertion into the inner membrane requires Tim22 but not functional Tim23. Thus, biogenesis of the Tim23•17 complex depends on the Tim22 complex, which is the translocase identified as mediating the import of carrier proteins.

Keywords: mitochondrial protein import/TIM complex

Introduction

The import and sorting of nuclear-encoded mitochondrial preproteins into the mitochondria are mediated by translocases which are located in the mitochondrial outer and inner membranes (Glick *et al*., 1991; Pfanner *et al*., 1992). Most preproteins that are imported into the mitochondrial matrix are synthesized with an N-terminal presequence (Neupert, 1997). The presequence is recognized by receptors on the mitochondrial surface (Lithgow *et al*., 1995; Mayer *et al*., 1995a) and then translocated across the outer membrane through the TOM complex (Mayer *et al*., 1995b). Subsequently, the Tim23·17 complex mediates translocation of the presequence across the inner membrane in a $\Delta\Psi$-dependent fashion (Berthold *et al*., 1995; Horst *et al*., 1995; Bauer *et al*., 1996). At the inner side of the inner membrane the preprotein is received by Tim44, mt-Hsp70 and Mge1p which then mediate the coordinated translocation of the precursor across both membranes in an ATP-dependent manner (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994, 1996).

Members of the mitochondrial carrier family, such as the ADP–ATP carrier, are synthesized without a cleavable presequence. To import these proteins into the inner membrane the TOM machinery cooperates with the Tim22 complex (Sirrenberg *et al*., 1996). This pathway does not require functional Tim23 and mt-Hsp70. Outer membrane proteins and certain proteins of the intermembrane space are imported by the TOM complex independent of the translocases of the inner membrane (Neupert, 1997).

In this study, we investigated the biogenesis of Tim23 and Tim17, components of the inner membrane translocase for matrix-targeted preproteins. Tim23 and Tim17 are two structurally related polytopic membrane proteins, which are synthesized without a cleavable presequence (Dekker *et al*., 1993; Emtage and Jensen, 1993; Ryan and Jensen, 1993; Maarse *et al*., 1994; Ryan *et al*., 1994). The results demonstrate that Tim17 and Tim23 contain internal import signals in their C-terminal portion which mediate the import of the precursors and insertion into the inner membrane in a membrane-potential-dependent fashion. These import signals are positively charged and resemble N-terminal presequences of matrix-targeted precursors. When placed at the N-terminus of a passenger protein, they direct import of the chimeric precursors into the matrix. Tim23, which has a hydrophilic N-terminal domain in the intermembrane space, contains an additional targeting signal which directs import of the precursor independent of the membrane potential. The signal is located within the first 62 amino acid residues of Tim23. It targets Tim23 as well as heterologous proteins to the mitochondria and promotes their translocation across the outer membrane. Such a signal is not present in Tim17, which lacks a domain in the intermembrane space.

We show that Tim23 is inserted into the inner membrane via the Tim22 complex. Import of Tim17 is partially affected by both compromised Tim23 and compromised Tim22 function. Fusion proteins containing the C-terminal targeting signals of Tim23 and Tim17 at the N-terminus of a passenger protein are imported via the Tim23•17 complex independent of Tim22.

Results

Import and membrane insertion of Tim17 and Tim23

To characterize the biogenesis of Tim17 and Tim23, the radiolabelled precursor proteins were synthesized *in vitro*

and incubated with isolated yeast mitochondria. When a membrane potential, ΔΨ, was present during incubation, the Tim17 precursor was inaccessible to added proteinase K (PK), demonstrating that it was translocated across the outer membrane (Figure 1A, left panel). When mitoplasts were generated and treated with PK a protease-resistant fragment, Tim17′, of ~14 kDa was formed. Tim17′ was resistant to the extraction of membranes with carbonate, pH 11 (Figure 1C), indicating that Tim17 was inserted into the inner membrane. Tim17 was not translocated across the outer membrane when ΔΨ was dissipated (Figure 1A, right panel). This indicates that the import of Tim17 into mitochondria is strictly dependent on ΔΨ.

Radiolabelled Tim23 precursor protein was incubated with energized mitochondria in the presence of ΔΨ (Figure 1B, left panel). Tim23 became inaccessible to added trypsin, demonstrating that it was translocated across the outer membrane. When mitoplasts were generated and treated with trypsin a protease resistant fragment, Tim23′, of ~ 15 kDa was formed. Endogenous Tim23 yielded the same fragment, which corresponds to the C-terminal membrane-integrated portion of Tim23 (not shown). Radiolabelled Tim23′ was not extractable by carbonate, pH 11 (Figure 1C). These results indicate that imported Tim23 became inserted into the inner membrane in a similar fashion to the endogenous protein.

When the Tim23 precursor was incubated with mitochondria in the absence of ΔΨ it was completely translocated across the outer membrane to a significant extent. Another fraction was partially imported as indicated by the generation of a distinct fragment (f) upon treatment of mitochondria with trypsin (Figure 1B, right panel). A fragment of the same size was obtained when a Tim23 precursor containing a C-terminal his-tag was imported (Figure 1D), indicating that the C-termini were clipped off the translocation intermediates. A fragment of similar size was observed when PK was used instead of trypsin. Using Tim23(1–180), a C-terminally truncated version of Tim23, as a molecular weight standard in SDS–PAGE, we estimate that the fragments generated by PK and trypsin correspond to the N-terminal 180–190 amino acid residues of Tim23. Thus, translocation across the outer membrane of the C-terminus of Tim23 was slowed down at a distinct step in the absence of ΔΨ. When mitoplasts were generated and treated with trypsin Tim23 was degraded, indicating that it was not inserted into the inner membrane. Thus, in the absence of ΔΨ, Tim23 is translocated across the outer membrane but is not inserted into the inner membrane.

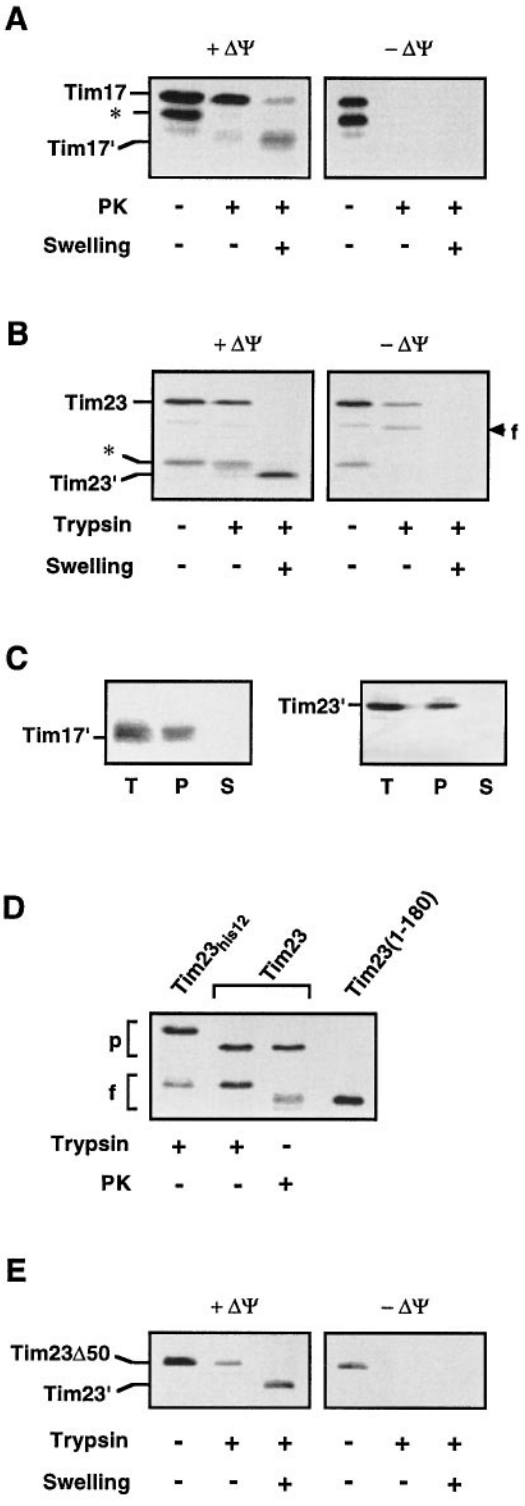

Fig. 1. Import of the precursors of Tim17 and Tim23 into mitochondria. Import and membrane insertion of (**A**) Tim17 and (**B**) Tim23. Radiolabelled precursors were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine and imported into isolated yeast mitochondria for 20 min at 25°C. Mitochondria were either energized (+ΔΨ) or the mitochondrial membrane potential was dissipated prior to import (–ΔΨ). When indicated, the samples were treated with 100 μg/ml proteinase K (PK) or 200 μg/ml trypsin (Trypsin) or mitoplasts were generated (Swelling) and treated with protease. Samples were analysed by SDS–PAGE and autoradiography. *: shorter translation product of Tim17 and Tim23 corresponding to Tim23Δ50; Tim17′ and Tim23′: proteolytic fragments of Tim17 and Tim23 in mitoplasts; f: proteolytic fragment of import intermediate of Tim23 in mitochondria. (**C**) Carbonate extraction of imported Tim17 and Tim23. Radiolabelled Tim17 and Tim23 were imported into mitochondria. Mitoplasts were generated and treated with protease as described above. Samples were halved and mitoplasts were then re-isolated. One half was directly processed for PAGE (T). The second half was extracted with 100 mM Na_2CO_3, pH 11, and subsequently collected by centrifugation. Pellet (P) and supernatant (S) were analysed. (**D**) Import of $Tim23_{his12}$, which contains a C-terminal his12-tag, and Tim23. Radiolabelled precursors were imported into mitochondria in the absence of ΔΨ and samples were treated with trypsin or PK as indicated. p: precursor forms, f: fragments of import intermediates of $Tim23_{his12}$ and Tim23 generated by protease treatment of mitochondria. To indicate the size of the proteolytic fragments, Tim23(1–180) is shown as a molecular weight standard. (**E**) Import and membrane insertion of Tim23Δ50.

In reticulocyte lysate, methionine 50 of Tim23 is used as internal translation initiation site giving rise to Tim23Δ50, an N-terminally truncated version of Tim23 (see Figure 1B,*). Tim23Δ50 was apparently imported into the mitochondria in the presence of ΔΨ, whereas it was not imported when ΔΨ was dissipated. To analyse whether Tim23Δ50 is inserted into the inner membrane, we constructed a plasmid which encodes Tim23Δ50 for *in vitro* transcription and translation. Tim23Δ50 was imported into energized mitochondria (Figure 1E, left panel). When mitoplasts were generated and treated with trypsin the Tim23′ fragment was generated, indicating that Tim23Δ50 was inserted into the inner membrane in a manner similar to the full size Tim23. In the absence of ΔΨ, Tim23Δ50 was not imported into the mitochondria (Figure 1E, right panel).

Tim23 and Tim23Δ50 were both imported into mitoplasts in a strictly ΔΨ-dependent fashion (not shown), indicating that the ΔΨ-dependent import signal is recognized by the TIM-machinery.

To characterize ΔΨ-independent translocation of Tim23 across the outer membrane, we constructed Tim23(1–101), a version of Tim23 where the C-terminal membrane-integrated portion of Tim23 was deleted. Tim23(1–101) was incubated with mitochondria in the presence and absence of ΔΨ. A substantial fraction of the added precursor was either completely or partially translocated across the outer membrane in a ΔΨ-independent fashion (Figure 2A).

To characterize the N-terminal import signal further, we constructed a chimeric precursor where the first 62 amino acid residues of Tim23 were fused to a his-tagged DHFR moiety (Figure 2B). The fusion protein was expressed in yeast from a multicopy plasmid under the control of the Gal10 promoter. The chimeric protein was localized to the mitochondria by subcellular fractionation of yeast cells (Figure 2C). When the isolated mitochondria were treated with PK, a fraction of the protein was not degraded, whereas the protein was completely degraded when mitoplasts were prepared and treated with PK (Figure 2D). This indicates that Tim23(1–62)DHFR was imported *in vivo* into the intermembrane space of the mitochondria.

Thus, Tim23 contains two independent signals for import into mitochondria. The N-terminal portion of Tim23 comprises a signal for translocation of the precursor across the outer membrane, independent of ΔΨ. The signal for ΔΨ-independent import is destroyed by deletion of the first 50 amino acid residues of Tim23. The C-terminal portion of Tim23 contains a signal for import and insertion of the precursor into the inner membrane. Import mediated by this signal is strictly dependent on ΔΨ.

Characterization of the membrane insertion signal

Tim23 and Tim17 are predicted to contain four membrane-spanning segments. Residues 186–197 of Tim23 and 103–112 of Tim17, which are located between the third and fourth predicted membrane-spanning segments of the two related proteins, exhibit features reminiscent of N-terminal presequences of preproteins targeted to the matrix. Thus, these sequences contain basic, hydroxylated and hydrophobic amino acid residues and have the potential to form a positively charged, amphipathic α-helix. Neither Tim17 nor Tim23 contain further apparent segments with such features.

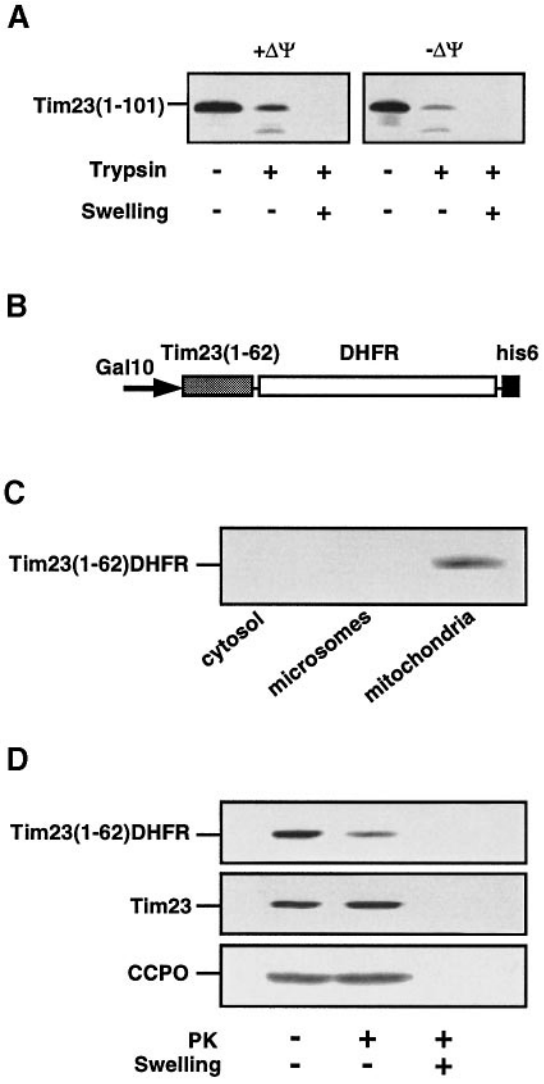

Fig. 2. The N-terminal portion of Tim23 contains a ΔΨ-independent import signal. (**A**) Import of Tim23(1–101) into isolated mitochondria. The radiolabelled protein was imported into mitochondria for 20 min at 25°C as described in Figure 1. (**B**) The structure of the chimeric protein Tim23(1–62)DHFR. DNA encoding the fusion protein was cloned into Yep51-derivative, where its expression was under control of the Gal10 promoter. (**C**) Subcellular localization of Tim23(1–62)DHFR expressed in *S.cerevisiae*. Yeast cells were transformed with the plasmid Yep51–Tim23(1–62)DHFR. Cells expressing the fusion protein were prepared and fractionated into cytosol, microsomes and mitochondria. Samples were analysed by Western blotting using anti-Tim23 IgG. (**D**) Localization of Tim23(1–62)DHFR within the mitochondria. Mitochondria (100 μg) isolated from cells expressing the fusion protein were kept on ice or treated with 50 μg/ml proteinase K (+PK) or mitoplasts were generated (Swelling) and treated with PK. Samples were analysed by SDS–PAGE and immunoblotting with antibodies against Tim23(1–100) and cytochrome c peroxidase (CCPO), a protein of the intermembrane space. The upper panel was exposed for 10 times longer than the others to detect Tim23(1–62).

We constructed Tim23(1–180), a truncated version of Tim23, where 42 amino acid residues were deleted from the C-terminus. Radiolabelled Tim23(1–180) was synthesized *in vitro* and incubated with isolated mitochondria (Figure 3A). The truncated precursor was efficiently imported into the mitochondria in the presence and in the absence of

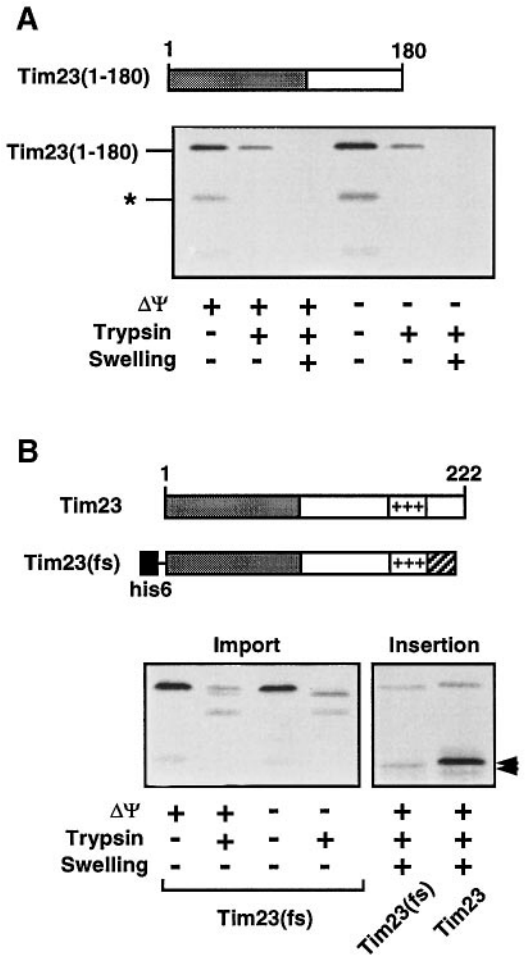

Fig. 3. An import and insertion signal is located in the C-terminal portion of Tim23. (**A**) Import into mitochondria of Tim23(1–180). *: translation product corresponding most likely to initiation at Met50. Tim23(1–180) is outlined schematically. The precursor was imported into mitochondria and samples were analysed as described in Figure 1. (**B**) Tim23 and Tim23(fs) are outlined schematically. Left panel, import of Tim23(fs) into mitochondria. Right panel, insertion into the inner membrane of Tim23(fs) in comparison to Tim23. Precursors were imported into isolated mitochondria for 20 min at 25°C in the presence or absence of ΔΨ. When indicated, mitoplasts were generated (Swelling) and treated with trypsin to monitor the insertion of precursors into the inner membrane. Arrowheads: proteolytic fragments of Tim23(fs) and of Tim23 inserted into the inner membrane. Equal amounts of radiolabelled Tim23(fs) and of Tim23 were used.

ΔΨ. When mitoplasts were generated and treated with trypsin, Tim23(1–180) was degraded, indicating that it was not inserted into the inner membrane. Neither was the truncated precursor inserted in mitoplast preparations (not shown). An internal translation product, which most likely corresponds to residues 50–180 of Tim23, was not imported into the mitochondria (Figure 3A, *). Thus, the C-terminal 42 amino acid residues of Tim23 are required for the ΔΨ-dependent import and insertion of the precursor into the inner membrane. A truncated version of Tim17, where residues 101–158 were deleted, was not imported either in the presence or in the absence of ΔΨ (not shown), indicating that the C-terminal 58 amino acid residues of Tim17 mediate its ΔΨ-dependent import.

To characterize membrane insertion of Tim23 further, we used an *in vitro* synthesized precursor of Tim23(fs). Owing to a translational frame shift after methionine 198 (Berthold *et al.*, 1995), the C-terminal transmembrane segment of Tim23 is replaced by 16 amino acid residues (HGLFLGNGGRCVRRLV) which do not have a predicted probability to form a membrane-spanning segment. Tim23(fs) is functional *in vivo*, indicating that the C-terminal segment of Tim23 is not strictly required for import of the protein into the inner membrane. Tim23(fs) precursor was synthesized *in vitro* and incubated with isolated mitochondria. It was translocated across the outer membrane in the presence and in the absence of ΔΨ (Figure 3B, left panel). In both cases, a significant number of translocation intermediates accumulated where the N-terminus was in the intermembrane space, while the C-terminus was accessible to added trypsin. In the presence of ΔΨ, the efficiency of insertion into the inner membrane of Tim23(fs) was significantly reduced as compared with authentic Tim23 (Figure 3B, right panel). In the absence of ΔΨ, Tim23(fs) was not inserted into the inner membrane (not shown). This indicates that the C-terminal transmembrane segment of Tim23 is required for efficient insertion of the protein into the inner membrane.

To test whether the positively charged segment between residues 103 and 112 of Tim17 and the corresponding region in Tim23 (residues 186–198) have the capacity to target heterologous proteins to the mitochondria, we constructed the chimeric precursor proteins IS17–DHFR and IS23–DHFR (Figure 4A). At the N-terminus, these precursors contain the putative import signal of Tim17 (residues 103–112) and of Tim23 (residues 186–198). These are fused with d2-20, an import-incompetent N-terminally truncated version of pb_2Δ19(167)DHFR which still contains the processing site for the matrix processing peptidase, MPP (Klaus *et al.*, 1996).

When IS17–DHFR and IS23–DHFR were incubated with energized mitochondria, processed forms of the preproteins were generated and their amounts increased over a period of 30 min (Figure 4B). The processed species were protected from degradation by PK and their appearance was strictly dependent on ΔΨ. This indicates that the chimeric precursors were imported and processed by MPP in the mitochondrial matrix. Thus, residues 103–112 of Tim17 and 186–198 of Tim23 have the capacity to target a passenger protein to the mitochondria.

Characterization of import pathways

To characterize the import pathways of Tim23 and Tim17 we used mitochondria that were compromised in the function of either the Tim23•17 or Tim22 complex. To monitor Tim22-dependent import we prepared Tim22↓ mitochondria which contain reduced levels of Tim22 (Sirrenberg *et al.*, 1996). To follow Tim23-dependent import we prepared Tim23(fs) mitochondria (Berthold *et al.*, 1995) which contain strongly reduced levels of the mutant Tim23(fs) protein. When these mitochondria were preincubated at 37°C the Tim23(fs) protein was degraded resulting in virtually complete depletion of the protein (Figure 5A).

Tim23 precursor was translocated in a ΔΨ-independent fashion across the outer membrane of Tim23(fs) mitochondria pretreated at 37°C and Tim22↓ mitochondria (not shown), indicating that neither Tim22 nor Tim23 are required for translocation across the outer membrane.

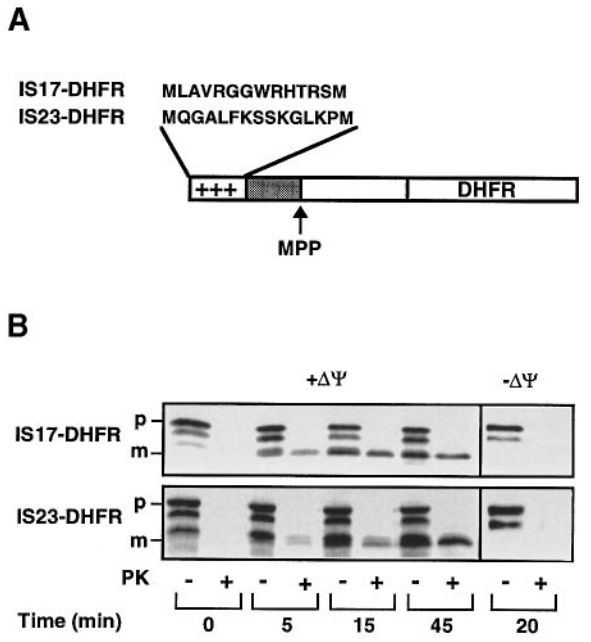

Fig. 4. IS17 and IS23, positively charged segments of Tim17 and Tim23, direct the import of heterologous proteins. (**A**) Schematic outline of the chimeric proteins IS17–DHFR and IS23–DHFR. The amino acid sequence of IS17 and IS23 is given in single letter code. The segments were fused to d2-20, a truncated version of $pb_2\Delta19(167)$DHFR, where the N-terminal mitochondrial targeting signal was deleted (Klaus *et al.*, 1996). MPP: cleavage site in d2-20 for the mitochondrial processing peptidase. (**B**) Import of IS17–DHFR and IS23–DHFR. Radiolabelled IS17–DHFR and IS23–DHFR were imported at 25°C into isolated mitochondria for the periods indicated in the presence (+ΔΨ) or absence (–ΔΨ) of a membrane potential. The samples were halved, one half was kept on ice (–PK) and the other half was treated with 100 μg/ml proteinase K (+PK). Samples were subjected to SDS–PAGE and analysed by autoradiography. p: precursor and m: MPP-processed forms of IS17–DHFR and IS23–DHFR.

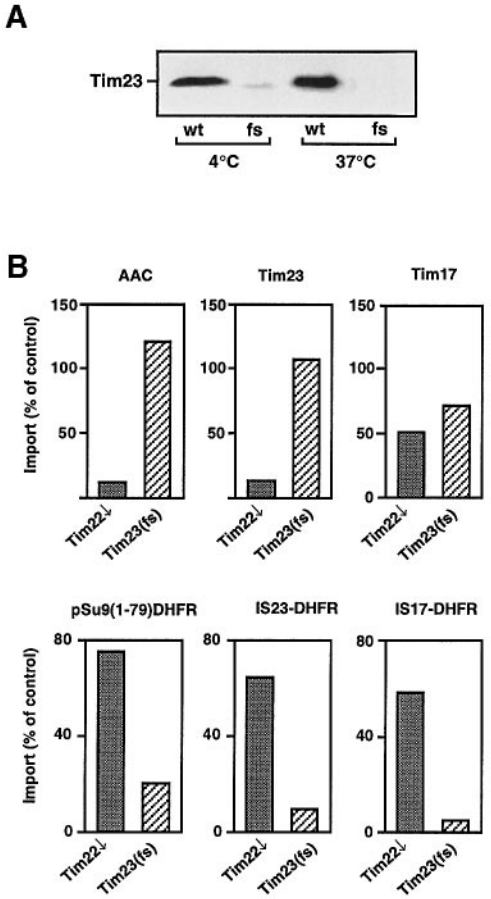

Fig. 5. Tim22 but not Tim23 is required for the import of Tim23. (**A**) Tim23(fs) mitochondria contain reduced levels of the labile Tim23(fs) protein. Wild-type (wt) and Tim23(fs) mitochondria (100 μg) were incubated for 30 min at the indicated temperatures. Samples were analysed by SDS–PAGE and immunoblotting with antibodies against Tim23. (**B**) Comparison of the import of AAC, Tim23, Tim17, pSu9(1–79)DHFR, IS23–DHFR and IS17–DHFR into Tim22↓ and Tim23(fs) mitochondria. Radiolabelled precursors were imported for 20 min into energized wild-type mitochondria, mitochondria which were depleted in Tim22 (Tim22↓) and Tim23(fs) mitochondria, which were preincubated for 30 min at 37°C. After import, mitochondria were subjected to hypotonic swelling and treated with 200 μg/ml trypsin (AAC, Tim23, Tim17) or 100 μg/ml PK [IS17–DHFR, IS23–DHFR, pSu9(1–79)DHFR]. Samples were analysed by SDS–PAGE and autoradiography, and quantified with a phosphor imaging system. Data are expressed as % import of the precursors into wild-type mitochondria (% of control).

However, ΔΨ-dependent insertion into the inner membrane did not occur in Tim22↓ mitochondria whereas the precursor was efficiently inserted into the inner membrane in Tim23(fs) mitochondria (Figure 5B). This indicates that Tim23 is imported via the Tim22 pathway in a manner similar to members of the mitochondrial carrier family (Sirrenberg *et al.*, 1996). Tim17 precursor was imported in a ΔΨ-dependent fashion into both types of mitochondria with reduced efficiency.

Interestingly, IS17–DHFR and IS23–DHFR were not imported into Tim23(fs) mitochondria, whereas both chimeric precursors were imported and processed when Tim22↓ mitochondria were used (Figure 5B). This indicates that functional Tim23 is required for the import of chimeric preproteins into the matrix. Apparently, IS17–DHFR and IS23–DHFR are imported by the same pathway as matrix-targeted preproteins with an N-terminal presequence.

Discussion

We have analysed the import and insertion pathways of Tim23 and Tim17, components of the TIM machinery specific for matrix targeted precursors, into the inner membrane.

Tim23 requires the Tim22 complex for its import. The Tim22 complex was shown to mediate insertion into the inner membrane of its own precursor and members of the mitochondrial carrier family, such as the ADP–ATP carrier (Sirrenberg *et al.*, 1996, 1998). Like mitochondrial carrier proteins, Tim23 is synthesized without an N-terminal presequence and it spans the membrane several times. Also like carrier proteins, Tim23 is a component which has apparently no counterpart in prokaryotes, unlike other polytopic inner membrane proteins that are imported via N-terminal targeting signals.

Two different targeting signals can be distinguished in Tim23. An internal, positively charged import signal is present in the C-terminal portion of Tim23. This import signal, termed IS23, resembles N-terminal presequences of matrix-targeted precursors. It directs translocation across the outer membrane and insertion into the inner

membrane of the precursors in a strictly ΔΨ-dependent fashion. A positively charged internal import signal has also been identified in the BCS1 protein (Fölsch *et al.*, 1996). When IS23 was placed at the N-terminus of a passenger protein, the chimeric precursor was imported via the Tim23•17 complex. Apparently, Tim23•17 and Tim22 complexes recognize similar import signals at the ΔΨ-dependent step of translocation across, or insertion into, the inner membrane. N-terminal positively charged presequences of matrix-targeted preproteins are presumably recognized by an acidic segment in the N-terminal portion of Tim23 (Bauer *et al.* 1996). Tim22 which is structurally related to Tim23 contains a segment in the N-terminal portion (residues 21–33) that carries five negatively charged residues. This acidic segment may have a corresponding role in the recognition of precursor proteins with internal targeting signals such as IS23.

In addition to IS23, Tim23 harbours a mitochondrial-targeting signal in its hydrophilic N-terminal domain. In the assembled protein, this domain is exposed to the intermembrane space. This targeting signal promotes translocation of the Tim23 precursor across the outer membrane in a ΔΨ-independent fashion. It also mediates translocation across the outer membrane of Tim23(1–180), a C-terminally truncated version of Tim23 that lacks the ΔΨ-dependent import signal IS23. Notably, in the absence of ΔΨ, complete translocation across the outer membrane of full-size Tim23 was less efficient than translocation of Tim23(1–180). A significant fraction of full-size Tim23 accumulated as a specific import intermediate. Translocation of Tim23 is apparently slowed down when IS23 is about to be translocated across the outer membrane. In this situation, IS23 appears to be specifically bound to the TOM complex which retards the complete translocation of the C-terminus of the Tim23 precursor in the absence of ΔΨ. This binding of IS23 may occur at the so-called *trans*-site of the TOM complex, which interacts with positively charged presequences (Mayer *et al*., 1995b; Bolliger *et al*., 1995; Court *et al*., 1996; Rapaport *et al*., 1997). The interaction of IS23 with the TOM complex is stable at high ionic strength, a characteristic feature of *trans*-site binding (Rapaport *et al*., 1997). We have recently shown that functional TOM complexes are present in excess over Tim23•17 complexes and Tim22 complexes (Sirrenberg *et al*., 1997). Thus, a tight interaction of the *trans*-site of the TOM complex with targeting signals for the inner membrane or for the matrix may hold precursor proteins until a TIM complex is recruited to mediate ΔΨ-dependent insertion into or translocation across the inner membrane.

Why would Tim23 contain two independent import signals? Tim23Δ50, which lacks the ΔΨ-independent targeting signal, is imported across the outer membrane and inserted into the inner membrane. Thus, IS23 is sufficient to mediate import of the N-terminally truncated precursor across the outer membrane. The requirement for an additional targeting signal may arise when larger domains have to be imported into the intermembrane space. Insertion of membrane spanning segments into the inner membrane mediated by IS23-like signals might then not provide sufficient driving force to promote translocation of such domains across the outer membrane. The N-terminal 62 amino acid residues of Tim23 are sufficient to direct import of a passenger protein into the intermembrane space, yet the nature of the targeting signal is not clear. Signals similar to the one in the N-terminal portion of Tim23 may facilitate ΔΨ-independent import into the intermembrane space of other proteins such as heme lyases that contain internal targeting signals (Lill *et al*., 1992). Components of this import pathway as well as corresponding targeting signals remain to be identified.

Tim17 is structurally related to the C-terminal portion of Tim23. It lacks an N-terminal domain that is exposed to the intermembrane space. Its import is strictly ΔΨ-dependent. IS17, a region in the C-terminal portion of Tim17 that apparently corresponds to IS23, has features of an N-terminal presequence. IS17 functions as a mitochondrial targeting signal when placed at the N-terminus of a passenger protein. This chimeric preprotein was then imported by the Tim23-dependent pathway. Despite its similarity to Tim23, the import pathway of Tim17 is not clear. Tim17 precursor was imported with reduced efficiency into both mitochondria which were depleted of Tim23 by preincubation of Tim23(fs) mitochondria at 37°C and mitochondria depleted of Tim22; but import was not abolished. As preincubated Tim23(fs) mitochondria do not contain detectable levels of Tim23(fs) it appears unlikely that Tim17 requires Tim23 for its import. However, it remains open whether Tim17 is imported via Tim22, or via Tim17, which is present at normal levels in Tim23(fs) mitochondria. Furthermore, the Tim17 precursor could use both pathways or even a yet unidentified pathway for its import.

In conclusion, we have shown that the Tim22 translocase is not only required for import of members of the carrier family but has a broader substrate specificity which includes other integral inner membrane proteins that are synthesized without an N-terminal matrix targeting signal. Not only is Tim22 structurally related to Tim17 and Tim23, suggesting that both translocation systems of the inner membrane have a common ancestor, but in addition, the two translocases also recognize related signals. The position of these signals within a precursor and/or additional signals may specify which import pathway is taken.

Materials and methods

Construction of Tim23 mutants

pGEM4–Tim23Δ50 was constructed by digestion of pGEM4–Tim23 (Berthold *et al.*, 1995) with *Bam*HI and *Aoc*I to delete a fragment encoding the translation start and the N-terminal portion of Tim23. The ends were filled in with Pfu polymerase and the DNA was religated. The first Met-codon in the resulting construct corresponds to Met51 in Tim23.

For the construction of pGEM4–Tim23(1–101), *Bam*HI and *Pvu*II fragments were isolated from pQE13–Tim23(1–101) (Bauer *et al.*, 1996) and subcloned into pGEM4.

Tim23(1–180) DNA was amplified from pGEM4–Tim23$_{his12}$ (Bauer *et al.*, 1996) by PCR using the upstream primer 5′-CCC CAA GCT TCA AGA TAT CTG CAT GCC CCC AGC GCC AAT G-3′ and the downstream primer 5′-GAT TTA GGT GAC ACT A TA-3′. The PCR product was digested with *Eco*RI and *Hin*dIII and subcloned into pGEM4.

For the construction of pGEM4–IS17–DHFR, Tim17(103–112) was amplified by PCR by using the upstream primer 5′-CCC CAA GCT TCA ACA CGT GAT CGC CAT GGA CCT TGT ATG CCT CCA AC-3′, the downstream primer 5′-CCC CGA ATT CAG CAT GCT AGC TGT AAG AGG TGG TTG-3′ and pGEM4–Tim17 as a template. To introduce an *Nco*I site into pGEM4–d2-20 (Klaus *et al.*, 1996), a PCR fragment was amplified from pGEM4–pb$_2$Δ19(167)DHFR (Koll *et al.*, 1992) using the upstream primer 5′-TAA TAC GAC TCA CTA TAG

GG-3′ and the downstream primer 5′-CCC CGA ATT CGC CAC CAT GGC GTC TAA GAC TAG AT-3′. The fragment was cloned into pGEM4 vector using the cloning sites *Eco*RI and *Hin*dIII. The Tim17(103–112) PCR fragment was digested with *Eco*RI and *Nco*I and subcloned into the modified pGEM4–d2-20 vector.

For the construction of pGEM4–IS23–DHFR, Tim23(184–222) was first amplified by PCR using the upstream primer 5′-TAA TAC GAC TCA CTA TAG GG-3′, the downstream primer 5′-CCC CGA ATT CAG CAT GCA GGG CGC TTT GTT CAA G-3′ and pGEM4–Tim23$_{his12}$ as a template. The PCR fragment was digested with *Eco*RI and *Nco*I and subcloned into the modified pGEM4–d2-20 vector.

For the construction of the plasmid Yep51–Tim23(1–62)DHFR$_{his6}$, the pQE16 vector was digested with *Bam*HI, the sticky ends were filled in with Pfu polymerase and the DNA fragment encoding DHFR$_{his6}$ was cut out with *Hin*dIII. Yep51–Tim23 (Bauer *et al.*, 1996) was digested with *Xba*I, the sticky ends were filled in with Pfu polymerase, the plasmid was cut with *Hin*dIII to remove the region encoding residues 63–222 and the DHFR$_{his6}$ fragment was subcloned into the resulting vector.

Expression and localization of Tim23(1–62)DHFR$_{his6}$ in Saccharomyces cerevisiae

Saccharomyces cerevisiae strain 334 (Hovland *et al.*, 1989) was transformed with Yep51–Tim23(1–62)DHFR$_{his6}$ using DMSO-enhanced transformation (Hill *et al.*, 1991). Transformants were grown on selective lactate medium in the presence of 1% galactose. Mitochondria were isolated as described previously (Berthold *et al.*, 1995) with the exception that buffer A containing 0.6 M sorbitol, 1 mM PMSF and MES–KOH, pH 6.0, was used for disrupting the cells. Isolated mitochondria were resuspended in the buffer A without PMSF. Mitochondria (50–75 mg) were overlaid onto an 18/14.5% Nycodenz gradient (5/5 ml, dissolved in buffer A) and subjected to ultracentrifugation (30 min at 220 000 *g* and 2°C). Mitochondria at the 18/14.5% interface were removed and washed with 10 vol 0.6 M sorbitol, 20 mM HEPES–KOH, pH 7.2. Proteinase K treatment of mitochondria was carried out either in a buffer containing 0.6 M sorbitol, 3% BSA and 20 mM HEPES–KOH, pH 7.2, or in swelling buffer containing 60 mM sorbitol, 0.3% BSA and 12 mM HEPES–KOH, pH 7.2. After protease treatment mitochondria were washed with a buffer containing 0.6 M sorbitol, 1 mM EDTA, 2 mM protease inhibitor PMSF and 20 mM HEPES–KOH, pH 7.2. To separate cytosol and microsomal fractions, the post-mitochondrial supernatant was centrifuged for 30 min at 100 000 *g* and 2°C, pellet and supernatant were considered as microsomal and cytosolic fractions, respectively.

In vitro synthesis of precursor proteins and import into mitochondria

Precursor proteins were synthesized by coupled transcription–translation in reticulocyte lysate in the presence of [^{35}S]-methionine (Promega) as described (Pelham and Jackson, 1976). Mitochondria were isolated as described (Berthold *et al.*, 1995; Sirrenberg *et al.*, 1996).

Import reactions were carried out for the periods indicated at 25°C in 100 µl of import buffer (600 mM sorbitol, 3 mg/ml BSA, 80 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 2 mM KH_2PO_4, 2 mM ATP, 5 mM NADH, 50 mM HEPES–KOH, pH 7.2). An assay contained 30–50 µg of mitochondria and 1–3% reticulocyte lysate with the radiolabelled precursor protein. To generate the membrane potential mitochondria were preincubated for 5 min at 25°C in the presence of 2.5 mM malate, 2.5 mM succinate, 5 mM phosphocreatine, 50 µg/ml creatine kinase and 2 mM ATP. Membrane potential was dissipated by the addition of 1 µM valinomycin and 25 mM KCl. For the generation of mitoplasts the import assay was diluted in 10 volumes of swelling buffer (10 mM HEPES–KOH, pH 7.2, 0.3% BSA). Protease treatment of mitochondria and mitoplasts were carried out on ice for 20 min.

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