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Tim9p, an essential partner subunit of Tim10p for the import of mitochondrial carrier proteins

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Tim10p, a protein of the yeast mitochondrial intermembrane space, was shown previously to be essential for the import of multispanning carrier proteins from the cytoplasm into the inner membrane. We now identify Tim9p, another essential component of this import pathway. Most of Tim9p is associated with Tim10p in a soluble 70 kDa complex. Tim9p and Tim10p co-purify in successive chromatographic fractionations and co-immunoprecipitated with each other. Tim9p can be cross-linked to a partly translocated carrier protein. A small fraction of Tim9p is bound to the outer face of the inner membrane in a 300 kDa complex whose other subunits include Tim54p, Tim22p, Tim12p and Tim10p. The sequence of Tim9p is 25% identical to that of Tim10p and Tim12p. A Ser_{67}→Cys_{67} mutation in Tim9p suppresses the temperature-sensitive growth defect of *tim10-1* and *tim12-1* mutants. Tim9p is a new subunit of the TIM machinery that guides hydrophobic inner membrane proteins across the aqueous intermembrane space.

Keywords: extragenic suppression/mitochondrial protein import/*Saccharomyces cerevisiae*/TIM complex

Introduction

Most mitochondrial proteins are synthesized with a cleavable N-terminal targeting sequence and imported into mitochondria by a general import pathway composed of cytosolic chaperones and two hetero-oligomeric membrane complexes: a TOM complex in the outer membrane and a TIM complex in the inner membrane (Ryan and Jensen, 1995; Schatz and Dobberstein, 1996; Neupert, 1997; Pfanner, 1998). Import across both membranes into the matrix is effected by the dynamic interaction of the TOM and TIM complexes (Glick *et al*., 1991; Pfanner *et al*., 1996; Pfanner and Meijer, 1997). Transfer of a precursor from the TOM complex to the TIM complex is facilitated by stepwise interactions of the precursor's positively charged presequence with negatively charged regions on the two complexes (Dietmeier *et al*., 1997; Schatz, 1997; Komiya *et al*., 1998).

Initially, it was assumed that yeast mitochondria contain only a single TIM complex. However, recent work has uncovered the existence of two different TIM complexes, each with a distinct function (Sirrenberg *et al*., 1996, 1998; Kerscher *et al*., 1997; Koehler *et al*., 1998). One TIM complex contains the transmembrane subunits Tim17p and Tim23p associated with an ATP-driven protein translocation motor; this complex mediates the import of all matrix proteins and of several intermembrane space proteins. A second TIM complex contains the transmembrane subunits Tim22p and Tim54p as well as the peripheral subunits Tim10p and Tim12p; this second complex mediates import and insertion of multispanning carrier proteins into the inner membrane. Import of these carriers is independent of the Tim17p–Tim23p complex.

Members of the mitochondrial carrier family contain six membrane-spanning segments. Inspection of the yeast genome suggests that least 34 such carriers reside in the mitochondrial inner membrane (Palmieri *et al*., 1996; el Moualij *et al*., 1997). Insertion of these highly hydrophobic proteins into the inner membrane requires cytosolic chaperones, the TOM system and the second TIM complex mentioned above (Sirrenberg *et al*., 1996, 1998; Kerscher *et al*., 1997; Koehler *et al*., 1998; Kübrich *et al*., 1998). As most carriers are synthesized without a cleaved targeting signal, their targeting information must reside in one or several regions of the mature protein, but these regions have not yet been clearly identified (Pfanner *et al*., 1987; Smagula and Douglas, 1988).

Recent work by Sirrenberg *et al*. (1998) and by us (Koehler *et al*., 1998) has suggested that Tim10p binds carriers as they emerge from the TOM channel into the intermembrane space, and transfers them to Tim12p which is peripherally bound to Tim22p and Tim54p on the outer face of the inner membrane. Our work with yeast mutants expressing temperature-sensitive (ts) Tim10p or Tim12p has shown that inactivation of Tim10p blocks transfer of carriers across the outer membrane whereas inactivation of Tim12p blocks their proper insertion into the inner membrane. An interaction between Tim12p and Tim22p was shown by genetic and biochemical experiments (Koehler *et al*., 1998): *TIM22* acts as a multicopy suppressor of a ts *tim12-1* mutation, and Tim12p and Tim22p co-purify.

Here we describe yet another protein of the TIM system that is responsible for the import of carrier proteins. We discovered the protein in a screen for extragenic suppressors of the ts *tim10-1* mutation. One of the suppressors proved to be a mutant allele of a small, and

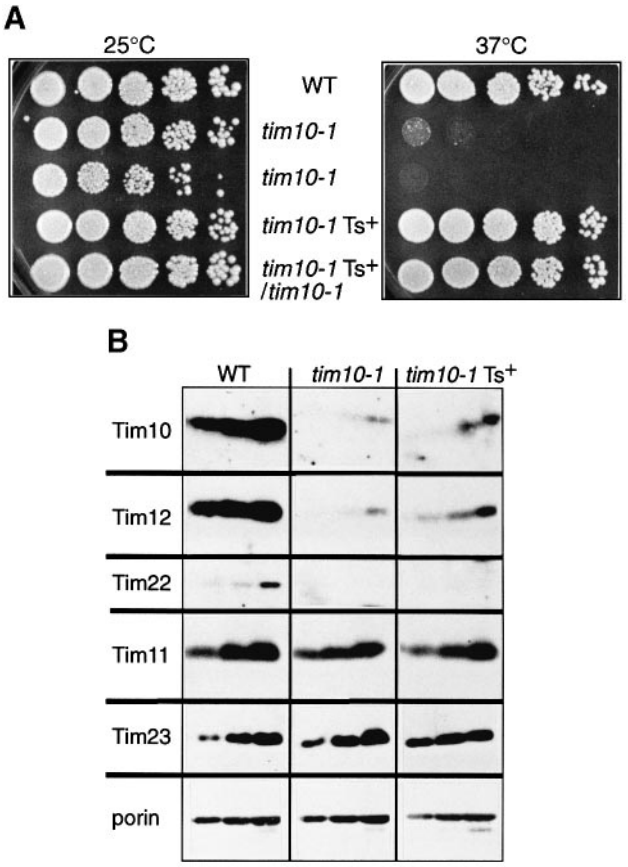

Fig. 1. An extragenic suppressor restores growth at 37°C to the *tim10-1* mutant. (**A**) The indicated yeast strains (Table I) were grown to mid-log phase at 25°C in liquid YPD. Cultures were serially diluted by a factor of 5 and spotted onto YPD plates. Plates were incubated for 3 days at 25 or 37°C. WT, GA74-1A (Jarosch *et al*., 1996); *tim10-1*, ts *tim10-1* allele integrated at the *LEU2* or *TRP1* locus; *tim10-1* Ts^+, spontaneous extragenic suppressor recovered at 37°C in one of the *tim10-1* colonies; *tim10-1* Ts^+/*tim10-1*, extragenic suppressor in a diploid that is Δ*tim10*/Δ*tim10 tim10-1* Ts^+/*tim10-1*. (**B**) The parental wild-type strain (WT), the *tim10-1* mutant and the *tim10-1* mutant carrying the second-site suppressor (*tim10-1* Ts^+) were grown in lactate medium for 16 h at 25°C and then shifted for 8 h to 37°C. Isolated mitochondria (50, 100 and 200 μg) were analysed by SDS–PAGE and immunoblotting with monospecific rabbit antisera for Tim10p, Tim12p, Tim22p, Tim11p, Tim23p and porin. Blots were decorated with [^{125}I]protein A and autoradiographed.

hence previously unidentified gene encoding a protein that we term Tim9p. Tim9p binds a translocation intermediate of the ADP/ATP carrier (AAC) in the intermembrane space, is essential for viability, has a mass of 10.2 kDa and displays 25% sequence identity with Tim10p and Tim12p. Most of Tim9p is associated with Tim10p in a soluble 70 kDa complex in the yeast mitochondrial intermembrane space. We suggest that this 70 kDa complex containing Tim9p and Tim10p is the functional unit that guides the hydrophobic carriers through the aqueous intermembrane space to the inner membrane.

Results

An extragenic suppressor restores growth to the tim10-1 mutant at 37°C

To identify proteins interacting with Tim10p, we screened for spontaneous extragenic suppressors that restored growth to the ts *tim10-1* mutant at 37°C (Figure 1A). One of the suppressors we isolated was dominant and unlinked to the *tim10-1* locus. The suppressed strain *tim10-1* Ts^+ had regained wild-type levels of the AAC and of the phosphate carrier, and grew as well as the wild-type on fermentable or non-fermentable carbon sources (unpublished data).

In principle, the suppressor mutation could alter the sequence or the steady-state level of an already known Tim protein. We checked this possibility by two methods. First, we measured the abundance of those Tim proteins that specifically mediate import of metabolite carriers into the inner membrane (Figure 1B). Although the suppressed *tim10-1* Ts^+ mutant had slightly higher levels of Tim10p and Tim12p than the unsuppressed mutant, the levels were still 85% lower than those of the wild-type parental strain. The amount of Tim22p in the suppressed strain was below the detection limit, as noted for the *tim10-1* mutant, whereas the abundance of the outer membrane protein porin and of the inner membrane proteins Tim23p and Tim11p was unaffected. In a second approach, we amplified the *TIM12*, *TIM22* or *TIM54* genes from the suppressor strain *tim10-1* Ts^+ and tested whether their overexpression suppressed the *tim10-1* mutation. Although the amplified *TIM22* gene suppressed the *tim12-1* mutant, as noted previously, suppression of the *tim10-1* mutant was not observed (Koehler *et al*., 1998; unpublished data). These results made it unlikely that the suppressor gene encoded a known protein of the TIM system that mediates import of carrier proteins into the mitochondrial inner membrane. Also, Tim23p was not overexpressed in the *tim10-1* Ts^+ strain. Thus, it is unlikely that suppression involves carrier import via the Tim17p–Tim23p machinery.

The extragenic suppressor encodes a point mutant of Tim9p, an essential protein in the mitochondrial intermembrane space

We speculated that the suppressor gene might encode a Tim10p partner protein whose sequence resembles that of Tim10p. The *Saccharomyces cerevisiae* chromosome V contains, next to the *URA3* gene, an open reading frame (ORF) encoding a small protein of 87 amino acids. This predicted protein is 25% identical to both Tim10p and Tim12p and has the same conserved cysteine motif that we term the 'twin CX_3C' motif (Figure 2A). We amplified this ORF from both the suppressed and the original *tim10-1* mutant by PCR with a proof-reading polymerase and transformed the two amplified DNA fragments into the original *tim10-1* mutant, using a centromeric-based vector. Four independent isolates of the amplified gene from the suppressor strain specifically restored heat-resistant growth to both the *tim10-1* mutant and *tim12-1* mutant at 37°C (Figure 2B). In contrast, the DNA fragment isolated from the unsuppressed *tim10-1* mutant suppressed both mutants only weakly, and only when overexpressed from a 2 μ-based vector. As the predicted mass of the protein product (10.2 kDa) is slightly less than that of Tim10p (10.3 kDa), we termed the wild-type allele of this gene *TIM9* and the corresponding protein Tim9p, consistent with the unified nomenclature for proteins of the mitochondrial protein import system (Pfanner *et al*., 1996). The suppressor allele of *TIM9* carries an A→T mutation at nucleotide 199 that changes Ser67 to cysteine ($Tim9p^{S67C}$; Figure 2A).

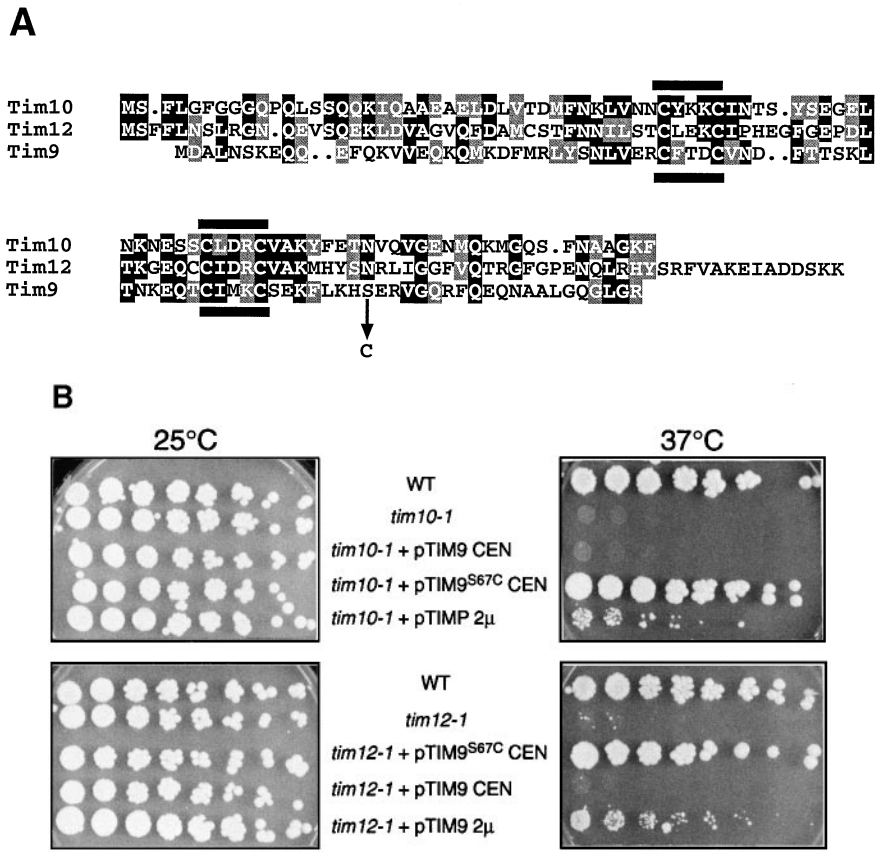

Fig. 2. The extragenic suppressor of the *tim10-1* mutation is a point mutant of Tim9p and also suppresses the *tim12-1* mutation. (**A**) Sequence comparison of Tim9p, Tim10p and Tim12p. Black boxes: identical amino acids; grey boxes: similar amino acids; arrow: the Ser→Cys suppressor mutation in Tim9p. (**B**) A mutation in Tim9p restores growth at 37°C to the *tim10-1* and *tim12-1* mutants. The indicated yeast strains were grown to mid-log phase at 25°C in liquid YPD media, serially diluted by a factor of 3, spotted onto YPD agar plates and incubated for 3 days at 25 or 37°C. WT, GA74D-1A (Jarosch *et al*., 1996); *tim10-1*, original *tim10-1* ts mutant; *tim12-1*, original *tim12-1* ts mutant; pTIM9 CEN, the wild-type *TIM9* allele on a centromeric plasmid; pTIM9^{S67C} CEN, the *TIM9* suppressor allele on a centromeric plasmid; pTIM9 2μ, the wild-type *TIM9* allele on a multicopy plasmid.

To investigate whether Tim9p is essential for viability, one *TIM9* gene of a diploid strain was disrupted with the *TRP1* marker, the strain was sporulated, and the haploid spores dissected from individual asci were incubated on YPD plates at 25 and 30°C. Of the >40 complete tetrads analysed, none yielded more than two viable spores (Figure 3A), and none of the viable spores carried the *TRP1* marker indicative of a disrupted *TIM9* gene. We conclude that *TIM9* is essential for viability. Spores inferred to have a disrupted *TIM9* gene germinated, but stopped growing after at most 10 cell divisions. The suppressor allele of *TIM9* had no detectable phenotype in the absence of another *tim* mutation, nor did it restore growth to a strain lacking the *TIM10* or *TIM12* gene (unpublished data). Therefore, the mutant Tim9p does not bypass the requirement for Tim10p or Tim12p.

To determine the intracellular location of Tim9p, we raised a monospecific polyclonal antibody against the protein. The antibody did not cross-react with Tim10p or Tim12p, nor did our antibodies against Tim10p or Tim12p cross-react with Tim9p (unpublished data). From subcellular fractionation, Tim9p was enriched in the mitochondrial fraction to the same extent as Tim10p or cytochrome b_2 (unpublished data). When the mitochondrial outer membrane was ruptured selectively by osmotic shock, Tim9p became accessible to added proteinase K (Figure 3B). The same behaviour was observed with the intermembrane space marker, cytochrome b_2, as well as with Tim10p. In contrast, the outer membrane marker Tom70p was fully protease accessible even in intact mitochondria, and the matrix marker α-ketoglutarate dehydrogenase remained inaccessible even after severe osmotic shock. As expected, all proteins were equally protease accessible upon addition of Triton X-100 (unpublished data). Based on these results, we conclude that Tim9p is located in the mitochondrial intermembrane space.

This conclusion was confirmed in an alternative approach in which radiolabelled Tim9p or Tim10p was imported into isolated intact mitochondria (Figure 3C). When mitochondria that had imported either Tim9p or Tim10p were subjected to osmotic shock, radiolabelled Tim9p and Tim10p became protease accessible. Although associated with the inner membrane after import, both Tim9p and Tim10p were peripherally associated with the membrane because they were recovered in the alkali-soluble supernatant. (Non-specific losses of Tim10p during alkali extraction or osmotic shock may be caused by inefficient acid precipitation or by adsorption to tube surfaces.) In addition, import into the intermembrane space did not depend on a membrane potential, implying that the import of Tim9p and Tim10p does not require either of the TIM complexes. Therefore, Tim9p and Tim10p

may be transferred directly from the TOM complex to the intermembrane space.

Tim9p and Tim10p form a soluble 70 kDa complex in the intermembrane space

The genetic interactions of *TIM9* with *TIM10* or *TIM12* suggested that Tim9p is associated with other proteins. To address this question, we solubilized mitochondria with non-ionic detergent, subjected the extract to two-dimensional electrophoresis and monitored the mobility of Tim9p, Tim10p, Tim12p, Tim22p and Tim54p by immunoblotting. Electrophoresis in the first dimension was in non-denaturing 'blue native gels' (Schägger and von Jagow, 1991; Schägger *et al*., 1994); electrophoresis in the second dimension was in Tricine-SDS–polyacrylamide gels (Schägger and von Jagow, 1987). Approximately 95% of Tim9p and Tim10p co-migrated as a 70 kDa complex and 5% as a 300 kDa complex (Figure 4A and B). Occasionally, some Tim10p also migrated with the dye front in blue native gels. In contrast, Tim12p, Tim22p and Tim54p (Figure 4A) were present exclusively in a 300 kDa complex. Fractionation of mitochondria established that the 300 kDa complex was membrane associated (unpublished data), whereas the 70 kDa complex was soluble in the intermembrane space (Figure 4B).

The facts that Tim9p and Tim10p interact genetically and co-migrate as a 70 kDa complex suggest that the two proteins are physically associated. To test this directly, we purified the 70 kDa species. We isolated mitochondria, released the soluble intermembrane space contents by osmotic shock, and fractionated the intermembrane space proteins by cation-exchange chromatography on a Mono S column, anion-exchange chromatography on a Mono Q column and gel filtration on a Superose 12 column. After each purification step, we monitored Tim9p, Tim10p and the intact 70 kDa complex by SDS–PAGE and blue native gel electrophoresis followed by immunoblotting. Tim9p and Tim10p co-purified through the entire fractionation procedure as a 70 kDa complex. Specifically, both flowed directly through the Mono S column, eluted at 60–80 mM NaCl on a Mono Q column and eluted in the 60–70 kDa mass range from the gel filtration column. Figure 5A depicts the eluted fractions from the final purification step on a Superose 12 column. Note that Tim9p and Tim10p are enriched and most of the polypeptides are a 70 kDa complex. A fraction of the Tim10p also migrated with the dye front, but the abundance of this presumably monomeric Tim10p species varied between experiments.

Analysis of the enriched complex by sedimentation to equilibrium in the analytical ultracentrifuge yielded a mass of 68 kDa (unpublished data), in good agreement with the results obtained by gel filtration or blue native gel electrophoresis.

To assess the purity and the subunit composition of the 70 kDa complex, we compared 5, 10 and 15% of fraction

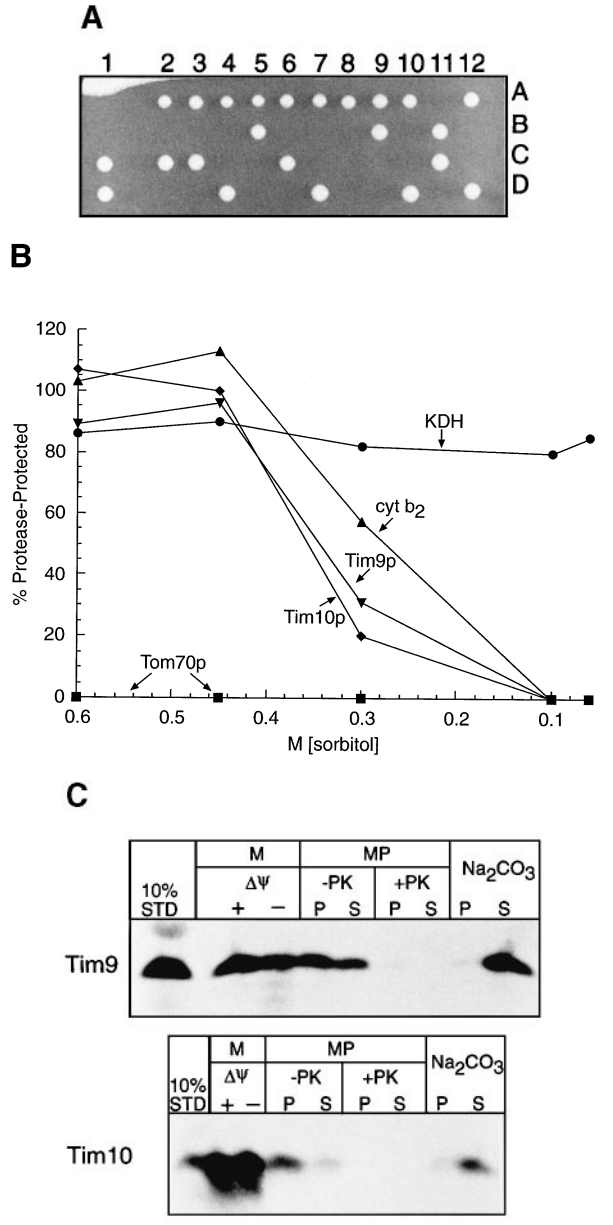

Fig. 3. Tim9p is an essential protein of the mitochondrial intermembrane space. (**A**) Tim9p is essential for viability. Twelve asci from the heterozygous diploid *TIM9*/Δ*tim9::TRP1* were microdissected and the four spores (A–D) from each of the 12 asci were allowed to grow at 30°C for more than one week on YPD plates. (**B**) Tim9p resides in the intermembrane space. Isolated mitochondria were incubated in 20 mM HEPES-KOH pH 7.4, 100 μg/ml proteinase K and the indicated sorbitol concentrations at 4°C for 30 min, followed by addition of 1 mM PMSF. After centrifugation, the pellet was analysed by SDS–PAGE and immunoblotting for Tim9p, Tim10p, cytochrome b_2 (cyt b_2), α-ketoglutarate dehydrogenase (KDH) and Tom70p. Antigen protected from protease was quantified by densitometry and expressed as a percentage of the antigen measured in the corresponding experiment performed without protease. In a separate control (unpublished data), the effectiveness of the protease was verified by addition of Triton X-100. (**C**) The Tim9p and Tim10p precursors are imported into the intermembrane space of isolated mitochondria. Radiolabelled Tim9p and Tim10p were synthesized *in vitro* and incubated for 10 min at 25°C with wild-type mitochondria in the presence or absence of a membrane potential (ΔΨ). The mitochondria (M) were treated with trypsin first to digest non-imported precursor, then with soybean trypsin inhibitor, and analysed by SDS–PAGE and fluorography. Equal aliquots of the mitochondria that had imported precursor in the presence of a membrane potential were analysed further by osmotic shock or alkali extraction. For osmotic shock, mitochondria were converted to mitoplasts (MP) in the absence or presence of 50 μg/ml proteinase K (PK). After addition of 1 mM PMSF, samples were centrifuged at 14 000 *g* for 10 min to separate pellet (P) and supernatant (S). For alkali extraction, mitochondria were incubated with 100 mM Na_2CO_3 for 30 min, followed by centrifugation at 100 000 *g* for 15 min to separate pellet (P) and supernatant (S). STD, 10% of the radioactive precursor present in each assay.

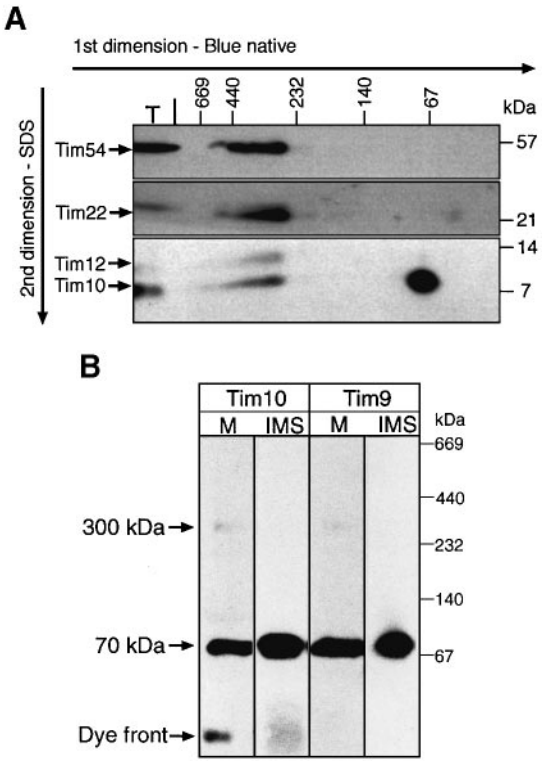

Fig. 4. Two distinct TIM complexes for the import of mitochondrial carrier proteins: a 70 kDa soluble complex and a 300 kDa membrane complex. (**A**) Mitochondria were solubilized in 0.16% *n*-dodecylmaltoside and subjected first to blue native gel electrophoresis (6–16% acrylamide) and then to Tricine-SDS–PAGE (10% acrylamide) (Schägger *et al*., 1994; Dekker *et al*., 1996). Tim10p, Tim12p, Tim22p and Tim54p were detected by immunoblotting and decoration with [^{125}I]protein A. T, a sample of the total detergent-solubilized mitochondria. (**B**) Mitochondria solubilized by *n*-dodecylmaltoside (M) and the intermembrane space obtained after hypotonic shock (IMS) were separated by blue native gel electrophoresis as in (A) and blotted to PVDF membranes. Tim9p and Tim10p were identified by immunoblotting and decoration with [^{125}I]protein A.

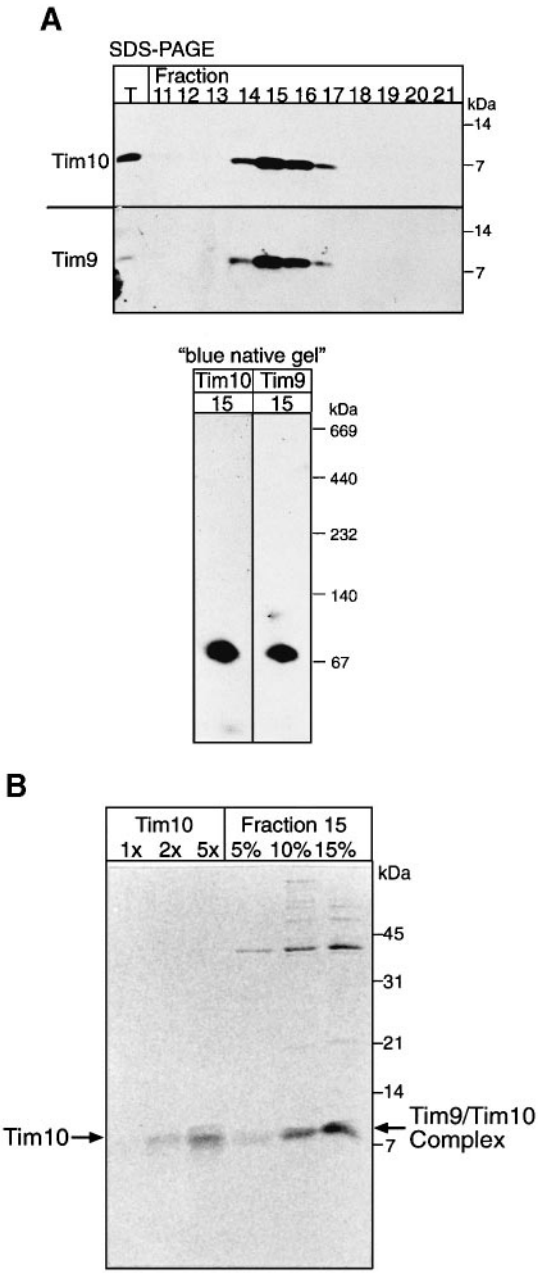

Fig. 5. Tim9p and Tim10p co-purify as a 70 kDa complex from the mitochondrial intermembrane space. (**A**) The soluble intermembrane space fraction from 200 mg of wild-type mitochondria was subjected to ion-exchange chromatography on Mono S followed by Mono Q Sepharose. The fractions eluting from Mono Q Sepharose (selected based on Tim9p–Tim10p enrichment and minimal contamination from other proteins) were pooled and fractionated on a Superose 12 column. The fractions from the Superose 12 column were analysed for Tim9p and Tim10p by SDS–PAGE (upper part) or blue native gel electrophoresis (lower part) followed by immunoblotting. T, aliquot of total protein that had been loaded onto the Superose 12 column. (**B**) Aliquots (50, 100 and 150 µl, corresponding to 5, 10 and 15%) from fraction 15 of the Superose 12 column [see (A)] and purified recombinant Tim10p (1x, 2x and 5x, with x = 1 µg) were subjected to electrophoresis on a 16% Tricine-SDS gel and stained with Coomassie Blue.

15 from the gel filtration column with pure, recombinant Tim10p after separation by SDS–PAGE and visualization by Coomassie Blue staining (Figure 5B). On this basis, we concluded that the 70 kDa complex was 70–80% pure and contained one major 10 kDa protein band which immunoblotting identified as the overlapping bands of Tim9p and Tim10p (unpublished data). A contaminant with a mass near 40 kDa eluted from the gel filtration column in fractions 15–18; thus, it is unlikely to be a component of the 70 kDa complex because it did not have the same elution profile as Tim9p and Tim10p. Furthermore, results from peptide sequencing of tryptic fragments indicated that Tim9p and Tim10p were the only proteins present in the 10 kDa band from the Coomassie-stained gel (unpublished data). Quantitative immunoblot analysis with purified recombinant Tim10p as the standard showed that isolated yeast mitochondria contain 1 µg of Tim10p per mg of protein (unpublished data). Since we recovered 200–400 µg of the complex from 200 mg of mitochondria, the recovery of the Tim9p–Tim10p complex in the purification procedure was in the range of 50–75%. Tim9p and Tim10p were present in equimolar amounts in the purified complex as deduced from quantitative immunoblot analysis with purified recombinant Tim10p and maltose-binding protein (MBP)–Tim9p fusion protein as standards (unpublished data). The standards had been quantified by amino acid analysis. We propose that the 70 kDa complex is a $(Tim9p)_3$–$(Tim10p)_3$ hetero-hexamer or $(Tim9p)_4$–$(Tim10p)_4$ hetero-octamer.

The existence of a stable complex containing both

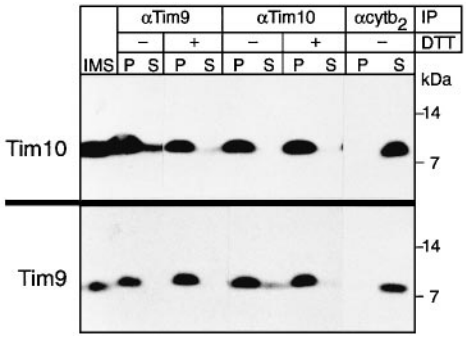

Fig. 6. Tim9p and Tim10p co-immunoprecipitate with each other. An intermembrane space fraction (IMS) was prepared by osmotic shock of mitochondria in the absence or presence of 1 mM DTT and incubated at 4°C for 2 h with protein A–Sepharose that had been coupled to IgGs against Tim9p (αTim9p), Tim10p (αTim10p) or cytochrome b_2 (αcytb$_2$). After centrifugation, 200 μg aliquots of unbound (S) and bound proteins (P) were analysed by SDS–PAGE and immunoblotting for Tim10p (upper part) or Tim9p (lower part).

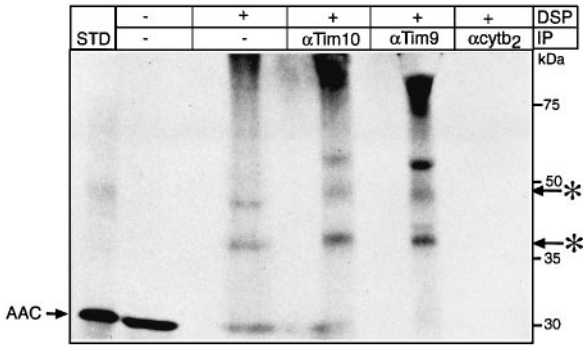

Fig. 7. Tim9p and Tim10p can be cross-linked to a translocation intermediate of the AAC precursor. Radiolabelled AAC was synthesized *in vitro* and imported into uncoupled (–ΔΨ) wild-type mitochondria. One-seventh of the assay was precipitated with trichloroacetic acid (–DSP) whereas the remainder was subjected to cross-linking with DSP. Two-sevenths of the cross-linked sample was precipitated with trichloroacetic acid and the remainder was denatured with SDS and subjected to immunoprecipitation with antiserum against Tim9p (αTim9p), Tim10p (αTim10p) or (as a negative control) cytochrome b_2 (αcytb$_2$). After centrifugation, bound proteins were eluted with SDS-containing sample buffer and analysed by SDS–PAGE and fluorography. The arrow marked 'AAC' denotes the position of the monomeric AAC, and the arrows marked with the asterisks denote the two major cross-linked products that were immunoprecipitated specifically with antiserum against Tim9p and Tim10p. STD, 10% of the amount of AAC precursor that was added to the non-cross-linked import assay.

Tim9p and Tim10p was confirmed by co-immunoprecipitation. Mitochondria were exposed to osmotic shock, the released intermembrane space fraction was subjected to immunoprecipitation with antibodies against Tim9p or Tim10p, and the immunoprecipitated proteins were analysed for Tim9p and Tim10p by immunoblotting. The antibodies to Tim9p or Tim10p quantitatively immunoprecipitated their cognate antigen (Figure 6). They also precipitated 80–90% of the partner protein (Tim10p or Tim9p, respectively), provided dithiothreitol (DTT) was present. When DTT was replaced by EDTA, precipitation of the cognate antigen was still quantitative, but co-immunoprecipitation of the partner protein diminished to 50–60% (unpublished data). An antiserum against cytochrome b_2, an abundant intermembrane space protein, precipitated neither Tim9p nor Tim10p, confirming the specificity of the Tim9p–Tim10p interaction. As expected, Tim12p was not released by osmotic shock and thus not present in the soluble intermembrane space fraction. Tim9p and Tim10p also co-migrated during isoelectric focusing under non-denaturing conditions (unpublished data). In conclusion, a direct interaction between Tim9p and Tim10p is shown by including co-purification, co-migration in non-denaturing electrophoresis or isoelectric focusing, co-immunoprecipitation and functional interaction of the corresponding genes.

Tim9p binds a translocation intermediate of radiolabelled AAC

When radiolabelled AAC is imported into isolated de-energized mitochondria, it binds transiently to Tim10p (Koehler *et al*., 1998; Sirrenberg *et al*., 1998). The same approach was used to determine whether Tim9p binds directly to AAC during translocation. We imported radiolabelled AAC into uncoupled mitochondria, reacted the mitochondria with a cleavable cross-linker, subjected the solubilized mitochondria to immunoprecipitation with antibodies against Tim10p, Tim9p or, as a control, cytochrome b_2, and analysed the immunoprecipitates by SDS–PAGE and fluorography (Figure 7). The antibodies to Tim10p and Tim9p precipitated the same radiolabelled cross-linked products (highlighted with asterisks in Figure 7), indicating that Tim9p and Tim10p bind AAC in a similar manner. Cleavage of the cross-links by 2-mercaptoethanol converted all radioactive cross-linked products to the monomeric AAC species (unpublished data). The non-specific cross-linked products that were immunoprecipitated may reflect a thiol-dependent association between AAC and IgGs under non-reducing conditions.

Discussion

The present work has identified Tim9p, a new component of the TIM machinery that transports multispanning carrier proteins from the cytoplasm to the mitochondrial inner membrane. Like all other known members of this TIM machinery, Tim9p is essential for viability. This fact is not surprising as the correct insertion of metabolite carriers into the inner membrane is probably essential for the interaction of mitochondria with the rest of the cell.

Tim9p interacts with both Tim10p and Tim12p. Most of Tim9p is found associated with Tim10p in the intermembrane space in a soluble 70 kDa complex whose probable subunit composition is $(Tim9p)_3(Tim10p)_3$ or $(Tim9p)_4(Tim10p)_4$. Our cross-linking data suggest that this complex is the functional unit that accepts carrier precursors from the inner mouth of the TOM channel in the outer membrane. The possibility that other proteins may be present in this complex cannot be overlooked, but after three successive chromatographic steps, Tim9p and Tim10p were enriched specifically in comparison with a few other higher molecular weight proteins that showed different elution patterns on the final gel filtration column. A small fraction of Tim9p is bound to the inner membrane, probably as a subunit of the membrane-bound 300 kDa complex that also contains Tim10p, Tim12p, Tim22p and Tim54p. In this 300 kDa complex, Tim9p probably

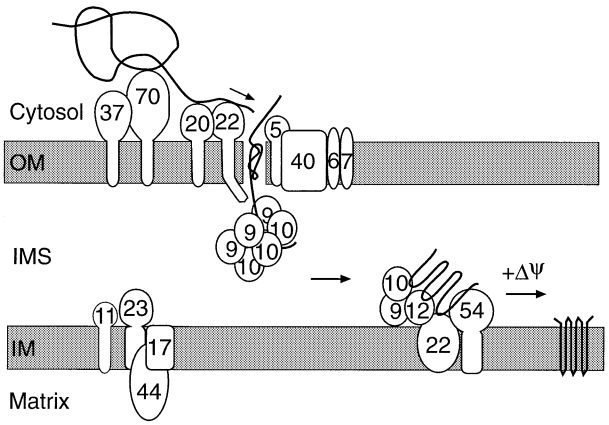

Fig. 8. The TIM complexes mediating import of mitochondrial carrier proteins. Outer membrane (OM) proteins 5, 6, 7, 20, 22, 37, 40 and 70 are subunits of the TOM system that transports proteins across the outer membrane. Proteins 9, 10, 12, 22 and 54 are subunits of the TIM system that mediates import of multispanning carrier proteins into the inner membrane (IM). A carrier precursor exiting the TOM channel is captured by the 70 kDa Tim9p–Tim10p complex in the intermembrane space and transferred to the 300 kDa inner membrane complex that contains Tim9p, Tim10p, Tim12p, Tim22p and Tim54p. Binding to the membrane complex triggers the ΔΨ-dependent insertion of the multispanning carrier into the inner membrane. Inner membrane proteins 11, 17, 23 and 44 are members of, or closely adjacent to, the second TIM (Tim17p–Tim23p) system that mediates transport of precursors carrying a targeting presequence.

interacts directly with Tim12p because the Ser→Cys mutant of Tim9p suppresses a ts mutation in Tim12p. Tim9p, however, does not substitute for either Tim10p or Tim12p, indicating that the requirement for Tim10p and Tim12p cannot be bypassed. Rather, Tim9p may have a stabilizing effect or may have additional functions not shared by Tim10p and Tim12p.

Interaction of Tim9p and Tim10p with each other is somewhat labile when the complex is released from the intact mitochondria and may well be dynamic *in vivo*. For example, the 70 kDa Tim9p–Tim10p complex, when released from the intermembrane space, dissociates upon freezing in liquid nitrogen (unpublished data) or upon incubation in the absence of DTT (Figure 6). Some stabilization is also provided by micromolar concentrations of Zn^{2+}, in agreement with the observation that the closely related Tim10p and Tim12p are Zn^{2+}-binding proteins (Sirrenberg *et al*., 1998). This fact could also explain our unpublished observation that EDTA labilizes the Tim9p–Tim10p complex. A fraction of Tim10p is detected in the dye front during blue native gel electrophoresis (Figure 4B), but Tim9p is never detected in that position. When the 70 kDa complex is released from the context of the 'intact' mitochondrion, Tim10p seemingly dissociates from the 70 kDa complex or a fraction of Tim10p may be stable without Tim9p. Conversely, Tim9p may be degraded preferentially by a protease such as Yme1p (Yta11p) (Leonhard *et al*., 1996; Weber *et al*., 1996) or, alternatively, may be lost by aggregation after dissociation from Tim10p.

We suggest the following model for the import of a multispanning carrier from the mitochondrial surface into the inner membrane (Figure 8). The carrier precursor binds to mitochondrial import receptors, passes through the TOM channel in the outer membrane and binds to the 70 kDa Tim9p–Tim10p complex in the intermembrane space. Binding to the 70 kDa complex is reversible. The complex then shuttles to the surface of the inner membrane and transfers the bound precursor to the membrane-associated Tim12p–Tim22p–Tim54p complex. Transfer of the precursor may involve direct interaction of Tim9p with Tim12p. Tim22p, which is a multicopy suppressor of both *tim54* and *tim12-1* mutants (Kerscher *et al*., 1997; Koehler *et al*., 1998), mediates the ΔΨ-dependent insertion of the carrier into the inner membrane. Finally, the Tim9p–Tim10p complex dissociates from the membrane complex and moves back into the intermembrane space for another round of precursor transfer. It also is possible that the 70 kDa complex dissociates reversibly into smaller units when it docks onto the inner membrane.

Tim9p and Tim10p are the first known essential proteins of the intermembrane space. The requirement for the Tim9p–Tim10p complex may have evolved specifically to chaperone the hydrophobic carrier proteins to the inner membrane, thus preventing aggregation in the aqueous intermembrane space or non-specific insertion into the outer membrane. An analogous system is present in the chloroplast stroma (Cline and Henry, 1996). The light-harvesting chlorophyll *a*/*b*-binding protein (LHCP) is maintained in a soluble form in the stroma prior to integration into the thylakoid membrane by the protein 54CP, a chloroplast homologue of the 54 kDa protein of the mammalian signal recognition particle (SRP) (Payan and Cline, 1991; Li *et al*., 1995). Unlike mammalian SRP, 54CP interacts with precursors, including cytochrome *f* and the Rieske FeS protein (High *et al*., 1997), post-translationally in the chloroplast stroma. 54CP binds to hydrophobic membrane-spanning domains on LHCP and escorts the bound precursor to the thylakoid membrane where it mediates insertion into the membrane; other unidentified proteins, including a 43 kDa polypeptide cpSRP43, also are required for this process (Li *et al*., 1995; Schuenemann *et al*., 1998).

Until now, the function of the mitochondrial intermembrane space has remained elusive. We suggest that Tim9p–Tim10p is the functional unit that is a novel molecular chaperone, guiding the polytopic carriers from the TOM complex to a 300 kDa TIM complex in the inner membrane. Import of the carriers is independent of the Tm17p–Tim23p complex. Further analyses of this pathway should yield interesting insights into the ways in which hydrophobic membrane proteins are sorted within a eukaryotic cell and inserted into the inner membrane.

Materials and methods

Yeast strains and extragenic suppressor screen

Standard genetic techniques were used for growth, manipulation and transformation of yeast strains (Gietz and Sugino, 1988; Guthrie and Fink, 1991). The *S.cerevisiae* strains used in this study are listed in Table I. For the extragenic suppressor screen and multicopy suppression analysis, the previously characterized *tim10-1* and *tim12-1* ts alleles were used (Koehler *et al*., 1998). The *tim10-1* allele was cloned into the integrative vectors pRS305 (*LEU2*) and pRS304 (*TRP1*) (Sikorski and Hieter, 1989) to generate pRS305:*tim10-1* and pRS304:*tim10-1*, respectively. These plasmids were integrated into the *LEU2* or *TRP1* locus in Δ*tim10* strains derived from GA74-1A and GA74-6A (kept viable by *TIM10* on a CEN, *URA3* plasmid). The wild-type *TIM10* plasmid was removed by selection on 5-fluoro-orotic acid; the resulting

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source	Comments
GA74-1A	MAT**a** *ade8 his3 leu2 trp1 ura3*	Jarosch *et al*. (1996)	parental strain
GA74-6A	MATα *ade8 his3 leu2 trp1 ura3*	Jarosch *et al*. (1996)	parental strain
GA74-1A.d	MAT**a**/α *ade8/ade8 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3*	Jarosch *et al*. (1996)	parental strain
CK13	MATα *ade8 his3 leu2 trp1 Δ::HIS3 tim10-1:LEU2*	this study	used for segregation analysis of second-site suppressor locus
CK14	MAT**a** *ade8 his3 leu2 trp1 ura3 Δtim10::HIS3 tim10-1:TRP1*	this study	original mutant used to generate second-site suppressors
CK18	MAT**a** *ade8 his3 leu2 trp1 ura3 Δtim10::HIS3 tim10-1:TRP1 tim9*$^{199A\rightarrow T}$	this study	extragenic suppressor strain identified in this study
CK19	MAT**a**/α *ade8/ade8 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 Δtim10::HIS3/ Δtim10::HIS3 tim10-1:TRP1/tim10-1:LEU2 TIM9/tim9*$^{199A\rightarrow T}$	this study	CK18×CK13
CK16	MATα *ade8 his3 leu2 trp1 ura3 Δtim12::HIS3 tim12-1:LEU2*	this study	*tim12-1* mutant for multicopy suppression analysis

strains were CK13 and CK14. The same approach was used to construct CK16 that expresses only the *tim12-1* allele. Strain CK14 was plated on YPD and spontaneous mutants were selected that grew at 37°C. After 5 days, 30 spontaneous suppressors were identified. After crossing to CK13 followed by tetrad analysis, one suppressing mutation was unlinked to the *tim10-1:LEU2* locus and dominant; this strain was named CK18 and the genotype designated as *tim10-1* Ts^+.

Potential genes coding for the suppressor (including *TIM9*) were amplified from CK18 and the control CK14 by PCR using the proof-reading Expand™ High Fidelity system (Boehringer Mannheim). The amplified fragments were subcloned into pRS316 (CEN, *URA3*). Plasmids were transformed back into the original ts mutant CK14 and suppression was analysed by growing cells in SD media at 25°C to mid-log phase and then serially diluting on YPD and SD plates followed by incubation at 25 and 37°C for 5 days. The *TIM9* gene was sequenced from each independent plasmid. The mutant allele contained an A→T substitution at nucleotide 199 in the coding region, resulting in the replacement of Ser67 by cysteine. The mutant allele is designated *tim9*$^{199A\rightarrow T}$ and the mutant protein Tim9p^{S67C}.

Recombinant DNA techniques and plasmid construction

For *in vitro* transcription/translation, the DNA fragments encoding *TIM9* and *TIM10* were subcloned into pSP65 (Promega). *TIM9* was cloned into pMAL-c2 (Life Technologies) and expressed as a fusion with MBP. Tim9p was cleaved from MBP with factor Xa (Life Technologies) and, after removal of MBP by FPLC over a Mono Q column, injected into rabbits to raise polyclonal antibodies. For cross-linking radiolabelled AAC to Tim9p, AAC was synthesized *in vitro* from pSP65-AAC2.

Import of preproteins into isolated mitochondria

Mitochondria were purified from lactate-grown yeast cells (Glick and Pon, 1995) and assayed for *in vitro* protein import as described previously (Hines *et al*., 1990; Wachter *et al*., 1994; Rospert and Schatz, 1998). Precursors were synthesized in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine after *in vitro* transcription by SP6 polymerase. Import reactions were performed by incubating the reticulocyte lysate containing radiolabelled precursor at 25°C for 10 min with isolated mitochondria in import buffer [2% w/v bovine serum albumin (BSA), 0.6 M sorbitol, 150 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 2 mM ATP, 2 mM NADH, 20 mM HEPES–KOH pH 7.4]. Where indicated, the potential across the mitochondrial inner membrane was dissipated with 1 μM valinomycin and 25 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). Non-imported precursor was removed by treatment with 100 μg/ml trypsin for 30 min at 4°C, and trypsin was inactivated with 200 μg/ml soybean trypsin inhibitor for 5 min at 4°C. For generation of mitoplasts by hypotonic swelling, import reactions were diluted with 9 vols of 20 mM HEPES–KOH, pH 7.4 in the absence or presence of 100 μg/ml proteinase K and incubated for 30 min at 4°C (Glick *et al*., 1992). For alkali extraction, mitochondria from import reactions were sedimented by centrifugation, resuspended at 0.1 mg/ml in 100 mM Na_2CO_3, and incubated for 30 min at 4°C (Fujiki *et al*., 1982). Supernatant and pellet were separated by centrifugation at 100 000 *g* for 15 min. Cross-linking of an AAC translocation intermediate to Tim proteins with dithio-bis(succinimidylpropionate) (DSP) followed by immunoprecipitation was as described previously (Koehler *et al*., 1998).

Purification of the Tim9p–Tim10p complex

Mitochondria (200 mg, 40 mg/ml in 0.6 M sorbitol, 20 mM HEPES–KOH, pH 7.4) were shocked hypotonically by dilution with 9 vols of buffer A (1 mM DTT, 20 mM HEPES–KOH, pH 7.4) and incubated for 1 h at 4°C. After removing mitoplasts by two centrifugation steps (10 min at 10 000 *g*), the soluble intermembrane space was loaded onto a Mono S cation exchange column (0.5×5 cm; Amersham Pharmacia Biotech). The column was washed with buffer A at a flow rate of 0.5 ml/min and bound proteins were eluted with 30 ml of a linear gradient (0–150 mM NaCl) in buffer A. Fractions of 1 ml were collected and analysed by SDS–PAGE followed by immunoblotting or silver staining, or by blue native gel electrophoresis (Schägger *et al*., 1994; Dekker *et al*., 1996). The flow-through that contained Tim9p and Tim10p was loaded onto a Mono Q anion exchange column (0.5×5 cm; Amersham Pharmacia Biotech). The column was washed with buffer A at a flow rate of 0.5 ml/min and bound proteins were eluted with 30 ml of a linear gradient (0–200 mM NaCl) in buffer A. Fractions of 1 ml were collected and analysed as described above. Tim9p and Tim10p co-eluted at 60–80 mM NaCl. These fractions were concentrated to 200 μl in buffer A containing 100 mM NaCl and loaded onto a Superose 12 gel filtration column (1.0×30 cm; Amersham Pharmacia Biotech). The column was developed with buffer A containing 100 mM NaCl at a flow rate of 0.2 ml/min. Again, 1 ml fractions were collected and analysed as described above. The molecular mass standards for the Superose 12 column were RNase A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), BSA (67 kDa) and aldolase (158 kDa).

Stoichiometry of Tim9p and Tim10p in the 70 kDa Tim9p–Tim10p complex was determined by comparative immunoblot analysis. For Tim9p, the standard was pure MBP–Tim9p fusion protein; for Tim10p, the standard was Tim10p that had been cleaved from a GST–Tim10p fusion protein and purified by FPLC. The concentrations of the standards were determined by quantitative amino acid analysis.

Co-immunoprecipitation of Tim9p with Tim10p

Monospecific antibody (10–20 μl per mg of mitochondria) against Tim9p or Tim10p, or against the unrelated control protein cytochrome b_2, was bound to protein A–Sepharose (30 μl wet volume per mg of mitochondria; Amersham Pharmacia Biotech) for 1 h in 1.0 ml wash buffer [20 mM HEPES–KOH pH 7.4, 0.2 M sucrose, 75 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The beads were washed twice to remove unbound antiserum. Mitochondria (20 mg/ml) were converted to mitoplasts by dilution in 9 vols of 20 mM HEPES–KOH pH 7.4, alone or supplemented with 1 mM DTT, 1 mM EDTA or 25 μM $ZnCl_2$. After incubation for 30 min at 4°C and after centrifugation at 10 000 *g* for 10 min, the released intermembrane space fraction was separated from mitoplasts and incubated with the antibody-bound protein A–Sepharose beads by gentle rotation for 2 h at 4°C. After washing the beads twice with wash buffer, bound proteins were extracted at 65°C with SDS-containing sample buffer and analysed by Tricine-SDS–PAGE.

Miscellaneous

Sub-mitochondrial localization of proteins was determined as described previously (Glick *et al*., 1992). Detergent solubilization of mitochondria (2.5 mg/ml) was in 20 mM HEPES–KOH pH 7.4, 50 mM NaCl, 1 mM EDTA and 2.5 mM $MgCl_2$ supplemented with 0.16% *n*-dodecylmaltoside

(Boehringer Mannheim) or 1.0% digitonin (Sigma Chemical); insoluble material was removed by centrifugation at 100 000 *g* for 10 min. Tim9p and Tim10p were analysed by SDS–PAGE using a 10 or 16% polyacrylamide gel and a Tricine-based running buffer (Schägger and von Jagow, 1987). The Tim9p–Tim10p complex was analysed 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). Proteins were detected by immunoblotting using nitrocellulose or PVDF membranes and visualization of immune complexes with either [^{125}I]protein A or the ECL detection kit (for chromatographic purification; Amersham Pharmacia Biotech). Protein concentration was assayed by the bicinchoninic acid method (Pierce) using BSA as the standard.

Sedimentation equilibrium measurements were performed at 20°C in an analytical ultracentrifuge (Beckman XLA) equipped with an optical absorption system. Sedimentation equilibrium was measured with 100 µl samples at 19 000 r.p.m.; absorbance was recorded at 225 nm. The molecular mass was determined using a linear regression computer program that adjusts the baseline absorption to obtain the best linear fit of ln$A$ versus r^2 (A = absorption, r^2 = radial distance from the rotor centre). Measurements were in the presence of 20 mM HEPES-KOH pH 7.4, 100 mM NaCl and 0.5 mM DTT.

Nucleotide sequence accession number

The nucleotide sequence of *TIM9* has been submitted to the DDBJ/EMBL/GenBank database under accession number AF093244.

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