Tim9, a new component of the TIM22-54 translocase in mitochondria

Alexander Adam, Maxi Endres,
Christian Sirrenberg, Friedrich Lottspeich¹,
Walter Neupert and Michael Brunner²

Institut für Physiologische Chemie der Universität München,
Goethestraße 33, 80336 München and ¹Max-Planck-Institut für
Biochemie, Am Klopferspitz 18, 82152 Martinsried, Germany

²Corresponding author
e-mail: brunner@bio.med.uni-muenchen.de

We have identified Tim9, a new component of the TIM22-54 import machinery, which mediates transport of proteins into the inner membrane of mitochondria. Tim9, an essential protein of *Saccharomyces cerevisiae*, shares sequence similarity with Tim10 and Tim12. Tim9 is located in the mitochondrial intermembrane space and is organized into two distinct heterooligomeric assemblies with Tim10 and Tim12. One complex contains Tim9 and Tim10. The other complex contains Tim9, Tim10 and Tim12 and is tightly associated with Tim22 in the inner membrane. The TIM9-10 complex is more abundant than the TIM9-10-12 complex and mediates partial translocation of mitochondrial carrier proteins across the outer membrane. The TIM9-10-12 complex assists further translocation into the inner membrane in association with TIM22-54.

Keywords: mitochondria/protein translocation/Tim9/
TIM22-54 complex

Introduction

Mitochondria contain preprotein translocases in their outer and inner membranes which mediate import and sorting of nuclear-encoded precursor proteins to mitochondrial subcompartments. The outer membrane contains a general preprotein translocase, the TOM complex, which is composed of surface-exposed preprotein receptors and a protein-conducting channel across the outer membrane (Schatz, 1996; Dietmeier *et al.*, 1997; Neupert, 1997; Pfanner and Meijer, 1997). The inner membrane contains two preprotein translocases, the TIM17-23 complex and the TIM22-54 complex, which differ in their specificity for preproteins (Sirrenberg *et al.*, 1996, 1997, 1998; Kerscher *et al.*, 1997; Koehler *et al.*, 1998). Both TIM complexes cooperate with the TOM complex in the outer membrane. Precursors which contain a positively charged N-terminal matrix targeting signal are imported by the TIM17-23 complex (Bauer *et al.*, 1996; Sirrenberg *et al.*, 1996). The import of such precursors into the matrix requires the membrane potential $\Delta\psi$ across the inner membrane and ATP in the matrix (Schatz, 1996; Neupert, 1997; Pfanner and Meijer, 1997). The $\Delta\psi$ is required for translocation of the presequence across a channel that

contains Tim17 and Tim23 (Berthold *et al.*, 1995; Bauer *et al.*, 1996). The peripheral membrane protein Tim44 is associated with the outlet of this channel (Schneider *et al.*, 1994; Berthold *et al.*, 1995). It recruits the mitochondrial chaperone mt-Hsp70 to the sites of protein import, which drives further translocation with ATP as an energy source (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994, 1996). Precursors that contain either N-terminal or internal matrix targeting signals together with sorting signals for the intermembrane space or the inner membrane are also imported via the TIM17-23 complex (Fölsch *et al.*, 1996; Sirrenberg *et al.*, 1996).

Many integral inner membrane proteins, such as the ADP/ATP carrier, AAC, and other members of the mitochondrial carrier family, are synthesized without a classical matrix targeting signal (Smagula and Douglas, 1988a,b; Kübrich *et al.*, 1998). Such precursors are imported via the TIM22-54 complex (Sirrenberg *et al.*, 1996, 1997, 1998; Kerscher *et al.*, 1997; Koehler *et al.*, 1998).

Import of carrier proteins can be dissected into distinct stages (Pfanner and Neupert, 1987; Wachter *et al.*, 1992; Sirrenberg *et al.*, 1996; Kübrich *et al.*, 1998). The precursors are recognized by specialized receptors of the TOM complex. They are then transferred across the TOM channel and interact with Tim10 in the intermembrane space (Koehler *et al.*, 1998; Sirrenberg *et al.*, 1998). The precursor is then transferred to Tim12, which is associated with Tim22 at the outer face of the inner membrane (Sirrenberg *et al.*, 1996, 1998; Koehler *et al.*, 1998). When the mitochondrial membrane potential $\Delta\psi$ is dissipated, the AAC precursor accumulates as an intermediate (stage 3) that is largely translocated across the outer membrane, but not yet inserted into the inner membrane (Pfanner and Neupert, 1987). This reaction requires Tim10 but not Tim12 or Tim22 (Koehler *et al.*, 1998; Sirrenberg *et al.*, 1998). In the presence of a membrane potential, $\Delta\psi$, Tim22 mediates the insertion of the AAC precursor into the inner membrane (Sirrenberg *et al.*, 1996).

Here we report the identification and characterization of Tim9, a new component of the import machinery for carrier proteins. Tim9 is located in the intermembrane space and shares sequence similarity with Tim10 and Tim12. Tim9 is essential and it occurs in a complex with Tim10 in the intermembrane space and in a complex with Tim10 and Tim12 which is associated with the inner membrane. The TIM9-10 complex is required for import of the precursor across the outer membrane at an early stage of the import pathway.

Results

Interaction of Tim10 and Tim12 with Tim9

To identify components of the mitochondrial import machinery that interact with Tim10, yeast cells were

grown in the presence of [35 S]sulfate and mitochondria were prepared. The mitochondria were solubilized with Triton X-100 and immunoprecipitation with antibodies against Tim10 was performed (Figure 1A). The immunoprecipitate contained three radiolabelled proteins with an electrophoretic mobility corresponding to mol. wts of 10, 11 and 12 kDa. By immunoblotting, the 10 and 12 kDa proteins were identified as Tim10 and Tim12, respectively (Figure 1A), indicating that both components are in a complex. Tryptic peptides were generated from the 11 kDa protein and subjected to sequencing. Two peptide sequences were obtained which matched a small unidentified open reading frame (ORF) of 87 amino acid residues on chromosome V. The ORF encodes a putative protein with a predicted molecular mass of 10.2 kDa. As the protein is smaller than Tim10, it will henceforth be referred to as Tim9. Tim9 shares significant sequence similarity with Tim10 and Tim12 (Figure 1B). In particular, four cysteine residues which constitute a metal-binding site in Tim10 and in Tim12 (Sirrenberg *et al.*, 1998) are also conserved in the Tim9 sequence, suggesting that Tim9 may be a zinc finger protein.

Genetic interaction of Tim9 with the Tim22-dependent import machinery

To study the function of Tim9, we disrupted one *TIM9* allele in the diploid strain MB2 (Maarse *et al.*, 1992) by integration of the *HIS3* gene. The cells were then allowed

Fig. 1. Identification of Tim9. (A) Co-immunoprecipitation of Tim9 and Tim12 with Tim10. Mitochondria from yeast cells that were metabolically labelled with [35 S]sulfate (left panel) and mitochondria from unlabelled cells (right panels) were solubilized with Triton X-100 and subjected to immunoprecipitation with antiserum against Tim10. The precipitates were analysed by SDS/urea-PAGE and phosphorimaging, and by Western blotting and immunodecoration with Tim12 and Tim10 antibodies. Tim9 was excised from a gel after preparative immunoprecipitation using 1 mg of mitochondrial protein and subjected to tryptic digestion. Sequences of two peptides are shown. (B) Sequence alignment of Tim12, Tim10 and Tim9. Shaded areas indicate similarities. The four conserved cysteine residues that constitute the metal-binding site in Tim12 and Tim10 are indicated by asterisks. The underlined regions correspond to the amino acid sequences of tryptic peptides derived from Tim9 (see A).

to sporulate and spores were subjected to tetrad analysis. Upon dissection, each tetrad yielded only two viable spores (Figure 2A). All viable spores carried the non-disrupted *TIM9* allele (not shown). This indicates that *TIM9* is essential for the viability of *Saccharomyces cerevisiae*.

Using a chromosomal replacement cassette (Sirrenberg *et al.*, 1996, 1998), we constructed the haploid yeast strain Tim9(gal), which expressed *TIM9* under control of the *Gal10* promoter (Figure 2B). When these cells were shifted to galactose-free medium, they stopped growing after 3–4 days (Figure 2C). In the presence of galactose in the growth medium, Tim9(gal) cells grew like the parental MB2 strain (not shown).

Mitochondria were prepared from Tim9(gal) cells which had been shifted for 2 days to galactose-free growth medium (Tim9 $\downarrow$). At this stage, the cells still grew like control cells (WT). The mitochondria were analysed by Western blotting for their content of selected proteins (Figure 2D). Tim9 $\downarrow$ mitochondria contained normal levels of the outer membrane protein Tom40, of the intermembrane space protein cytochrome *b*₂ and of the matrix protein Tim44. Furthermore, the levels of AAC and Tim23 were normal in these mitochondria. Tim9 $\downarrow$ mitochondria contained reduced levels of Tim9, Tim10, Tim12 and Tim22. Thus, Tim9 is required to maintain normal levels of components of the Tim22-dependent import machinery.

When Tim9(gal) cells were cultured for 4 days in the absence of galactose, they contained reduced levels of AAC and Tim23 (not shown), which are both imported via the Tim22-dependent import pathway (Sirrenberg *et al.*, 1996; Káldi *et al.*, 1998).

Requirement for Tim9 for import of mitochondrial carrier proteins

Tim9 $\downarrow$ mitochondria from Tim9(gal) cells grown for 2 days in the absence of galactose were tested for their ability to import mitochondrial precursor proteins *in vitro*. Radiolabelled precursor proteins were synthesized in reticulocyte lysate and incubated with energized mitochondria. Precursor proteins with a cleavable N-terminal matrix targeting signal, which are imported via the TIM17-23 complex (Sirrenberg *et al.*, 1996, 1997, 1998), were found to be imported into Tim9 $\downarrow$ mitochondria (Figure 3A) and sorted correctly to the mitochondrial matrix [pSu9 (1–79)DHFR, pb Δ 19(1–167)DHFR] and the inner membrane (pCyt. *c*₁-DHFR). In contrast, the ADP/ATP carrier, AAC, the phosphate carrier, PiC, and Mrs3, a member of the carrier family with unknown function (Wiesenberger *et al.*, 1991), were imported into Tim9 $\downarrow$ mitochondria with greatly reduced efficiency. This indicates that Tim9 is required specifically for import of precursors via the Tim22-dependent import pathway.

To investigate at which stage along the import pathway Tim9 is required, the AAC precursor was imported into mitochondria in the presence and absence of a membrane potential, $\Delta\psi$. In WT mitochondria, the AAC precursor became imported efficiently across the outer membrane if $\Delta\psi$ was present, indicated by the resistance of the precursor to treatment of mitochondria with high levels of proteinase K (PK) (Figure 3B, upper left panel). When these mitochondria were converted to mitoplasts and treated with PK, the AAC was clipped to a fragment (f), which is

indicative of its insertion into the inner membrane (Pfanner and Neupert, 1987; Sirrenberg *et al.*, 1996; Kübrich *et al.*, 1998). In the absence of a $\Delta\psi$, the AAC precursor was partially resistant to treatment of mitochondria with PK but was degraded in mitoplast preparations (Figure 3B, upper right panel), demonstrating accumulation at stage 3. When Tim9 $\downarrow$ mitochondria were incubated with the AAC precursor, it associated efficiently with the mitochondria. However, it was degraded by PK in the presence as well as in the absence of $\Delta\psi$ (Figure 3B, lower panels). This indicates that the AAC cannot reach stage 3 in mitochondria that lack Tim9.

When arrested at stage 3, the AAC precursor was cross-linked efficiently to Tim10 (Sirrenberg *et al.*, 1998), while cross-linking of AAC with Tim9 under such conditions was not observed (not shown). This may be due to

inefficiency of chemical cross-linking or it may reflect that Tim9 does not interact directly with the AAC precursor.

Organization of Tim9, Tim10 and Tim12 in the intermembrane space

Mitochondria were incubated in hypotonic buffer to generate mitoplasts. The mitoplasts were then either left untreated or were treated with 0.5 M NaCl to release peripheral membrane proteins. Subsequently, the mitoplasts were re-isolated by centrifugation, and proteins in the membrane pellet and in the supernatant were analysed (Figure 4A). Tim12 was recovered exclusively with the mitochondrial membranes. It was not released from the membranes by 0.5 M NaCl, indicating that it was tightly associated with the mitoplasts. The majority of Tim10 was released from the intermembrane space upon swelling of the mitochondria; however, a small fraction remained with the mitoplasts. This fraction was not released from the membranes by treatment of mitoplasts with buffer containing 0.5 M NaCl. Tim9 behaved like Tim10; the majority of the protein was released in mitoplast preparations, while a fraction remained membrane associated in a salt-resistant manner. Thus, the mitochondrial intermembrane space contains one species of Tim12 which is tightly associated with the inner membrane, but apparently two species of Tim9 and of Tim10; the majority of Tim9 and Tim10 are released readily when the intermembrane space is opened, while a small fraction of both proteins remains tightly associated with mitochondrial membranes.

To characterize the organization of Tim9, Tim10 and Tim12 in the intermembrane space, mitochondria were prepared from yeast cells that were metabolically labelled with [35 S]sulfate. The mitochondria were solubilized with digitonin, which preserves the TIM22-54 complex (Sirrenberg *et al.*, 1996, 1998), and the detergent extract was subjected to gel filtration. Tim22 eluted in a fraction corresponding to a molecular mass of 300 kDa (not shown). Fractions were subjected to immunoprecipitation with antibodies against Tim10 (Figure 4B). Tim12 co-eluted with Tim22 in the 300 kDa form, confirming that Tim12 is associated with Tim22

Fig. 2. *TIM9* is required for viability of *S. cerevisiae*. (A) Dissection of tetrads after sporulation of a *TIM9* heterozygous diploid (a/a MB2 *TIM9/tim9::HIS3*). (B) Construction of a *TIM9* replacement cassette. A PCR fragment corresponding to the *LEU2* gene and the *Gal10* promoter with extensions corresponding to the *TIM9* 5'-untranslated region and the *TIM9* ORF, respectively, is schematically outlined. The fragment was amplified from Yep51 using the oligonucleotide primers Us and Ls. Upper strand: 50 nucleotides correspond to the 5'-untranslated region of *TIM9* and 20 nucleotides to the 5'-untranslated region of the *LEU2* gene of Yep51. Lower strand: 49 nucleotides correspond to the N-terminus of the *TIM9* ORF followed by 25 nucleotides corresponding to the transcription start of the *Gal10* promoter in Yep51. An *S. cerevisiae* MB2 haploid was transfected with the PCR fragment. *LEU*⁺ clones were selected and analysed for proper integration of the *Gal10* promoter in front of the *TIM9* ORF. The cells are referred to as Tim9(gal) cells. (C) Down-regulation of *TIM9* affects cell growth. Tim9(gal) cells and control cells (WT) were shifted to galactose-free medium. Cell growth was monitored by increase in absorbance at 600 nm. The cell number at day 2 was set equal to 1. (D) Depletion of Tim9 affects the Tim22-dependent import machinery. Mitochondria were prepared from Tim9(gal) cells (Tim9 $\downarrow$) and from WT cells which were shifted for 2 days to galactose-free medium. Mitochondria were probed by immunoblotting for the indicated antigens. Cyt. *b*₂, cytochrome *b*₂; AAC, ADP/ATP carrier.

(Koehler *et al.*, 1998; Sirrenberg *et al.*, 1998). In contrast, only ~25% of Tim9 and of Tim10 eluted in the 300 kDa form. The remaining Tim10 and Tim12 eluted corresponding to a molecular mass of ~70 kDa. When mitochondria were solubilized with Triton X-100, which disrupts the Tim22-54 complex, Tim12, Tim10 and Tim9 were found exclusively in 70 kDa complexes (not shown).

To analyse the composition of the 70 kDa complexes, mitochondria were solubilized with Triton X-100 and immunoprecipitation was performed. Antibodies against Tim12 and Tim10 precipitated the same set of three radiolabelled proteins (Figure 4C, left panel), which were identified immunologically as Tim9, Tim10 and Tim12 (data not shown). The quantities and stoichiometries of Tim9, Tim10 and Tim12 were, however, different in the two immunoprecipitates, Tim10 antibodies depleted Tim12 from mitochondrial extracts, but Tim12 antibodies did not deplete Tim10 (Figure 4C, right panel). This

suggests that there are two forms of 70 kDa complexes which are composed of different subunits. To estimate the molar ratios of the subunits in the 70 kDa complexes, we quantified the precipitated Tim components with a phosphorimaging system (Sirrenberg *et al.*, 1997). Correcting the values for the different contents of methionines and cysteines of the three proteins, the molar ratio of Tim12:Tim10:Tim9 was ~1:1.8:3.2 in the immunoprecipitate with Tim12 antibodies and ~1:8.3:13.6 when antibodies against Tim10 were used (Figure 4C, left panel). As mitochondria were solubilized with detergent, Tim10 antibodies precipitated both the membrane-associated and the soluble forms of 70 kDa complexes. The majority of Tim10 was not tightly associated with mitochondrial membranes. To analyse this fraction, radiolabelled mitochondria were incubated with hypotonic buffer to rupture the outer membrane by osmotic shock. The mitochondrial membranes were then removed by centrifugation, and the Tim proteins released from the intermembrane space were analysed by immunoprecipitation (Figure 4D, left panel). Antibodies against Tim10 precipitated Tim10 and Tim9, but no Tim12. Likewise, Tim9 antibodies precipitated Tim9 and Tim10, but no Tim12. The molar ratio of Tim10:Tim9 was ~1:1.35 in the immunoprecipitate with antibodies against Tim10, and 1:1.15 with antibodies against Tim9 (Figure 4D, right panel).

Together, the data indicate that the mitochondria contain two species of 70 kDa complexes, which may be composed of six or seven subunits. The TIM9-10-12 complex contains Tim9, Tim10 and Tim12 and is tightly associated with the mitochondrial inner membrane. The TIM9-10 complex contains Tim9, Tim10, but no Tim12. It is more abundant than the TIM9-10-12 complex and it appears to be mobile in the intermembrane space.

Discussion

We report the identification and characterization of Tim9, a new component of the Tim22-dependent import pathway for nuclear-encoded mitochondrial preproteins without a presequence (Sirrenberg *et al.*, 1996). Like the other components of this translocation machinery, Tim9 is essential for the viability of *S.cerevisiae* (Jarosch *et al.*, 1996, 1997; Sirrenberg *et al.*, 1996, 1998; Kerscher *et al.*, 1997; Koehler *et al.*, 1998). Tim9 is structurally related to Tim10 and Tim12. In particular, the proteins share four conserved cysteine residues, which constitute a metal-binding site in Tim10 and in Tim12 (Sirrenberg *et al.*, 1998). Tim9 is therefore probably also a zinc finger protein. Like Tim10 and Tim12, Tim9 is located in the mitochondrial intermembrane space. These three proteins form two types of assemblies with apparent molecular masses of ~70 kDa. One complex, the TIM9-10 complex, may consist of 3–4 molecules of Tim9 and 2–3 molecules of Tim10. The second complex, the TIM9-10-12 complex, contains Tim9, Tim10 and Tim12 and may consist of three or four subunits of Tim9, two Tim10 and one Tim12 molecule. The TIM9-10-12 complex is present in equimolar amounts with Tim22 (C.Sirrenberg, unpublished) and it is tightly associated with mitochondrial membranes. The TIM9-10 complex is 3- to 4-fold more abundant than the TIM9-10-12 complex. The association of the TIM9-10 complex with mitochondrial membranes seems to be much

Fig. 3. Tim9 is required for import of carrier proteins. (A) Import of precursors into Tim9 mitochondria. Radiolabelled precursor proteins with a matrix targeting signal, pCyt. c₁-DHFR, pSu9(1-79)DHFR and pb₂Δ19(1-167)DHFR, and precursors of mitochondrial carrier proteins, AAC, PiC (phosphate carrier) and Mrs3, were imported into energized WT and Tim9 mitochondria. Mitochondria were treated with proteinase K (PK), samples were analysed by SDS-PAGE and the imported precursors were quantified with a phosphorimaging system. Import into Tim9 mitochondria is expressed as a percentage of import into WT mitochondria (% of control). (B) Tim9 is required for accumulation of AAC at stage 3. The AAC precursor was imported into WT and Tim9 mitochondria in the presence and absence of a membrane potential, Δψ. Left panels: mitochondria were treated with PK (200 μg/ml) to measure translocation of AAC across the outer membrane, or mitoplasts (MP) were generated and treated with PK (200 μg/ml) to determine insertion into the inner membrane, indicated by formation of fragment f. Right panels: samples were treated with PK (30 μg/ml) to measure stage 3 import, indicated by PK-resistant precursor and formation of f*.

looser than that of the TIM9-10-12 complex. Thus, Tim12, which is not present in the TIM9-10 complex, appears to confer the tight association of the TIM9-10-12 complex with Tim22 in the inner membrane. Both Tim9-containing complexes are required for import of carrier proteins into the inner membrane.

What drives the translocation of precursors across the outer membrane and what is the role of these two 70 kDa complexes? The TIM9-10 complex, which is present in excess over the TIM9-10-12 complex, may be mobile in the intermembrane space and screen the TOM complexes for precursors that use the Tim22-dependent import pathway into the inner membrane (Figure 5). It then binds the

translocation intermediates and thereby mediates their stable association with the mitochondria and partial translocation across the outer membrane at an early step in the import pathway (stage 3a). For further import of carrier proteins, the TIM9-10 complex then cooperates with the TIM9-10-12 complex on the outer face of the inner membrane. The interaction of the TIM9-10 complex with the membrane could be triggered by precursor binding. At present, it cannot be distinguished whether the transfer of AAC precursor to the inner membrane is accompanied by movement of Tim9 and Tim10 of the TIM9-10 complex into the TIM9-10-12 or whether only the precursor is transferred to the TIM9-10-12 complex. Finally, the carrier is passed on to TIM22-54 which mediates its $\Delta\psi$ -dependent insertion into the inner membrane (Sirrenberg *et al.*, 1996; Kerscher *et al.*, 1997).

The putative zinc fingers of Tim10 and of Tim12 have been suggested to interact with precursors of carrier proteins via a pattern of charged and apolar amino acid residues (Sirrenberg *et al.*, 1998). This pattern in Tim10 and Tim12 is complementary to the so-called carrier signature, a conserved element that is repeated three times in all members of the carrier family (Saraste and Walker, 1982; Palmieri, 1994; Nelson *et al.*, 1998). Tim9 does not contain such a pattern, and thus may not interact with the carrier signature. Tim9 could bind to a different, as yet unrecognized motif, it might be involved in an interaction

Fig. 4. Interaction of Tim9 with components of the Tim22-54 import machinery. **(A)** Membrane association of Tim12, Tim9 and Tim10. Mitochondria were washed with SEM buffer and mitoplasts were generated by incubation of the mitochondria in 10 mM HEPES-KOH, pH 7.2. The samples were then halved. One half received an equal volume of 1 M NaCl HEPES-KOH, pH 7.2 and the second half received HEPES-KOH, pH 7.2. Samples were incubated further for 30 min on ice. Mitoplasts and NaCl-treated mitoplasts were then re-isolated by centrifugation. Proteins from the supernatants subsequently were precipitated with trichloroacetic acid (TCA). Membranes were washed with 10 mM HEPES-KOH, pH 7.2 and then solubilized in sample buffer. Mitochondria (M), mitoplasts (MP) and TCA-precipitated supernatants (S) were analysed by SDS/urea-PAGE and Western blotting. **(B)** Gel-filtration analysis. Mitochondria (0.5 mg) prepared from [35 S]sulfate-labelled yeast cells were solubilized with 1% digitonin, subjected to a clarifying spin and chromatographed on a Superose 6 gel filtration column (25 ml bed volume, 0.2 ml/min). Fractions were subjected to immunoprecipitation with antibodies against Tim10. The precipitates were analysed by SDS/urea-PAGE (upper part) and quantified with a phosphorimaging system (lower panels). The column was calibrated using the following molecular mass standards: thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa). **(C)** Immunoprecipitation of the TIM9-10 complex and the TIM9-10-12 complex. Radiolabelled mitochondria were solubilized with Triton X-100 and subjected to immunoprecipitation with antibodies against Tim12 and Tim10, and with control IgG. The immunoprecipitated proteins were analysed by SDS/urea-PAGE and phosphorimaging (left panel) and the precipitated proteins were quantified (right panel). To determine molar ratios of proteins, the phosphorimager signals were corrected for differences in the contents of sulfur-containing amino acid residues. The amount of Tim12 precipitated with Tim12 antibodies was set equal to 1. Error bars represent the standard deviation of five immunoprecipitations. **(D)** Immunoprecipitation of the TIM9-10 complex. 35 S-labelled mitochondria were converted into mitoplasts as described in (A). The mitoplasts were removed by centrifugation and the supernatant was subjected to immunoprecipitation with antibodies against Tim9 and Tim10. Samples were analysed and quantified as described in (C). Columns represent the mean of two immunoprecipitations. The amount of Tim10 in each precipitate was set equal to 1.

Fig. 5. Model for import of the AAC precursor. The cytosolic AAC precursor (stage 1) binds to receptors of the TOM machinery on the surface of the mitochondria (stage 2). Partial translocation of AAC across the outer membrane is facilitated by the TIM9-10 complex which binds to the translocation intermediate on the inner side of the outer membrane (stage 3a). The precursor is then transferred to the TIM9-10-12 complex (stage 3b) on the outer face of the inner membrane. In this step, only the precursor could be transferred or the transfer may be accompanied by movement of Tim9 and Tim10 from the TIM9-10 complex into the TIM9-10-12 complex. The TIM9-10-12 complex is associated with TIM22-54 complex which mediates then insertion of the precursor into the inner membrane (stage 4) in a reaction that requires the membrane potential, $\Delta\Psi$.

with the TOM machinery or it could be required for the transfer of precursors from the TIM9-10 complex to the TIM9-10-12 complex. As Tim9 is necessary for maintaining normal levels of Tim10 and Tim12, it could serve a role in import of these proteins into the intermembrane space.

Materials and methods

Disruption of TIM9

A PCR fragment was amplified from pFA6a-HIS3MX6 (Wach *et al.*, 1997) which carries the *HIS3* gene of *Scizosaccharomyces pombe* using the following oligonucleotide primers: primer 1, CAA ATT AGA GCT TCA ATT TAA TTA TAT CAG TTA TTA CCC GGG AAT CTC GGT CGT AAT GAT CGT ACG CTG CAG GTC GAC; and primer 2, CTA AAA ATG GGC TAC ACC AGA GAT ACA TAA TTA GAT ATA TAT ACG CCA GTA CAC CAT CGA TGA ATT CGA GCT CG. Nucleotides in bold hybridize to the *HIS3* transcription unit. The 3' extensions of primers 1 and 2 correspond to the 5'- and 3'-untranslated region of *TIM9*, respectively. The diploid *S.cerevisiae* strain MB2 was transfected with the PCR fragment and transformants were selected on selective minimal medium without histidine (Maarse *et al.*, 1992).

Integration of the *Gal10* promoter in front of *TIM9*

The construction of a replacement cassette is described in Figure 2B. The replacement cassette was amplified by PCR using Yep51 as DNA template. The primers used were primer 3, CAA ATT AGA GCT TCA ATT TAA TTA TAT CAG TTA TTA CCC GGG AAT CTC GGT GTG GGA ATA CTC AGG TAT C; and primer 4, CCA CTA CTT TTT GGA ACT CTT GTT GTT CTT TGG AGT TCA ATG CGT CCA TTG AAT TTT CAA AAA TTC TTA CTT TT. *Saccharomyces cerevisiae* MB2 (Maarse *et al.*, 1992) was transfected with the replacement cassette. Transformants were selected on SD lactate medium without leucine and in the presence of 1% galactose. Integration of the *LEU2* marker and the *Gal10* promoter in front of *TIM9* was verified by PCR. Mitochondria were isolated as previously described (Berthold *et al.*, 1995).

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 (Káldi *et al.*, 1998). Import reactions were carried out for 5 min at 25°C in 100 μ l of import buffer [600 mM sorbitol, 3 mg/ml bovine serum albumin (BSA), 80 mM KCl, 10 mM MgCl₂, 2.5 mM EDTA, 2 mM KH₂PO₄, 2 mM ATP, 5 mM NADH, 50 mM HEPES-KOH, pH 7.2]. An assay contained 30–50 μ g of mitochondria and 1–3% reticulocyte lysate with the radiolabelled precursor protein. For

generation of a high membrane potential, mitochondria were pre-incubated for 5 min at 25°C in the presence of 2.5 mM malate, 2.5 mM succinate, 5 mM phosphocreatine, 50 μ g/ml creatine kinase and 2 mM ATP. Membrane potential was dissipated by the addition of 1 μ M valinomycin and 25 mM KCl. For generation of mitoplasts, the import assay was diluted in 10 vols of swelling buffer (10 mM HEPES-KOH pH 7.2, 0.3% BSA). Protease treatment of mitochondria and mitoplasts was carried out on ice for 20 min.

Immunoprecipitation

Yeast cells were grown overnight at 30°C in the presence of [35 S]sulfate (300 mCi/mol). Mitochondria were prepared and aliquots corresponding to 50 μ g of protein were solubilized in 20 mM HEPES-KOH pH 7.4, 100 mM KCl. After a clarifying spin, the supernatant was subjected to immunoprecipitation with anti-Tim9 IgG (10 μ g), with affinity-purified anti-Tim12 IgG (0.5 μ g) or affinity-purified anti-Tim10 IgG (0.5 μ g), respectively. After gel filtration, 1% Triton X-100 was added to the fractions prior to immunoprecipitation. Immunoprecipitates were analysed by SDS/urea-PAGE (Künkele *et al.*, 1998) and quantified with a phosphorimaging system.

Acknowledgements

We thank C.Kotthoff for expert technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 184 (Teilprojekt B2 and B12), the Human Frontiers of Science Program, the Fonds der Chemischen Industrie and the Münchener Medizinische Wochenschrift (to M.B.).

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Received October 8, 1998; revised November 13, 1998;
accepted November 16, 1998