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Multiple interactions of components mediating preprotein translocation across the inner mitochondrial membrane

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The protein transport machinery of the inner mitochondrial membrane contains three essential Tim proteins. Tim17 and Tim23 are thought to build a preprotein translocation channel, while Tim44 transiently interacts with the heat shock protein Hsp70 to form an ATP-driven import motor. For this report we characterized the biogenesis and interactions of Tim proteins. (i) Import of the precursor of Tim44 into the inner membrane requires mtHsp70, whereas import and inner membrane integration of the precursors of Tim17 and Tim23 are independent of functional mtHsp70. (ii) Tim17 efficiently associates with Tim23 and mtHsp70, but only weakly with Tim44. (iii) Depletion of Tim44 does not affect the co-precipitation of Tim17 with antibodies directed against mtHsp70. (iv) Tim23 associates with both Tim44 and Tim17, suggesting the presence of two Tim23 pools in the inner membrane, a Tim44–Tim23-containing sub-complex and a Tim23–Tim17-containing sub-complex. (v) The association of mtHsp70 with the Tim23–Tim17 sub-complex is ATP sensitive and can be distinguished from the mtHsp70–Tim44 interaction by the differential influence of an amino acid substitution in mtHsp70. (vi) Genetic evidence, suppression of the protein import defect of a tim17 yeast mutant by overexpression of mtHsp70 and synthetic lethality of conditional mutants in the genes of Tim17 and mtHsp70, supports a functional interaction of mtHsp70 with Tim17. We conclude that the protein transport machinery of the mitochondrial inner membrane consists of dynamically interacting sub-complexes, each of which transiently binds mtHsp70.

Keywords: Hsp70/mitochondria/protein import/ Saccharomyces cerevisiae/Tim complex

Introduction

The mitochondrial outer and inner membranes contain specific transport machineries for the import of nuclear encoded precursors (Pfanner et al., 1994, 1996; Lithgow et al., 1995; Ryan and Jensen, 1995; Lill and Neupert, 1996; Schatz and Dobberstein, 1996). Preproteins, which often carry N-terminal targeting sequences (presequences), are recognized by receptor proteins anchored in the mitochondrial outer membrane. The receptors assemble with other outer membrane proteins to form a dynamic complex, the translocase of the outer mitochondrial membrane (Tom). The Tom machinery includes a general import pore that is responsible for membrane translocation of preproteins. Recent studies indicate that the Tom machinery, consisting of at least nine distinct polypeptides, is not a static protein complex. Several sub-complexes can be distinguished that assemble and dissociate in a dynamic fashion (Alconada et al., 1995b; Gratzer et al., 1995; Mayer et al., 1995; Bömer et al., 1996a; Haucke et al., 1996; Hönlinger et al., 1996).

The preprotein translocase of the inner mitochondrial membrane (Tim) can operate independently of the Tom machinery. This was shown by the direct import of preproteins across the inner membrane of mitochondria with a ruptured outer membrane (Ohba and Schatz, 1987; Hwang et al., 1989). The Tom and Tim machineries can be transiently connected via a precursor polypeptide in transit (Hörst et al., 1995). Three essential proteins of the Tim machinery have been identified in the yeast Saccharomyces cerevisiae: Tim44 (Maarse et al., 1992; Scherer et al., 1992), Tim23 (Dekker et al., 1993; Emtage and Jensen, 1993) and Tim17 (Maarse et al., 1994; Ryan et al., 1994) (the new uniform nomenclature for the proteins is described in Pfanner et al., 1996). Tim17 and Tim23 are integral membrane proteins, whereas Tim44 behaves as a peripheral membrane protein that is mainly located on the matrix side of the inner membrane. All three Tim proteins have been shown to be in close contact with a preprotein in transit by the use of cross-linking reagents (Scherer et al., 1992; Blom et al., 1993; Hörst et al., 1993; Ryan and Jensen, 1993; Kübrich et al., 1994; Berthold et al., 1995).

The initial stage of transport of a preprotein into or across the inner membrane is driven by the membrane potential Δψ, possibly by exerting an electrophoretic effect on the positively charged presequence (Martin et al., 1991). The unfolded precursor polypeptide chain emerging on the matrix side is then bound by mtHsp70, an abundant 70 kDa heat shock protein of the matrix (Kang et al., 1990; Scherer et al., 1990). mtHsp70 is essential for the viability of yeast (Craig et al., 1987) and for driving further import of the bound precursor polypeptide (Gambill et al., 1993; Glick et al., 1993; Voos et al., 1993, 1996; Stuart et al., 1994; Ungermann et al., 1996). A fraction (~10–15%) of mtHsp70 was found to transiently interact with Tim44 (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). The complex between Tim44 and mtHsp70 is dissociated by binding of ATP (von Ahsen
et al., 1995; Horst et al., 1996). In addition, complex formation between Tim23, Tim17 (Berthold et al., 1995; Blom et al., 1995) and Tim44 was reported (Berthold et al., 1995). It was concluded that a preprotein transport channel formed by Tim17 and Tim23 is coupled to the ATP-driven import motor mtHsp70 via association with Tim44.

For this report we studied the biogenesis and assembly of the Tim proteins and found that import and membrane integration of the precursors of Tim17 and Tim23 into the inner membrane did not depend on functional mtHsp70. We then analyzed the interactions of the membrane-integrated form of Tim17. Surprisingly, we observed that Tim17 was specifically co-precipitated with antibodies directed against mtHsp70 in a Tim44-independent manner. Genetic results support a functional interaction of Tim17 with mtHsp70. We conclude that Tim17 (or a closely associated protein) functions as a second membrane anchor for mtHsp70 at the Tim complex. The results suggest a dynamic view of the protein transport machinery of the mitochondrial inner membrane: the Tim complex can dissociate into sub-complexes (Tim17–Tim23 and Tim23–Tim44), each of which transiently interacts with a fraction of mtHsp70 in the course of preprotein translocation into mitochondria.

Results

Mitochondrial import and integration of Tim17 and Tim23 into the inner membrane are independent of mtHsp70 function

The precursors of yeast Tim17, Tim23 and Tim44 were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine/[35S]cysteine and incubated with isolated yeast mitochondria (Blom et al., 1993; Dekker et al., 1993; Maarse et al., 1994; Alconada et al., 1995a). To test for the dependence of mitochondrial import on the function of mtHsp70, mitochondria were used from a S.cerevisiae strain containing a point mutation within the gene SSC1 encoding mtHsp70. The scc1-3 allele is conditional lethal and confers a temperature-sensitive phenotype for viability. At non-permissive temperatures the mutant mtHsp70 (Scc1-3p) does not interact with precursor polypeptides and thus import of mtHsp70-dependent preproteins is blocked in scc1-3 mitochondria (Gambill et al., 1993; Voos et al., 1993, 1996; Gärtner et al., 1995a,b). The scc1-3 mitochondria (and control wild-type mitochondria) were preincubated at 37°C to induce the mutant phenotype (Gambill et al., 1993). The import phenotype of the mutant mitochondria was determined with the precursor of the β subunit of mitochondrial F1-ATPase. Import of F1β to a protease-protected location was inhibited in scc1-3 mitochondria (Figure 1A, lanes 6–9; compare with lanes 1–4, wild-type).

Similarly, import of Tim44 into scc1-3 mitochondria was blocked (Figure 1A, lanes 6–9, and B, left panel), indicating that import of Tim44 depended on functional mtHsp70. The import of Tim17 and Tim23 to a protease-protected location, however, was not affected by the scc1-3 mutation (Figure 1A, lanes 6–9). Quantification revealed identical import kinetics for Tim23 and Tim17 into scc1-3 and wild-type mitochondria (Figure 1B, middle and right panels). Dissipation of the membrane potential Δψ across the inner membrane reduced the import of Tim23 and Tim17 into wild-type and scc1-3 mitochondria in a comparable manner (Figure 1A, lanes 5 and 10; see also Figure 1D below), demonstrating specific import and excluding the possibility that the protease-protection of Tim17 and Tim23 observed with scc1-3 mitochondria was due to non-specific aggregation. Independence of import from the scc1-3 mutation has previously been found for some proteins that are sorted at the inner membrane, in particular for proteins transported to the intermembrane space, as shown here with a fusion protein between part of cytochrome b and dihydrofolate reductase (Figure 1A; Voos et al., 1993, 1996; Gärtner et al., 1995b).

We then tested if Tim17 and Tim23 imported into scc1-3 mitochondria were correctly inserted into the inner membrane. The correct topology of the imported proteins can be assessed by a characteristic proteolytic fragmentation of the proteins after opening of the intermembrane space (by swelling of the mitochondria) and addition of proteinase K. Tim23 is cleaved to an ~14 kDa fragment (Tim23') by removal of an N-terminal domain exposed to the intermembrane space and Tim17 is cleaved to an ~14 kDa fragment (Tim17') by removal of the C-terminal tail (Figure 1C, lanes 2 and 8; Kübrich et al., 1994; Bömer et al., 1996b). These typical fragments were also generated with scc1-3 mitochondria (Figure 1C, lanes 5 and 11) in a Δψ-dependent manner (Figure 1C, compare lanes 5 and 6 with lanes 11 and 12). The efficiency of fragment formation was comparable between wild-type and scc1-3 mitochondria (Figure 1D). This demonstrates the correct topogenesis of Tim17 and Tim23 independently of functional mtHsp70.

Since Ssc1-3p does not interact with incoming precursor polypeptides (Gambill et al., 1993; Voos et al., 1993, 1996), we conclude that mitochondrial import and integration of Tim17 and Tim23 into the inner membrane does not involve interaction with mtHsp70. This conclusion is supported by results with mitochondria from a different mutant of mtHsp70 (scc1-2); Tim17 and Tim23 are correctly imported and integrated into the membrane in scc1-2 mitochondria, yet the mutant protein Ssc1-2p does not interact at all with the Tim proteins (see below).

Association of Tim proteins with mtHsp70

We then asked if the fully imported Tim proteins interact with mtHsp70. Wild-type mitochondria with imported Tim17, Tim23 and Tim44 were lysed with digitonin and subjected to co-immunoprecipitation. Antibodies directed against Tim23 co-precipitated both Tim44 and Tim17 (Figure 2A, lane 3), whereas antibodies directed against Tim44 efficiently co-precipitated Tim23, but only minute amounts of Tim17 (Figure 2A, lane 5; the relative amounts of Tim proteins in the mitochondrial extract are shown in lane 1 of Figure 2A). Surprisingly, antibodies directed against mtHsp70 co-precipitated all three Tim proteins, Tim44, Tim23 and Tim17 (Figure 2A, lane 4). The efficiency of co-precipitation of Tim17 with anti-mtHsp70 was ~50% of the efficiency observed for co-precipitation of Tim44 with anti-mtHsp70 (Figure 2B, column 2); the efficiency for Tim23 was ~30% of that for Tim44 (Figure 2B, column 1).

We performed a series of controls to test the specificity
Mitochondrial import and membrane integration of Tim17 and Tim23 are not affected by the ssc1-3 mutation. (A) Precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of \[^{35}S\]methionine/\[^{35}S\]cysteine and incubated with isolated \textit{S.cerevisiae} mitochondria (80 µg protein in a total volume of 100 µl) at 25°C for the times indicated as described in Materials and methods. The mitochondria were preincubated at 37°C for 10 min to induce the ssc1-3 mutant phenotype. In lanes 5 and 10 the membrane potential had been dissipated prior to import by including 1 µM valinomycin (-Δψ). The import reactions were stopped by addition of valinomycin and cooling on ice. After treatment with protease K (100 µg/ml final concentration), mitochondria were re-isolated and imported proteins were analyzed by tricine–SDS–PAGE. \(b_2\)--DHFRR, fusion protein between the 167 N-terminal amino acid residues of the precursor of cytochrome \(b_2\) and dihydrofolate reductase (Voos et al., 1993); \(F_1\)β, \(F_1\)-ATPase subunit β; i, m, intermediate and mature-sized forms of a protein respectively; WT, wild-type mitochondria. (B) The experiment was performed as described for (A). Quantification was performed by digital autoradiography. The amount of protein imported into wild-type mitochondria after 30 min was set at 100% (control). (C) After 10 min preincubation at 37°C, isolated wild-type and ssc1-3 mitochondria were incubated with radiolabeled Tim23 and Tim17 for 30 min at 25°C. The import reactions were stopped by addition of valinomycin and cooling on ice. One aliquot was diluted with cold SEM buffer and left on ice (samples 1, 4, 7 and 10; 7 and 10 were also treated with proteinase K). The other aliquot (samples 2, 5, 8 and 11) was subjected to hypotonic swelling by dilution with EM buffer and treated with proteinase K for 30 min. Re-isolated mitochondria were washed and analyzed by tricine–SDS–PAGE. In parallel, samples 3, 6, 9 and 12 received valinomycin from the beginning (-Δψ) and were subjected to swelling in the presence of proteinase K. Tim23’, Tim17’, proteolytic fragments of Tim23 and Tim17. (D) The experiment was performed as described for (C) with the indicated import times. The amounts of the fragments Tim23’ and Tim17’ were quantified and corrected for the different number of radiolabeled amino acids compared with the full-length proteins. The amounts of full-length Tim23 and Tim17 respectively transported to a protease-protected location in wild-type mitochondria after 30 min were set as 100% (control).
Fig. 2. Co-precipitation of Tim proteins with mtHsp70. (A) Co-immunoprecipitation of Tim44, Tim23 and Tim17 by antibodies directed against Tim23, mtHsp70 or Tim44. Radiolabeled Tim44, Tim23, Tim17 and the ADP/ATP carrier (AAC) were imported into isolated yeast mitochondria for 30 min at 25°C. Non-imported material was removed by treatment with proteinase K (100 µg/ml) and the washed mitochondria were lysed in buffer containing 1% digitonin. After a clarifying spin, aliquots were subjected to immunoprecipitation with antibodies directed against Tim23, mtHsp70, Tim44 and AAC or with preimmune antibodies (described in Materials and methods). Precipitated proteins were analyzed by tricine–SDS–PAGE. As a control, 5% of the material added to the antibodies was directly analyzed (sample 1). (B) Efficiency of co-precipitation of Tim23, Tim17 and AAC with antibodies directed against mtHsp70. The experiment was performed as described in (A). The efficiency of co-precipitation of radiolabeled proteins was determined by digital autoradiography in comparison with the total amount of imported material; the efficiency for co-precipitation of Tim44 with anti-mtHsp70 (Rassow et al., 1994) was set as 100% (control). (C) Radiolabeled Tim23 (upper panel) and Tim17 (lower panel) were imported into yeast mitochondria as in (A). The mitochondria were treated with proteinase K, re-isolated and dissolved in lysis buffer. The soluble material was split into halves and subjected to immunoprecipitation with antibodies directed against mtHsp70 or preimmune antibodies. In parallel, another sample was incubated with the radiolabeled preprotein after dissipation of the membrane potential (Δψ); treatment with proteinase K was omitted. Then co-immunoprecipitation with antibodies directed against mtHsp70 was performed. As in (A), controls were directly analyzed without co-precipitation (samples 1 and 2). (D) Imported Tim23 and Tim17 bind to mtHsp70 prior to lysis of mitochondria. Mitochondria from wild-type or the ssc1-3 mutant were incubated with radiolabeled Tim17 and Tim23 as in (A) and non-imported material was removed by treatment with proteinase K. Re-isolated mitochondria were resuspended in SEM buffer and shifted to 37°C to induce the mutant phenotype. Samples were divided into four aliquots, two of which were diluted with a 4-fold excess of mock-treated mitochondria from wild-type or the ssc1-3 mutant (i.e. without radiolabeled preprotein). The mitochondria were re-isolated and lysed with 1% digitonin. Insoluble material was removed by ultracentrifugation and mitochondrial extracts were subjected to immunoprecipitation with preimmune antibodies or antibodies directed against mtHsp70 as in (A). (E) Co-precipitation of the membrane-integrated fragments of Tim17 and Tim23. Radiolabeled Tim17 and Tim23 were imported into isolated wild-type mitochondria as described in the legend to Figure 1C (half of the samples were subjected to swelling; all samples were treated with proteinase K). Aliquots were directly analyzed. Mt, mitochondria; Mp, mitoplasts (swelling). Then a co-precipitation with anti-mtHsp70 or preimmune antibodies was performed as described for (A). The efficiencies of co-precipitation (with anti-mtHsp70) of full-length Tim17 and Tim23 were set as 100% (control).
Translocase of inner mitochondrial membrane

of the co-immunoprecipitation assay. (i) Preimmune antibodies did not precipitate any of the Tim proteins (Figure 2A, lane 2). (ii) Antibodies directed against the most abundant mitochondrial inner membrane protein, the ADP/ATP carrier, did not co-precipitate any of the Tim proteins (Figure 2A, lane 6) and imported ADP/ATP carrier was not co-precipitated with antibodies against Tim44, Tim23 or mtHsp70 (Figure 2A, lanes 3–5, and B, column 3). (iii) To exclude an interaction of Tim17 or Tim23 with mtHsp70 after lysis of the mitochondria, the precursors of Tim17 and Tim23 were incubated with mitochondria in the absence of a membrane potential $\Delta \psi$ across the inner membrane and thereby accumulated at the outer membrane (Figure 2C, lane 2). No co-immunoprecipitation of these precursor forms was observed with anti-mtHsp70 (Figure 2C, lane 5). (iv) As a further control to exclude a post-lysis binding of Tim17 or Tim23 to mtHsp70, the Tim proteins were imported into ssc1-3 mitochondria, where they were correctly integrated into the inner membrane but did not bind to the mutant Hsp70 (Figure 2D, lane 6).

Lysis of the ssc1-3 mitochondria was then performed in the presence of a 4-fold excess of lysed wild-type mitochondria, but no binding of Tim17 or Tim23 to wild-type mtHsp70 was detectable (Figure 2D, lane 7). This confirms that the interaction of Tim17 and Tim23 with mtHsp70 shown in Figures 2A (lane 4), B (columns 1 and 2) and D (lanes 2–4) occurred in the intact mitochondria prior to lysis. (v) The results with the yeast mutants of mtHsp70 demonstrated that functional mtHsp70 is not required for the biogenesis pathway of Tim17 and Tim23. To provide independent evidence for a co-precipitation of imported, i.e. mature Tim17 and Tim23, with mtHsp70, mitochondria with imported Tim17 or Tim23 were subjected to swelling and treatment with proteinase K. The characteristic fragments Tim17' and Tim23', which demonstrate correct membrane integration of the Tim proteins (Figure 1C; Kübrich et al., 1994; Bömer et al., 1996b), were formed. The fragments Tim17' and Tim23' were efficiently co-precipitated with antibodies directed against mtHsp70 (Figure 2E, columns 4 and 8), demonstrating that the mature Tim proteins associated with mtHsp70.

In a gel filtration analysis of digitonin-lysed mitochondria, most Tim17 and Tim44 migrated in separate fractions, whereas Tim23 co-migrated with both Tim17 and Tim44 (Figure 3A). Tim23 may thus be present in two pools, one in association with Tim44, the other in association with Tim17. To substantiate this model, co-precipitation analysis with Tim17 was performed. Since antibodies that efficiently precipitated Tim17 were not available, we constructed a yeast strain that expressed Tim17 with a His$_9$-tag. Tim17-His complemented a lack of authentic Tim17. In parallel, a strain expressing Tim23 with a His$_7$-tag was constructed; Tim23-His similarly complemented a lack of authentic Tim23. Digitonin extracts of mitochondria containing the His$_9$-tagged Tim proteins were subjected to Ni-nitrotriacetate (Ni-NTA) affinity chromatography. Bound proteins were eluted and analyzed by immunodecoration with antibodies directed against mtHsp70, Tim44 and Tim23. In the co-purification with Tim17-His, Tim44 was present only in minute amounts compared with mtHsp70 and Tim23 (Figure 3B, lane 3). In contrast, Tim44 efficiently co-purified with Tim23-His (Figure 3B, lane 2). When extracts of wild-type mitochondria were applied to Ni-NTA agarose only background binding was observed (Figure 3B, lane 1), indicating the specificity of the affinity chromatography. As an independent control, a yeast strain expressing Tim17 with a c-Myc epitope was used (Kübrich et al., 1994; Maarse et al., 1994). Antibodies directed against c-Myc co-precipitated imported Tim23, but not Tim44 (Figure 3C, lane 3). The co-precipitation of Tim23 with anti-c-Myc was specific, as it was not observed with control antibodies (Figure 3C, lane 2) or wild-type Tim17 (Figure 3C, lane 5).

In summary, our results suggest that the Tim machinery can dissociate into sub-complexes. Tim23 seems to be present in both sub-complexes, Tim44–Tim23 and Tim23–Tim17. mtHsp70 not only interacts with Tim44–Tim23, but also with Tim23–Tim17.

Co-precipitation of Tim17 with anti-mtHsp70 does not involve Tim44

Since previous studies had reported an interaction of mtHsp70 with Tim44, the possibility that co-precipitation
Depletion of Tim44 does not influence co-precipitation of Tim17 with anti-mtHsp70. (A) Isolated yeast mitochondria were incubated with radiolabeled Tim44 and Tim17 and then treated with proteinase K to remove non-imported proteins as described in the legend to Figure 2A. The mitochondria were re-isolated, washed, dissolved in lysis buffer, divided into halves and subjected to immunodepletion with preimmune or anti-Tim44 antibodies (see Materials and methods). The resulting extracts were clarified by ultracentrifugation and co-immunoprecipitation was performed with preimmune, anti-mtHsp70 and anti-Tim44 antibodies. (B) The experiment was performed as described for (A) except that Tim23 was also imported and that co-precipitation with anti-Tim23 was included. Quantification was by digital autoradiography. The reduction of co-precipitation by immunodepletion of Tim44 was determined by subtracting the amount of protein co-precipitated after depletion with anti-Tim44 from the amount of protein co-precipitated after depletion with preimmune antibodies. The reduction for Tim44 was set as 100% (control).

Genetic evidence for functional interaction of Tim17 with mtHsp70

Overexpression of mtHsp70 was reported to suppress a mitochondrial protein import defect of tim44 yeast mutants in vivo, providing genetic evidence for the functional importance of the interaction of mtHsp70 with Tim44 (Rassow et al., 1994). We thus asked if overexpression of mtHsp70 affected protein transport defects of tim17 or tim23 mutants. We used a strain where the cytosolic enzyme orotidine 5'-phosphate decarboxylase (OMP decarboxylase), encoded by URA3, was tagged with an N-terminal mitochondrial targeting sequence (that of superoxide dismutase, SOD), leading to efficient mis-targeting into mitochondria (Maarse et al., 1992). The resulting cells were unable to grow in uracil-free medium due to lack of cytosolic OMP decarboxylase activity. Mutant cells with defective mitochondrial protein import, including tim17 and tim23 mutants, at least partially blocked import of the SOD–OMP decarboxylase chimeric protein and thereby allowed growth of the cells in the absence of added uracil (Dekker et al., 1993; Maarse et al., 1994). Expression of wild-type Tim17 in the tim17 mutant (Figure 5A, left plate) or expression of wild-type Tim23 in the tim23 mutant (Figure 5B, left plate) restored mitochondrial protein import and thereby conferred uracil dependence. The dependence of growth on the addition of uracil can be taken as an assay for import of the chimeric protein into mitochondria in vivo.

tim17-1 and tim23-2 mutant cells containing the tagged OMP decarboxylase were transformed with the gene SSC1 (encoding mtHsp70), either on the centromeric vector YCplac111 or the multicopy vector YEplac181. When mtHsp70 was expressed from the multicopy vector, growth on uracil-free medium was strongly diminished with the tim17-1 mutant (Figure 5A, left plate), but not with the tim23-2 mutant (Figure 5B, left plate). This demonstrates that overexpression of mtHsp70 suppresses the protein import defect of a tim17 mutant, providing genetic evidence for a functional relation of mtHsp70 and Tim17. It should be emphasized that overexpression of mtHsp70 did not suppress the lethal phenotype of a complete deletion of the TIM17 gene, demonstrating that mtHsp70 cannot replace the full function of Tim17.

A synthetic growth phenotype is found when a combination of mutations in two genes displays a growth phenotype not observed with the single mutations. Synthetic growth defects, for example observed for tim44 and ssc1 mutants, can be used as independent genetic evidence for a functional interaction of two gene products (Huffaker et al., 1987; Kaiser and Schekman, 1990; Scidmore et al., 1993; Rassow et al., 1994). We crossed the tim17-1 mutant with the mtHsp70 mutant ssc1-2 or ssc1-3 (each of the

Fig. 4. Depletion of Tim44 does not influence co-precipitation of Tim17 with anti-mtHsp70. (A) Isolated yeast mitochondria were incubated with radiolabeled Tim44 and Tim17 and then treated with proteinase K to remove non-imported proteins as described in the legend to Figure 2A. The mitochondria were re-isolated, washed, dissolved in lysis buffer, divided into halves and subjected to immunodepletion with preimmune or anti-Tim44 antibodies (see Materials and methods). The resulting extracts were clarified by ultracentrifugation and co-immunoprecipitation was performed with preimmune, anti-mtHsp70 and anti-Tim44 antibodies. (B) The experiment was performed as described for (A) except that Tim23 was also imported and that co-precipitation with anti-Tim23 was included. Quantification was by digital autoradiography. The reduction of co-precipitation by immunodepletion of Tim44 was determined by subtracting the amount of protein co-precipitated after depletion with anti-Tim44 from the amount of protein co-precipitated after depletion with preimmune antibodies. The reduction for Tim44 was set as 100% (control).

of Tim17 and Tim23 with anti-mtHsp70 occurred via co-precipitated Tim44 was of concern. The inefficient co-precipitation of Tim17 with anti-Tim44 rendered this possibility unlikely, at least in the case of Tim17. To directly determine the role of Tim44 in the interaction of Tim17 and Tim23 with mtHsp70, mitochondria were lysed with digitonin-containing buffer and depleted of Tim44 by immunoadsorption. Most Tim44 was depleted by this method (Figure 4A, lanes 4–6, and B, columns 1, 4, 6 and 7), while preimmune antibodies did not lead to depletion of Tim44 (Figure 4A, lanes 1–3). Then co-precipitations with antibodies directed against mtHsp70, Tim23 or Tim44 were performed. Depletion of Tim44 influenced neither the amount of Tim17 co-precipitated with anti-mtHsp70 (Figure 4A, lane 5, and B, column 3) nor the amount of Tim17 co-precipitated with anti-Tim23 (Figure 4B, column 5). The co-precipitation of Tim23 with anti-mtHsp70, however, was reduced by ~35% after depletion of Tim44 (Figure 4B, column 2). As expected, precipitations with anti-Tim44 were in the background range (Figure 4A, lane 6, and B, columns 6 and 7).

We conclude that co-precipitation of Tim17 with anti-mtHsp70 does not involve Tim44. A part of the observed association of Tim23 with mtHsp70 is sensitive to depletion of Tim44, whereas the remainder is independent of the presence of Tim44.
conditional alleles confers a temperature-sensitive growth phenotype; the single mutants grow well at 23°C, but not at 37°C. The heterozygous diploids were sporulated and viability of the spores was tested at 23°C. The genotypes of the viable spores were analyzed. The double mutation ssc1-2/tim17-1 was inviable (Figure 5C, panel 1; synthetic lethality) and the double mutant strain ssc1-3/tim17-1 was strongly impaired in growth (Figure 5C, panel 2; strong synthetic growth defect). In contrast, the double mutant strains ssc1-2/tim23-2 (Figure 5C, panel 3) and ssc1-3/tim23-2 (Figure 5C, panel 4) were only moderately reduced in growth at 23°C. We conclude that synthetic growth phenotypes provide genetic evidence for a functional interaction of Tim17 with mtHsp70.

Co-precipitation of Tim17 with anti-mtHsp70 is ATP sensitive and differentially affected by the ssc1-3 mutation compared with the mtHsp70–Tim44 interaction

We then investigated the nucleotide dependence of the interaction between Tim proteins and mtHsp70. The co-precipitations described above were performed in the absence of Mg-ATP. When Mg-ATP was added, Tim17 was not found in association with mtHsp70 (Figure 6A, lane 6 versus lane 4, and B, column 2 versus column 1). The total amount of Tim17 in the mitochondrial extract was not changed by the addition of ATP (Figure 6A, lane 2 versus lane 1). The addition of Mg-ATP similarly disrupted the association of Tim23 and Tim44 with mtHsp70 (Figure 6A, lane 6, and B, columns 6 and 8). Addition of ATP did not, however, disrupt the association of Tim23 with Tim17 (Figure 6B, column 4). The non-hydrolyzable ATP analog AMP-PNP led to dissociation of the Tim17–mtHsp70 interaction (not shown), as previously found for the Tim44–mtHsp70 interaction, indicating that binding of ATP is sufficient for complex dissociation (von Ahsen et al., 1995; Horst et al., 1996). The association of Tim17 with mtHsp70 is thus regulated by ATP, resembling the interaction of Hsp70 with other partner proteins (summarized in Rothman, 1989; Rassow et al., 1995; Hartl, 1996).

The mutant protein Ssc1-3p contains an amino acid change (Gly56→Ser) in the ATPase domain of mtHsp70 (Gambill et al., 1993). After preincubation at 37°C, Ssc1-3p does not bind to Tim44 (Figure 7B, column 5), whereas in the absence of a temperature shift Ssc1-3p stably binds to Tim44 (Figure 7D, column 5; Rassow et al., 1994; Schneider et al., 1994; Voos et al., 1996). We asked how fig.5
Fig. 7. Differential effect of the ssc1-3 mutation on association of mtHsp70 with Tim44 and Tim17. (A) Tim17 and Tim23 were imported into wild-type or ssc1-3 mitochondria at 25°C. After treatment with proteinase K, the mitochondria were re-isolated and resuspended in SEMP buffer and incubated for 10 min at 37°C to induce the mutant phenotype. The mitochondria were lysed and, after a clarifying spin, co-immunoprecipitation was performed with antibodies directed against mtHsp70 or preimmune antibodies. (B) The experiment was performed as described for (A). In addition, co-precipitation with antibodies directed against Tim23 or Tim44 was performed as described in the legend to Figure 2A. The co-precipitated proteins were quantified for wild-type and ssc1-3 mitochondria. Shown is the ratio of co-precipitation between wild-type for each protein. (C and D) The experiments were performed as described for (A) and (B) except that the incubation at 37°C was omitted. We conclude that in the absence of a temperature shift the ssc1-3 mutation differentially affects the association of mtHsp70 with Tim17 and substrates of Ssc1-3p with Tim44 (Figure 7D, column 5). Co-precipitation of Tim23 with Ssc1-3p was partially reduced (Figure 7D, column 3). The interactions of Tim23 with Tim17 and Tim44 were not disturbed (Figure 7D, columns 2 and 4).

Differential effect of the ssc1-2 mutation on association of mtHsp70 with Tim17 and substrates

It was conceivable that Tim17 or a closely associated protein may represent a chaperone–substrate that only bound to the peptide binding site of mtHsp70. To analyze this we used a different allele of SSC1, ssc1-2. The mutant...
chaperone Ssc1-2p, which carries an amino acid exchange (Pro419→Ser) in the C-terminal domain of mtHsp70, is known to bind substrate proteins efficiently, whereas binding to Tim44 is blocked, distinguishing binding to substrate from binding to Tim-44 (Gambill et al., 1993; Voos et al., 1993, 1994, 1996). The Tim proteins were imported into ssc1-2 mitochondria after preincubation at the non-permissive temperature of 37°C. Tim17 and Tim23 yielded the characteristic fragments Tim17′ and Tim23′ after proteolytic treatment of mitoplasts (Figure 8A, lanes 4 and 8) and co-precipitation of Tim17 with anti-Tim23 and Tim23 with anti-Tim44 occurred with efficiencies close to those of the wild-type (Figure 8B, columns 2 and 4), indicating correct membrane integration of Tim17 and Tim23. Co-precipitations with antibodies directed against mtHsp70 were then performed. As reported (Schneider et al., 1994; Voos et al., 1996), Tim44 from ssc1-2 mitochondria was not co-precipitated with anti-mtHsp70 (Figure 8B, column 5). Additionally, neither Tim17 nor Tim23 were found in association with Ssc1-2p (Figure 8B, columns 1 and 3). In contrast, co-precipitation of substrate proteins with Ssc1-2p, shown here with a fusion protein between the presequence of F0-ATPase subunit 9 and dihydrofolate reductase (Su9-DHFR), was of high efficiency (Figure 8B, column 6; Gambill et al., 1993; Voos et al., 1993, 1994, 1996). This demonstrates that the interaction of mtHsp70 with Tim17 is different from the interaction of mtHsp70 with substrate proteins.

In summary, we conclude that the Tim machinery contains a second membrane anchor for mtHsp70. Tim17 (or a closely associated protein) represents a novel binding partner for mtHsp70, distinct from the properties of the binding partner Tim44 and substrate proteins.

**Discussion**

We report that the preprotein translocon of the inner mitochondrial membrane Tim has two independent membrane anchors for matrix Hsp70. Previous studies had identified Tim44 as a specific binding partner of mtHsp70 (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). We show that the three essential Tim proteins Tim44, Tim23 and Tim17 are not permanently associated, but that they can form sub-complexes. Specific association of mtHsp70 with a Tim17–Tim23-containing sub-complex is also observed after depletion of Tim44. Interestingly, Tim23 can efficiently associate with Tim44, suggesting that two pools of Tim23 exist in the inner membrane, a Tim17–Tim23 sub-complex and a Tim23–Tim44 sub-complex. Each sub-complex interacts with a fraction of mtHsp70 in an ATP-sensitive manner. A mutant form of mtHsp70 (Ssc1-3p without temperature shift) efficiently binds to Tim44, but not to the other sub-complex, suggesting a different type of interaction of mtHsp70 with the two membrane anchors.

Tim17 (or a closely associated protein) is the most likely candidate for the second membrane anchor [the presence of additional Tim proteins in the sub-complexes is conceivable, yet has not been analyzed at a molecular level (Blom et al., 1995; Berthold et al., 1995)]. Since we observed efficient co-precipitation of Tim23 with mtHsp70, Tim23 could also serve as second membrane anchor. We consider it to be more likely, however, that co-precipitation of Tim23 with mtHsp70 is indirect and mediated in two ways, via Tim44 and via Tim17. About 35–40% of co-precipitation of Tim23 depends on the presence of Tim44; it is sensitive to depletion of Tim44 and not affected by the ssc1-3 mutation (without temperature shift). The remaining 60–65% of co-precipitation of Tim23 with mtHsp70 shows the characteristics of the Tim17–mtHsp70 interaction, i.e. independence of the presence of Tim44 and sensitivity to the ssc1-3 mutation. In agreement with the biochemical results, genetic evidence (suppression of mutant defects by overexpression of mtHsp70 and synthetic growth defects of mutations in two genes) indicates a functional in vivo interaction of mtHsp70 with Tim44 (Rassow et al., 1994) and with Tim17, but not with Tim23.

The second membrane anchor of mtHsp70 does not behave as a substrate protein that binds to the peptide binding site of mtHsp70, since another mutant form of mtHsp70 (Ssc1-2p) efficiently binds substrates but is blocked in association with the Tim17–Tim23 sub-complex. As Ssc1-2p is also able to interact with the co-chaperone Mge1p (Voos et al., 1994), a homolog of bacterial GrpE (Bolliger et al., 1994; Laloraya et al., 1994), we conclude that the second membrane anchor...
represents a novel partner for mtHsp70 with binding properties distinct from Tim44, Mge1p and substrates.

Our study gives insights into the role of mtHsp70 in the biogenesis of the proteins of the Tim machinery. The import of Tim44, a peripheral membrane protein synthesized with an N-terminal presequence (Blom et al., 1993), strictly requires mtHsp70, as is the case with most presequence-containing preproteins of inner membrane or matrix proteins analyzed so far (Kang et al., 1990; Scherer et al., 1990; Cyr et al., 1993; Ungermann et al., 1994; Horst et al., 1995). The import and membrane insertion of Tim17 and Tim23, integral membrane proteins that are synthesized without a cleavable presequence (Dekker et al., 1993, 1994), however, are independent of mtHsp70. Tim17 and Tim23 thus join a small group of intermembrane space and inner membrane proteins with a mtHsp70-independent import pathway (Wachter et al., 1992; Glick et al., 1993; Voos et al., 1993, 1996; Stuart et al., 1994). Tim17 and Tim23 contain four membrane-spanning segments each (Kübrich et al., 1994; Pfanner et al., 1994). We propose that each preprotein forms two loops (each loop consisting of two membrane-spanning segments) that insert into the inner membrane from the intermembrane space without the aid of mtHsp70.

We suggest a dynamic model of the functional architecture of the Tim machinery. Like the Tom machinery of the outer mitochondrial membrane (Alconada et al., 1995b; Höninger et al., 1996), the Tim machinery consists of reversibly interacting sub-complexes. Association between the Tim44–Tim23 sub-complex and the Tim23–Tim17 sub-complex may be very transient (as evidenced by the weak co-precipitation efficiency), but the demonstration of genetic interactions between all three Tim proteins (Blom et al., 1995) suggests an important role in vivo. The model implies that at least two molecules of Tim23 should be present in a complete Tim complex, yet is in contrast to the report by Berthold et al. (1995) that only a single Tim23 molecule is found in a Tim complex. Very recently, however, Bauer et al. (1996) showed that Tim23 can form transient dimers in the inner membrane, in full agreement with our model. Interestingly, the existence of sub-complexes of a protein transport complex has also been shown for the endoplasmic reticulum (ER) (Brodsky and Schekman, 1993; Panzner et al., 1995; Brodsky, 1996; Finke et al., 1996). As observed here for Tim23, the ER protein Ssr1p is a subunit of two distinct complexes (Finke et al., 1996). The ER Hsp70 (Bip, Kar2p) binds to the membrane protein Sec63p (Sadler et al., 1989; Brodsky and Schekman, 1994) and Tim44 contains a short segment with limited similarity to Sec63p (Rassow et al., 1994). It is unknown if Kar2p can bind to a second membrane anchor. Except for the Hsp70 homology and the very limited similarity between Sec63p and Tim44, however, there is no primary structure similarity found between the subcomplexes of the protein transport machineries of the ER and mitochondrial membranes. Future studies will have to address to what extent possible analogy between the protein transport machineries of the ER and mitochondria can be envisaged.

### Materials and methods

#### Construction of plasmids and manipulation of S.cerevisiae strains

The *S.cerevisiae* strains used in this study are listed in Table I. The test for complementation of tim17 and tim23 yeast mutants by expression of *SSC1, TIM17 and TIM23 was performed as described by Maarse et al. (1992). Double-deficient yeast strains were constructed by crossing the *ssc1-1* and *ssc1-3* mutants. The resulting diploid cells were sporulated and analyzed for growth on selective medium. For manipulation of DNA and yeast strains, standard procedures were used (Ausubel et al., 1989; Guthrie and Fink, 1991).

Plasmid YCplac111–TIM17 (BstEII–HindIII + His<sub>6</sub> [N]) was constructed in two steps. A BamHI site was created after the ATG initiation codon by site-directed mutagenesis of TIM17. A 24 bp SacI3A fragment from pQE30 (Qagen) which encodes the His<sub>6</sub> tag was cloned in-frame into this BamHI site. YCplac111–TIM17 (BstEII–HindIII + His<sub>6</sub> [N]) was introduced into yeast strain MB26. Double transformants were cured from YCplac33–TIM17 (URA3 + BstEII–HindIII + His<sub>6</sub> [N]) similarly constructed and introduced into yeast strain MB29 by plasmid shuffling. The resulting strains carrying a His<sub>6</sub> tag at the N-terminus of Tim17 or Tim23 respectively grew like the wild-type.

#### Import of preproteins into isolated mitochondria

Yeast cells (Table I) were grown in YPG medium (1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol) and mitochondria were prepared according to published procedures (Daum et al., 1982; Gambill et al., 1993). Preproteins were synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine/[35S]cysteine after in vitro transcription by SP6 polymerase (Amersham or Stratagene) or by coupled transcription/translation with T7 polymerase (Promega).

Import reactions were performed by incubation of reticulocyte lysate with isolated mitochondria in import buffer (3% w/v bovine serum albumin, 250 mM sucrose, 60 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM sodium malate, 2 mM ATP, 20 mM potassium phosphate, 10 mM MOPS–KOH, pH 7.2) at 25°C. Samples with a dissipated membrane potential received 1 µM valinomycin prior to incubation. For generation of mitoplasts by hypotonic swelling, import reactions were diluted with 9 vol. EM buffer (1 mM EDTA, 10 mM MOPS–KOH, pH 7.2) and left on ice for 30 min.

The phenotypes of the mitochondria from the mutants *ssc1-2* and *ssc1-3* were induced by incubation for 10 min at 37°C and were stable.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK82</td>
<td>MATa his4-713 lys2 ura3-52 Δplo1 leu2-3,112</td>
<td>Gambill et al. (1993)</td>
</tr>
<tr>
<td>PK81</td>
<td>MATα ade2-101 lys2 ura3-52 Δplo1 leu2-3,112 ssc1-2 (LEU2)</td>
<td>Gambill et al. (1993)</td>
</tr>
<tr>
<td>PK83</td>
<td>MATα ade2-101 lys2 ura3-52 Δplo1 leu2-3,112 ssc1-3 (LEU2)</td>
<td>Gambill et al. (1993)</td>
</tr>
<tr>
<td>MB1-33</td>
<td>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-LYS2 tim17-1</td>
<td>Dekker et al. (1993)</td>
</tr>
<tr>
<td>MB1-46</td>
<td>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-LYS2 tim23-2</td>
<td>Dekker et al. (1993)</td>
</tr>
<tr>
<td>MB3-33</td>
<td>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-LYS2 tim17-1 (TRP1)</td>
<td>Blom et al. (1995)</td>
</tr>
<tr>
<td>MB6-46</td>
<td>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-LYS2 tim23-2 (LEU2)</td>
<td>Blom et al. (1995)</td>
</tr>
<tr>
<td>MB16</td>
<td>ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 tim17-1 (LYS2 + YCplac11-TIM17-c-myc (LEU2))</td>
<td>Maarse et al. (1994)</td>
</tr>
<tr>
<td>MB26</td>
<td>MATα ade2 his3 leu2 lys2 ura3 trp1 tim17-LYS2 + YCplac33-TIM17 (URA3)</td>
<td>this study</td>
</tr>
<tr>
<td>MB29</td>
<td>MATα ade2 his3 leu2 lys2 ura3 trp1 tim23-LYS2 + YCplac33-TIM23 (URA3)</td>
<td>this study</td>
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<tr>
<td>YPH499</td>
<td>MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1 Δ63 lys2-801</td>
<td>Sikorski and Hieter (1989)</td>
</tr>
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</table>
in subsequent reactions at lower temperature (Kang et al., 1990; Gambill et al., 1993).

Proteinase K treatment, re-isolation of mitochondria and separation by tricine–SDS–PAGE have been described previously (Sollier et al., 1991; Bömmel et al., 1998b). Autoradiographs were obtained and quantified using a storage Phosphor Imaging System (Molecular Dynamics Inc.).

Co-immunoprecipitations of radio-labelled Tim complexes

Specific antibodies (6 µl serum) were pre-bound to protein A-Sepharose (10 µl wet volume; Pharmacia Biotech Inc.) for 1 h in 480 µl lysis buffer [1% digitonin (1× recrystallized from ethanol), 10% w/v glycerol, 50 mM NaCl, 2 mM EDTA, 30 mM HEPES–KO H, pH 7.4]. Import reactions were performed for 30 min with 60 µg mitochondrial protein per lane and 20–30% (v/v) reticulocyte lysate. Mitochondria were treated with proteinase K (100 µg/ml final concentration) to remove non-imported preproteins and re-isolated. After washing with SEMP buffer [250 mM sucrose, 1 mM EDTA, 10 mM MOPS–KO H, pH 7.2, 0.2 mM freshly added phenylmethylsulfonyl fluoride (PMSF)], mitochondria were resuspended in lysis buffer supplemented with protease inhibitors (2 µg/ml antipain, 5 µg/ml aprotinin, 0.25 µg/ml leupeptin, 1.25 µg/ml leupeptin, 0.5 µg/ml pepstatin A, 0.2 mM PMSF) and shaken end-over-end for 10 min at 8°C. Insoluble material was removed by ultracentrifugation (30 min at 100 000 g) and the supernatants were incubated for 45 min at 8°C by end-over-end shaking with antibodies pre-bound to protein A-Sepharose. After three washing cycles with lysis buffer, the protein A-Sepharose pellets were boiled in sample buffer and applied to tricine–SDS–PAGE gels.

For immunodepletion of Tim44, lysed mitochondria were incubated for 30 min at 8°C with anti-Tim44 antibodies or preimmune antibodies pre-bound to protein A-Sepharose (40 µl antiserum and 65 µl wet volume protein A-Sepharose were used per 100 µg mitochondrial protein). After a further incubation for 10 min with fresh protein A-Sepharose to remove unbound IgGs and removal of insoluble material by ultracentrifugation (30 min at 100 000 g), the supernatants were subjected to immunoprecipitation as described above.

ATP depletion of mitochondria before lysis was performed by incubation with 20 U/ml apyrase and 20 µM oligomycin in SEMP buffer (10 min on ice). ATP loading was performed by incubation in SEMP buffer supplemented with 7 mM MgCl2 and 2 mM ATP.

Gel filtration and metal affinity chromatography

Mitochondria (250 µg protein) were suspended in cold gel filtration buffer (1% digitonin, 50 mM NaCl, 5 mM MgCl2, 2 mM ATP, 30 mM HEPES–NaOH, pH 7.4) and shaken end-over-end for 4°C for 10 min. Insoluble material was removed by ultracentrifugation (30 min at 100 000 g) and the supernatant was applied to a Sepharocryl HR-300 column (30/0.7 cm) that was first pre-conditioned with gel filtration buffer at 0.1 ml/min and fractions of 0.2 ml were collected. Mitochondria (250 µg protein) were lysed in 750 µl cold binding buffer (1% digitonin, 10% glycerol, 100 mM NaCl, 30 mM HEPES–NaOH pH 7.4). After ultracentrifugation (30 min at 100 000 g), the mitochondrial extract was incubated with 5 µl Ni2+-NTA agarose (Qiagen) for 40 min at 4°C. The agarose was washed three times with 750 µl binding buffer supplemented with 10 mM imidazole. Bound material was eluted with 250 mM imidazole (in binding buffer, 2×50 µl). After TCA precipitation in the presence of 0.5% sodium deoxycholate samples were analyzed by tricine–SDS–PAGE and immunoblotting.

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