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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 matrix 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 preproteins (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 (Horst *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; Horst *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 $\Delta\psi$, 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 [^{35}S]methionine/[^{35}S]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 *ssc1-3* allele is conditional lethal and confers a temperature-sensitive phenotype for viability. At non-permissive temperatures the mutant mtHsp70 (Ssc1-3p) does not interact with precursor polypeptides and thus import of mtHsp70-dependent preproteins is blocked in *ssc1-3* mitochondria (Gambill *et al.*, 1993; Voos *et al.*, 1993, 1996; Gärtner *et al.*, 1995a,b). The *ssc1-3* 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 F_1-ATPase. Import of $F_1\beta$ to a protease-protected location was inhibited in *ssc1-3* mitochondria (Figure 1A, lanes 6–9; compare with lanes 1–4, wild-type).

Similarly, import of Tim44 into *ssc1-3* 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 *ssc1-3* mutation (Figure 1A, lanes 6–9). Quantification revealed identical import kinetics for Tim23 and Tim17 into *ssc1-3* 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 *ssc1-3* 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 *ssc1-3* mitochondria was due to non-specific aggregation. Independence of import from the *ssc1-3* 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_2 and dihydrofolate reductase (Figure 1A; Voos *et al.*, 1993, 1996; Gärtner *et al.*, 1995b).

We then tested if Tim17 and Tim23 imported into *ssc1-3* 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 *ssc1-3* 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 *ssc1-3* 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 (*ssc1-2*); Tim17 and Tim23 are correctly imported and integrated into the membrane in *ssc1-2* 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

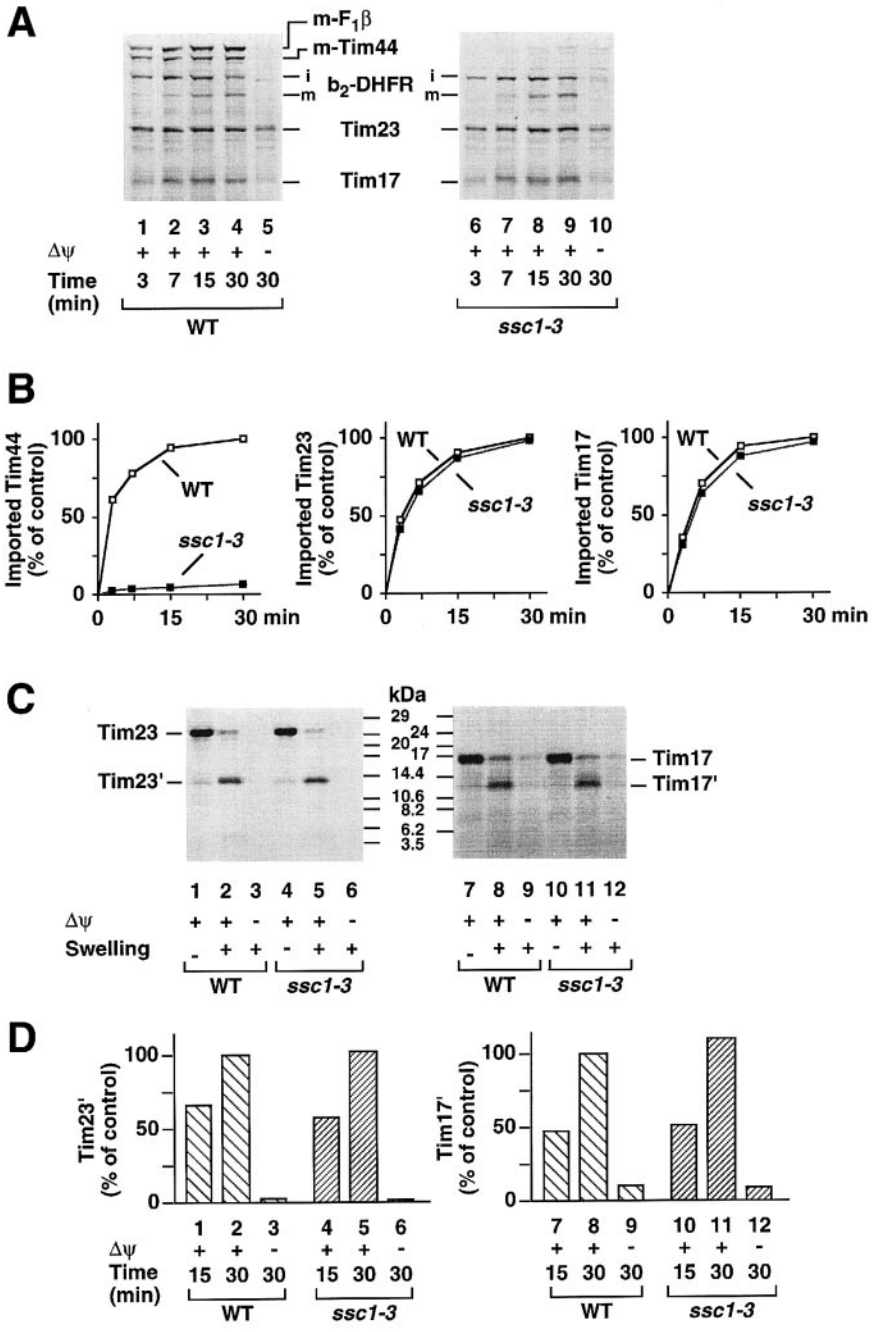

Fig. 1. Mitochondrial import and membrane integration of Tim17 and Tim23 are not affected by the *ssc1-3* mutation. (**A**) Precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine/[^{35}S]cysteine and incubated with isolated *S.cerevisiae* mitochondria (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 proteinase K (100 μg/ml final concentration), mitochondria were re-isolated and imported proteins were analyzed by tricine–SDS–PAGE. b_2–DHFR, fusion protein between the 167 N-terminal amino acid residues of the precursor of cytochrome b_2 and dihydrofolate reductase (Voos *et al*., 1993); $F_1\beta$, 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 divided into halves. 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).

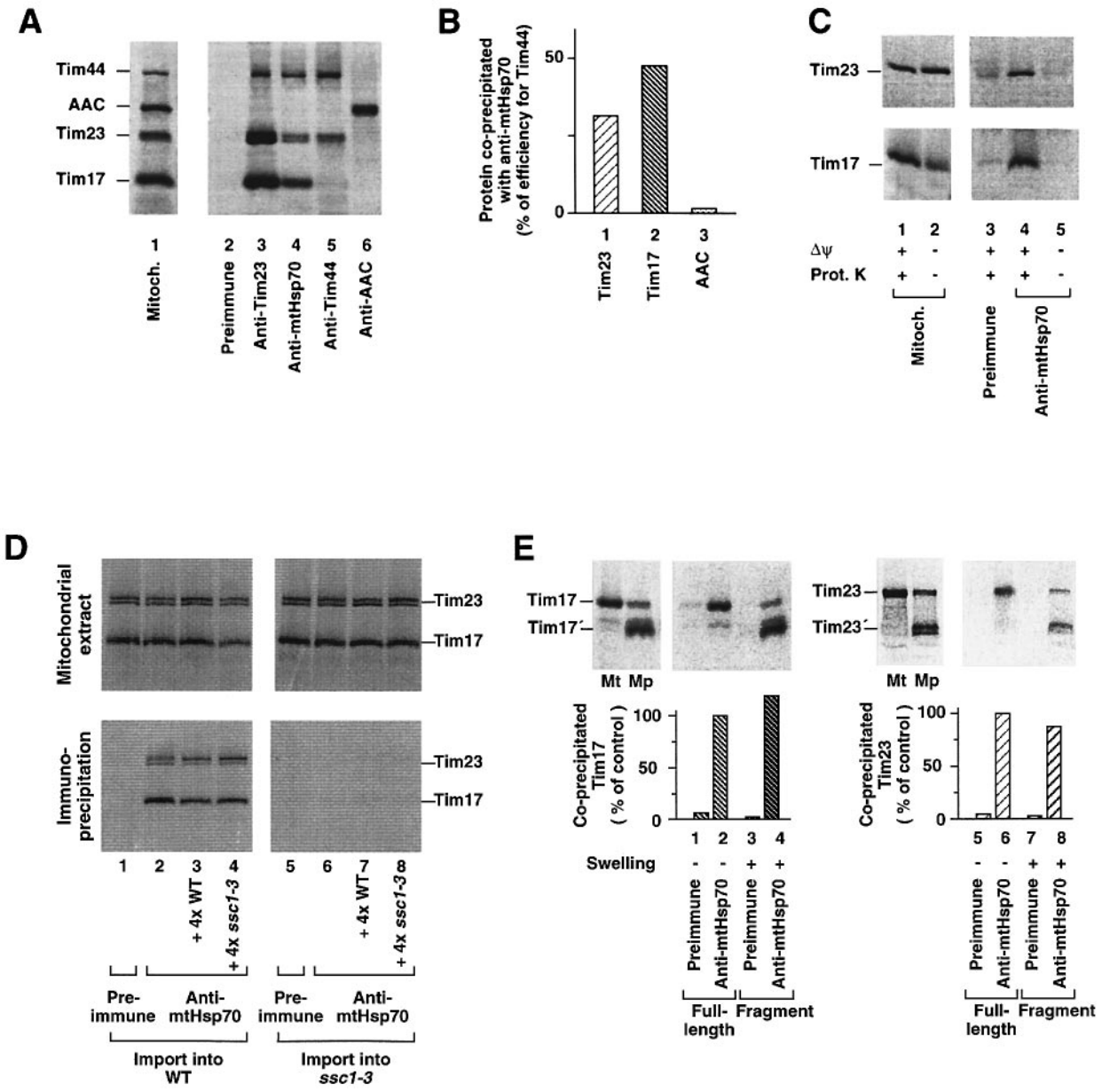

Fig. 2. Co-precipitation of Tim proteins with mtHsp70. (**A**) Co-immunoprecipitation of Tim44, Tim23 and Tim17 by antibodies directed against Tim23, mtHsp70 or Tim44. Radiolabeled Tim44, Tim23, Tim17 and the ADP/ATP carrier (AAC) were imported into isolated yeast mitochondria for 30 min at 25°C. Non-imported 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 for (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 precursor proteins). 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).

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_6-tag. Tim17-His complemented a lack of authentic Tim17. In parallel, a strain expressing Tim23 with a His_6-tag was constructed; Tim23-His similarly complemented a lack of authentic Tim23. Digitonin extracts of mitochondria containing the His-tagged Tim proteins were subjected to Ni–nitrilotriacetate (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).

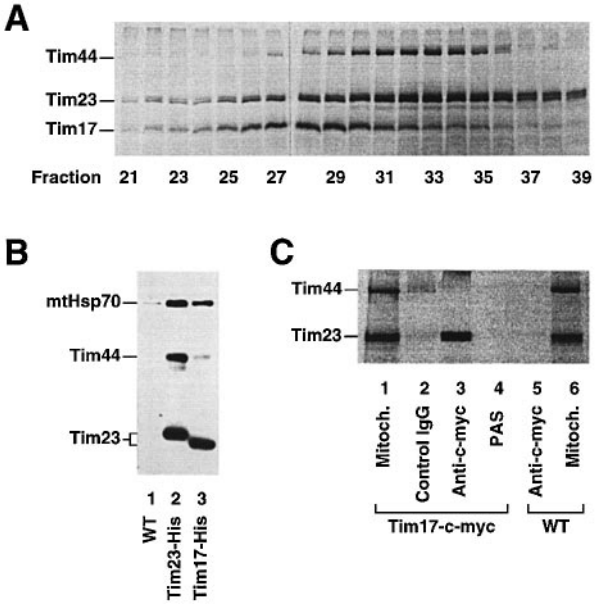

Fig. 3. Association of Tim17 with mtHsp70 and a fraction of Tim23. (**A**) Gel filtration. Import of radiolabeled Tim17, Tim23 and Tim44 into mitochondria was performed as described in the legend to Figure 2A. After treatment with proteinase K and a washing step, mitochondria were lysed with 1% digitonin as described in Materials and methods. After a clarifying spin, the mitochondrial extract was subjected to gel filtration analysis. Fractions were analyzed by tricine–SDS–PAGE and autoradiography. The peak for blue dextran (2000 kDa) was found in fraction 20 and for alcohol dehydrogenase (150 kDa) in fraction 31. (**B**) Mitochondria isolated from wild-type and strains containing Tim17 or Tim23 with an N-terminal His_6 tag were lysed with digitonin and incubated with Ni–NTA agarose as described in Materials and methods. The bound material was eluted with imidazole, TCA-precipitated, and analyzed by tricine–SDS–PAGE and immunodecoration. (**C**) Radiolabeled Tim23 and Tim44 were imported into mitochondria isolated from strain MB16 containing Tim17 with a C-terminal c-Myc tag. Mitochondrial extracts were prepared as described in the legend to Figure 2A. Aliquots were subjected to immunoprecipitation with antibodies directed against the c-Myc tag (lane 3) or control IgG prebound to protein A–Sepharose or with protein A–Sepharose only. In parallel, reactions were performed with mitochondria from a wild-type strain (without the c-Myc tag) and immunoprecipitation was performed with antibodies directed against the c-Myc tag (lane 5). Fractions of the mitochondrial extracts were directly analyzed (lanes 1 and 6).

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

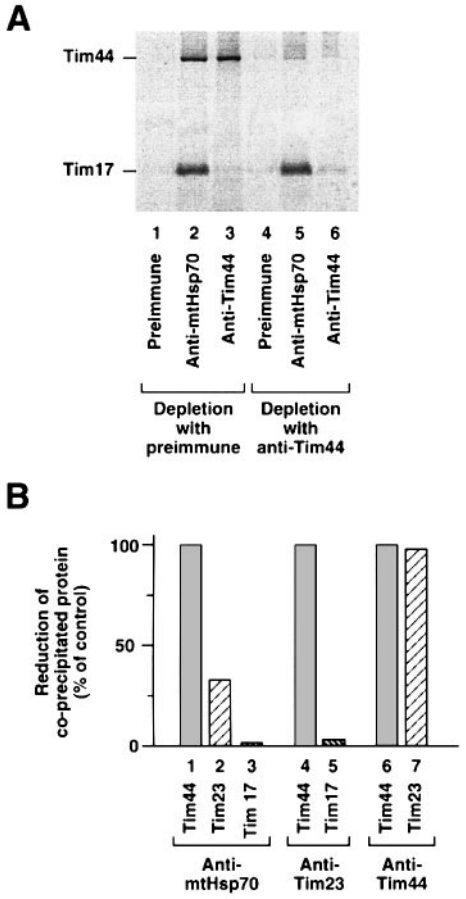

Fig. 4. Depletion of Tim44 does not influence co-precipitation of Tim17 with anti-mtHsp70. (**A**) Isolated yeast mitochondria were incubated with radiolabeled Tim44 and Tim17 and then treated with proteinase K to remove non-imported proteins as described in the legend to Figure 2A. The mitochondria 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.

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 mistargeting 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

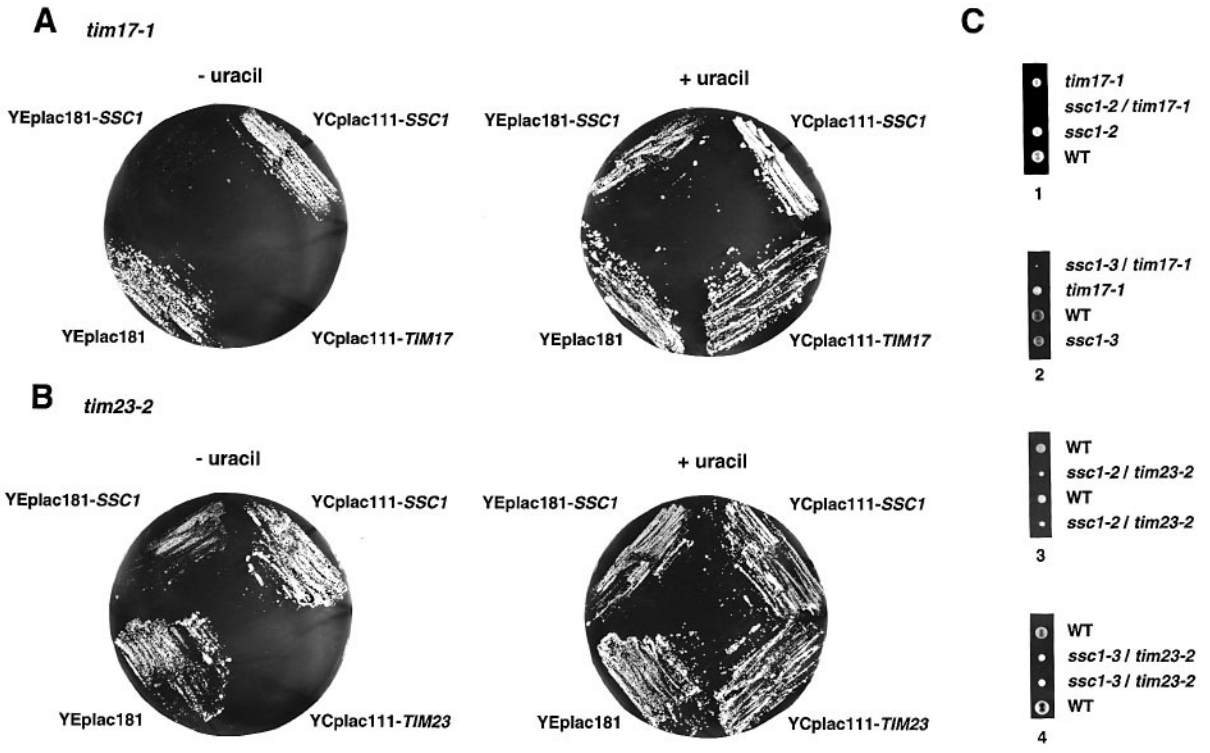

Fig. 5. Genetic evidence for interaction of Tim17 with mtHsp70. (**A** and **B**) Suppression of *S.cerevisiae tim17* mutant by overexpression of mtHsp70 (Ssc1p). Strains MB3-33 (*tim17-1*) and MB3-46 (*tim23-2*) have a complete deletion of the chromosomal *URA3* coding region (see Table I) and contain the *SOD–URA3* test plasmid, encoding OMP decarboxylase with a mitochondrial targeting sequence, as described by Maarse *et al.* (1992). Construction of the additional plasmids was as described in Rassow *et al.* (1994). Growth was determined on selective minimal medium plates lacking uracil at 23°C (left plates). Expression of genes *TIM17* or *TIM23* respectively led to complementation of the mutants (i.e. no growth after 6 days); expression of *SSC1* from the multicopy vector YEplac181 led to partial complementation of the *tim17* mutant (i.e. very slow growth after 4–5 days), but not of the *tim23* mutant (i.e. growth after 2–3 days); expression of *SSC1* from the centromeric vector YCplac111 did not lead to complementation (growth after 2–3 days). All transformants grew well after 2–3 days in the presence of 40 μg/ml uracil (right plates). (**C**) Synthetic growth defects of *tim17* and *ssc1* mutants. Strain MB33-14 (*tim17-1*) was crossed with strains PK81 (*ssc1-2*) and PK83 (*ssc1-3*). PK81 and PK83 were crossed back with YPH499 to change the mating type and then crossed with MB46-4 (*tim23-2*). The resulting diploid yeast cells were sporulated and the asci were dissected. The viable spores were analyzed for their phenotype by growth on selective medium to assign the genotype. Samples of 30–50 tetrads were analyzed per cross. Typical tetratype tetrads (for *tim17-1* crossed with *ssc1-2* and *ssc1-3*) and non-parental ditype tetrads (for *tim23-2* crossed with *ssc1-2* and *ssc1-3*) are shown.

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

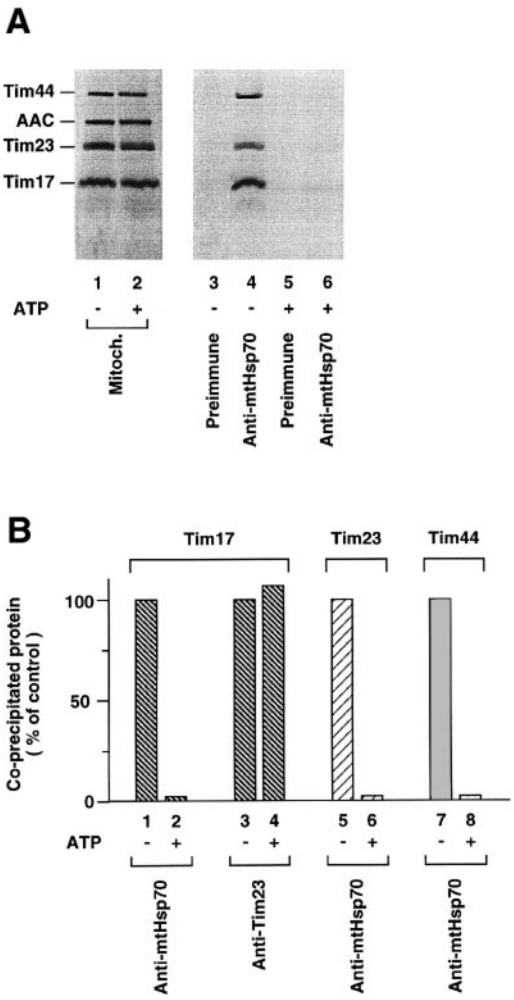

Fig. 6. The association of Tim17 with mtHsp70 is sensitive to ATP. (**A**) Radiolabeled Tim44, Tim23, Tim17 and AAC were imported into isolated wild-type mitochondria as described in the legend to Figure 2A. After treatment with proteinase K, the sample was divided into halves and mitochondria were re-isolated. One half (–ATP) was depleted of ATP by incubation with apyrase and oligomycin and lysed in lysis buffer (containing 2 mM EDTA); the other half (+ATP) was incubated with Mg-ATP and lysed in buffer supplemented with 7 mM $MgCl_2$ and 2 mM ATP. After a clarifying spin, each sample was divided into two aliquots and subjected to immunoprecipitation with antibodies directed against mtHsp70 or preimmune antibodies. (**B**) The experiment was performed as described for (A). An aliquot with co-precipitation of Tim17 with anti-Tim23 was included. The amount of protein co-precipitated in the absence of ATP was set as 100% (control).

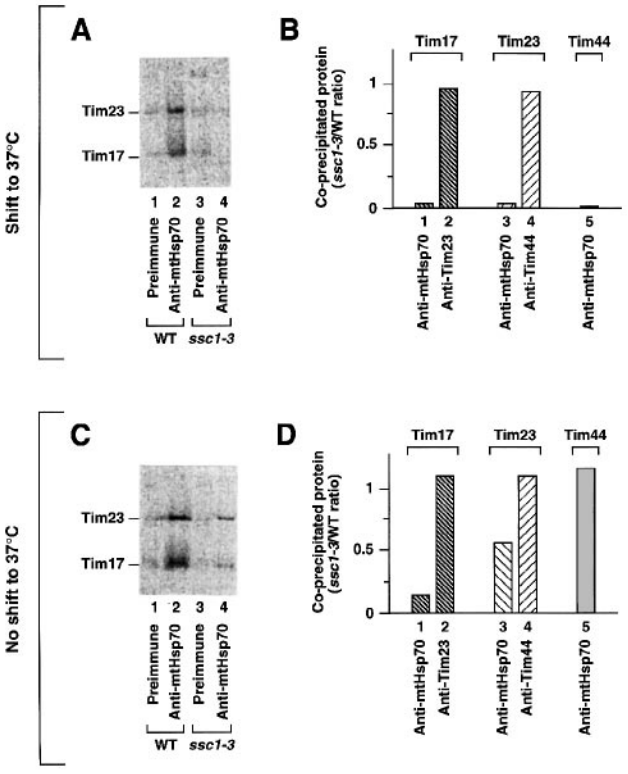

Fig. 7. Differential effect of the *ssc1-3* mutation on association of mtHsp70 with Tim44 and Tim17. (**A**) Tim17 and Tim23 were imported into wild-type or *ssc1-3* mitochondria at 25°C. After treatment with proteinase K, the mitochondria were re-isolated and resuspended in SEMP buffer and incubated 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-precipitation of Tim44 with anti-mtHsp70 was determined by immunodecoration (Rassow *et al*., 1994; Voos *et al*., 1996). The co-precipitated proteins were quantified for wild-type and *ssc1-3* mitochondria. Shown is the ratio of co-precipitation between *ssc1-3* and 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.

the *ssc1-3* mutation affected the association of mtHsp70 with Tim17 and Tim23. After preincubation at 37°C, Ssc1-3p did not bind to Tim17 or Tim23 (Figure 7A, lane 4, and B, columns 1 and 3), while the interactions of Tim23 with Tim17 and Tim44 were still observed with *ssc1-3* mitochondria (Figure 7B, columns 2 and 4).

Without a temperature shift, the association of Ssc1-3p with Tim17 was still strongly reduced (Figure 7C, lane 4, and D, column 1), in contrast to the efficient assocation of Ssc1-3p with Tim44 (Figure 7D, column 5). Co-precipitation of Tim23 with Ssc1-3p was partially reduced (Figure 7C, lane 4, and D, column 3). The interactions of Tim23 with Tim17 and Tim44 were not disturbed (Figure 7D, columns 2 and 4).

We conclude that in the absence of a temperature shift the *ssc1-3* mutation differentially affects the association of mtHsp70 with Tim44 and Tim17. In comparison with the co-precipitation efficiencies of Tim17 and Tim44 with Ssc1-3p (Figure 7D, columns 1 and 5), a part (~60%) of the co-precipitation of Tim23 with mtHsp70 is affected by the *ssc1-3* mutation similarly to the Tim17–mtHsp70 interaction (Figure 7D, column 3). The remainder (~40%) of the Tim23–mtHsp70 association then resembles the Tim44–mtHsp70 interaction. This agrees well with the results obtained with depletion of Tim44 (Figure 4), where ~35% of co-precipitation of Tim23 with mtHsp70 was sensitive to removal of Tim44.

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

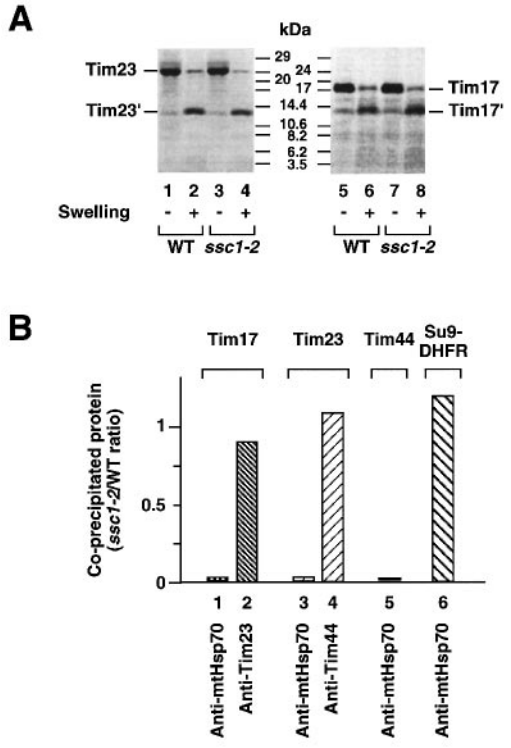

Fig. 8. Differential effect of the *ssc1-2* mutation on association of mtHsp70 with Tim17 and substrate proteins. (**A**) *ssc1-2* mitochondria correctly import Tim17 and Tim23. Radiolabeled Tim17 and Tim23 were imported into isolated mitochondria subjected to swelling and treatment with proteinase K as described in the legend to Figure 1C except that *ssc1-2* mitochondria were used instead of *ssc1-3* mitochondria. (**B**) Co-precipitation of Tim17, Tim23, Tim44 and a substrate protein (Su9–DHFR) with anti-mtHsp70 from *ssc1-2* mitochondria. The experiment was performed and quantified as described in the legend to Figure 7B except that *ssc1-2* mitochondria were used instead of *ssc1-3* mitochondria. In addition, the preprotein Su9–DHFR was imported and analyzed by co-precipitation with anti-mtHsp70 (Gambill *et al.*, 1993; Voos *et al.*, 1994).

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 Tim44 (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 F_0-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 translocase 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

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

Strain	Genotype	Source
PK82	MATα *his4-713 lys2 ura3-52 Δtrp1 leu2-3,112*	Gambill *et al.* (1993)
PK81	MATα *ade2-101 lys2 ura3-52 Δtrp1 leu2-3,112 ssc1-2*(*LEU2*)	Gambill *et al.* (1993)
PK83	MATα *ade2-101 lys2 ura3-52 Δtrp1 leu2-3,112 ssc1-3*(*LEU2*)	Gambill *et al.* (1993)
MB3-33	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3*::*LYS2 tim17-1*	Dekker *et al.* (1993)
MB3-46	MATα *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3*::*LYS2 tim23-2*	Dekker *et al.* (1993)
MB33-14	MAT**a** *ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 tim17-1*(*TRP1*)	Blom *et al.* (1995)
MB46-4	MATα *ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 tim23-2*(*LEU2*)	Blom *et al.* (1995)
MB16	*ade2-101 his3 leu2 lys2-801 trp1-289 ura3-52 tim17*::*LYS2* + YCplac111-*TIM17*-c-*myc* (*LEU2*)	Maarse *et al.* (1994)
MB26	MAT**a** *ade2 his3 leu2 lys2 ura3 trp1 tim17*::*LYS2* + YCplac33-*TIM17*(*URA3*)	this study
MB29	MAT**a** *ade2 his3 leu2 lys2 ura3 trp1 tim23*::*LYS2* + YCplac33-*TIM23*(*URA3*)	this study
YPH499	MAT**a** *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*	Sikorski and Hieter (1989)

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; Maarse *et al.*, 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; Hönlinger *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 Sss1p 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 components of the preprotein 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 descibed by Maarse *et al.* (1992). Double-deficient yeast strains were constructed by crossing the *tim17-1* and *tim23-2* mutants with the *ssc1-2* 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* [*Bst*E2–*Hin*dII + His_6(N)] was constructed in two steps. A *Bam*HI site was created after the ATG initiation codon by site-directed mutagenesis of *TIM17*. A 24 bp *Sau*3A fragment from pQE30 (Qiagen) which encodes the His_6 tag was cloned in-frame into this *Bam*HI site. YCplac111-*TIM17* [*Bst*E2–*Hin*dII + His_6 (N)] was introduced into yeast strain MB26. Double transformants were cured from YCplac33-*TIM17* by growth overnight in YPD medium (or rich broth) and plating a sample on minimal selective medium containing 5-fluoro-orotic acid (0.6 g/l). Plasmid YCplac111-*TIM23* [*Bst*E2–*Hin*dII + His_6(N)] was similarly constructed and introduced into yeast strain MB29 by plasmid shuffling. The resulting strains carrying a His_6 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 [^{35}S]methionine/[^{35}S]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 $MgCl_2$, 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

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 (Söllner *et al.*, 1991; Bömer *et al.*, 1996b). Autoradiographs were obtained and quantified using a storage PhosphorImaging system (Molecular Dynamics Inc.).

Co-immunoprecipitations of radiolabeled Tim complexes

Specific antibodies (6 µl serum) were pre-bound to protein A–Sepharose (10 µl wet volume; Phamacia 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–KOH, 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–KOH, 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 chymostatin, 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*). The supernatants were incubated for 45 min at 8°C by end-over-end shaking with antibodies prebound 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 prebound 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 $MgCl_2$ 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 $MgCl_2$, 2 mM ATP, 30 mM HEPES–NaOH, pH 7.4) and shaken end-over-end at 4°C for 10 min. Insoluble material was removed by ultracentrifugation (30 min at 100 000 *g*) and the supernatant was applied to a Sephacryl HR-300 column (30×0.7 cm). The column was run 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 Ni–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 0.05% sodium deoxycholate samples were analyzed by tricine–SDS–PAGE and immunoblotting.

Acknowledgements

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