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Separation of structural and dynamic functions of the mitochondrial translocase: Tim44 is crucial for the inner membrane import sites in translocation of tightly folded domains, but not of loosely folded preproteins

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The essential gene *TIM44* encodes a subunit of the inner mitochondrial membrane preprotein translocase that forms a complex with the matrix heat-shock protein Hsp70. The specific role of Tim44 in protein import has not yet been defined because of the lack of means to block its function. Here we report on a *Saccharomyces cerevisiae* mutant allele of *TIM44* that allows selective and efficient inactivation of Tim44 *in organello*. Surprisingly, the mutant mitochondria are still able to import preproteins. The import rate is only reduced by ~30% compared with wild-type as long as the preproteins do not carry stably folded domains. Moreover, the number of import sites is not reduced. However, the mutant mitochondria are strongly impaired in pulling folded domains of preproteins close to the outer membrane and in promoting their unfolding. Our results demonstrate that Tim44 is not an essential structural component of the import channel, but is crucial for import of folded domains. We suggest that the concerted action of Tim44 and mtHsp70 drives unfolding of preproteins and accelerates translocation of loosely folded preproteins. While mtHsp70 is essential for import of both tightly and loosly folded preproteins, Tim44 plays a more specialized role in translocation of tightly folded domains.
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Introduction

The passage of nuclear-encoded preproteins from the cytosol to the mitochondrial matrix is mediated by Tom proteins in the mitochondrial outer membrane and by Tim proteins in the mitochondrial inner membrane (Ryan and Jensen, 1995; Schatz, 1996; Neupert, 1997; Pfanner and Meijer, 1997). The mechanism which drives the preproteins across the outer membrane seems to depend on a stepwise interaction of the positively charged presequences with patches of negatively charged amino acids of the Tom proteins (Dietmeier *et al*., 1997; Schatz, 1997). Translocation across the inner membrane is driven by two different mechanisms. The first step is mediated by the presequences which are pulled into the translocation channel by the mitochondrial membrane potential (Martin *et al*., 1991; Bauer *et al*., 1996). The presequences subsequently are cleaved off and translocation of the mature parts of the preproteins is membrane potential-independent. The final steps of translocation are mediated by mitochondrial Hsp70 (mtHsp70) and are coupled to the hydrolysis of ATP (Kang *et al*., 1990; Scherer *et al*., 1990; Hwang *et al*., 1991; Rassow and Pfanner, 1995a; Ungermann *et al*., 1996).

The inner membrane proteins Tim44, Tim23 and Tim17 have been identified in the yeast *Saccharomyces cerevisiae* as components of the machinery which mediates the translocation of presequence-targeted preproteins into the matrix (Maarse *et al*., 1992, 1994; Scherer *et al*., 1992; Horst *et al*., 1993; Dekker *et al*., 1993; Emtage and Jensen, 1993; Ryan *et al*., 1994; for the uniform nomenclature, see Pfanner *et al*., 1996). Tim23 and Tim17 are integral membrane proteins and components of a 90 kDa complex which associates with preproteins during translocation across the inner membrane. It has been suggested that both proteins directly participate in the formation of the inner membrane protein import channel (Dekker *et al*., 1993, 1997; Ryan and Jensen, 1993; Kübrich *et al*., 1994; Bauer *et al*., 1996; Lohret *et al*., 1997; Ryan *et al*., 1998).

Tim44 is encoded by an essential gene (Maarse *et al*., 1992). A mammalian homologue was identified recently in mice (Wada and Kanwar, 1998). The elucidation of its functions has been hampered by the lack of a means to block its activities. The role of Tim44 in mitochondrial protein import is, therefore, still ambiguous. It has been suggested that Tim44 might participate directly in the formation of a protein import channel since it can be cross-linked efficiently to preproteins in transit across the mitochondrial membranes and co-purified with translocation intermediates (Scherer *et al*., 1992; Blom *et al*., 1993; Berthold *et al*., 1995; Horst *et al*., 1995, 1996; Kanamori *et al*., 1997). Interactions of Tim44 with Tim17 and Tim23 have been demonstrated by both biochemical and genetic means (Berthold *et al*., 1995; Blom *et al*., 1995; Bömer *et al*., 1997). However, the contribution of Tim44 to the formation of the channel is difficult to conceive because the primary structure of Tim44 does not show putative membrane-spanning segments, and in fractionations Tim44 behaves like a peripheral membrane protein (Maarse *et al*., 1992; Blom *et al*., 1993; Berthold *et al*., 1995).

According to its accessibility for proteases, Tim44 is

exposed to the matrix side of the inner membrane (Blom *et al*., 1993; Rassow *et al*., 1994). In this location, Tim44 serves as a membrane anchor for mtHsp70, and the complex of Tim44 with mtHsp70 was suggested to represent the core of the mitochondrial protein import machinery (Kronidou *et al*., 1994; Rassow *et al*., 1994, 1995; Schneider *et al*., 1994; von Ahsen *et al*., 1995; Horst *et al*., 1996; Ungermann *et al*., 1996). The functional relevance of this interaction was addressed using a mutant form of mtHsp70, Ssc1-2p, that is unable to bind to Tim44 but still binds to preproteins (Voos *et al*., 1996). However, more recent data indicate that mtHsp70 binds not only to Tim44, but also directly to the Tim17–Tim23 complex. The mutant form Ssc1-2p is defective in the interaction with both membrane-binding sites (Bömer *et al*., 1997). Therefore, it could not be decided if the defects in protein translocation which were observed with the mutant allele *ssc1-2* were indeed caused by the lack of interaction with Tim44. Up to now, the individual functions of the two membrane anchors in the mechanism of protein translocation could not be discerned, and the contribution of Tim44 to the activity of mtHsp70 is unclear.

In this study, we have examined the function of Tim44, using a temperature-sensitive mutant of Tim44. For the first time, this mutant allows investigation of the effects of a selective inactivation of functional Tim44. We found that the number of translocation sites in the inner membrane is not reduced by depletion of Tim44, suggesting that Tim44 is not a constitutive component of the protein import channel. The mutant mitochondria show a reduced rate of import reactions, most pronounced in the case of tightly folded protein domains. We conclude that Tim23 and Tim17 can form the protein import channel in the absence of Tim44. Tim44 is part of a separate functional unit which acts as the dynamic element of the Tim machinery.

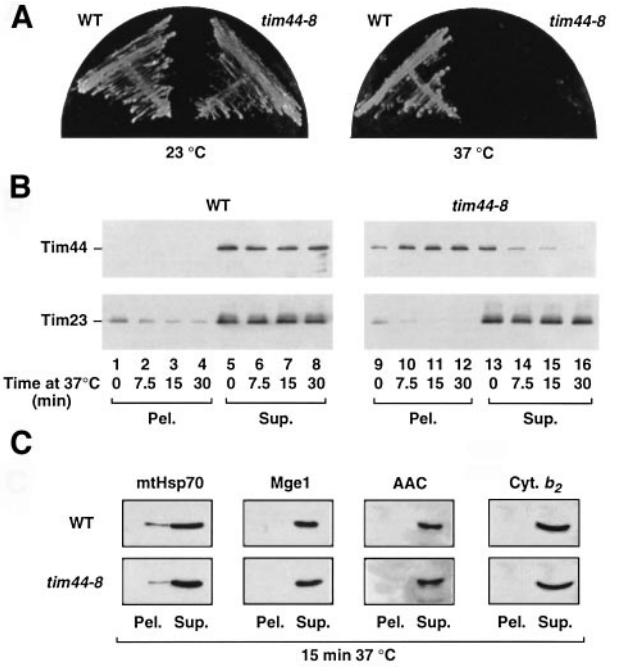

Fig. 1. Temperature-sensitive mutant of Tim44. (**A**) Growth defect of a yeast strain containing the allele *tim44-8*. Wild-type *TIM44* and the mutant allele *tim44-8* were cloned into the single-copy vector YCplac111 and expressed in a *tim44* deletion strain. The strains were grown on glycerol plates at 23 or 37°C, respectively. (**B**) Aggregation of mutant Tim44 after heat shock. Isolated mitochondria were incubated in import buffer (including 2 mM ATP; BSA was omitted) at 37°C for different time periods as indicated. Subsequently, all samples were shifted to 25°C for 5 min. In parallel, mitochondria were kept on ice and then incubated at 25°C. Triton X-100 was added to a final concentration of 0.1%, and the samples were shaken end-over-end at 8°C for 10 min. Aggregates were pelleted for 1 h at 106 000 g_{max} and analysed by SDS–PAGE and immunoblotting. (**C**) Mitochondria were treated as in (B) and tested for aggregation of mtHsp70, Mge1, ADP/ATP carrier (AAC) and cytochrome b_2 (Cyt. b_2).

Results

A temperature-sensitive mutant of Tim44

We generated mutated forms of *TIM44* by low fidelity PCR and expressed the mutated genes in *S.cerevisiae* in a single-copy vector under the control of their own promoters. The clone containing the allele *tim44-8* was isolated and used in this study (Figure 1A). At 23°C, the mutant strain grows similarly to the wild-type on glucose and on glycerol, whereas at 37°C it grows only very poorly on these media. We isolated mitochondria from both the mutant and the wild-type strain after growth at 23°C. We found that the Tim44 protein was present in the same amounts in both strains. After lysis of the mitochondria in the presence of detergent, we found that the mutant Tim44 differed from the wild-type protein by a dramatically enhanced tendency to aggregate (Figure 1B). After a pre-incubation of the isolated mitochondria at 37°C, the mutant *tim44-8* protein aggregated efficiently (Figure 1B, lanes 9–16) while the wild-type Tim44 protein remained soluble (Figure 1B, lanes 1–8). The solubility of other mitochondrial proteins which were tested was not affected by the *tim44-8* mutation, including Tim23 (Figure 1B), the matrix proteins mtHsp70 and Mge1p, the ADP/ATP carrier (AAC) of the inner membrane and the intermembrane space protein cytochrome b_2 (Figure 1C). The mutant Tim44 protein seems to have a labile tertiary structure which collapses at higher temperature and renders the protein prone to aggregation. Hence, the allele *tim44-8* provides the opportunity to deplete mitochondria of functional Tim44. As the mutant phenotype can be induced by a pre-incubation of isolated mitochondria without affecting other known components of the protein import machinery (and does not require a temperature shift *in vivo*), indirect effects of the inactivation of Tim44 on mitochondrial structure and function are minimized.

The number of translocation sites for preproteins carrying a presequence is not reduced upon depletion of functional Tim44

To determine the role of Tim44 in mitochondrial protein import, we isolated mitochondria from the *tim44-8* mutant and the corresponding wild-type, and imported several different preproteins. To deplete the *tim44-8* mitochondria of Tim44, the mitochondria were subjected to a heat shock prior to the import reactions. We synthesized the precursors of the α-subunit of the matrix-processing peptidase (MPP), the β-subunit of the ATP synthase ($F_1\beta$), and AAC in reticulocyte lysate in the presence of [^{35}S]methionine/[^{35}S]cysteine and imported the proteins into the isolated mitochondria (Figure 2). Complete translocation of αMPP and $F_1\beta$ across the mitochondrial membranes was assayed

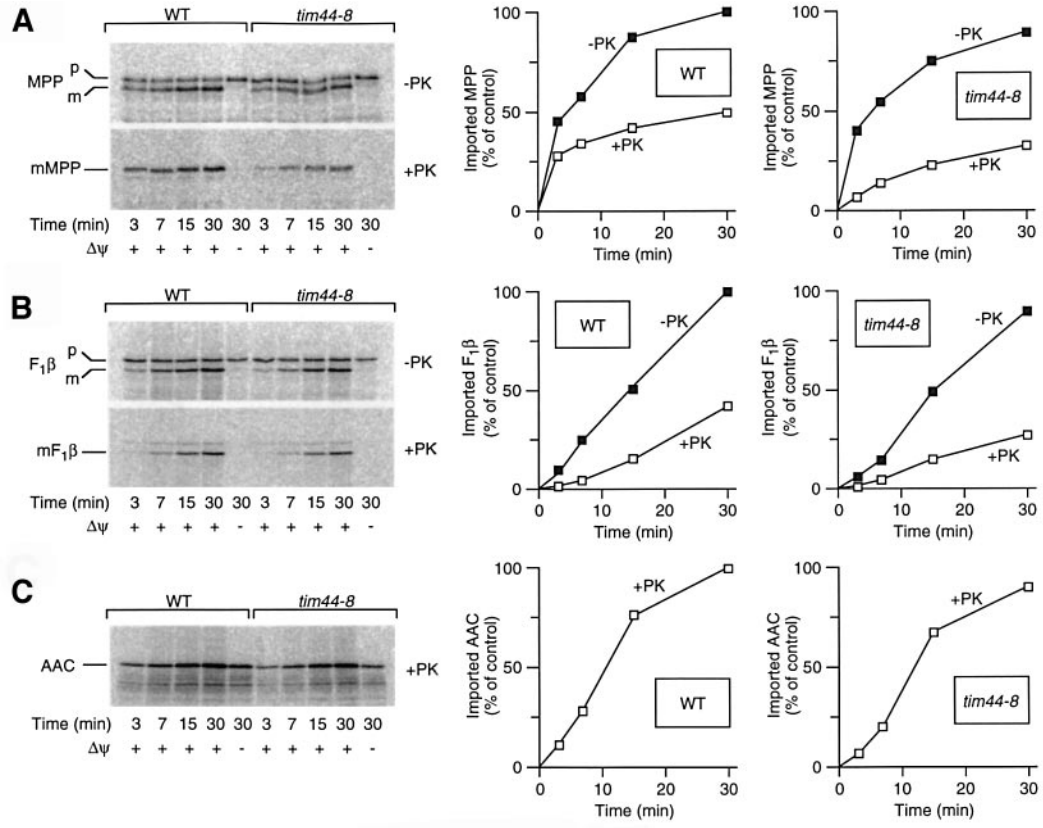

Fig. 2. Mitochondria of the *tim44-8* mutant show efficient import of several preproteins. (**A**) Import of the α-subunit of the matrix-localized mitochondrial processing peptidase MPP. The preprotein was synthesized in a reticulocyte lysate in the presence of [^{35}S]methionine/[^{35}S]cysteine and incubated with isolated mitochondria from the *tim44-8* mutant strain and the corresponding wild-type (WT) at 25°C for the times indicated. Prior to import, the mitochondria of both strains were incubated at 37°C for 15 min to induce the mutant phenotype. The import reactions were stopped by addition of valinomycin and cooling on ice. The samples were split into halves and treated with proteinase K to remove preproteins outside the mitochondrial membranes (+ PK) or left without protease (– PK). After reisolation of the mitochondria by centrifugation, the imported proteins were separated by SDS–PAGE and analysed using a Phosphorimager. p, precursor protein; m, mature protein. The graphs show the relative amounts of processed proteins. The amount of processed protein obtained after 30 min in the absence of proteinase K was set to 100% (control). (**B**) Import of the β-subunit of the mitochondrial ATP synthase ($F_1\beta$). The experiment was performed as described in (A). (**C**) Import of the ADP/ATP carrier (AAC). Since the AAC does not contain a cleavable presequence, only the protease-protected AAC is shown. For quantification of the Δψ-dependent imported AAC, the amount of protease-protected AAC which accumulated in the absence of a membrane potential was subtracted.

by resistance to externally added protease. In this assay, the import efficiencies in the *tim44-8* mutant mitochondria were reduced by ~20–40% (Figure 2A and B, samples + PK). However, processing of the imported proteins reached 80–95% of the wild-type levels, demonstrating that the inactivation of Tim44 has only minor effects on the translocation of the presequences through the protein import channels of the membranes (Figure 2A and B, samples – PK). Considering that the mutant Tim44 was depleted by >95%, the reduction in the import efficiencies of αMPP and $F_1\beta$ was less pronounced than expected. In particular, the insertion of the presequences into the Tim channel seems to be Tim44-independent. Tim44 appears to act primarily in late stages of protein translocation.

A slight reduction in the import kinetics was also observed with the AAC, the import of which is regarded as completely independent of the Tim17/Tim23/Tim44 machinery (Sirrenberg *et al.*, 1996, 1998; Dekker *et al.*, 1997; Koehler *et al.*, 1998). However, the import of the AAC requires the membrane potential Δψ across the mitochondrial inner membrane. We found that the membrane potential was slightly weaker in the *tim44-8* mutant mitochondria than in the wild-type mitochondria (see below, Figure 6), suggesting that the reduced import efficiencies may be due in part to this.

To study the import defects of the mutant mitochondria in more detail, we used several hybrid proteins containing a DHFR (dihydrofolate reductase) domain which were designed specifically to characterize the mechanisms of mitochondrial protein import. First we imported the hybrid protein Su9(70)–DHFR (containing the presequence of subunit 9 of the ATP synthase fused to the entire DHFR) which is used commonly to characterize the import of matrix-targeted proteins (Figure 3A). The import efficiency was reduced by 20–30%, again demonstrating that the inactivation of Tim44 still allows considerable activity of the import machinery.

The possibility existed that the radiochemical amounts of the preprotein synthesized in the reticulocyte lysate may be imported by a small number of sites still containing intact Tim44. We therefore tested the proximity of the translocating Su9(70)–DHFR to Tim44 by chemical cross-linking (Figure 3B). Using the reagent disuccinimidyl suberate (DSS), the preprotein was cross-linked efficiently to Tim44 in wild-type mitochondria as observed previously (Blom *et al.*, 1993; Kübrich *et al.*, 1994). Two cross-

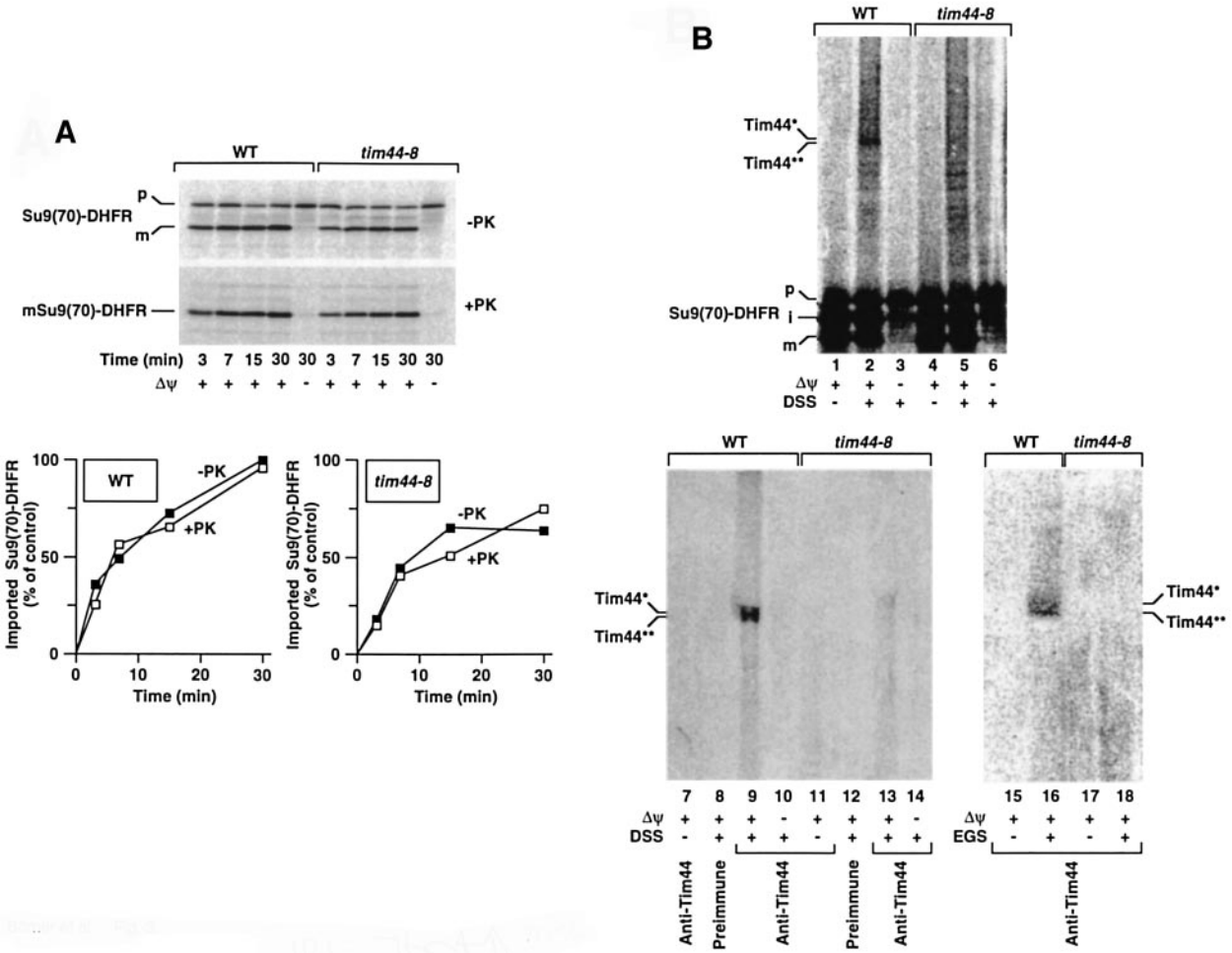

Fig. 3. The hybrid protein Su9(70)–DHFR can be imported in the absence of functional Tim44. (**A**) Import of Su9(70)–DHFR. The preprotein contains the N-terminal residues 1–70 of Su9 fused to the entire DHFR of the mouse. After synthesis of the ^{35}S-labelled protein in a reticulocyte lysate, the preprotein was imported as described in the legend to Figure 2A. p, preprotein; i, processing intermediate; m, mature protein. (**B**) Cross-linking of Su9(70)–DHFR. The preprotein was accumulated in translocation sites by import into ATP-depleted mitochondria. In parallel samples, the membrane potential Δψ was dissipated by addition of valinomycin prior to import. The mitochondria were reisolated and the cross-linking reagent DSS was added. For details of the cross-linking protocol, see Materials and methods. Lanes 1–6, SDS–PAGE and fluorography of total cross-linked products; lanes 7–14, SDS–PAGE and fluorography of immunoprecipitated proteins; lanes 15–18, immunoprecipitation of proteins after cross-linking by EGS. Tim44* and Tim44**, cross-linking products containing unprocessed or processed Su9–DHFR, respectively.

linking products were formed which could be immunoprecipitated by antibodies raised against Tim44, representing cross-linking to the preprotein and to the processing intermediate (Figure 3B, bands labelled Tim44* and Tim44** in lanes 2 and 9). In contrast, Su9(70)–DHFR imported into the mutant mitochondria was not cross-linked (Figure 3B, lane 5 versus lane 2, and lane 13 versus lane 9). The same result was obtained with ethylene glycolbis succinimidyl-succinate (EGS; Figure 3B, lanes 15–18), a reagent with a longer spacer arm than DSS (1.61 nm versus 1.14 nm). These results thus confirm that preproteins can be imported in the absence of Tim44.

We then asked whether the mitochondria with inactivated Tim44 were able to import large amounts of preproteins. To obtain a soluble preprotein in chemical amounts, we expressed the hybrid protein b_2(167)Δ–DHFR in *Escherichia coli* and purified it in functional form (Dekker *et al*., 1997). The construct consists of the first 167 residues of cytochrome b_2 and complete DHFR (Koll *et al*., 1992). Due to the deletion (Δ) of a segment within the presequence, the preprotein is targeted to the mitochondrial matrix space (Koll *et al*., 1992). The preprotein was applied at concentrations close to saturation of the mitochondrial import sites (Dekker *et al*., 1997). The import kinetics of this protein are shown in Figure 4A. The import efficiency in the mutant mitochondria was reduced to a similar extent as the import of the radiochemical amounts of preproteins from reticulocyte lysate shown in Figures 2 and 3.

The preprotein b_2(167)Δ–DHFR is processed in two stages. Immediately after translocation across the inner membrane, the first part of the presequence is cleaved off and the processing intermediate i is formed. In a subsequent step, ~8 additional amino acids are removed, yielding the intermediate i*, indicating a further maturation step of the preprotein (Laloraya *et al*., 1995). Interestingly, the formation of the i* intermediate is reduced drastically in the *tim44-8* mitochondria while the formation of the i intermediate is less affected (Figure 4A, upper panel). The same effect has been observed with mutants of the mtHsp70/Mge1 system (Voos *et al*., 1993, 1994; Laloraya *et al*., 1995), suggesting that Tim44 and the mtHsp70

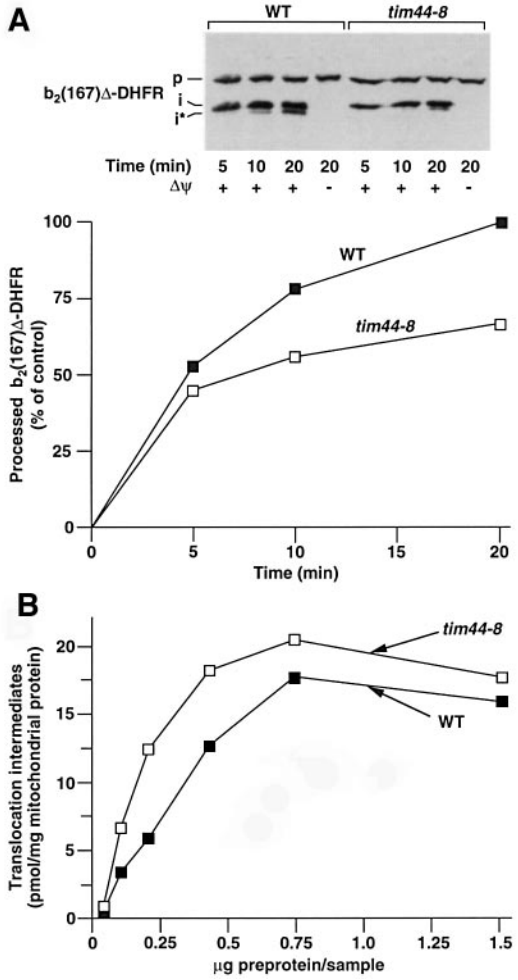

Fig. 4. Import of the purified preprotein b_2(167)Δ–DHFR in chemical amounts after inactivation of Tim44. (**A**) Import of b_2(167)Δ–DHFR (in the absence of MTX). The preprotein b_2(167)Δ–DHFR contains the N-terminal 167 residues of cytochrome b_2 fused to the complete DHFR. b_2(167)Δ–DHFR was expressed in *E.coli*, purified and incubated with isolated mitochondria of the *tim44-8* mutant and the corresponding wild-type (WT), pre-treated at 37°C, at 25°C for the times indicated. In parallel samples, valinomycin was added to dissipate the membrane potential $\Delta\psi$. The mitochondria were reisolated and the amounts of imported protein were analysed by SDS–PAGE, immunoblotting and densitometry. The amounts of b_2(167)Δ–DHFR processed in wild-type mitochondria after 20 min were set to 100% (control). p, precursor protein; i, processing intermediate; i*, second processing intermediate. (**B**) Titration of protein import sites. Mitochondria were pre-treated at 37°C and incubated in the presence of increasing concentrations of b_2(167)Δ–DHFR. MTX was included to accumulate the preprotein as a translocation intermediate spanning both mitochondrial membranes. The import reactions were stopped by addition of valinomycin and cooling on ice. The mitochondria were reisolated and the relative amounts of processed translocation intermediates of b_2(167)Δ–DHFR were determined by SDS–PAGE, immunoblotting and densitometry. In both (A) and (B), the import-competent preprotein was added in excess; <10% of the total amount of added preprotein was imported into the mitochondria.

system act on the translocating polypeptide at a similar stage of import.

The remarkably high import efficiencies observed after depletion of Tim44 raise the question of whether Tim44 is an intrinsic component of the protein import channel of the inner membrane or if it is involved in the import reaction in a different way. We took advantage of the possibility of accumulating the b_2(167)Δ–DHFR in translocation sites by import in the presence of the DHFR ligand methotrexate (MTX). It has been shown previously that the number of translocation intermediates which can be accumulated by this method is limited by the number of available import sites in the inner membrane (Dekker *et al.*, 1997). We now titrated the maximal number of translocation intermediates in parallel for the *tim44-8* mutant mitochondria and the corresponding wild-type mitochondria. Surprisingly, we found similar numbers for mitochondria from both strains (Figure 4B). About 20 pmol of preprotein could be accumulated per mg of mitochondrial protein, in agreement with previous studies with wild-type mitochondria (Vestweber and Schatz, 1988; Rassow *et al.*, 1989; Dekker *et al.*, 1997). We conclude that Tim44 is not an essential factor in the formation of functional protein translocation channels. The involvement of Tim44 increases the efficiency of protein import, but the amount of Tim44 does not determine the number of import sites.

Function of Tim44 in unfolding and import of a tightly folded protein domain

To assess the features of a preprotein which determine its dependence on Tim44, we investigated the import of cytochrome b_2–DHFR hybrid proteins and compared constructs which differ in their requirement for unfolding of defined protein domains (Figure 5A). We first tested two different constructs, containing N-terminal parts of cytochrome b_2, residues 1–220 or 1–85, respectively. The presequences of both constructs are identical; however, the longer construct [b_2(220)-DHFR] contains the complete haem-binding domain of cytochrome b_2 (residues 81–180) which is tightly folded and requires unfolding to allow translocation across the mitochondrial membranes. The haem-binding domain is missing in b_2(85)–DHFR. Thus, both preproteins differ in the requirement for energy-dependent unfolding of a distinct protein domain; the rate of import is limited by the efficiency of unfolding of the domain (Gambill *et al.*, 1993; Glick *et al.*, 1993; Voos *et al.*, 1993, 1996). The DHFR domain is less stably folded and, in the absence of MTX, does not cause effective restrictions in import reactions (Pfanner *et al.*, 1990; Voos *et al.*, 1996). We found that both preproteins were imported efficiently into mitochondria of the wild-type (Figure 5B, lanes 1–4). In contrast, in mitochondria of the *tim44-8* mutant, the import of b_2(220)–DHFR was considerably delayed while the import of b_2(85)–DHFR showed only a minor reduction (Figure 5B, lanes 6–9). The effect was most pronounced after treatment of the mitochondria with protease (+ PK). The comparison of the import efficiencies of both constructs thus demonstrates a role for Tim44 in the unfolding of the cytochrome b_2 haem-binding domain. The unfolding and translocation of more loosely folded protein domains, as in b_2(85)–DHFR [and b_2(167)Δ–DHFR, Figure 4A], is far less affected.

The preprotein b_2(220)–DHFR contains an intact cytochrome b_2 presequence. This presequence directs the processed proteins to the intermembrane space (Guiard, 1985). A substitution of alanine in position 63 by proline entails mistargeting of the imported proteins into the mitochondrial matrix (Beasley *et al.*, 1993). We now tested whether in *tim44-8* mitochondria the defect in

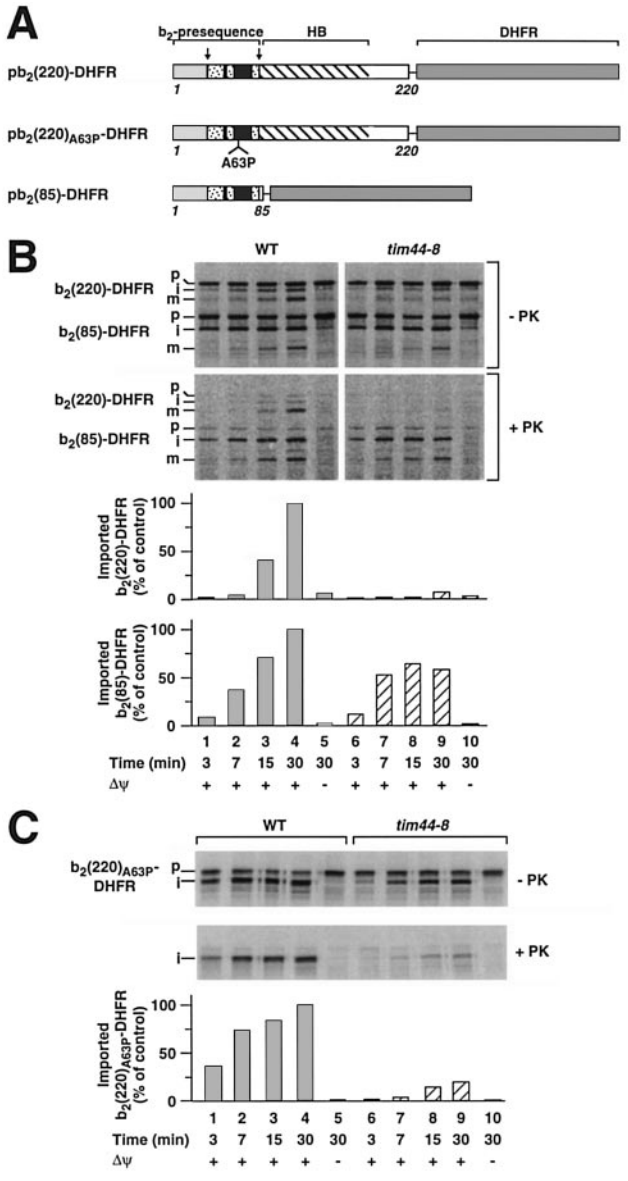

Fig. 5. Import of the cytochrome b_2 haem-binding domain is inhibited in *tim44-8* mitochondria. (**A**) Cytochrome b_2–DHFR fusion proteins. In contrast to b_2(85)–DHFR, the preprotein b_2(220)–DHFR contains a complete haem-binding domain (residues 81–180). In $b_2(220)_{A63P}$–DHFR, the intermembrane space sorting signal is inactivated by substitution of the alanine in position 63 for proline. (**B**) The preproteins b_2(85)–DHFR and b_2(220)–DHFR were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine/[^{35}S]cysteine and imported into isolated mitochondria for the times indicated. To dissipate the membrane potential, two samples received valinomycin. After the import reaction, the mitochondria were treated with proteinase K (+ PK) or left without protease (– PK), reisolated and analysed by SDS–PAGE and fluorography. The relative amounts of processed and proteinase K-protected protein were determined using a phosphorimager. The amount of protein imported into wild-type mitochondria in 30 min was set to 100% (control). p, precursor protein; i, processing intermediate; m, mature protein. (**C**) Import of the cytochrome b_2 haem-binding domain as part of a matrix-targeted preprotein. ^{35}S-labelled $b_2(220)_{A63P}$–DHFR was synthesized in reticulocyte lysate and imported into isolated mitochondria for the times indicated. Due to the amino acid substitution in the presequence, the preprotein (p) is only processed to the intermediate (i). The mitochondria were treated with proteinase K (+ PK) or left without protease (– PK), reisolated and analysed by SDS–PAGE and fluorography. The relative amounts of processed and proteinase K-protected protein were quantified.

unfolding of the haem-binding domain is also observed with a preprotein containing the modified presequence. We used the hybrid protein $b_2(220)_{A63P}$–DHFR (Figure 5A) and found that its import in *tim44-8* mutant mitochondria was inhibited similarly to the import of b_2(220)–DHFR (Figure 5C). This result shows that intact Tim44 facilitates the import of the tightly folded cytochrome b_2 haem-binding domain, independently of whether this domain is targeted to the intermembrane space or to the matrix.

We then wondered if the strong effect of the *tim44-8* mutation on the translocation of the haem-binding domain could be caused by a weakening of the mitochondrial membrane potential $\Delta\psi$ and a differential $\Delta\psi$ dependence of the preproteins used. For this purpose, we compared the membrane potential of isolated wild-type and mutant mitochondria (Figure 6A). As an assay, we used the quenching of the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$]. We found a minor reduction in the membrane potential in the mutant mitochondria which may account for the slightly reduced import rate of preproteins in the $\Delta\psi$-dependent steps (shown in Figures 2 and 3). In order to exclude that differential effects of the *tim44-8* mutation were mediated indirectly via a differential $\Delta\psi$ dependence of the preproteins, we determined the influence of the membrane potential on the import rate of several preproteins (Figure 6B). We compared the import of the preproteins in the presence of increasing concentrations of the uncoupling reagent carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP; Figure 6B). The preprotein $F_1\beta$ showed a strong reduction in import already at low concentrations of CCCP, demonstrating a strong dependence on $\Delta\psi$. In contrast, the import of the fusion proteins $b_2(220)_{A63P}$–DHFR and b_2(85)–DHFR revealed only a weak dependence on the membrane potential; the AAC showed an intermediate behaviour. There was no obvious correlation between these differences in the CCCP titrations and the differences observed in the import experiments with *tim44-8* mitochondria shown in Figures 2 and 5. In particular, the construct $b_2(220)_{A63P}$–DHFR, which in the import experiments was strongly affected by the inactivation by Tim44, showed the highest resistance to the reduction of $\Delta\psi$ in the CCCP titrations. We conclude from these data that the membrane potential does not account for the delayed translocation of the haem-binding domain into the *tim44-8* mitochondria. The characterization of the import defects as shown in Figures 2–6 suggests that Tim44 is not a structural part of the import channel but acts in later steps of import.

Function of Tim44 in a translocation step after insertion of the presequence into the inner membrane

We then investigated the possible role of Tim44 in the $\Delta\psi$-independent steps of the import process. The preprotein Su9(70)–DHFR can be accumulated spanning both mitochondrial membranes by import in the presence of MTX (Schwarz *et al*., 1993; Ungermann *et al*., 1994). An active protein import machinery is capable of pressing the MTX-stabilized DHFR domain against the outer membrane, rendering the intermediate resistant to externally added proteases, even after subsequent dissipation of the membrane potential (the DHFR domain of the hybrid protein

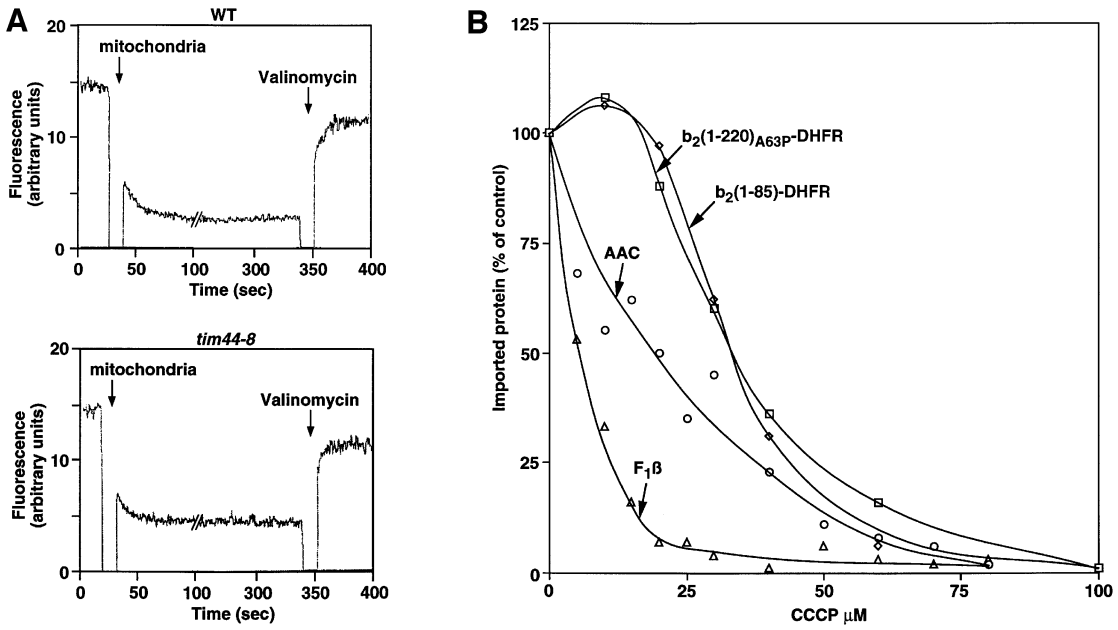

Fig. 6. Inhibition of import of the cytochrome b_2 haem-binding domain in *tim44-8* mitochondria is not caused by a reduced membrane potential. (**A**) Assessment of mitochondrial membrane potential $\Delta\psi$. Wild-type mitochondria (WT) and *tim44-8* mitochondria (200 µg of protein) were pre-incubated at 37°C for 15 min and subsequently incubated with the membrane potential-sensitive dye $DiSC_3(5)$. The fluorescence changes were recorded by a fluorescence spectrophotometer at 25°C. Valinomycin (Val) was added as indicated to dissipate the membrane potential. The difference between the fluorescence prior to and after the addition of valinomycin represents a relative assessment of the membrane potential. (**B**) Sensitivity of import efficiency to reduction of the membrane potential. The preproteins were imported as described in the legend to Figure 2A except that increasing amounts of CCCP were included to reduce the mitochondrial membrane potential. All import reactions were stopped after 4 min and analysed by SDS–PAGE and fluorography. The amounts of processed protein were quantified using a phosphorimager, the amount of processed preprotein in the absence of CCCP was set to 100% (control).

stays outside the membranes but is resistant to proteolysis). A reduction in the activity of the import machinery leads to partial release of the translocation intermediate, and the Su9 part of the hybrid protein becomes accessible to proteases. We now followed this principle to determine the activity of the protein import machinery in the *tim44-8* mitochondria (Figure 7). After import in the presence of an intact membrane potential and MTX into mitochondria of both the wild-type and the mutant, the Su9(70)–DHFR precursor protein was resistant to proteinase K (Figure 7B, lanes 2 and 8). The membrane potential was sufficient to keep the preproteins in the import channel. After dissipation of the membrane potential, the preproteins remained intact in the wild-type mitochondria and were released only very slowly (lanes 3–6). In the mutant mitochondria, however, the intermediates were degraded rapidly after addition of the protease (lanes 9–12). These data indicate that Tim44 is required to establish a membrane potential-independent force which acts on polypeptides in transit across the mitochondrial membranes.

To determine the location in which Tim44 functionally gains access to the translocating polypeptide, we constructed a series of hybrid proteins containing N-terminal parts of different lengths of subunit 9, fused to the DHFR (Figure 8A). The constructs were imported in the presence of MTX and the Su9 part was taken as a molecular ruler to determine the distance between the DHFR domain outside the mitochondrial membranes and the location at which Tim44 starts to act on the translocation intermediate, using the assay of the previous experiment. In wild-type mitochondria, the stability of the constructs in the import channels was dependent on the length of the membrane-spanning segment of the translocation intermediates. After dissipation of the membrane potential, the precursor form of Su9(70)–DHFR and the processing intermediate of Su9(81)–DHFR were released only slowly from the import sites, demonstrating a $\Delta\psi$-independent retention mechanism which acts on the polypeptide (Figure 8, upper panel, lane 5 versus lane 4, and lane 11 versus lane 10). The shorter constructs were released and digested rapidly by the added protease (Figure 8, precursor form in lanes 1–3, and the processing intermediate in lanes 4–6). A comparison of the different constructs indicates that ~45–50 amino acid residues are required to keep the polypeptides in the import channel. In contrast, in the *tim44-8* mutant mitochondria, no $\Delta\psi$-independent retention of polypeptides in the import sites was observed with any of the constructs (Figure 8, lower panel).

The assay not only confirms that Tim44 is required in the retention of polypeptides in the import channels, but also demonstrates that the constructs have to be of sufficient length to encounter the Tim44-dependent activity. Since it is known from independent studies that ~50 amino acid residues are required to span the mitochondrial membranes (Rassow *et al.*, 1990; Ungermann *et al.*, 1994), we conclude that Tim44 acts on preproteins which have already partially traversed the inner membrane and are accessible at the matrix side of the membrane.

Discussion

Tim44 was the first protein identified as a component of the protein translocation machinery of the mitochondrial inner membrane (Maarse *et al.*, 1992; Scherer *et al.*,

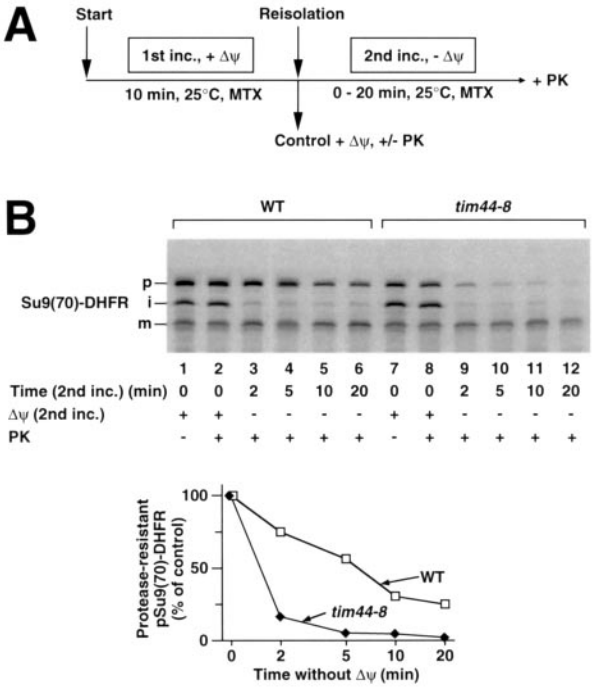

Fig. 7. Reduced activity of the protein import machinery and enhanced mobility of translocation intermediates in *tim44-8* mitochondria. (**A**) Scheme of the experiment. (**B**) As illustrated in (A), the hybrid protein Su9(70)–DHFR (containing residues 1–70 of Su9 fused to the entire DHFR, see Figure 8A) was synthesized in a reticulocyte lysate in the presence of [^{35}S]methionine/[^{35}S]cysteine and imported into mitochondria of the *tim44-8* mutant and of the corresponding wild-type in the presence of MTX. Subsequently, the mitochondria containing the accumulated translocation intermediate were washed and then incubated at 25°C in the presence of valinomycin (to dissipate the membrane potential Δψ) and MTX (to stabilize the native folding state of the DHFR domain). After the times indicated, the mitochondria were cooled on ice and treated with proteinase K (PK). The mitochondria were reisolated and analysed by SDS–PAGE and fluorography. The activity of the mitochondrial protein import machinery leads to pressing of the DHFR domain against the outer membrane and thus to resistance to the externally added protease. Reduced activity of the import machinery allows access for the protease. The presequence of Su9 contains two sites for processing, at position 35 (i-form) and at position 66 (m-form). p, precursor protein; i, processing intermediate; m, mature protein. The relative amounts of the protease-resistant pSu9(70)–DHFR translocation imtermediates were determined using a phosphorimager. The amounts retained after the first incubation in the presence of the membrane potential and reisolation, prior to dissipation (time 0), were set to 100% (control).

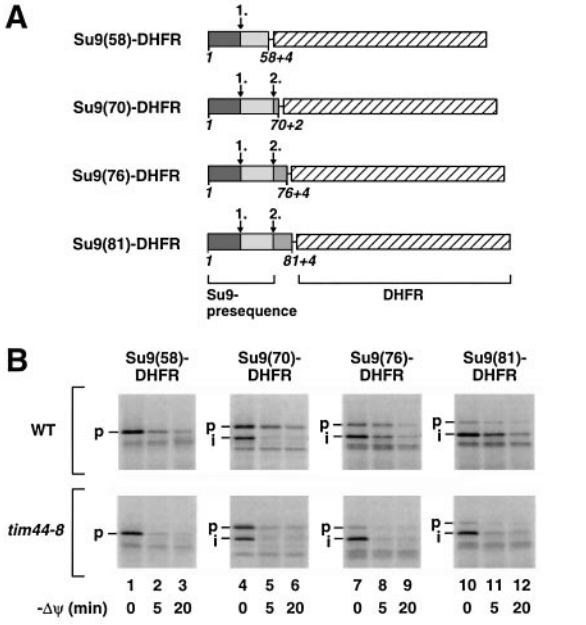

Fig. 8. Su9–DHFR fusion proteins require membrane-spanning segments of ~45 amino acids for stable and Tim44-dependent insertion in translocation sites. (**A**) Su9–DHFR fusion proteins. The constructs contain different N-terminal parts of Su9 of the mitochondrial ATP synthase fused to the entire DHFR of the mouse. The proteins contain two processing sites (designated 1. and 2.) between residues 35 and 36 and between residues 66 and 67, respectively. The numbers shown in the figure refer to the C-terminal residue of the Su9 part of the respective construct. Both parts of the fusion proteins are connected by a linker of two (Gly, Ile) or four residues (Gly, Ser, Gly, Ile), as indicated. (**B**) Resistance of Su9–DHFR fusion proteins to proteinase K after accumulation in mitochondrial protein import sites. The fusion proteins were synthesized in a reticulocyte lysate in the presence of [^{35}S]methionine and imported into mitochondria of the *tim44-8* mutant and of the corresponding wild-type in the presence of MTX. After reisolation and washing, the mitochondria were resuspended in a buffer containing valinomycin (to dissipate the membrane potential Δψ) and MTX (to stabilize the native folding state of the DHFR). The mitochondria were incubated at 25°C for the times indicated and then cooled to 0°C. The samples were treated with proteinase K to digest unstable translocation intermediates. The mitochondria were reisolated and analysed by SDS–PAGE and fluorography.

1992). Since then, only little information has accumulated concerning the precise function of Tim44. It has been speculated that Tim44 might be involved essentially in the formation of the protein import pore; alternatively, it has been suggested that Tim44 together with mtHsp70 might constitute the motor of mitochondrial protein import (Maarse *et al*., 1992; Horst *et al*., 1993; Glick, 1995; Pfanner and Meijer, 1995; Rassow and Pfanner, 1995b; Neupert, 1997). In this study, we have addressed these alternatives by a selective inactivation of Tim44 in mitochondria from a temperature-sensitive mutant strain and the characterization of the consequences for preprotein translocation.

Surprisingly, the inactivation of Tim44 allows quite efficient import of many preproteins, even at concentrations close to saturation of import sites. This observation thus challenges the concept both of Tim44 as a component of the import channel and of Tim44 as an essential part of the import motor. Indeed we found that the number of translocation sites was not affected by the inactivation of Tim44, indicating that Tim44 is not required for the formation of the protein import pore. This conclusion is corroborated by the observation that the formation of the 90 kDa complex of Tim proteins which is tightly associated with translocating polypeptides also is not reduced by the depletion of Tim44 (unpublished data). The formation of the inner membrane protein import channel seems to be the function of proteins other than Tim44; candidates are Tim23 and Tim17 which are intrinsic components of the 90 kDa complex (Bauer *et al*., 1996; Dekker *et al*., 1997; Lohret *et al*., 1997).

The insertion of Tim23 and Tim17 into the inner membrane does not require mtHsp70 or Tim44 (Bömer *et al*., 1997; Káldi *et al*., 1998). However, we cannot rule out the possibility that Tim44 may play a role in the import of other proteins which may be involved in initial steps of channel formation. Once established, the maintenance of functional import channels appears to be completely Tim44-independent.

Our functional data obtained with the *tim44-8* mutant indicate that a preprotein has to traverse the inner membrane and reach the matrix side to encounter the consequences of an inactivation of Tim44. Considering that Tim44 is not a structural component of the protein import pore and that the amino acid sequence of Tim44 shows a hydrophilic protein which does not reveal any potential membrane-spanning segments, we suggest that Tim44 acts at the inner side of the inner membrane, in cooperation with other Tim proteins which form the import channel.

Excluding a role for Tim44 as a pore-forming protein, what is its role in mitochondrial protein import? After it had been observed that Tim44 can form a complex with mtHsp70, it was speculated that Tim44 might be required to deliver mtHsp70 to the translocating polypeptide (Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994). It was known from other studies that mtHsp70 binds to translocating preproteins and is an essential component of the import machinery (Kang *et al*., 1990; Scherer *et al*., 1990). However, it was not possible to investigate whether Tim44 actually plays a role in this process. We now find that several preproteins which are strictly mtHsp70-dependent in their translocation (Gambill *et al*., 1993; Glick *et al*., 1993; Voos *et al*., 1993; Stuart *et al*., 1994) are still imported with reasonable efficiencies into *tim44-8* mitochondria. We conclude that the transfer of mtHsp70 to the polypeptides emerging at the import sites is not entirely dependent on Tim44. At least to some degree, Tim44-independent binding of mtHsp70 seems to be possible, and this binding appears to be functional in the mechanism of protein import.

However, in testing for the consequences of a depletion of active Tim44 in protein import, we observed a drastic reduction in the import of a tightly folded protein domain. In contrast to the import of more loosely folded domains, the unfolding and translocation of a tightly folded domain require the involvement of Tim44. This result is remarkable in the context of data which were obtained previously with mutant forms of mtHsp70 (von Ahsen *et al*., 1995; Voos *et al*., 1996; Bömer *et al*., 1997). The mtHsp70 which is encoded by the allele *ssc1-2* shows strong binding to preproteins but impaired binding to Tim44 and to Tim17–Tim23. This defect correlates with a delayed import of tightly folded protein domains, raising the question of whether the complex of mtHsp70 with Tim44 or the complex with Tim17–Tim23 is involved in the import reaction. The results with the *tim44-8* mutant demonstrate that it is Tim44 which is required in the translocation of the tightly folded domain, and they show that the mtHsp70–Tim17–Tim23 complexes of mitochondria do not substitute for the mtHsp70–Tim44 complexes after the inactivation of Tim44. We conclude that the complexes of mtHsp70 with Tim17–Tim23 and with Tim44 play different roles in the protein import mechanism. The strong 'pulling' function which is required for unfolding of tightly folded domains and has been attributed to mtHsp70 (Glick, 1995; Pfanner and Meijer, 1995; Schatz, 1996; Matouschek *et al*., 1997) is not exerted by the Tim17–Tim23-bound mtHsp70 but by mtHsp70 in cooperation with Tim44. Our data do not exclude, however, that the mtHsp70–Tim17–Tim23 complex could be involved in the formation of a weaker pulling activity required for import of more loosely folded preproteins (Glick *et al*., 1993; Voos *et al*., 1996; Matouschek *et al*., 1997).

The stability of the Su9–DHFR translocation intermediates in import sites shows a striking dependence on the presence of Tim44. We suggest that Tim44 strongly facilitates the transfer of mtHsp70 to the translocating polypeptide directly at the membrane. In the presence of Tim44, the mobility of the accumulated translocation intermediates is reduced drastically, suggesting that polypeptide segments which emerge at the import sites immediately receive a mtHsp70 molecule. This high Tim44-dependent fidelity of the import motor appears to be required in the unfolding and import of tightly folded domains. In the absence of Tim44 function, mtHsp70 can drive the import of loosely folded preproteins even though the bound mtHsp70s are more distant from the membrane (the model protein which shows the high mobility in the import channel in the experiment of Figure 7B is the same preprotein that is imported efficiently in Figure 3A). Our results suggest that in mitochondria the functions of channel formation and the generation of a strong force in vectorial protein movement are mediated by different subcomplexes of the import machinery. Tim23 and Tim17 seem to constitute the import pore; Tim44 together with mtHsp70 appears to be the dynamic element of the import machinery. A similar situation has emerged in the elucidation of the protein transport mechanisms of the endoplasmic reticulum. Here, the corresponding functions are exerted by complexes of Sec61p or Sec63p, respectively (Brodsky and Schekman, 1993; Panzner *et al*., 1995; Corsi and Schekman, 1997; Lyman and Schekman, 1997).

In summary, we find that Tim44 is not a structural constituent of the inner membrane protein import channel. Tim44 rather acts at the inner side of the inner membrane and is an element of an independent motor unit driving unfolding and translocation of preproteins. Mitochondrial Tim44 plays only a stimulatory role in import of loosely folded preproteins, but is crucial for pulling in folded domains.

Materials and methods

Construction of plasmids and manipulation of S.cerevisiae strains

Temperature-sensitive alleles of *TIM44* were generated by a low fidelity PCR technique as published previously (Rassow *et al*., 1994). The mutant alleles were cloned into the single-copy vector YCplac111 and introduced into the diploid strain MB2-22 (Maarse *et al*., 1992). After random sporulation, haploid cells with a disrupted nuclear *TIM44* gene and harbouring a rescuing plasmid were tested for temperature sensitivity. One of the isolated alleles was designated *tim44-8*. A corresponding strain containing the wild-type *TIM44* gene was used as the wild-type strain. Hybrid proteins containing N-terminal parts of Su9 fused to murine DHFR (Viebrock *et al*., 1982; Ungermann *et al*., 1994) were obtained by *in vitro* transcription/translation using cDNA constructs cloned into the vector pGEM4 (Promega). To assemble Su9(58)–DHFR, Su9(76)–DHFR and Su9(81)–DHFR, the corresponding Su9 parts were obtained by PCR and ligated into a pGEM4 plasmid containing a DHFR cDNA, using the *Bam*HI site of the cDNA upstream of the DHFR start ATG and the *Eco*RI site of the vector. Su9 and DHFR parts are thereby connected by a linker of four residues (Gly, Ser, Gly, Ile). The construct Su9(70)–DHFR was described previously (Pfanner *et al*., 1987).

Import of preproteins into isolated mitochondria and cross-linking to Tim44

Yeast cells were grown in YPG medium (1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol) at 30°C. Mitochondria were isolated

essentially according to the protocol of Daum *et al*. (1982), with modifications as published by Gambill *et al*. (1993). To induce the *tim44-8* phenotype, the mitochondria were pre-incubated at 37°C for 15 min and then cooled to 25°C for subsequent import experiments. Preproteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine/[^{35}S]cysteine (Amersham) after *in vitro* transcription by SP6 RNA polymerase (Stratagene). To import the preproteins, the reticulocyte lysates were diluted with import buffer [3% (w/v) bovine serum albumin (BSA, fatty acid-free; Sigma), 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] and isolated mitochondria were added to a final concentration of 25 µg of mitochondrial protein per 100 µl assay. Under these conditions, the amount of mitochondria was limiting in the import experiments. Some samples received 1 µM valinomycin from a 100-fold concentrated stock solution in ethanol to dissipate the membrane potential. The samples were then incubated at 25°C for up to 30 min. To accumulate translocation intermediates, hybrid proteins containing a DHFR domain were imported in the presence of 2 µM MTX (Sigma). For treatment of the mitochondria after the import reactions, proteinase K was used at a final concentration of 40 µg/ml for 10 min at 0°C. Mitochondria were reisolated by centrifugation at 16 000 *g* for 10 min. Proteins were analysed by SDS–PAGE and autoradiography. Relative amounts of ^{35}S-labelled proteins were quantified using a storage PhosphorImaging system (Molecular Dynamics Inc.). Chemical amounts of the soluble preprotein b_2(167)Δ–DHFR were purified after expression in *E.coli* as published previously (Dekker *et al*., 1997). The amount of imported protein was determined by immunoblotting, using an antiserum raised against the DHFR moiety and comparison with standard amounts of the purified preprotein (Dekker *et al*., 1997). Blue native electrophoresis of mitochondrial proteins was performed following a published procedure (Schägger and von Jagow, 1991; Dekker *et al*., 1996, 1997).

For cross-linking experiments, the hybrid protein Su9(70)–DHFR was accumulated in import sites by import into ATP-depleted mitochondria. Mitochondria (in the presence of 20 µM oligomycin to inhibit the F_0F_1-ATPase) and reticulocyte lysate were ATP depleted by incubation with apyrase (10 U/ml; Sigma) for 10 min at 0°C; the import was performed in the presence of 20 µM oligomycin. The mitochondria were washed by centrifugation through a sucrose cushion (500 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) and resuspended in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH pH 7.2 (SEM buffer). For cross-linking with EGS (Pierce), the mitochondria (50 µg protein) were incubated at 0°C for 20 min in 1 ml of SEM containing EGS at a final concentration of 200 µM. The reaction was stopped by addition of 100 mM Tris–HCl pH 7.2 and a second incubation of 20 min at 0°C. Cross-linking by DSS (Pierce) was performed following basically the same protocol, using DSS at a final concentration of 400 µM. Details of the procedure were published previously (Blom *et al*., 1993; Kübrich *et al*., 1994). Proteins were precipitated by addition of 10% trichloroacetic acid in the presence of 0.0125% deoxycholate. For immunoprecipitation, the precipitates were lysed in 1% SDS, 60 mM Tris–HCl pH 6.8. The samples were diluted 40-fold by addition of 1% (w/v) Triton X-100, 0.3 M NaCl, 10 mM Tris–HCl pH 7.5 and the antibodies were added bound to protein A–Sepharose (Pharmacia).

Assessment of the mitochondrial membrane potential

The Δψ of isolated yeast mitochondria was assessed by recording the fluorescence decrease of the voltage-sensitive dye $DiSC_3(5)$ (Molecular Probes) essentially as described previously (Sims *et al*., 1974; Gärtner *et al*., 1995). An aliquot of 200 µg of mitochondrial protein was used per assay. The mitochondria were pre-incubated at 37°C for 15 min to induce the phenotype of the *tim44-8* mutant. The measurements were then performed at 25°C.

Co-immunoprecipitations

All antibodies used in this study were polyclonal antibodies raised in rabbits against isolated proteins. For co-immunoprecipitations, antibodies (6 µl of serum) were pre-bound to protein A–Sepharose (10 µl wet volume; Pharmacia Biotech Inc.) for 1 h in 480 µl of 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]. In parallel, a preprotein containing a DHFR domain was synthesized in a reticulocyte lysate and imported in the presence of MTX into isolated mitochondria (20 µg of mitochondrial protein). 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)], the 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 pre-bound to protein A–Sepharose. After three washing cycles with lysis buffer, the protein A–Sepharose pellets were boiled in sample buffer and analysed by SDS–PAGE.

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