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Pam17 and Tim44 act sequentially in protein import into the mitochondrial matrix

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Abstract

Import of proteins into the matrix is driven by the Tim23 presequence translocase-associated import motor PAM. The core component of PAM is the mitochondrial chaperone mtHsp70, which ensures efficient translocation of proteins across the inner membrane through interactions with the J-protein complex Pam16–18 (Tim16-Tim14) and its cochaperone Tim44. The recently identified non-essential Pam17 is a further member of PAM. Genetic and biochemical analyses reveal synthetic interactions between *PAM17* and *TIM44*. Pam17 is involved in an early stage of protein translocation whereas Tim44 assists in a later step of transport, suggesting that both proteins can cooperate in a complementary manner in protein import.

1. Introduction

Since most of the mitochondrial proteins are nuclear-encoded and synthesized on cytosolic ribosomes, efficient protein import mechanisms into mitochondria are essential. Mitochondrial preproteins carry an N-terminal presequence that is recognized by receptors on the outer membrane. Translocation across the mitochondrial membranes is accomplished by the TOM (translocase of the outer mitochondrial membrane) complex and the TIM23 (translocase of the inner membrane) complex (Neupert and Herrmann, 2007). Import of matrix proteins requires both a membrane potential across the inner membrane and the ATP-consuming presequence translocase-associated import motor PAM in the matrix (Neupert and Brunner, 2002).

PAM couples the action of the mitochondrial Hsp70 chaperone Ssc1 to the translocation of a preprotein across the inner membrane. Ssc1 binds to preproteins in the ATP-bound state. ATP-hydrolysis, stimulated by the J-complex Pam16-Pam18 (also known as Tim16-Tim14) stabilizes this interaction (Bolender et al., 2008). The membrane-associated co-chaperone Tim44 can act as a membrane anchor for Ssc1. A purified Tim44-Ssc1(ATP) complex is destabilized upon binding of a peptide substrate to Ssc1, suggesting that in vivo another Ssc1(ATP) binds to freed Tim44 thus promoting further protein translocation (Liu et al., 2003). Recently two non-essential proteins, Tim21 and Pam17, which bind to the Tim23 channel, have been identified. Tim21 is involved in sorting of preproteins into the inner membrane, whereas Pam17 plays a role in import of preproteins into the matrix (Hutu et al., 2008; Mokranjac et al., 2005; Popov- eleketi et al., 2008; van der Laan et al., 2005, 2006).

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The role of Pam17 in protein import is only poorly understood. *pam17* Δ mitochondria display an import defect with selected matrix-targeted precursors, but *pam17* Δ cells grow like wild type under most conditions. To understand this apparent discrepancy between the *in vivo* and the *in vitro* phenotype, I performed genetic and biochemical analyses of the *PAM17* gene. Pam17 is required for efficient posttranslational protein import. The deletion of *PAM17* in combination with mutants of essential genes of the import motor, *SSC1* and *TIM44*, led to a synthetic enhancement of the single phenotypic effects. The data show that Pam17 is involved in an early step of import, thereby facilitating the interaction of Ssc1 with the incoming polypeptide, while Tim44 promotes further translocation of the protein into the matrix suggesting an overlapping functional cooperation of Pam17 and Tim44 in protein import.

2. Materials and methods

2.1. Strains used in this study

Yeast strains deleted for *TIM44* and *SSC1* have been constructed as described (Rassow et al., 1994; Voisine et al., 1999). Deletion of the *PAM17* gene was carried out by replacing the open reading frame of *PAM17* with the *HPH* gene from pAG32 (Goldstein and McCusker, 1999). Cell colonies resistant to hygromycin B were verified by PCR. Strains deleted for *PAM17* and *TIM44* or *PAM17* and *SSC1* were obtained by mating of the single deletion haploids. The resulting diploids were sporulated and the desired haploids were isolated by dissecting tetrads. The strain *pam17* Δ /*tim44* Δ carrying pRS314-*tim44*(R180A) was obtained by transforming *pam17* Δ /*tim44* Δ carrying pRS316-*TIM44* with pRS314-*tim44*(R180A) (Schiller et al., 2008). Strain *pam17* Δ /*ssc1* Δ carrying pRS314-*ssc1*-2 was obtained by transforming *pam17* Δ /*ssc1* Δ cells carrying pRS316-*SSC1* with pRS314-*ssc1*-2. In both cases transformants were selected on 5-fluoroorotic acid to select for candidates having lost the wild type copy of *TIM44* and *SSC1*, respectively. Viable colonies were plated onto YPD plates and incubated at 23, 30, and 37°C.

2.2. Testing for in vivo phenotypes

To test for the accumulation of precursors in vivo, wild type and mutant strains were grown in YPD at 23°C to mid-logarithmic phase and then shifted to 30°C and 37°C for 6 h, respectively. Cell extracts (optical density at 600 nm (OD₆₀₀), 0.2) were prepared and analyzed by SDS-PAGE and immunoblotting using antibodies specific for the mitochondrial matrix protein Hsp60.

2.3. Mitochondrial assays

Mitochondria were isolated from strains grown in liquid YPG medium (1% yeast extract, 2% Bacto peptone, 3% glycerol) at 25°C as described (Liu et al, 2001). For analysis of the steady state protein levels, 5 μ g of mitochondrial protein was analyzed by SDS-PAGE and immunoblotting using antibodies specific for components of the TIM23 translocase and the PAM import motor. Recombinant precursor proteins were purified from *Escherichia coli* cells as described (Lim et al., 2001) and added to isolated mitochondria at a final concentration of 1 nmol precursor per mg mitochondrial protein. For import assays the purified precursor was added to energized mitochondria in import buffer (10 mM morpholinepropanesulfonic acid [MOPS]-KOH pH 7.2, 80 mM KCl, 5 mM KP_i pH 7.2, 5 mM MgCl₂, 250 mM sucrose, 1% (w/v) BSA fatty acid-free) including an ATP-regenerating system (4 mM ATP, 2 mM NADH, 5 mM creatine phosphate, and 0.1 mg/ml creatine kinase). At times indicated the import reaction was stopped by addition of 1 mM valinomycin and cooling on ice. Following protease treatment with proteinase K (ratio of mitochondrial protein to protease mass was 10:1) for 20 min on ice to remove unimported precursor, protease resistant recombinant Cytochrome b2-DHFR was detected by SDS-

PAGE and immunoblotting using affinity-purified antibodies specific for DHFR. For arresting a precursor in a membrane-spanning fashion, the precursor was incubated with 10 μ M methotrexate (MTX) and import was carried out in the presence of 10 μ M MTX in the import buffer. For import into mitoplasts, the outer membrane was disrupted by hypoosmotic swelling in ice-cold buffer (10 mM MOPS-KOH pH 7.2, 1mM EDTA) prior to import. Mitoplasts were sedimented and washed once in ice-cold swelling buffer. The mitoplasts were resuspended in import buffer and energized for 10 min before adding precursor. The efficiency of disrupting the outer membrane was tested by monitoring the presence of the inter membrane space protein Cytochrome b2 using Cyt b2-specific antibodies. Import of denatured precursor was carried out by incubating the precursor in 8 mM Urea, 50 mM DTT, 10 mM Tris/HCl pH 7.2 for 15 min at 23°C prior to adding to energized mitochondria.

2.4. Coimmunoprecipitation in mitochondrial lysates

To analyze the interaction of mtHsp70 with the presequence of an arrested precursor, Cytochrome b2(47)-DHFR was imported into mitoplasts in the presence of MTX under high (4 mM ATP, 2 mM NADH) and low (10 U apyrase, 20 μ M oligomycin) mitochondrial ATP levels. Import was stopped by cooling the reaction on ice. Mitochondria were sedimented and resuspended in lysis buffer (20 mM Tris/HCl pH 7.2, 80 mM KCl, 1 mM PMSF) and incubated at 4°C for 20 min. Insoluble material was sedimented and the supernatant was incubated with anti Ssc1 antibodies cross-linked to proteinA beads as described (Schiller et al., 2008). Immunoprecipitated precursor was detected by SDS-PAGE and immunoblotting using affinity-purified antibodies specific for DHFR.

2.5. Precursor accumulation and processing in vivo

The accumulation and processing of mitochondrial precursors in vivo was tested by growing cells in liquid YPD to early-logarithmic phase. After addition of the protonophore carbonylcyamide *m*-chlorophenylhydrazine [CCCP] to a final concentration of 20 μ M, cells were further incubated. Samples ($OD_{600} = 0.1$) were withdrawn and resuspended in double-concentrated SDS-PAGE sample buffer containing 1 mM PMSF. The samples were boiled for 3 min, centrifuged to sediment insoluble material and the supernatant was subjected to SDS-PAGE and western-blot analysis using antibody against the mitochondrial matrix protein Mdj1. To compare the processing efficiency cells were treated like described and further incubated for 30 min in the presence of CCCP. 10 mM β -mercaptoethanol was added to reverse the effect of the uncoupler (Reid and Schatz, 1982) and samples were analyzed as described.

2.6. Miscellaneous

Affinity purification of antibody against Ssc1 and cross-linking to protein A beads by the use of dimethylpimelimidate dihydrochloride was carried out as described previously (Liu et al., 2003). Immunoblot analysis was carried out using an ECL system (Amersham Pharmacia Biotech) according to the manufacturer's instructions, using polyclonal antibodies specific for Tim17, Tim23, Tim50, Tim44, Pam16, and Pam18. For quantifying the amount of imported precursor proteins into mitochondria calibration curves with defined amounts of purified precursor proteins were determined by densitometry using antibodies specific for DHFR and compared to the signal intensity of detected imported protein. The relative membrane potential of mutant mitochondria was assessed by quenching the fluorescence of the membrane potential-sensitive dye DiSC₃(5) (Geissler et al., 2000). All results presented were obtained from a minimum of two completely independent experiments.

3. Results

3.1. Pam17 is required for posttranslational protein import

To test the role of Pam17 in protein import I accumulated preproteins in the cytosol of *pam17Δ* cells by reducing the mitochondrial membrane potential with the protonophore CCCP and monitored subsequent import of precursors after restoration of the membrane potential $\Delta\Psi$ (Reid and Schatz, 1982). Cell lysates were analyzed by western-blot using antibodies against Mdj1, a mitochondrial J-protein involved in folding, but not in import of matrix proteins (Rowley et al., 1994). In wild type cells the amount of accumulated precursor decreased from 21.3% to 2.7% within 45 minutes, compared to a decrease from 28.3% to 14.9% of preMdj1 in *pam17Δ* cells in this period (Fig. 1). I concluded that *PAM17* is involved in posttranslational protein import into mitochondria.

3.2. PAM17 genetically interacts with SSC1 and TIM44

I combined the genomic deletion of *PAM17* with mutant alleles of *SSC1* and *TIM44*, encoding for two essential central components of the import motor involved in binding and translocation of mitochondrial matrix proteins. The single mutants *ssc1-2* and *tim44(R180A)* display temperature-sensitive phenotypes and are defective in protein import, due to a deregulated Tim44-Ssc1 interaction in the presence of polypeptide substrate, as described (D'Silva et al., 2004; Schiller et al., 2008). The double mutant *pam17Δ/ssc1-2* was inviable (Supplemental Fig. S1). *pam17Δ tim44Δ* cells, however, having Tim44(R180A) as the only functional Tim44 were compromised in growth at all temperatures (Fig. 2A). In contrast, *tim44(R180A)* cells alone showed a growth defect at 34°C and 37°C, while cells lacking *PAM17* were not compromised in growth. I concluded that the *tim44* and *ssc1* mutations in the *pam17Δ* background cause a synthetic enhancement of the single phenotypic effects.

I asked whether the observed phenotype was due to a defective import of mitochondrial preproteins. Lysates from cells grown at different temperatures were analyzed by immunoblotting using antibody directed against the matrix chaperonin Hsp60 (Fig. 2B). Consistent with the observed growth phenotype, the precursor form of Hsp60 was present in lysates of *pam17Δ tim44(R180A)* cells under all temperatures tested, while *tim44(R180A)* cells accumulated the precursor only at 37°C, as described (Schiller et al., 2008). No precursor could be detected in lysates of *pam17Δ* cells. The steady state levels of mitochondrial proteins were not significantly reduced in the mutant mitochondria (Supplemental Fig. S2).

I compared the import efficiencies of a recombinant fusion protein consisting of the first 167 amino acids of cytochrome *b₂* fused to mouse dihydrofolate reductase (DHFR) into the matrix of mutant mitochondria (cf. Supplemental Fig. S3). Deletion of 19 amino acids in the membrane sorting signal directs this precursor into the matrix. Import of *b₂(167) Δ* ₉-DHFR into both *pam17Δ* (van der Laan et al., 2005), and *pam17Δ/tim44(R180A)* mitochondria was strongly impaired, even though a membrane potential like wild type was generated (Fig. 2C and Fig. S4). In contrast, *tim44(R180A)* mitochondria imported the precursor similar to wild type, suggesting that the absence of *PAM17* in *pam17Δ/tim44(R180A)* mitochondria causes a dominant negative effect in the double mutant. Interestingly, facilitating the import by providing an extended substrate upon Urea-induced unfolding of the entire precursor prior to import did not alter the import efficiencies in the single mutant mitochondria (Supplemental Fig. S5 G). Since import was not compromised in *tim44(R180A)*, but defective in *pam17Δ* mitochondria, both proteins assist in import of the precursor *b₂(167) Δ* ₁₉-DHFR in a different manner.

3.3. Pam17 and Tim44 are involved at different stages of preprotein translocation

To analyze at what stage of protein translocation the mutants are defective, I tested a precursor whose presequence is already cleaved by the matrix processing peptidase (MPP) while the remainder of the protein is still being imported. In the import assay the ratio of processed precursor before and after treatment with proteinase K indicates whether an early, i.e. import of the presequence, or later step (i.e. translocation of the folded carrier protein) is compromised. I used purified $b_2(220)_{\Delta 19}$ -DHFR consisting of the first 220 amino acids of cytochrome b_2 with the folding-competent full-length heme binding domain preceding DHFR (cf. Supplemental Fig. S3). In control mitochondria, the amount of processed precursor after 30 minutes was comparable before and after protease treatment, indicating that all mitochondria-associated precursor was imported into the protease-protected matrix (Fig. 3A upper panel). In contrast, in *tim44*(R180A) mitochondria, only a fraction of processed protein was detectable after proteinase K treatment, whereas the amount of processed precursor before protease treatment was comparable to wild type, indicating an inefficient import of this precursor into the matrix (Fig. 3A, middle panel). In *pam17* Δ mitochondria, the amount of processed protein was low (Fig. 3A, lower panel). Thus, Pam17 is involved in an early stage of import i.e. before processing of the presequence by the matrix-localized processing peptidase occurs, while Tim44 acts in a later step of preprotein import, when import motor-catalyzed unfolding of extra mitochondrial protein domains is required.

Ssc1(ATP) can bind to a translocated presequence in the mitochondrial matrix even before its processing occurs (Ungermann et al., 1994). To test whether such an early Ssc1-presequence interaction appears in mutant *tim44* and *pam17* mitochondria, $b_2(47)$ -DHFR was imported into mitoplasts and immunoprecipitated with antibodies against Ssc1 (Fig. 3B). This precursor was chosen because of its short N-terminal sequence followed by the folded DHFR domain. Since about 20 amino acids of a helix are required to span the inner membrane, in this assay only about 25 amino acids of the presequence would emerge into the matrix, while the folded DHFR domain remained outside, since a methotrexate(MTX)-stabilized DHFR domain cannot be unfolded by the mitochondrial protein import motor under these conditions (Eilers and Schatz, 1986; Rassow et al., 1990). To arrest the precursor in transit, the folded state of DHFR was stabilized by the addition of the ligand methotrexate (MTX). When importing $b_2(47)$ -DHFR(MTX) into energized mitoplasts, only wild type mitochondria were capable of a stable Ssc1- $b_2(47)$ -DHFR(MTX) interaction due to a fully functional import motor at the exit of the Tim23 channel (Fig. 3B). In *tim44*(R180A) mitochondria, however, a functional Ssc1-precursor interaction fails most likely due to the stable complex that is formed between Ssc1 and Tim44(R180A) even in the presence of peptide substrate preventing the binding of Ssc1 to the precursor, as previously reported (Schiller et al., 2008). Interestingly also in *pam17* Δ mitochondria no stable Ssc1-precursor interaction could be detected, even though the peptide substrate regulated dissociation of a Ssc1-Tim44 complex was similar to wild type when immunoprecipitating the protein complex in mitochondrial lysates, suggesting that the interaction of Ssc1 with Tim44 per se is not affected by the absence of Pam17 (Supplemental Fig. S6). Thus I concluded that the impaired Ssc1-presequence interactions close to the exit of the Tim23 channel in both *pam17* Δ and *tim44* mitochondria have different causes, since efficient import of preproteins into *tim44*(R180A) mitochondria but not in the *pam17* mutant occurs (cf. Fig. 2C). To further test this hypothesis I measured the ATP-driven inward-driving force on an arrested precursor in transit in isolated mitochondria. In these experiments the precursor spans both mitochondrial membranes while the N-terminal sequence interacts with the import motor such that the extra mitochondrial folded domain is pulled against the outer membrane preventing its proteolytic degradation by externally added proteinase K (Krayl et al., 2007). When testing the arrested precursor $b_2(167)_{\Delta 19}$ -DHFR(MTX), a functional

interaction of its N-terminal portion with the import motor could be detected as judged by the resistance of the stabilized extra mitochondrial DHFR domain to proteolytic degradation. In contrast, no stable interaction of Ssc1 with the precursor could be detected in *pam17Δ* mitochondria, whereas *tim44(R180A)* mitochondria still exerted a moderate import-driving force (Supplemental Fig. S7). Thus, consistent with the observed import defect, no detectable efficient interaction of Ssc1 with the preprotein in transit occurs in *pam17Δ* mitochondria.

3.4. Different import modes reveal complementary functions of PAM17 and TIM44

tim44(R180A) mitochondria are defective in import of $b_2(220)_{\Delta 19}$ -DHFR, whereas the shorter version $b_2(167)_{\Delta 19}$ -DHFR is imported with wild type efficiency, suggesting a selective defect depending on the required import mode for a given preprotein (cf. Fig. 2C). I asked whether such variability in import occurs in *pam17Δ* mitochondria and tested different precursors varying in both the length of the N-terminal sequence preceding the carrier protein and the nature of the carrier protein. Interestingly I found precursors which were efficiently imported into *pam17Δ* mitochondria (Supplemental Fig. S8). To further analyze this apparent precursor-dependent import efficiency I imported the same precursor in kinetically saturating amounts but under 3 different conditions (Fig. 4A). First I imported native $b_2(47)$ -DHFR into mitochondria. Since 50 amino acids are required to span both mitochondrial membranes, interaction of the N-terminal segment of $b_2(47)$ -DHFR with matrix Ssc1 and subsequent import can only occur upon spontaneous unfolding of the native DHFR domain prior to import (Krayl et al., 2007; Rassow et al., 1990). Under this condition *pam17Δ* and *tim44(R180A)* mitochondria imported native $b_2(47)$ -DHFR with an efficiency of about 90% compared to the control reaction (Fig. 4A, upper panel). Import into *pam17Δ/tim44(R180A)* mitochondria, however, was strongly impaired. Next, I unfolded the native precursor by urea treatment prior to import to provide a substrate, whose presequence easily reaches the matrix since the otherwise folded DHFR-domain is absent (Fig. 4A, middle panel). Only *tim44(R180A)* mitochondria imported the unfolded precursor similar to wild type, whereas import into both *pam17Δ* and *pam17Δ/tim44(R180A)* mitochondria was compromised. Finally, I combined the characteristics of the two preceding assays, by providing a binding site for Ssc1 to the presequence in the matrix while the folded DHFR domain remained outside (cf. Fig. 3B). Therefore, I imported native $b_2(47)$ -DHFR into mitoplasts (Fig. 4A, lower panel). As expected, no efficient import into either mutant compared to wild type was observed. This is consistent with the observed import defects of *tim44(R180A)* and *pam17Δ* mitochondria when using $b_2(220)_{\Delta 19}$ -DHFR as a substrate (cf. Fig. 3A), where the import motor needs to unfold first the cytochrome b_2 heme binding domain (HBD) before the subsequent DHFR domain can be translocated (cf. supplemental Fig. S3). I hypothesized that if import motor-catalyzed unfolding of HBD is the rate-limiting step in import of native $b_2(220)_{\Delta 19}$ -DHFR, then transport of the unfolded precursor should only be impaired in the *pam17Δ* mutant. Indeed, only *pam17Δ* and *pam17Δ/tim44(R180A)*, but not *tim44(R180A)* mitochondria were defective in import of denatured $b_2(220)_{\Delta 19}$ -DHFR (Fig. 4B).

4. Discussion

The data presented here show that Pam17 and Tim44 act in a complementary fashion with Pam17 being involved in an early stage of protein import, i.e. the translocation of the presequence across the mitochondrial inner membrane and Tim44 in a later stage of protein import, catalyzing the complete translocation of the mitochondrial matrix protein. The import efficiencies of recombinant cytochrome b_2 -DHFR preproteins into *tim44* and *pam17* mitochondria depend on the required translocation mode dictated by the nature of the respective precursor (Fig. 4C): Three import modes were tested: (i) If the presequence provides binding site(s) for mtHsp70 in the matrix, while the folded carrier protein is still

outside, then import motor-initiated unfolding prevails and the extra-mitochondrial domain of DHFR is subsequently translocated (Fig. 4C, right panel). Since both proteins are required for an efficient early Ssc1-presequence interaction (cf. Fig. 3B), Tim44 and Pam17 are required for this import mode independently of each other. (ii) If translocation of an unfolded precursor is required, only *pam17* mutants are defective, suggesting an exclusive role for Pam17 in this process (Fig. 4C, middle panel). (iii) If the initiation of the precursor import is dependent on spontaneous unfolding of the extra-mitochondrial DHFR domain first before further import can be catalyzed, both proteins are required, suggesting that Tim44 and Pam17 cooperate in a complementary manner (Fig. 4C, left panel). This cooperation does not require a direct physical interaction of the two proteins. As a matter of fact a more dynamic sequential assembly and functional rearrangement of the import motor with the Tim23 translocase has been proposed, recently (Hutu et al., 2008; Popov- eleketi et al., 2008; Wiedemann et al., 2007). In this regard the observations made herein are conclusive with a working model in which Pam17 associates transiently with the import-motor-free Tim23 translocase first, while the Pam16-Pam18 complex assembles with the import channel before Tim44 with Pam17 presumably having already left the complex. This view is supported by a recent observation in which the association of Pam17 and Pam16-Pam18 with the TIM23 complex shows an opposite dependence on the functionality of Tim44, with inactivated Tim44 leading to an increased binding of Pam17 while the Pam16-Pam18 association with the import channel is reduced (Hutu et al., 2008). This model is also in line with the observed lethality of the double mutant *pam17Δ/ssc1-2*. In the *ssc1-2* mutant the interaction of Ssc1 with Tim44 is defective in a similar way as has been observed for the *tim44*(R180) mutant, namely a failure of the Tim44-Ssc1 complex to dissociate in the presence of peptide substrate (D'Silva et al., 2004; Schiller et al., 2008). It is tempting to speculate that Pam17 might act as a membrane adaptor for matrix Ssc1 at the Tim23 channel, initiating a Tim44-independent early interaction of Ssc1 with incoming polypeptide and thus promoting the functional assembly of the Pam16-Pam18 complex and Tim44 to complete the translocation of the matrix protein. The existence of such a potential additional membrane adaptor for Ssc1 has been proposed recently (Bömer et al., 1997). However a direct interaction of Pam17 with Ssc1 has not been reported so far and might not be required under most growth conditions, since -dependent on the precursor- efficient protein import into *pam17Δ* mitochondria can occur (cf. Supplemental Fig. S8).

In conclusion Pam17 contributes to the translocation of the presequence across the inner membrane, whereas Tim44 is required for completing the initiated import of the processed protein into the matrix. Each protein assists at a different import stage so that the single *tim44* and *pam17* mutations are complemented by the respective wild type allele, but only their combination is deleterious *in vivo*. A complementary functional cooperation of Pam17 and Tim44 would ensure efficient import of hundreds of mitochondrial proteins under any given growth condition, regardless of whether a post- or cotranslational import mode prevails.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at

Fig. 1.

Pam17 facilitates posttranslational protein import defect *in vivo*. (A) Cells were grown to mid-logarithmic phase. After addition of the uncoupler CCCP incubation was continued for 30 minutes. β -mercaptoethanol was added to reverse the effect of CCCP and to restore the membrane potential across the mitochondrial inner membrane. At the times indicated, cell extracts were analyzed by immunoblotting using antibodies against the mitochondrial matrix protein Mdj1. (B) The signal intensities from four independent experiments were quantified by densitometry and the amount of preMdj1 was plotted against the incubation time using the formula: $\text{preMdj1}/(\text{preMdj1} + \text{mMdj1})$. p, premature; m, mature Mdj1. BME, β -mercaptoethanol.

Fig. 2.

Synthetic phenotype of *tim44(R180A)* in the *pam17Δ* strain background. (A) Growth phenotypes of mutant strains *pam17Δ*, *tim44(R180A)*, or *pam17Δ/tim44(R180A)*. Tenfold serial dilutions were plated onto rich medium and incubated for 3 days (23°C) or 2 days (30°C, 34°C, and 37°C). (B) Accumulation of a preprotein *in vivo*. Cells were grown at 23°C to mid-logarithmic phase and subsequently shifted to 30°C or 37°C. Cell extracts were analyzed by immunoblotting using Hsp60-specific antibodies. p, premature Hsp60; m, mature Hsp60. (C) Import of the recombinant precursor cytochrome $b_2(167)_{\Delta 19}$ -DHFR into isolated mitochondria. Kinetically saturating amounts (1 nmol/mg of mitochondrial protein) of purified preprotein was incubated with energized mitochondria for the indicated times. Following proteinase K treatment to remove unimported precursor, the amount of imported processed protein was determined by immunoblotting using antibodies against DHFR. The amount of precursor processed in wild type mitochondria after 10 minutes was set to 100% (control). The data points represent the average value of at least two independent experiments.

Fig. 3.

PAM17 is involved in an early stage of preprotein translocation. (A) The recombinant precursor cytochrome $b_2(220)_{\Delta 19}$ -DHFR was imported into isolated mitochondria for the times indicated. Samples were split in half and treated with proteinase K (+PK) or left untreated (-PK). The amount of processed precursor in each assay was determined and plotted against the incubation time. Inset: western blot of mitochondria-associated precursor after 15 minutes of import (-PK and +PK, respectively). (B) Coimmunoprecipitation of $b_2(47)$ -DHFR as arrested precursor in mitoplasts. $b_2(47)$ -DHFR was incubated in the presence or absence of 10 μ M MTX with energized mitoplasts for 10 minutes in the presence of an ATP-regenerating system (+ATP) or in the absence of ATP (-ATP). After lysis the supernatant was incubated with antibodies against Ssc1 cross linked to protein A beads. The immunoprecipitate was subjected to immunoblotting using antibodies against

DHFR. p, premature b₂(47)DHFR; m, mature b₂(47)DHFR. The data points represent the average value of at least two independent experiments.

Fig. 4. Import of $b_2(47)$ -DHFR under different conditions. (A) $b_2(47)$ -DHFR was imported into energized mitochondria: wild type (closed circles), $pam17\Delta$ (open circles), $tim44(R180A)$ (closed triangles), and $pam17\Delta/tim44(R180A)$ (open triangles). Import of the native precursor into intact mitochondria (upper panel). The precursor was denatured by Urea-treatment prior to import (middle panel). The native precursor was imported into mitoplasts, whose outer membrane has been disrupted by hypo-osmotic swelling prior to import (lower panel). (B) Import of denatured radiolabeled $b_2(220)_{\Delta 19}$ -DHFR into mitochondria. The precursor was unfolded by Urea-treatment prior to import. (C) Evaluation of the different import modes of $b_2(47)$ -DHFR into mutant mitochondria: The native (I and III) or unfolded (II) domain of DHFR (gray) and the N-terminal presequence (black rectangle) are shown. The relative import of $b_2(47)$ -DHFR as native (I and III) or denatured (II) precursor is shown. +, efficient import; -, inefficient import. OM, outer membrane, IM, inner membrane. The data points represent the average value of at least two independent experiments.