Mitochondrial Protein Import Motor: Differential Role of Tim44 in the Recruitment of Pam17 and J-Complex to the Presequence Translocase

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The presequence translocase of the mitochondrial inner membrane (TIM23 complex) mediates the import of preproteins with amino-terminal presequences. To drive matrix translocation the TIM23 complex recruits the presequence translocase-associated motor (PAM) with the matrix heat shock protein 70 (mtHsp70) as central subunit. Activity and localization of mtHsp70 are regulated by four membrane-associated cochaperones: the adaptor protein Tim44, the stimulatory J-complex Pam18/Pam16, and Pam17. It has been proposed that Tim44 serves as molecular platform to localize mtHsp70 and the J-complex at the TIM23 complex, but it is unknown how Pam17 interacts with the translocase. We generated conditional *tim44* yeast mutants and selected a mutant allele, which differentially affects the association of PAM modules with TIM23. In *tim44-804* mitochondria, the interaction of the J-complex with the TIM23 complex is impaired, whereas unexpectedly the binding of Pam17 is increased. Pam17 interacts with the channel protein Tim23, revealing a new interaction site between TIM23 and PAM. Thus, the motor PAM is composed of functional modules that bind to different sites of the translocase. We suggest that Tim44 is not simply a scaffold for binding of motor subunits but plays a differential role in the recruitment of PAM modules to the inner membrane translocase.

INTRODUCTION

The vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes and subsequently imported into the organelle. Virtually all precursor proteins initially enter the mitochondria via the general translocase of the outer membrane, the TOM complex. At the intermembrane space side of the TOM complex several import pathways diverge. The presequence translocase of the inner membrane (TIM23 complex) is dedicated to the import of preproteins with amino-terminal presequences (Jensen and Johnson, 2001; Koehler, 2004; Dolezal *et al.*, 2006; Bohnert *et al.*, 2007; Neupert and Herrmann, 2007). The channel-forming Tim23 protein and its partner protein Tim17 constitute the membrane-embedded core of the TIM23 complex (Dekker *et al.*, 1997; Truscott *et al.*, 2001). Tim21 and Tim50 expose domains to the intermembrane space and are involved in the transfer of preproteins from the TOM complex to the TIM23 complex (Geissler *et al.*, 2002; Yamamoto *et al.*, 2002; Mokranjac *et al.*,

2003a; Chacinska *et al.*, 2003, 2005; Oka and Mihara, 2005; Perry and Lithgow, 2005; Albrecht *et al.*, 2006). In the absence of a preprotein substrate, Tim50 maintains the Tim23 channel in a closed state (Meinecke *et al.*, 2006).

Translocation of the presequences across the inner membrane depends on the electrochemical gradient (Δp) that activates the Tim23 channel and exerts an electrophoretic effect on the positively charged presequences (Geissler *et al.*, 2000; Truscott *et al.*, 2001; Huang *et al.*, 2002). The ATP-driven presequence translocase-associated motor (PAM) is essential for full translocation of preproteins into the matrix (Jensen and Johnson, 2001; Endo *et al.*, 2003; Koehler, 2004; Dolezal *et al.*, 2006; Bohnert *et al.*, 2007; Dudek *et al.*, 2007; Neupert and Herrmann, 2007). Recent studies have demonstrated that the TIM23 complex exists in two different forms. 1) For preproteins, which carry an inner membrane-sorting signal and are laterally released from the TIM23 complex into the lipid phase, Δp can be used as the only external energy source (van der Laan *et al.*, 2007). This form of the presequence translocase consists of Tim23/Tim17, Tim50, and Tim21 and is termed TIM23^{sort} (Chacinska *et al.*, 2005; Oka and Mihara, 2005; Perry and Lithgow, 2005; van der Laan *et al.*, 2006, 2007). Tim21 binds to the proton-pumping respiratory chain complexes III and IV to stimulate the Δp -driven preprotein insertion into the inner membrane (van der Laan *et al.*, 2006; Wiedemann *et al.*, 2007). 2) The majority of presequence-carrying preproteins, however, are fully translocated into the matrix. For these preproteins, the motor PAM associates with the TIM23 complex, whereas Tim21 is released, and ATP is used as external energy source in addition to Δp (Chauwin *et al.*, 1998; Chacinska *et al.*, 2005;

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Abbreviations used: Δp , proton-motive force; J-complex, Pam18-Pam16 complex of mitochondria; mtHsp70, mitochondrial heat shock protein 70; PAM, presequence translocase-associated motor; TIM, presequence translocase of inner mitochondrial membrane

Oka and Mihara, 2005; Perry and Lithgow, 2005; Bohnert *et al.*, 2007; Neupert and Herrmann, 2007).

The central component of PAM is the mitochondrial heat shock protein 70 (mtHsp70), which generates an inward-directed import driving activity at the expense of ATP. MtHsp70 binds to the TIM23 complex via the adaptor protein Tim44 (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). Pam18 belongs to the J-protein family, a family of cochaperones that stimulate the ATPase activity of Hsp70 proteins (Walsh *et al.*, 2004; Young *et al.*, 2004; Bukau *et al.*, 2006). Pam18 is an integral membrane protein, which exposes its J-domain to the mitochondrial matrix to stimulate the activity of mtHsp70 at protein import sites (D'Silva *et al.*, 2003; Mokranjac *et al.*, 2003b; Truscott *et al.*, 2003; Li *et al.*, 2004). Pam18 is associated with the partner protein Pam16 (Frazier *et al.*, 2004; Kozany *et al.*, 2004), which has been classified as J-like protein, as it shows significant homology to members of the J-protein family, but lacks the conserved signature sequence HPD (Walsh *et al.*, 2004). Pam18 and Pam16 form a heterodimeric complex, which is defined here as J-complex (Frazier *et al.*, 2004; Kozany *et al.*, 2004; Li *et al.*, 2004; D'Silva *et al.*, 2005; Mokranjac *et al.*, 2006). Another regulatory subunit of the PAM complex, Pam17, is required for efficient protein import and influences the association of Pam18 and Pam16 in the J-complex (van der Laan *et al.*, 2005).

Different views have been reported on how the motor PAM is recruited to the TIM23 complex. Only one direct interaction site between PAM and the TIM23 complex has been reported so far, the binding of the intermembrane space domain of Pam18 to Tim17 (Chacinska *et al.*, 2005). The significance of this interaction has been questioned by Mokranjac *et al.* (2006, 2007). A recent study, however, demonstrated that Pam18 binds to Tim17 and additionally interacts with the presequence translocase via a second site that involves Pam16 and likely Tim44 (D'Silva *et al.*, 2008). Tim44 is thought to function as a scaffold, which does not only bind mtHsp70 but also the further PAM subunits, in particular the J-complex (Kozany *et al.*, 2004; Mokranjac *et al.*, 2007; D'Silva *et al.*, 2008). Direct experimental evidence that the activity of Tim44 is needed for the recruitment of Pam proteins has not been obtained so far. Moreover, it is unknown how Pam17 interacts with the presequence translocase.

For this study, we have screened for conditional mutants of *TIM44* in order to assess the function of Tim44 for the organization of the PAM complex. We describe a conditional allele, termed *tim44-804*, which displays a selective import defect for precursors destined for the mitochondrial matrix. The inactivation of Tim44 leads to a reorganization within the TIM23-PAM machinery. The J-complex dissociates from the translocase, while Pam17 binding to Tim23 is strongly enhanced. We propose that Tim44 fulfills a critical function in the dynamics of the mitochondrial import motor, while Tim23 not only functions as protein import channel but also as binding site for the regulatory subunit Pam17.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

Saccharomyces cerevisiae strain YPH499 (Sikorski and Hieter, 1989) was used as wild-type strain throughout this study. For generation of the mutant strain *tim44-804* (YPH-BG-0804) (*MATa*, *ade2-101_ochre*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801_amber*, *tim44::ADE2*, [*pBG-TIM44-0804*]) we constructed a *tim44Δ* strain that was complemented by a plasmid-borne copy of *TIM44* under control of the *MET25* promoter and followed by the *CYC1* terminator. PCR mutagenesis was used to introduce random nucleotide sequence alterations into the *TIM44* gene (Leung *et al.*, 1989). The mutated PCR fragment

was transformed together with a gapped plasmid into the complemented *tim44Δ* strain. Transformants were selected and subsequently cured from plasmids carrying the wild-type *TIM44* by selection on plates containing 5-fluoroorotic acid (5-FOA; Sikorski and Boeke, 1991). Plasmids that led to a temperature-sensitive growth phenotype were isolated from the transformants and reintroduced into the *tim44Δ* strain to confirm the authenticity of the phenotype. Selected mutants were subjected to a biochemical screening protocol. Mitochondria were isolated from cells that were grown under permissive conditions. Isolated mitochondria were analyzed for steady-state protein composition, wild-type-like membrane potential, import of mitochondrial precursor proteins into various subcompartments, stability of mitochondrial protein complexes, and intactness of mitochondria by protease treatment. Sequence analysis of the *tim44-804* allele revealed the following amino acid alterations compared with the wild-type Tim44 protein: K59E, Q66R, N240I, P249S, F276S, and V397A.

pam16-1, *tom22-2*, *pam17Δ*, and *tim21Δ* yeast mutant strains have been described (Frazier *et al.*, 2003, 2004; Chacinska *et al.*, 2005; van der Laan *et al.*, 2005). Mutant and corresponding wild-type cells were grown at 24°C (*tim44-804*, *pam16-1*, *pam17Δ*) or 30°C (*tom22-2*, *tim21Δ*) in YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] Bacto Peptone, and 3% [vol/vol] glycerol).

Isolation of Mitochondria and In Vitro Import of ³⁵S-labeled Preproteins

Mitochondria were isolated by differential centrifugation (Meisinger *et al.*, 2006) and resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) at a protein concentration of 10 mg/ml. For preincubation at high temperature mitochondria were incubated for 15 min at 37°C. ³⁵S-labeled preproteins were synthesized by in vitro transcription and translation using the TNT SP6 Quick Coupled kit (Promega, Madison, WI) in the presence of [³⁵S]methionine (GE Healthcare, Waukesha, WI) and imported into isolated mitochondria in BSA import buffer (250 mM sucrose, 80 mM KCl, 3% [wt/vol] bovine serum albumin, 5 mM MgCl₂, 2 mM KH₂PO₄, 5 mM methionine, 10 mM MOPS-KOH, pH 7.2) supplemented with 2 mM ATP and 2 mM NADH. Where indicated the proton-motive force (Δp) was dissipated by addition of 1 μM valinomycin, 8 μM antimycin, and 20 μM oligomycin. For protease treatment mitochondria were incubated with 50 μg/ml proteinase K for 15 min on ice. The protease was subsequently inhibited by the addition of 2 mM phenylmethylsulfonyl fluoride.

Coimmunoprecipitation

Polyclonal antibodies raised against Tim23 were covalently coupled to protein A-Sepharose beads using dimethyl pimelimidate. For coimmunoprecipitations, mitochondria were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, and 1% digitonin) and gently shaken for 20 min at 4°C. After a clarifying spin, supernatants were incubated with antibody-coated beads for 1 h at 4°C. Bound proteins were eluted with 100 mM glycine, pH 2.5, precipitated with 10% trichloroacetic acid (TCA), and analyzed by SDS-PAGE and Western blotting.

Chemical Cross-Linking

The homobifunctional, amine-reactive agents disuccinimidyl glutarate (DSG, 0.5 mM final concentration) and ethylene glycol bis-succinimidyl succinate (EGS, 0.25 mM final concentration) were used for chemical cross-linking experiments. Mitochondria were resuspended in import buffer without BSA for DSG cross-linking or KPS buffer (250 mM sucrose, 50 mM potassium phosphate, pH 8.5) for EGS treatment. Cross-linking reagents were added from 100-fold stock solutions in dimethylsulfoxide (DMSO). Control reactions without cross-linker received the identical amount of solvent. Samples were incubated for 30 min at 4°C, and reactions were stopped by addition of glycine, pH 8.0 (0.1 M final concentration).

Miscellaneous

Mitochondrial protein complexes were analyzed by blue native electrophoresis essentially as described (Dekker *et al.*, 1997). After protein separation on SDS polyacrylamide gels, proteins were transferred to PVDF membranes. Standard techniques were applied for Western blotting and protein-antibody complexes were detected by enhanced chemiluminescence (GE Healthcare).

RESULTS

A Conditional Mutant of *TIM44* with a Selective Defect in Preprotein Import into the Matrix

Cross-linking experiments showed that Pam18/Pam16 are in close proximity to Tim44 (Kozany *et al.*, 2004; Mokranjac *et al.*, 2007), yet the function of Tim44 in recruiting Pam proteins has so far only been studied by the use of *Tim44*-depletion mutants (Mokranjac *et al.*, 2003b; Kozany *et al.*, 2004) and suppressor mutants of *TIM44* in a *pam16* mutant

background (D'Silva *et al.*, 2008). Because in the depletion experiments the cells have to grow for many hours to gradually decrease the amounts of Tim44, indirect effects of the loss of Tim44 cannot be excluded. We thus screened for temperature-conditional mutants of the essential gene *TIM44*. The mutants should allow growth of the cells at permissive (low) temperature to minimize indirect effects on mitochondrial composition and function. The mutant phenotype would then be induced by a short temperature shift of the isolated mitochondria.

We generated mutants of *TIM44* in the yeast *S. cerevisiae* by error-prone PCR and plasmid shuffling (Leung *et al.*, 1989; Sikorski and Boeke, 1991). Mutants were tested for their growth behavior at 24 versus 37°C by replica plating. Mitochondria were isolated from different mutant strains and analyzed for steady-state levels of proteins and protein complexes, for integrity of the membrane potential across the inner mitochondrial membrane, and for preprotein import activity (data not shown). We selected the *tim44-804* allele, which conferred a temperature-sensitive growth phenotype. *tim44-804* cells grew similar to wild-type cells at 24°C, but were strongly impaired in growth at 37°C (Figure 1A). When the mutant cells were grown at permissive temperature, isolated mitochondria contained a steady-state protein composition that was similar to that of wild-type mitochondria, including the subunits of PAM, TIM23 complex, and the control proteins Tom40 of the outer membrane and ADP/ATP carrier of the inner membrane (Figure 1B). Because the mutant Tim44 protein, as well as the other subunits of PAM and TIM23, remained stable under these growth conditions, we could use the mutant mitochondria to analyze the function of Tim44.

To examine the activity of the PAM machinery in *tim44-804* mitochondria, we analyzed the import of the matrix-targeted model preprotein $b_2(167)_\Delta$ -DHFR that consists of the N-terminal portion of cytochrome b_2 , in which the inner membrane sorting signal is inactivated by deletion of 19-amino acid residues, and mouse dihydrofolate reductase (Koll *et al.*, 1992; Voos *et al.*, 1993). The Δp -dependent import of $b_2(167)_\Delta$ -DHFR, determined by processing of the N-terminal presequence and transport to a protease-protected location, was strongly inhibited in *tim44-804* mitochondria compared with wild-type mitochondria (Figure 2A). A similar defect was observed, when the matrix-targeted preprotein of the β -subunit of the F_1F_0 -ATPase was imported (Figure 2B).

To determine if *tim44-804* mutant mitochondria displayed a general import defect, we studied the transport of the inner membrane-sorted preproteins $b_2(167)$ -DHFR and cytochrome c_1 , which are transported by the TIM23^{SORT} complex in a PAM-independent manner (Glick *et al.*, 1992; Voos *et al.*, 1993; Chacinska *et al.*, 2005; van der Laan *et al.*, 2007). In contrast to the matrix-destined precursor proteins, Δp -dependent import of $b_2(167)$ -DHFR and cytochrome c_1 was similar in wild-type and *tim44-804* mutant mitochondria (Figure 3, A and B). We conclude that the *tim44-804* mutant mitochondria are selectively impaired in the import of matrix-targeted precursor proteins.

The J-Complex Is Stable in *tim44* Mutant Mitochondria But Impaired in Association with the TIM23 Complex

To analyze the organization of the TIM23 complex in *tim44-804* mutant mitochondria, we used blue native electrophoresis. In wild-type mitochondria, lysed with the nonionic detergent digitonin, the Tim21-containing but motor-free TIM23^{SORT} form migrates in two high-molecular-weight species (Figure 4A, lanes 1 and 3; Chacinska *et al.*, 2005; van der Laan *et al.*, 2007). The motor PAM is released from the

Figure 1. A temperature-conditional yeast mutant of *TIM44*. (A) Ten-fold serial dilutions of wild-type (WT) and *tim44-804* cells were spotted on YPD plates and incubated at the indicated temperatures. (B) Steady-state protein levels. Mitochondria (10 and 20 µg of protein) isolated from WT and *tim44-804* cells were analyzed by SDS-PAGE and Western blotting. AAC, ADP/ATP carrier.

TIM23 complex during blue native electrophoresis and thus the Tim21-free core of the TIM23 complex migrates as a 90-kDa subcomplex (TIM23^{CORE}; Figure 4A, lane 1; Dekker *et al.*, 1997; Chacinska *et al.*, 2005; van der Laan *et al.*, 2007). In *tim44-804* mutant mitochondria, both TIM23^{CORE} and TIM23^{SORT} complexes were present (Figure 4A, lanes 2 and

Figure 2. *tim44-804* mutant mitochondria are defective in preprotein import into the matrix. ^{35}S -labeled precursors of $b_2(167)\Delta$ -DHFR (A) and the β -subunit of F_1F_0 ATPase ($F_1\beta$; B) were imported into wild-type (WT) and *tim44-804* mutant mitochondria in the presence or absence of a proton-motive force (Δp). Where indicated, mitochondria were subsequently treated with proteinase K (Prot. K). Samples were analyzed by SDS-PAGE and digital autoradiography. p, precursor; i, intermediate; m, mature.

4), indicating that the general architecture of the presequence translocase was not disrupted by the mutation of *TIM44*. *tim44-804* mitochondria contained an additional species migrating at ~ 130 kDa, which was detected with antibodies against Tim23 but not with antibodies against Tim21 (Figure 4A, lanes 2 and 4). The Pam18/Pam16 J-complex forms a separate complex on blue native electrophoresis (Figure 4A, lane 5; Frazier *et al.*, 2004; van der Laan *et al.*, 2005). The J-complex was indistinguishable between wild-type and *tim44-804* mitochondria (Figure 4A, lanes 5 and 6). Thus, the additional 130-kDa complex detected with Tim23 antibodies did not include the J-complex.

We used two approaches to study the association of the J-complex with the TIM23 complex, chemical cross-linking and coimmunoprecipitation. 1) In wild-type mitochondria, Pam18 and Pam16 can be cross-linked to Tim44 (Kozany *et al.*, 2004; Mokranjac *et al.*, 2007), as shown here with the cross-linking reagent DSG and immunodecoration with antibodies against Pam16, Pam18, and Tim44 (Figure 4B, lanes 1, 3, and 5). In *tim44-804* mitochondria, cross-linking of Tim44 to Pam16 and Pam18 was almost completely abolished (Figure 4B, lanes 8 and 10). 2) We lysed the mitochondria with digitonin and performed coimmunoprecipitation under mild conditions with antibodies directed against Tim23. Tim17 was efficiently coprecipitated with Tim23 in both wild-type and *tim44-804* mitochondria (Figure 5, lanes 3 and 4). The amount of Tim44 coprecipitated with Tim23 was only moderately reduced in *tim44-804* mitochondria, whereas the coprecipitation of Pam18 was strongly diminished compared with wild-type mitochondria (Figure 5, lanes 3 and 4).

Taken together, the results described in Figures 4 and 5 indicate that the interaction of Pam18 and Pam16 in the J-complex is stable in *tim44-804* mitochondria, but that the

Figure 3. Sorting of preproteins into the inner membrane is not affected in *tim44-804* mitochondria. ^{35}S -labeled precursors of $b_2(167)\Delta$ -DHFR and cytochrome c_1 (Cyt. c_1) were incubated with wild-type (WT) and *tim44-804* mitochondria in the presence or absence of a proton-motive force (Δp). After the import reactions mitochondria were treated with proteinase K (Prot. K) as indicated. Samples were analyzed by SDS-PAGE and digital autoradiography. p, precursor; i, intermediate; m, mature.

association of the J-complex with the TIM23 complex is strongly impaired.

Pam17 Accumulates at the TIM23 Complex in tim44 Mutant Mitochondria

The coprecipitation with antibodies against Tim23 revealed an unexpected and massive effect on Pam17. The coprecipitate from *tim44-804* mitochondria contained considerably larger amounts of Pam17 than that from wild-type mitochondria (Figure 5, lanes 3 and 4), although the total amount of Pam17 was similar in wild-type and mutant mitochondria (Figures 1B and 5, lanes 1 and 2).

Thus, the alteration of Tim44 led to opposite effects on the association of the J-complex and Pam17 with the TIM23 complex. We asked if a reduced binding of the J-complex to TIM23 induced an increase in the binding of Pam17. We utilized *pam16-1* mutant mitochondria that are characterized by loss of the J-complex from the TIM23 complex (Figure 6, lane 4; Frazier *et al.*, 2004). However, Tim44 and Pam17 were recovered in the immunoprecipitates from *pam16-1* mitochondria in similar amounts as from wild-type mitochondria, i.e., the yield of copurification of Pam17 was not increased (Figure 6). We conclude that the loss of the J-complex per se does not affect the association of Pam17 with the TIM23 complex, whereas the inactivation of Tim44 causes a dual effect, diminished J-complex association but strongly increased association of Pam17 with the TIM23 complex.

Pam17 Is in Proximity to Tim23

Because the association of Pam17 with the TIM23 complex was increased in *tim44-804* mitochondria in which the func-

Figure 4. Interaction between Tim44 and Pam18/Pam16 is inhibited in *tim44-804* mitochondria. (A) Wild-type (WT) and *tim44-804* mitochondria were solubilized in buffer containing 1% digitonin and subjected to blue native electrophoresis and Western blotting. TIM23', additional form of the TIM23 complex in *tim44-804* mitochondria. (B) Mitochondria from WT and *tim44-804* mitochondria were incubated in the presence or absence of 0.5 mM DSG and subsequently analyzed by SDS-PAGE and Western blotting. Asterisks, cross-linking products.

tion of Tim44 was compromised and Pam18/Pam16 were released from the translocase, we wondered which binding partner mediated the interaction of Pam17 with the translocase. We performed chemical cross-linking in intact mitochondria and searched for cross-linking products of the TIM23 constituents with a protein of ~15–20 kDa. Using the cross-linking reagent EGS, we identified a product of Tim23 that migrated at 40 kDa (Figure 7A, lane 1). Tom22 and Tim21 were potential candidates for the cross-linking partners of Tim23. We thus used *tom22-2* mitochondria, which contain a truncated form of Tom22 (Frazier *et al.*, 2003), as

well as *tim21Δ* mitochondria (Chacinska *et al.*, 2005). In both mutants, the 40-kDa cross-linking product was present and not altered in its size (Figure 7A, lanes 2 and 4). However, when cross-linking was performed in *pam17Δ* mitochondria (van der Laan *et al.*, 2005), the cross-linking product was lost (Figure 7B, lane 6 vs. lane 5), suggesting that Tim23 was cross-linked to Pam17. To verify that Pam17 was a component of the cross-linking product and did not indirectly affect cross-linking of Tim23 to another protein, immunodecoration of Tim23 to another protein, immunodecoration with anti-Pam17 antibodies was carried out. Indeed, antibodies against Pam17 specifically decorated the 40-kDa cross-linking product

Figure 5. Tim44 differentially affects the association of the J-complex and Pam17 with the TIM23 complex. Mitochondria from wild-type (WT) and *tim44-804* mitochondria were solubilized in digitonin buffer and subjected to immunoprecipitation with antibodies against Tim23. Samples were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. Mito, 5% total mitochondrial extract; Tim23-precipitate, 100%.

in wild-type mitochondria but not in *pam17* Δ mitochondria (Figure 7B, lanes 3 and 4). We conclude that Pam17 is in close proximity to Tim23 in intact mitochondria.

The Pam17-Tim23 cross-linking product provided a direct assay to determine if the coimmunoprecipitation data from detergent-lysed mitochondria, where the yield of association of Pam17 with the TIM23 complex was strongly increased (Figure 5), reflected the in organello situation. We thus performed cross-linking in *tim44-804* mutant mitochondria and wild-type mitochondria in parallel. Indeed, the formation of the Tim23-Pam17 cross-linking product was significantly enhanced in the mutant mitochondria (Figure 7C, lane 2 vs. lane 1). We conclude that inactivation of Tim44 enhances the association of Pam17 with the TIM23 complex. Pam17 binds to Tim23 or in close proximity of this channel-forming subunit of the TIM23 complex.

DISCUSSION

Our study extends the view of the function of Tim44 in the mitochondrial protein import machinery. Although it has been assumed that Tim44 simply functions as adaptor/scaffold for the association of mtHsp70 and further PAM subunits with the presequence translocase (Kozany *et al.*, 2004; Dudek *et al.*, 2007; Neupert and Herrmann, 2007; Mokranjac *et al.*, 2007; D'Silva *et al.*, 2008), we report that Tim44 plays a differential role in the recruitment of distinct PAM modules to the TIM23 complex.

We generated a conditional yeast mutant of *TIM44* that allowed the analysis of Tim44 function with isolated mitochondria, whereas the mutant cells were grown at permissive conditions and thus indirect effects of the *tim44* mutation on mitochondrial composition and function were minimized. The *tim44* mutant mitochondria were impaired in the association of the Pam18/Pam16 J-complex with the TIM23 complex, providing direct evidence for the view that Tim44 is important for recruiting the J-complex to the trans-

Figure 6. Inactivation of the J-complex does not affect Pam17 binding to the TIM23 complex. Wild-type (WT) and *pam16-1* mutant mitochondria were solubilized in digitonin-containing buffer and TIM23 complexes were immunoprecipitated with antibodies against Tim23. Samples were analyzed by SDS-PAGE and Western blotting. Mito, 5% total mitochondrial extract; Tim23-precipitate, 100%.

location channel (Kozany *et al.*, 2004; Mokranjac *et al.*, 2007; D'Silva *et al.*, 2008). Together with the work by D'Silva *et al.* (2008), our study thus sheds light on the controversially discussed issue of how Pam18 associates with the TIM23 complex. We conclude that three distinct interactions contribute to this association: 1) the amino-terminal intermembrane space domain of Pam18 interacts with Tim17 (Chacinska *et al.*, 2005; D'Silva *et al.*, 2008); 2) the transmembrane domain of Pam18 stabilizes the interaction with the presequence translocase (Mokranjac *et al.*, 2007); and 3) the J-complex interacts with Tim44 most likely via Pam16 (this study; D'Silva *et al.*, 2008). If only one of these interactions is disturbed, reduced binding of Pam18 is observed, whereas interfering with two interaction sites appears to be deleterious (Mokranjac *et al.*, 2007; D'Silva *et al.*, 2008).

Surprisingly, a further regulatory factor of the motor, Pam17, was strongly enhanced in binding to the TIM23 complex in *tim44* mutant mitochondria. Because Pam17 has been shown to be involved in the association of the J-complex with the TIM23 complex (van der Laan *et al.*, 2005), we wondered if the increased binding of Pam17 to the TIM23 complex was not directly caused by the inactivation of Tim44 but an indirect consequence of the impaired interaction of the J-complex with the TIM23 complex, i.e., the increased binding of Pam17 would represent a futile attempt to rescue the disturbed interaction of the J-complex with the TIM23 complex. We thus made use of *pam16* mutant mitochondria, where the J-complex was blocked in its association

Figure 7. Cross-linking of Pam17 to Tim23. (A) Wild-type (WT), *tom22-2* and *tim21Δ* mitochondria were incubated in the presence or absence of 0.25 mM EGS. Samples were analyzed by SDS-PAGE and immunodecoration with antibodies against Tim23. (B) Mitochondria from WT and *pam17Δ* cells were treated with EGS and analyzed as described for A with the indicated antibodies. (C) WT and *tim44-804* mitochondria were subjected to EGS treatment and analyzed as described in A. Asterisks, cross-linking products.

with the TIM23 complex, whereas the binding of Tim44 to the translocase was not affected (Frazier *et al.*, 2004). Importantly, in *pam16* mutant mitochondria, the binding of Pam17

to the TIM23 complex was not affected, i.e., was not enhanced in contrast to the situation in *tim44* mutant mitochondria. We conclude that the increased binding of Pam17 to the TIM23 complex in *tim44* mutant mitochondria was not indirectly caused by the release of the J-complex. Thus, Tim44 is not simply a platform for binding of other motor subunits but shows both stimulatory and inhibitory influence on the association of PAM modules with the TIM23 complex. Tim44 binds mtHsp70 and the J-complex and thus promotes the interaction of these motor modules with the TIM23 complex. In contrast, Tim44 keeps the level of TIM23-bound Pam17 low, whereas inactivation of Tim44 stimulates binding of Pam17 to the translocase.

Our findings shed new light on a puzzling observation regarding the relation of Pam17 and the J-complex. Wiedemann *et al.* (2007) showed that a fraction of the J-complexes but not Pam17 were recruited to the respiratory chain in early steps of preprotein translocation. This observation implied that the localization of Pam17 and J-complex were differently regulated; however, it was unclear whether this just represented a special situation concerning the recruitment to the respiratory chain or whether a separate localization of Pam17 and J-complex was a general principle of the motor function. We report that Pam17 binds to a new interaction site at the presequence translocase, at or in close proximity to the channel-forming protein Tim23. Thus, Pam17 and J-complex bind to the TIM23 complex via different sites and show an opposite dependence on the functionality of Tim44.

We propose that the assembly of the mitochondrial protein import motor involves a regulated interplay of several membrane-bound cochaperones: Tim44, Pam18/Pam16, and Pam17. The cochaperones do not form one stable complex but differently interact with the presequence translocase. Interestingly, the cochaperones not only possess stimulatory activities but also in part inhibitory characteristics. Pam16 was shown to promote association of Pam18 with the presequence translocase (Frazier *et al.*, 2004; Kozany *et al.*, 2004; D'Silva *et al.*, 2005, 2008; Mokranjac *et al.*, 2007) but also to reduce the stimulatory function of Pam18 on the ATPase activity of mtHsp70 (Li *et al.*, 2004; D'Silva *et al.*, 2005). We found that Tim44 promotes the binding of the J-complex to the presequence translocase but impairs the binding of Pam17. The multistep regulation of mtHsp70 function at the protein translocation channel by four membrane-bound cochaperones identifies the mitochondrial protein import motor as one of the most complicated chaperone systems known.

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