Pam17 Is Required for Architecture and Translocation Activity of the Mitochondrial Protein Import Motor

Martin van der Laan,^{1†} Agnieszka Chacinska,^{1†} Maria Lind,^{1‡} Inge Perschil,¹ Albert Sickmann,²
Helmut E. Meyer,³ Bernard Guiard,⁴ Chris Meisinger,¹ Nikolaus Pfanner,^{1*}
and Peter Rehling^{1*}

Institut für Biochemie und Molekularbiologie, Universität Freiburg, Freiburg,¹ Rudolf-Virchow-Center for Experimental Biomedicine, Universität Würzburg, Würzburg,² and Medizinisches Proteom-Center, Ruhr-Universität Bochum, Bochum,³ Germany, and Centre de Génétique Moléculaire, Laboratoire propre du CNRS associé à l'Université Pierre et Marie Curie, Gif-sur-Yvette, France⁴

Received 29 April 2005/Returned for modification 7 June 2005/Accepted 10 June 2005

Import of mitochondrial matrix proteins involves the general translocase of the outer membrane and the presequence translocase of the inner membrane. The presequence translocase-associated motor (PAM) drives the completion of preprotein translocation into the matrix. Five subunits of PAM are known: the preprotein-binding matrix heat shock protein 70 (mtHsp70), the nucleotide exchange factor Mge1, Tim44 that directs mtHsp70 to the inner membrane, and the membrane-bound complex of Pam16-Pam18 that regulates the ATPase activity of mtHsp70. We have identified a sixth motor subunit. Pam17 (encoded by the open reading frame YKR065c) is anchored in the inner membrane and exposed to the matrix. Mitochondria lacking Pam17 are selectively impaired in the import of matrix proteins and the generation of an import-driving activity of PAM. Pam17 is required for formation of a stable complex between the cochaperones Pam16 and Pam18 and promotes the association of Pam16-Pam18 with the presequence translocase. Our findings suggest that Pam17 is required for the correct organization of the Pam16-Pam18 complex and thus contributes to regulation of mtHsp70 activity at the inner membrane translocation site.

Mitochondria possess about 900 different proteins in yeast and about 1,500 different proteins in humans (34, 42, 44). Ninety-nine percent of the proteins are synthesized on cytosolic ribosomes. Studies of the past years revealed the existence of multiple protein import pathways into the four mitochondrial subcompartments: outer membrane, intermembrane space, inner membrane, and matrix (8, 15, 16, 18, 20, 29, 33). A major pathway is used by preproteins carrying the classical amino-terminal presequences that direct translocation into the matrix and are removed by the mitochondrial processing peptidase. These cleavable preproteins use the general translocase of the outer membrane (TOM complex), which contains surface receptors and the import channel Tom40, and then engage the specific presequence translocase of the inner membrane (TIM23 complex). The membrane potential $\Delta\psi$ across the inner membrane drives the translocation of the presequences through the TIM23 complex. Some cleavable preproteins contain a hydrophobic stop-transfer signal behind the positively charged presequence (13). Those preproteins are laterally released from the TIM23 complex and are thus sorted into the inner membrane. Most preproteins with a pre-

sequence, however, do not carry hydrophobic sorting signals and are thus completely translocated into the mitochondrial matrix. This transport strictly requires the presequence translocase-associated motor (PAM) (26, 36).

The presequence translocase contains four different integral proteins of the inner mitochondrial membrane (4, 10, 18, 30, 36, 48): the channel-forming protein Tim23; Tim50 that cooperates with Tim23 in binding of preproteins on the intermembrane space side; Tim17 that plays a dual role in promoting protein sorting into the inner membrane and binding of PAM subunits to the translocase; and Tim21 that transiently connects the TIM23 complex to the TOM complex. The TIM23 complex switches between two functional states: a sorting-competent form and a matrix-import form. While Tim17, Tim23, and Tim50 are present in both forms of the TIM23 complex, Tim21 is present in the sorting-competent form but absent in the PAM-associated matrix-import form of the TIM23 complex (4). The TIM23 complex thus alternates between binding of Tim21 and PAM.

Five subunits of PAM are known. Three subunits, mtHsp70, Pam18 (Tim14), and Mge1, share significant homology with the classical Hsp70 system of bacteria, consisting of DnaK, DnaJ, and GrpE (2, 14, 27). The matrix heat shock protein 70 (mtHsp70) forms the ATP-driven core of the motor and binds the precursor polypeptide chain like DnaK (24, 32, 46). Pam18 contains a J-domain that stimulates the ATPase activity of mtHsp70 similar to the bacterial DnaJ (7, 31, 45). The mitochondrial GrpE (Mge1) promotes nucleotide exchange like its bacterial homolog (27). In contrast to the soluble Hsp70 system of bacteria, however, Pam18 is a membrane protein which is associated with the TIM23 complex via interaction with

* Corresponding author. Mailing address: Institut für Biochemie und Molekularbiologie, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg, Germany. Phone: 49-761-203-5224. Fax: 49-761-203-5261. E-mail for Nikolaus Pfanner: Nikolaus.Pfanner@biochemie.uni-freiburg.de. E-mail for Peter Rehling: Peter.Rehling@biochemie.uni-freiburg.de.

† M.V.D.L. and A.C. contributed equally to this work.

‡ Present address: Department of Comparative Physiology, Evolutionary Biology Centre, Uppsala University, SE-75236 Uppsala, Sweden.

Tim17 (4) and thus activates mtHsp70 close to the preprotein exit site of the presequence translocase. Moreover, PAM shows a significantly higher complexity than the DnaK system since two further subunits have been identified, both of which are proteins specifically associated with the presequence translocase. Tim44 functions as a dynamic membrane binding site for mtHsp70 molecules and thus directs the chaperone to the preprotein channel (24, 26, 32, 46). Pam16 (Tim16) forms a complex with Pam18 and controls the activity of this stimulating subunit (9, 21, 23). Recently, a further matrix protein, Zim17 (Tim15/Hep1), was identified that interacts with mtHsp70; Zim17 is not associated with the presequence translocase but plays a more general role in preventing aggregation of matrix Hsp70s, including mtHsp70 (Ssc1) and Ssq1 (3, 41, 49).

Different views exist about the molecular mechanism of mtHsp70 function, either as a molecular ratchet or as a more complicated multistep motor (8, 12, 24, 26, 32, 36, 46). A mechanistic characterization of the mitochondrial import motor, however, requires that all components of PAM have been identified and their functional contribution to the import-driving activity has been characterized. Here we report the unexpected identification of a further presequence translocase-associated protein that classifies as a PAM protein. Pam17 is selectively required for protein translocation into the mitochondrial matrix. Pam17 plays a specific role in organizing the Pam16-Pam18 complex and thus is required for the import-driving activity of the import motor. Therefore, the mitochondrial motor is one of the most complicated Hsp70 systems known.

MATERIALS AND METHODS

Yeast strains and growth conditions. *Saccharomyces cerevisiae* strains were grown in YP medium (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone) containing either 3% (wt/vol) glycerol (YPG) or 2% sucrose (YPS). For plate growth assays, synthetic medium containing 3% glycerol was used. Deletion of *PAM17* was achieved by replacing the corresponding chromosomal open reading frame YKR065c (*FMP18*) in the *S. cerevisiae* strain YPH499 with a *HIS5* marker, yielding the strain *pam17Δ* (IP18Δ) (MATa *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 pam17::HIS5*). *pam17Δ* cells were grown at 24°C unless indicated otherwise. Strains containing a C-terminal protein A tag on Tim18 (37), Tim21 (4), or Tim23 (10) have been described before. For purification of the TIM23 complex, a plasmid encoding Tim23 with a C-terminal protein A tag (10) was transformed into *pam17Δ*. For purification of the TOM-TIM23-PAM supercomplex, the yeast strain MR103 containing histidine-tagged Tom22 (28) was used. The temperature-sensitive mutant allele *tim17-5* has been described previously (4). The *tim17-5* mutant strain harboring a protein A-tagged version of Tim23 and the corresponding wild type were grown in synthetic medium containing 2% sucrose at 24°C.

Isolation and analysis of mitochondria. Mitochondria were isolated by differential centrifugation. For protein localization studies, mitochondria were treated with proteinase K after osmotic swelling, sonication, or lysis with Triton X-100 or were treated with sodium carbonate (pH 11.5) as described previously (9, 38). Steady-state levels of mitochondrial proteins were analyzed by separation via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Mitochondrial membrane protein complexes were analyzed by blue native electrophoresis (BN-PAGE) on 6 to 16.5% gradient gels as described previously (6).

Purifications of TIM23 and TIM22 complexes via protein A tags on Tim23, Tim21, or Tim18, respectively, were performed according to previously published methods (4, 10, 37). Purification of the TOM-TIM23-PAM supercomplex was carried out as described previously (5).

In vitro protein import. ³⁵S-labeled precursor proteins were synthesized in rabbit reticulocyte lysate and imported into isolated mitochondria as described previously (38). *b₂(120)*-DHFR (B40) was constructed from the fusion protein

b₂(220)-DHFR (47) by deleting the region coding for amino acid residues 86 to 185 (containing the heme-binding domain of cytochrome *b₂*). For generation of the TOM-TIM23-PAM supercomplex, cytochrome *b₂(167)*_Δ-DHFR was imported in the presence of 5 μM methotrexate (5). Inward-directed import driving activity assays were performed as described previously (4).

Miscellaneous. In gel digestion, nano-high-performance liquid chromatography separation of peptides and mass spectrometry were performed as described previously (10). Anti-Pam17 antibodies were raised in rabbits against the mature protein purified from inclusion bodies. Membrane potential measurements were carried out as described previously (11). Amino acid sequence alignments were made using the ClustalW software. In some figures, irrelevant gel lanes were excised by digital processing. Figures were prepared by using ImageQuant 5.2 (Amersham), Adobe Photoshop CS 8.0, and Adobe Illustrator CS 11.0.0.

RESULTS

Identification of the mitochondrial inner membrane protein

Pam17. We purified the TIM23 complex and associated PAM from *S. cerevisiae* mitochondria carrying a protein A tag at Tim23 (Fig. 1A, lane 2) (4, 9, 10, 45). Besides the known subunits of TIM23-PAM, we identified a novel protein by mass spectrometry, encoded by the open reading frame YKR065c. This protein, designated Pam17, migrates like Tim17 on most gels and thus has escaped detection in previous studies. In the high-resolution gel used here, Pam17 migrates in a separate band. Additional protein bands found in the purified fraction included fragments of larger TIM23-PAM proteins and a contaminating fraction of the highly abundant inner membrane protein ADP/ATP carrier (Fig. 1A, lane 2) (9, 10). The deduced primary structure of Pam17 consists of 197 amino acid residues, including a putative mitochondrial presequence and two predicted hydrophobic segments of sufficient length to function as transmembrane segments (Fig. 1B) (19). Pam17 shows homology to proteins of other eukaryotic species (Fig. 1C).

Upon treatment of isolated yeast mitochondria at pH 11.5, Pam17 fractionated in the membrane pellet like the integral proteins Tim23 and Tim50, while the peripheral membrane protein Tim44 was extracted (Fig. 1D). For an experimental determination if Pam17 carried a cleavable presequence, we synthesized and radiolabeled the precursor of Pam17 in rabbit reticulocyte lysate. The precursor migrated more slowly on SDS-PAGE than mature Pam17 detected by Western blotting (Fig. 1E, lanes 1 and 2). Upon incubation of the precursor with isolated mitochondria in the presence of a membrane potential ($\Delta\psi$), Pam17 was processed to the mature-sized form (Fig. 1E, lanes 3 to 5). Upon dissipation of $\Delta\psi$, the processing of Pam17 was inhibited (Fig. 1E, lane 6). Thus, Pam17 behaves as an inner membrane protein synthesized with a presequence. When mitochondria were subjected to swelling in order to open the intermembrane space, Pam17 remained protected against added protease like the matrix-exposed Tim44 while Tim23 and Tim21 became accessible to the protease (Fig. 1F, lanes 5 and 6). Upon sonication of mitochondria to open the matrix, Pam17 was digested by the protease (Fig. 1F, lanes 8 and 9). We conclude that Pam17 is anchored in the mitochondrial inner membrane and exposed to the matrix.

Pam17 cofractionates with the import motor. The purification of tagged Tim23 from digitonin-lysed mitochondria leads to the copurification of TIM23 and PAM subunits (4, 9, 10, 45). To obtain evidence of whether Pam17 is a TIM or a PAM protein, we purified the sorting-competent but PAM-free form

FIG. 1. Pam17 is a mitochondrial inner membrane protein associated with the TIM23 complex. (A) Isolation of the presequence translocase from *S. cerevisiae* mitochondria. Mitochondria from wild-type (WT) yeast and yeast cells expressing protein A-tagged Tim23 were lysed in digitonin-containing buffer. Digitonin extracts were subjected to immunoglobulin G affinity chromatography. Bound proteins were eluted with ammonium acetate buffer, pH 3.5, separated by SDS-PAGE, and stained with colloidal Coomassie. Proteins were identified by mass spectrometry (10). AAC, ADP/ATP carrier. (B) Predicted primary structure of the Pam17 precursor protein of *S. cerevisiae*. Broken line, predicted presequence; boxes, predicted transmembrane segments. (C) Sequence alignment of Pam17 from *S. cerevisiae* (Sc) to proteins from *Schizosaccharomyces pombe* (Sp), *Neurospora crassa* (Nc), and *Caenorhabditis elegans* (Ce). Black or gray boxes indicate amino acid residues that are identical or similar, respectively, in at least three out of four sequences. (D) Mitochondria were subjected to treatment with sodium carbonate at pH 11.5. Samples were left on ice (total, T) or separated into pellet (P) and supernatant (S) by centrifugation at $100,000 \times g$. (E) ^{35}S -labeled Pam17 precursor (sample 1) was incubated with wild-type mitochondria for the indicated times (samples 3 to 6) in the presence or absence of a membrane potential ($\Delta\psi$). Samples 3 to 6 were subsequently treated with proteinase K and analyzed by SDS-PAGE and digital autoradiography. As a control, a Western blot of the mature Pam17 protein is shown (lane 2). p, precursor; m, processed mature protein. (F) Isolated yeast mitochondria (Mitoch.) were subjected to osmotic swelling (samples 4 to 6), sonication (samples 7 to 9), or lysis with Triton X-100 (samples 10 to 12) and were treated with proteinase K (Prot. K) as indicated. Samples were analyzed by SDS-PAGE and Western blotting.

FIG. 2. Pam17 is a motor subunit and is present in an active protein import complex. (A) Mitochondria were isolated from yeast strains expressing protein A-tagged versions of Tim18, Tim23, or Tim21. Mitochondria were lysed in digitonin-containing buffer in the presence of 80 mM KCl, and protein extracts were subjected to immunoglobulin G (IgG) affinity chromatography. Bound proteins were eluted by tobacco etch virus protease treatment (45), and samples were subjected to SDS-PAGE and Western blot analysis. L, 20% of load; E, 100% of eluate. AAC, ADP/ATP carrier. (B) Mitochondria from strains expressing protein A-tagged Tim23 in a wild-type (WT) or *tim17-5* mutant background were lysed in digitonin buffer. Proteins were incubated with IgG-Sepharose, and TIM23 complexes were eluted with SDS sample buffer. L, 5% of load; U, 5% of unbound; E, 100% of eluate. (C) Mitochondria carrying Tom22 with a C-terminal His₁₀ tag were incubated for 15 min at 25°C with either b₂(167)_Δ-DHFR and methotrexate (MTX) or MTX alone. Mitochondria were solubilized with digitonin buffer, and protein extracts were subjected to Ni-nitrilotriacetic acid agarose affinity chromatography. L, 10% of load; W, 100% of three wash fractions; E, 100% of eluate. Proteins copurifying with Tom22_{His} in the presence or absence of b₂(167)_Δ-DHFR were quantified. The yield of copurification of Tim50 in the presence of b₂(167)_Δ-DHFR was set to 100% (control).

of the TIM23 complex via tagged Tim21 (4). While the subunits of the TIM23 complex like Tim17 and Tim50 copurified with Tim21, none of the motor subunits was found in the purified fraction in significant amounts, shown here with Pam18, Tim44, and mtHsp70 (Fig. 2A, lane 6). Pam17 fractionated like the motor subunits, i.e., was copurified in significant amounts together with Tim23 but not with Tim21 (Fig. 2A, lanes 4 and 6). As a further control, we purified the second translocase complex of the inner membrane, the TIM22 complex (carrier translocase), via tagged Tim18 (37). None of the subunits of TIM23 or PAM, including Pam17, copurified with the TIM22 complex (Fig. 2A, lane 2).

To obtain independent evidence for the cofractionation of Pam17 with the import motor, we made use of a specific yeast mutant of Tim17. Chacinska et al. (4) reported that Tim17 is required for the interaction of PAM with the TIM23 complex. In *tim17-5* mitochondria, the association of PAM subunits, including Pam16, Pam18, Tim44, and mtHsp70, with the TIM23 complex is strongly impaired. Pam17 behaved like the other PAM subunits since its copurification with tagged Tim23

was strongly reduced in *tim17-5* mitochondria, while TIM subunits, such as Tim21 and Tim50, remained associated with the TIM23 complex (Fig. 2B, lane 6).

To determine if Pam17 was present in an active protein import complex, we accumulated chemical amounts of a preprotein in mitochondrial import sites. When the model preprotein b₂(167)_Δ-DHFR, consisting of a matrix-targeted amino-terminal portion of the precursor of cytochrome b₂ and the passenger protein dihydrofolate reductase (DHFR), is incubated with the DHFR ligand methotrexate, the DHFR moiety cannot be unfolded by the mitochondrial import machinery. Thus, the amino-terminal portion of the preprotein accumulates in a two-membrane-spanning fashion in mitochondria, leading to the formation of a stable preprotein-TOM-TIM23-PAM supercomplex (5, 9, 10, 45). Under these conditions TIM23 and PAM are copurified with tagged Tom22, shown here with the copurification of Tim50 and Tim44 (Fig. 2C, columns 11 and 15), while in the absence of the two-membrane-spanning preprotein the yield of copurification is strongly reduced (Fig. 2C, columns 12 and 16) and close to the background level observed with the control proteins ADP/ATP carrier and porin (Fig. 2C, columns 17 to 20). Pam17 was specifically copurified with tagged Tom22 in the presence of the accumulated preprotein (Fig. 2C, samples 5 and 13), demonstrating its presence in the preprotein-TOM-TIM23-PAM supercomplex. We conclude that Pam17 is present in an active protein import complex and cofractionates with PAM subunits.

Pam17 is required for protein transport to the matrix but not the inner membrane. We generated a yeast mutant lacking the open reading frame of *PAM17*. The resulting cells grew slowly on nonfermentable medium (Fig. 3A). Mitochondria were isolated from the mutant cells grown at 24°C. The steady-state levels of TIM proteins and PAM proteins were comparable between wild-type and mutant mitochondria (Fig. 3B). The import of matrix proteins, however, was strongly impaired in *pam17Δ* mitochondria, including the F₁-ATPase subunit β (F₁β) and b₂(167)_Δ-DHFR (Fig. 3C and D). Upon re-expression of Pam17 from a plasmid in *pam17Δ* cells, full protein import activity was restored (data not shown).

To exclude that the import inhibition in *pam17Δ* mitochondria was indirectly caused by a reduction of Δψ, we assessed the membrane potential with the potential-sensitive dye 3,3'-dipropylthiadicarbocyanine iodide [DiSC₃(5)] (11). Wild-type and *pam17Δ* mitochondria showed a similar efficiency of fluorescence quenching (Fig. 4A), indicating that the mutant mitochondria were able to generate a Δψ. We then analyzed two cleavable preproteins, cytochrome c₁ and b₂(120)-DHFR, that are inserted into the inner membrane by the TIM23 complex but do not require PAM. Both preproteins contain a hydrophobic stop-transfer sequence behind the positively charged matrix-targeting signal and are thus arrested in the inner membrane (1, 9, 13, 47), while the stop-transfer signal has been deleted in b₂(167)_Δ-DHFR. Cytochrome c₁ and b₂(120)-DHFR were imported into *pam17Δ* and wild-type mitochondria with similar efficiency (Fig. 4B and C).

Thus, mitochondria lacking Pam17 show a characteristic defect of PAM, i.e., inhibition of matrix import while inner membrane sorting can take place. Together with the cofractionation of Pam17 with the PAM subunits, we conclude that Pam17 is a new subunit of the mitochondrial import motor.

FIG. 3. Pam17 is required for protein import into the mitochondrial matrix. (A) Serial dilutions of wild-type (WT) and *pam17*Δ cells were spotted on synthetic glycerol medium and incubated at 30°C or 24°C. (B) Mitochondria (Mito) were isolated from WT and *pam17*Δ cells grown at 24°C in YPG medium. Indicated amounts of mitochondrial proteins were separated by SDS-PAGE and analyzed by Western blotting. AAC, ADP/ATP carrier. (C) The ³⁵S-labeled precursor of F₁β was incubated with WT and *pam17*Δ mitochondria for the indicated times in the presence or absence of a membrane potential (Δψ). Subsequently, samples were left on ice or treated with proteinase K (Prot. K) and analyzed by SDS-PAGE and digital autoradiography. For quantification of imported proteins, the amount of protease-resistant processed F₁β in WT mitochondria after the longest import time was set to 100% (control). p, precursor; m, processed mature protein. (D) Radiolabeled b₂(167)_Δ-DHFR was imported into WT and *pam17*Δ mitochondria as described for panel C. The amount of protease-resistant processed b₂(167)_Δ-DHFR in WT mitochondria after the longest import time was set to 100% (control). p, precursor; i, intermediate.

Pam17 is required for organization of the Pam16-Pam18 complex and import motor activity. To obtain evidence for a possible role of Pam17 in vivo, *pam17*Δ cells and the corresponding wild-type cells were grown at elevated temperature (37°C) and the protein levels of isolated mitochondria were compared. We observed a reduction in the steady-state level of Pam18 in *pam17*Δ mitochondria, while the levels of all other proteins analyzed were not significantly decreased, including

Pam16, mtHsp70, Tim44, all subunits of the TIM23 complex, and control proteins for the outer and inner membranes (Fig. 5A). This finding pointed to a possible role of Pam17 in maintaining the stability of Pam18. To exclude indirect effects due to lowered levels of Pam18, all other experiments with *pam17*Δ mitochondria reported here were performed with mitochondria isolated from cells grown at low temperature.

We asked if Pam17 may be involved in the association of

FIG. 4. Pam17 is not required for protein sorting at the inner mitochondrial membrane. (A) Assessment of the membrane potential of wild-type (WT) and *pam17Δ* mitochondria with a fluorescence quenching assay using the potential-sensitive dye DiSC₃(5). (B) The ³⁵S-labeled precursor of cytochrome c_1 was imported into WT and *pam17Δ* mitochondria as described in the legend to Fig. 3C. p, precursor; i, intermediate. (C) Radio-labeled $b_2(120)$ -DHFR was imported into WT and *pam17Δ* mitochondria as described in the legend to Fig. 3C. The amount of protease-resistant processed $b_2(120)$ -DHFR in WT mitochondria after the longest import time was set to 100% (control). p, precursor; i, intermediate; m, mature; Prot. K, proteinase K; Mitoch., mitochondria; cyt., cytochrome.

PAM subunits with the TIM23 complex. TIM23-PAM was purified from wild-type and *pam17Δ* mitochondria via tagged Tim23 (Fig. 5B). While the yield of copurification of the TIM23 subunits as well as Tim44 and mtHsp70 was similar between wild-type and *pam17Δ* mitochondria, the amount of Pam16 and Pam18 recovered with tagged Tim23 was significantly decreased (Fig. 5B and C). Thus, Pam17 is involved in the association of Pam16 and Pam18 with the TIM23 complex.

Pam16 and Pam18 migrate as a distinct PAM subcomplex of about 80 kDa on blue native electrophoresis (BN-PAGE) of digitonin-lysed mitochondria (Fig. 6A, lane 1) (9, 23). In *pam17Δ* mitochondria, the amount of Pam16-Pam18 complex was strongly reduced (Fig. 6A, lane 2). The total mitochondrial levels of Pam16 and Pam18, as determined by SDS-PAGE, as well as the extractability of both proteins by digitonin were not changed between wild-type and *pam17Δ* mitochondria (Fig. 6A, lanes 1 and 2, and data not shown), indicating that the dissociated Pam16 and Pam18 of *pam17Δ* mitochondria migrated out of the separation range of the BN-PAGE. Other translocase complexes analyzed by BN-PAGE were present in both wild-type and *pam17Δ* mitochondria, including TIM23

complex, TIM21 complex, TIM22 complex, and TOM complex (Fig. 6A, lanes 5 to 12). We conclude that the Pam16-Pam18 complex is significantly destabilized in *pam17Δ* mitochondria.

The residual Pam16-Pam18 complex in the mutant mitochondria showed the same mobility on BN-PAGE as the wild-type complex (Fig. 6A, lanes 1 and 2), indicating that Pam17 itself is not a genuine subunit of this complex. Indeed, Pam17 migrated in a BN-PAGE band of about 50 kDa and thus separately from the Pam16-Pam18 complex (Fig. 6A, lane 3).

To determine if Pam17 was required for the PAM import-driving activity, we analyzed the $\Delta\psi$ -independent motor activity with a two-membrane-spanning preprotein. Since preprotein translocation into the matrix was strongly impaired in *pam17Δ* mitochondria, we used the sorted preprotein $b_2(220)$ -DHFR (47). In the presence of methotrexate, the folded DHFR remains on the cytosolic side while the cytochrome b_2 portion spans across the mitochondrial membranes and the amino-terminal presequence is cleaved to the intermediate-sized form by the matrix processing peptidase (4). Two import-driving activities, $\Delta\psi$ and the ATP-driven PAM, promote an inward movement of the preprotein such that the DHFR is so

FIG. 5. Pam16 and Pam18 dissociate from the presequence translocase in *pam17Δ* mitochondria. (A) Mitochondria were isolated from wild-type (WT) and *pam17Δ* cells grown at 37°C in YPG medium. Indicated amounts of mitochondrial proteins were separated by SDS-PAGE and analyzed by Western blotting. Asterisk, unspecific band. (B) Mitochondria containing protein A-tagged Tim23 in a WT and *pam17Δ* background were isolated from cells grown at 24°C, solubilized in digitonin buffer in the presence of 150 mM NaCl, and subjected to immunoglobulin G affinity chromatography. Bound TIM23 complex was eluted with SDS sample buffer. Samples were analyzed by SDS-PAGE and Western blotting. Twenty percent of load and 100% of eluate are shown. (C) Proteins purified from *pam17Δ* mitochondria via tagged Tim23 (as described for panel B) were quantified. The amount purified from WT mitochondria was set to 100% for each protein. Standard errors of the means were calculated from at least three independent experiments. Mito, mitochondria; AAC, ADP/ATP carrier.

closely apposed to the outer membrane that it cannot be removed by externally added proteinase K (9, 40, 46). To selectively determine the PAM activity, the preprotein was first accumulated across both membranes, and then $\Delta\psi$ was dissipated by addition of the potassium ionophore valinomycin in the presence of potassium in the medium (4). Upon a second

incubation, the protease resistance of intermediate-sized $b_2(220)$ -DHFR was determined (Fig. 6B, flow diagram). In wild-type mitochondria, a significant fraction of $b_2(220)$ -DHFR was not degraded by protease, while in *pam17Δ* mitochondria most of the intermediate-sized $b_2(220)$ -DHFR was degraded (Fig. 6B, quantification). Thus, Pam17 is required for the import-driving activity of PAM.

DISCUSSION

We have identified a new subunit of the presequence translocase-associated motor of mitochondria. Pam17 migrates like Tim17 on most gel systems and thus has escaped detection in previous studies that characterized the subunits of TIM23 and PAM (4, 7, 9, 10, 21, 30, 31, 45, 48). We identified Pam17 by use of a high-resolution gel and mass spectrometry and found that it cofractionates with the known PAM subunits, Tim44, Pam16, Pam18, and mtHsp70, upon isolation of TIM23-PAM.

Yeast cells lacking Pam17 show growth defects, and the resulting mitochondria are strongly impaired in import of presequence-carrying matrix proteins. Presequence-carrying preproteins with a hydrophobic stop-transfer sequence, however, can be efficiently inserted into the inner membrane of *pam17Δ* mitochondria. This import phenotype is characteristic for subunits of the mitochondrial import motor (9, 21, 31, 45, 47). Two forces drive protein translocation across the mitochondrial inner membrane, the membrane potential $\Delta\psi$ and the ATP-powered PAM (17, 25, 32, 43, 46). By dissipation of $\Delta\psi$ upon accumulation of a two-membrane-spanning preprotein, we could show that Pam17 is required for the import-driving activity of PAM.

Previous studies showed that the PAM subunits are organized in two distinct subcomplexes, Tim44-mtHsp70 and Pam16-Pam18 (9, 21, 22, 23, 35, 39, 46). While the Tim44-mtHsp70 complex does not migrate as a distinct band on BN-PAGE (4, 6), the Pam16-Pam18 complex forms a BN complex of about 80 kDa (9). Pam17 migrates in a separate BN band of about 50 kDa, indicating that PAM consists of three subcomplexes that are all associated with the TIM23 complex. Though Pam17 is not stably associated with the Pam16-Pam18 complex, it plays a critical role in the organization of this complex. In mitochondria lacking Pam17, the BN-stable association of Pam16 and Pam18 is strongly impaired. Moreover, the association of Pam16 and Pam18 with the TIM23 complex is significantly reduced in *pam17Δ* mitochondria. Our findings suggest that in *pam17Δ* mitochondria the organization of the Pam16-Pam18 complex is disturbed, leading to an impairment of the import-driving activity of PAM.

Tim17 plays a critical role in the association of the various PAM subunits with the TIM23 complex (4 and this study). The association of the stimulatory J-protein Pam18 with the TIM23 complex involves several PAM components. The purified amino-terminal domain of Pam18 that is exposed to the intermembrane space binds to Tim17 in vitro (4). However, this interaction alone is not sufficient for the stable interaction of Pam18 with the TIM23 complex in *organello*, since the adaptor protein Pam16 has been shown to be required for the stable association of Pam18 with the TIM23 complex (9, 21). We show here that Pam17 is required for an efficient association of Pam16

FIG. 6. Dissociation of the Pam16-Pam18 complex in *pam17Δ* mitochondria and impairment of the import-driving motor activity. (A) Wild-type (WT) and *pam17Δ* mitochondria isolated from cells grown in YPG at 24°C were solubilized in digitonin buffer in the presence of 150 mM NaCl and analyzed by BN-PAGE (75 μ g protein per lane) or SDS-PAGE (20 μ g protein per lane) and Western blotting. Protein complexes separated by BN-PAGE were detected with antibodies against Pam18 (Pam16/18 complex), Pam17, Tim23 (TIM23^{core} complex), Tim21 (TIM23* complex) (4), Tim22 (TIM22 complex), or Tom40 (TOM complex). (B) In order to analyze the inward-directed import-driving activity, WT and *pam17Δ* mitochondria were incubated with radiolabeled $b_2(220)$ -DHFR in the presence of methotrexate (MTX) for 30 min at 25°C. Samples were either left untreated or, after dissipation of the membrane potential by addition of valinomycin, further incubated at 25°C for the indicated times (Δt), subsequently treated with proteinase K on ice, and analyzed by SDS-PAGE and digital autoradiography (p, precursor; i, intermediate; m, mature). The amount of i- $b_2(220)$ -DHFR in non-protease-treated samples at $\Delta t = 0$ was set to 100%. Standard errors of the means were calculated from at least three independent experiments. Prot. K, proteinase K.

and Pam18 with the TIM23 complex, probably via the role of Pam17 in organizing the Pam16-Pam18 complex. The relevance of Pam17 for Pam18 stability *in vivo* became evident when *pam17Δ* cells were grown at elevated temperature, since under these conditions the steady-state level of Pam18 was selectively reduced.

The mitochondrial import motor is thus one of the most complicated Hsp70 systems known. mtHsp70 is a soluble protein of the mitochondrial matrix, and four membrane proteins, Tim44, Pam16, Pam17, and Pam18, are required to coordinate the action of mtHsp70 at the TIM23 complex, i.e., at the exit site for preproteins at the inner membrane. Pam17 is the only

one of those proteins that is resistant to an extraction at pH 11.5, indicating that it is firmly embedded in the lipid phase of the inner membrane. This agrees with the presence of two predicted transmembrane segments in Pam17 while the other PAM subunits contain either no or one predicted hydrophobic segment. In summary, Pam17 is an integral protein of the mitochondrial inner membrane required for the stable organization of the Pam16-Pam18 complex that stimulates the activity of mtHsp70 at the presequence translocase. Thus, Pam17 is involved in regulating the activity of mtHsp70 at the preprotein translocation channel of the inner membrane. We conclude that mtHsp70 together with the four membrane-bound co-

chaperones and soluble Mge1 forms a multistep motor for preprotein translocation.

ACKNOWLEDGMENTS

We are grateful to R. E. Jensen and S. Rospert for antisera and to A. Schulze-Specking for expert technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 388, Gottfried Wilhelm Leibniz Program, Max Planck Research Award, Alexander von Humboldt Foundation, Bundesministerium für Bildung und Forschung, Nationales Genomforschungsnetz, and the Fonds der Chemischen Industrie. M.V.D.L. and M.L. were supported by postdoctoral fellowships from EMBO and the Wenner-Gren foundations, respectively.

REFERENCES

1. Arnold, I., H. Fölsch, W. Neupert, and R. A. Stuart. 1998. Two distinct and independent mitochondrial targeting signals function in the sorting of an inner membrane protein, cytochrome *c*₁. *J. Biol. Chem.* **273**:1469–1476.
2. Bukau, B., and A. L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. *Cell* **92**:351–366.
3. Burri, L., K. Vascotto, S. Fredersdorf, R. Tiedt, M. N. Hall, and T. Lithgow. 2004. Zim17, a novel zinc finger protein essential for protein import into mitochondria. *J. Biol. Chem.* **279**:5024–5029.
4. Chacinska, A., M. Lind, A. E. Frazier, J. Dudek, C. Meisinger, A. Geissler, A. Sickmann, H. E. Meyer, K. N. Truscott, B. Guiard, N. Pfanner, and P. Rehling. 2005. Mitochondrial presequence translocase: switching between TOM tethering and motor recruitment involves Tim21 and Tim17. *Cell* **120**:817–829.
5. Chacinska, A., P. Rehling, B. Guiard, A. E. Frazier, A. Schulze-Specking, N. Pfanner, W. Voos, and C. Meisinger. 2003. Mitochondrial translocation contact sites: separation of dynamic and stabilizing elements in formation of a TOM-TIM-preprotein supercomplex. *EMBO J.* **22**:5370–5381.
6. Dekker, P. J., F. Martin, A. C. Maarse, U. Bömer, H. Müller, B. Guiard, M. Meijer, J. Rassow, and N. Pfanner. 1997. The Tim core complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70-Tim44. *EMBO J.* **16**:5408–5419.
7. D'Silva, P. D., B. Schilke, W. Walter, A. Andrew, and E. A. Craig. 2003. J protein cochaperone of the mitochondrial inner membrane required for protein import into the mitochondrial matrix. *Proc. Natl. Acad. Sci. USA* **100**:13839–13844.
8. Endo, T., H. Yamamoto, and M. Esaki. 2003. Functional cooperation and separation of translocators in protein import into mitochondria, the double-membrane bounded organelles. *J. Cell Sci.* **116**:3259–3267.
9. Frazier, A. E., J. Dudek, B. Guiard, W. Voos, Y. Li, M. Lind, C. Meisinger, A. Geissler, A. Sickmann, H. E. Meyer, V. Bilanchone, M. G. Cumsky, K. N. Truscott, N. Pfanner, and P. Rehling. 2004. Pam16 has an essential role in the mitochondrial protein import motor. *Nat. Struct. Mol. Biol.* **11**:226–233.
10. Geissler, A., A. Chacinska, K. N. Truscott, N. Wiedemann, K. Brandner, A. Sickmann, H. E. Meyer, C. Meisinger, N. Pfanner, and P. Rehling. 2002. The mitochondrial presequence translocase: an essential role of Tim50 in directing preproteins to the import channel. *Cell* **111**:507–518.
11. Geissler, A., T. Krimmer, U. Bömer, B. Guiard, J. Rassow, and N. Pfanner. 2000. Membrane potential-driven protein import into mitochondria: the sorting sequence of cytochrome *b*₂ modulates the $\Delta\psi$ -dependence of translocation of the matrix-targeting sequence. *Mol. Biol. Cell* **11**:3977–3991.
12. Glick, B. S. 1995. Can Hsp70 proteins act as force-generating motors? *Cell* **80**:11–14.
13. Glick, B. S., A. Brandt, K. Cunningham, S. Müller, R. L. Hallberg, and G. Schatz. 1992. Cytochromes *c*₁ and *b*₂ are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism. *Cell* **69**:809–822.
14. Hartl, F. U., and M. Hayer-Hartl. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**:1852–1858.
15. Herrmann, J. M., and W. Neupert. 2000. Protein transport into mitochondria. *Curr. Opin. Microbiol.* **3**:210–214.
16. Hoogenraad, N. J., L. A. Ward, and M. T. Ryan. 2002. Import and assembly of proteins into mitochondria of mammalian cells. *Biochim. Biophys. Acta* **1592**:97–105.
17. Huang, S., K. S. Ratliff, and A. Matouschek. 2002. Protein unfolding by the mitochondrial membrane potential. *Nat. Struct. Biol.* **9**:301–307.
18. Jensen, R. E., and C. D. Dunn. 2002. Protein import into and across the mitochondrial inner membrane: role of the TIM23 and TIM22 translocases. *Biochim. Biophys. Acta* **1592**:25–34.
19. Kim, H., K. Melén, and G. von Heijne. 2003. Topology models for 37 *Saccharomyces cerevisiae* membrane proteins based on C-terminal reporter fusions and predictions. *J. Biol. Chem.* **278**:10208–10213.
20. Koehler, C. M. 2004. New developments in mitochondrial assembly. *Annu. Rev. Cell Dev. Biol.* **20**:309–335.
21. Kozany, C., D. Mokranjac, M. Sichting, W. Neupert, and K. Hell. 2004. The J domain-related cochaperone Tim16 is a constituent of the mitochondrial TIM23 preprotein translocase. *Nat. Struct. Mol. Biol.* **11**:234–241.
22. Kronidou, N. G., W. Oppliger, L. Bolliger, K. Hannavy, B. S. Glick, G. Schatz, and M. Horst. 1994. Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. *Proc. Natl. Acad. Sci. USA* **91**:12818–12822.
23. Li, Y., J. Dudek, B. Guiard, N. Pfanner, P. Rehling, and W. Voos. 2004. The presequence translocase-associated protein import motor of mitochondria: Pam16 functions in an antagonistic manner to Pam18. *J. Biol. Chem.* **279**:38047–38054.
24. Liu, Q., P. D'Silva, W. Walter, J. Marszalek, and E. A. Craig. 2003. Regulated cycling of mitochondrial Hsp70 at the protein import channel. *Science* **300**:139–141.
25. Martin, J., K. Mahlke, and N. Pfanner. 1991. Role of an energized inner membrane in mitochondrial protein import: $\Delta\psi$ drives the movement of presequences. *J. Biol. Chem.* **266**:18051–18057.
26. Matouschek, A., N. Pfanner, and W. Voos. 2000. Protein unfolding by mitochondria: the Hsp70 import motor. *EMBO Rep.* **1**:404–410.
27. Mayer, M. P. 2004. Timing the catch. *Nat. Struct. Mol. Biol.* **11**:6–8.
28. Meisinger, C., M. T. Ryan, K. Hill, K. Model, J. H. Lim, A. Sickmann, H. Müller, H. E. Meyer, R. Wagner, and N. Pfanner. 2001. Protein import channel of the outer mitochondrial membrane: a highly stable Tom40-Tom22 core structure differentially interacts with preproteins, small Tom proteins, and import receptors. *Mol. Cell. Biol.* **21**:2337–2348.
29. Mihara, K. 2003. Cell biology: moving inside membranes. *Nature* **424**:505–506.
30. Mokranjac, D., S. A. Paschen, C. Kozany, H. Prokisch, S. C. Hoppins, F. E. Nargang, W. Neupert, and K. Hell. 2003. Tim50, a novel component of the TIM23 preprotein translocase of mitochondria. *EMBO J.* **22**:816–825.
31. Mokranjac, D., M. Sichting, W. Neupert, and K. Hell. 2003. Tim14, a novel key component of the import motor of the TIM23 protein translocase of mitochondria. *EMBO J.* **22**:4945–4956.
32. Neupert, W., and M. Brunner. 2002. The protein import motor of mitochondria. *Nat. Rev. Mol. Cell Biol.* **3**:555–565.
33. Pfanner, N., N. Wiedemann, C. Meisinger, and T. Lithgow. 2004. Assembling the mitochondrial outer membrane. *Nat. Struct. Mol. Biol.* **11**:1044–1048.
34. Prokisch, H., C. Scharfe, D. G. Camp II, W. Xiao, L. David, C. Andreoli, M. E. Monroe, R. J. Moore, M. A. Gritsenko, C. Kozany, K. K. Hixson, H. M. Mottaz, H. Zischka, M. Ueffing, Z. S. Herman, R. W. Davis, T. Meitinger, P. J. Oefner, R. D. Smith, and L. M. Steinmetz. 2004. Integrative analysis of the mitochondrial proteome in yeast. *PLoS Biol.* **2**:e160.
35. Rassow, J., A. C. Maarse, E. Krainer, M. Kübrich, H. Müller, M. Meijer, E. A. Craig, and N. Pfanner. 1994. Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *J. Cell Biol.* **127**:1547–1556.
36. Rehling, P., K. Brandner, and N. Pfanner. 2004. Mitochondrial import and the twin-pore translocase. *Nat. Rev. Mol. Cell Biol.* **5**:519–530.
37. Rehling, P., K. Model, K. Brandner, P. Kovermann, A. Sickmann, H. E. Meyer, W. Kühlbrandt, R. Wagner, K. N. Truscott, and N. Pfanner. 2003. Protein insertion into the mitochondrial inner membrane by a twin-pore translocase. *Science* **299**:1747–1751.
38. Ryan, M. T., W. Voos, and N. Pfanner. 2001. Assaying protein import into mitochondria. *Methods Cell Biol.* **65**:189–215.
39. Schneider, H. C., J. Berthold, M. F. Bauer, K. Dietmeier, B. Guiard, M. Brunner, and W. Neupert. 1994. Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature* **371**:768–774.
40. Schwarz, E., T. Seytter, B. Guiard, and W. Neupert. 1993. Targeting of cytochrome *b*₂ into the mitochondrial intermembrane space: specific recognition of the sorting signal. *EMBO J.* **12**:2295–2302.
41. Sichting, M., D. Mokranjac, A. Azem, W. Neupert, and K. Hell. 2005. Maintenance of structure and function of mitochondrial Hsp70 chaperones requires the chaperone Hep1. *EMBO J.* **24**:1046–1056.
42. Sickmann, A., J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H. E. Meyer, B. Schönlisch, I. Perschil, A. Chacinska, B. Guiard, P. Rehling, N. Pfanner, and C. Meisinger. 2003. The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl. Acad. Sci. USA* **100**:13207–13212.
43. Stuart, R. A., A. Gruhler, I. van der Klei, B. Guiard, H. Koll, and W. Neupert. 1994. The requirement of matrix ATP for the import of precursor proteins into the mitochondrial matrix and intermembrane space. *Eur. J. Biochem.* **220**:9–18.
44. Taylor, S. W., E. Fahy, B. Zhang, G. M. Glenn, D. E. Warnock, S. Wiley, A. N. Murphy, S. P. Gaucher, R. A. Capaldi, B. W. Gibson, and S. S. Ghosh. 2003. Characterization of the human heart mitochondrial proteome. *Nat. Biotechnol.* **21**:281–286.
45. Truscott, K. N., W. Voos, A. E. Frazier, M. Lind, Y. Li, A. Geissler, J. Dudek, H. Müller, A. Sickmann, H. E. Meyer, C. Meisinger, B. Guiard, P. Rehling, and N. Pfanner. 2003. A J-protein is an essential subunit of the presequence translocase-associated protein import motor of mitochondria. *J. Cell Biol.* **163**:707–713.
46. Voisine, C., E. A. Craig, N. Zufall, O. von Ahsen, N. Pfanner, and W. Voos.

1999. The protein import motor of mitochondria: unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. *Cell* **97**: 565–574.
47. Voos, W., B. D. Gambill, B. Guiard, N. Pfanner, and E. A. Craig. 1993. Presequence and mature part of preproteins strongly influence the dependence of mitochondrial protein import on heat shock protein 70 in the matrix. *J. Cell Biol.* **123**:119–126.
48. Yamamoto, H., M. Esaki, T. Kanamori, Y. Tamura, S. Nishikawa, and T. Endo. 2002. Tim50 is a subunit of the TIM23 complex that links protein translocation across the outer and inner mitochondrial membranes. *Cell* **111**:519–528.
49. Yamamoto, H., T. Momose, Y. Yatsukawa, C. Ohshima, D. Ishikawa, T. Sato, Y. Tamura, Y. Ohwa, and T. Endo. 2005. Identification of a novel member of yeast mitochondrial Hsp70-associated motor and chaperone proteins that facilitates protein translocation across the inner membrane. *FEBS Lett.* **579**:507–511.