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Mitochondrial Presequence Translocase: Switching between TOM Tethering and Motor Recruitment Involves Tim21 and Tim17

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Summary

The presequence translocase of the inner mitochondrial membrane (TIM23 complex) operates at a central junction of protein import. It accepts preproteins from the outer membrane TOM complex and directs them to inner membrane insertion or, in cooperation with the presequence translocase-associated motor (PAM), to the matrix. Little is known of how the TIM23 complex coordinates these tasks. We have identified Tim21 (*YGR033c*) that interacts with the TOM complex. Tim21 is specific for a TIM23 form that cooperates with TOM and promotes inner membrane insertion. Protein translocation into the matrix requires a switch to a Tim21-free, PAM bound presequence translocase. Tim17 is crucial for the switch by performing two separable functions: promotion of inner membrane insertion and binding of Pam18 to form the functional TIM-PAM complex. Thus, the presequence translocase is not a static complex but switches between TOM tethering and PAM binding in a reaction cycle involving Tim21 and Tim17.

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Introduction

Most proteins of eukaryotic cells are encoded by nuclear genes and synthesized on cytosolic ribosomes. About half of these proteins must be translocated into or across biological membranes to reach their functional destination in cell organelles or at the cell surface (Schnell and Hebert, 2003). Organelles like the endoplasmic reticulum, mitochondria, chloroplasts, and peroxisomes contain specific proteinaceous machineries (translocases) that mediate the translocation of preproteins across the organelle membrane. Mitochondria and chloroplasts pose a further complexity, as they are surrounded by more than one membrane, yet little is known about how the outer and inner membrane translocases cooperate in protein transport.

The major mitochondrial import pathway is followed by preproteins with N-terminal presequences (Bauer et al., 2000; Jensen and Johnson, 2001; Koehler, 2004; Rehling et al., 2004). The preproteins are transported by the translocase of the outer membrane (TOM complex). The channel-forming protein Tom40 is associated with three receptors, Tom20, Tom22, and Tom70, and small Tom proteins. After passing through the Tom40 channel, the presequences interact with the intermembrane space (IMS) domain of the central receptor Tom22 and then direct the preproteins via the presequence translocase of the inner membrane (TIM23 complex) to the matrix, where they are proteolytically removed.

The TIM23 complex contains three integral membrane proteins, Tim17, Tim23, and Tim50. Tim23 is the channel-forming subunit and possesses an IMS domain that functions as a presequence receptor (Bauer et al., 2000; Truscott et al., 2001). Tim50 binds directly to the IMS domain of Tim23 and plays an early role in protein transport to the inner membrane by interacting with preproteins emerging from the TOM complex and directing them to the Tim23 channel (Geissler et al., 2002; Yamamoto et al., 2002; Mokranjac et al., 2003a). Though the third subunit, Tim17, was identified a decade ago and shown to be essential for cell viability and protein import, its function has remained enigmatic (Jensen and Johnson, 2001; Rehling et al., 2004). The membrane potential $\Delta\psi$ serves as the initial driving force for protein translocation across the inner membrane by activating the Tim23 channel and promoting translocation of the positively charged presequences. For translocation of the entire preprotein into the matrix, a second driving force, the presequence translocase-associated motor (PAM), is essential. The matrix heat shock protein 70 (mtHsp70) binds the preproteins and forms the ATP-powered core of PAM (Bauer et al., 2000; Jensen and Johnson, 2001; Koehler, 2004; Reh-

ling et al., 2004). Three inner membrane-associated cochaperones are required for PAM function by coordinating the activity of mtHsp70 at the channel exit of the TIM23 complex. The J protein Pam18 (Tim14) stimulates the ATPase activity of mtHsp70 (D'Silva et al., 2003; Mokranjac et al., 2003b; Truscott et al., 2003), while Pam16 (Tim16) controls the activity of Pam18 (Frazier et al., 2004; Kozany et al., 2004; Li et al., 2004). Tim44 loads mtHsp70 onto the precursor polypeptide emerging from the TIM23 channel. Finally, the matrix cochaperone Mge1 functions as a nucleotide exchange factor for mtHsp70.

The presequence translocase not only transports preproteins that end up in the matrix but also preproteins that contain a hydrophobic sorting signal behind the positively charged matrix targeting sequence (Glick et al., 1992). The sorting signal arrests the preproteins in the inner membrane. This sorting into the inner membrane can still occur when PAM subunits are inactivated (Voos et al., 1993; Mokranjac et al., 2003b; Truscott et al., 2003; Frazier et al., 2004; Kozany et al., 2004). The TIM23 complex thus operates at a central junction of the mitochondrial presequence pathway by taking over preproteins from the TOM complex and mediating their transfer to either the matrix or the inner membrane. The molecular mechanisms of cooperation between TOM, TIM23, and PAM are largely unknown. A preprotein in transit can stably connect the translocases to form a TOM-TIM23-PAM supercomplex (Chacinska et al., 2003; Frazier et al., 2004), but no direct interaction between Tim and Tom proteins has been observed to date. Neither of the IMS-exposed Tim proteins, Tim23 and Tim50, seems to bind to the TOM complex. In contrast to the elusive TOM-TIM23 interaction, TIM23 and the associated PAM are generally seen as a stable functional unit, although it is not known which subunits of PAM and TIM23 interact with each other.

We report that the TIM23 complex coordinates the tasks of TOM interaction, PAM interaction, and inner membrane sorting by switching between different modular states. We have identified Tim21 that directly interacts with the TOM complex and is specific for a sorting-competent but PAM-free form of TIM23. Protein translocation into the matrix requires a switch to a PAM bound Tim21-free TIM23 form. Tim17 plays a crucial role in this switch, as it is required for both inner membrane sorting and direct interaction with PAM. These findings indicate that the presequence translocase is a dynamic machinery that adapts to the needs of the preprotein that is transported.

Results

Tim21, a Subunit of the Presequence Translocase

We identified a 21 kDa subunit in purified TIM23-PAM by mass spectrometry and found that it is encoded by the *Saccharomyces cerevisiae* open reading frame *YGR033c*, referred to as *TIM21* (Figure 1A). When TIM23-PAM was isolated from a yeast strain lacking *TIM21*, the Tim21 band was no longer present, confirming its identification. Tim21 could be crosslinked to Tim23, supporting its presence in the TIM23 complex (see Figure S1A in the Supplemental Data available with this article online). The deduced primary structure of Tim21 predicts the presence of 239 amino acids with a single transmembrane segment (Figure 1B). The N-terminal segment of Tim21 shows the characteristics of a positively charged mitochondrial presequence with a predicted cleavage site after residue 42. Tim21 is well conserved among eukaryotes (Figure S1B). *tim21*Δ cells grew well on glucose but were significantly affected at elevated temperatures on the nonfermentable carbon source glycerol (Figure 1C). Similarly, cells overexpressing Tim21 from a high-copy number plasmid displayed significantly reduced growth on glycerol.

The ^{35}S-labeled precursor of Tim21 was imported into isolated mitochondria in the presence of a $\Delta\psi$ and processed to the mature form (Figure 1D), indicating that Tim21 contains a cleavable presequence. A truncated Tim21 lacking the first 40 amino acids migrated close to mature Tim21. The size difference between precursor and mature protein agrees with the predicted cleavage site, suggesting that mature Tim21 consists of ~197 amino acids.

Upon treatment of mitochondria at pH 11.5, Tim21 remained in the membrane pellet like the integral membrane proteins Tom70 and Tim23 (Figure 1E). Tim21 was protected from protease in intact mitochondria but, like Tim23, became accessible upon opening of the outer membrane by swelling (Figure 1F). In swollen mitochondria, a 20K protease-resistant fragment of Tim21 was generated. Tim21 was further degraded to fragments of 19K and 17K when the inner membrane was opened by sonication. When the membranes were solubilized in Triton X-100, Tim21 was more efficiently but not fully degraded, pointing to the presence of a folded domain with limited protease accessibility (Figure 1F). Together with the $\Delta\psi$ dependence of Tim21 import, these results indicate that Tim21 spans the inner membrane with its predicted single transmembrane segment. To determine the orientation of Tim21 in the inner membrane, a strain expressing Tim21 with a C-terminal myc_{13} tag was generated. Upon swelling of mitochondria, $Tim21_{myc}$ was clipped by added protease to a 20K fragment like authentic Tim21 (Figure 1G), demonstrating that the C terminus is exposed to the IMS. The data suggest that mature Tim21 consists of an N-terminal segment of about 3 kDa in the matrix (corresponding to the clipping from 20K to 17K after sonication), a transmembrane segment and a C-terminal domain of about 16 kDa in the IMS with limited accessibility to protease.

Tim21 Interacts with the TOM Complex via Tom22

We expressed the IMS domain of Tim21 with a histidine tag in *Escherichia coli*. Purified $Tim21_{IMS}$ and $Tim50_{IMS}$ as a control were immobilized on Ni-NTA agarose. Isolated mitochondria were solubilized in Triton X-100 and incubated with the immobilized IMS domains. $Tim50_{IMS}$ selectively interacted with Tim23 as reported (Figure 2A, lane 8) (Geissler et al., 2002). Unexpectedly, $Tim21_{IMS}$ efficiently associated with Tom40 but not with Tim23, Tim44, or control proteins (Figure 2A, lane 9). To study if $Tim21_{IMS}$ interacted with the TOM complex, we made use of the differential detergent sensitivity of the TOM

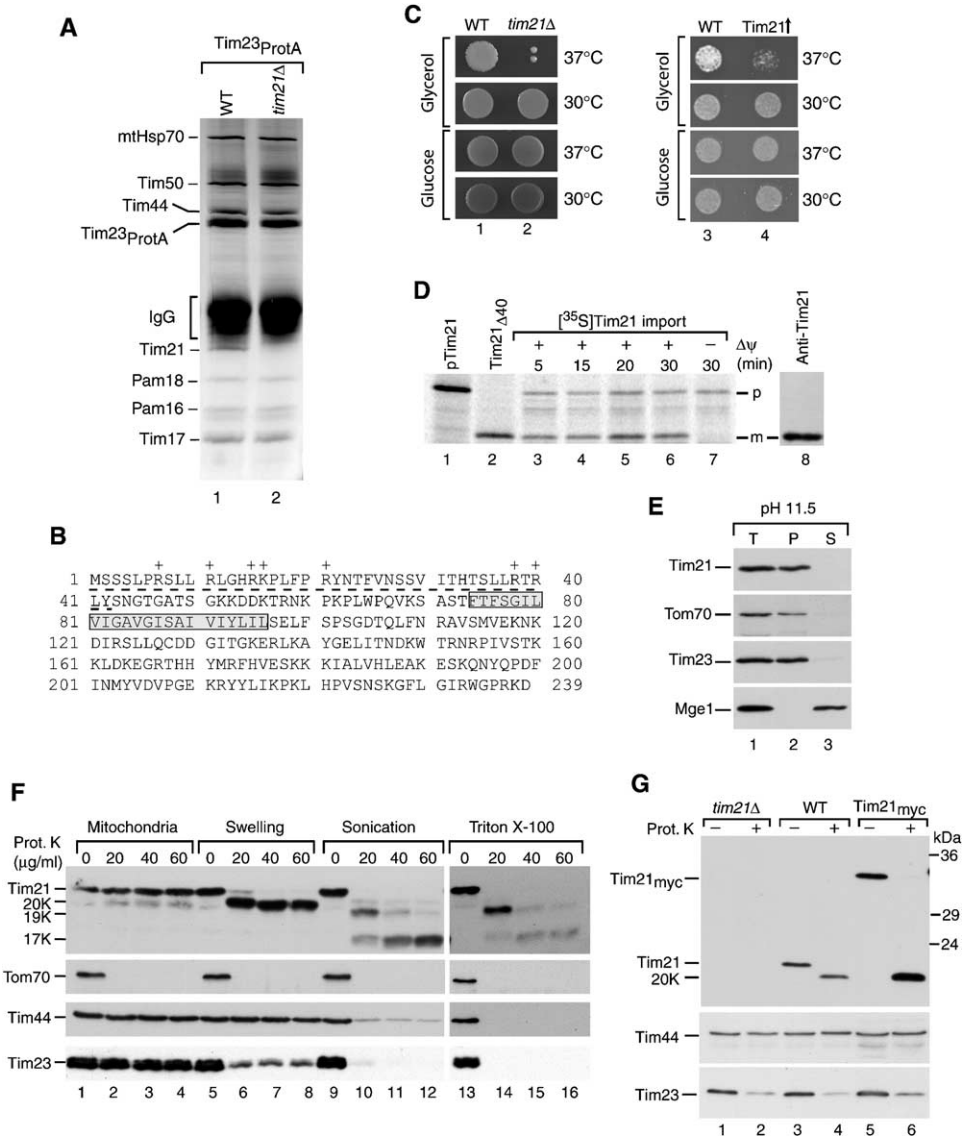

Figure 1. Identification and Localization of Tim21

(A) Isolation of protein A-Tim23 from wild-type (wt) and *tim21*Δ yeast mitochondria lysed with digitonin. Bound proteins were eluted with sample buffer, separated by urea SDS-PAGE, and stained with colloidal Coomassie. Proteins were identified by mass spectrometry and Western blotting.

(B) Deduced amino acid sequence of Tim21. Box, predicted transmembrane segment; dashed line, presequence.

(C) Wt, *tim21*Δ (left panel), and wt cells transformed with YEp352 or YEp352-Tim21 (Tim21↑) (right panel) were grown on glucose or glycerol-containing synthetic media.

(D) ^{35}S-labeled Tim21 was imported into isolated wt mitochondria for the indicated times, treated with proteinase K, and analyzed by SDS-PAGE. For reference, ^{35}S-labeled Tim21, Tim21$_{\Delta 40}$ (lacking aa 2–40), and Tim21 detected by Western blotting are shown. p, precursor; m, mature.

(E) Mitochondria were extracted at pH 11.5, separated into supernatant (S) and pellet (P) fraction, and then processed for Western blot analysis. T, total.

(F) Mitochondria were swollen, sonicated, or lysed with 1% Triton X-100 and/or treated with proteinase K prior to analysis by Western blot.

(G) Mitoplasts were treated with proteinase K where indicated and subjected to Western blotting.

complex. In digitonin, the full TOM complex with Tom40, receptors and small Tom proteins can be isolated. In Triton X-100, Tom40 and Tom22 form a stable core while the other receptors and the small Tom proteins are released. In Triton X-100, Tom40 and Tom22 were indeed associated with Tim21$_{IMS}$ while, in digitonin, the further subunits of the TOM complex were also associated (Figure S2A). Thus, Tim21$_{IMS}$ apparently associates with the core of the TOM complex, i.e., Tom40 or Tom22.

Tom22 exposes a C-terminal domain of 4 kDa to the IMS (Tom22$_{IMS}$). *tom22-2* mutant mitochondria selec-

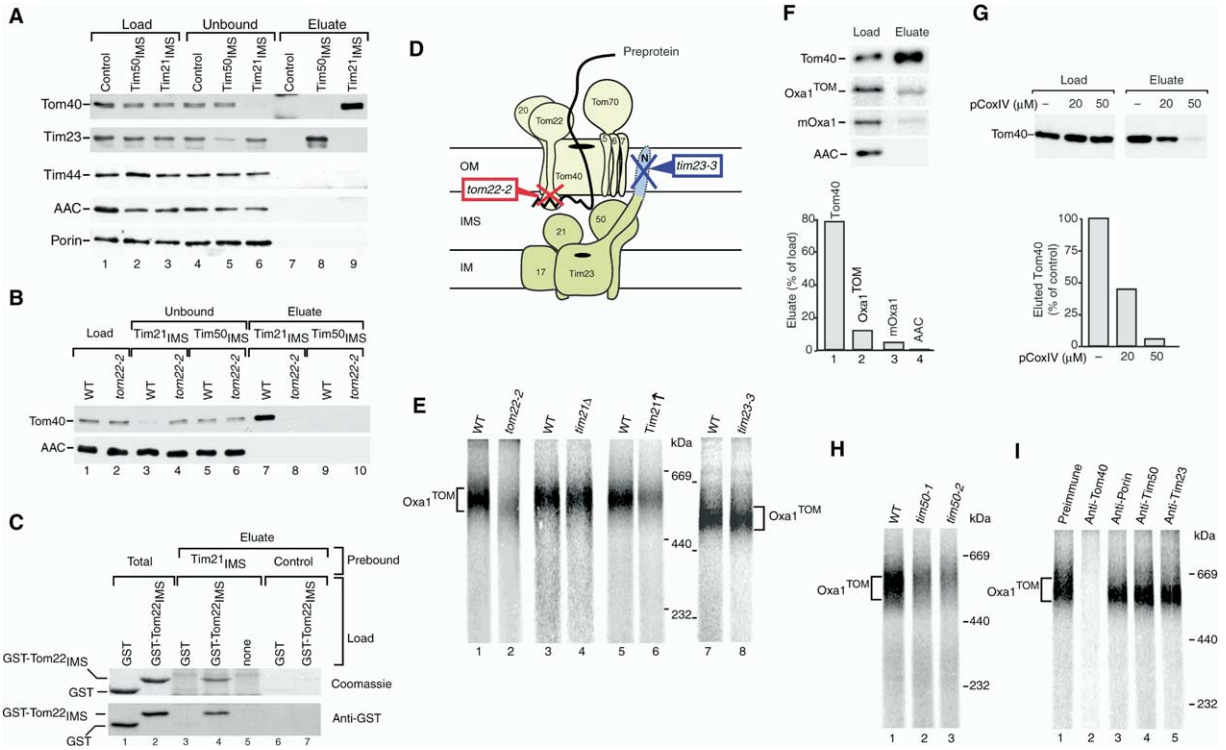

Figure 2. Tim21 Binds the TOM Complex and Affects Formation of a Preprotein-TOM Intermediate

(A) Ni-NTA agarose was left untreated (control) or loaded with purified $Tim21_{IMS}$ and $Tim50_{IMS}$ prior to incubation with Triton X-100 solubilized mitochondrial extracts. Samples were analyzed by Western blotting. Load and unbound, 2.5%; eluate, 100%. AAC, ADP/ATP carrier.

(B) Wt and *tom22-2* mitochondria were used as described in (A).

(C) Purified $Tim21_{IMS}$ was immobilized on Ni-NTA agarose and incubated with the indicated proteins. Bound proteins were eluted and analyzed by SDS-PAGE, Coomassie blue staining, and Western blotting. Total, 5%; eluate, 100%.

(D) Schematic presentation of Tom and Tim proteins with accumulated preprotein and yeast mutants. OM, outer membrane; IM, inner membrane.

(E) Isolated mitochondria were incubated with ^{35}S-labeled Oxa1 in the absence of $\Delta\psi$ and subjected to BN-PAGE and digital autoradiography. $Oxa1^{TOM}$ amount: *tom22-2*, ~35% of wt; *tim21Δ*, ~98% of wt; Tim21↑, ~40% of wt.

(F) ^{35}S-labeled Oxa1 was imported into wt mitochondria in the presence or absence of $\Delta\psi$. Triton X-100 extracts were incubated with immobilized $Tim21_{IMS}$. Bound proteins were analyzed by Western blotting or digital autoradiography after SDS PAGE. Load, 30%; eluate, 100%.

(G) Purified $Tim21_{IMS}$ was incubated with Triton X-100 solubilized mitochondria in the absence or presence of CoxIV presequence peptide. Samples were analyzed by Western blotting. Load, 10%; eluate, 100%.

(H) Isolated mitochondria were assayed for $Oxa1^{TOM}$ formation as in (E). *tim50-1* and *tim50-2* accumulate ~30% of wt.

(I) Wt mitochondria were incubated with Oxa1 in the absence of $\Delta\psi$. After swelling, purified IgG was added. Mitochondria were reisolated and analyzed by BN-PAGE.

tively lack $Tom22_{IMS}$ while other Tom, Tim, and Pam proteins are present in wild-type amounts (Chacinska et al., 2003; Frazier et al., 2003). Lysed *tom22-2* mitochondria were incubated with immobilized $Tim21_{IMS}$. Although Tom40 was present in similar amounts in *tom22-2* mitochondria as in wild-type mitochondria, it did not associate with $Tim21_{IMS}$ (Figure 2B), indicating that the association of the TOM core with Tim21 requires $Tom22_{IMS}$. For a direct analysis, we purified an *E. coli*-expressed fusion protein between $Tom22_{IMS}$ and glutathione S-transferase (GST). GST-$Tom22_{IMS}$ but not GST itself bound to immobilized $Tim21_{IMS}$ (Figure 2C). Thus, $Tim21_{IMS}$ interacts with the TOM complex via direct binding to $Tom22_{IMS}$.

Differential Effect of Tim21 and Tim50 on a Preprotein-TOM Intermediate

To address the function of the Tim21-Tom22 interaction, we asked if Tim21 played an early role in protein import. We made use of a translocation intermediate that accumulates at the TOM complex (Figure 2D) (Frazier et al., 2003). The presequence-carrying precursors of multispanning inner membrane proteins, such as Oxa1, can be stably arrested at the TOM core in the absence of a $\Delta\psi$ and directly analyzed by blue native electrophoresis (BN-PAGE) of digitonin-lysed mitochondria. $Tom22_{IMS}$ has been shown to be required for stabilizing the $Oxa1^{TOM}$ intermediate (Figure 2E, lane 2) (Frazier et al., 2003). The formation of $Oxa1^{TOM}$ was not affected in *tim21Δ* mitochondria (Figure 2E, lane 4), indicating that Tim21 was required neither for generation nor stabilization of this transport intermediate at $Tom22_{IMS}$. However, overexpression of Tim21 significantly reduced the amount of $Oxa1^{TOM}$ (Figure 2E, lane 6). An inhibitory role of Tim21 on the preprotein-TOM interaction was also observed with wild-type mitochondria. The recovery of the preprotein-carrying TOM complex ($Oxa1^{TOM}$) with purified $Tim21_{IMS}$ was strongly

reduced (Figure 2F, column 2) while the TOM complex without preprotein efficiently interacted with $Tim21_{IMS}$ (Figure 2F, column 1). To address a possible competition between preprotein and $Tim21_{IMS}$ in binding to $Tom22_{IMS}$, we studied the interaction of the TOM complex with $Tim21_{IMS}$ in the presence of a presequence peptide. The presequence indeed strongly inhibited the binding of $Tim21_{IMS}$ to the TOM complex (Figure 2G), while peptides derived from a carrier precursor did not (data not shown). These findings support the view that Tim21 plays an inhibitory/antagonistic function on the preprotein-TOM interaction.

We thus analyzed if the two further subunits of the presequence translocase that are exposed to the IMS, Tim23 and Tim50, played an active/stimulatory role in formation of $Oxa1^{TOM}$. The N-terminal 50 residue segment of Tim23, which has been reported to span the outer membrane (Donzeau et al., 2000), is absent in *tim23-3* mitochondria (Figure 2D) (Chacinska et al., 2003). The formation of $Oxa1^{TOM}$, however, was not affected in *tim23-3* mitochondria compared to wild-type mitochondria (Figure 2E, lane 8). Together with the finding that this N-terminal segment of Tim23 is not conserved in evolution (data not shown), this supports the view that the predicted outer membrane-spanning topology of Tim23 is not critical for preprotein import or accumulation (Chacinska et al., 2003).

Tim50 has been crosslinked to a preprotein in the absence of a $\Delta\psi$, i.e., under conditions where a portion of the preprotein was accumulated in the TOM complex and another portion was located in the IMS (Yamamoto et al., 2002; Mokranjac et al., 2003a). However, it was unknown if Tim50 was functionally required for accumulation of a preprotein at the TOM complex. To address if Tim50 played such an early role, we used Tim50-depleted mitochondria that were isolated from yeast cells carrying the *TIM50* gene under the control of a regulatable promoter (Geissler et al., 2002). The formation of $Oxa1^{TOM}$ was partially reduced but not blocked in the Tim50-depleted mitochondria (Figure S2B), although the amount of Tim50 was below detectable levels (less than 3% of wild-type amount) (Geissler et al., 2002). Two explanations were conceivable. Either the depletion of Tim50, which takes 24–36 hr, only indirectly affected the formation of $Oxa1^{TOM}$ or Tim50 did not play a structural but a dynamic/catalytic role in generation of $Oxa1^{TOM}$. To circumvent possible indirect effects of the depletion approach, we generated conditional alleles of the essential *TIM50* gene (*tim50-1, tim50-2*). The isolated mutant mitochondria contained wild-type amounts of all proteins analyzed (Figure S2C). The import of matrix-targeted proteins into the mutant mitochondria was selectively inhibited (Figure S2D). Strikingly, the formation of $Oxa1^{TOM}$ was strongly reduced in *tim50-1* and *tim50-2* mitochondria (Figure 2H), suggesting a catalytic role for Tim50 in formation of the TOM intermediate.

To further exclude that Tim50 was present as a structural component of the $Oxa1^{TOM}$ complex on BN-PAGE, we used an antibody-shift assay (Frazier et al., 2003). Upon accumulation of $Oxa1^{TOM}$, the outer membrane was opened by swelling, and different antibodies were added. Antibodies against Tom40 completely shifted the $Oxa1^{TOM}$ band out of the separation range of the BN-PAGE, while antibodies against Tim50 or Tim23 had no effect (Figure 2I). As the polyclonal antibodies used efficiently recognize their respective antigens (Geissler et al., 2002), these results indicate that neither Tim50 nor Tim23 is present in the $Oxa1^{TOM}$ BN complex. We conclude that Tim50 is crucial for generation but not for stabilization of the preprotein-TOM intermediate. Since the abundance and mobility of $Oxa1^{TOM}$ are not affected in *tim21Δ* mitochondria, Tim21 is also not a structural part of this intermediate but seems to function in an antagonistic manner to Tim50.

Two Different Forms of the Presequence Translocase

We asked if Tim21 also exerted an effect on the TIM23 complex itself. Upon separation of digitonin-lysed mitochondria by BN-PAGE, Tim23 and Tim17 are found in a 90 kDa core complex ($TIM23^{core}$) and additionally in larger TIM23* complexes (Dekker et al., 1997; Chacinska et al., 2003) (Figure 3A, lanes 1 and 5). The relevance and composition of the larger TIM23* complexes have not been known. Remarkably, in *tim21Δ* mitochondria, the larger TIM23* complexes were largely absent (Figure 3A, lanes 2 and 6). Western blotting with anti-Tim21 showed that Tim21 comigrated only with the larger TIM23* complexes and not with the $TIM23^{core}$ complex (Figure 3A, lane 3). The BN mobility of neither the TOM complex nor the TIM22 complex was altered in *tim21Δ* mitochondria (Figure 3A, lanes 8 and 10). These findings suggested the presence of two pools of Tim23/Tim17: TIM23* complexes containing Tim21 and the $TIM23^{core}$ complex without Tim21. To exclude that the $TIM23^{core}$ complex was simply formed by a dissociation of TIM23* complexes during the electrophoretic run, we isolated the TIM23 complexes by two different means, via protein A-tagged Tim21 and via protein A-tagged Tim23. While the purification via tagged Tim23 yielded both $TIM23^{core}$ and TIM23*, the purification via tagged Tim21 yielded only TIM23* (Figure 3B), demonstrating that the TIM23* complexes were not dissociated during the BN-PAGE. Overexpression of Tim21 shifted the ratio of the TIM23 complexes toward the larger TIM23* complexes, while a lack of Tim21 increased the amount of $TIM23^{core}$ (Figure 3C).

We asked if additional subunits of the TIM23-PAM machinery were able to influence the ratio between the TIM23 complexes and, surprisingly, found that mitochondria with a mutant Pam16 (*pam16-3*) shifted the ratio toward the larger TIM23* complexes on BN-PAGE (Figure 3D). This finding was unexpected for two reasons. First, while the subunits of TIM23 and PAM can be copurified by a mild one-step affinity purification as shown in Figure 1A, BN-PAGE analysis leads to a release of the PAM subunits as well as Tim50 from the Tim23-Tim17 complexes (Dekker et al., 1997; Geissler et al., 2002; Chacinska et al., 2003). Second, the *pam16-3* mutation destabilizes the PAM machinery, leading to a release of Pam18 from TIM23 as analyzed by affinity purification via tagged Tim23 (Figure 3E) (Frazier et al., 2004). An explanation was provided by the finding that, in contrast to Pam18, the yield of Tim21 copurifying with tagged Tim23 was significantly increased in *pam16-3* mitochondria (Figure 3E), suggesting an antagonistic behavior of Tim21 and PAM in interaction with Tim23.

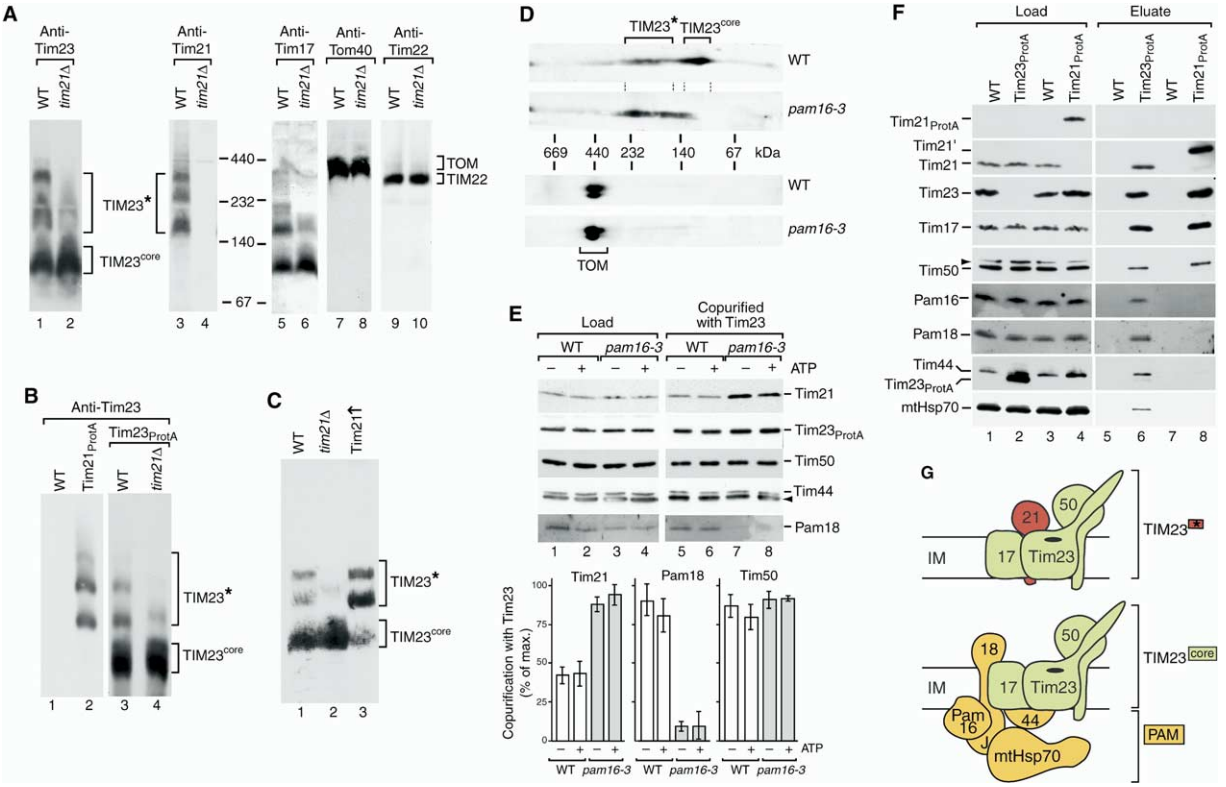

Figure 3. Tim21 Affects the Architecture of the TIM23 Complex

(A) Mitochondrial protein complexes were analyzed by BN-PAGE and Western blotting.

(B) The TIM23 complex was isolated from digitonin-solubilized mitochondria via protein A-tagged Tim21 or Tim23, eluted from IgG Sepharose by TEV-protease cleavage, and analyzed by BN-PAGE and Western blotting.

(C) Mitochondria were analyzed by BN-PAGE and immunodecorated with anti-Tim23.

(D) Mitochondria were subjected to BN-PAGE followed by SDS-PAGE and immunodecoration with anti-Tim23 and anti-Tom40.

(E) Protein A-Tim23 was expressed in wt and *pam16-3* cells. Mitochondria were lysed in digitonin and Tim23 was isolated. After elution with SDS, samples were subjected to SDS-PAGE and Western blotting. Load, 5%; eluate, 100%; arrowhead, protein A-Tim23. The quantified values were standardized to the amount of purified protein A-Tim23, and the maximal amount of copurification was set to 100% for each protein. SEM of at least four independent experiments.

(F) TIM23 complex isolations via protein A-tagged Tim23 or Tim21. Samples were subjected to Western blot analysis. Load, 5%; eluate, 100%. Tim21′, larger form of Tim21 due to TEV cleavage; arrowhead, unspecific band.

(G) Schematic presentation of the two forms of the TIM23 complex.

We thus directly compared which components of TIM23-PAM were copurified with either tagged Tim23 or tagged Tim21 (Figure 3F). Both assays led to a co-purification of Tim17, Tim21, Tim23, and Tim50. However, the PAM subunits, including Pam16, Pam18, Tim44, and mtHsp70, were only copurified in significant amounts via tagged Tim23, not via tagged Tim21. Together with the BN-PAGE analysis, these results suggest the presence of two types of TIM23 complexes (Figure 3G). Tim21 is specific for TIM23 complexes that migrate as TIM23* on BN-PAGE and do not contain PAM components even after a mild affinity purification, thus representing a motor-free form of the presequence translocase. The second form of the presequence translocase is the Tim21-free $TIM23^{core}$ complex, which is associated with the PAM subunits, as shown by affinity purification. In agreement with the idea of different TIM23 complexes, we observed a partial separation of TIM23 complexes in mitochondrial membrane vesicles on a sucrose gradient. The amount of Tim21 but not PAM was significantly reduced in one of the Tim23-containing peaks (Figure S3).

To test if, in addition to their different composition, $TIM23^{core}$-PAM and TIM23* were also functionally different, we analyzed if both translocases were involved in the translocation of matrix-targeted preproteins. The matrix-destined model preprotein $b_2(220)_\Delta$-DHFR (consisting of an N-terminal portion of cytochrome b_2 and dihydrofolate reductase) was accumulated in mitochondrial import sites in the presence of the DHFR ligand methotrexate (MTX). MTX-stabilized DHFR cannot be unfolded and remains on the cytosolic side while the b_2 portion spans across outer and inner membranes into the matrix. Following digitonin lysis of mitochondria with either tagged Tim23 or tagged Tim21, the presequence translocase-preprotein complex was isolated by affinity purification. While the accumulated matrix-targeted preprotein was copurified together with tagged Tim23 and PAM (Figure 4, columns 1 and 3), it was virtually absent in the complex isolated with

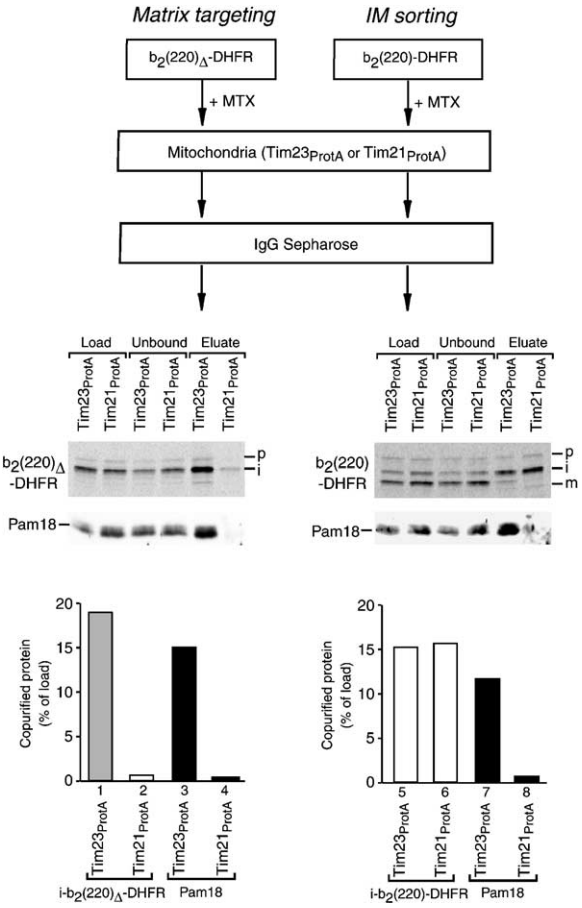

Figure 4. TIM23^{core}-PAM but Not TIM23* Associates with Matrix Preproteins

Mitochondria containing protein A-tagged Tim23 or Tim21 were incubated with ^{35}S-labeled $b_2(220)_\Delta$-DHFR or $b_2(220)$-DHFR in the presence of MTX. After reisolation and solubilization, the TIM23 complex was purified, eluted with SDS, and analyzed by SDS-PAGE. Load and unbound, 5%; eluate, 100%.

tagged Tim21 (Figure 4, column 2). In contrast, when a similar experiment was performed using the precursor $b_2(220)$-DHFR, which contains an inner membrane-sorting signal, the preprotein was copurified with Tim23 as well as Tim21 with similar efficiency (Figure 4, columns 5 and 6). Thus, the Tim21-containing PAM-free presequence translocase (TIM23*) was found in association with a two-membrane-spanning sorted preprotein but not a two-membrane-spanning matrix-targeted preprotein.

Tim21 Impairs the Import Motor Function

In agreement with the view that deletion of *TIM21* will rather promote PAM association to the TIM23 complex, mitochondria isolated from *tim21*Δ cells did not display defects in import of any matrix protein analyzed, as shown here with the model preprotein $b_2(167)_\Delta$-DHFR (Figure 5A; see also Figure S4A). We therefore investigated the effect of Tim21 overexpression on protein import. The import of proteins into the matrix (Figure 5B; Figure S4B) was indeed significantly delayed in Tim21↑ mitochondria compared to wild-type mitochondria. The increased levels of Tim21 had no effect on the steady-state amounts of other Tim or Pam components or marker proteins (Figure S4C) and did not decrease the inner membrane potential $\Delta\psi$ (Figure S4D), excluding the possibility that the reduced protein import was indirectly caused by a reduction of $\Delta\psi$.

To test whether Tim21 influenced the activity of PAM, we used an assay with a two-membrane-spanning preprotein. $b_2(167)_\Delta$-DHFR was accumulated in the mitochondrial import sites in the presence of MTX. Both $\Delta\psi$ and PAM generate an import-driving activity on the preprotein such that the folded DHFR domain becomes so closely apposed to the outer membrane that it cannot be cleaved off by proteinase K (Frazier et al., 2004). When $\Delta\psi$ is dissipated, PAM is responsible for generating the import-driving activity and preventing backsliding of the preprotein. Overexpression of Tim21 significantly reduced the import-driving activity of PAM, as indicated by increased protease accessibility of the processed preprotein (Figure 5C, right panel). In contrast, in *tim21*Δ mitochondria, the protease resistance of the arrested preprotein was even moderately increased compared to wild-type mitochondria (Figure 5C, left panel). Thus, the presence of Tim21 exerts a negative effect on the import-driving activity of PAM.

Since the Tim21-containing translocase TIM23* was found in association with a sorted preprotein, we asked if Tim21 itself was required for the sorting of preproteins at the inner membrane. However, the precursor $b_2(167)$-DHFR, which contains an inner membrane-sorting signal, was imported and processed to the mature sorted form in both *tim21*⊿ and Tim21↑ mitochondria with an efficiency close to that of wild-type mitochondria (Figures 5D and 5E). Other sorted preproteins behaved similarly (Figures S5A–S5C). These results provide further evidence that overexpression of Tim21 does not damage mitochondrial protein import nonspecifically but selectively affects the PAM-driven import of matrix proteins. Thus, Tim21 is present in a sorting-active form of the presequence translocase but is itself not rate limiting for the sorting process.

Tim17 Is Required for Both Inner Membrane Sorting and Matrix Import

Our findings raised two questions with regard to the steps of protein transport at the presequence translocase. (1) The lack of an effect of Tim21 on preprotein sorting at the inner membrane was puzzling. Thus far, eight different components (Tim21, Tim23, Tim50, Pam16, Pam18, Tim44, mtHsp70, and Mge1) of TIM23-PAM have been analyzed for a possible role in protein sorting at the inner membrane, but none of them was actively required for the sorting process. This raised the possibility that protein sorting may represent a default pathway that does not require the active participation of a TIM or PAM component. (2) When transition of the TIM23 complex from a motor-free (TIM23*) into a motor bound form occurs, which component acts as the physical link for PAM binding in response to Tim21 dissociation?

The essential protein Tim17 is the only stoichiometric component of TIM23-PAM whose function has not

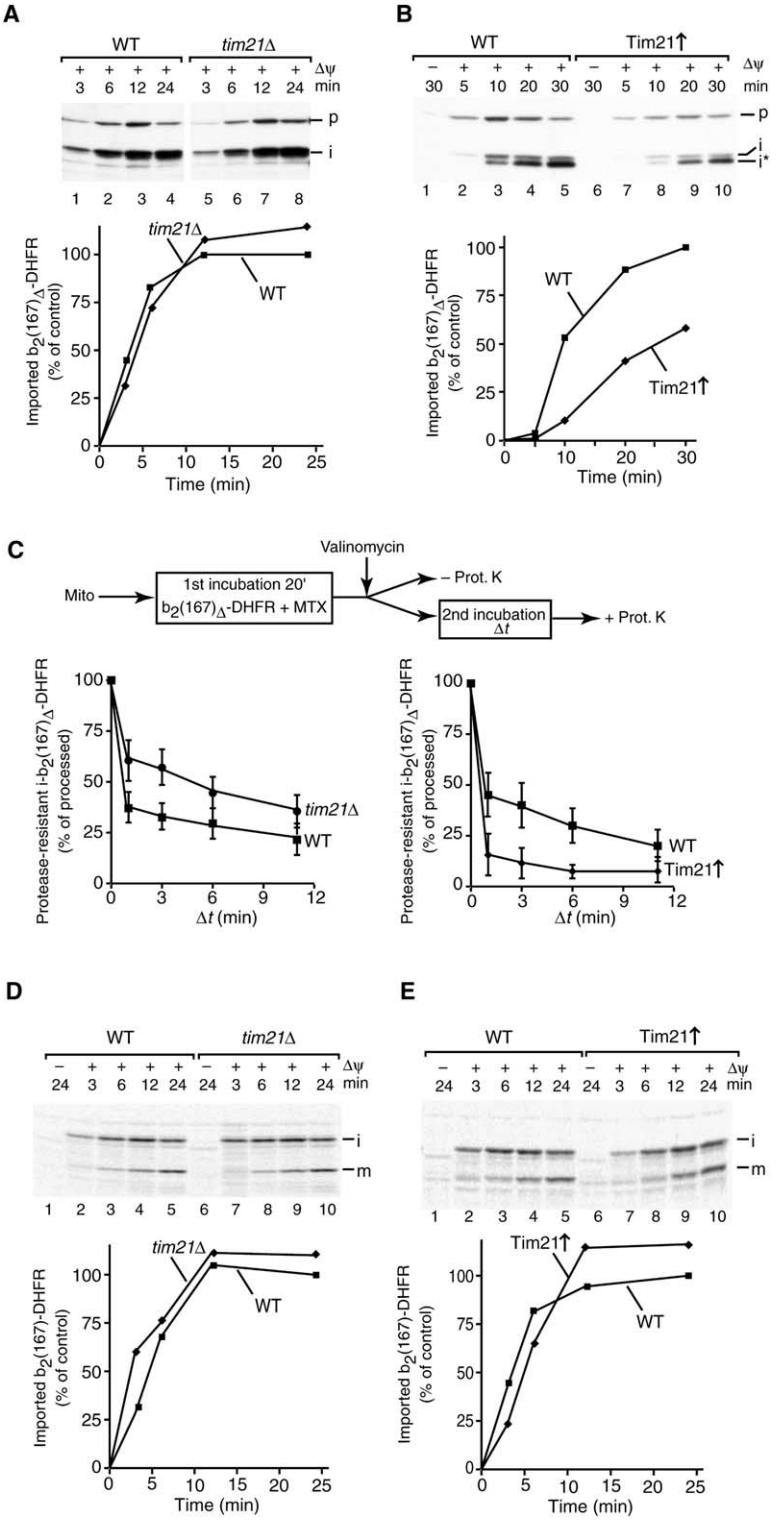

Figure 5. Tim21 Impairs Matrix Translocation but Not Inner Membrane Sorting

(A) Wt and *tim21⊿* mitochondria were incubated with purified $b_2(167)_\Delta$-DHFR, proteinase K treated, and analyzed by SDS-PAGE and Western blotting with anti-DHFR. Import into wt mitochondria after 24 min was set to 100%.

(B) Purified $b_2(167)_\Delta$-DHFR was incubated with mitochondria followed by proteinase K treatment, SDS-PAGE, and Western blotting with anti-DHFR. Import into wt mitochondria after 30 min was set to 100%.

(C) Mitochondria were incubated with purified $b_2(167)_\Delta$-DHFR in the presence of MTX, then $\Delta\psi$ was dissipated. Samples were subjected to a second incubation for Δt at 25°C prior to treatment with proteinase K. The amount of i-form in the non-protease-treated samples was set to 100%. SEM of at least five independent experiments.

(D and E) ^{35}S-labeled $b_2(167)$-DHFR was imported into wt, *tim21⊿*, and Tim21↑ mitochondria followed by protease treatment, SDS-PAGE, and autoradiography.

been characterized so far. The major reason was the lack of suitable mutants of this protein for an in organello analysis. We generated conditional yeast mutants of Tim17 and selected the mutant strains *tim17-4* and *tim17-5* (Figure S6A). The steady-state protein levels, BN complexes, and generation of a $\Delta\psi$ by the mutant mitochondria were comparable to that of wild-type mitochondria (Figures S6B–S6E). The import of F_1-ATPase subunit β ($F_1\beta$), determined by both processing to the mature form and protease protection, was moderately affected in *tim17-4* mitochondria and strongly reduced in *tim17-5* mitochondria (Figure 6A), while import of a

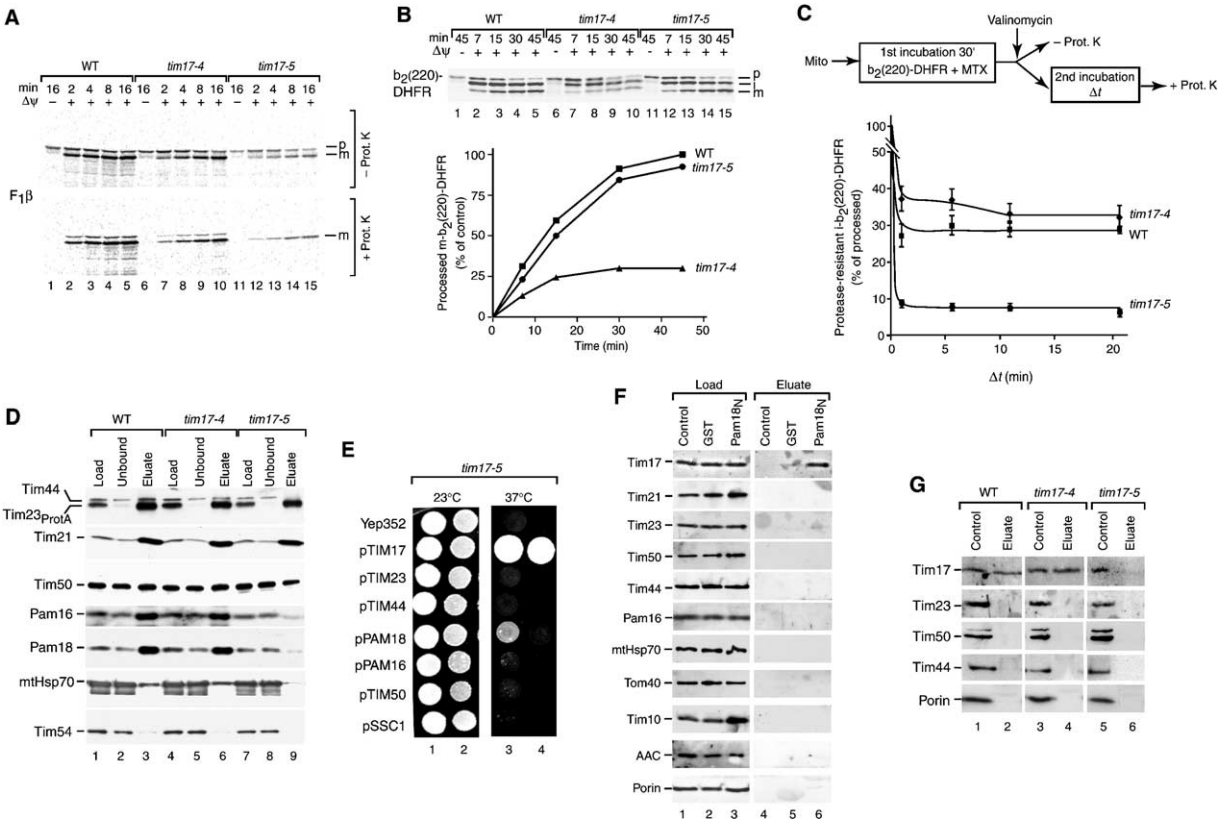

Figure 6. Dual Role for Tim17 in Inner Membrane Sorting and PAM Binding/Matrix Import

(A) ^{35}S-labeled $F_1\beta$ was imported into isolated mitochondria.

(B) b_2(220)-DHFR was imported into mitochondria in the presence of MTX. The m-form was quantified.

(C) b_2(220)-DHFR was imported into mitochondria in the presence of MTX. $\Delta\psi$ was dissipated followed by a second incubation at 4°C for Δt prior to proteinase K treatment. The amount of i-form in non-protease-treated samples was set to 100%. SEM of at least three independent experiments.

(D) The TIM23 complex was isolated from wt, *tim17-4*, and *tim17-5* mitochondria and analyzed by Western blotting. Load and unbound, 5%; eluate, 100%.

(E) Dilutions of *tim17-5* cells transformed with the indicated genes on 2μ plasmids were plated on YPD and grown at the temperatures indicated.

(F) GSH-Sepharose was left untreated (control) or loaded with GST or GST-Pam18$_N$ prior to incubation with wt mitochondrial extracts. Bound proteins were analyzed by Western blotting. Load, 2.5%; eluate, 100%.

(G) Immobilized GST-Pam18$_N$ was incubated with mitochondrial extracts from wt, *tim17-4*, and *tim17-5* mitochondria as in (F).

carrier protein was not reduced in the mutant mitochondria (Figure S6F). Taken together, these results indicate that the *tim17* mutant mitochondria are specifically affected in the function of the presequence translocase.

When the sorted preprotein b_2(220)-DHFR was used, however, unusual phenotypes were observed. In wild-type mitochondria, b_2(220)-DHFR is processed twice. First, the positively charged matrix-targeting signal is removed, indicated by generation of the intermediate form. The hydrophobic sorting signal becomes arrested in the inner membrane and, upon completion of inner membrane sorting, is removed by a second processing step that releases the mature protein to the IMS (Glick et al., 1992; Voos et al., 1993). *tim17-5* mitochondria generated both intermediate- and mature-sized b_2(220)-DHFR, while *tim17-4* mitochondria were predominantly impaired in generation of the mature protein (Figure 6B). Thus, *tim17-4* mitochondria are severely affected in protein sorting at the inner membrane.

The efficient inner membrane sorting but defective matrix import observed with *tim17-5* mitochondria resembled the phenotype of mutants in PAM components (Voos et al., 1993; Mokranjac et al., 2003b; Truscott et al., 2003; Frazier et al., 2004; Kozany et al., 2004). We thus asked if *tim17-5* mitochondria were impaired in PAM activity by assaying for the $\Delta\psi$-independent import-driving activity with a two-membrane-spanning preprotein. Since the severe matrix import defect of *tim17-5* mitochondria did not allow the use of established matrix-targeted precursors for the assay, we accumulated the sorted preprotein b_2(220)-DHFR in the presence of MTX and determined the protease resistance of the intermediate-sized, i.e., two-membrane-spanning preprotein (Figure 6C). Upon dissipation of $\Delta\psi$, the protease resistance of i-b_2(220)-DHFR was not reduced in *tim17-4* mitochondria compared to wild-type mitochondria, while, in *tim17-5* mitochondria, it was strongly impaired. We conclude that the mutant

mitochondria reveal separable functions of Tim17 in matrix import and inner membrane sorting. On the one hand, *tim17-4* mitochondria but not *tim17-5* mitochondria are impaired in inner membrane sorting. On the other hand, *tim17-5* mitochondria but not *tim17-4* mitochondria are affected in generating an import-driving activity, suggesting that the function of PAM is impaired in *tim17-5* mitochondria.

Tim17 Links PAM to the TIM23 Complex

The *tim17-5* mutant phenotype indicated a connection between Tim17 and PAM. To directly investigate a possible relationship, we isolated the presequence translocase via tagged Tim23 from digitonin-lysed wild-type and *tim17* mutant mitochondria. The isolated complex from wild-type and both mutant mitochondria contained comparable amounts of the components of the TIM23 complex, including Tim23, Tim50, and Tim21 (Figure 6D, lanes 3, 6, and 9). While the subunits of PAM copurified with Tim23 in the *tim17-4* mitochondria (Figure 6D, lane 6), the yield of PAM copurification with Tim23 was strongly reduced in *tim17-5* mitochondria (Figure 6D, lane 9). Therefore, association between the TIM23 complex and PAM is disturbed in *tim17-5* mutant mitochondria, explaining the observed defect in matrix protein import.

We speculated that Tim17 represented the missing link that promotes the physical interaction between TIM23 and PAM. To screen for possible interacting proteins in vivo, we looked for suppression of the temperature-sensitive lethal growth phenotype of *tim17* cells by overexpression of mtHsp70 (Ssc1) or any of the three inner membrane bound cochaperones, Pam16, Pam18, and Tim44. Of all proteins tested, only the overexpression of Pam18 led to partial suppression of the growth defect of *tim17-5* cells but not of *tim17-4* cells at the nonpermissive temperature (Figure 6E and data not shown). Thus, Pam18 was a candidate for the putative Tim17 interaction partner of PAM.

The C-terminal J domain of Pam18 is exposed to the matrix and performs two functions, stimulation of the ATPase activity of mtHsp70 (D'Silva et al., 2003; Truscott et al., 2003) and interaction with the regulatory/inhibitory partner protein Pam16 (Frazier et al., 2004; Kozany et al., 2004; Li et al., 2004). When purified $Pam18_J$ was incubated with lysed mitochondria, it bound Pam16 as reported (Frazier et al., 2004) but neither Tim17 nor other Tim proteins (Figure S6G).

Pam18 exposes an N-terminal domain to the IMS (Mokranjac et al., 2003b; Truscott et al., 2003). We expressed and purified $Pam18_N$ and incubated it with lysed mitochondria. $Pam18_N$ selectively bound Tim17 but none of the other proteins tested, including various Tim and Pam proteins (Figure 6F). In order to assay for the binding of individual Tim proteins, the mitochondria had been lysed with Triton X-100 to completely dissociate the TIM23 complex (in digitonin, the TIM23 complex remains intact, excluding a determination of individual binding partners). Thus, Tim17 specifically interacts with $Pam18_N$.

The binding studies indicated that PAM is recruited to the TIM23 complex by a direct interaction of Pam18 with Tim17. If the Pam18-Tim17 interaction represents the critical link between PAM and TIM23, it should also explain the differential behavior of *tim17-4* and *tim17-5* mitochondria in PAM binding. $Pam18_N$ indeed bound the mutant protein Tim17-4 from lysed mitochondria with the same efficiency as wild-type Tim17 but was strongly inhibited in the binding of Tim17-5 (Figure 6G). We conclude that the interaction between $Pam18_N$ and Tim17 plays a crucial role in the recruitment of PAM to the TIM23 complex.

Discussion

We report that the presequence translocase of the mitochondrial inner membrane is a dynamic machinery that coordinates the tasks of TOM tethering, inner membrane sorting of preproteins, and motor recruitment for protein translocation into the matrix by switching between two modular states (Figure 7). The core of the presequence translocase, the channel-forming protein Tim23, is associated with the essential proteins Tim17 and Tim50 as well as Tim21. These proteins play multiple but distinct roles in coordinating the reaction cycle of the presequence translocase.

Tim21 directly contacts the TOM complex by binding to the IMS domain of the receptor Tom22. What is the function of this TOM/TIM tethering? We show that Tim50 initially plays an active role in generating an early transport intermediate of a preprotein at $Tom22_{IMS}$, leading to the transaccumulation of the preprotein at the TOM complex (Figure 7). Subsequently, Tim21 functions in an antagonistic manner to Tim50 by reducing preprotein accumulation at the TOM complex. We suggest that tethering of Tim21 to Tom22 allows the preprotein to be released from the TOM complex and engage with the Tim23 channel. It has been suggested that the N-terminal segment of Tim23 spans the outer membrane and, although no association with the TOM complex was found, should promote the transfer of preproteins from the TOM complex to the TIM23 complex (Donzeau et al., 2000). However, this N-terminal segment of Tim23 is dispensable for both protein import and formation of a TOM-TIM-preprotein supercomplex (Chacinska et al., 2003). In support of this, we found that the N-terminal segment of Tim23 is dispensable for formation of the preprotein-TOM intermediate and, in contrast to Tim50 and Tim21, not conserved in evolution. We suggest that the functional interplay between $Tom22_{IMS}$, Tim50, and Tim21 represents the early form of TOM-TIM23 cooperation.

Tim21 is not only involved in contacting the TOM complex but also promotes dissociation of the motor PAM from the TIM23 complex. Tim21 is selectively found in a PAM-free form of the TIM23 complex that is competent in protein sorting at the inner membrane but not in import of matrix proteins. Consequently, the PAM bound form of the TIM23 complex that translocates matrix proteins does not contain Tim21. By manipulating the amount of Tim21 experimentally, the equilibrium between the two TIM23 forms can be shifted toward the PAM-free state (TIM23*) resulting in import defects for matrix proteins. Tim21 is not essential for viability of yeast cells under all growth conditions, but both deletion and overexpression of Tim21 impair cell growth

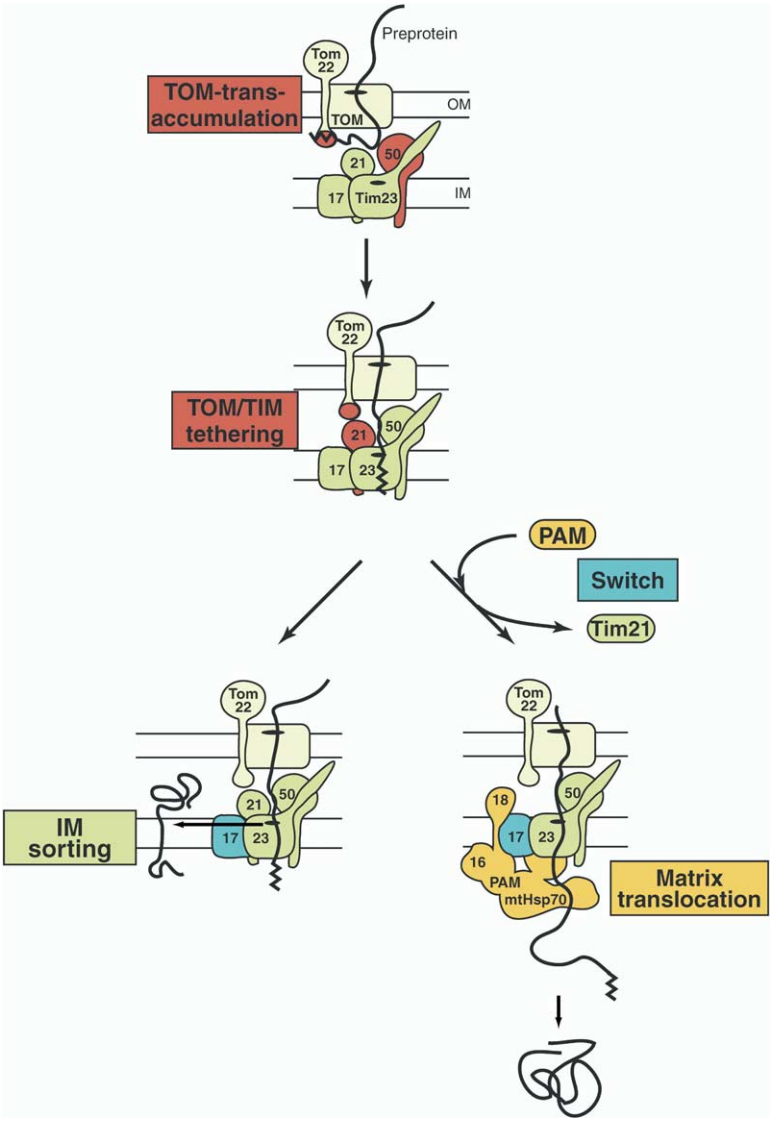

Figure 7. Stages of Protein Transport along the Presequence Pathway: A Hypothetical Model

Binding of the preprotein to the IMS domain of Tom22 is promoted by Tim50. Subsequently, Tim21 tethers the presequence translocase to Tom22, leading to dissociation of the preprotein and its transfer toward the Tim23 pore. The Tim21-containing TIM23 complex can promote inner membrane sorting. Matrix translocation requires a module switch of TIM23 by recruitment of PAM to Tim17 and release of Tim21.

at elevated temperature, indicating that the regulatory effect of Tim21 on the cooperation of TIM23 with TOM and PAM is of particular importance for rapidly growing cells. The conservation of Tim21 from yeast to man supports the view that its regulatory function is important for mitochondrial activity.

The transition from TIM23* to a PAM bound state of the translocase raises the question as to how PAM is recruited to the TIM23 complex. Interestingly, the so-far enigmatic Tim17 was found to bind specifically to the IMS domain of the cochaperone Pam18 and is thus essential for recruiting PAM to its functional position at the protein import channel. Tim17 represents a missing link for the direct interaction between TIM23 and PAM. We suggest that Tim17 acts as a molecular switch for PAM association to the $TIM23^{core}$ upon Tim21 dissociation (Figure 7).

Although Tim21 is present in the sorting-competent form of the TIM23 complex, Tim21 itself is not rate limiting for the sorting of presequence-carrying proteins with a hydrophobic inner membrane-sorting signal. Thus, none of the eight TIM or PAM components analyzed to date, including Tim21, Tim23, Tim50, Tim44, mtHsp70, Pam16, Pam18, and Mge1, has been found to play an active role in protein sorting at the inner membrane (Voos et al., 1993; Geissler et al., 2002; Mokranjac et al., 2003a; Mokranjac et al, 2003b; Truscott et al., 2003; Frazier et al., 2004; Kozany et al., 2004). We observed that the essential protein Tim17 is crucial for sorting of presequence-carrying preproteins. Thus, Tim17 apparently plays two essential but separate functions at the TIM23 complex: promotion of protein sorting and a function as a docking point for the PAM machinery.

In summary, we suggest a molecular mechanism of how TOM, TIM23, and PAM cooperate in protein import (Figure 7). We identified a physical chain of interactions between import components from the cytosol to the matrix (Tom22-Tim21-TIM23 complex/Tim17-Pam18-mtHsp70). However, this is not a static chain but one in

which the TIM23 complex functions in a dynamic manner and alternates between TOM tethering and PAM recruitment. Tim50 is the first inner membrane protein that is functionally required for a presequence-carrying preprotein emerging from the Tom40 channel and promotes the interaction of the preprotein with the IMS domain of the receptor Tom22. Tim21 functions in an antagonistic manner by directly binding to this IMS domain of Tom22 and disfavoring preprotein binding. In the case of cleavable preproteins with a hydrophobic sorting signal, a PAM-free TIM23 complex promotes inner membrane sorting. Tim17 plays an active role in the sorting process. The majority of cleavable preproteins, however, are translocated across the inner membrane into the matrix. This requires a module switch of the TIM23 complex by recruiting PAM. While Tim21 must be released to perform this switch, Tim17 is essential for anchoring the PAM machinery to the TIM23 complex via Pam18. After completion of transport, PAM is released, and the initial steps of TOM tethering of the presequence translocase will occur for the next round of protein import.

Experimental Procedures

Manipulation of Yeast and Analysis of Mitochondria

Temperature-conditional alleles *tim50-1* (YPH-BG-50-2A), *tim50-2* (YPH-BG-50-3A), and *tim17* (YPH-BG-17-9A; YPH-BG-17-21A) were generated by error-prone PCR and used to replace the plasmid-born wild-type gene in the corresponding gene deletion strains derived from *S. cerevisiae* YPH499 by gap repair and plasmid shuffling. The *tim17* alleles were integrated into the *TIM17* locus leading to *tim17-4* (YPH-BG17-9d) and *tim17-5* (YPH-BG17-21-7). Plate growth assays were done on synthetic media containing 2% glucose or 3% glycerol. Deletion of *TIM21* and C-terminal myc_{13} or protein A tagging of Tim21 was performed in YPH499. The *TIM21* open reading frame, including 305 bp upstream and 234 bp downstream, was cloned into Yep352 and transformed into YPH499 (Tim21↑). Other strains/plasmids were published previously: AG55-Gal (Tim50↓) (Geissler et al., 2002), *pam16-3* (Frazier et al., 2004), *tom22-2* (Frazier et al., 2003), *tim23-3* (Chacinska et al., 2003), and protein A-Tim23 (Geissler et al., 2002; Li et al., 2004).

Yeast cells were grown in YPG medium at 30°C or 24°C (temperature-conditional mutants), and mitochondria were isolated. Protein localization by protease treatment after osmotic swelling, sonication, Triton X-100 lysis, and carbonate treatment were performed essentially as described (Ryan et al., 2001; Frazier et al., 2004).

Binding Studies

Binding of purified proteins was performed essentially as described (Frazier et al., 2004). For peptide competition experiments, 20 or 50 μM CoxIV presequence peptide (Truscott et al., 2001) was preincubated with mitochondrial extracts for 30 min on ice prior to the binding assay. GST, GST-$Pam18_J$, and GST-$Pam18_N$ were immobilized on GSH-Sepharose equilibrated in PDG buffer (20 mM HEPES [pH 7.5], 100 mM KOAc, 10 mM $Mg[OAc]_2$, 10% glycerol). Mitochondria were solubilized at 1 mg/ml protein concentration in PDG containing 0.5% Triton X-100 (or 1% digitonin as indicated) and 2 mM PMSF. Cleared lysates were used directly ($Tim21_{IMS}$ binding) or diluted in PDG buffer to 0.25% Triton X-100 (Pam18 binding). Immobilized proteins were incubated with the lysates and washed, and bound proteins were eluted first with PDE buffer (PDG, 10 mM reduced glutathione) and then with SDS sample buffer. Eluates were combined and analyzed by SDS-PAGE.

In Vitro Protein Import

^{35}S labeling of proteins in rabbit reticulocyte lysate and import into isolated mitochondria were done as described (Ryan et al., 2001). The recombinant precursors of $b_2(167)_\Delta$-DHFR and $b_2(220)_\Delta$-DHFR were purified and imported as described (Ryan et al., 2001; Chacinska et al., 2003). For translocation arrest, DHFR fusion proteins were imported in the presence of 5 μM MTX. The import-driving activity was assayed as in Frazier et al. (2004). $Oxa1^{TOM}$ was generated according to Frazier et al. (2003).

Cloning, Expression, and Protein Purification

For expression of His_{10}-Tim21 (aa 42–239) and His_{10}-$Tim21_{IMS}$ (aa 103–239) in *E. coli*, PCR-amplified DNA fragments were cloned into pET10N. Purification was performed under denaturating conditions from inclusion bodies (Truscott et al., 2001) or under native conditions as described (Qiagen). A DNA fragment encoding $Tom22_{IMS}$ (aa 120–152) was cloned into pGEX-4T-3 (Amersham). GST and GST-$Tom22_{IMS}$ were purified according to the manufacturer (Amersham). Cloning and purification of $Tim50_{IMS}$ were described previously (Geissler et al., 2002). GST-$Pam18_N$ was generated by fusion of GST to Pam18 (aa 2–65) in pGEX-4T-2 and purified as described for GST-$Pam18_J$ (Frazier et al., 2004).

Miscellaneous

Rabbit antibodies were raised against purified His_{10}-Tim21 (aa 42–239). Antibodies were purified using $Tim21_{IMS}$ coupled to CNBr-activated Sepharose. To raise antibodies against Tim17, a peptide (CEAPSSQPLQA) was used for immunization. In some gels, irrelevant lanes were removed digitally. The following techniques were carried out as previously published: $\Delta\psi$ measurements using $DiSC_3(5)$, mass spectrometric analysis, BN-PAGE, and antibody-shift BN-PAGE (Dekker et al., 1997; Geissler et al., 2002; Frazier et al., 2003, 2004).

Supplemental Data

Supplemental Data include six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/120/6/817/DC1/.

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