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Active remodelling of the TIM23 complex during translocation of preproteins into mitochondria

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The TIM23 (translocase of the mitochondrial inner membrane) complex mediates translocation of preproteins across and their insertion into the mitochondrial inner membrane. How the translocase mediates sorting of preproteins into the two different subcompartments is poorly understood. In particular, it is not clear whether association of two operationally defined parts of the translocase, the membrane-integrated part and the import motor, depends on the activity state of the translocase. We established conditions to *in vivo* trap the TIM23 complex in different translocation modes. Membrane-integrated part of the complex and import motor were always found in one complex irrespective of whether an arrested preprotein was present or not. Instead, we detected different conformations of the complex in response to the presence and, importantly, the type of preprotein being translocated. Two non-essential subunits of the complex, Tim21 and Pam17, modulate its activity in an antagonistic manner. Our data demonstrate that the TIM23 complex acts as a single structural and functional entity that is actively remodelled to sort preproteins into different mitochondrial subcompartments.

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Introduction

Almost all eukaryotic proteins are synthesized on cytoplasmic ribosomes. To reach the sites of their function, roughly half of them are synthesized as preproteins with targeting signals, which lead to their subsequent transport into or across organellar membranes in the cell (Schnell and Hebert, 2003; Wickner and Schekman, 2005). Similarly, in bacterial cells all proteins are synthesized in the cytosol irrespective of where their site of function is. Protein translocases present in the membranes of cell organelles and bacteria are responsible for recognition and translocation of preproteins. Whereas some translocases appear to have specialized for either translocation of proteins across or their insertion into a membrane, some of them can perform both functions (Schnell and Hebert, 2003; Alder and Johnson, 2004). However, very little is known about the mechanisms involved in switching between translocation and insertion modes.

The TIM23 (translocase of the mitochondrial inner membrane) complex is one of the translocases in the cell that can operate both in translocation and in insertion modes. It collects preproteins carrying N-terminal, positively charged presequences as soon as they emerge at the outlet of the TOM (translocase of the mitochondrial outer membrane) complex. Using energy of the membrane potential across the inner mitochondrial membrane and ATP in the mitochondrial matrix, it mediates translocation of preproteins across and their insertion into the mitochondrial inner membrane (Endo *et al*, 2003; Koehler, 2004; Rehling *et al*, 2004; Neupert and Herrmann, 2007). Tim50 is the receptor subunit of the complex that takes over preproteins from the TOM complex and directs them to the translocation channel in the inner membrane formed by Tim23 and Tim17 (Geissler *et al*, 2002; Yamamoto *et al*, 2002; Mokranjac *et al*, 2003a). Tim23 also has a domain located in the intermembrane space that serves as an additional preprotein receptor of the complex (Bauer *et al*, 1996). At the matrix face of the channel, preproteins are recognized by Tim44 and mtHsp70 (Neupert and Brunner, 2002). mtHsp70 belongs to the Hsp70 family of chaperones and is the ATP-consuming subunit of the complex. It cycles between ATP- and ADP-bound states, which correspond to low- and high-affinity states for incoming preproteins. mtHsp70 hydrolysis of ATP to ADP and thereby the tight binding of the chaperone to the preprotein is regulated by the J and J-like proteins, Tim14 (Pam18) and Tim16 (Pam16) (D'Silva *et al*, 2003; Mokranjac *et al*, 2003b, 2006; Truscott *et al*, 2003; Frazier *et al*, 2004; Kozany *et al*, 2004). The nucleotide exchange factor, Mge1 facilitates the release of ADP from mtHsp70 and thereby starts a new cycle of the chaperone binding. In this way, the energy of ATP hydrolysis is converted into unidirectional transport of preproteins into mitochondria. Operationally, subunits of the TIM23 complex are divided into those which form the membrane-integrated part of the complex (Tim17, Tim23 and Tim50) and those which form the import motor/PAM (presequence translocase-associated motor) (Tim14, Tim16, Tim44, mtHsp70 and Mge1).

Recently, two further subunits of the TIM23 complex were discovered. In contrast to all other subunits described above, these are not essential for viability of yeast cells. Tim21 was described as a subunit of the membrane-integrated part of the translocase involved in the cooperation of TOM and TIM23 complexes (Chacinska *et al*, 2005; Mokranjac *et al*, 2005). Pam17 was proposed to be part of the import motor (van der Laan *et al*, 2005), but its function has remained largely unclear. Another protein, Tam41/Mmp37 was recently

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demonstrated to affect preprotein translocation through the TIM23 complex; however, no direct interaction with the complex has been observed (Gallas *et al*, 2006; Tamura *et al*, 2006).

Nearly all known substrates of the TIM23 complex have an N-terminal presequence that is necessary and sufficient to target a passenger protein into the mitochondrial matrix (Hurt *et al*, 1984; Ostermann *et al*, 1989). Such preproteins contain no other targeting/sorting signals indicating that transport into the matrix is the default mode of the translocase. A group of TIM23 substrates have, in addition to the presequence, a stop-transfer signal which, when recognized by the TIM23 complex, halts the transport into the matrix. The complex then laterally opens to insert the hydrophobic segment into the inner membrane (Glick *et al*, 1992). Recently, a model was proposed as to how the TIM23 complex manages this differential sorting of preproteins (Chacinska *et al*, 2005). According to this model, translocation into the matrix requires reversible assembly of the TIM23 complex with the import motor, whereas insertion into the inner membrane is mediated by a motor-free complex. Tim21 was identified as a component specifically present in the TIM23 complex that promotes insertion into the inner membrane. Preprotein translocation into the matrix was suggested to require a switch to a Tim21-free but the motor-bound form of the TIM23 complex. On the other hand, another group showed that Tim21 is present in the TIM23 complex that also contains the components of the import motor (Tamura *et al*, 2006). Evidently, how the TIM23 complex manages to sort preproteins into two different compartments remains unclear.

Here, we describe experiments in which we analysed the TIM23 complex trapped *in vivo* in different modes of translocation. We found no evidence for the existence of a motor-free form of the translocase. In contrast, our results show that the TIM23 complex undergoes a series of conformational changes in response to the presence and the type of the translocating preprotein. Furthermore, we found that both non-essential components of the TIM23 complex, Tim21 and Pam17, bind to the Tim17–Tim23 core of the translocase. Unexpectedly, we obtained evidence that Tim21 and Pam17 are functionally connected and have antagonistic roles in the TIM23 complex. Our data show that the TIM23 complex functions as a single structural and functional entity that is actively remodelled to sort different types of preproteins into the matrix or the inner membrane.

Results

Composition of the TIM23 complex during protein translocation

To address the question as to how the TIM23 complex sorts preproteins into different mitochondrial subcompartments, we have set out to analyse its composition and conformation in different states of activity. To this end, we developed a method to *in vivo* trap the TIM23 complex in different translocation states. First, we generated the empty state of the translocase by treating yeast cells with puromycin to terminate protein synthesis and allow the truncated polypeptide chains to be completely imported (+PUR) (Figure 1A). Mitochondria were isolated also from cells grown under standard conditions, that is, without any further treatment.

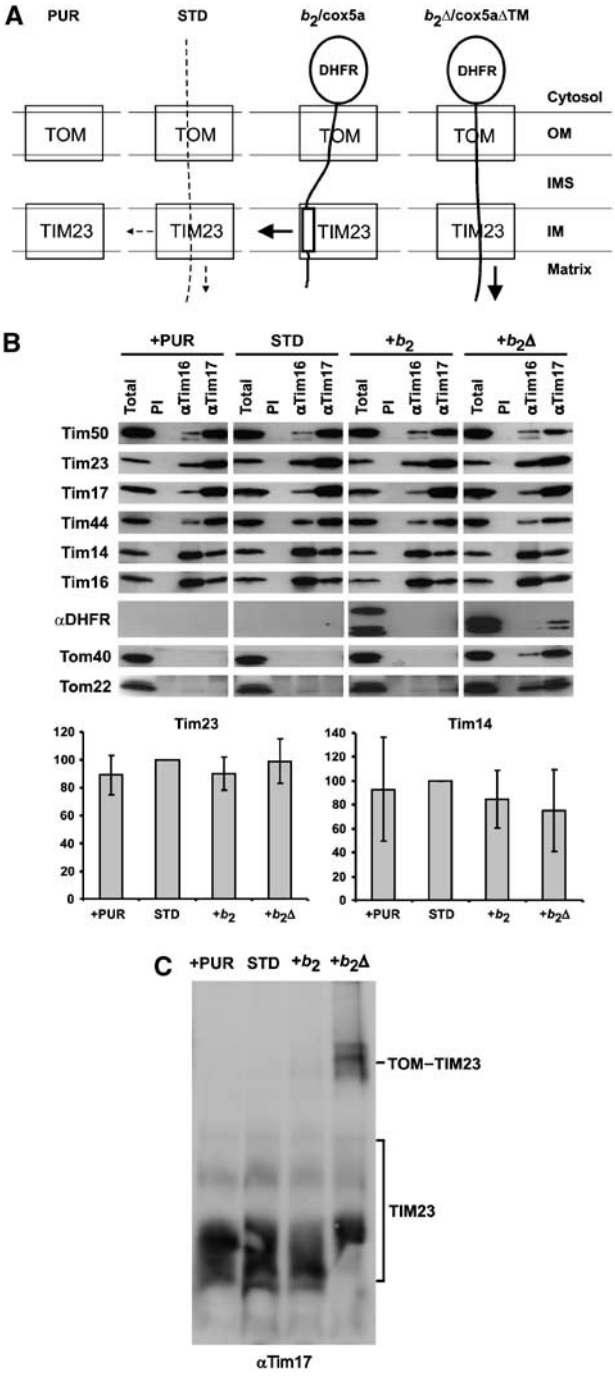

Figure 1 Composition of the TIM23 complex during translocation of preproteins. (**A**) Schematic representation of the different states of the TIM23 complex analysed. See text for details. OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner mitochondrial membrane. (**B**) Mitochondria were solubilized with digitonin and immunoprecipitated with affinity-purified antibodies to Tim16 or Tim17 or preimmune (PI) serum as a control. Samples were analysed by SDS–PAGE and immunodecoration. Here, 20% of the material used for immunoprecipitations was loaded as total. The amounts of Tim23 and Tim14 precipitated with antibodies to Tim16 and Tim17, respectively, under these conditions were quantified from three independent experiments (lower panels). Precipitation in STD, 100%. (**C**) Mitochondria were solubilized with digitonin and analysed by BN-PAGE and immunodecoration with antibodies to Tim17.

This served as a control for the state of the TIM23 complex prevailing under the usual conditions of analysis of preprotein import (STD). To investigate the effects of translocating

preproteins on the TIM23 complex, we trapped in the complex different hybrid preproteins whose import pathways were described previously (Geissler *et al*, 2000 and references therein). The first one, abbreviated as b_2, consists of the N-terminal 167 amino-acid residues of yeast cytochrome b_2 fused to full-length dihydrofolate reductase (DHFR) from mouse. This preprotein has a matrix-targeting signal at its N terminus followed by a hydrophobic stop-transfer signal, which directs it into the inner membrane. The second preprotein, indicated as $b_2\Delta$, lacks the hydrophobic-sorting signal and the preprotein is completely translocated into the matrix. When expression of these proteins is induced in the presence of the folate analogue, aminopterine, the DHFR moieties fold stably in the cytosol (Wienhues *et al*, 1991). This prevents complete import into mitochondria leading to accumulation of both types of preproteins as intermediates that span and connect both TOM and TIM23 complexes (Figure 1A). In case of b_2, the TIM23 complex is locked in the state of lateral sorting and in the case of $b_2\Delta$ in the state of translocation into the matrix. Both preproteins were expressed though the expression levels were somewhat higher for $b_2\Delta$ (Supplementary Figure S1A). The import of radioactive preproteins by the TIM23 complex was strongly inhibited, demonstrating that both preproteins were efficiently arrested and that the majority of complexes contained trapped preproteins (Supplementary Figure S1B). Interestingly, import of both laterally sorted and matrix-targeted preproteins was inhibited to a similar extent, indicating that the same pool of the TIM23 complexes mediates both functions. In contrast, import of TIM23-independent substrates, such as ADP–ATP carrier, was affected only mildly, likely due to the partial occupancy of the TOM complexes.

To analyse possible changes in the composition of the TIM23 complex in response to protein translocation, we performed immunoprecipitation experiments. Mitochondria were solubilized with digitonin and incubated with affinity-purified antibodies to Tim17 and Tim16 or preimmune immunoglobulins bound to ProteinA-Sepharose. We have chosen antibodies to Tim17 and Tim16, as we have previously shown that they are able to immunodeplete their respective antigens and also to precipitate all other known components of the TIM23 complex with, however, different efficiencies due to the reported instability of the complex upon solubilization (Kozany *et al*, 2004). Under these conditions, the precipitation patterns of the essential components of the TIM23 complex, Tim50, Tim23, Tim17, Tim44, Tim14 and Tim16 were essentially identical in all four types of mitochondria analysed (Figure 1B). In the empty state of the translocase, the import motor and the membrane-integrated part were clearly present in one complex. Apparently, the translocating chain is not required for association of the two parts of the complex. Furthermore, translocating preproteins did neither stabilize nor destabilize the association of various TIM23 components. Translocating preproteins were, however, required to connect TIM23 and TOM complexes. In the presence of $b_2\Delta$, TOM and TIM23 complexes were associated stably enough to be detectable as a supercomplex both by coimmunoprecipitation (Figure 1B) and by Blue Native electrophoresis (BN-PAGE) (Figure 1C). Interestingly, the interaction of b_2 with the TIM23 complex is not strong enough to allow its coisolation with the complex upon solubilization of mitochondria. This was not unexpected in view of the partitioning of such substrates between the TIM23 channel and the lipid phase of the membrane. This behaviour did not depend on the length of the laterally sorted preprotein in front of the DHFR passenger; none of the tested b_2 constructs ranging from 87 to 220 residues was coisolated with the TIM23 complex. However, laterally sorted preproteins were found stably associated with the TOM complex (see below) and they were apparently present in close vicinity to the TIM23 complex as they could be crosslinked to it (data not shown).

These results strongly argue against any model according to which the membrane part and the motor associate only in the presence of a preprotein in transit into the matrix. Rather, they support a mechanistic model of the TIM23 complex in which all essential components form a non-transient assembly with the various components undergoing dynamic rearrangements depending on the state of activity.

Tim23 inserts into the outer membrane in response to preprotein translocation

Can one use mitochondria containing the TIM23 complex trapped in various states of its function to show that these states are indeed different and if so, to obtain insights into how they look like? We have previously shown that the N-terminal segment of Tim23 is exposed on the surface of mitochondria and have suggested that it contributes to the coordinated action of TOM and TIM23 complexes during translocation of preproteins into mitochondria (Donzeau *et al*, 2000). However, this view has been challenged (Chacinska *et al*, 2005). We have therefore analysed whether the association of Tim23 with the outer membrane is influenced by the translocation activity of the TIM23 complex. After 10-min incubation with proteinase K, ca. 5% of Tim23 was accessible to protease added to mitochondria isolated from puromycin-treated cells and ca. 10% in control mitochondria (Figure 2A). In contrast, in mitochondria with arrested b_2 roughly 35% of Tim23 was clipped by externally added protease and ca. 45% in mitochondria with arrested $b_2\Delta$. The intactness of mitochondria was not compromised under these conditions as the accessibilities of marker proteins of the outer membrane (Tom70), the intermembrane space (Tim50) and the matrix (Hep1) were not changed. To confirm that the accessibility of Tim23 correlates with an increased translocation load, we incubated mitochondria isolated from puromycin-treated cells with recombinant preprotein $b_2\Delta$. Indeed, addition of increasing amounts of purified $b_2\Delta$ to mitochondria led to increased insertion of Tim23 into the outer membrane as documented by the strongly increased efficiency of clipping of Tim23 (Figure 2B). Upon removal of the translocating chain from the TIM23 complex, clipping of Tim23 returned to the level observed with mitochondria containing empty TIM23 complex (Figure 2C). Taken together, these data clearly show that the N-terminal segment of Tim23 actively responds to translocation of preproteins through the TIM23 complex (Figure 2D).

Conformational changes of the TIM23 complex during translocation of preproteins

The different exposure of Tim23 on the mitochondrial surface in the empty and occupied states of the TIM23 complex demonstrates that the translocase reacts to the presence of

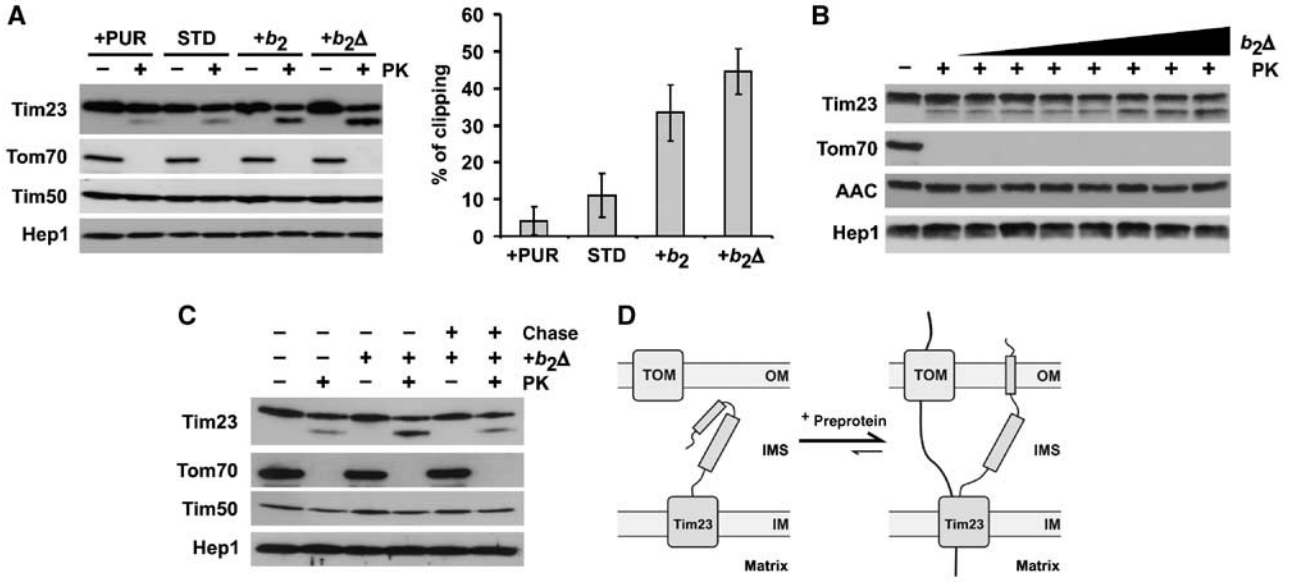

Figure 2 Exposure of Tim23 on the mitochondrial surface during translocation of preproteins. (**A**) Mitochondria were treated with proteinase K (PK) and analysed by SDS–PAGE and immunodecoration. The percentage of Tim23 clipped under these conditions was quantified from three independent experiments (right panel). (**B**) Mitochondria isolated from puromycin-treated cells were incubated with increasing amounts of recombinant preprotein $b_2\Delta$ (0–100 μg), treated with PK and analysed as described in (A). (**C**) Mitochondria isolated from puromycin-treated cells were incubated with or without recombinant preprotein $b_2\Delta$ in the presence of dihydrofolate and NADPH. Dihydrofolate and NADPH were subsequently removed from one portion of the sample (chase) and further incubated to clear the TIM23 complex from preproteins. Samples were then treated with PK and analysed as described in (A). (**D**) Schematic presentation showing the dynamic interaction of the N-terminal segment of Tim23 with the outer membrane during translocation of preproteins.

the translocating preprotein. However, does the response differ if the translocating preprotein is destined to the matrix or to the inner membrane? We used protein crosslinking in intact mitochondria to probe the molecular environment of TIM23 components in the empty and occupied states of the translocase. In mitochondria under standard condition of analysis, Tim23 gives one major crosslinking adduct to a protein of ca. 17 kDa (Figure 3A; Bauer *et al*, 1996). Using mitochondria containing His-tagged Pam17, we identified this major crosslinking product as an adduct of Tim23 to Pam17 (Supplementary Figure S2), an unexpected result in view of the recently reported role of Pam17 as a component of the import motor (van der Laan *et al*, 2005). Does this crosslinking pattern change in different functional states of the TIM23 complex? Crosslinking of Tim23 in mitochondria from control cells and cells treated with puromycin gave essentially the same result. Together with the above-described accessibility of Tim23 to the protease this shows that the TIM23 complex is largely empty in mitochondria isolated under standard conditions (Figure 2B). In contrast, clear differences were visible in mitochondria containing arrested preproteins. Arrest of laterally sorted b_2 led to a pronounced increase in the visibility of previously observed Tim23 dimers (Bauer *et al*, 1996). On the other hand, Tim23 was not crosslinked to any protein upon arrest of matrix-targeted $b_2\Delta$. The TIM23 complex thus clearly undergoes conformational changes in response to the translocating chain. Furthermore, these changes seem to depend on the type of the translocating preprotein.

To confirm that these various crosslinking patterns of Tim23 indeed represent different conformations of the complex in different modes of translocation and are not just a reflection of specific interactions of a certain preprotein with the complex, we analysed another couple of differentially sorted preproteins. The hybrid protein Cox5aDHFR, abbreviated Cox5a, is laterally sorted into the inner membrane by the TIM23 complex, whereas Cox5a(ΔTM)DHFR, abbreviated Cox5aΔTM, lacks the transmembrane domain and is targeted to the matrix (Gärtner *et al*, 1995). Arrest of these two proteins induced essentially the same changes in the crosslinking patterns of Tim23 as the arrest of b_2 and $b_2\Delta$ (Figure 3B). This clearly demonstrates that the observed changes represent genuine differences in the conformation of the complex due to the different translocation modes.

Are other components of the TIM23 complex rearranging during translocation of preproteins as well? We analysed the molecular environments of Tim44 and Tim16, two proteins whose crosslinking patterns we have previously characterized. Analysis of the environment of Tim44 revealed several changes in the active translocase as compared with its empty state (Figure 3C). In mitochondria containing arrested b_2 or $b_2\Delta$, crosslinks between two Tim44, crosslinks between Tim44 and Tim14 as well as Tim44 and Tim16 were strongly decreased. The prominent crosslinking product of ca. 80 kDa in mitochondria containing arrested $b_2\Delta$ represented an adduct of Tim44 to the preprotein. This was demonstrated by arresting His-tagged $b_2\Delta$ *in vitro* followed by crosslinking and NiNTA-Agarose pull down (Supplementary Figure S3). In case of Tim16, the most prominent difference between control mitochondria and mitochondria saturated with preproteins was the reduced crosslinking to Tim14, in particular in mitochondria containing arrested $b_2\Delta$ (Figure 3D).

Taken together, the crosslinking experiments demonstrate that the TIM23 complex actively responds to the translocating preproteins and that the nature of this response depends on the type of preprotein being translocated.

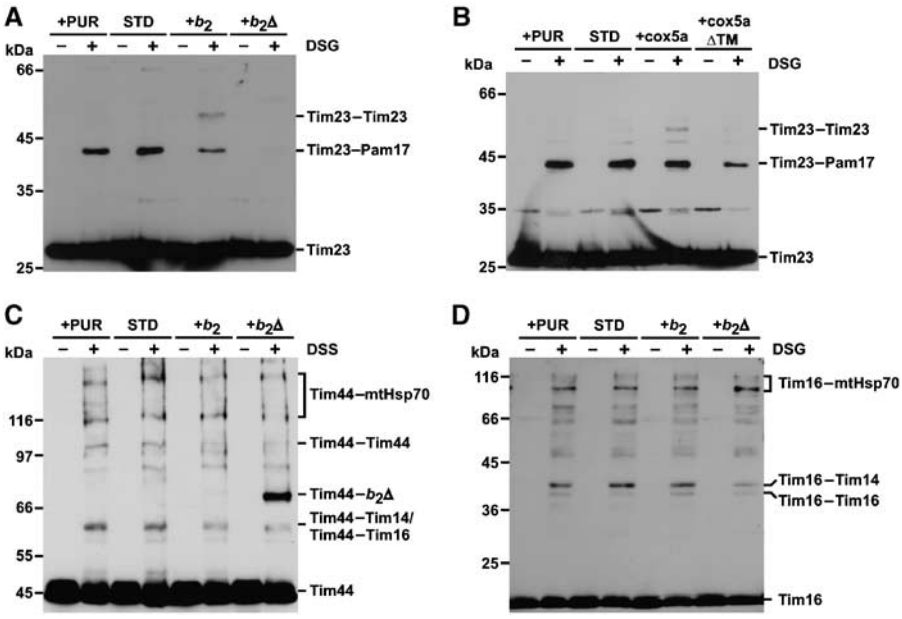

Figure 3 Conformation of the TIM23 complex during translocation of preproteins. (**A**–**D**) Mitochondria containing TIM23 complex in different translocation states were crosslinked with disuccinimidyl glutarate (DSG) (A, B, D) or disuccinimidyl suberate (DSS) (C). Samples were analysed by SDS–PAGE and immunodecoration with affinity-purified antibodies to Tim23 (A, B), Tim44 (C) or Tim16 (D). Mitochondria containing accumulated b_2 and $b_2\Delta$ (A, C, D) or Cox5a and Cox5aΔTM (B) were used.

Behaviour of Tim21 and Pam17 during translocation of preproteins through the TIM23 complex

So far we have analysed only the components of the TIM23 complex that are essential for the function of the translocase and therefore are all also essential for the viability of yeast cells. What is happening with the two non-essential components, Tim21 and Pam17? Conflicting results were obtained concerning the presence of Tim21 in the TIM23 complex actively involved in translocation of preproteins into the matrix. In one study, Tim21 was absent from such a complex (Chacinska *et al*, 2005), whereas in the other it was present (Mokranjac *et al*, 2005). As in both cases tagged forms of Tim21 were analysed, we have reassessed the issue. We used immunoprecipitation experiments to analyse the association of Tim21 and Pam17 with the TIM23 complex in various states of its function. We found Tim21 associated with the complex in all states of activity tested (Figure 4A). In contrast, Pam17 was present in the empty complex and in the complex saturated with b_2, but was absent in the complex saturated with $b_2\Delta$. This is in agreement with the observed crosslinking pattern of Tim23 to Pam17 under the same conditions (Figure 3A).

To further study the behaviour of Tim21 and Pam17 in response to the presence of a translocating preprotein, we expressed and arrested His-tagged versions of b_2 and $b_2\Delta$ in intact cells, isolated the mitochondria, lysed them with detergent and analysed for interacting components by binding to NiNTA-Agarose. Indeed, Tim21 was specifically retained on the beads together with the components of the TOM–TIM23 supercomplex linked by a translocating matrix-destined preprotein. Pam17 was absent from the TIM23 complex actively translocating preproteins into the matrix also when analysed by this procedure (Figure 4B). On the other hand, the TOM–TIM23 supercomplex generated in the presence of laterally sorted preprotein b_2 is apparently less stable as compared with the one generated in the presence of $b_2\Delta$, in agreement with above-described results obtained by coimmunoprecipitation and BN-PAGE. In this case, the TIM23 components were recovered on the beads only slightly above background. Interestingly, the TOM complex components, exemplified here by TOM40, were equally efficiently associated with both types of preproteins, demonstrating that it is the association of the laterally sorted preprotein with the TIM23 complex that is labile. This further supports the notion of different conformations of the TIM23 complex involved in lateral insertion and matrix translocation.

Both Tim21 and Pam17 interact with the Tim17–Tim23 core of the TIM23 complex

In view of a rather surprising absence of Pam17 from TIM23 complex engaged in translocation into the matrix, a process which requires the activity of the import motor, and of the observed crosslink of Pam17 to Tim23, we decided to analyse the association Pam17 and Tim21 with the TIM23 complex in more detail. Wild-type mitochondria were lysed with digitonin and subjected to immunoprecipitation with antibodies to Tim16, Tim17 and Tim23 (Figure 5A). The majority of Tim21 in the mitochondrial extract was co-precipitated with antibodies to Tim17 and Tim23. Small but detectable amounts could also be precipitated with antibodies to Tim16. In contrast, the majority of Pam17 remained in the supernatant, irrespective of which antibody was used for precipitation, and only small amounts were detected in the pellets after precipitation with antibodies to Tim17 and Tim23, but not with antibodies to the motor component Tim16. Thus, it appears that the majority of Tim21 but only minor amounts of Pam17 are associated with the TIM23 complex or that the association of the latter one is very weak.

To study the modes of interaction of Tim21 and Pam17 with the TIM23 complex, we analysed mitochondria isolated from cells depleted of each one of the essential TIM23 components. In mitochondria depleted of Tim17

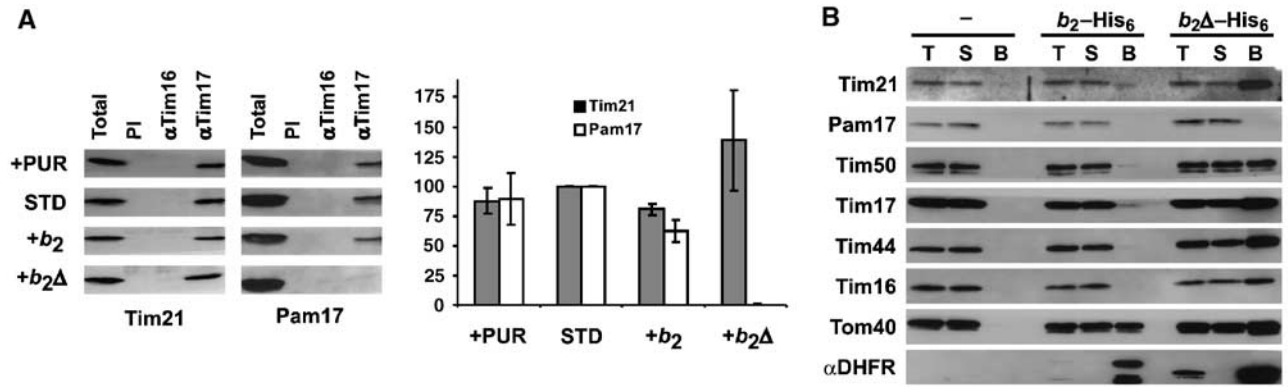

Figure 4 Association of Tim21 and Pam17 with the TIM23 complex in different states of translocation. (**A**) Mitochondria containing the TIM23 complex in various states of activity were solubilized with digitonin and subjected to immunoprecipitation with affinity-purified antibodies to Tim16, Tim17 or preimmune (PI) serum as a control. Total (20%) and immunoprecipitated material were analysed by SDS–PAGE and immunodecoration with antibodies to Tim21 and Pam17. Right panel: amounts of Tim21 and Pam17 precipitated under these conditions were quantified from three independent experiments. Precipitation in STD, 100%. (**B**) Mitochondria containing *in vivo*-arrested His-tagged versions of b_2 or $b_2\Delta$ were solubilized with digitonin and incubated with NiNTA-Agarose beads. T, total; S, supernatant and B, bound fractions were analysed by SDS–PAGE and immunodecoration. Total and supernatant, 5%.

(Figure 5B) or Tim23 (Figure 5C), association of Tim21 with the remaining parts of the TIM23 complex was lost. Essentially the same observation was made for Pam17. In contrast, depletion of any of the other essential components of the complex had no effect on the association of these two proteins with the Tim17–Tim23 core (Figure 5D–G). Apparently, both Tim21 and Pam17 interact with the assembled Tim17–Tim23 core of the complex.

We then asked which TIM23 components can be coisolated with Tim21 and Pam17. We constructed a yeast strain containing C-terminally His_6-tagged Tim21. Upon solubilization of mitochondria with digitonin, Tim23, Tim17, Tim50 but also Tim44 were specifically retained in significant amounts on the NiNTA beads together with the tagged Tim21 (Figure 5H, left panel). This result is in clear contrast to the result of the study in which no motor component was found to be present in the Tim21-containing TIM23 complex (Chacinska *et al*, 2005; van der Laan *et al*, 2005). Could the different tags used in the experiments be responsible for this discrepancy? We constructed a yeast strain containing ProteinA-tagged Tim21. When compared with the pull-down experiment with the His-tagged Tim21, we obtained essentially the same result as far as Tim17 and Tim23 were concerned (Figure 5H, right panel). However, the amounts of co-precipitated Tim50 and Tim44 were considerably lower compared to the precipitations observed with Tim21His_6. Thus, motor components, exemplified here by Tim44 and Tim14, are indeed not found in the same complex with ProteinA-tagged Tim21, in agreement with previous observations (Chacinska *et al*, 2005; van der Laan *et al*, 2005). These results, however, also suggest that the nature of the tag on Tim21 can have adverse effects on the coisolation of TIM23 components. Interestingly, we did not observe any coisolation of Pam17 with either His- or ProteinA-tagged Tim21. When NiNTA pull down was performed with mitochondria isolated from a yeast strain expressing His-tagged Pam17, coisolation of Tim50, Tim23 and Tim17 was observed (Figure 5I). Significant amounts of Tim21, Tim44, Tim16 and Tim14 were not recovered in the bound fraction, though it cannot be excluded that this is due to the generally weak/labile interaction of Pam17 with the complex and/or possible effect of the tag.

In summary, both Tim21 and Pam17 bind to the Tim17–Tim23 core of the TIM23 complex and this association is not dependent on any other known essential component of the complex. Furthermore, no evidence was obtained for a direct association of Pam17 with the TIM23 subunits characterized as components of the import motor. Also, we could not observe any coisolation of Tim21 and Pam17.

Opposing roles of Tim21 and Pam17 in the TIM23 complex

In the light of the results on the association of Tim21 and Pam17 with the TIM23 complex, we investigated their roles in the translocase. As previously shown, the deletion of *TIM21* had virtually no effect on the efficiency of protein import through the TIM23 complex, and the deletion of *PAM17* reduced import motor-dependent transport (Chacinska *et al*, 2005; van der Laan *et al*, 2005 and our data, not shown). However, we did not observe any destabilization of the TIM23 complex in mitochondria lacking Tim21, Pam17 or both, suggesting that neither of the two proteins has an essential role in the assembly of the essential components of the TIM23 complex (Supplementary Figure S4A). The reported destabilization of the TIM23 complex in mitochondria lacking Pam17 may be due to the harsher method employed for analysis and/or additional effects of the tag used (van der Laan *et al*, 2005). Absence of Pam17 did, however, produce observable changes in the conformation of the TIM23 complex as judged by altered crosslinking patterns of Tim44 and Tim16 but also of Tim23 (Supplementary Figure S4B–D). The observed import defect in mitochondria lacking Pam17 can therefore be due either to an altered conformation of the import motor or of the membrane part.

We then analysed the effects of overexpression of Tim21 and Pam17 on the TIM23 complex. Interestingly, the degree of their overexpression differed markedly even though they were expressed from the same promoter (Figure 6A). The levels of Pam17 were increased 3- to 4-fold as compared with wild type. In contrast, the levels of Tim21 were increased

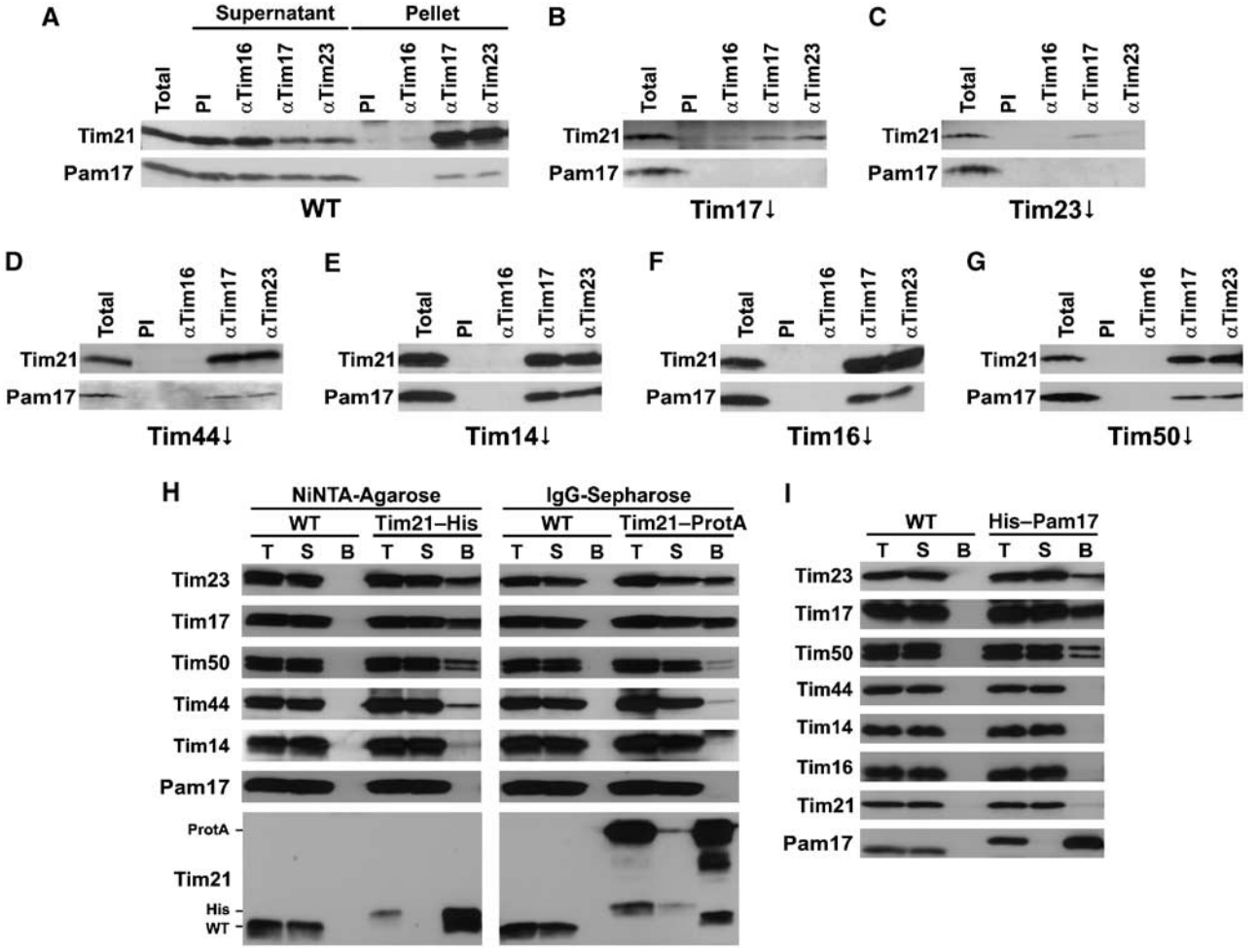

Figure 5 Tim21 and Pam17 interact with the Tim17–Tim23 core of the TIM23 complex. (**A**) Wild-type mitochondria were solubilized with digitonin and immunoprecipitated with the affinity-purified antibodies to Tim16, Tim17 or antibodies from preimmune (PI) serum as a control. Samples were analysed by SDS–PAGE and immunodecoration. For simplicity reasons, only decorations with the antibodies to Tim21 and Pam17 are shown. Total and supernatant fractions represent 20% of the material used for immunoprecipitations. (**B–G**) Mitochondria were isolated from cells depleted of the indicated TIM23 components and analysed by immunoprecipitation as described under (A). Only total and immunoprecipitated materials are shown. (**H**) Mitochondria, isolated from wild-type cells and cells expressing Tim21 with C-terminal His or ProteinA tag, were solubilized with digitonin and incubated with NiNTA-Agarose or IgG-Sepharose beads. Samples were analysed by SDS–PAGE and immunodecoration. T, total and S, supernatant fractions contain 5% of the material present in B, bound fraction. (**I**) Mitochondria isolated from wild type and cells expressing Pam17 with an N-terminal His tag were solubilized with digitonin and incubated with NiNTA-Agarose beads. T, total; S, supernatant and B, bound fractions were analysed by SDS–PAGE and immunodecoration. T and S represent 20% of the material present in B.

several dozen fold. The levels of other mitochondrial proteins analysed were not influenced under these conditions. When we analysed the molecular environment of Tim23, overexpression of Tim21 was found to lead to a decreased efficiency of Tim23 crosslinking to Pam17 and also to a significantly increased efficiency of crosslinking of two Tim23 (Figure 6B). Intriguingly, overexpression of Pam17 counteracted this effect. In mitochondria in which both proteins were overexpressed, adducts of two Tim23 were virtually absent and the intensity of the Tim23-Pam17 adduct was restored to almost wild-type level. This result brings Pam17 and Tim21 into a functional connection and suggests that they have antagonistic effects on the TIM23 complex.

To further analyse this effect, we performed immunoprecipitation experiments using digitonin-solubilized mitochondria containing overexpressed Pam17, Tim21 or both (Figure 6C). The overexpression of Pam17 noticeably reduced the amounts of Tim21 that could be precipitated with the TIM23 complex. Conversely, upon upregulation of Tim21 virtually complete removal of Pam17 from the TIM23 complex was observed. And indeed, in mitochondria with both Pam17 and Tim21 overexpressed, precipitation of Pam17 with the TIM23 complex was again visible showing that the increased levels of Pam17 led to partial removal of the overexpressed Tim21 from the complex. This is in full agreement with the crosslinking experiments described above. It should be noted that the effects of overexpression of Tim21 and/or Pam17 were not due to defects in the assembly of the essential TIM23 components as they were precipitated in the same amounts with all four types of mitochondria analysed (data not shown). This argues against the suggestion that the overexpression of Tim21 promotes dissociation of the motor from the membrane part (Chacinska *et al*, 2005), but rather verifies that Tim21 and Pam17 are functionally connected in an antagonistic manner. In addition, it suggests that Tim21 and Pam17 do not bind to the TIM23 complex at the same time.

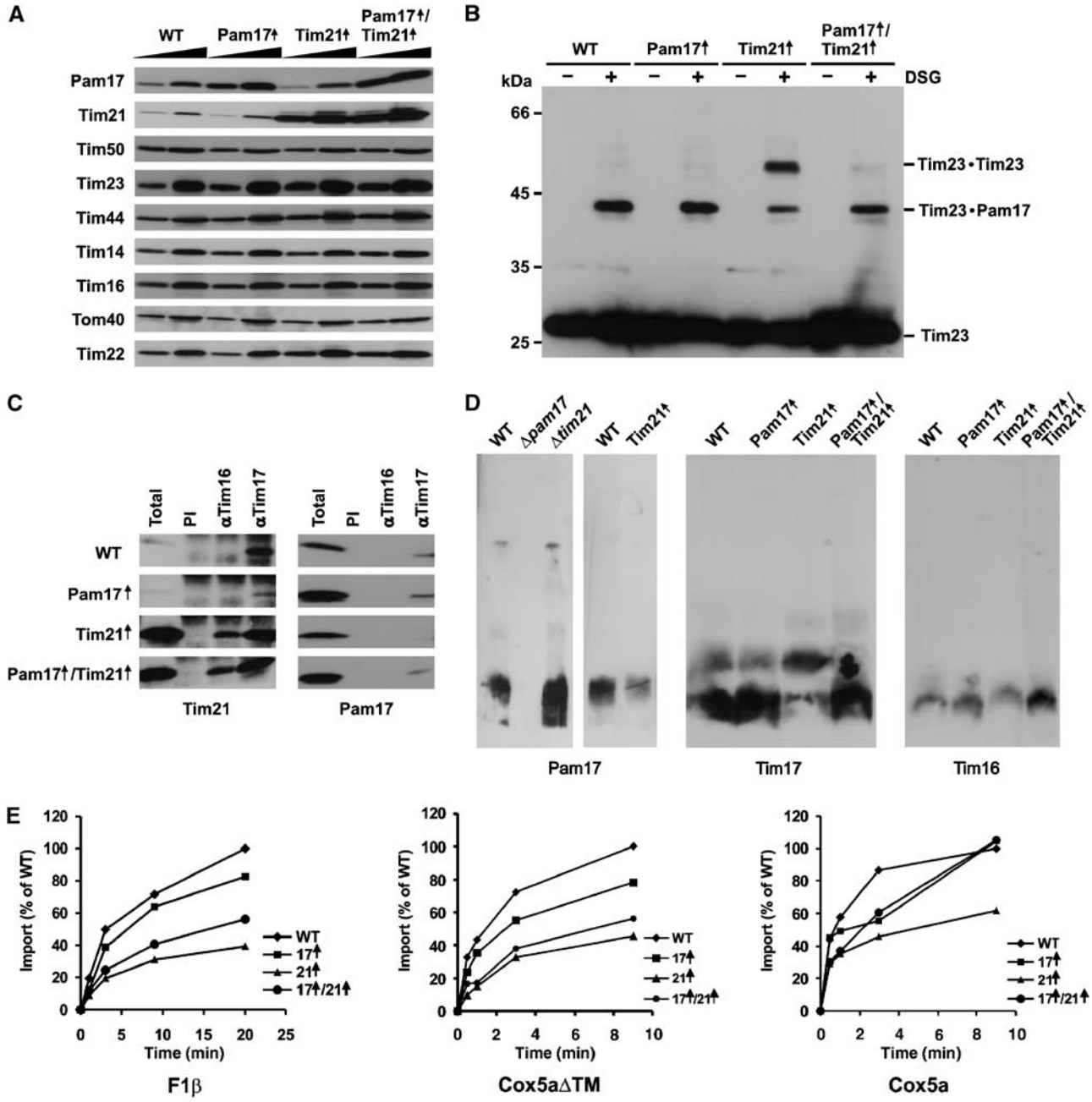

Figure 6 Opposing functions of Tim21 and Pam17 in the TIM23 complex. (**A**) Mitochondria (10 and 50 μg) were analysed by SDS–PAGE and immunodecoration. (**B**) Mitochondria were crosslinked with DSG and analysed by SDS–PAGE and immunodecoration with antibodies to Tim23. (**C**) Mitochondria (labelled on the left) were solubilized with digitonin and subjected to immunoprecipitation with antibodies to Tim16, Tim17 or antibodies from preimmune (PI) serum as a control (labelled on top). Total (20%) and precipitated materials were analysed by SDS–PAGE and immunodecoration with antibodies to Tim21 (left panel) and Pam17 (right panel). (**D**) Mitochondria were solubilized in digitonin and subjected to BN-PAGE followed by immunodecoration with antibodies to Pam17, Tim17 or Tim16. (**E**) Various ^{35}S-labelled preproteins as indicated were imported into isolated mitochondria followed by protease treatment, SDS–PAGE and autoradiography.

The same functional connection was observed upon analysis by BN-PAGE. The TIM23 complex dissociates into several subcomplexes when analysed by this method (Frazier *et al*, 2004; Chacinska *et al*, 2005; van der Laan *et al*, 2005; Tamura *et al*, 2006) and Pam17 runs as a ca. 50 kDa subcomplex (van der Laan *et al*, 2005 and Figure 6D). The formation of this subcomplex was greatly impaired in mitochondria containing overexpressed Tim21, whereas it was promoted in mitochondria lacking Tim21. The shift of Tim17–Tim23 subcomplexes to higher molecular weights observed in Tim21-upregulated mitochondria (Figure 6D and Chacinska *et al*, 2005) was counteracted by simultaneous overexpression of Pam17. In addition, slight destabilization of the BN-PAGE complex of Tim14–Tim16 observed in mitochondria containing overexpressed Tim21 was relieved upon co-overexpression of Pam17. Intriguingly, a similar destabilization of BN-PAGE Tim14–Tim16 subcomplex was observed in mitochondria lacking Pam17 (van der Laan *et al*, 2005), again suggesting that Tim21 exerts its effects by removing Pam17 from the complex. Taken together, these results speak

strongly for antagonistic functions of Pam17 and Tim21 in the TIM23 complex and their mutual displacement from the translocase.

These opposing effects of Tim21 and Pam17 correlated well with the ability of isolated mitochondria to import preproteins through the TIM23 complex (Figure 6E). Mitochondria containing overexpressed Tim21 showed a reduced ability to import preproteins into the matrix, exemplified here by F1β and Cox5aΔTM, in agreement with previously published results (Chacinska *et al*, 2005). However, this defect was, at least partially, relieved by overexpression of Pam17. The same results were obtained with laterally sorted preproteins whose import requires the import motor, exemplified here by Cox5a.

Taken together, these results show that Tim21 and Pam17 have mutually antagonistic effects and modulate the function of the TIM23 complex in a complementary manner.

Discussion

How does the TIM23 complex accomplish to sort preproteins into two different mitochondrial subcompartments? We have generated homogeneous populations of the TIM23 complex in various states of its function. Thereby, for the first time we were able to directly analyse their composition and conformation. In our hands, all essential components of the TIM23 complex are and remain associated irrespective of whether the complex is in its empty state or is actively involved in translocation of preproteins. The complex does, however, actively respond to the incoming preproteins. First, we observed that the exposure of the N-terminal segment of Tim23 on the mitochondrial surface increases with the increased translocation load. This unambiguously shows its active involvement in the preprotein-triggered dynamic cooperation between the TIM23 and TOM complexes even if a direct interaction of Tim23 with the TOM complex has not been shown so far. A direct interaction, at least *in vitro*, between Tim21 and the TOM complex has been described (Chacinska *et al*, 2005; Mokranjac *et al*, 2005). Also, Tim50 interacts with preproteins as soon as they emerge from the outlet of the TOM complex (Yamamoto *et al*, 2002; Mokranjac *et al*, 2003a). Interestingly, in the absence of Tim50, Tim23 does not reach the surface of mitochondria (Yamamoto *et al*, 2002). We thus suggest that at least three components of the TIM23 complex, Tim50, Tim23 and Tim21, are involved in the active cooperation of TOM and TIM23 complexes during the early steps of preprotein translocation through the TIM23 complex.

We further show that the way in which the TIM23 complex responds to the incoming preproteins depends on whether the translocating preprotein is to be transported into the matrix or is to be laterally sorted into the inner membrane. In the latter case, the TIM23 complex has to open laterally to insert the transmembrane segment into the lipid bilayer. We report that this process is accompanied by a distinct change in the positioning of the intermembrane space domains of two Tim23 molecules relative to each other. This provides first clues about the conformation of the TIM23 complex actively involved in lateral insertion. We also demonstrate a series of rearrangements during the transfer of preproteins from the TOM complex through the translocation channel in the inner membrane to mtHsp70 in the matrix. Matrix-targeted preproteins rearrange the channel components in a way that they no longer interact with Pam17. We suggest that at the matrix face of the channel, Tim44 has an active role in transfer of preproteins to mtHsp70 likely by undergoing an initial conformational change, which is then conveyed to the Tim14–Tim16 pair. Changes in the Tim14–Tim16 interaction relieve the inhibitory effect of Tim16 (Li *et al*, 2004; Mokranjac *et al*, 2006) so that Tim14 can stimulate the ATPase activity of mtHsp70 and thus enable the tight binding of the chaperone to the incoming preprotein.

Data presented here demonstrate that the membrane-integrated part and the import motor, two operational parts of the TIM23 complex, function as a single entity that undergoes extensive remodelling upon engaging in import of different types of preproteins. In contrast, a model was recently proposed (Chacinska *et al*, 2005) according to which, in the absence of a translocating preprotein, the TIM23 complex exists as a Tim21-containing, but motor-free form. This form was proposed to mediate insertion of preproteins into the inner membrane. Translocation of preproteins into the matrix, on the other hand, would require a switch necessary to release Tim21 and recruit the import motor. The model implies the existence of a signal in matrix-targeted preproteins that would cause this switch. However, no clue was offered as to what such a signal could be. The presequence itself clearly cannot be this signal as it is present both in matrix-targeted as well as in laterally sorted preproteins. In fact, it is difficult to envisage such a signal in the above-mentioned preproteins, which consist only of the N-terminal presequence and a non-mitochondrial passenger protein. Moreover, the model cannot explain the sorting pathway of a number of laterally inserted preproteins whose import clearly depends on the import motor (Voos *et al*, 1993; Stuart *et al*, 1994; Gärtner *et al*, 1995). The study in which the model was proposed largely relied on the analysis of the yeast strain containing ProteinA-tagged Tim21. We find that this tag has an adverse effect on the association of Tim21 with the TIM23 complex both in its empty and active forms. Indeed, none of the other tags used on Tim21 had a similar effect as the ProteinA tag (Mokranjac *et al*, 2005; Tamura *et al*, 2006). Furthermore, the lack of coisolation of Pam17 with ProteinA-tagged Tim21 apparently led to the classification of Pam17 as the import motor component (van der Laan *et al*, 2005). In contrast, our data demonstrate that Tim21 and Pam17 bind directly to the Tim17–Tim23 core of the TIM23 complex. Interestingly, we found a functional connection between the two non-essential subunits of the TIM23 complex. Tim21 and Pam17 modulate the function of the TIM23 complex in an opposite manner. Both Tim21 and Pam17 are likely to interact with the TIM23 complex in a dynamic, on/off manner and binding of one of them, directly or indirectly, may prevent binding of the other one. Our data suggest that Pam17 is needed for optimal positioning of the Tim17–Tim23 core with respect to the import motor. Pam17 is likely to assist in inducing a conformational change of the complex in response to the entry of a translocating preprotein. Once this conformational change has taken place, Pam17 is no longer required and its presence in the complex may have an adverse effect. As translocation into the mitochondrial matrix can occur in the absence of Pam17, these conformational changes take place even in the absence of Pam17.

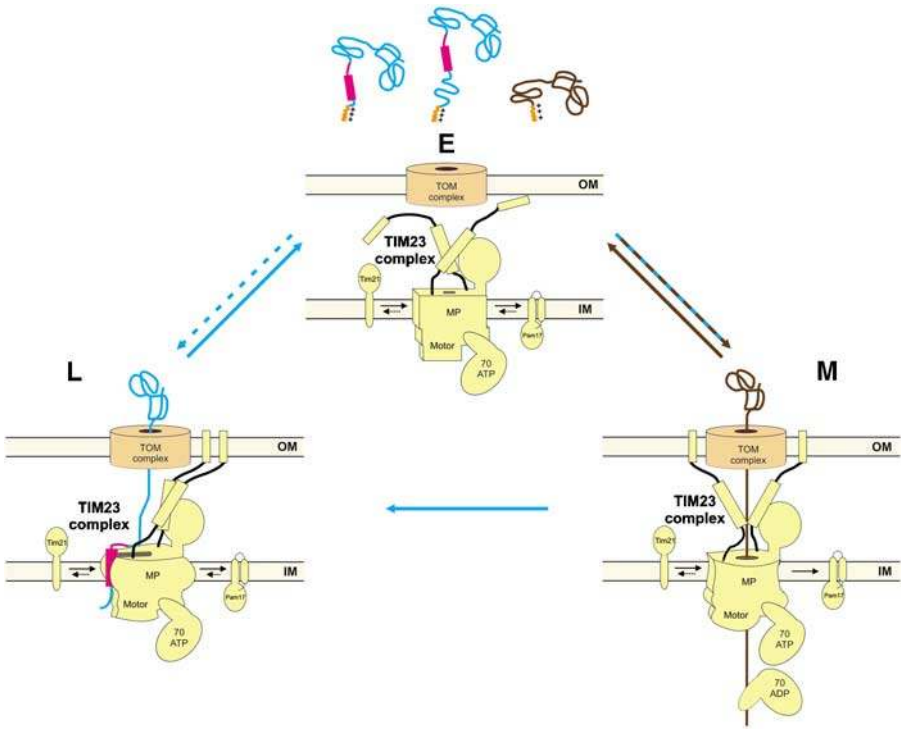

Figure 7 Remodelling of the TIM23 complex during preprotein translocation—a model. See text for details. Matrix-targeted preproteins are shown in brown and laterally sorted ones in cyan, presequences are in orange and stop-transfer signals in magenta. MP, membrane-integrated part of the TIM23 complex; OM, outer mitochondrial membrane; IM, inner mitochondrial membrane.

On the basis of all available data, we propose a model presented in Figure 7. In the absence of a preprotein, the TIM23 complex is in its empty or E state. Interaction with a presequence induces a series of conformational changes until the membrane part adopts the conformation required for passage of the unfolded preprotein into the matrix (brown/cyan arrow in E to M direction). If no other signal appears within the preprotein, translocation into the matrix will be completed by a number of cycles of the import motor. This mode of the translocase is defined as matrix translocation (M). Upon completion of translocation of the preprotein into the matrix, the complex returns to its E state (brown arrow in M to E direction). If, however, a sorting signal is recognized in the preprotein in transit, the translocase undergoes additional conformational changes (cyan arrow in M to L direction) that trigger lateral opening of the TIM23 complex (L mode) and insertion of the transmembrane domain into the inner membrane. These changes are induced only after a certain number of cycles of the import motor. These cycles are not only needed to translocate into the matrix the segment between the targeting signal and the sorting signal but also for transport of tightly folded domains across the outer membrane into the intermembrane space (Voos *et al*, 1993; Stuart *et al*, 1994; Gärtner *et al*, 1995). As all known preproteins that are laterally sorted through the TIM23 complex contain a single transmembrane domain, the translocase is likely to change from the L mode directly back to the E state (cyan arrow in L to E direction). Some laterally sorted preproteins, which have a sorting signal directly after the presequence, are inserted without the apparent involvement of the import motor. It is thus possible that the translocase directly changes from its E state to the L mode (broken cyan line pointing from E to L), though it cannot be excluded that these preproteins also require one or a few cycles of the import motor. Tim21 and Pam17 bind to the complex in a dynamic manner (depicted by bidirectional black arrows) and affect the transitions between E state and M and L modes in an antagonistic way. The essential components of the membrane part and the import motor of TIM23 complex operate as a single entity during the entire reaction cycle.

The approach taken in here is likely applicable to other protein translocases in the cell. In the absence of high-resolution structures of translocases in various modes of transport, such experiments may shed light on how the translocases are changing during translocation and insertion of proteins.

Materials and methods

Plasmids, yeast strains and growth conditions

Wild-type yeast strain YPH499 was used for all genetic manipulations. Yeast strains in which expression of Tim50 (50↓), Tim14 (14↓) or Tim16 (16↓) is under the control of the *GAL* promoter were published previously (Mokranjac *et al*, 2003a, b; Kozany *et al*, 2004). Strains 17↓, 23↓ and 44↓ were generated in YPH499 in essentially the same manner. Δ*pam17* and Δ*tim21* were constructed by replacing the corresponding genes with a *HIS3* cassette by homologous recombination. Strain Δ*pam17*/Δ*tim21* was generated by replacing *TIM21* with a *KAN* cassette in Δ*pam17* strain. C-terminal His_6 and ProteinA tagging of Tim21 were performed by homologous recombination into the chromosome using pYM9 and pYM7 vectors, respectively. His_6 Pam17 is the Δ*pam17* strain transformed with pRS314 plasmid coding for the N-terminally His_6-tagged Pam17 under its endogenous promoter. For the creation of overexpression strains, *PAM17* and *TIM21* were cloned under the *ADH* promoter in yeast vectors pVT-W and pVT-U, respectively, and the resulting plasmids, alone or in combination, were transformed into YPH499.

Yeast cells were grown in lactate medium containing 0.1% glucose unless otherwise stated. Depletion of individual TIM23 components was performed as described before (Mokranjac *et al*, 2003a). For the overexpression of Pam17 and/or Tim21, corresponding cells were grown in selective lactate medium containing 0.1% glucose.

Saturation of the TIM23 complex with preproteins in intact cells

Constructs cytb_2(1–167)DHFR (b_2) and cytb_2(1–167)Δ19DHFR ($b_2\Delta$) were previously described (Rassow *et al*, 1989; Koll *et al*, 1992). They were subcloned into pYES2 vector (Invitrogen) to enable expression of the proteins in yeast from a regulatable *GAL1* promoter. C-terminal His tags were introduced into b_2 and $b_2\Delta$ by PCR. Plasmids were subsequently transformed into wild-type yeast strain YPH499. Cells were grown in selective lactate medium containing 0.1% glucose. To induce expression of the hybrid proteins and saturate the translocase, cells were washed, transferred to selective lactate medium containing 0.5% galactose and 0.2 mM aminopterine and grown for 2 h before mitochondria were isolated. To deplete the translocases of preproteins, 100 μg/ml puromycin was added to the growing culture of the wild-type cells 1 h prior to isolation of mitochondria.

Treatment of mitochondria with proteinase K

Isolated mitochondria were incubated for 10 min on ice with proteinase K (500 μg/ml). Protease digestion was stopped by addition of phenylmethylsulphonyl fluoride. Mitochondria were reisolated and analysed by SDS–PAGE and immunodecoration.

Antibodies

Tim21(97–239) and Pam17(124–197) were expressed from pQE30 (Qiagen) and pMALcRI (NEB) plasmids and purified on NiNTA-Agarose and Amilose resin, respectively. Purified proteins were injected into rabbits for generation of specific antibodies. All antibodies were affinity purified before use.

Miscellaneous

The following procedures were as previously described: chemical crosslinking and manipulation of ATP levels in the mitochondrial matrix (Mokranjac *et al*, 2003b), protein import into isolated mitochondria (Mokranjac *et al*, 2003a) and BN-PAGE (Waizenegger *et al*, 2004). For immunoprecipitation experiments, mitochondria (1 mg/ml) were solubilized in 20 mM TRIS, 80 mM KCl, pH 7.5 with 1% digitonin and further processed as described previously (Mokranjac *et al*, 2003a).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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