EMBO reports

scientific report

Assembly of the three small Tim proteins precedes docking to the mitochondrial carrier translocase

Natalia Gebert[1,2], Agnieszka Chacinska[1], Karina Wagner[1,2], Bernard Guiard[3], Carla M. Koehler[4], Peter Rehling[1,5], Nikolaus Pfanner[1+] & Nils Wiedemann[1]

[1]Institut für Biochemie und Molekularbiologie, Zentrum für Biochemie und Molekulare Zellforschung, and [2]Fakultät für Biologie, Universität Freiburg, Freiburg, Germany, [3]Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France, [4]Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, California, USA, and [5]Abteilung Biochemie II, Universität Göttingen, Göttingen, Germany

The mitochondrial intermembrane space contains a family of small Tim proteins that function as essential chaperones for protein import. The soluble Tim9–Tim10 complex transfers hydrophobic precursor proteins through the aqueous intermembrane space to the carrier translocase of the inner membrane (TIM22 complex). Tim12, a peripheral membrane subunit of the TIM22 complex, is thought to recruit a portion of Tim9–Tim10 to the inner membrane. It is not known, however, how Tim12 is assembled. We have identified a new intermediate in the biogenesis pathway of Tim12. A soluble form of Tim12 first assembles with Tim9 and Tim10 to form a Tim12-core complex. Tim12-core then docks onto the membrane-integrated subunits of the TIM22 complex to form the holo-translocase. Thus, the function of Tim12 in linking soluble and membrane-integrated subunits of the import machinery involves a sequential assembly mechanism of the translocase through a soluble intermediate complex of the three essential small Tim proteins.

Keywords: MIA; mitochondria; *Saccharomyces cerevisiae*; Tim12; TIM22

EMBO reports (2008) **9**, 548–554. doi:10.1038/embor.2008.49

INTRODUCTION

There are two main pathways of protein transport to the mitochondrial inner membrane. Preproteins, which are synthesized on cytosolic ribosomes with a cleavable amino-terminal presequence, are imported by means of the general translocase of the outer mitochondrial membrane (TOM complex) and the presequence translocase of the inner membrane (TIM23 complex). Most of the presequence-carrying preproteins are imported into the matrix. Some of the preproteins contain a hydrophobic sorting signal that arrests translocation in the TIM23 complex and induces a lateral release into the lipid phase (Jensen & Johnson, 2001; Koehler, 2004; Rehling *et al*, 2004; Neupert & Herrmann, 2007).

Most of the hydrophobic inner membrane proteins, including the metabolite carriers, contain internal targeting information and use a different import mechanism. The precursors are not directly transferred from the TOM complex to the carrier translocase of the inner membrane (TIM22 complex) but are bound to chaperones of the intermembrane space (IMS; Koehler, 2004; Webb *et al*, 2006; Davis *et al*, 2007; Gentle *et al*, 2007; Neupert & Herrmann, 2007). The chaperones are formed by the family of small Tim proteins. Of the five small Tim proteins, four are found in soluble hexameric complexes of the IMS, the essential Tim9–Tim10 complex and the Tim8–Tim13 complex. A fifth small Tim protein, Tim12, has been found only as a membrane-bound form (Koehler *et al*, 1998a; Sirrenberg *et al*, 1998; Adam *et al*, 1999; Baud *et al*, 2007; Gentle *et al*, 2007). Tim12 is an essential subunit of the TIM22 complex and provides an interaction site for a portion of Tim9 and Tim10 at the inner membrane. In addition to the small Tim proteins, the TIM22 complex contains three integral membrane proteins, the pore-forming proteins Tim22, Tim54 and Tim18 (Jensen & Johnson, 2001; Koehler, 2004; Rehling *et al*, 2004; Neupert & Herrmann, 2007).

The import pathway of the four soluble small Tim proteins has been characterized. The precursors are transported through the TOM complex and use a specific machinery of the IMS for import and assembly (MIA). The IMS receptor Mia40 forms transient disulphide bonds with the cysteine residues of the precursors and, with the help of the sulphydryl oxidase Erv1, promotes folding of the imported proteins (Chacinska *et al*, 2004; Neupert & Herrmann, 2007). The folded monomers then assemble to form the hexameric chaperone complexes. However, it is not known how the soluble Tim9–Tim10 chaperone is converted into a

[1]Institut für Biochemie und Molekularbiologie, Zentrum für Biochemie und Molekulare Zellforschung, and [2]Fakultät für Biologie, Universität Freiburg, Hermann-Herder-Strasse 7, 79104 Freiburg, Germany
[3]Centre de Génétique Moléculaire, CNRS, 91190 Gif-sur-Yvette, France
[4]Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, California 90095-1569, USA
[5]Abteilung Biochemie II, Universität Göttingen, 37073 Göttingen, Germany
[+]Corresponding author. Tel: +49 761 203 5224; Fax: +49 761 203 5261;
E-mail: nikolaus.pfanner@biochemie.uni-freiburg.de

Received 23 November 2007; revised 18 February 2008; accepted 3 March 2008; published online 18 April 2008

membrane-bound form at the TIM22 complex. The main reason for this is that little is known about the assembly pathway of the precursor of Tim12. At present, it is assumed that the precursor of Tim12 first binds to the membrane-integrated TIM22 complex, and subsequently recruits Tim9 and Tim10 molecules to the TIM22 complex; however, there is no experimental evidence to support this.

Here, we analysed the import pathway of Tim12. We found that, after interaction with the MIA system, Tim12 forms a new assembly intermediate. In contrast to the present view, the precursor of Tim12 associates with Tim9 and Tim10 in a soluble complex in the IMS that subsequently docks onto the membrane-integrated TIM22 complex subunits. These findings provide a new mechanism for conversion of the soluble IMS chaperone into an inner membrane-bound chaperone. The Tim9–Tim10 chaperone does not simply bind to Tim12 at the TIM22 complex, instead the three small Tim proteins first assemble into a soluble intermediate form and this preformed complex is recruited to the TIM22 complex *en bloc*.

RESULTS AND DISCUSSION

Formation of a core complex during the import of Tim12

We imported the radiolabelled precursor of Tim12 into isolated yeast mitochondria. The mitochondria were lysed in digitonin and protein complexes were separated by using blue native polyacrylamide gel electrophoresis (BN-PAGE). In addition to the mature TIM22 complex of approximately 300 kDa, the precursor of Tim12 was found in two other forms that migrated at 180 and 70 kDa (Fig 1A, lanes 1–3). Mature, endogenous Tim12 was found only in the 300 kDa complex (Fig 1A, lane 4; Koehler *et al*, 1998b; Adam *et al*, 1999; Kovermann *et al*, 2002). We show here that the 180 kDa form is the Mia40-bound intermediate of Tim12 and that the 70 kDa form represents a core complex of Tim12, referred to as Tim12-core. Tim12-core was dissociated by treatment under denaturing conditions (SDS; Fig 1B, lane 2). On treatment with dithiothreitol, the Mia40-intermediate was dissociated, whereas the Tim12-core was largely resistant, similar to the mature TIM22 complex (Fig 1B, lanes 6–8).

To exclude the possibility that Tim12-core was simply released from the TIM22 complex during the electrophoretic run, Tim12 was imported into mitochondria containing Tim18 with a cleavable Protein A-tag (Fig 1C, lane 3; Rehling *et al*, 2003). The mitochondria were lysed in digitonin and the TIM22 complex was isolated by IgG affinity chromatography. Neither the Mia40-intermediate nor the Tim12-core was found in the eluate (Fig 1C, lane 4), excluding that Tim12-core was generated during electrophoresis. The gel mobility of Tim12-core seemed to be similar to the mobility reported for the hexameric Tim9–Tim10 complex (Koehler *et al*, 1998b; Chacinska *et al*, 2004). Thus, we compared the mobility of Tim9 and Tim12 directly, and found that the Tim12-core migrated slightly faster than the Tim9–Tim10 complex (Fig 1C, lanes 1,3), indicating that it was different from the Tim9–Tim10 complex. When Tim22 was imported into mitochondria, it was efficiently co-purified with tagged Tim18 and was not present in the Tim12-core (Fig 1D).

We investigated whether Tim18 and Tim54 were required for the formation of Tim12-core. When the precursor of Tim12 was imported into mitochondria isolated from a *tim18*Δ yeast strain, Tim12-core was formed efficiently and Tim12 assembled into the smaller, Tim18-deficient TIM22′ complex (Fig 1E). As the size and amount of Tim12-core were not altered in *tim18*Δ mitochondria, we conclude that Tim18 is not required for the formation of Tim12-core. In mitochondria lacking Tim54, the TIM22 complex is dissociated (Kovermann *et al*, 2002); however, the formation of Tim12-core was not impaired but rather found to increase (Fig 1F). Thus the formation of Tim12-core does not require the presence of the mature TIM22 complex, and Tim12-core accumulates when the TIM22 complex cannot be formed.

Association of Tim9, Tim10 and Tim12 in Tim12-core

To determine whether the formation of Tim12-core required Mia40, we used *mia40-4* mitochondria that accumulate precursors at the mutant Mia40 and inhibit the subsequent assembly steps (Chacinska *et al*, 2004). In *mia40-4* mitochondria, the formation of both Tim12-core and the mature TIM22 complex was strongly impaired (Fig 2A). Similarly, the formation of Tim12-core and the TIM22 complex was inhibited in mutant mitochondria of Tim10 (*tim10-2*; Truscott *et al*, 2002; Fig 2B). To study whether Mia40 and Tim10 were required for the formation of Tim12-core or represented subunits of Tim12-core, we used antibody-depletion BN-PAGE. On import of Tim12 and lysis of mitochondria with digitonin, antibodies directed against either Mia40 or Tim10 were added and antibody–antigen complexes were removed before BN-PAGE. Antibodies directed against Mia40 did not affect the Tim12-core but removed the 180 kDa band, indicating that it contained Mia40 (Fig 2C, lane 2). By contrast, antibodies specific for Tim10 did not affect the Mia40-intermediate but depleted the Tim12-core as well as the mature TIM22 complex (Fig 2C, lane 3). We conclude that Mia40 is required for the formation of Tim12-core but is not part of the core complex, whereas Tim10 is present in Tim12-core and the TIM22 complex. Thus, Tim12 cannot directly associate with the TIM22 complex but depends on the presence of functional Tim10.

We wondered whether all three small Tim proteins were present in Tim12-core and imported the precursors in parallel. We used a high-resolution blue native gel to separate the Tim9–Tim10 complex from the Tim12-core. Tim9 and Tim10 were not only found in the Tim9–Tim10 complex but also a fraction of each co-migrated with Tim12 at the position of the Tim12-core, although the amount of Tim10 in the Tim12-core was significantly lower than that of Tim9 (Fig 2D). For the TOM complex, it was shown that the detergent Triton X-100 led to a partial dissociation into subcomplexes (Dekker *et al*, 1998). We treated the purified TIM22 complex—containing radiolabelled Tim9 and Tim12—with Triton X-100, leading to a release of the small Tim proteins. The released Tim9 co-migrated with Tim12 but not with the Tim9–Tim10 complex (Fig 1C), indicating that Tim9 in the TIM22 complex is distinct from that in the Tim9–Tim10 complex. When the precursor of Tim9 was imported into *tim10-2* mitochondria, the Mia40-intermediate was generated (Chacinska *et al*, 2004); however, the formation of the Tim9–Tim10 complex and also that of Tim12-core and TIM22 complex was blocked (Fig 2E). Thus, Tim10 is required for integrating Tim9 into the Tim12-core and the TIM22 complex. To establish directly the presence of the three small Tim proteins in Tim12-core, we imported Tim9 into mitochondria and performed antibody-depletion BN-PAGE. Antibodies directed against Tim12 and Tim10 removed Tim12-core as well as the TIM22 complex, whereas the Mia40 antibody depleted

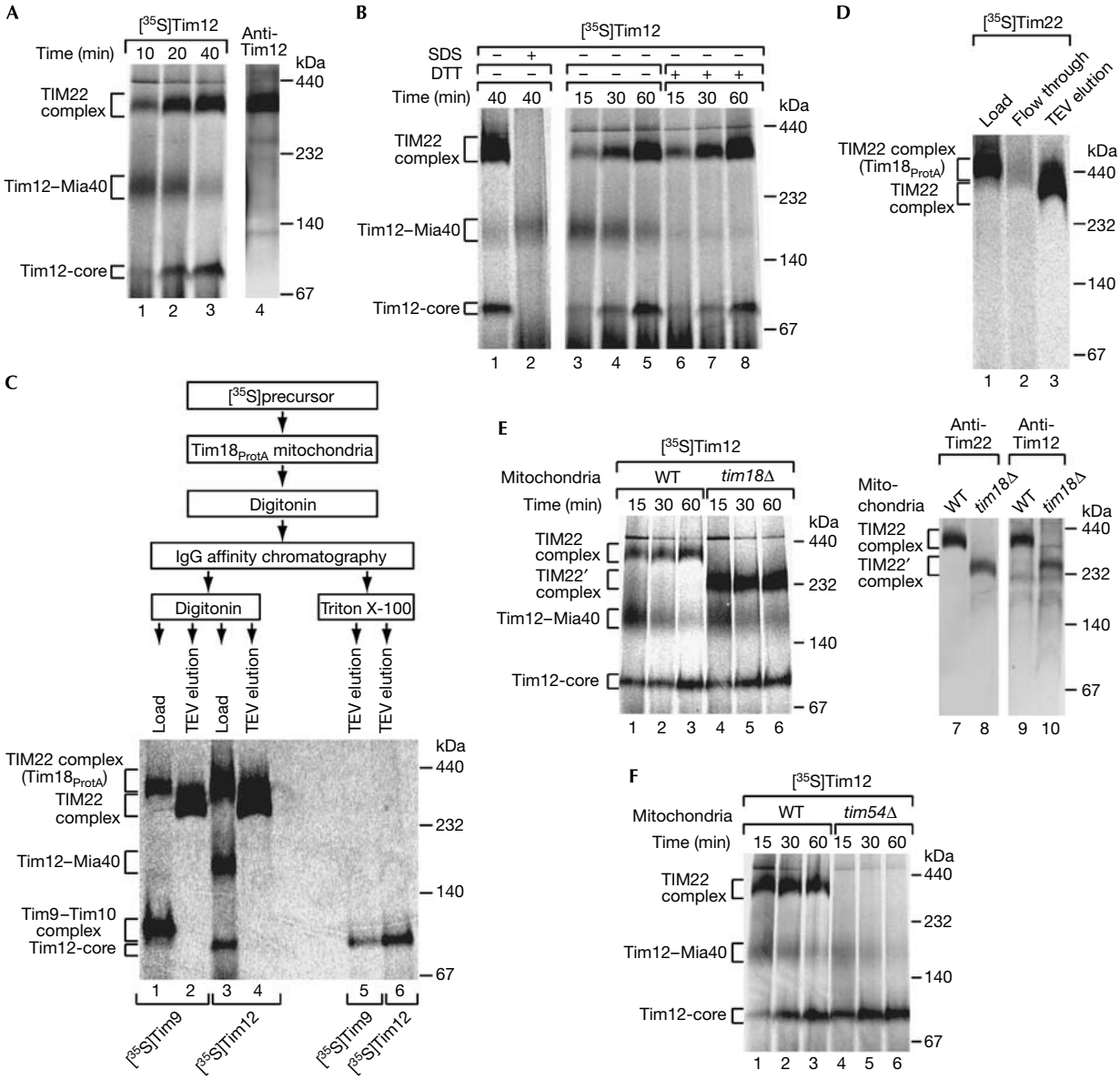

Fig 1 | Formation of the Tim12-core complex. (**A**) [^{35}S]Tim12 was incubated with isolated yeast mitochondria for the indicated time periods at 35 °C (lanes 1–3). Mitochondria were solubilized with digitonin, and analysed by using blue native polyacrylamide gel electrophoresis and autoradiography. Lane 4, shows immunodecoration. (**B**) Tim12 was imported into mitochondria. Mitochondria were lysed with digitonin in the presence of 0.2% SDS or 10 mM dithiothreitol (DTT) as indicated. (**C,D**) Tim9, Tim12 and Tim22 were imported into mitochondria with a cleavable Protein-A tag ($Tim18_{ProtA}$) for 60 min at 35 °C. Mitochondria were solubilized with digitonin and subjected to IgG affinity chromatography. Purified TIM22 complex was eluted with tobacco etch virus (TEV) protease in digitonin or, where indicated, with Triton X-100. Load, 30%; flow through, 30%; elution, 100%. (**E**) Tim12 was imported into wild-type (WT) and *tim18*Δ mitochondria, and analysed by autoradiography (lanes 1–6). Lanes 7–10 show immunodecoration. (**F**) Tim12 was imported into WT and *tim54*Δ mitochondria and analysed by autoradiography.

only the Mia40-intermediate of Tim9 (Fig 2F). Thus, Tim9, Tim10 and Tim12 are associated with each other not only in the TIM22 complex but also in the Tim12-core.

The high-resolution structure of the hexameric Tim9–Tim10 complex shows an alternating association of Tim9 and Tim10 molecules (Webb *et al*, 2006). A sequence analysis by Gentle *et al* (2007) suggested that yeast Tim12 is a modified form of Tim10 and Baud *et al* (2007) reported that purified Tim12 binds to Tim9 but not to Tim10. These findings suggest a model in which one or two Tim10 molecules in the Tim12-core are replaced by Tim12 and thus the Tim9 molecules should be arranged in an alternating manner with Tim10/Tim12. To test this model in intact mitochondria, we used chemical crosslinking of the small Tim proteins at the TIM22 complex. In the alternating model, Tim9 has to be in association with Tim10 as well as Tim12. We observed two crosslinking products, Tim9–Tim10 and Tim9–Tim12 (Fig 2G),

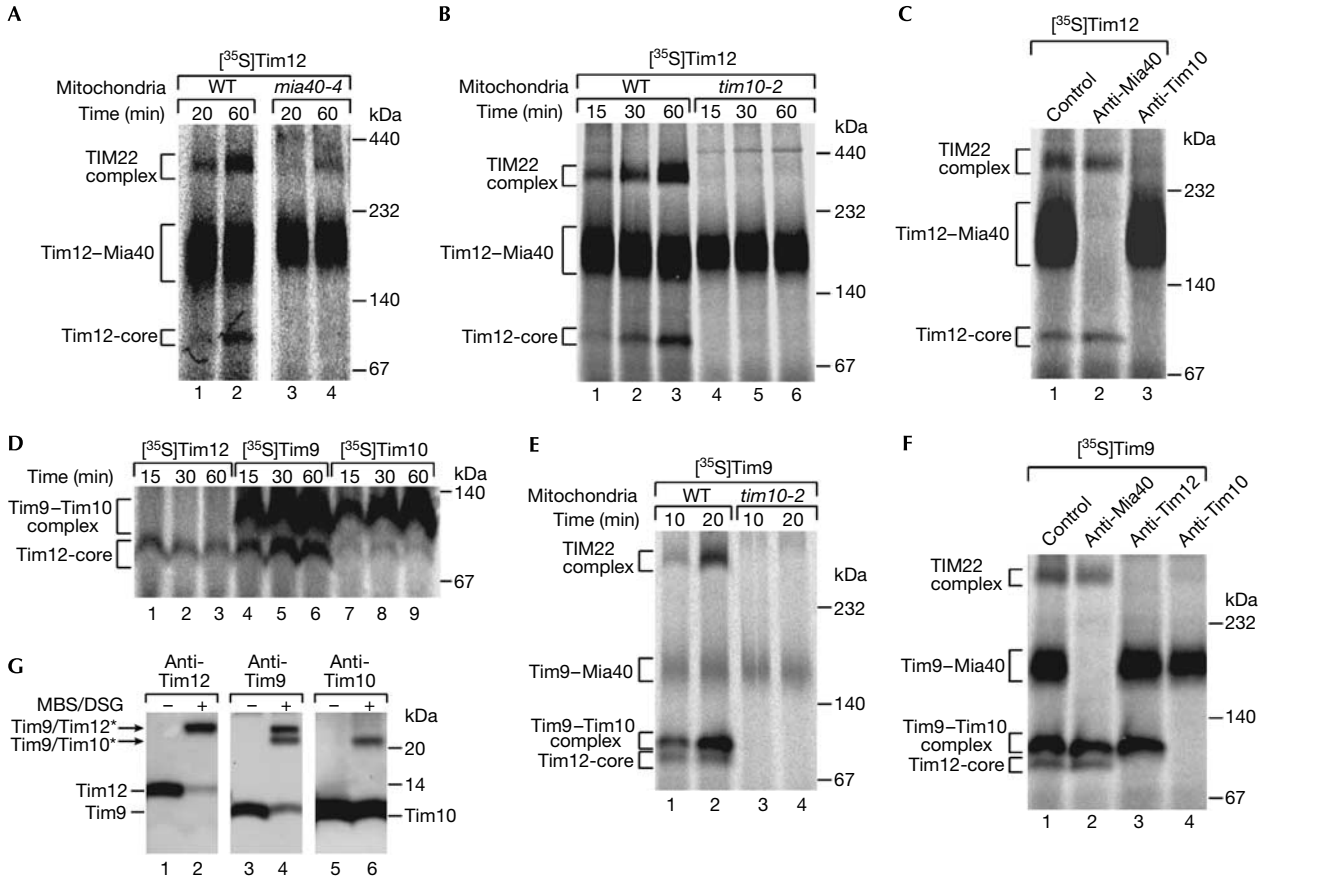

Fig 2 | The Tim12-core complex contains Tim9, Tim10 and Tim12. (**A**) Wild-type (WT) and *mia40-4* mitochondria (isolated from cells grown at 19 °C) were incubated with Tim12 at 30 °C. The mitochondria were lysed with digitonin and separated by BN-PAGE. (**B**) WT and *tim10-2* mitochondria (cells grown at 24 °C) were incubated with Tim12. (**C**) Tim12 was imported into WT mitochondria for 60 min at 30 °C. On lysis with digitonin, purified IgGs were added (15 min), followed by protein A–Sepharose (10 min). The supernatants were separated by using BN-PAGE. (**D**) Tim12, Tim9 and Tim10 were imported into WT mitochondria and analysed by BN-PAGE. (**E**) Tim9 was imported into WT and *tim10-2* mitochondria. (**F**) Tim9 was imported into WT mitochondria and treated as described for (**C**). (**G**) After incubation with MBS and DSG, $Tim18_{ProtA}$ mitochondria were solubilized with digitonin, subjected to IgG affinity chromatography, Tris–tricine SDS–polyacrylamide gel electrophoresis and immunodecoration. The asterisks indicate crosslinking products. BN-PAGE, blue native polyacrylamide gel electrophoresis; DSG, disuccinimidyl glutarate; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.

which supported this model. The stoichiometry of the Tim12-core was analysed by two methods—radiolabelled Tim proteins and standardized western blotting using purified small Tim proteins as references (Fig 2D; data not shown). The Tim9–Tim10 complex contains equal amounts of radiolabelled Tim9 and Tim10, in agreement with the 3:3 stoichiometry determined in the high-resolution structure (Webb *et al*, 2006), whereas in the Tim12-core Tim9 and Tim10 are present at a 3:1 ratio. Standardized western blotting of all three subunits of the Tim12-core at the TIM22 complex supported the conclusion of a stoichiometry of Tim9/Tim10/Tim12 at a ratio of 3:1:2.

Tim12-core is a soluble intermediate

To obtain further evidence that the Tim12-core is an import intermediate on the way to the TIM22 complex, we performed a pulse–chase analysis. The precursor of Tim12 was accumulated in mitochondria after a short-term import of 3 min, leading preferentially to the formation of the Mia40-intermediate. Subsequently, the mitochondria were re-isolated and incubated for up to 90 min. During this chase reaction, Tim12 was first observed in the Tim12-core and later in the mature TIM22 complex (Fig 3A), supporting the conclusion that the Tim12-core represents an intermediate stage in the assembly of the TIM22 complex.

To determine the intramitochondrial localization of Tim12-core, mitochondria containing radiolabelled Tim12 were treated with proteinase K. Tim12-core was not digested by the protease and thus behaved similarly to the mature TIM22 complex (Fig 3B), indicating that the small Tim proteins present in the Tim12-core were fully translocated into mitochondria.

The mature form of Tim12 in the TIM22 complex is stably associated with the inner mitochondrial membrane (Koehler *et al*, 1998b; Sirrenberg *et al*, 1998; Adam *et al*, 1999; Rehling *et al*, 2003). We investigated whether Tim12-core was membrane-associated or a soluble intermediate. We imported Tim12 into isolated mitochondria and opened the outer membrane by hypotonic swelling. The resulting mitoplasts were pelleted by

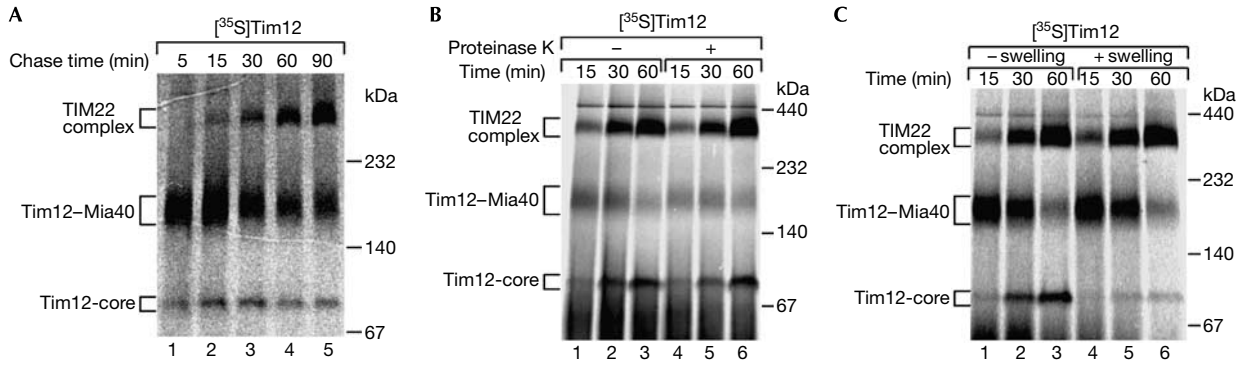

Fig 3 | The Tim12-core complex is released from the intermembrane space. (**A**) Tim12 was imported into wild-type (WT) mitochondria for 3 min at 25 °C. Mitochondria were re-isolated, incubated at 30 °C as indicated and analysed by using blue native polyacrylamide gel electrophoresis. (**B**) Tim12 was imported into WT mitochondria at 35 °C, followed by a treatment with 50 μg/ml proteinase K. (**C**) Tim12 was imported into WT mitochondria, followed by hypotonic swelling. Mitochondria and mitoplasts were pelleted.

centrifugation. The TIM22 complex and Mia40-intermediate remained in the mitoplast fraction, as expected (Chacinska *et al*, 2004); however, a major fraction of Tim12-core was released on swelling (Fig 3C), similar to the soluble Tim9–Tim10 complex (Chacinska *et al*, 2004). We conclude that Tim12-core represents a soluble intermediate on the biogenesis pathway of Tim12.

Tim9–Tim10 complex assembly does not require Tim12

To study the role of Tim12 in protein assembly, we generated conditional yeast mutants by using error-prone PCR, and selected the mutant strains *tim12-4* and *tim12-21*. Mitochondria were isolated and the protein levels were analysed by western blot analysis. Most of the proteins analysed, including various Tim proteins of the IMS and inner membrane, Mia40 and mtHsp70 (HSP for heat-shock protein), were present in similar amounts in wild-type and mutant mitochondria with the exception of Tim12 and Tim22, which were present in reduced amounts in the mutant mitochondria, *tim12-4* (Fig 4A; antibodies were generated against a Tim12 peptide that was not affected by the mutations). BN-PAGE showed that the endogenous TIM22 complex was present in reduced amounts in *tim12-21* mitochondria and almost completely absent in *tim12-4* mitochondria (Fig 4B). Import of the precursor of the ADP/ATP carrier to the assembled dimer (AAC_2) was impaired in the mutant mitochondria (Fig 4C), which was in agreement with the role of Tim12 in carrier import (Koehler *et al*, 1998a; Sirrenberg *et al*, 1998). As a control, we imported the β-subunit of the F_1F_o-ATP synthase, a model substrate of the presequence pathway. This preprotein was efficiently imported into both mutant mitochondria (Fig 4D), excluding that the mutants were generally defective in protein import.

We then imported the precursors of small Tim proteins. The wild-type precursor of Tim12 was efficiently imported into the mutant mitochondria and the formation of Tim12-core was increased compared with wild-type mitochondria (Fig 4E). In *tim12-4* mitochondria, radiolabelled Tim12 formed a TIM22 complex, although with reduced stability. In *tim12-21* mitochondria, the integration of radiolabelled Tim12 into the TIM22 complex was strongly increased compared with wild-type mitochondria (Fig 4E). How can the increased efficiency of complex formation in mutant mitochondria be explained? The assembly of individual subunits into multi-subunit complexes requires either the replacement of pre-existing subunits or the association with non-assembled subunits. Mutant complexes are more labilized and lead to larger pools of non-assembled subunits that can rapidly associate with imported wild-type precursors. As the formation of Tim12-core with wild-type Tim12 precursor was increased in both mutant mitochondria, we conclude that increased pools of the partner subunits Tim9 and Tim10 were available in the mutants. The mutant precursors Tim12-4 and Tim12-21 neither assembled into Tim12-core nor the TIM22 complex of wild-type mitochondria (Fig 4F). Thus, the mutant precursors were not able to compete with the pre-existing wild-type Tim12, confirming that the mutations affected the generation of Tim12-core and TIM22 complex.

To study whether the Tim12-core was also needed for the biogenesis of the soluble Tim9–Tim10 complex, we imported the precursor of Tim9. In *tim12-4* mitochondria, neither Tim12-core nor mature TIM22 complex were generated; in *tim12-21* mitochondria, Tim12-core and the mature TIM22 complex were formed, although with reduced stability (Fig 4G). However, Tim9 formed the Mia40-intermediate and the mature Tim9–Tim10 complex in all mitochondria (Fig 4G). The Tim9–Tim10 complex was efficiently generated in *tim12-4* mitochondria despite the lack of Tim12-core. We conclude that the biogenesis pathway of Tim9 is split before formation of Tim12-core. The association of Tim9 with the TIM22 complex, but not the formation of the Tim9–Tim10 complex, is dependent on the Tim12-core.

CONCLUSIONS

The small Tim proteins form essential chaperones in the mitochondrial IMS as well as at the inner membrane. However, it is not known how the soluble subunits of the IMS chaperone are converted into a membrane-bound form. We have analysed the biogenesis pathway of the precursor of Tim12 and found a new assembly intermediate. This Tim12-core complex changes the view of how the small Tim proteins are recruited to the TIM22 complex. Although it has been assumed that Tim12 is exclusively located at the inner membrane in a non-soluble form, and then recruits the soluble Tim9 and Tim10 subunits (Koehler *et al*, 1998a; Sirrenberg *et al*, 1998; Adam *et al*, 1999; Kovermann *et al*,

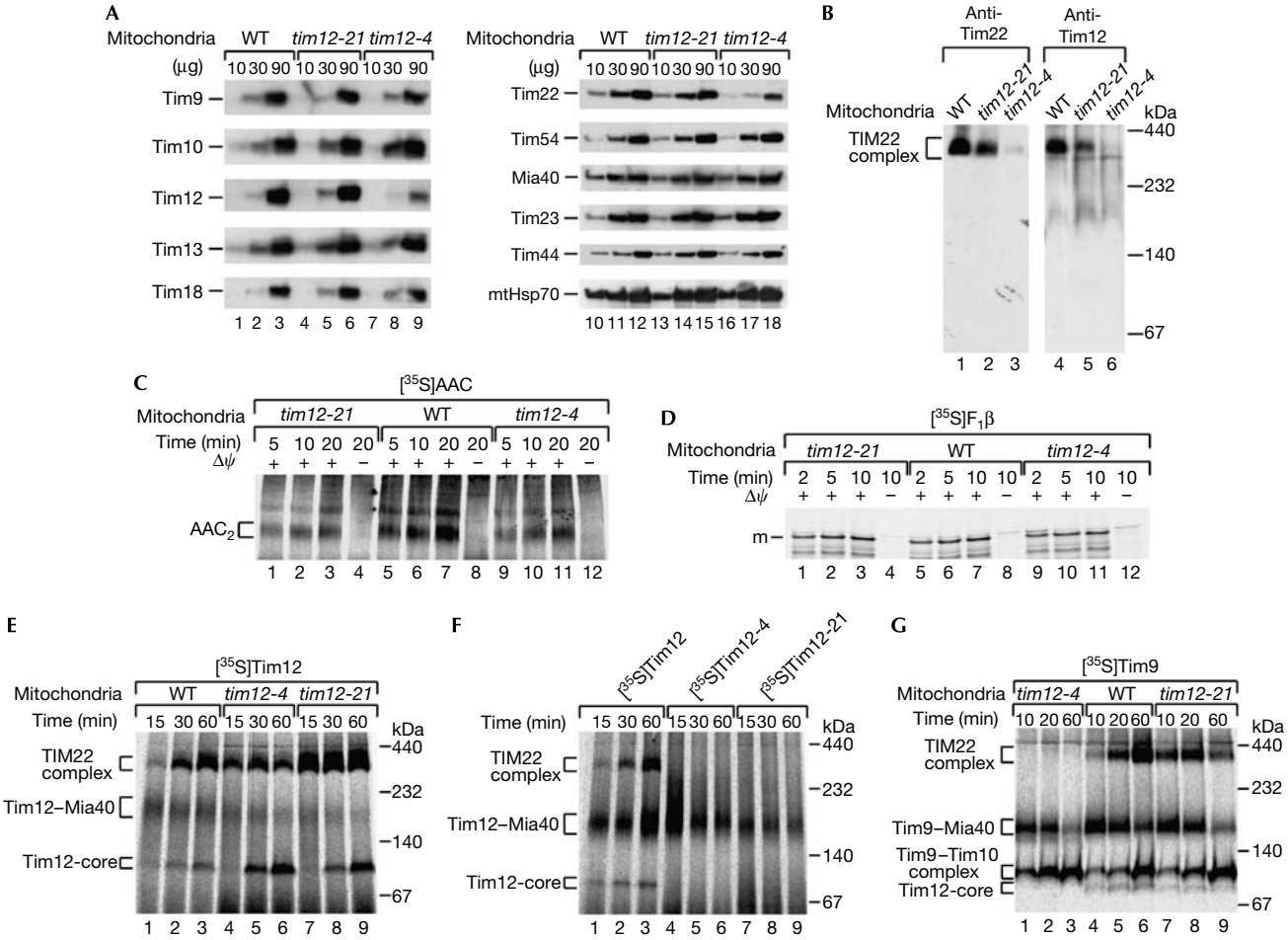

Fig 4 | Assembly of Tim proteins in *tim12* mutant mitochondria. (**A**) Mitochondria (μg protein) from wild-type (WT), *tim12-21* and *tim12-4* yeast grown at 24 °C were analysed by using Tris–tricine SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunodecoration. (**B**) Mitochondria were separated by BN-PAGE. (**C**) Mitochondria were incubated with ADP/ATP carrier (AAC) at 30 °C, followed by treatment with proteinase K and BN-PAGE. AAC_2, assembled dimer of AAC; $\Delta\psi$, membrane potential. (**D**) Mitochondria were incubated with $F_1\beta$, followed by treatment with proteinase K and SDS–PAGE. m, mature. (**E–G**) Tim12, Tim12 mutant precursors and Tim9 were imported into WT or indicated mutant mitochondria and analysed by BN-PAGE. BN-PAGE, blue native polyacrylamide gel electrophoresis; $F_1\beta$, F_1F_o-ATP synthase.

2002), we have shown that the three small Tim proteins first assemble into the soluble core complex before stable binding to the TIM22 complex. Thus, the conversion of Tim9–Tim10 to the membrane-bound form does not occur simply by binding of the soluble proteins to the mature inner membrane complex, rather the assembly of Tim9, Tim10 and Tim12 into the core complex must occur in the IMS. The preformed chaperone complex then docks onto the membrane complex.

METHODS

Yeast strains and growth conditions. The *Saccharomyces cerevisiae* strains YPH499 (wild type), *mia40-4*, *tim10-2*, *tim54*Δ and $Tim18_{ProtA}$ have been described previously (Kovermann *et al*, 2002; Truscott *et al*, 2002; Rehling *et al*, 2003; Chacinska *et al*, 2004). Conditional mutants of *TIM12* were generated by error-prone PCR (Truscott *et al*, 2002). The strains *tim12-21* (YPH-BG-tim12-1) and *tim12-4* (YPH-BG-tim12-4) (MATa *ade2-101 his3-200 leu2-1 ura3-52 trp1-63 lys2-801 tim12*::*ADE2* (pFL39-TIM12-1*ts* or pFL39-TIM12-4*ts*)) were selected. The mutations led to the following amino-acid changes: Tim12-4: I63K, N90S; Tim12-21: S7G, V14D, A22E, D64A. The strains were grown in YPG or YPS at 19–30 °C. Mitochondria were isolated by differential centrifugation.

Import of precursor proteins. Precursor proteins were synthesized in reticulocyte lysate in the presence of [35S]methionine. Import into isolated mitochondria was performed in the presence of 2 mM ATP, 2 mM NADH and an ATP-regeneration system (5 mM creatine phosphate and 0.1 mg/ml creatine kinase) in 3% (w/v) fatty acid-free BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 5 mM methionine, 2 mM KH_2PO_4 and 10 mM MOPS/KOH, pH 7.2, at 30–35 °C unless otherwise indicated. Where indicated, samples were treated with proteinase K (25–50 μg/ml) for 15 min on ice. To generate mitoplasts, mitochondria were incubated for 15 min in hypo-osmotic buffer (25 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2).

Miscellaneous. Isolation of the TIM22 complex from digitonin-lysed $Tim18_{ProtA}$ mitochondria by IgG affinity chromatography and elution with tobacco etch virus protease have been described previously (Rehling *et al*, 2003). Where indicated, TIM22 complex was eluted with 0.5% (v/v) Triton X-100. BN-PAGE and antibody-depletion assay were performed as described previously (Truscott *et al*, 2002). For crosslinking, mitochondria were incubated with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) or disuccinimidyl glutarate (DSG) (Pierce, Rockford, IL, USA) for 1 h on ice, followed by quenching with 50 mM Tris–HCl (pH 7.4) and 50 mM cysteine for 15 min.

ACKNOWLEDGEMENTS

We are grateful to M.T. Ryan and M.J. Baker (La Trobe University) for constructs and purified Tim proteins. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, Gottfried Wilhelm Leibniz Program, Max Planck Research Award, Alexander von Humboldt Foundation and the Fonds der Chemischen Industrie.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Adam A, Endres M, Sirrenberg C, Lottspeich F, Neupert W, Brunner M (1999) Tim9, a new component of the TIM22–54 translocase in mitochondria. *EMBO J* **18:** 313–319

Baud C, de Marcos-Lousa C, Tokatlidis K (2007) Molecular interactions of the mitochondrial Tim12 translocase subunit. *Protein Pept Lett* **14:** 597–600

Chacinska A, Pfannschmidt S, Wiedemann N, Kozjak V, Sanjuán Szklarz LK, Schulze-Specking A, Truscott KN, Guiard B, Meisinger C, Pfanner N (2004) Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. *EMBO J* **23:** 3735–3746

Davis AJ, Alder NN, Jensen RE, Johnson AE (2007) The Tim9p/10p and Tim8p/13p complexes bind to specific sites on Tim23p during mitochondrial protein import. *Mol Biol Cell* **18:** 475–486

Dekker PJT, Ryan MT, Brix J, Müller H, Hönlinger A, Pfanner N (1998) Preprotein translocase of the outer mitochondrial membrane: molecular dissection and assembly of the general import pore complex. *Mol Cell Biol* **18:** 6515–6524

Gentle IE *et al* (2007) Conserved motifs reveal details of ancestry and structure in the small TIM chaperones of the mitochondrial intermembrane space. *Mol Biol Evol* **24:** 1149–1160

Jensen RE, Johnson AE (2001) Opening the door to mitochondrial import. *Nat Struct Biol* **8:** 1008–1010

Koehler CM (2004) New developments in mitochondrial assembly. *Annu Rev Cell Dev Biol* **20:** 309–335

Koehler CM, Jarosch E, Tokatlidis K, Schmid K, Schweyen RJ, Schatz G (1998a) Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. *Science* **279:** 369–373

Koehler CM, Merchant S, Oppliger W, Schmid K, Jarosch E, Dolfini L, Junne T, Schatz G, Tokatlidis K (1998b) Tim9p, an essential partner subunit of Tim10p for the import of mitochondrial carrier proteins. *EMBO J* **17:** 6477–6486

Kovermann P, Truscott KN, Guiard B, Rehling P, Sepuri NB, Müller H, Jensen RE, Wagner R, Pfanner N (2002) Tim22, the essential core of the mitochondrial protein insertion complex, forms a voltage-activated and signal-gated channel. *Mol Cell* **9:** 363–373

Neupert W, Herrmann JM (2007) Translocation of proteins into mitochondria. *Annu Rev Biochem* **76:** 723–746

Rehling P, Model K, Brandner K, Kovermann P, Sickmann A, Meyer HE, Kühlbrandt W, Wagner R, Truscott KN, Pfanner N (2003) Protein insertion into the mitochondrial inner membrane by a twin-pore translocase. *Science* **299:** 1747–1751

Rehling P, Brandner K, Pfanner N (2004) Mitochondrial import and the twin-pore translocase. *Nat Rev Mol Cell Biol* **5:** 519–530

Sirrenberg C, Endres M, Fölsch H, Stuart RA, Neupert W, Brunner M (1998) Carrier protein import into mitochondria mediated by the intermembrane proteins Tim10/Mrs11 and Tim12/Mrs5. *Nature* **391:** 912–915

Truscott KN, Wiedemann N, Rehling P, Müller H, Meisinger C, Pfanner N, Guiard B (2002) Mitochondrial import of the ADP/ATP carrier: the essential TIM complex of the intermembrane space is required for precursor release from the TOM complex. *Mol Cell Biol* **22:** 7780–7789

Webb CT, Gorman MA, Lazarou M, Ryan MT, Gulbis JM (2006) Crystal structure of the mitochondrial chaperone TIM9–10 reveals a six-bladed α-propeller. *Mol Cell* **21:** 123–133