Lateral release of proteins from the TOM complex into the outer membrane of mitochondria

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The TOM complex of the outer membrane of mitochondria is the entry gate for the vast majority of precursor proteins that are imported into the mitochondria. It is made up by receptors and a protein conducting channel. Although precursor proteins of all subcompartments of mitochondria use the TOM complex, it is not known whether its channel can only mediate passage across the outer membrane or also lateral release into the outer membrane. To study this, we have generated fusion proteins of GFP and Tim23 which are inserted into the inner membrane and, at the same time, are spanning either the TOM complex or are integrated into the outer membrane. Our results demonstrate that the TOM complex, depending on sequence determinants in the precursors, can act both as a protein conducting pore and as an insertase mediating lateral release into the outer membrane.

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Introduction

The protein translocase of the outer membrane, the TOM complex, is the entry site for almost all mitochondrial proteins that are encoded by nuclear DNA and synthesized in the cytosol. The TOM complex comprises the receptors Tom20 and Tom70, the core components Tom40, Tom22, as well as three smaller proteins, Tom5, Tom6 and Tom7. The β -barrel protein Tom40 likely forms the pore of the TOM complex which represents a protein conducting channel (Hill *et al.*, 1998; Künkele *et al.*, 1998; Rapaport, 2005; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009). Currently, it is not known whether the TOM pore has also the capacity to open laterally, resulting in release of proteins

into the outer membrane. In fact, the mechanism of insertion of outer membrane proteins has largely remained an open question (Neupert and Herrmann, 2007; Walther and Rapaport, 2009; Dukanovic and Rapaport, 2011). This process has a certain degree of complexity, since outer membrane proteins fall into different classes. Many of these proteins have single transmembrane segments but there are also a number of proteins spanning the outer membrane twice or several times. Some single spanning proteins are integrated with the C-terminus facing the cytosol and the N-terminus facing the intermembrane space (IMS), others in the opposite orientation (Walther and Rapaport, 2009).

In the present study, we have addressed the question whether the TOM complex is competent for lateral release of certain precursor polypeptides into the lipid phase of the outer membrane. To this end, we took advantage of a rather unusual property of yeast Tim23, an essential subunit of the TIM23 translocase in the inner mitochondrial membrane (Dekker *et al.*, 1993; Emtage and Jensen, 1993; Ryan and Jensen, 1993; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009). Three parts of Tim23 can be distinguished: (i) The C-terminal part of the protein (residues 97–222) consists of four predicted transmembrane helices that anchor the protein in the inner membrane and form a presequence conducting, membrane potential-sensitive channel (Bauer *et al.*, 1996; Truscott *et al.*, 2001; Alder *et al.*, 2008). (ii) The rather hydrophilic and negatively charged part of Tim23 (residues 51–96) is located in the IMS where it serves as part of the preprotein receptor site of the TIM23 complex (Bauer *et al.*, 1996; Gevorkyan-Airapetov *et al.*, 2009; Mokranjac *et al.*, 2009; Tamura *et al.*, 2009). (iii) The N-terminus of yeast Tim23 (residues 1–50) is able to reversibly insert into the outer mitochondrial membrane despite the absence of a canonical α -helical transmembrane segment (Donzeau *et al.*, 2000). The exposure of the N-terminal part of Tim23 was found to depend on the presence of Tim50 and on the translocation activity of the TIM23 complex (Popov-Čeleketić *et al.*, 2008a; Yamamoto *et al.*, 2002; Tamura *et al.*, 2009). Tim23 itself is imported via the TOM/TIM22 pathway in analogy to mitochondrial carrier proteins (Kerscher *et al.*, 1997; Káldi *et al.*, 1998; Davis *et al.*, 2000; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009).

In order to address the question of lateral release from the TOM pore, we employed fusion constructs of GFP with Tim23 (GFP–Tim23). When expressed in cells in which the TIM23 gene was deleted, the GFP domain was present on the mitochondrial surface and the protein inserted into the outer membrane. As we show here, however, this was only true when the cells were grown at 24°C; at 30 and 37°C, the GFP domain was partly or completely localized to the IMS. Furthermore, we studied the targeting of GFP–Tim23 in which the N-terminal 20 residues of Tim23 were replaced either by a random hydrophilic segment of an outer membrane protein, Tom22, or by transmembrane segments of outer membrane proteins spanning the membrane once,

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Mim1 and Tom22, both in a C-in and N-out orientation. When replacement was by the hydrophilic segment, no release from the TOM channel was observed. In contrast, when replacement was by the transmembrane segments, the fusion proteins were present in the outer membrane and had left the TOM channel. Our results demonstrate that the TOM complex can open laterally to release proteins provided a segment is present in these proteins that can insert into the membrane. We suggest that in case of the fusion proteins studied, the rate of folding/unfolding of the GFP moiety is determining the rate of translocation versus lateral release. Furthermore, our results show that GFP as a passenger protein can be a useful tool for studying important aspects of mechanisms and energetics of translocation of proteins across membranes.

Results

The topology of GFP-Tim23 depends on the growth conditions

It was previously found that the fusion protein GFP-Tim23, in which GFP is present at the N-terminus of full-length Tim23, was anchored to both outer and inner mitochondrial membranes (Vogel *et al*, 2006). This two membrane spanning topology raised several intriguing questions, such as where and how this fusion protein traverses the outer membrane, and why the GFP domain was not translocated like the many other mitochondrial proteins that are anchored to the inner membrane and extend large domains into the IMS. We expressed GFP-Tim23 in cells in which the *TIM23* gene was deleted. In the isolated mitochondria the GFP-Tim23 fusion protein and no wild-type Tim23 was detectable (Figure 1). When the mitochondria were incubated with proteinase K only about one half of the GFP-Tim23 was cleaved whereas the other half remained uncleaved. Three cleavage products of 25, 23 and 13 kDa were generated by this protease treatment, as visualized by immunodecoration with antibodies against the C-terminal sequence of Tim23. The first two of these bands corresponded in size to full-length Tim23, and a fragment lacking ca. 20 amino-acid residues which was expected on the basis of the previously reported clipping of authentic Tim23 in intact mitochondria (Donzeau *et al*, 2000). The weak 13 kDa band represents the

membrane part of Tim23 which is formed in the small amount of mitochondria with an open outer membrane. When the mitochondria were treated with proteinase K and then removed by centrifugation, the resulting supernatant contained intact GFP (not shown, but see Supplementary Figure S2). Hypo-osmotic swelling was applied to rupture the outer membrane and obtain access to the IMS. Under these conditions, GFP-Tim23 was also efficiently cleaved and the only product was the 13-kDa C-terminal part of Tim23 that spans the inner membrane. Controls with marker proteins for the various mitochondrial subcompartments demonstrated the reliability of these localization experiments.

We then addressed potential causes for the ambiguous distribution of the GFP domain between the surface of the mitochondria and the IMS. As variable parameters, we tested the influence of growth temperature and carbon source for growth of the cells. Upon growth at 24°C, GFP-Tim23 was almost completely susceptible to cleavage by added protease. When the cells were grown at 37°C, however, GFP-Tim23 was not susceptible at all and after growth at 30°C an intermediate situation was observed (Figure 2A). This effect was stronger when cells were grown in lactate medium instead of glucose (Figure 2B). To confirm that the insusceptibility to protease upon growth at 37°C was due to localization to the IMS and not to aggregation of GFP-Tim23 at the mitochondrial surface, we performed proteinase K treatment of intact mitochondria, of mitochondria in which the outer membrane was opened (mitoplasts) and of detergent lysed mitochondria. Indeed, the fusion protein was degraded efficiently with mitoplasts and lysed mitochondria, but not with intact mitochondria (Supplementary Figure S1A). Notably, the topology of the N-terminus of wild-type Tim23 did not depend on the growth conditions. Only small differences in the percentage of cleavable Tim23 were observed. Also, there was no significant dependence on growth temperature or growth medium (Figure 2C and D; see also Supplementary Figure S1B).

In conclusion, the GFP domain representing the N-terminal half of GFP-Tim23 can adopt alternative topologies depending on the growth temperature and the composition of the growth medium (Figure 2E). This dependency is due to the GFP moiety of the fusion protein and not due to an intrinsic property of Tim23.

Figure 1 The N-terminal GFP domain of GFP-Tim23 expressed under standard conditions is partly located on the mitochondrial surface and in the IMS. Strain W303ΔTim23 harbouring plasmid pRS315-GFP-TIM23 with the endogenous *TIM23* promoter was grown in YP liquid medium containing 2% glucose at 30°C until an OD_{600 nm} of 0.5 was reached. Mitochondria were isolated, treated as indicated and subjected to SDS-PAGE and western blotting. The calculated molecular masses of GFP-Tim23, Tim23 and the C-terminal inner membrane segment of Tim23 are 51, 23 and 13 kDa, respectively. PK, proteinase K; SW, swollen mitochondria; TX-100, Triton X-100; α, antibodies against the respective proteins; IM, inner mitochondrial membrane; IMS, intermembrane space; OM, outer mitochondrial membrane.

Figure 2 The submitochondrial localization of the N-terminus of GFP-Tim23 depends on the growth conditions. Strain W303ΔTim23 harbouring plasmid pRS315-GFP-TIM23 with the endogenous TIM23 promoter and, as control, wild-type strain W303 were grown in YP liquid medium containing 2% glucose or lactate medium at 24, 30 and 37°C to an OD_{600 nm} of ~0.7. Mitochondria were isolated and analysed as in Figure 1. Proteolytic treatment of mitochondria to analyse the presence or absence of the GFP domain on the surface (clipping) was performed with mitochondria from cells grown on YP and lactate medium and are shown in (A) and (B), respectively. Mitochondria from wild type were analysed in parallel (C, D) (see also Supplementary Figure S1B). (E) Depicts the influence of the growth conditions on the topology of GFP-Tim23.

The topology of GFP-Tim23 and the growth phenotype of cells are correlated

The growth phenotype of yeast cells expressing GFP-Tim23 at different temperatures under the control of the endogenous promoter or the strong *TPI* promoter was analysed on agar plates and in liquid medium. On agar plates (Figure 3A), wild-type cells and cells expressing GFP-Tim23 were growing at both 24 and 30°C at very similar rates. At 37°C, however, growth of GFP-Tim23 expressing cells was strongly reduced, in particular, on lactate medium and when GFP-Tim23 was overexpressed in relation to wild-type Tim23. These observations were confirmed by growth in liquid media (Figure 3B). In summary, localization of the GFP moiety to the IMS parallels impaired growth.

To explain the observed growth phenotype, we first determined the topology of GFP-Tim23 when overexpressed. The same temperature dependence was observed for overexpressed GFP-Tim23[↑] as for GFP-Tim23 expressed from the endogenous promoter (Supplementary Figure S2). Then, we checked the effect of expression of GFP-Tim23 on mitochondrial protein composition. The levels of Tim17, Tom40, Tom20, aconitase and the ADP/ATP carrier did not vary with the topology of GFP-Tim23 (Figure 4). The ratio between wild-type Tim23, upregulated GFP-Tim23[↑]:24°C, GFP-Tim23:24°C (both with their GFP domain facing the cytosol) and GFP-Tim23:37°C (with its GFP domain in the IMS) under control of the endogenous promoter was roughly 3:9:1:1. Interestingly, overexpression of GFP-Tim23 at 24°C led to a specific increase of Tim17, suggesting that the expression of Tim17 or the turnover of Tim17 is regulated by a feedback mechanism depending on the level of Tim23.

Functionality of mitochondrial protein import as a criterion for the topology of GFP-Tim23

How do topology and expression levels of GFP-Tim23 affect mitochondrial protein import? Import of none of several precursors that are representative of the various import pathways was altered when the GFP moiety faced the cytosol (GFP-Tim23:24°C; Figure 5A–E). In contrast, when the GFP moiety was located in the IMS, the amount of all imported proteins was strongly reduced as compared with the precursors imported into wild-type mitochondria (GFP-Tim23:37°C). The import defect was similar among precursors using the TOM/TIM23 pathway (Figure 5A and B) and was more pronounced than for precursors that are imported in a TIM23-independent manner (Figure 5C–E).

We conclude that GFP-Tim23 as a subunit of the TIM23 complex and the TOM complex are fully functional when the GFP moiety at the N-terminus faces the cytosol. When present in the IMS, the GFP moiety might prevent proper interactions of TIM23 components, such as that of Tim50 with the IMS domain of Tim23, or obstruct the TOM pore from the inside (Figure 5F). Then, this would be detrimental but not lethal (cf. Figure 3) because Tim23, and therefore GFP-Tim23 (cf. Figure 4), is less abundant than the TOM complex (Dekker *et al*, 1997; Sirrenberg *et al*, 1997). Accordingly, the remaining free TOM complexes would still be used for import of substrates (Figure 5F).

Next, we studied the effect of the overexpression of GFP-Tim23 on mitochondrial import. With mitochondria containing GFP-Tim23[↑]:24°C import of all precursors tested was comparable to that in wild-type mitochondria (Figure 5G–K).

Figure 3 Growth phenotype of *GFP-TIM23* expressing strains. Wild-type W303 (WT) and strain W303Δ*Tim23* harbouring either plasmid pRS315-*GFP-TIM23* with the endogenous *TIM23* promoter (*GFP-Tim23*) or plasmid pYX242-*GFP-TIM23* under the control of a *TPI* promoter to achieve overexpression of *GFP-Tim23* (*GFP-Tim23*↑) were cultured at 24, 30 and 37°C in YP liquid medium containing 2% glucose or lactate liquid medium. Each culture was subcultured 3–4 times in the logarithmic growth phase. (A) Liquid cultures were diluted to an OD_{600 nm} of 0.3. Dilution series (1:10, 1:100, 1:1000 and 1:10000) were prepared and 3 μl of each dilution was spotted on either YP or lactate agar plates and incubated for 36 or 90 h, respectively. (B) Alternatively, liquid cultures were diluted to an OD_{600 nm} of 0.2 and the optical density was recorded upon further growth at the respective temperatures. *GFP-Tim23* containing strains that were grown at 37°C on lactate medium were only diluted to an OD_{600 nm} of 0.6 since at lower OD the doubling time was strongly increased. WT, closed circles; *GFP-Tim23*, closed squares; *GFP-Tim23*↑, open triangles.

Figure 4 Protein content of *GFP-TIM23* expressing cells. Mitochondria were purified from wild-type strain W303 (WT) and strain W303Δ*Tim23* harbouring either plasmid pYX242-*GFP-TIM23* under control of the *TPI* promoter (*GFP-Tim23*↑:24°C) or plasmid pRS315-*GFP-TIM23* under control of the endogenous *TIM23* promoter (*GFP-Tim23*:24°C) on lactate medium at 24°C. Strain W303Δ*Tim23* containing the pRS315 construct was also grown at 37°C (*GFP-Tim23*:37°C). The protein content was compared by SDS-PAGE and western blot analysis (using the indicated antibodies) and was quantified using shorter exposure times.

This result is surprising. *Tim23* itself is imported via the TOM/*Tim22* pathway and *GFP-Tim23*, therefore, passes through the TOM pore (except the GFP moiety remaining outside). If the outer membrane spanning segment of upregulated *GFP-Tim23* was still located in the TOM pore (after the assembly of the C-terminal part of *GFP-Tim23* in the inner membrane), the majority of the TOM pores would be blocked (Figure 5L). Pronounced defects in protein import are to be expected when *GFP-Tim23* is about three-fold

more abundant than *Tim23* in wild-type mitochondria (cf. Figure 4). Considering the absence of import defects as shown in Figure 5G–K, we conclude that *GFP-Tim23*:24°C crossing both membranes is not any longer present in the TOM pores. To strengthen this conclusion, we performed *in vitro* import experiments using chemical amounts of recombinantly expressed matrix-targeted precursor of Su9-DHFR (Supplementary Figure S3). Again, a reduction of import compared with wild-type mitochondria was not detectable with mitochondria expressing *GFP-Tim23*↑:24°C or *GFP-Tim23*:24°C. In contrast, import was strongly impaired when the GFP moiety was localized to the IMS (*GFP-Tim23*:37°C; Supplementary Figure S3).

These results suggest that *GFP-Tim23* leaves the TOM complex during the import process. This is likely to happen by lateral opening of the TOM pore and release of the TOM pore-spanning segment into the outer membrane.

Lateral release of *GFP-Tim23* from the TOM complex depends on the sequence of the N-terminal segment present on *Tim23*

If the outer membrane spanning segment of *GFP-Tim23*:24°C was indeed released from the TOM complex, it should not be possible to co-isolate components of the TOM complex with components of the *TIM23* complex. This was actually the case; neither *Tom40* nor other TOM proteins were found in association with the Ni-NTA bound fraction of His6-*GFP-Tim23* (Figure 6A). On the other hand, components of the *TIM23* complex were successfully co-isolated. If the N-terminal segment of *GFP-Tim23*:24°C was responsible for the lateral release from the TOM complex, replacing this segment with an unrelated hydrophilic sequence should lead to trapping of the protein in the pore. In fact, *Tom40* and *Tom22* were co-purified upon expression at 24°C of a construct in which the N-terminal 20 residues of *GFP-Tim23* were replaced by a hydrophilic sequence (*GFP-Tom22*_{SOL}-Δ20*Tim23*; Figure 6B). Moreover, increased expression of the latter construct significantly increased the amount of TOM complex pulled down (data not shown). In contrast, when the first 20 residues of *Tim23* were replaced

by the hydrophobic transmembrane segment of Tom22 or Mim1 (GFP-Tom22^{TMS}- Δ 20Tim23 and GFP-Mim1^{TMS}- Δ 20Tim23), the amounts of co-purified Tom40 and Tom22 were much lower (Figure 6C and D) than those co-purified with the trapped construct (cf. Figure 6B). Figure 6E shows a quantification of the results. The folded GFP moiety of all these constructs was present at the outer surface of the outer membrane (Supplementary Figure S4A). The steady-

state levels of the various GFP-Tim23 constructs were comparable to the exception of GFP-Tom22^{sol}- Δ 20Tim23, which was somewhat lower (Supplementary Figure S4B). Moreover, intact TOM complex was present in all strains in similar amounts (Supplementary Figure S4C). These results strongly support the conclusion that lateral release did occur. To further confirm these results, we performed protein import experiments using recombinantly expressed

Su9(1-69)-DHFR. Mitochondria containing GFP-Tom22^{SOL}- Δ 20Tim23:24°C showed a strong import defect compared with wild-type mitochondria, whereas no import defect was detectable in GFP-Mim1^{TMS}- Δ 20Tim23:24°C mitochondria (Figure 6F).

In summary, these results suggest that the TOM complex is competent for releasing the N-terminus of Tim23 in a lateral manner into the outer membrane. Furthermore, lateral release from the TOM pore does not seem to be restricted to the N-terminus of Tim23 but can take place with transmembrane segments, at least of those proteins investigated here, Mim1 and Tom22, which are authentic outer membrane components.

Discussion

In this study, we have addressed the question as to whether precursor proteins imported into mitochondria can be laterally released into the outer membrane from the TOM complex. To our knowledge, this study is the first to prove that indeed lateral release can occur. The ability of the TOM complex to open laterally has a number of important implications. It bears relevance to the organization and dynamics of the TOM complex and to the mechanism of topogenesis of mitochondrial proteins. In addition, it relates to the interaction of outer and inner membranes and the interaction of TOM and TIM complexes.

For our analysis, we have used fusion proteins of GFP with Tim23, a component of the TIM23 protein translocase. Tim23 was shown before to contain an N-terminal sequence that can reversibly be exposed on the surface of the outer membrane. GFP was fused to the N-terminus of Tim23 to halt translocation when the folded GFP domain meets the TOM complex. Surprisingly, the GFP domain turned out to be present either on the mitochondrial surface or in the IMS, depending on the growth conditions of the cells. The main factor determining these alternative topologies is temperature of growth, another, minor one, is composition of the growth medium. There are at least three, mutually not exclusive, explanations: (i) The rate of folding of the GFP domain of the precursor in the cytosol is lower than the rate of import at 37°C, but not at 24°C. (ii) The rate of unfolding of GFP is higher at higher temperature, due to an increased spontaneous unfolding (breathing). (iii) GFP is 'pulled in' by lateral movement of the membrane part of Tim23 in the inner membrane relative to the TOM complex in the outer membrane and this lateral movement is more efficient at higher temperatures. The

topological distribution of the GFP domain of GFP-Tim23 between mitochondrial surface and IMS could well depend on the rate of passage of the precursor across the outer membrane. A longer presence in the TOM channel could lead to a higher rate of lateral release. Likewise, the rate of folding/unfolding of GFP could influence the distribution. This explanation might pertain to the observed higher rate of lateral release in cells growing more slowly on lactate as carbon source.

Lateral release from the TOM complex most likely depends on particular sequence elements of the precursor proteins. Apparently, such an element is present at the N-terminus of Tim23. When this sequence was exchanged by an unrelated hydrophilic sequence of an outer membrane protein there was no release, and, as to be expected, this construct was arrested in the TOM channel under conditions, when the GFP domain was folded. In contrast, if the sequence was exchanged by the transmembrane domain of Tom22 or Mim1, there was release from the TOM channel. Structure prediction programs for the N-terminal 20 residues of Tim23 suggest β -structural elements rather than an α -helical conformation as it is the case with the transmembrane domains of Tom22 and Mim1. Sequence similarity to Tom22 and Mim1 could not be recognized. Tom22 and Mim1 carry flanking sequences that are negatively charged and might perhaps play a role. Altogether, it is an open question whether there is a specific signal or whether segregation into the outer membrane or a protein lipid interface in the outer membrane is a possible mechanism for lateral release.

Our findings have intriguing implications on possible mechanisms of TOM complex function (Figure 6G). Translocation of precursor proteins might occur through pores of single Tom40 subunits. Such a mechanism would imply opening of the β -barrel structure. Conceptually, the reaction that leads to closing of a β -barrel ring structure during its biogenesis could be a reversible one, depending on the free energies which are involved in the hydrogen bonding between the N- and C-terminal β -strands of the ring. Whether this is a realistic scenario remains open. It seems also possible that several Tom40 subunits, perhaps in conjunction with other components of the TOM complex, are forming the channel. The TOM complex is a large structure which according to electron microscopy data contains two pores (Künkele *et al*, 1998; Model *et al*, 2002). This would then favour a mechanism by which rings made up of β -barrel and possibly other subunits can open. Interestingly, previous experiments have shown that TOM channels can exchange

Figure 5 Influence of the GFP topology on the kinetics of protein import. (A–F) Import of proteins into mitochondria from cells grown at 37°C, but not at 24°C, is impaired. Wild-type cells W303 (WT) and cells from W303 Δ Tim23 harbouring plasmid pRS315–GFP–TIM23 under control of the endogenous TIM23 promoter were grown on lactate medium at 24 or 37°C. Mitochondria were isolated and import of radiolabelled precursor proteins was performed. Control, 50% of total lysate (left panels). Averages of three import reactions are shown. Error bars represent the standard deviation of the three experiments (right panels). (A) Su9(1-69)-DHFR, targeted to matrix; (B) Dld1, IM; (C) AAC, IM; (D) CCHL, IMS; (E) porin, OM. Wild-type Tim23, open circles; Tim23–GFP:24°C, open triangles; Tim23–GFP:37°C, open squares. (F) Models of disturbed Tim23 function in GFP–Tim23 whose GFP moiety is present in the IMS (37°C). Upper panel: Disturbed Tim23–Tim50 interaction. Lower panel: Block of a subpopulation of the TOM complexes. Due to the excess of the TOM complex over Tim23, a fraction of pores is still functional, but overall import is reduced. (G–L) The TOM pore is open in GFP–Tim23 overexpressing cells grown at 24°C. Wild-type strain W303 (WT) and strain W303 Δ Tim23 harbouring plasmid pYX242–GFP–TIM23 under control of the TPI promoter (GFP–Tim23[↑]) were grown on lactate medium at 24°C and import experiments with isolated mitochondria were performed in parallel with the experiments in Figure 5A–E. Control, 50% of total lysate (left panels). Averages of three import reactions are shown. Error bars represent the standard deviation of the three experiments (right panels). (G–K) Su9(1-69)-DHFR, Dld1, AAC, CCHL and porin. Wild-type Tim23, open circles; overexpressed GFP–Tim23[↑]:24°C, closed triangles. (L) Model of possible topologies of GFP–Tim23 in cells grown at 24°C. Vertical arrows indicate vacant TOM pores. Upper panel: GFP–Tim23 occupies the majority of the TOM channels. Lower panel: GFP–Tim23 is laterally released from the TOM pore. The results strongly support the latter model.

Figure 6 Replacement of the N-terminal segment of Tim23 in GFP-Tim23. (**A–D**) Mitochondria were isolated from W303ΔTim23 cells expressing His6-GFP-Tim23, His6-GFP-Tom22sol-Δ20Tim23, His6-GFP-Tom22TMS-Δ20Tim23 or His6-GFP-Mim1TMS-Δ20Tim23 as well as the corresponding untagged proteins as controls. Cells were grown on lactate medium at 24°C. The various constructs are schematically shown on top of each panel. T, total; S, supernatant fraction; B, bound/eluate fraction. Sequences replacing the 20 N-terminal residues of Tim23: SOL, 35 residues from the hydrophilic (soluble) part of Tom22; TMS, transmembrane sequence of Tom22 or Mim1 (34 and 39 residues, respectively). Arrow head, crossreaction of Tom70 antibody. (**E**) Quantification of co-isolated Tom40 with the indicated fusion proteins by binding to Ni-NTA matrix. (**F**) Import of recombinantly expressed Su9(1-69)-DHFR-His6 into mitochondria of W303 WT, GFP-Tom22sol-Δ20Tim23 and GFP-Mim1TMS-Δ20Tim23 cells grown on lactate medium at 24°C. (**G**) Hypothetical models of alternative mechanisms of pore opening of the TOM complex leading to release of precursor proteins into the outer membrane. Left panel, opening of N- and C-terminal β-strands of a Tom40 subunit. Right panel, opening of a channel formed by several Tom40 subunits. Please note that the depicted β-barrel subunits are just a schematic representation for visualization and do not reflect an actual molecular model of Tom40.

subunits and thus represent dynamic structures, in equilibrium with transiently open forms (Rapaport *et al*, 2001). Affinity of the hydrophobic segments in the precursors

for the hydrophobic lipid phase of the outer membrane might determine the degree of release by equilibrium distribution.

Translocation into the IMS, on the other hand, is mechanistically different from translocation across the inner membrane into the matrix. The latter process also works at lower temperatures, even at 5–10°C (Gaume *et al*, 1998), most likely due to the activity of the import motor which, driven by ATP hydrolysis, has the capacity to bind firmly to incoming segments of precursor proteins. During topogenesis of Tim23, its C-terminal segment is inserted into the inner membrane by the TIM22 translocase, a process powered by the membrane potential. Then, translocation of the N-terminus seems to be 'passive'. When GFP is fused to the N-terminus of Tim23, C-terminal segments of GFP that are unfolded due to thermal breathing can slide through the TOM complex. To achieve vectorial movement, however, a so far unknown component in the IMS, other than the mtHsp70 machinery, would be required. On the other hand, lateral movement of the transmembrane domain of Tim23 in the inner boundary membrane might represent a driving force. Whether such lateral diffusion by itself would be sufficient to support translocation across the outer membrane is an open question. Likewise, the mechanism of driving the passage of proteins of up to 70 kDa across the outer membrane is also not clear. Those proteins are anchored in the inner membrane by an α -helical segment close to the N-terminus, exposing large domains into the IMS. In these cases, a possible ratchet would have to rely on the fixation of the N-terminal part in the inner membrane.

The results presented here also have interesting implications on the problem of how proteins or a subclass of proteins of the outer membrane of mitochondria are inserted into the lipid bilayer. The answer to this question is complex, since different subclasses of outer membrane proteins appear to follow different pathways. Precursors of proteins spanning the outer membrane once, either with the C-terminus or the N-terminus in the IMS, such as Tom22 and Tom20, respectively, have been shown to use the receptors of the TOM complex (Schneider *et al*, 1991; Keil and Pfanner, 1993; Yamano *et al*, 2008). It seems not unlikely that, as other precursors, they are subsequently directed to the TOM channel. Lateral release by opening of the channel then would be necessary for membrane insertion.

A question related to the insertion of membrane proteins regards the mechanism of insertion of the N-terminus of authentic Tim23 into the outer membrane. At least two pathways for this insertion process can be imagined. First, the N-terminus of Tim23 inserts directly, perhaps like membrane insertion of some viral proteins to initiate fusion with the attacked host cell (Harrison, 2008). Alternatively, the N-terminus has the ability to enter the TOM channel from the IMS side, possibly with the help of Tim50 and Tom22, to undergo lateral release. The latter explanation might be more realistic not only in view of the results presented here but also in view of the reported requirement for interaction of Tim50 and the IMS domain of Tim23 (Popov-Čeleketić *et al*, 2008a; Yamamoto *et al*, 2002; Tamura *et al*, 2009).

Finally, our results bear relevance to the use of GFP-fusion proteins for localization studies in general. If folding of the GFP domain in the cell is a process that depends on parameters such as growth rate, temperature or perhaps metabolic conditions, GFP-fusion proteins might well be useful tools to study alternative targeting *in vivo* and *in vitro*.

Materials and methods

Cloning of GFP-TIM23 constructs

The GFP sequence in all constructs was identical to the sequence of wild-type GFP with the exception of residues Thr65 and Arg80 (Tsien, 1998). The Tim23 sequence was identical to the SGD entry Ynr017wp. Plasmids were amplified in *E. coli* strain X11Blue and selected with ampicillin. All constructs were checked by restriction analysis and sequencing of both strands.

Cloning of GFP-fusion constructs of wild-type Tim23: Plasmid pRS315-GFP-TIM23 encoding GFP-(GGSAGAGS)-Tim23 under the control of the endogenous TIM23 promoter was generated previously (Vogel *et al*, 2006). Plasmid pRS315-HIS6-GFP-TIM23 encoding His6-GFP-(GGSAGAGS)-Tim23 under the control of the endogenous TIM23 promoter was cloned in two steps. First, the TIM23 promoter (proTim23), comprising 564 bp upstream the start codon, was cloned into pRS315 (Sikorski and Hieter, 1989) using the *SacI* and *XmaI* restriction sites. Then, HIS6-GFP-TIM23 was PCR amplified using a 6 × His-tag encoding primer, and the PCR product was cloned into the *XmaI* and *HindIII* restriction sites of pRS315 containing the endogenous TIM23 promoter. Plasmid pYX242-GFP-TIM23 encoding GFP-(GGSAGAGS)-Tim23 under the control of a TPI promoter was cloned using the *HindIII* and *XhoI* restriction sites of pYX242 (Novagen).

Cloning of GFP-fusion replacement constructs encoding N-terminally truncated versions of Tim23 with the transmembrane segments of Tom22 and Mim1 (TMS) or a soluble segment of Tom22 (SOL): GFP was inserted into pDrive (Qiagen) using the restriction sites *KpnI* and *PstI*. Sequences encoding the TMS of Tom22 (residues 92–126, Tom22^{TMS}), the TMS of Mim1 (residues 29–68, Mim1^{TMS}) or a soluble segment of Tom22 (residues 22–57, Tom22^{SOL}) were PCR amplified and cloned into pDrive-GFP via the *PstI* and *SacI* restriction sites. To prevent oligomerization of the fusion protein containing Mim1^{TMS}, two point mutations (Gly63 → Ile and Ala67 → Ile) were introduced by PCR (Popov-Čeleketić *et al*, 2008b). TIM23 lacking the first 20 amino-acid residues (Δ 20TIM23) was PCR amplified and subsequently inserted using the restriction sites *SacI* and *NotI*. The pDrive constructs GFP-Tom22^{TMS}- Δ 20TIM23, GFP-Mim1^{TMS}- Δ 20TIM23 and GFP-Tom22^{SOL}- Δ 20TIM23 were used as PCR templates for cloning into pRS315 containing the endogenous TIM23 promoter (see above). The PCR was performed with 5' primers with or without 6 × His-tag encoding segment, and the product was inserted via the *XmaI* and *HindIII* restriction sites resulting in plasmids pRS315-GFP-Tom22^{TMS}- Δ 20TIM23, pRS315-HIS6-GFP-Tom22^{TMS}- Δ 20TIM23, pRS315-GFP-Mim1^{TMS}- Δ 20TIM23, pRS315-HIS6-GFP-Mim1^{TMS}- Δ 20TIM23, pRS315-GFP-Tom22^{SOL}- Δ 20TIM23 and pRS315-HIS6-GFP-Tom22^{SOL}- Δ 20TIM23.

Yeast strains and media

The coding region of TIM23 was replaced by a HIS3 cassette in strain W303 Δ Tim23 carrying an episomal copy of TIM23 on the URA3-containing plasmid pVT-100U (Vernet *et al*, 1987) followed by plasmid shuffling as described previously for strain YPH499 (Mokranjac *et al*, 2005). All experiments were performed with strains W303 {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*} and W303 Δ Tim23 harbouring the episomal GFP-TIM23 constructs described above (see Supplementary Experimental Procedures for details). For growth curve analysis and mitochondrial preparations, all strains were cultured as indicated at 24, 30 or 37°C in either 2% lactate medium (containing 3 g yeast extract, 1 g NH₄Cl, 1 g KH₂PO₄, 0.5 g CaCl₂·2H₂O, 0.5 g NaCl, 1 g MgSO₄·7H₂O and 3 mg FeCl₃ per litre) or YP 2% glucose medium (containing 1% yeast extract, 2% peptone) (Sherman, 1991).

Proteolytic susceptibility assay

Mitochondria were purified as described previously (Herrmann *et al*, 1994). The proteolytic susceptibility of mitochondrial proteins was analysed with proteinase K under conditions resulting in intact, swollen or lysed mitochondria (see Supplementary Experimental Procedures for details). GFP-Tim23 and marker proteins were visualized after SDS-PAGE and western blot analysis using the indicated primary antibodies, goat anti-rabbit IgG-HRP conjugate secondary antibody (Bio-Rad), ECL solutions and X-ray film RX (Fuji).

Import of radioactive and non-radioactive proteins

Radiolabelled proteins were synthesized using pGEM4 constructs under control of the SP6 promoter. The labelled precursors were

imported into isolated mitochondria (see Supplementary Experimental Procedures for details). Imported proteins were visualized by SDS-PAGE, blotting and autoradiography using Kodak BioMax MR films (Sigma). Several series of exposure times were evaluated and quantified in the linear range of detection using the software 'image master' (Amersham Bioscience). Recombinant precursor, pSu9(1-69)-DHFR-6 × His, was purified from *E. coli* via Ni-NTA affinity chromatography. The protein was eluted with buffer containing 300 mM NaCl, 300 mM imidazole, 50 mM Tris, pH 8.0, and diluted with 100 mM NaCl, 20 mM HEPES, pH 7.4, resulting in a final concentration of 1.7 mg protein/ml. Import was performed with isolated mitochondria (50 µg) and recombinant preprotein (1 µg) under the same conditions as described for radiolabelled precursors. Quantitation of imported protein was performed by western blot analysis as described above.

Co-isolation (pull-down) assays

For co-isolation of proteins with His-tagged GFP-Tim23, GFP-Tom22TMS-Δ20TIM23, GFP-Mim1TMS-Δ20TIM23 or GFP-Tom22sol-Δ20TIM23, 1 mg mitochondria isolated from the corresponding strains were lysed with 1% (w/v) digitonin. After Ni-NTA affinity chromatography (see Supplementary Experimental Procedures for details), fractions were analysed by SDS-PAGE,

and co-purified components of the TIM23 and TOM complex were detected via western blot analysis.

Supplementary data

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

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Author contributions: MH performed the experiments; WN conception of the project, supervised and wrote the manuscript; MD supervised and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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