Role of Tim50 in the Transfer of Precursor Proteins from the Outer to the Inner Membrane of Mitochondria

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Transport of essentially all matrix and a number of inner membrane proteins is governed, entirely or in part, by N-terminal presequences and requires a coordinated action of the translocases of outer and inner mitochondrial membranes (TOM and TIM23 complexes). Here, we have analyzed Tim50, a subunit of the TIM23 complex that is implicated in transfer of precursors from TOM to TIM23. Tim50 is recruited to the TIM23 complex via Tim23 in an interaction that is essentially independent of the rest of the translocase. We find Tim50 in close proximity to the intermembrane space side of the TOM complex where it recognizes both types of TIM23 substrates, those that are to be transported into the matrix and those destined to the inner membrane, suggesting that Tim50 recognizes presequences. This function of Tim50 depends on its association with TIM23. We conclude that the efficient transfer of precursors between TOM and TIM23 complexes requires the concerted action of Tim50 with Tim23.

INTRODUCTION

Biogenesis of mitochondria depends on the import of many hundreds of different proteins from the cytosol (Neupert and Herrmann, 2007; Rehling et al., 2004; Endo et al., 2003; Koehler, 2004). These proteins are encoded in the nuclear genome, synthesized on cytosolic ribosomes as precursor proteins, and, by virtue of specific targeting signals, subsequently transported into the organelle. Almost all mitochondrial precursor proteins use the translocase of the outer mitochondrial membrane (TOM complex) to cross the outer membrane. At the intermembrane space (IMS) side of the TOM complex the transport pathways of precursors carrying different types of targeting signals diverge. Precursors which carry an N-terminal, positively charged matrix-targeting signal or, shortly, a presequence, are directed to the TIM23 complex (translocase of the inner mitochondrial membrane).

The TIM23 complex is an intricate molecular machine consisting of at least 10 subunits (Endo et al., 2003; Koehler, 2004; Rehling et al., 2004; Neupert and Herrmann, 2007; Alder et al., 2008a). Tim17, Tim23, and Tim50 make up the translocation channel and the receptor(s) of the complex. This membrane-embedded part of the complex is sufficient for recognition of precursors and the initial, membrane-potential dependent translocation of the presequence across the inner membrane. Complete translocation of precursors into the matrix requires repeated cycles of their binding to and release from mitochondrial heat shock protein 70 (mtHsp70). mtHsp70 is recruited to the translocase by Tim44 and binds to the incoming precursors in an ATP-dependent manner that is regulated by nucleotide exchange factor Mge1 and the J-complex Tim14-Tim16. In addition to these eight subunits essential for cell viability and function of the translocase, the TIM23 complex contains two recently identified nonessential subunits, Tim21 and Pam17 (Chacinska et al., 2005; Mokranjac et al., 2005; van der Laan et al., 2005), which appear to modulate the activity of the translocase in an antagonistic manner (Popov-Čeleketić et al., 2008).

The repertoire of TIM23 substrates is broad (Endo et al., 2003; Rehling et al., 2004; Koehler, 2004; Neupert and Herrmann, 2007). Precursors of soluble matrix proteins whose transport into mitochondria is governed by the N-terminal presequence form the major group of TIM23 substrates. One group of inner membrane proteins are transported into mitochondria by a conservative sorting pathway that involves N-terminal presequence-driven translocation into the matrix via TIM23 and subsequent insertion into the inner membrane from the matrix side. Another group use a stop-transfer mechanism for mitochondrial import and sorting. An N-terminal presequence initiates translocation into the matrix but a hydrophobic stop-transfer signal stalls translocation, leading to lateral release at the level of the TIM23 complex. A third group of inner membrane proteins that use the TIM23 complex have an internal targeting signal that is
composed of a transmembrane domain directly followed by a presequence-like element. Whether switching between these different transport modes involves conformational changes of the complex or assembly and disassembly processes is currently under debate (Chacinska et al., 2005; Popov-Čeleketić et al., 2008).

A receptor function in the translocase has been ascribed mainly to Tim50 because it can be cross-linked to precursors of soluble matrix proteins that were only partially translocated through the TOM complex (Yamamoto et al., 2002; Mokranjac et al., 2003a). At this stage of translocation, no cross-links of translocating precursors to any other TIM23 subunit were observed so far. The topology of Tim50 is also in agreement with such a function as it is integrated into the inner membrane with a single transmembrane domain and exposes a conserved domain into the IMS (Geissler et al., 2000, 2002). The receptor function of Tim50 remained, however, poorly characterized.

Here we have analyzed Tim50 in regard to its association with other components of the TIM23 complex, the importance of various domains for its functionality, and have studied its function as a receptor for precursor proteins.

MATERIALS AND METHODS

Plasmids, Yeast Strains, and Cell Growth

A centromeric yeast plasmid pRS315 (Sikorski and Hieter, 1989) was used to clone full-length Tim30 and all truncation mutants of Tim30 under the control of the endogenous promoter and 3UTR using standard genetic manipulations. Tim30Δamrix is Tim30 (109–476) behind the presequence (residues 1–69) of subunit nine of Neurospora crassa FFe ATPase to allow mitochondrial targeting. DLDTim30 consists of the IMS domain of Tim30 (residues 132–476) present behind the first 72 residues of yeast α-lactate dehydrogenase to allow mitochondrial targeting and integration into the inner membrane. bTim30 contains residues 1–167 of yeast cytochrome b followed by the IMS domain of Tim30. All constructs were confirmed by sequencing.

Wild-type yeast strains W334 and YPH499 were used (Hovland et al., 1989; Sikorski and Hieter, 1989). The deletion strain of Tim30 in a diploid W303 background was described before (Mokranjac et al., 2003a). It was transformed with a pYT-U plasmid (Vernet et al., 1987) carrying a full-length wild-type Tim30 and subsequently sporulated to obtain a haploid yeast strain with a chromosomal deletion of TIM30 and a wild-type copy of Tim30 on the URA plasmid. The haploid strain obtained was transformed with the above described plasmids and the empty pRS315 plasmid as a control. The wild-type copy of Tim30 on the URA plasmid was chased out on medium containing 5-fluoroorotic acid to obtain yeast strains carrying only the Tim30 version on pRS315 plasmid.

Yeast cells were normally grown on lactate medium containing 0.1% glucose at 30°C, except for tim23Δ, which was grown at 24°C. For down-regulation of Tim30, Tim50, and Mia40, published strains and procedures were used (Mokranjac et al., 2003a; Terziyska et al., 2005; Popov-Čeleketić et al., 2008).

Cross-Linking

Cross-linking followed by immunoprecipitation was performed essentially as described before (Mokranjac et al., 2003a). Briefly, mitochondria were incubated in import buffer (0.6 M sorbitol, 80 mM KCl, 10 mM Mg-acetate, 2 mM K-phosphate, 2.5 mM EDTA, 2.5 mM MnCl2, and 50 mM HEPES, pH 7.2) in the presence of 25 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), 8 μM Valinomycin, and 0.5 μM valinomycin for 10 min at 25°C to dissipate the membrane potential. 35S-labeled mitochondrial precursors were then added, and the samples incubated for further 15 min at 25°C or 4°C, as indicated. Samples were then transferred to 4°C for cross-linking. One aliquot received 50 μM homobifunctional, amino group-reactive agent 1,4-difluoro-2,4-dinitrobenzene (DFDNB; Perbio Sciences, Bonn, Germany) freshly dissolved in SHK buffer and was incubated with SHK buffer (0.6 M sorbitol, 20 mM HEPES, and 100 mM NaCl, pH 7.4), and mitochondria were resolated by centrifugation. For immunoprecipitation, samples were solubilized with 1% SDS in 50 mM Na-phosphate, 100 mM NaCl, pH 8.0, diluted with the same buffer containing 0.2% Triton X-100 and subsequently incubated with Tim50 antibodies (Mokranjac et al., 2003a) or antibodies from preimmune serum bound to Protein A-Sepharose (GE Healthcare, Freiburg, Germany). Beads were washed, and bound material was eluted with Laemmli buffer. Total (20%) and immunoprecipitated material (100%) were analyzed by SDS-PAGE and immunodecoration with the depicted antibodies. (C) As in A and B, with the exception that digitonin-lysed mitochondria from wild-type and Tim50-depleted cells were used and that antibodies to Tim16 were additionally used for precipitation.

Figure 1. Association of Tim50 with the TIM23 complex. Wild type (A) or mitochondria depleted of Tim23 (B) were solubilized with digitonin and incubated with affinity-purified antibodies to Tim17, Tim23, or Tim50 or antibodies from preimmune serum (PI) bound to Protein A Sepharose beads. Beads were washed, and bound material was eluted with Laemmli buffer. Total (20%) and immunoprecipitated material (100%) were analyzed by SDS-PAGE and immunodecoration with the depicted antibodies. (C) As in A and B, with the exception that digitonin-lysed mitochondria from wild-type and Tim50-depleted cells were used and that antibodies to Tim16 were additionally used for precipitation.

Miscellaneous

Previously described procedures were used for immunoprecipitation experiments from digitonin-solubilized mitochondria (Popov-Čeleketić et al., 2008) and synthesis of precursor proteins in the presence of [35S]methionine using standard or transcription-translation–coup­led reticulocyte lysate system (Promega, Madison, WI; Mokranjac et al., 2003a).

RESULTS

Association of Tim50 with the TIM23 Complex

Previous work suggested Tim23 to be the binding partner of Tim50 in the TIM23 complex (Geissler et al., 2002; Yamamoto et al., 2002; Mokranjac et al., 2003a,b; Alder et al., 2008b). It remained unclear, however, whether Tim23 is the only interaction partner of Tim50 within the complex. To gain more insight into the association of Tim50 with the TIM23 complex, we performed coimmunoprecipitation experiments using wild-type yeast mitochondria. Mitochondria were lysed with digitonin and subjected to immunoprecipitation with affinity-purified antibodies to Tim17, Tim23, and Tim50. Tim50 was equally well precipitated with antibodies to Tim17 and to Tim23 (Figure 1A). In addition, the efficiencies of precipitation of Tim17 and Tim23 with antibodies to Tim50 were essentially the same. This suggested that Tim17 may be an additional binding partner of Tim50 in the TIM23
complex. To analyze whether there is a subcomplex between Tim17 and Tim50 in the absence of Tim23, we isolated mitochondria from yeast cells depleted of Tim23. Depletion of Tim23 did not influence the levels of Tim17 and Tim50; however, a stable subcomplex between these two proteins was not observed upon coimmunoprecipitation from digitonin-solubilized mitochondria (Figure 1B). In contrast, the subcomplex consisting of Tim23 and Tim50 was previously found to be stable in the absence of Tim17 (Mokranjac et al., 2003b). Also, depletion of Tim44, Tim14, or Tim16 had no effect on the association of Tim50 with Tim17-Tim23 (Mokranjac et al., 2003a,b) nor did deletions of Pam17 and Tim21 (Popov-Čeleketić et al., 2008). This suggests that the only stable interaction of Tim50 in the TIM23 complex is to Tim23, at least when analyzed by this method.

We further studied the assembly of the TIM23 complex in the absence of Tim50. In lysates of wild-type mitochondria antibodies to Tim16, Tim17, Tim23, and Tim50 precipitate all known components of the complex, however, with different efficiencies due to the reported instability of the complex upon solubilization (Figure 1C). When the same experiment was performed with mitochondria from cells depleted of Tim50, all subunits of the complex analyzed were precipitated with antibodies to Tim16, Tim17, and Tim23 with virtually the same efficiency as in wild type. As expected, only residual amounts of Tim50 and none of the other TIM23 subunits were precipitated with antibodies to Tim50, confirming the specificity of precipitation. Thus, the assembly of the TIM23 complex appears to be largely unaffected in the absence of Tim50.

In summary, Tim23 appears to be the major component that recruits Tim50 to the TIM23 complex. The lack of Tim50 does not greatly affect the assembly of the rest of the translocase.

**Domain Analysis of Tim50**

To analyze the contributions of the various segments and domains of Tim50 to its function, we generated several truncation mutants (Figure 2A). The first mutant, denoted Tim50Δmatrix, lacked the matrix localized N-terminal segment in front of the transmembrane domain. In the second mutant, DLDTim50, we exchanged the N-terminal and the transmembrane segments of Tim50 with the corresponding segments of yeast d-lactate dehydrogenase. The third mutant, b2Tim50, consisted of first 167 residues of yeast cytochrome b2 fused to the IMS domain of Tim50 (residues 132–476). In this mutant, the b2-sorting signal was expected to be cleaved off at the IMS side of the inner membrane by Imp1. Thereby, only the IMS domain of Tim50 would be present soluble in the IMS. When expressed in yeast cells all truncation mutants were able to support growth of yeast cells in the absence of the wild-type copy of Tim50 (Figure 2B). Yeast cells carrying the truncation mutants were essentially indistinguishable from wild type when analyzed for growth on fermentable and nonfermentable carbon sources at 24, 30, and 37°C (Supplementary Figure S1). Likewise endogenous levels of various mitochondrial proteins and ability to import proteins into mitochondria in vitro were similar to those in wild type. Therefore, the only essential part of Tim50 appears to be its IMS domain. Mitochondria harboring wild-type or truncation mutants of Tim50 were subjected to carbonate extraction to differentiate between soluble and integral membrane proteins. Full-length Tim50, Tim50Δmatrix, and DLDTim50 were found in the pellet fraction as was the integral membrane protein Tim17 (Figure 2C). In contrast, b2Tim50 was recovered in the soluble fraction like Mge1. This result proves that the b2-sorting signal was properly processed and that b2Tim50 was not anchored in the membrane. We conclude that the soluble IMS domain of Tim50 is sufficient to support the function of the full-length protein.

**Tim50 Recognizes All Types of TIM23 Substrates**

We next asked which types of substrates of the TIM23 complex interact with Tim50. Various TIM23 substrates were added to de-energized wild-type yeast mitochondria. Under these conditions precursors are only partially translocated across the outer membrane. To analyze which types of TIM23 substrates interacted with Tim50, samples were incubated with the cross-linking reagent DFDNB and subsequently subjected to immunoprecipitation with antibodies to Tim50 or preimmune serum as a control. We first analyzed the interaction of Tim50 with precursors of soluble matrix proteins. A construct consisting of the first 55 residues of cytochrome b2, which contain its matrix targeting signal, fused to mouse DHFR was efficiently cross-linked to Tim50 (Figure 3A). The same was observed with the precursor of the matrix protein Jac1 (Figure 3B). Likewise, precursors of inner membrane proteins that are conservatively
of [35S]methionine and incubated with mitochondria in the absence of matrix pOxa1 (C) and pRieske (D) were synthesized in the presence of inner membrane proteins conservatively sorted through the inner membrane? We used a precursor consisting of the first 167 residues of cytochrome b2 fused to DHFR (A) and pJac1 (B) and of inner membrane proteins conservatively sorted through the matrix pOxa1 (C) and pRieske (D) were synthesized in the presence of [35S]methionine and incubated with mitochondria in the absence of membrane potential so that they could only partly cross the outer membrane. To examine their interaction with Tim50, samples were treated with cross-linker DFDNB followed by immunoprecipitation with antibodies to Tim50 and analysis by SDS-PAGE and autoradiography. Antibodies from preimmune serum (PI) were used as a control.

Figure 3. Tim50 recognizes matrix-targeted proteins. Precursors of soluble, matrix-destined proteins pD(1–55)DHFR (A) and pJac1 (B) and of inner membrane proteins conservatively sorted through the matrix pOxa1 (C) and pRieske (D) were synthesized in the presence of [35S]methionine and incubated with mitochondria so that they only partially crossed the outer membrane. To examine their interaction with Tim50, samples were treated with cross-linker DFDNB followed by immunoprecipitation with antibodies to Tim50 and analysis by SDS-PAGE and autoradiography. Antibodies from preimmune serum (PI) were used as a control.

sorted, such as Oxa1 and Rieske FeS protein, were cross-linked to Tim50 (Figure 3, C and D). On reestablishment of the membrane potential all of these precursors were efficiently chased into mitochondria with the concomitant loss of cross-linking adducts to Tim50, demonstrating that Tim50-bound species are productive import intermediates (Supplementary Figure S2, A–D). We conclude that apparently all matrix-targeted precursors are recognized by Tim50.

Is Tim50 also involved in recognition of precursors that are laterally sorted by the TIM23 complex? We first used a precursor consisting of the first 72 residues of DLD fused to DHFR which is laterally sorted and remains anchored in the inner membrane. A second precursor, which consisted of the first 167 residues of cytochrome b2 fused to DHFR, is laterally sorted, cleaved by Imp1 and released into the IMS. These precursors were incubated with de-energized mitochondria so that they only partially crossed the outer membrane, as described above. Cross-linking followed by immunoprecipitation showed them to be recognized by Tim50 (Figure 4, A and B).

Are these species productive intermediates on the way to the inner membrane? We used a precursor consisting of the first 220 residues of cytochrome b2 fused to DHFR and bound it to de-energized mitochondria. On reestablishment of membrane potential one aliquot was analyzed for cross-linking to Tim50 and another one for further transport into mitochondria. Precursor initially bound to mitochondria was efficiently chased to its final destination as observed by processing first by MPP and then by Imp1 (Figure 4C, bottom panel). At the same time, all three adducts to Tim50 observed with precursor bound to de-energized mitochondria disappeared in a time-dependent manner (Figure 4C, top panel). Interestingly, the adduct with the lowest mobility disappeared first followed by the middle one. In contrast, the adduct with the highest mobility first increased in its intensity and then disappeared like the other ones. Similar observations were made for the other two laterally sorted precursors used in this study (Supplementary Figure S2, E and F). Thus, interaction of laterally sorted precursors with Tim50 represents the initial step of their transport via the TIM23 complex. The presence of multiple cross-linking adducts suggests that transfer to and release from Tim50 occur in distinct steps in which precursors exist in different conformations. It should be noted that multiple cross-linking adducts were also observed for precursors cross-linked to Tom40 (Esaki et al., 2004).

We also analyzed whether Tim50 serves as a receptor for TIM23 substrates which are targeted to mitochondria by a signal that consists of a transmembrane domain followed by a presequence-like segment. The precursor of Tim14, as a representative of this class of substrates, was cross-linked to Tim50 (Figure 4D) on its way to the mitochondrial inner membrane (Supplementary Figure S2G). In contrast, precursors independent of the TIM23 complex, like ATP/ADP carrier (AAC) and Tim23, were not cross-linked to Tim50 (Figure 4E), demonstrating the specificity of interactions.

In summary, Tim50 recognizes all known types of TIM23 substrates showing that it serves as a general receptor of the TIM23 complex and that it most likely binds to the presequence or presequence-like elements.

Receptor Function of Tim50 Requires Its Association with the TIM23 Complex

Does the receptor function of Tim50 depend on its association with the TIM23 complex or can Tim50 function in a manner independent of TIM23? Cross-linking of precursors to Tim50 in mitochondria depleted of Tim23 yielded virtually no cross-linking adducts to Tim50 (Figure 5A). In contrast, cross-linking of precursors to Tim50 was indistinguishable from wild type in control mitochondria (Supplementary Figure S2G). In contrast, cross-linking of precursors to Tim23 was indistinguishable from wild type in control mitochondria depleted of Mia40, an essential mitochondrial protein whose function is unrelated to the TIM23 import pathway (Figure 5B). This demonstrates specificity of the removal of Tim23 and argues against secondary effects due to mere depletion of an essential mitochondrial protein. To obtain additional support for this finding, we used mitochondria carrying a temperature-sensitive mutant of Tim23 in which specifically the interaction between Tim50 and Tim23 is affected (Gevorkyan-Airapetov et al., 2008). Cross-linked adducts of Tim50 to precursors partially translocated across the outer membrane were virtually absent (Figure 5B). In conclusion, the receptor function of Tim50 in mitochondria appears to depend on its association with TIM23.

Tim50 Interacts with Precursors at a Very Early Stage of Translocation

When are the presequences seen by Tim50? The precursor consisting of the first 55 residues of cytochrome b2 and full-length DHFR was incubated with de-energized mito-
chondria at 25°C in the presence of methotrexate to prevent unfolding of the DHFR domain. The efficiency of cross-linking to Tim50 was reduced to ca. 30% of the cross-linking efficiency observed in the absence of methotrexate (Figure 6A). This demonstrates that the DHFR domain had to be at least partially unfolded to allow movement of the targeting signal sufficiently far in to be seen by Tim50. A similar, though less pronounced effect, was seen when the incubation was performed in the absence of methotrexate but at a temperature of 4°C, at which unfolding of DHFR is greatly reduced because of the reduced thermal fluctuations. There are five lysine residues in the 35-resides long \( b_2 \) presequence that could participate in cross-linking: Four of them are among the first 12 residues (residues 3, 5, 9, and 12). Therefore, a stretch of at least 55 residues can be trapped within the TOM complex before becoming accessible to the TIM23 complex. This result predicts that extending the cytochrome \( b_2 \) segment from 55 to 68 residues in the \( p_{b2}(1–87) \) DHFR should abolish these effects. Indeed, the precursor \( p_{b2}(1–167) \) DHFR, which has 148 residues in front of DHFR (first 167 residues of cytochrome \( b_2 \) with the 19 residues of the sorting signal deleted) was cross-linked to Tim50 with essentially the same efficiency irrespective of whether it was preincubated with methotrexate or not (111 vs. 100%). Likewise, no difference in cross-linking was observed when the incubation with mitochondria was performed at 25 or at 4°C (Figure 6B).

How many residues of the polypeptide chain can be bound to the TOM complex before they are seen by Tim50? We extended the \( b_2 \) segment from 55 to 68 residues in the \( p_{b2}(1–87) \) DHFR. The efficiencies of cross-linking to Tim50 in the presence and absence of methotrexate were essentially the same (96 compared with 100%; Figure 6C). Therefore, 55 residues can be accommodated in the TOM complex and are not accessible to Tim50, in contrast to 68 residues that cannot be contained within the TOM complex and are already exposed to the translocation machinery of the inner membrane. Apparently, at this stage of translocation, matrix targeted precursors are not yet in a fully extended state because ca. 60 residues are sufficient to span both TOM and TIM23 complexes when the import motor of...
the TIM23 complex has engaged in driving vectorial movement into the matrix (Ungermann et al., 1994).

**DISCUSSION**

Transport of proteins into the mitochondrial matrix depends on the coordinated action of the translocases in the outer and inner membranes, the TOM and TIM23 complexes. In the present study we analyzed Tim50, subunit of the TIM23 complex implicated in the transfer of precursors from TOM to TIM23. Earlier experiments had demonstrated that Tim50 is cross-linked to the precursors destined to matrix that were...
only partially translocated across the TOM complex, before any other TIM23 component (Mokranjac et al., 2003a). This had led to the suggestion that Tim50 functions as a receptor of the TIM23 complex. Efficient cooperation of the translocases of the two membranes is, however, not only required to sort precursors into the mitochondrial matrix but also into the inner membrane and IMS. Such precursors have a presequence and an additional sorting signal. It has been suggested that Tim50 is only required for transport into the matrix but not for lateral insertion (Geissler et al., 2002), raising the possibility that it is not the presequence that is recognized by Tim50 but some other element in the precursor. Here we present evidence that precursors that become laterally integrated into the inner membrane by TIM23 are recognized by Tim50. Therefore Tim50 appears to serve as the initial binding partner of all TIM23 substrates. Our results strongly suggest that Tim50 recognizes the presequence part of the precursors. Interestingly, even precursors which use the TIM23 complex but have internal targeting signals with a presequence-like element are recognized by Tim50. Such precursors cross the outer membrane in a loop conformation (Fölsch et al., 1996; Stan et al., 2003), suggesting that Tim50 can recognize a presequence even in such a context.

The function of Tim50 in collecting substrates from the TOM complex seems to depend on its association with the TIM23 complex. Tim23 is the protein that recruits Tim50 to the complex. Most likely the IMS domains of the two proteins form the major interacting surface, because the IMS domain of Tim50 is sufficient to support the function of the full-length protein. However, it is likely that the transmembrane helices of Tim50 and Tim23 have a stabilizing effect on their interaction. Indeed, Alder et al. (2008b) recently identified residues in the first transmembrane domain of Tim23 that are in close proximity to Tim50. The identification of the exact binding surfaces will, however, require a high-resolution structure of the complex. From the data presented here it seems clear that Tim50 on its own cannot function as a receptor of the TIM23 complex. In mitochondria lacking Tim23 or carrying a mutation in Tim23 that impairs the interaction with Tim50, we did not observe any cross-linking of precursors to Tim50. One possible scenario is that Tim50 and Tim23 together form the recognition site for a presequence, even if only cross-links of precursor to Tim50 are visible and not to Tim23. It is also possible that precursors are transferred to Tim23 from Tim50 in consecutive steps but that cross-linking adducts to Tim50 are only visible in the presence of Tim23. Previous experiments suggested that presequence peptides can bind to the isolated IMS domain of Tim23 (Bauer et al., 1996). Similar experiments have not been reported so far with the isolated IMS domain of Tim50. In any case, the interaction between the IMS domains of Tim50 and Tim23 appears not only to be important for maintaining the permeability barrier of the inner membrane (Meinecke et al., 2006) but also for transfer of precursors between translocation channels of the two membranes.

How close to each other are the two translocases? We observed that a presequence-containing polypeptide segment of 68 residues but not one comprising 55 residues in length, can be cross-linked to Tim50 without the necessity to unfold the following domain. Intriguingly, Endo and colleagues found that presequences longer than 63 residues, but not those shorter than 58 residues, can reach the IMS side of the TOM complex, the so-called trans site (Esaki et al., 2004). Even though the two studies used different presequences, the numbers fit surprisingly well. Taken together, these data suggest that ca. 60 residues can be accommodated in the TOM complex before being bound to Tim50. They also demonstrate the presence of Tim50 in very close proximity to the trans site of the TOM complex. It remains to be investigated whether there exists a subunit of the TOM complex that recruits Tim50. If so, the most likely candidates are the IMS domain of Tom22, Tom7, or Tom40, three proteins so far implicated in the formation of the trans site of the TOM complex (Endo et al., 2003; Koehler, 2004; Rehling et al., 2004; Neupert and Herrmann, 2007).

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