Mitochondrial Protein Import

Tom40 PLAYS A MAJOR ROLE IN TARGETING AND TRANSLOCATION OF PREPROTEINS BY FORMING A SPECIFIC BINDING SITE FOR THE PRESEQUENCE*

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During preprotein transport across the mitochondrial outer membrane, the N-terminal presequence initially binds to a surface-exposed site, termed cis site, of the protein translocation complex of this membrane (the TOM complex). The presequence then moves into the translocation pore and becomes exposed at the intermembrane space side. Membrane passage is driven by specific interaction of the presequence with the trans site. We have used chemical cross-linking to identify components in the vicinity of the translocating presequence. Preproteins bound to the surface-exposed cis site can be cross-linked via their N-terminal presequence to Tom20 and Tom22, demonstrating their direct association with this part of the preprotein. In addition, the presequence establishes an early contact to Tom40, a membrane-embedded protein of the TOM complex. Upon further entry of the preprotein into the translocation pore, the presequence loses its contact with Tom20/Tom22, but remains in firm association with Tom40. Our study suggests that Tom40 plays an important function in guiding the presequence of a preprotein across the mitochondrial outer membrane. We propose that Tom40 forms a major part of the trans site.

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‡ The abbreviations used are: TOM, translocon of the outer membrane of mitochondria; TIM, translocon of the inner membrane of mitochondria; OMV, outer membrane vesicle; PAGE, polyacrylamide gel electrophoresis; DHFR, dihydrofolate reductase; MOPS, 4-morpholinepropanesulfonic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; HSW, high salt washing buffer; LSW, low salt washing buffer; DSG, diisuccinimidyl glutarate; EDC, ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.
The results presented here demonstrate that, in addition to the well known presequence binding partners Tom20 and Tom22, Tom40 is in close contact with the presequence part of a translocating polypeptide chain. This intimate contact is established early on in the translocation event, when the preprotein is still bound to the surface-exposed cis site. Upon entering the translocation pore, the presequence looses its contact to the cis site-forming proteins Tom20/Tom22, but remains bound in the close vicinity of Tom40. Our data suggest that Tom40 plays an important role in presequence interaction during the passage of preproteins across the mitochondrial outer membrane.

MATERIALS AND METHODS

General Biochemical Procedures—The following published procedures were used: growth of Neurospora crassa wild type strain 74A and purification of mitochondria and mitochondrial OMV (20); treatment of OMV with trypsin, raising antisera, and purification of IgG (21), with the alteration that IgG were concentrated by ultrafiltration in Centricon tubes (Amicon); transcription and translation reactions in reticulocyte lysate using [35S]methionine (ICN Radiochemicals) as radiolabeled precursor (22); analysis of proteins by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography of the resulting gels (23); blotting of proteins onto nitrocellulose and immunostaining of blotted proteins using the ECL chemiluminescence detection system (Amer sham Corp.) (20). The amount of radioactive protein was quantitated on a PhosphorImager FUJI X BAS 1500. Protein concentrations were determined by the Coomassie dye-binding assay using IgG as a standard (Bio-Rad).

Preprotein Synthesis in Reticulocyte Lysates—The following fusion proteins were synthesized in reticulocyte lystate: PreSub9-dihydrofolate reductase (DHFR) containing the first 69 amino acids of subunit 9 of the mitochondrial F<sub>0</sub> -ATPase (pSu9) in front of mouse DHFR; this protein was termed pSu9-DHFR and contains cleavage sites for matrix processing peptidase after residues 35 and 66 (24). A truncated version of pSu9-DHFR termed pSu9-DHFR<sup>Δ7</sup> was generated by cleaving the plasmid pGEM5 harboring the coding region of pSu9-DHFR at unique site with AccI/BstEII restriction enzyme before transcription. This results in a linearized DNA containing the coding region for amino acid residues 1–69 of pSu9 plus the first 13 residues of DHFR. A protein termed pSu9(Δ7) containing amino acid residues 1–69 of pSu9 plus 7 additional residues was generated by linearizing plasmid pGEM5 harboring the coding region for pSu9-DHFR at unique site with EcoRI restriction enzyme before transcription. This results in a linearized DNA containing the coding region for amino acid residues 1–32 of the precursor of the subunit 9 protein. In both cases, pSu9-DHFR and pSu9(Δ7) were synthesized by run-off translation for 1 h at 25 °C in reticulocyte lysate. Puramycin (250 μg/ml) was added at the end of the synthesis reaction, and incubation was continued for 3 min at 25 °C to release nascent protein from the ribosomes.

In Vitro Import of Preproteins, Cross-linking, and Co-immunoprecipitation—For import or binding experiments, all vials were coated with fatty acid-free bovine serum albumin (1 mg/ml) before use to reduce unspecific interactions of preprotein with tube walls. OMV were suspended in import buffer (0.25 mg/ml bovine serum albumin, 20 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 mM MOPS/KOH, pH 7.2) in the absence or presence of 1 mM NADPH and 1 μM met horectox (MTX). In experiments using mitochondria, the import buffer was supplemented with 290 mM sucrose, and with 30–45 μM carbonyl cyanide m-chlorophenyldihydrazone (CCCP) to dissipate the membrane potential across the inner membrane. Reticulocyte lysate containing the radiolabeled precursor proteins was then added and incubated with OMV or mitochondria for the desired times at various temperatures. Samples were diluted with high (HSW) or low salt (LSW) washing buffer (10 mM Tris-HCl, pH 7.2). After incubation for 5 min at 25 °C, the lysed material was diluted 40-fold with lysis buffer lacking SDS. Under a clarifying spin (15 min at 20,000 × g), the supernatant was incubated with antibodies that were coupled to protein A-Sepharose beads. Co-immunoprecipitation of the TOM complex with bound translocation intermediates was performed as described earlier using antibodies directed against Tom40 (15).

RESULTS

To identify the components involved in forming the previously defined cis and trans sites of the TOM complex (7), a fusion protein comprised of the presequence part of subunit 9 of the F<sub>0</sub>-ATPase and mouse DHFR termed pSu9-DHFR was used. cis site binding was established by incubating isolated OMV with the fusion protein at 0 °C in the presence of 1 μM MTX followed by a wash of the OMV with LSW (20 mM KCl). Under this condition, the preprotein was loosely bound to the OMV and dissociated at higher ionic strength (8) (see also Fig. 1A, lanes Total). trans site interaction was established by performing the binding reaction at 25 °C in the absence of MTX. In this case, the majority of the preprotein was bound in a stable, salt-resistant fashion. Residual cis site-bound material could be removed by a wash with HSW (120 mM KCl), thus resulting in an almost exclusive accumulation of trans site-bound preprotein. This latter material was firmly associated with the TOM complex and could be isolated by co-immunoprecipitation using antibodies against, e.g., Tom40 (Fig. 1A), in contrast to what was found for cis site-bound material. Using these two binding conditions, we analyzed the molecular environment of the bound preprotein by a cross-linking approach.

In an initial cross-linking experiment, the homobifunctional reagent DSG was added to OMV carrying pSu9-DHFR bound to either the cis or the trans site. Several specific cross-linking products were formed under both conditions (Fig. 1B). Some of the bands were obtained also by using the “zero length” cross-linking reagent EDC (not shown; see below), indicating that the preprotein is in intimate contact to these cross-linking partners. The pattern of the cross-linking products in the two binding conditions was similar, despite the fundamentally different character of binding. Addition of chemical amounts of the presequence peptide F<sub>1</sub>β (corresponding to amino acids 1–32 of the precursor of the β-subunit of yeast F<sub>0</sub>-ATPase) after the incubation of preprotein with OMV, but before the cross-linking reaction largely reduced the formation of the specific cross-linking products in both the cis and trans binding conditions (Fig. 1B), indicating that the cross-links were occurring with a preprotein that is specifically bound via its presequence.

The sizes of the cross-linking products suggested Tom20, Tom22, and Tom40 as possible partners. This expectation was confirmed by immunoprecipitation using antibodies directed against these TOM complex proteins (Fig. 1C). Two Tom40-specific cross-linking products were observed which differed in their electrophoretic mobility. These two cross-linking species correspond to different conformations of the preprotein-Tom40 complex.2 None of the cross-linking products was immunoprecipitated with antibodies derived from preimmune serum or with antibodies against porin, the most abundant protein of the mitochondrial outer membrane. We conclude from these results that a preprotein bound to the surface-exposed cis site is also in the vicinity of Tom40, a membrane-embedded component. Most likely, this early contact with Tom40 occurs through the presequence part of the preprotein (see also below). After transfer of the presequence into and across the outer membrane, the translocating polypeptide chain remains in the vicinity of both Tom40 and the receptors Tom20/Tom22.

To more precisely analyze the role of the presequence for

2 D. Rapaport, unpublished results.
preprotein binding to the TOM machinery, we constructed two additional polypeptides. The first is a truncated version of pSu9-DHFR termed pSu9-DHFR13, consisting of the presequence of subunit 9 and the N-terminal 13-amino acid residues of DHFR. The second, pSu9(17), consists of the presequence of subunit 9 plus 7 additional amino acid residues resulting from the cloning procedure. Cross-linking of these polypeptides to the TOM components by DSG or EDC can occur only via the presequence part, as the short segments following the presequence lack any lysyl residues. Addition of the cross-linkers to OMV that were incubated with pSu9(17) under cis binding conditions resulted in the formation of several specific cross-linking products (Fig. 2A), while under conditions favoring trans site binding, reduced amounts of pSu9(17) were bound to OMV, and only a few specific cross-linking bands were detected. Similar results were obtained with pSu9-DHFR13 (data not shown). As seen by immunoprecipitation, pSu9(17) bound at the cis site became cross-linked to Tom20, Tom22, Tom40, and Tom70 (Fig. 2B). Cross-linking to the latter two components is consistent with results from a previous cross-linking study that utilized a presequence peptide to identify new components of the mitochondrial protein translocation machinery.

**Fig. 1.** A preprotein bound to OMV under cis and trans conditions can be cross-linked to various TOM components. A, a preprotein can be co-immunoprecipitated with antibodies against Tom40, only when bound to the trans site. Radioactively labeled pSu9-DHFR was bound to OMV for 20 min at 0 or 25 °C in the presence or absence of MTX/NADPH, respectively. The reaction mixtures were chilled on ice and diluted with LSW or HSW buffers (containing 20 or 120 mM KCl, respectively). OMV together with cis or trans site-bound material were reisolated (20 min, 125,000 × g) and resuspended in SEM buffer with or without MTX/NADPH. Co-immunoprecipitation with antibodies against Tom40 or with antibodies derived from preimmune serum was performed. As a control for the input, an aliquot (Total) representing 25% of the material used for co-immunoprecipitation is shown. B, pSu9-DHFR was bound to OMV under cis and trans conditions and the OMV were washed as described in A. To the indicated samples, 13 µM of F1β presequence peptide was added. The cross-linking reagent DSG (final concentration 117 µM) was added, and samples were incubated for 40 min at 0 °C. Aliquots were removed before (−DSG) and after the cross-linking reaction (40 min at 0 °C; +DSG). Further analysis was as in Fig. 1B. B, identification of the cross-linking products by immunoprecipitation. OMV were incubated with pSu9(17) for 15 min at 0 °C, diluted with LSW buffer, and resolated (20 min, 125,000 × g). The pellets were resuspended in SEM buffer, and DSG (117 µM) was added. Aliquots were removed before (−DSG) and after the cross-linking reaction (40 min at 0 °C; +DSG). Further analysis and immunoprecipitation with antibodies directed against Tom20, Tom22, Tom40, and Tom70 or antibodies derived from preimmune serum were as in Fig. 1C. Apparent molecular masses are given on the left.
Tom40 Forms a Specific Binding Site

The association (reviewed in Ref. 26). Chemical amounts of the purified presequence peptide F1β (see above) were bound to OMV. The OMV were washed with high or low salt buffers and cross-linking with EDC was performed. Immunostaining analysis revealed specific cross-linking of this presequence peptide to Tom22 and to Tom40 (Fig. 4, A and B, marked b). In addition, cross-linking of Tom22 and Tom40 to one of the small TOM complex proteins was seen (Fig. 4, A and B, marked a). Weak cross-linking of the presequence peptide was also observed to Tom20, but no products were recognized for porin (not shown). Strikingly, the cross-link to Tom22 was absent, when OMV were washed with high salt buffer, in contrast to the cross-link to Tom40. It appears that F1β is bound to Tom22 and Tom40 in a distinct fashion, a situation presumably reflecting the different binding properties of preproteins such as pSu9-DHFR to the cis and trans sites (see above). Cross-linking was specific for mitochondrial presequences, as no reaction was observed for a control peptide (CH4), corresponding to amino acids 1–25 of N. crassa cytochrome c heme lyase, which does not resemble a presequence (Fig. 4A) (cf. Ref. 27). The cross-linking product of F1β with Tom40 was observed at a similar efficiency also after trypsin pretreatment of the OMV (Fig. 4B), corroborating our results from above that the presequence-Tom40 contact can occur independently of receptors. Taken together, our data show that a presequence peptide bound to OMV is in intimate contact with the surface receptors Tom20 and Tom22 only in the presence of low salt, whereas the interaction with Tom40 may persist even after a high salt wash. Since the results were obtained with a purified presequence peptide, no soluble factors are necessary for establishing these interactions.

[Image 317x487 to 555x729]

FIG. 4. A purified presequence peptide can be cross-linked to Tom22 and Tom40. The F1β presequence peptide or the control peptide CH4 (35 μM) were incubated with OMV that were pretreated with or without trypsin for 20 min at 0 °C as indicated. As a control, an aliquot was removed before the addition of the peptides. The reaction mixture was diluted with LSW and HSW buffers before resolation of the OMV. The pellets were resuspended in SEM buffer, and 1 mM EDC was added as indicated. After quenching of the cross-linker the proteins were precipitated with trichloroacetic acid, solubilized with sample buffer, and separated by SDS-PAGE. After blotting onto nitrocellulose membranes, immunostaining analysis was performed for A, Tom22 or B, Tom40. In both panels the cross-linking product with a small TOM component is marked with a and the one with the presequence peptide with b.

We next investigated whether establishing a direct contact between the presequence of a preprotein and Tom40 requires the participation of the cytoplasmic domains of Tom20/Tom22 receptors. For that purpose, we performed the cross-linking of pSu9-DHFR and pSu9+7 with OMV that were pretreated with trypsin. This pretreatment quantitatively removes the surface receptors and completely abolishes cis site binding (8), but leaves Tom40 intact. Binding to trypsin-treated OMV under the trans site conditions was reduced to about 20–30% of the binding to untreated OMV (Fig. 3, A and B). Using the zero-length cross-linking reagent EDC, adducts between both preproteins and Tom40 were observed with trypsin-pretreated OMV. As quantitated by PhosphorImager analysis, the efficiency of cross-linking to Tom40 relative to bound precursor protein was about 2-fold higher than in the presence of surface receptors. This suggests that a preprotein, via its presequence, can bind to Tom40, even in the absence of surface receptors. Thus, the surface receptors appear to be essential for efficient accumulation of preproteins at the trans site (7), but they are not necessary for establishing and maintaining the interaction with Tom40.

We asked whether the interaction of the presequence with the different TOM components is dependent on soluble factors that are contained in the lysate mixture. Factors such as MSF and Hsp70 have been reported to be important for establishing the association (reviewed in Ref. 26). Chemical amounts of the purified presequence peptide F1β (see above) were bound to OMV. The OMV were washed with high or low salt buffers and cross-linking with EDC was performed. Immunostaining analysis revealed specific cross-linking of this presequence peptide to Tom22 and to Tom40 (Fig. 4, A and B, marked b). In addition, cross-linking of Tom22 and Tom40 to one of the small TOM complex proteins was seen (Fig. 4, A and B, marked a). Weak cross-linking of the presequence peptide was also observed to Tom20, but no products were recognized for porin (not shown). Strikingly, the cross-link to Tom22 was absent, when OMV were washed with high salt buffer, in contrast to the cross-link to Tom40. It appears that F1β is bound to Tom22 and Tom40 in a distinct fashion, a situation presumably reflecting the different binding properties of preproteins such as pSu9-DHFR to the cis and trans sites (see above). Cross-linking was specific for mitochondrial presequences, as no reaction was observed for a control peptide (CH4), corresponding to amino acids 1–25 of N. crassa cytochrome c heme lyase, which does not resemble a presequence (Fig. 4A) (cf. Ref. 27). The cross-linking product of F1β with Tom40 was observed at a similar efficiency also after trypsin pretreatment of the OMV (Fig. 4B), corroborating our results from above that the presequence-Tom40 contact can occur independently of receptors. Taken together, our data show that a presequence peptide bound to OMV is in intimate contact with the surface receptors Tom20 and Tom22 only in the presence of low salt, whereas the interaction with Tom40 may persist even after a high salt wash. Since the results were obtained with a purified presequence peptide, no soluble factors are necessary for establishing these interactions.

[Image 79x557 to 277x729]

FIG. 3. Cross-linking of preproteins to Tom40 occurs independently of surface receptors. A, pSu9-DHFR or B, pSu9+7 were incubated with OMV that were pretreated with trypsin (30 μg/ml) or were mock-treated. The binding incubation was for 20 min at 25 °C (pSu9-DHFR) or 0 °C (pSu9+7). The reaction mixture was chilled on ice and diluted with HSW (pSu9-DHFR) or LSW (pSu9+7) buffers before resolation of the OMV. The pellets were resuspended in SEM buffer, and the cross-linker EDC was added as indicated for 40 min at 0 °C.Further analysis was as in Fig. 1. Apparent molecular masses are given on the left.

(25). The additional band at an apparent molecular mass of 18 kDa (Fig. 2A) corresponds most likely to the cross-linking product of the preprotein with one of the small Tom proteins. Thus, a surface-bound presequence is in intimate contact with various TOM complex proteins including the receptors. After the high salt wash only 13% of the preprotein remained bound to OMV as estimated by PhosphorImager analysis. The cross-link to Tom40 relative to bound protein occurred to a similar extent under the cis binding condition (Fig. 2A). This result shows that a presequence polypeptide bound in a salt-resistant fashion stays in the vicinity of Tom40. Further, the data suggest that stable binding to the trans site requires a contribution of the mature part of the preprotein.

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It was now of particular interest whether the presequence-TOM protein interactions observed with isolated OMV can be identified during the import of preproteins into intact mitochondria. For that purpose, import intermediates were accumulated with isolated mitochondria. To avoid translocation across the inner membrane, both matrix ATP and the membrane potential \( \Delta W \) across the inner membrane were depleted (29). pSu9-DHFR and pSu9(7) were bound under conditions that result in almost exclusive binding to the \( \text{cis} \) site and in binding to a state corresponding to the \( \text{trans} \) site (7, 30, 31). As seen with isolated OMV, the DHFR moiety became unfolded in the latter binding condition, a characteristic feature of \( \text{trans} \) site binding.\(^4\) The cross-linking pattern observed for binding of both preproteins under \( \text{cis} \) site conditions was similar to that obtained with OMV, i.e. specific cross-links to Tom20, Tom22, and Tom40 were formed (Fig. 5). After incubation of the preproteins with mitochondria at 25 °C and a high salt wash (\( \text{trans} \) condition), the cross-linking efficiency to the TOM components was reduced, even though the amount of bound preproteins was comparable to that under the \( \text{cis} \) site condition. This observation may be due to the further movement of the presequence into the intermembrane space or to the outer face of the inner membrane, a reaction that is not possible in OMV. Nevertheless, preproteins bound under this condition could be specifically cross-linked to Tom40 and to a lesser extent to Tom20 and Tom22 (Fig. 5). Thus, the nearest neighbors of a presequence during its transport across the outer membrane of isolated mitochondria are the same as those identified with OMV. In summary, our data suggest that the presequence of a translocating preprotein is in the proximity of Tom40 under both the \( \text{cis} \) and \( \text{trans} \) binding conditions. Since in our experiments, the zero length cross-linker EDC was used, Tom40 appears to be in direct contact with the presequence, especially when bound at the \( \text{trans} \) site.

**DISCUSSION**

Our present cross-linking study analyzes the neighborhood of presequence-containing preproteins bound to the protein import complex of the mitochondrial outer membrane. The data allow us to extend and refine our view of the molecular mechanism of preprotein translocation across this membrane (see, e.g. Ref. 5). Earlier investigations have shown that the preprotein first interacts with the surface receptors Tom20 and Tom22 (see Fig. 6, stage A). This has been demonstrated by various techniques including binding studies (8, 9) and cross-linking techniques (32). Our study now shows that it is the presequence part of the preprotein which is in close contact to the receptors. In this surface-bound stage previously defined as the \( \text{cis} \) site, the presequence is also in contact with Tom40, a component, which according to its high protease resistance, is embedded in the outer membrane and most likely forms the translocation channel or a major part thereof. This contact could occur either via a surface-exposed region of Tom40 (Fig. 6, stage A), or the presequence could be inserted at least partially into the translocation pore thereby initiating the translocation process (Fig. 6, stage B). Only N-terminal parts of the presequence may have entered the translocation channel in this surface-bound state, since the mature part of the preprotein remains fully folded, and added matrix processing peptidase can readily cleave off the presequence of \( \text{cis} \) site-bound preproteins (7) (see Fig. 6, stages A and B).

Deeper insertion of the presequence across the outer membrane requires the unfolding of the mature part of the preprotein, a reaction which does not depend on external energy sources such as ATP hydrolysis (Fig. 6, stage C) (7). Translocation is thought to be driven by the interaction of the presequence with a second presequence-specific binding site. This site was previously termed \( \text{trans} \) site, since the presequence is accessible from the intermembrane space side of the outer membrane. The matrix processing peptidase introduced into the lumen of OMV could cleave off the presequence of \( \text{trans} \) site-bound preproteins, but was unable to do so from the cytosolic side (Fig. 6, stages C and D) (7). Our cross-linking study now provides important information about the molecular environment forming this presequence-specific binding site. Tom40 appears to be the major partner of interaction for the presequence at this stage. The presequence-Tom40 interaction can be seen even after removal of the cytoplasmic domains of the receptor proteins, suggesting that these domains are not essential for the formation of this binding site. Moreover, cross-linking of a presequence peptide to Tom40 was observed also after removing loosely bound molecules by a high salt wash.

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4 R. Lill and D. Rapaport, unpublished results.
Tom40Forms a Specific Binding Site

![Diagram of the movement of preproteins across the mitochondrial outer membrane.](https://example.com/diagram.png)

**Fig. 6.** Working model for the movement of preproteins across the mitochondrial outer membrane. At the mitochondrial surface, the N-terminal presequence is recognized by the receptor proteins Tom20 and Tom22 (depicted as R), which together form the cis site. In this surface-bound stage, the mature domain adjacent to the presequence can remain folded, and the presequence cleavage site (filled circle) is accessible for externally added matrix processing peptidase (MPP). The early contact of the preprotein to Tom40 (hatched area) during the translocation reaction may be established either via an exposed loop of Tom40 (stage A) or by the reversible movement of the presequence into the translocation pore (B). Unfolding of the mature domain allows deeper insertion of the presequence into the outer membrane (OM) and its binding to the trans site in which the matrix processing peptidase cleavage site is accessible from the intermembrane space, but not from the cytosolic side (C and D). Under these conditions, bound preproteins interact with Tom40 mainly via their presequence and with the receptors via the mature part. Thus, Tom40 is in close vicinity to the presequence during all stages of translocation and forms a major part of the trans presequence binding site.

(Figs. 2 and 4). In contrast, cross-linking to Tom22 was almost completely abolished under such conditions. Thus, Tom40 appears to be directly involved in forming the second presequence binding site. Preproteins bound to this stage remain in close contact to Tom20 and Tom22. This contact is formed mainly through the mature part of the preprotein, as cross-linking of trans site-bound preproteins to Tom20 and Tom22 is only seen with preproteins containing a larger portion of the mature part. In addition to Tom40, other components of the TOM complex may be important for the formation of the trans presequence binding site. One such component is likely one of the small TOM complex proteins that was observed to become cross-linked to a presequence polypeptide.

The precise position of the trans presequence binding site on Tom40 remains unknown. Based on the accessibility of the matrix processing peptidase cleavage site of trans site-bound preproteins from the inner face of the outer membrane, it may be argued that the trans site is located toward the intermembrane space. However, a situation in which the presequence is in dynamic equilibrium between the translocation pore and the intermembrane space side seems also possible (Fig. 6, stages C and D). A prerequisite for defining the binding site in detail is the unraveling of the structure of Tom40.

Earlier investigations have addressed the role of the intermembrane space domain of Tom22 in presequence binding on the trans side of the outer membrane. Two studies found no significant alterations in preprotein translocation across the outer membrane upon deletion of this segment of Tom22 (33, 34). In another report, deletion of this piece had a dramatic influence on the growth of mutated yeast cells and largely affected protein import into mitochondria (10). In addition, an interaction between presequences and the C-terminal portion of yeast Tom22 was found. In contrast, no significant association between presequences or preproteins and the corresponding piece of N. crassa Tom22 was observed (34). Our present cross-linking study was unable to detect any cross-linking of Tom22 to presequence peptides, when they were bound at the salt-resistant trans site of the outer membrane. Thus, our previous (34) and present results suggest that the C terminus of Tom22 does not play an essential role in binding and translocation of mitochondrial presequences across the mitochondrial outer membrane.

Preprotein translocation across the outer membrane of mitochondria is similar in a number of aspects to protein transport across the chloroplast outer envelope membrane (35). The N-terminal targeting information is first recognized at the organelar surface. In chloroplasts, the outer envelope protein IAP66/OEP86 serves as the initial binding partner (36, 37). As in the case of the mitochondrial outer membrane, the surface-bound preprotein is in close proximity to a membrane-embedded component that possibly forms a translocation pore, namely IAP75/OEP75 (37, 38). Insertion of the targeting sequence leads to a stable import intermediate in which the presequence is recognized a second time. In both organelles, the membrane-embedded pore-forming components (Tom40 in mitochondria or IAP75/OEP75 in chloroplasts) are involved in the formation of these two binding sites.

What may be the relevance of a second presequence binding site for the protein import reaction? Repeated deciphering of the targeting information might be important to increase the accuracy of targeting. At the organelar surface, preproteins are bound in a rather labile fashion and readily equilibrate with the bulk solution. The labile character of interaction in this first binding site would be well suited for selecting the cognate preproteins from the large pool of cytosolic proteins. Erroneously bound proteins could readily dissociate from the organelar surface at this stage. Efficient insertion into the translocation pore and binding to the second presequence binding site is only feasible if the correct targeting information is attached to the N terminus of the preprotein. It is possible that different features of the targeting sequences are recognized at these two binding sites. Such a “double-check system” would thus guarantee the accurate selection of the correct preproteins.

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