Dual role of the receptor Tom20 in specificity and efficiency of protein import into mitochondria

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Mitochondria import most of their resident proteins from the cytosol, and the import receptor Tom20 of the outer-membrane translocator TOM40 complex plays an essential role in specificity of mitochondrial protein import. Here we analyzed the effects of Tom20 binding on NMR spectra of a long mitochondrial presequence and found that it contains two distinct Tom20-binding elements. In vitro import and cross-linking experiments revealed that, although the N-terminal Tom20-binding element is essential for targeting to mitochondria, the C-terminal element increases efficiency of protein import in the step prior to translocation across the inner membrane. Therefore Tom20 has a dual role in protein import into mitochondria: recognition of the targeting signal in the presequence and tethering the presequence to the TOM40 complex to increase import efficiency.

yeast | Tim50 | Tom22

A protein's function relies on its correct subcellular location. Newly synthesized proteins are delivered to their sites of actions by cellular protein transport systems. Because subcellular compartments are bounded by biological membrane(s) in eukaryotic cells, the protein transport needs to start with insertion into or translocation across the target membrane. Therefore most protein transport systems involve a targeting signal on the cargo protein and a multiprotein complex on the target membrane called a translocator (1).

Mitochondria are essential organelles in eukaryotic cells that consist of the outer and inner membranes and two aqueous compartments, the intermembrane space (IMS) and the matrix. Most mitochondrial proteins are encoded in the nuclear genome, synthesized in the cytosol, and subsequently imported into mitochondria. Mitochondrial protein import is mediated by translocators in the outer and inner membranes, including two TOM (translocase of the outer mitochondrial membrane) complexes, the TOM40 complex and the TOB (topogenesis of outer-membrane β-barrel proteins)/SAM (sorting and assembly machinery) complex, and two TIM (translocase of the inner mitochondrial membrane) complexes, the TIM23 complex and the TIM22 complex (2–5). The import pathway generally starts from the TOM40 complex and then branches out into several distinct intramitochondrial sorting pathways involving other translocators.

The targeting information for mitochondria is contained in the N-terminal cleavable presequence or within the mature part of precursor proteins. The targeting information as well as intramitochondrial sorting information is recognized by several receptor subunits of the TOM and TIM complexes along the import pathways. Among them, Tom20, a peripheral subunit of the TOM40 complex, is a general import receptor that recognizes mitochondrial targeting signals contained in presequences. Tom20 is anchored to the outer membrane by the N-terminal hydrophobic segment and exposes a receptor domain to the cytosol. The NMR and X-ray structures of the receptor domain of rat Tom20 in a complex with a presequence peptide showed that the bound presequence forms an amphiphilic helix with hydrophobic residues that align on one side to interact with a hydrophobic groove of Tom20 (6, 7). The size of the hydrophobic groove of Tom20 suggests that sequences recognized by Tom20 are as short as 8 amino acid residues, whereas the lengths of presequences are often longer than 40 amino acid residues. Further NMR identification of the Tom20-binding elements in various presequence peptides revealed a 5–6-residue common motif for Tom20 recognition (8, 9).

Tom20 is not the only presequence binding site on the TOM40 complex. The cytosolic acidic domain of Tom22 may also function as a receptor for mitochondrial proteins. Tom20 and Tom22 likely constitute a presequence binding site (*cis* site) on the cytosolic side of the TOM40 complex (10–14). The TOM40 complex has a presequence binding site (*trans* site) on the IMS side of the outer membrane, which may involve the IMS side of Tom40 and the IMS domains of Tom22 and Tom7 (10, 13, 15–18). The N-terminal presequence likely binds to the *cis* site first and subsequently moves through the channel in the TOM40 complex to reach the *trans* site (10, 13). In parallel, the mature part that is transiently unfolded may be trapped by the inner wall of the Tom40 channel, which functions like a molecular chaperone (10, 19).

In the present study, we analyzed by NMR a possible Tom20-binding element in a long (69-residue) presequence (pSu9) of the precursor to subunit 9 of *Neurospora crassa* F_o-ATPase and found that pSu9 contains two distinct Tom20-binding elements, one in the N-terminal half and the other in the C-terminal half of pSu9. The N-terminal Tom20-binding element is essential for targeting to mitochondria, whereas the C-terminal Tom20-binding element increases efficiency of protein import in the step prior to trans-

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location across the inner membrane. Therefore the receptor protein Tom20 has a dual role in protein import into mitochondria, recognition of the targeting signal in the presequences, and cooperation with the *trans* site for tethering the presequences to the TOM40 complex to increase the import efficiency.

Results

Identification of Tom20-Binding Elements in the pSu9 Presequence. Previous studies showed that binding elements for the mitochondrial import receptor Tom20 in mitochondrial presequences are 6–8 residues long, but that their positions are variable in the presequences consisting of 19–33 amino acid residues (6, 8). However, because many (>30%) mitochondrial presequences are longer than 40 residues (Fig. S1), we decided to localize the Tom20-binding element in pSu9, a long (69-residue) presequence of the precursor to subunit 9 of *Neurospora crassa* F_o-ATPase. For this purpose, we monitored chemical-shift changes in the [^{1}H, ^{15}N]-heteronuclear sequential quantum correlation (HSQC) spectra of the ^{15}N-labeled peptides corresponding to pSu9, its N-terminal half (pSu9N; residues 1–34), and C-terminal half (pSu9C; residues 35–69) (Fig. 1*A*) upon addition of the nonlabeled cytosolic receptor domain of rat Tom20 (dTom20) (6). Because residues 1–14 of pSu9 are important for guiding a passenger protein to mitochondria in vitro (18) and the proposed 5-residue motif for binding to Tom20 (8) is found in residues 8–12 of pSu9N, but not in pSu9C, we expected that pSu9N, but not pSu9C, would bind to dTom20. Indeed, dTom20 caused perturbation of a subset of the backbone amide resonances of pSu9N (Fig. 1*B*, *Left*), suggesting that pSu9N has a binding site for dTom20. However unexpectedly, dTom20 also caused perturbation in the backbone amide resonances of pSu9C (Fig. 1*B*, *Right*). The plot of chemical-shift changes against residue numbers show that dTom20 binds to residues 6–21 of pSu9N and residues 52–66 of pSu9C (Fig. 1*C*, *Upper*). Addition of nonlabeled pSu9N or pSu9C to ^{15}N-labeled dTom20, in turn, led to chemical-shift changes in nearly the same set of the backbone amide resonances of dTom20 (Fig. S2 *A* and *B*), suggesting that dTom20 has a common binding site for pSu9N and pSu9C.

When we used the full-length presequence peptide, pSu9, instead of the fragment peptide pSu9N or pSu9C, addition of dTom20 to the molar ratio of dTom20/pSu9 = 2/1 caused chemical-shift changes in the signals from both the N-terminal half and the C-terminal half of the peptide. The resulting chemical-shift perturbation patterns (Fig. 1*C*, *Lower*) are similar to those for pSu9N and pSu9C (Fig. 1*C*, *Upper*), suggesting that the N-terminal and C-terminal halves bind to dTom20 independently. The chemical-shift changes of dTom20 are larger for the N-terminal half than for the C-terminal half (Fig. 1*C*), and dissociation constants estimated from the analyses of the titration curves are smaller for pSu9N than pSu9C (Fig. S3). Therefore the pSu9 presequence contains two distinct binding elements for Tom20, and the affinity of dTom20 binding is higher for the element in the N-terminal half than that in the C-terminal half.

Second Tom20-Binding Element in pSu9 Enhances Its Import Ability. In an attempt to assess the roles of the presence of two Tom20-binding elements in pSu9 in mitochondrial protein import, we synthesized model precursor proteins containing mouse dihydrofolate reductase (DHFR) fused to a chimeric presequence consisting of pSu9N followed by pSu9N, pSu9C, or an unrelated sequence HBD (heme-binding domain, residues 111–144 of the heme-binding domain of cytochrome b_2) (Fig. 2*A*). HBD was confirmed by NMR not to interact with Tom20. Upon incubation with isolated yeast mitochondria, the fusion proteins with pSu9N at the N terminus of the presequence [pSu9N followed by pSu9N and DHFR (NN-DHFR), pSu9N followed by pSu9C and DHFR (NC-DHFR), and pSu9N followed by HBD and DHFR (NH-DHFR)] were imported into mitochondria efficiently, although processing

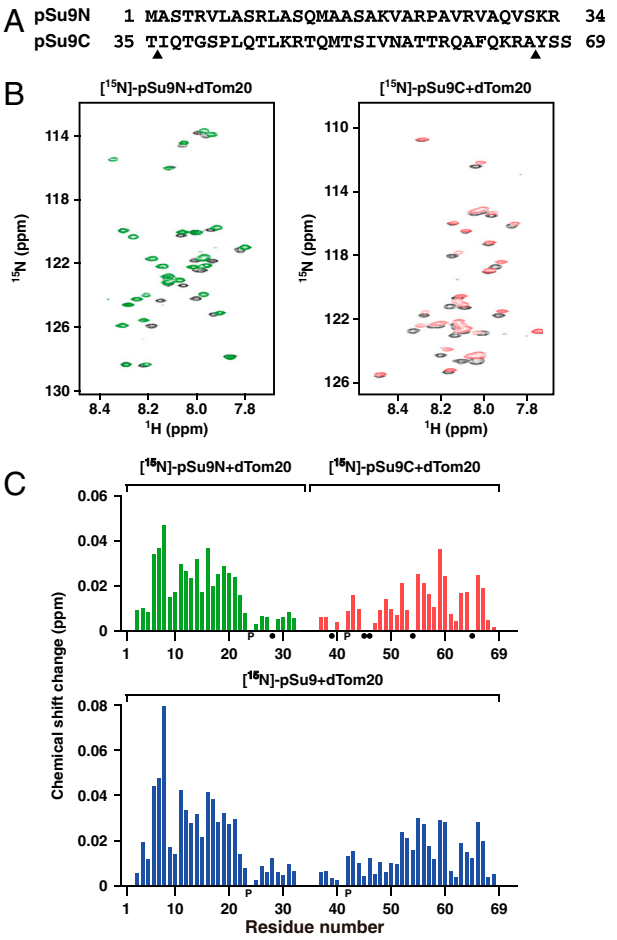

Fig. 1. Identification of the Tom20-binding elements in pSu9N, pSu9C, and pSu9. (*A*) Amino acid sequences of pSu9N and pSu9C. The triangles show the presequence cleavage sites. (*B*) Chemical-shift changes in [^{1}H, ^{15}N]-HSQC spectra of 0.5 mM ^{15}N-labeled pSu9N (*Left*) and 1.5 mM ^{15}N-labeled pSu9C (*Right*) upon addition of 1.5-M excess of dTom20 in 20 mM KPi, pH 6.7, 50 mM KCl, D_2O/H_2O (5/95) at 10 °C. Black spectra, before addition of dTom20; green spectrum, pSu9N after addition of dTom20; red spectrum, pSu9C after addition of dTom20. (*C*) The chemical-shift changes of each backbone amide in [^{1}H, ^{15}N]-HSQC spectra of 0.5 mM ^{15}N-labeled pSu9N and 1.5 mM ^{15}N-labeled pSu9C (*Upper*) and of 0.25 mM ^{15}N-labeled pSu9 (*Lower*) upon addition of 2-fold excess of rat dTom20 was calculated according to the equation $[\Delta\delta(^1H)^2 + (\Delta\delta(^{15}N)/15)^2]^{1/2}$. Resonances from residues M1, A2, K33, and R34 of pSu9N and those from T35 and I36 of pSu9C were not detected. Resonances from M1, A2, K33, R34, T35, and I36 were not assigned for pSu9. Chemical-shift changes were not followed for the residues indicated with a dot because of line broadening or signal overlapping. P, proline residue.

patterns and import rates differ among the three fusion proteins (Fig. 2*B*). We also tested other combinations of pSu9N, pSu9C, and HBD in the chimeric presequence of the DHFR fusion proteins (Fig. S4*A*) and found that pSu9C, but not HBD, at the N terminus of the presequence still has an ability to import the DHFR domain into mitochondria, but with much lower (0.07–0.1-fold) efficiencies than those with N-terminally attached pSu9N (Fig. S4*B*). Therefore the presence of the Tom20-binding element in the N-terminal half is essential for the presequence to guide proteins to mitochondria, although import rates vary significantly with different combinations of the N-terminal and C-terminal segments in the presequences. The N-terminal Tom20-binding element evidently promotes efficient binding of the presequence to Tom20 at the *cis* site of the TOM40 complex. Besides, the second Tom20-binding element appears to cooperate with the first Tom20-binding element in the N terminus of the presequences to enhance import into mitochondria.

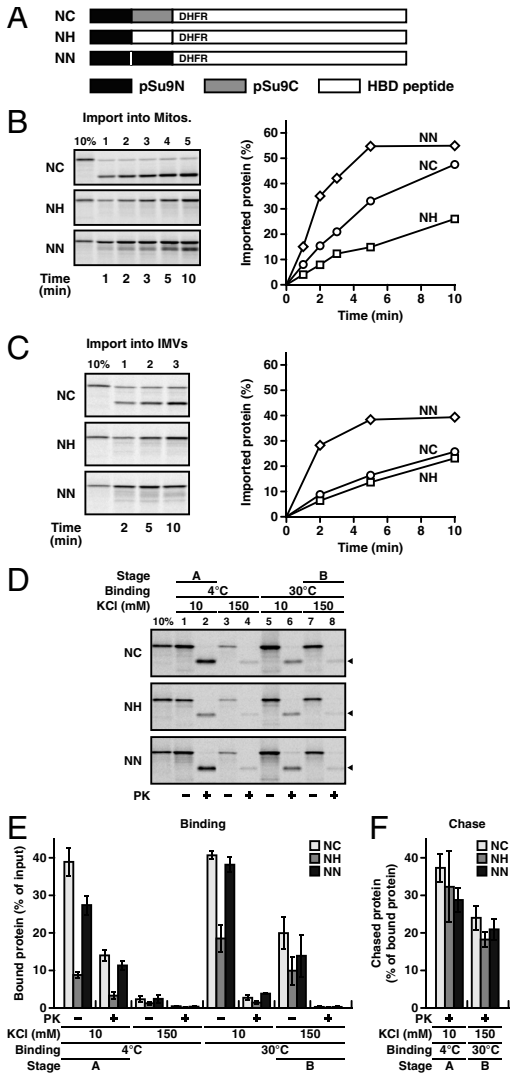

Fig. 2. In vitro import of pSu9-DHFR derivatives into mitochondria and IMVs. (*A*) Schematic representation of the fusion proteins, NC-DHFR (NC), NH-DHFR (NH), and NN-DHFR (NN). (*B*) NC, NH, and NN were incubated with isolated mitochondria for the indicated times at 25 °C. The mitochondria were treated with 200 μg/mL PK for 30 min on ice and reisolated by centrifugation. The proteins were analyzed by SDS-PAGE and radioimaging, and the imported, protease-protected proteins were plotted (*Right*). The amounts of the fusion proteins added to each reaction were set to 100%. (*C*) NC, NH, and NN were incubated with IMVs for the indicated times at 25 °C. The IMVs were treated with 50 μg/mL PK for 30 min on ice and reisolated by ultracentrifugation. The proteins were analyzed as in *B*. The amounts of the fusion proteins added to each reaction are set to 100%. (*D*) Two-step import of pSu9-DHFR derivatives into mitochondria. NC, NH, and NN were incubated with mitochondria without ΔΨ for 10 min at 4 °C or at 30 °C in binding buffer. The samples were divided into halves, which were diluted 5-fold with SMC buffer (250 mM sucrose, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-KOH, pH 7.2, 10 μM carbonyl cyanide *m*-chlorophenylhydrazone) containing 10 mM KCl or 150 mM KCl and incubated for 10 min at 4 °C. The samples were reisolated by centrifugation and treated with or without 100 μg/mL proteinase K (PK) in SMC buffer with 10 mM KCl, 1 μM methotrexate, and 1 mM NADPH. Arrowheads indicate protease-resistant DHFR-containing fragments. Ten percent, 10% of input. (*E*) Quantification of the bound forms in *D*. The amounts of the fusion proteins added to each reaction are set to 100%. (*F*) NC, NH, and NN were bound to mitochondria without ΔΨ at 4 °C with 10 mM KCl or at 30 °C with 150 mM KCl as in *D*. The mitochondria were reisolated by centrifugation and incubated for 10 min at 30 °C in chase buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2, 10 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 20 mg/ml BSA, 2 mM KPi, pH 7.2, 2 mM ATP, 2 mM NADH, 5 mM sodium malate). The mitochondria were treated with 100 μg/mL PK for 30 min on ice and reisolated by centrifugation. The amounts of the pSu9-DHFR derivatives bound to −ΔΨ mitochondria are set to 100%.

Second Tom20-Binding Element in pSu9 Facilitates Translocation Across the Outer and/or the Inner Membranes. Although Tom20 is a general presequence receptor, the presequence could interact with components other than Tom20 along the import pathway, e.g., subunits of the TOM40 and TIM23 complexes and MMC (mitochondrial Hsp70-associated motor and chaperone) (20) proteins and could also sense the membrane potential across the inner membrane (ΔΨ), which is essential for protein translocation across the inner membrane (3–5). To determine whether the second Tom20-binding element contributes to translocation across the outer membrane or the inner membrane, we compared the import of NN-DHFR, NC-DHFR, and NH-DHFR into mitochondria with import into purified inner-membrane vesicles (IMVs), which contain normal amounts of inner-membrane import machinery and the Hsp70 motor, Ssc1p, but contain significantly reduced amounts of the outer-membrane import machinery (Fig. S4*C*).

In the presence of ΔΨ, NH-DHFR and NC-DHFR were transported into IMVs to similar extents, whereas NN-DHFR was imported with a 3-fold higher efficiency (Fig. 2*C*). This is in contrast to the case of intact mitochondria, where NN-DHFR was imported with a 2-fold higher efficiency than NC-DHFR, but NC-DHFR with a 2-fold higher efficiency than NH-DHFR, also (Fig. 2*B*). Therefore, the presence of pSu9N, but not pSu9C, in the C-terminal half of the presequence enhances the rate of precursor translocation across the inner membrane, which could explain the reason for the higher rate of import of NN-DHFR into mitochondria than those of NC-DHFR and NH-DHFR. On the other hand, the presence of pSu9C in the C-terminal half of the presequence should accelerate the step prior to, but not the step of, the translocation across the inner membrane in the entire process of the mitochondrial import. When we specifically delete the cytosolic domain of Tom20, import of both NC-DHFR and NN-DHFR was significantly impaired (Fig. S5 *A*–*C*).

To test the above interpretation directly, we performed two-step import experiments for NC-DHFR, NH-DHFR, and NN-DHFR. In the two-step import, we can analyze the steps of binding to mitochondria and subsequent chase into the matrix, separately. The binding step can be assessed by incubation of the precursor proteins with mitochondria without ΔΨ to block the translocation across the inner membrane. The previous study showed that, in the absence of ΔΨ, long-presequence-containing precursor proteins are accumulated at the level of the TOM40 complex, forming two distinct intermediates at stage A or at stage B (13). At stage A, a positively charged N-terminal segment of the presequence binds to the *trans* site through electrostatic interactions without unfolding of the mature part. At stage B, the mature part is unfolded and trapped by the inner wall of the Tom40 channel mainly through hydrophobic interactions (13, 19), irrespective of additional interactions of the N-terminal segment of the presequence with the *trans* site. The subsequent chase step can be assessed by regeneration of ΔΨ of the mitochondria with bound precursor proteins (13).

NC-DHFR, NH-DHFR, and NN-DHFR were incubated with isolated mitochondria in the absence of ΔΨ at 4 °C or 30 °C, and subsequently, the mitochondria were washed with buffer containing 10 mM KCl or 150 mM KCl. Because spontaneous unfolding of DHFR depends on temperature, DHFR fusion proteins tend to generate the stage-A intermediate at 4 °C with low-salt wash and the stage-B intermediate at 30 °C with high-salt wash. Indeed at 4 °C, NN-DHFR, NC-DHFR, and NH-DHFR bound to mitochondria were sensitive to wash with high-salt buffer, but their DHFR domains were only moderately degraded after protease treatment (Fig. 2*D*), suggesting predominant accumulation of the intermediate at stage A. At 30 °C, the fusion proteins bound to mitochondria were not sensitive to wash with high-salt buffer, but their DHFR domains were nearly completely degraded by protease treatment (Fig. 2*D*), suggesting accumulation at stage B.

Binding of NH-DHFR to the mitochondrial surface was reduced, especially at stage A, as compared with that of NN-DHFR and NC-DHFR (Fig. 2*E*), suggesting that the second C-terminal Tom20-binding element in pSu9 is important for binding of the DHFR fusion proteins to the TOM40 complex. Binding of CC-DHFR, CH-DHFR, and CN-DHFR to the mitochondrial surface also showed similar dependence on the second C-terminal Tom20-binding element (Fig. S4 *D* and *E*).

The intermediate bound to deenergized mitochondria (without $\Delta\Psi$) at stage A or stage B can be chased into the matrix by replenishment of $\Delta\Psi$ (13). When $\Delta\Psi$ was regenerated, NC-DHFR, NH-DHFR, and NN-DHFR intermediates at stage A and stage B were chased into the matrix with similar efficiencies to one after another (Fig. 2*F*). This suggests that the presence of the second C-terminal Tom20-binding element in the presequence is not important for transfer from the TOM40 complex to the TIM23 complex, which is likely the rate-limiting step in the chase reaction.

Cooperation of the Two Tom20-Binding Elements in pSu9 in Tethering Precursor Proteins to the TOM40 Complex. To determine the translocator components that bind to the N-terminal and C-terminal halves of the pSu9 presequence, we performed site-specific photo-cross-linking experiments (13) for the fusion proteins bound to deenergized mitochondria. The binding process of presequence-containing precursor proteins to the TOM40 complex consists of two sequential steps involving presequence binding to the *cis* site on the cytosolic side and then to the *trans* site on the IMS side (2). The *trans*-site binding can be further dissected into two distinct steps, formation of the stage-A intermediate and subsequent shift to the stage-B intermediate as described above. A photoreactive cross-linking group, benzoylphenylalanine (BPA) moiety, was introduced in the N-terminal half or in the C-terminal half of the pSu9 presequence by in vitro translation in the presence of a suppressor tRNA carrying BPA (13). Although BPA at residue 15 or 18 in the N-terminal half of pSu9 was cross-linked to recombinant dTom20 (Fig. S6), we could not detect cross-linking between residue 15 or 18 in pSu9 and endogenous Tom20 in isolated yeast mitochondria due to difficulty in generation of a stable *cis*-site bound form with mitochondria (18). We thus analyzed only the *trans*-site bound form by incubation of BPA-carrying pSu9-DHFR with mitochondria in the absence of $\Delta\Psi$ at 4 °C with low-salt wash (for stage A) or at 30 °C with high-salt wash (for stage B) followed by UV irradiation. The cross-linked partner was identified by immunoprecipitation with antibodies against Tom20, Tom22, and Tim50. At stage A, residue 21 in the N-terminal half of pSu9 was cross-linked to Tom22 (Fig. 3*A*, *Upper*, lanes 1–5), whereas residue 65 in the C-terminal half of pSu9 was cross-linked to Tom20 (Fig. 3*A*, *Lower*, lanes 1–5). At stage B with unfolding of the DHFR domain, residue 21 was cross-linked to Tom22 slightly but mainly to Tim50 (Fig. 3*A*, *Upper* panel, lanes 11–15), and residue 65 was only slightly cross-linked to Tom20 (Fig. 3*A*, *Lower*, lanes 11–15). Cross-linking of residue 21 to Tim50 at stage B indicates that the presequence is already partly transferred from the *trans* site of the TOM40 complex to Tim50 of the TIM23 complex in the inner membrane, indicating that Tim50 is a presequence receptor in the inner membrane. Interestingly, cross-linking to Tim50 at stage B was observed only after wash with 150 mM KCl, but not with 15 mM KCl (Fig. 3*A*, *Upper*, lanes 6–10), suggesting that, although the presequence interacts with Tom22 in the IMS primarily through electrostatic interactions, the interactions between the presequence and Tim50 are hydrophobic rather than electrostatic ones.

We changed the positions of BPA systematically along the presequence of pSu9-DHFR and analyzed their cross-linked products at stages A and B (Fig. 4*B* and Fig. S7*A*). At stage A, the N-terminal half of the presequence bound to Tom22, Tom40,

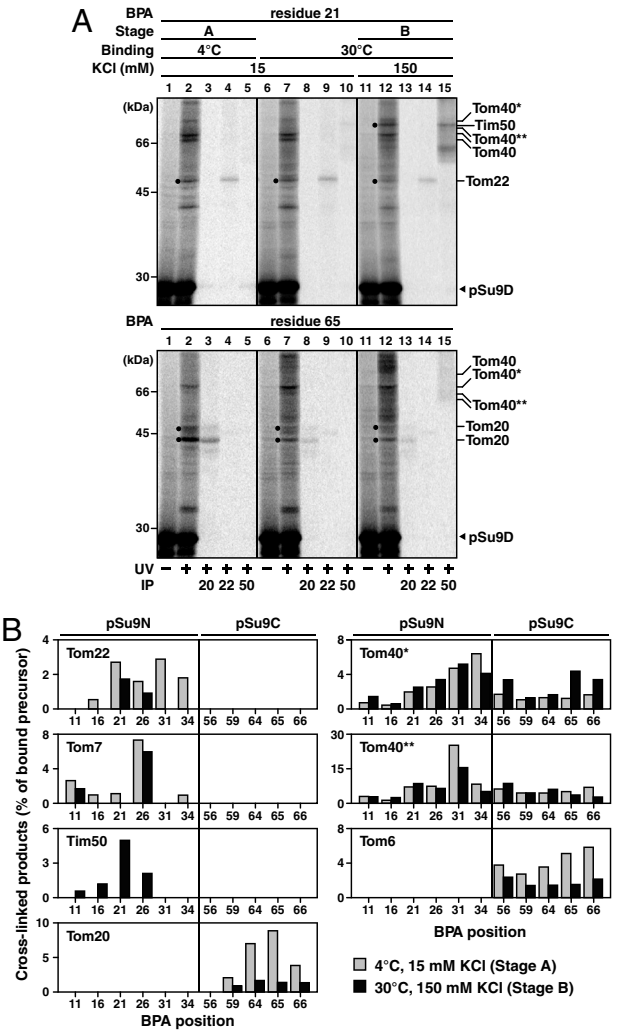

Fig. 3. Site-specific photo-cross-linking of pSu9-DHFR at stage A and stage B. (*A*) Cross-linking experiments and immunoprecipitation (IP) for pSu9-DHFR containing BPA with a photoreactive cross-linking group at residue 21 (*Upper*) or 65 (*Lower*) bound to deenergized mitochondria at 4 °C or at 30 °C. The mitochondria were washed with 15 mM KCl or 150 mM KCl, and UV-irradiated. IP was performed with antibodies against Tom20, Tom22, and Tim50. The samples loaded in lanes for IP are 3-fold excess over those in lanes for cross-linking. Tom40, Tom40*, and Tom40** indicate cross-linked products with Tom40 with different cross-linking configurations (13). Dots indicate cross-linked products precipitated with anti-Tom20, anti-Tom22, or anti-Tim50 antibodies. UV, UV irradiation. (*B*) Summary of the results of site-specific photo-cross-linking. The amounts of cross-linked products with Tom22, Tom7, Tim50, Tom40*/Tom40** (as in *A*), Tom20, and Tom6 were quantified and plotted against the positions of introduced BPA. Gray bars represent the stage-A intermediate and black bars represent the stage-B intermediate. The amounts of the precursor form of pSu9-DHFR recovered with mitochondria under the same conditions without UV irradiation were set to 100%.

and Tom7 in the *trans* site as found previously (13), whereas the C-terminal half generated cross-linked products with Tom20 and Tom40. We confirmed, by monitoring the effect of deletion of the cytosolic domain of Tom20, that the C-terminal half of the pSu9 presequence was cross-linked to the cytosolic domain, not the transmembrane segment, of Tom20 (Fig. S7*B*). We also found that the C-terminal residues in the pSu9 presequence were cross-linked to a small protein (Fig. S7*A*), which was identified as Tom6 (Fig. S7*C*).

When we compare the cross-linking patterns for stage B with those for stage A, the N-terminal half of the presequence moved from Tom22 and Tom7 to Tim50 upon transfer from stage A to

stage B, whereas the C-terminal residues shifted away from Tom20 and Tom6 (Fig. 3*B*). Deletion of the *TOM6* gene decreased the import of NN-DHFR, NC-DHFR, and NH-DHFR by 40% (Fig. S5*D*), yet it is at the moment unclear if this reflects a possible Tom6 function as a part of receptors in the TOM40 complex or altered organization of the TOM40 complex structure lacking Tom6 (21). These results suggest that release of the C-terminal part of the presequence from Tom20 of the *cis* site allows the entire presequence to cross the outer membrane through the Tom40 channel, so that it is passed onto the inner-membrane presequence receptor, Tim50. Therefore the interaction between the C-terminal Tom20-binding element and Tom20 likely facilitates the precursor binding to the TOM40 complex at stage A, but not at stage B, as observed in Fig. 2*E*.

Discussion

We found that sequential interactions of the general import receptor Tom20 with different parts of the presequence facilitate not only targeting specificity but also efficient protein import into mitochondria. Our results suggest that the long presequence of pSu9 contains two binding elements for Tom20 on the mitochondrial surface. Although the previously proposed Tom20-recognition motifs, $\phi\chi\chi\phi\phi$ (8) and $\sigma\phi\chi\beta\phi\phi$ (9) (ϕ is a hydrophobic/aromatic residue, σ is a hydrophilic residue, χ is an any amino acid residue, and β is a hydrophilic/basic residue), are found in the N-terminal half of pSu9 [e.g., residues 8–12 (ASRLA)], they are not found in the C-terminal half. However, we can extend the motifs to $\phi\chi\chi\beta\phi\phi$ [residues 7–12 (LASRLA) of Su9N and residues 62–67 (FQKRAY) of pSu9C] and $\phi\chi\chi\beta\chi\phi\phi$ [residues 56–62 (ATTRQAF) of pSu9C].

What are the roles of the two Tom20-binding elements in the long pSu9 presequence in protein import into mitochondria? The N-terminal binding element appears essential for precursor targeting to mitochondria because DHFR fusion proteins lacking this element were not imported into mitochondria (Fig. S4*B*). On the other hand, although the C-terminal Tom20-binding element is not essential for mitochondrial import, it affects the import efficiency of the DHFR fusion proteins significantly at least in vitro. Because NC-DHFR was imported into mitochondria more efficiently than NH-DHFR (Fig. 2*B*) while NC-DHFR and NH-DHFR were imported into IMVs with similar efficiencies (Fig. 2*C*), we conclude that the C-terminal Tom20-binding element accelerates the step prior to the translocation across the inner membrane, i.e., at the level of the TOM40 complex.

Binding of the C-terminal Tom20 element to the *cis* site for presequence binding together with binding of the N-terminal element to the *trans* site enhances tethering of the presequence to the TOM40 complex. This was demonstrated by taking "snapshots" of the presequence-translocator complex interactions at different steps of the translocation across the outer membrane by site-specific photo-cross-linking. After possible binding of the N-terminal half of the presequence of pSu9-DHFR to the *cis* site of the TOM40 complex, which mainly consists of Tom20 and Tom22 (14), the presequence is transferred quickly to the *trans* site of the TOM40 complex to form the stage-A intermediate (Fig. 4) (13, 18). At this stage A, the N-terminal half of the presequence is bound to or close to the IMS-facing regions of Tom40, Tom22, and Tom7, whereas the second Tom20-binding element in the C-terminal half of the presequence is bound to the cytosolic domain of Tom20 and Tom6 (Fig. 3*B*). Because formation of the stage-A intermediate largely requires the second Tom20-binding element in the presequence (Fig. 2*D*), the precursor protein is tethered to the TOM40 complex through not only interactions of the first Tom20-binding element in the presequence with the *trans* site but also those of the second C-terminal Tom20-binding element with the *cis* site at stage A (Fig. 4). This means that, although the *cis*-site bound form itself is not stable (18), cooperation of Tom20 with the *trans* site in binding to the

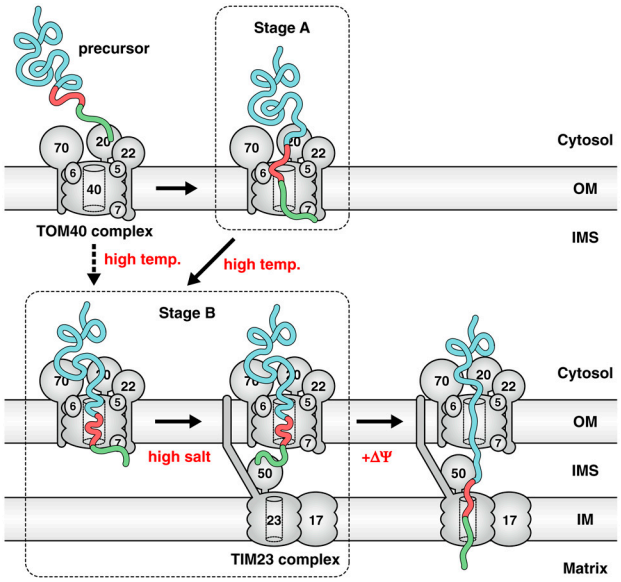

Fig. 4. Model of translocation of pSu9-DHFR across the outer mitochondrial membrane. Interactions between the presequence of translocating pSu9-DHFR and the components of the TOM40 and TIM23 complexes revealed by the site-specific photo-cross-linking experiments under the condition of $-\Delta\Psi$ are schematically shown for different steps along the import pathway. Green tubes indicate pSu9N, and red tubes pSu9C. Red letters indicate conditions to assess each step in vitro. OM, outer membrane; IM, inner membrane.

presequence leads to enhanced tethering of the precursor protein to the TOM40 complex at stage A, which will increase import efficiency. During the import, the stage-A intermediate is at least partly shifted to the stage-B intermediate (13), in which the unfolded DHFR domain is trapped by the Tom40 channel (Fig. 4). At stage B, the C-terminal half of the presequence is dissociated from Tom20 to cross the outer membrane, and the N-terminal half of the presequence partly moves from the *trans* site to Tim50 in the TIM23 complex, switching the interactions from electrostatic to hydrophobic ones (Figs. 3*B* and 4). Subsequently, ΔΨ drives further translocation of the presequence across the inner membrane (Figs. 2*F* and 4).

Is the presence of the second Tom20-binding element common to mitochondrial proteins with long presequences, such as subunit 9? Statistical analyses of the lengths of putative mitochondrial presequences in the database show that about 30% of mitochondrial presequences are longer than 40 amino acid residues (Fig. S1*A*) and that these long presequences also contain multiple Tom20-recognition motifs described above (Fig. S1 *B* and *C*). Besides, presequences with three or more Tom20-binding elements often have the elements located >20 residues apart (Fig. S1*D*). These multiple Tom20-recognition motifs could offer a second C-terminal Tom20-binding element, like the pSu9 presequence, to increase the import efficiencies. In this connection, it would be interesting to note that the presequence with a duplication of pSu9 further improved the import efficiency of mitochondrial precursor proteins (22). In order to further confirm that a second Tom20 binding element is not specific to the Su9 presequence, we picked up the presequence of Mprl3, a mitochondrial ribosomal protein of the large subunit 1, as one of the long presequences that was predicted to have second Tom20-binding elements. We found that the N-terminal and C-terminal peptides of the Mprl3 presequence (pMprl3N and pMprl3C, respectively) indeed bind to Tom20 like the pSu9 presequence (Fig. S2*C*). The observed chemical-shift changes of dTom20 for pMprl3C were small as compared to those for pSu9C partly because we could add only a lower concentration (0.2 mM, i.e., 1/3 of dTom20) of pMprl3C than pSu9C (1.4 mM, i.e., 2-fold

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excess over dTom20) due to its low solubility. Besides, pMprl3C-dTom20 interactions associated with precipitation, which also lowered the effective concentration of pMprl3C. Nevertheless, pMprl3C caused small but significant chemical-shift perturbation for dTom20.

In conclusion, the interactions between the presequence and the receptor Tom20 have a dual role, the targeting signal recognition and facilitation of the efficient tethering of the presequence to the TOM40 complex to increase efficiency of the import process, although its biological or in vivo relevance is still open to future studies. Presequences and the import receptor Tom20 have been likely optimized to increase efficiency as well as specificity of mitochondrial protein import during evolution.

Materials and Methods

Fusion Proteins. The DNA fragments for pSu9N and pSu9C were amplified by PCR by using pGEM-4Z/pSu9(1-69)-DHFR as a template. The DNA fragment for pb_2(111-144) was amplified by PCR by using pGEM-4Z/pb2-DHFR as a template. The obtained DNA fragments were introduced into the SalI/XhoI site of pGEM-4Z/pSu9(1-69)-DHFR to replace with the DNA fragment for pSu9(1-69), resulting in pGEM-4Z/pSu9(1-34)-DHFR, pGEM-4Z/pSu9(35-69)-DHFR, and pGEM-4Z/pb2(111-144)-DHFR. To construct the genes for NN-DHFR, NC-DHFR, HC-DHFR, NH-DHFR, CH-DHFR, and HH-DHFR (Fig. 2*A* and Fig. S3), the DNA fragments for pSu9N, pSu9N, pb_2(111-144), pSu9N, pSu9C, and pb_2(111-144) were introduced into the BamHI/SalI sites of pGEM-4Z/pSu9(1-34)-DHFR, pGEM-4Z/pSu9(35-69)-DHFR, pGEM-4Z/pSu9(35-69)DHFR, pGEM-4Z/pb2(111-144)-DHFR, pGEM-4Z/pb2(111-144)-DHFR, and pGEM-4Z/pb2(111-144)-DHFR, respectively.

Presequence Peptides. For the preparation of unlabeled or uniformly labeled pSu9, pSu9N, and pSu9C peptides, fusion protein consisting of the gene10 protein plus one glutamate followed by the presequences were expressed in *E. coli* cells using the vector pET-17xb (Novagen) in LB medium or M9-minimal medium containing $^{15}NH_4Cl$ (1.0 g/L) and/or [U-^{13}C]-glucose (2.0 g/L), respectively. The fusion proteins were recovered as inclusion bodies, solubilized in 4 M urea, and subjected to V8 protease treatment (1/50 wt/wt) to yield the presequence peptides, which were further purified by reversed-phase HPLC.

NMR Measurements. NMR spectra were recorded on a Bruker AVANCE600 NMR spectrometer. A series of 3D double and triple resonance experiments (6) were performed for spectral assignments of pSu9N and pSu9C peptides.

Import into Mitochondria and IMVs. Wild-type mitochondria were isolated from the yeast D273-10B strain. The radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysates by coupled transcription/translation in the presence of ^{35}S-methionine. Import reactions were preformed as described previously (23). IMVs were prepared from isolated mitochondria as described previously (24). Import buffer for IMVs is 0.6 M sorbitol, 20 mM KPi, pH 7.4, 5 mM $MgCl_2$, 0.3 mM EDTA, 0.6 mM DTT, 10 mM sodium succinate, 10 mM sodium malate, 1 mg/mL fatty acid-free BSA, 4 mM sodium ascorbate, 1 mM ATP, 0.6 mg/mL cytochrome *c*, 8 mM creatin phosphate, and 13 units/mL creatin kinase.

Miscellaneous. Two-step import was performed by using mitochondria treated with carbonyl cyanide *m*-chlorophenylhydrazone for 3 min at 4 °C to dissipate $\Delta\Psi$ for binding, and by subsequent incubation of the mitochondria with BSA, malate, and NADH to regenerate $\Delta\Psi$ for chase as described previously (13). Photo-cross-linking experiments with pSu9-DHFR derivatives bearing BPA as described previously (13).

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