Efficient Twin Arginine Translocation (Tat) Pathway Transport of a Precursor Protein Covalently Anchored to Its Initial cpTatC Binding Site*

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The thylakoid twin arginine protein translocation (Tat) system operates by a cyclical mechanism in which precursors bind to a cpTatC-Hcf106 receptor complex, which then recruits Tha4 to form the translocase. After translocation, the translocase disassembles. Here, we fine-mapped initial interactions between precursors and the components of the receptor complex. Precursors with (Tmd)Phe substitutions in the signal peptide and early mature domain were bound to thylakoids and photo-cross-linked to components. cpTatC and Hcf106 were found to interact with different regions of the signal peptide. cpTatC cross-linked strongly to residues in the immediate vicinity of the twin arginine motif. Hcf106 cross-linked less strongly to residues in the hydrophobic core and the early mature domain. To determine whether precursors must leave their initial sites of interaction during translocation, cross-linked precursors were subjected to protein transport conditions. tOE17 cross-linked to cpTatC was efficiently translocated, indicating that the mature domain of the precursor can be translocated while the signal peptide remains anchored to the receptor complex.

The thylakoid twin arginine protein translocation (Tat)3 systems are widely present in the cytoplasmic membranes of bacteria and archaea and in the thylakoid membrane of plant chloroplasts (1–3). Tat systems operate in parallel with the well studied Sec systems but have different energy requirements, a different mode of transport, and novel translocase components. Whereas the Sec system employs ATP and the SecA protein to transport folded protein substrates (2, 8), Tat pathway precursor proteins possess hydrophobic signal peptides similar to Sec pathway precursor proteins, with a charged amino-terminal N-domain, a hydrophobic H-domain, and a polar C-domain containing the signal peptidase “AXA” cleavage site. However, Tat signal peptides are distinctive in possessing an essential twin arginine motif in their N-domains (9–11). In thylakoids, three membrane protein components are essential for Tat translocation in vivo and in vitro (12–16). Tha4 and Hcf106 are homologous proteins with similar structure, each containing an amino proxi-

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2 The abbreviations used are: Tat, twin arginine translocation; (Tmd)Phe, -4’-3-[trifluoromethyl]-3H-diazirin-3-ylphenylalanine; PMF, protonmotive force; BN-PAGE, blue native-PAGE.

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cross-linked to Tat components. Our results were generally similar to the results with the E. coli Tat system but with important differences. The pattern of interactions showed that cpTatC and Hcf106 interact with distinct regions of the signal peptide, raising the possibility that these two components can simultaneously bind to the same precursor protein. Cross-links between precursor and Tha4 were not observed, even when the membranes were energized for translocation. As an alternative test of the hand-off mechanism for thylakoids, we asked whether it is necessary for the signal peptide to be passed from cpTatC or Hcf106 in order for the precursor to be translocated. Precursors cross-linked to cpTatC were efficiently transported across the thylakoid membrane. These results will be discussed in the context of current models for the mechanism of Tat pathway transport.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis**—DNA clones for the various precursors used in this study were constructed by PCR mutagenesis with the QuikChange mutagenesis kit from Stratagene and the clone for tOE17 (11) as template. DNA sequencing on both strands at the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Facility verified all constructs. The clone for tOE17M was prepared by replacing the TAG stop codon at the end of the tOE17 DNA sequence with an ATG codon. This allowed translation to continue to the next stop codon in the vector sequence, which added the amino acids MTQGLIMAM. tOE17M clones used for (Tmd)Phe translational incorporation were constructed by replacing the codons for amino acid residues shown in Fig. 1 with TAG.

**Preparation of Radiolabeled Precursors**—Capped mRNA was transcribed in vitro with SP6 polymerase (Promega) and was translated in the presence of [35S]methionine with a homemade wheat germ translation system (23). N-Pentenoyl-(Tmd)Phe-tRNA_{CUA}, the protected form of the suppressor (Tmd)Phe-tRNA_{CUA}, was purchased from Botanica PhotoProbes and prepared following the manufacturer’s instructions. 1 μl of the (Tmd)Phe-tRNA_{CUA} (0.1 μg/μl) was added per 25 μl of translation reaction.

**Preparation of Chloroplasts, Thylakoids, and Lysate**—Intact chloroplasts were isolated from 9- to 10-day-old pea seedlings as described by Cline et al. (24). Chloroplast pellets were lysed at 2 mg of chlorophyll/ml in 10 mM HEPES, KOH, pH 8.0, 10 mM MgCl2 at 0 °C for 10 min and adjusted to import buffer (IB: 50 mM HEPES, KOH, pH 8.0, 0.33 M sorbitol), 10 mM MgCl2. Thylakoids were obtained from lysates by centrifugation (3,300 × g) for 8 min. Stomatal extract was obtained from the supernatant by further centrifugation (100,000 × g) for 20 min to remove envelope membranes. Thylakoids were washed with IB and resuspended in IB to 1 or 2 mg of chlorophyll/ml before use. Chlorophyll concentrations were determined according to Arnon (25).

**Thylakoid Protein Binding, Chase, and Transport Assay**—Precursor binding assays were conducted as described (26). Basically, [35S]methionine-labeled in vitro translation products were incubated with apyrase for 10 min at 0 °C and then with an equivalent volume of washed thylakoid membranes for 15 min in darkness at 0 °C. Thylakoid membranes were then recovered by centrifugation, washed twice with IB, and further incubated for transport from the bound state (chase) by resuspension with a stoichiometric quantity of stromal extract, 5 mM Mg-ATP, and 2 mM dithiothreitol and incubation for 15 min at 25 °C in ~50 microeinstein/mg2/s white light. Thylakoid membranes were recovered by centrifugation and analyzed by SDS-PAGE and fluorography. For transport assays, in vitro translation products were incubated with an equivalent volume of chloroplast lysate (1 mg of chlorophyll/ml) for 15 min at 25 °C in the light. Recovered thylakoid membranes were further treated with thermolysin to remove non-transported precursor proteins (24).

**Bound Precursor Extraction Assays**—Precursor-bound thylakoid membranes (15 μg of chlorophyll) were resuspended in 30 μl of the extraction solution (prepared in 10 mM HEPES, KOH, pH 8.0, just prior to use) and incubated at 0 °C for 10 min. Samples were centrifuged at 100,000 × g for 2 min at 2 °C. Pellets and supernatants were analyzed by SDS-PAGE and fluorography. Radiolabeled proteins were quantified by scintillation counting of bands extracted from dried gels as described (23).

**Cross-linking of (Tmd)Phe-labeled Precursors**—Thylakoid membranes recovered from binding assays or binding reaction mixtures were washed with IB, and then with an equivalent volume of washed thylakoids. With the exception of the Gly24 substitution, all were indistinguishable from tOE17M in this assay (data not shown, see supplemental Fig. S1). Gly24, which places the bulky (Tmd)Phe group next to the RR motif, was chosen to bracket the RR motif. Residues Ala21 through Gly24 and Ala21 through Gly18 are also part of the early hydrophobic region. Substitutions in Val18, Gly17, and Phe30 are representative of the early mature sequence of OE17 (Fig. 1A). All substituted precursors were assayed for protein transport with isolated thylakoids. With the exception of the Gly24 substitution, all were indistinguishable from tOE17M in this assay (data not shown, see supplemental Fig. S1). Gly24, which places the bulky (Tmd)Phe group next to the RR,
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Some Tmd(Phe) Substitutions in the Signal Peptide Enhance the Binding Properties of tOE17—Although not significantly different in transport assays, substituted precursors showed variation in thylakoid binding assays. Precursors with (Tmd)Phe at positions Ala^21 (Fig. 1C, lane 2), Ala^21, Ile^19 (Fig. 1D, lanes 2 and 8, respectively), and Ala^8 (Fig. 1E, lane 8) had a reduced ability to stably bind to thylakoids. By contrast, precursors with (Tmd)Phe at the remaining positions bound strongly to thylakoids (Fig. 1, B and D–F, lanes designated "b"). To determine whether such binding reflected productive interaction, thylakoid-bound precursors were subjected to a chase assay, wherein conditions for protein translocation were re-established. All bound precursors were efficiently transported from the bound state (Fig. 1, B–F, lanes designated "c").

tOE17M precursors with Tmd(Phe) substitutions were also examined for association with the cpTatC-Hcf106 receptor complex by BN-PAGE and fluorography (Fig. 2A). Previous work showed that bound precursors are recovered with the ~700-kDa receptor complex when the precursor-bound thylakoids are dissolved with digitonin and subjected to BN-PAGE (15). All precursors analyzed could be detected at ~700 kDa. However tOE17M with substitutions in positions Val^20, Gly^18, Gly^10, Gly^8, Leu^7 produced dramatically more precursor-receptor complex on BN-PAGE (Fig. 2A, lanes 9, 13, 15, 19, respectively, compared with tOE17M lanes 1, 11; supplemental Fig. S2).

The binding reactions used for the above analyses were conducted with thylakoids in the absence of the PMF. We have recently found that precursors bind more tightly to membranes energized with the PMF and that this binding is characterized by greater stability of the precursor-receptor complex on BN-PAGE, similar to that observed for tOE17M Val^20 (upper panels) and unsubstituted TOE17 (lower panels) were bound to thylakoids, which were then subjected to washings with 50, 100, and 130 mM KCl (Fig. 2B, upper panel). The relevant Tmd(Phe)-containing precursors also appear more resistant to extraction with salt or urea solutions alone. The relevant Tmd(Phe)-containing precursors also appear more resistant to extraction with salt or urea solutions alone. The relevant Tmd(Phe)-containing precursors also appear more resistant to extraction with salt or urea solutions alone. The relevant Tmd(Phe)-containing precursors also appear more resistant to extraction with salt or urea solutions alone. The relevant Tmd(Phe)-containing precursors also appear more resistant to extraction with salt or urea solutions alone. The relevant Tmd(Phe)-containing precursors also appear more resistant to extraction with salt or urea solutions alone.

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protein translocation. The underlying physical basis for this tighter binding is not known; however, we note that the previously studied recombinant precursors DT17 and DT23, with a slightly higher hydrophobicity in their H-domain, exhibit the same behavior (15).

The Twin Arginine Region of the Signal Peptide Contacts cpTatC and the Hydrophobic Core Contacts Hcf106—For cross-linking analysis, precursor-bound thylakoids were subjected to UV irradiation for 5 min at 0 °C. Six of the nine precursors substituted in the signal peptide, Ala24, Ala31, Val36, Gly8, Gly9, Ala16, and Leu27 (panel B, substitutions Gly8, Gly9, Ala16, and Leu27 (panel C) and substitutions Val36, Gly8, Ala16, and Phe30 (panel D). The two panels in B and D were from different experiments but are displayed together as representing the same region from the signal peptide.

Cross-link partners were determined by co-immunoprecipitation of cross-link products directed by other residues. (Tmd)Phe-substituted tOE17M and cpTatC components.

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FIGURE 3. UV cross-linking of bound tOE17M (Tmd)Phe derivatives. In vitro translated (Tmd)Phe derivatives of tOE17M were bound to thylakoid membranes and recovered membranes resuspended in IB at 0.5 mg of chlorophyll/ml (see “Experimental Procedures”). Samples (10 μl) were irradiated (+) or not (−) for 5 min at 0 °C with a 365-nm UV lamp. Samples were analyzed by SDS-PAGE/fluorography. An asterisk indicates the position of the cross-link product obtained. UV cross-link of tOE17M (panel A), substitutions Ala24, Ala31, Val36, Ile19, and Gly18 (panel B), substitutions Gly8, Gly9, Ala16, and Leu27 (panel C), and substitutions Val36, substitutions Gly8, Ala16, and Phe30 (panel D). The two panels in B and D were from different experiments but are displayed together as representing the same region from the signal peptide.

FIGURE 4. The RR region of the signal peptide contacts cpTatC, whereas the H-domain and the early part of the mature domain contact Hcf106. Precursor-bound and UV-irradiated thylakoids (lanes 1) were subjected to co-immunoprecipitation under denaturing conditions (see “Experimental Procedures”) with antibodies to Tha4, Hcf106, cpTatC, or Alb3 as depicted above the panels. The antibody-bound (B) and unbound (U) fractions were analyzed by SDS-PAGE/fluorography. A, bound and UV cross-linked precursors Ala24, Ala31, and Val36 were specifically co-immunoprecipitated with αcpTatC IgGs, as represented by the co-immunoprecipitation of Val36, B, the faster migrating Gly8 cross-linked precursor was co-immunoprecipitated with αHcf106 IgGs; the slower migrating Gly18 cross-linked precursor was co-immunoprecipitated with αcpTatClgGs. C, cross-linked precursors Gly8 and Gly18 were specifically co-immunoprecipitated with αHcf106 IgGs, as represented by the co-immunoprecipitation of Gly8, D, summary of interactions between (Tmd)Phe-substituted tOE17M and cpTat components.

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for co-immunoprecipitation of cross-link products directed by other residues). Cross-link products from precursors with substitutions Gly8, Gly9, and Val36 were immunoprecipitated solely by antibodies to Hcf106 (see Fig. 4C, lane 4, for a representative co-immunoprecipitation and supplemental Fig. S3 for co-immunoprecipitation of cross-link products directed by other residues). The slower migrating cross-link product from the substitution in Gly18 was immunoprecipitated with antibodies to cpTatC (Fig. 4B, lane 6), whereas the faster migrating cross-link product from Gly18 was immunoprecipitated with antibodies to Hcf106 (Fig. 4B, lane 4). No cross-link product was immunoprecipitated by antibodies to Tha4 (Fig. 4A, lane 2; supplemental Fig. S3) or were any cross-link products immunoprecipitated by antibodies to Alb3 (Fig. 4A, C–E, lanes 8). Alb3 serves as a negative control for this experiment because Alb3 functions on a different thylakoid translocation pathway, the cpSRP pathway (28). Taken together, these results indicate that the region next to the RR motif interacts strongly with cpTatC and that the hydrophobic domain from position Gly18 up to and including the signal peptidase cleavage site interacts with Hcf106 (see Fig. 4D).

The Twin Arginine Motif of the Signal Peptide Is Essential for Interaction with cpTatC and Hcf106—The twin arginine motif RR is a key specificity element for interactions between the precursor and the
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**FIGURE 5.** The RR motif is required for productive binding and interaction with the cpTatC-Hcf106 receptor complex. A, in vitro translated tOE17M or KK-tOE17M with (Tmd)Phe substitutions in Val–20, Gly–10, and Gly–9 (see “Experimental Procedures”) were subjected to binding and chase reactions. Translation products (tp) equivalent to 2.5% of that added to binding reactions and thylakoids representing 10% of the assay were loaded in the lanes. The binding (b) and chase (c) lanes from each panel are from the same gel and film and are juxtaposed for clarity. The RR-containing precursors are in lanes 1–3; the KK precursors are in lanes 4–6. B, cross-link analysis of substituted precursors incubated with thylakoid membranes. In vitro translated precursors were incubated with thylakoid membranes at 0.5 mg of chlorophyll/ml for 15 min in darkness at 0 °C. Unfractionated reaction mixtures (10 μl) were then irradiated (+) or not (−) as depicted above the panel for 5 min at 0 °C. Thylakoid membranes were recovered by centrifugation, washed twice with IB, and analyzed by SDS-PAGE/fluorography.

**FIGURE 6.** tOE17M cross-linked to cpTatC via Val–20 can be transported across the thylakloid membranes. A, in vitro translated tOE17M Val–20 was bound to thylakloid membranes (lane 1). Aliquots (10 μl) of precursor-bound thylakoids at 0.5 mg of chlorophyll/ml were UV irradiated for 5 min at 0 °C (lanes 2–6) and recovered by centrifugation. Recovered thylakoids were resuspended with stromal extract, 5 μM Mg-ATP, and 2 μM dithiothreitol and incubated for 15 min at 25 °C with white light (chase, lanes 3–6) in the absence (lanes 3, 4) or presence (lanes 5, 6) of 0.5 μM nigericin and 1.0 μM valinomycin. Recovered thylakoids were analyzed directly (lanes 3 and 5) or treated with thermolysin (+, lanes 4 and 6) as shown below the panel. All samples were analyzed by SDS-PAGE/fluorography. B, schematic representation of transport of tOE17M Val–20 cross-linked to cpTatC. The amino terminus of cpTatC, which is degraded by thermolysin treatment of thylakoids (27), is shown as a dashed line on the left. After transport and thermolysin treatment, the truncated cpTatC cross-linked to the unprocessed precursor are resistant to further proteolysis.

cpTat machinery (9–11, 15, 17). On the other hand, weak interactions have been reported between twin lysine KK precursors and the thylakoid Tat machinery (29, 30), and Alami et al. (20) detected a cross-link product between a KK precursor and *E. coli* TatB. To determine whether the RR motif is necessary for the interactions described above, the RR motif was substituted by KK in the precursors in which (Tmd)Phe replaced Val–20, Gly–10, and Gly–9 (see “Experimental Procedures”). As expected from previous work, the KK substrates bind poorly to thylakoid membranes compared with the RR-containing precursors (Fig. 5A, lanes 5 compared with lanes 2). The KK precursors were neither transported directly (data not shown) nor chased from the bound state (Fig. 5A, lanes 6 compared with lanes 3). Because KK precursors do not stably bind to thylakoids, potential interactions with cpTat components were assayed by directly UV irradiating the binding reaction mixture, i.e. before recovering and washing the membranes. Fig. 5B shows the results of such an assay. None of the KK precursors exhibited cross-link products (lanes 4, 8, and 12). These observations indicate that precursors with KK substituted for RR do not interact in a measurable way with the thylakoid Tat receptor complex.

**Precursor Cross-linked to cpTatC Can Still Be Transported across the Membrane**—The pattern of interactions between tOE17M and the thylakoid Tat components bears some similarities to that reported between pre85F and *E. coli* Tat components (20). One important difference is that Alami et al. (20) also detected interactions between precursor and TatA. TatA interacted with the same signal peptide region as TatC, but cross-links to TatA were only observed in the presence of the PMF. This suggested that the signal peptide is transferred to TatA for the translocation step (20). In our experiments, cross-links between the signal peptide and Tha4 were not observed, even when activation of the cross-link agent was initiated at varying times following the start of a chase reaction (data not shown).

An alternative way of addressing this issue is to ask whether the signal peptide must be transferred from cpTatC or Hcf106 for translocation to occur. In the experiment shown in Fig. 6, precursor was cross-linked to Hcf106 or cpTatC, and the resulting membranes were subjected to a chase reaction. Because the [35S]methionine label is located at the extreme carboxyl terminus of the precursor and the cross-link occurs via the amino-terminal signal peptide, there are two possible outcomes if translocation occurs. If translocation were accompanied by processing by the thylakoid signal peptidase, the cross-link product would dis-
producing an thylakoids destroys the stromally exposed amino terminus of cpTatC, proteolysis of the amino terminus of cpTatC. Thermolysin treatment of /H11011 sor in the same reactions shows that the cross-link product was trans-
from the thylakoid signal peptidase. In addition, by being linked to cpTatC, the processing site is masked result indicates that tOE17M cross-linked to cpTatC is sufficiently must remain intact in order to be distinguishable in this analysis. This
ysis of the tOE17 adduct because, as mentioned above, the precursor drop in molecular mass of the cross-link product is not due to proteol-
ably interfere with transport of the mature domain.

Comparison with the binding and chase of non-cross-linked precursors in the same reactions shows that the cross-link product was transported with efficiency comparable with tOE17M (Fig. 6 A, lanes 2–4). This result suggests that even when the precursor is covalently bound to cpTatC by the twin arginine region, this interaction does not measurably interfere with transport of the mature domain.

**DISCUSSION**

Interactions detected between the tOE17M precursor protein and thylakoid Tat components were similar in two aspects to those between the preSufI precursor and *E. coli* Tat components (20) using the same (Tmd)Phe photo-cross-link approach. First, residues near the consensus twin arginine motif interacted strongly with cpTatC (TatC ortholog); specifically, the strongest cross-link product with cpTatC was from the same position relative to RR as the preSufI residue that ortholog); specifically, the strongest cross-link product with cpTatC was from the same position relative to RR as the preSufI residue that was strongly cross-linked to cpTatC.5 This verifies the conclusion of Alami et al. (20) that TatC (cpTatC) is the primary recognition component for the consensus RR motif. A second similarity is that tOE17 signal peptide H- and C-domains interacted with Hcf106 (TatB).

Our results also differ from Alami et al. (20). We found that cpTatC and Hcf106 interact primarily with different parts of the signal peptide. Only the weakly interacting Gly 19 cross-linked with both cpTatC and Hcf106. This raises the possibility that the precursor can simultaneously occupy binding sites of cpTatC and Hcf106. Another important difference is that we did not detect any interactions with Tha4 (TatA ortholog). The transient nature of Tha4 association with the translocase (17) might explain the absence of Tha4 cross-linking products. However, the efficient transport of tOE17M cross-linked to cpTatC (Fig. 6) argues that the tOE17M signal peptide does not need to entirely move from cpTatC to other components for the substrate protein to be translocated. cpTatC (TatC) has been suggested to provide the driving force for Tat protein translocation (31), and our results are consistent with that suggestion. Our results do not exclude the possibility that Tha4 interacts with the signal peptide at some stage of the process. For example, Tha4 might displace Hcf106 from its signal peptide binding site without displacing cpTatC. In addition, our results do not rule out a role for Tha4 as the protein-conducting channel. Rather, they simply suggest that the channel is attached to or includes cpTatC.

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