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Requirement of a Tha4-conserved Transmembrane Glutamate in Thylakoid Tat Translocase Assembly Revealed by Biochemical Complementation*

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Carole Dabney-Smith, Hiroki Mori‡, and Kenneth Cline§

From the Horticultural Sciences and Plant Molecular and Cellular Biology, University of Florida, Gainesville, Florida 32611

The thylakoid Tat system employs three membrane components and the pH gradient to transport folded proteins. The translocase is signal-assembled, *i.e.* a receptor complex containing cpTatC and Hcf106 binds the precursor protein, and upon membrane energization, Tha4 is recruited to the precursor-receptor complex to effect translocation. We developed a two-step complementation assay to examine the implied central role of Tha4 in translocation. The first step results in the inactivation of endogenous Tha4 with specific antibodies. The second step involves integrating exogenous Tha4 and presenting the system with precursor protein. We verified this approach by confirming the results obtained recently with the *Escherichia coli* Tha4 ortholog TatA, *i.e.* that the carboxyl terminus is dispensable and the amphipathic helix essential for transport. We then investigated a conserved Tha4 transmembrane glutamate in detail.

Substitution of glutamate 10 with alanine, glutamine, and even aspartate largely eliminated the ability of Tha4 to complement transport, whereas a conservative substitution elsewhere in the transmembrane domain was without effect. Chemical cross-linking assays showed that the mutated Tha4s failed to be recruited to the receptor complex under transport conditions, indicating a role for the transmembrane glutamate in translocase assembly. This assay promises an avenue into understanding the role of Tha4 in both the assembly and translocation steps of the Tat translocase.

The cytoplasmic membrane of bacteria and the thylakoid membrane of plant chloroplasts possesses parallel systems for transporting soluble proteins across the bilayer, namely the Sec system and the Tat[1] system. Both systems employ hydrophobic signal peptides on the transported precursor proteins. However, Tat system signal peptides have consensus and essential twin arginine residues on the amino-flanking side of the hydrophobic core, hence the name Tat for twin arginine translocation. These two systems also differ in their components and mechanism of action. The Sec system minimally employs a SecY/E integral membrane complex that serves as a protein-conducting channel and SecA, an ATP-driven motor that "pushes" proteins in unfolded conformation through the channel (1). The Sec system has been classified as possessing a "signal- gated" translocase because precursor binding to the machinery opens the channel (see Ref. 2 for review). The Tat system appears to employ, minimally, three integral membrane protein components, Tha4, Hcf106, and cpTatC, in thylakoids and TatA, TatB, and TatC, respectively, in bacteria (3, 4). Importantly, Tat systems transport proteins in a folded conformation, require neither cis-soluble factors nor NTPs, and rely entirely on the $\Delta\mu_{H+}$ for transport (ΔpH in the case of thylakoids).

Tat systems have been classified as possessing signal-assembled translocases (see Ref. 2 for review). The evidence for this is clearest in the thylakoid system. The three known components exist as subcomplexes in the membrane (5). Precursors bind to a receptor complex consisting of cpTatC and Hcf106 (5). Upon establishment of the pH gradient, Tha4 is recruited to the precursor-bound receptor complex forming the putative translocase, whereupon the precursor is transported to the lumen (6). The translocase then dissociates into starting complexes. It is thought that such a mechanism allows the translocase to form-fit the translocating precursor and thereby allows a large protein to cross the membrane without permitting uncontrolled ion leakage. One model envisions that Tha4 (TatA) oligomerizes to form a flexible and dynamic channel through which the substrate passes. Indeed, in thylakoids Tha4 is present in 8-fold excess over both cpTatC and the estimated number of translocation sites (7). In *Escherichia coli* TatA (Tha4 ortholog) is present in 40-fold excess over TatC (8).

The ability to introduce altered or tagged forms of Tha4 into the system would facilitate studies of the role of Tha4 in translocation. For example, it would permit the investigation of some interesting Tha4 signature motifs that may be important in recruitment or translocation steps. In addition, it would allow a functional comparison between the domains of Tha4 and Hcf106, which are very similar in structure and sequence. Hcf106 and Tha4 are each anchored to the membrane by amino-proximal transmembrane domains and expose predicted amphipathic helices and acidic carboxyl termini to the chloroplast stroma. Hcf106 and Tha4 have the highest sequence similarity in the amino proximal regions, especially in the transmembrane domain. A transmembrane proline (Pro)-glu-

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‡ Current address: Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan.

§ To whom correspondence should be addressed: Horticultural Sciences Dept., Box 110690, University of Florida, Gainesville, FL 32611. Tel.: 352-392-4711 (ext. 219); Fax: 352-392-5653; E-mail: kcline@ufl.edu.

[1] The abbreviations used are: Tat, twin arginine transport; PI, preimmune; IB, import buffer; DT17 or DT23, precursor form of the 17- or 23 kDa subunits of the oxygen-evolving complex of photosystem II containing a modified, truncated transit peptide; mOE17 or mOE23, mature form of the 17- or 23-kDa subunits of the oxygen evolving complex of photosystem II, respectively; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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tamate (Glu) motif is conserved among all Hcf106 and Tha4 proteins, as is a glycine (Gly)-Pro motif between the transmembrane helix and the amphipathic helix (3). Despite this similarity, Tha4 and Hcf106 appear to perform very different functions. Hcf106 participates in the first step of the reaction, precursor binding; Tha4 participates in later steps of the reaction that result in translocation. An assay that would simultaneously replace endogenous Tha4 and assay for steps of the transport reaction could yield considerable insight into the operation of the system.

Our initial strategy for biochemical complementation of Tha4, using thylakoids isolated from maize *tha4* null mutant plants, was to sequentially integrate Tha4 and then query the membrane for transport of a Tat pathway substrate. Initial results showed that biochemical complementation of Tha4 is feasible.[2] However, the limited availability of mutant thylakoids and a reduced capability of mutant membranes to generate and maintain a pH gradient prompted us to examine an alternative approach. Here we have developed an *in vitro* complementation assay with pea thylakoids that inactivates endogenous Tha4 with antibodies. Subsequent integration of *in vitro* translated Tha4 restores transport. To verify the validity of this approach, we examined Tha4 carboxyl-terminal truncations for activity. In agreement with findings from the *E. coli* Tat system (9), we were able to show that the nonconserved carboxyl-terminal region is not required for function, whereas the amphipathic helix is essential.

We further examined the necessity of the conserved transmembrane glutamate. Our results show that Tha4s, with a variety of substitutions, which include a charge-conserved aspartate and a structurally conserved glutamine, were unable to support transport. Moreover we showed that mutated proteins were unable to assemble with the cpTatC-Hcf106 receptor complex to form the translocase. Thus, in addition to providing a rapid assay for substituting endogenous Tha4 with engineered Tha4, our results provide insights into the molecular basis for ΔpH-induced assembly of the thylakoid Tat translocase.

EXPERIMENTAL PROCEDURES

Generation of cDNA mpsTha4 and Variants—Pea mTha4 and the mTha4E10Q variant in pGEM4Z under the control of the SP6 promoter were as described previously[2] (10). Other mutations of Tha4 employed the mTha4-pGEM4Z plasmid as template and the QuikChange mutagenesis kit (Stratagene). Mutations included substitutions of glutamate 10 by alanine (E10A) and aspartate (E10D) and substitution of the transmembrane leucine 20 with isoleucine (L20I). Internal stop codons were inserted with QuikChange for the generation of deletions Tha4ΔC29 and Tha4ΔC54. The Tha4ΔC29 mutation removed 29 amino acids from the carboxyl terminus, resulting in a protein 53 amino acids long ending with the amino acids . . . KEFETELK-Stop. The Tha4ΔC54 mutation removed 54 amino acids from the carboxyl terminus, resulting in a protein 28 amino acids long ending with . . . VFGPKKLP-Stop. The domain swaps of mTha4 (full-length 82 amino acids, 8.9 kDa) and mHcf106 (full-length 176 amino acids, 19 kDa) were generated by splicing by overlap extension -PCR (11). One fusion, called T6, consisted of the transmembrane domain of mTha4 (amino acids 1–22) and the stromal domain of mHcf106 (amino acids 23–176) to yield a protein of 175 residues with a molecular mass of 19 kDa containing the amino acid sequence VAAL/VFFGPKGL at the splice site. The second fusion, called 6T, consisted of the transmembrane domain of mHcf106 (amino acids 1–22) and the stromal domain of mTha4 (amino acids 23–82) to yield a protein of 83 residues with a molecular mass of 8.9 kDa containing the amino acids sequence VALL/VFGPKKLP at the splice site. All clones were verified by sequencing in both directions using ABI Prism Dye Terminator cycle sequencing protocols (Applied Biosystems, PerkinElmer Life Sciences) on an Applied Biosystems model 373 Stretch DNA sequencer by the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Facility.

Preparation of Chloroplasts, Lysates, Thylakoids, and Stroma—Intact chloroplasts were isolated from 9–10-day-old pea (*Pisum sativum* L. cv. Laxton's Progress 9) seedlings (12). Chloroplasts were resuspended to 1 mg of chlorophyll/ml in import buffer (IB) (50 mM HEPES-KOH, pH 8.0, 330 mM sorbitol) and kept on ice until used. Chloroplast lysates were prepared from pelleted, intact chloroplasts by osmotic lysis at 2 mg of chlorophyll/ml in 10 mM HEPES-KOH, pH 8.0, 10 mM $MgCl_2$ at 0 °C and subsequent adjustment to 1 mg of chlorophyll/ml with IB containing 10 mM $MgCl_2$ (13). Thylakoids were obtained from lysates by centrifugation at 3300 × *g* for 8 min followed by washing with IB, 10 mM $MgCl_2$ and resuspension of the pellet to 1 mg of chlorophyll/ml with IB, 10 mM $MgCl_2$. Stromal extract, equivalent to chloroplasts at 1 mg of chlorophyll/ml, was obtained from the supernatant of lysate centrifugation by further centrifugation (100,000 × *g*) for 20 min to remove envelope membranes (12).

Preparation of Radiolabeled mpsTha4 and Precursors—*In vitro* transcription plasmids for precursors DT17 and DT23 have been described previously (14). Radiolabeled Tha4 and precursors were translated in a wheat germ extract from capped RNA in the presence of [^{3}H]leucine. Tha4 translation products were diluted with an equal volume of 60 mM leucine in 2× IB (100 mM HEPES-KOH, pH 8.0, 660 mM sorbitol) and 1× IB, 0.5× wheat germ, and 30 mM leucine to give the desired dilutions before its addition to thylakoids, as indicated in the figure legends. Precursor translation products were diluted with an equal volume of 2× IB, 60 mM leucine and 2 volumes of stromal extract for a total dilution of 1:4 before their addition to transport reactions.

Biochemical Complementation of Transport by Exogenous Tha4—Thylakoids were pretreated with IgGs as described previously (15). Briefly, thylakoids were incubated with either preimmune (PI) IgG or anti-Tha4 (α-Tha4) IgG for 45 min at 0 °C and then washed with 2 volumes of IB, 10 mM $MgCl_2$. A typical preincubation contained 300 μg of chlorophyll and 75 μg of IgG in 20 mM HEPES-KOH, pH 8.0, 0.1 M sorbitol. Thylakoids were then recovered by centrifugation, washed with IB, 10 mM $MgCl_2$, resuspended in IB, 10 mM $MgCl_2$, and treated with protein A (5 μg/25 μg of chlorophyll) to clamp the bound IgG. Pretreated thylakoids were incubated with *in vitro* translated [^{3}H]Tha4, mutated [^{3}H]Tha4, mock translation mix (no mRNA added to the translation), or the α-Tha4 antigen, Tha4sd, for 15 min at 0 °C in the dark. Transport assays were initiated by the addition of precursor in stromal extract and transfer to light at temperatures listed in the figure legends. Typical assays contained 25 μl of thylakoids equivalent to 25 μg of chlorophyll, 25 μl of diluted [^{3}H]Tha4 component, and 25 μl of precursor protein in stromal extract (total assay volume 75 μl). Reactions were terminated after 20 min by transfer to 0 °C and recovery of the thylakoids by centrifugation. Recovered thylakoids were resuspended in 300 μl of IB and divided into two aliquots. Thylakoids in one aliquot were recovered by centrifugation and washed with IB containing 5 mM EDTA. Thylakoids in the other aliquot were treated with thermolysin at a final concentration of 0.1 mg/ml for 40 min at 4 °C. Proteolysis was terminated by adding an equal volume of IB containing 14 mM EDTA. Thylakoids were then recovered by centrifugation and were washed with IB containing 5 mM EDTA. Samples were subjected to SDS-PAGE and analyzed by fluorography.

Tha4 Recruitment—Tha4 assembly with the cpTatC-Hcf106 complex was determined by the chemical cross-linking and co-immunoprecipitation procedure of Mori and Cline (6) with the following modifications. Briefly, reaction mixtures containing thylakoids equivalent to 100 μg of chlorophyll (at 1 mg chlorophyll/ml) in transport buffer (330 mM sorbitol, 50 mM Hepes-KOH, 5.0 mM $MgCl_2$, pH 8.0) were preincubated for 10 min at 25 °C with 50 μl of [^{3}H]Tha4 or mutated [^{3}H]Tha4 in the light. Nigericin was added during the preincubation (after 5 min) to 0.5 μM to dissipate the ΔpH as indicated. Unlabeled precursor (DT23) was then added to a final concentration of 1 μM from a freshly prepared 50 μM stock solution in 8 M urea, 0.8 mM dithiothreitol, and the reactions were incubated for 5 min at 25 °C in the light. Then the cross-linking agent dithiobis(succinimidyl propionate) was added to 1 mM final concentration, and incubation was continued for an additional 5 min at 25 °C in the light. Cross-linking was then terminated with the addition of 50 mM Tris-HCl and incubation for 5 min at 25 °C. Co-immunoprecipitation under denaturing conditions was performed as described previously with either PI or α-Hcf106 IgG covalently cross-linked to protein A-Sepharose (6). Immunoprecipitates were eluted from the IgG-protein A-Sepharose beads with 8 M urea, 5% SDS, 125 mM Tris-HCl, pH 6.8, reduced with β-mercaptoethanol (5% final concentration), and analyzed by SDS-PAGE and fluorography.

Miscellaneous—Antibodies used here have been described elsewhere (5, 7, 15). Chlorophyll was determined according to Arnon (16). The reagents used are commercially available

[2] V. Fincher, C. Dabney-Smith, and K. Cline, submitted for publication.

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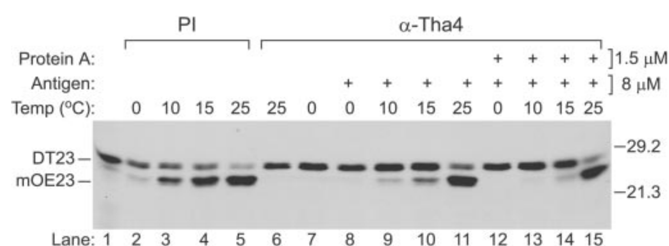

FIG. 1. **Protein A stabilizes the α-Tha4-endogenous Tha4 interaction.** Thylakoids equivalent to 25 μg of chlorophyll were preincubated with either preimmune IgG (*PI*) (*lanes 2–5*) or anti-Tha4 IgG (*α-Tha4*) (*lanes 6–15*) as described under "Experimental Procedures." Then the treated thylakoids were incubated at 0 °C for 10 min in the presence (+) (*lanes 12–15*) or absence (*lanes 2–11*) of 1.5 μM protein A prior to incubation with (+) (*lanes 8–15*) or without (*lanes 2–7*) 8 μM antigen (Tha4sd, final concentration) as designated above the panel. Transport reactions were then initiated by the addition of *in vitro* translated precursor ([^{3}H]DT23) and transfer to light (~70 microeinsteins/m^2/s) at the temperatures indicated. Thylakoids were recovered by centrifugation, washed with import buffer, and analyzed by SDS-PAGE and fluorography (see "Experimental Procedures"). *Lane 1* contains 1:40 dilution of the DT23 translation product. *Lanes 2–15* contain recovered thylakoids equivalent to ~3 μg of chlorophyll. An image of the fluorogram is shown. Transport is indicated by the appearance of the mature form of the 23-kDa subunit of the oxygen-evolving complex of photosystem II (*mOE23*). The migration of molecular weight markers is shown on the *right side* of the panel.

RESULTS

In Vitro Integrated Tha4 Can Restore Transport to Anti-Tha4-treated Membranes—Pretreatment of thylakoids with antibodies against Tha4 (α-Tha4) does not affect precursor binding to the cpTatC-Hcf106 receptor complex but rather inhibits the subsequent translocation step (5). Recent experiments have also demonstrated that physiological quantities of Tha4 can be integrated into isolated thylakoids (10). These data suggested the possibility that exogenous Tha4 could complement a Tha4 deficiency or Tha4 inactivated with antibodies. *i.e.* that transport capability to α-Tha4-treated membranes could be restored by integration of exogenous Tha4. An initial attempt to "biochemically complement" antibody-treated membranes was successful (data not shown). However, because exogenous Tha4 can interact with the Tha4 antibody, it was important to rule out competitive release of the antibody, which would free endogenous Tha4 to participate in the transport reaction.

An experiment to test for competitive release of antibody is shown in Fig. 1. Thylakoid membranes treated with PI or α-Tha4 IgGs were washed and then incubated with or without the Tha4 antigen Tha4sd, *i.e.* the stromal domain of Tha4, before addition of the precursor DT23 at various temperatures. As seen in Fig. 1, *lanes 2–5*, DT23 is increasingly transported across the PI IgG-treated membranes with increases in temperature from 0 to 25 °C. There was no transport across α-Tha4-treated membranes at either 0 or 25 °C (Fig. 1, *lanes 6* and *7*) unless 8 μM Tha4sd was also included in the assay mixture (*lanes 8–11*). The amount of transport in the presence of Tha4 antigen was significant at 10 °C and above. Thus, exogenous Tha4 antigen can promote release of α-Tha4, resulting in transport of the precursor. To stabilize the antibody-antigen complex, protein A was added to "clamp" the bound IgG (Fig. 1, *lanes 12–15*). Under these conditions, very little transport of DT23 occurred between the temperatures of 0 and 15 °C. It should be noted that 8 μM antigen is 30–50 times the concentration of Tha4 translation product included in our assays and thus represents a "worst case" scenario for competitive release of bound antibody.

As a result of the above experiment, we chose to conduct assays at 10–15 °C and to include routinely an 8 μM antigen control to verify that endogenous Tha4 was not released during the assay. The results of such a biochemical complementation assay are shown in Fig. 2. Two different precursors, DT23 (Fig. 2*A*) and DT17 (Fig. 2*B*), were used in these assays. DT23 and DT17 are modified forms of the OE23 and OE17 precursors, respectively (14). They were chosen because they are very efficiently transported by the thylakoid Tat system, even at reduced temperatures (17). Thylakoids were pretreated with PI IgG or α-Tha4 IgG, washed, and then treated with protein A and either mock translation mixture (Fig. 2, *lanes 2–4* and *8*) and Tha4sd antigen (*lane 4*) or varying amounts of *in vitro* translated mTha4 (*lanes 5–7*). Alternatively thylakoids were pretreated with buffer (Fig. 2, *lane 8*) before treatment with protein A and mock translation mixture. After 15 min on ice, the treated membranes were incubated with precursor in a transport assay. Inclusion of exogenous Tha4 enhanced transport of either DT23 or DT17 in a concentration-dependent manner (Fig. 2, *lanes 5–7*). A higher concentration of *in vitro* translated Tha4 (undiluted translation product) resulted in diminished translocation of the precursor, suggesting that the wheat germ used for *in vitro* translation contains inhibitory

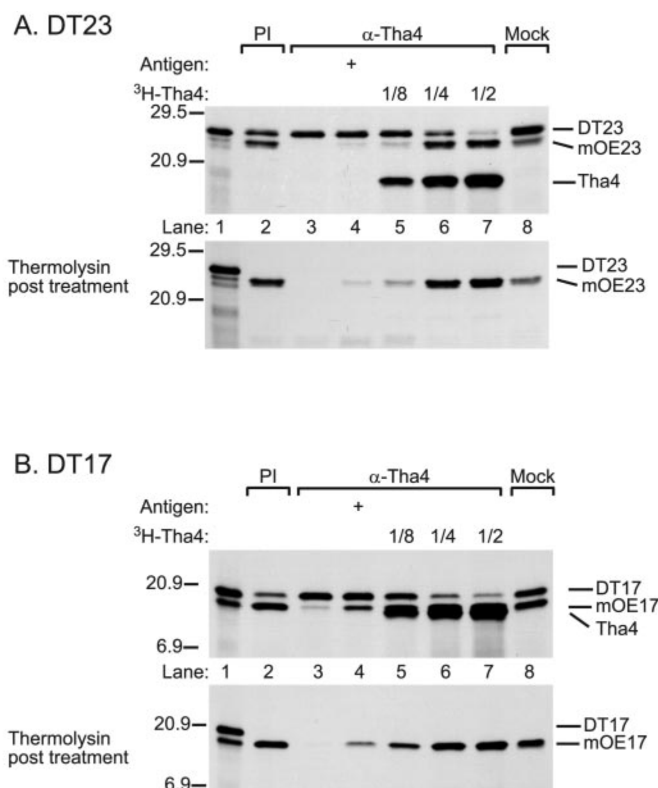

FIG. 2. **Exogenous Tha4 restores transport to antibody-inhibited thylakoid membranes.** Thylakoids treated with PI IgG plus protein A (*PI*) (*lane 2*), with α-Tha4 plus protein A (*lanes 3–7*), or with buffer (*lane 8*) were incubated with *in vitro* translated [^{3}H]Tha4 (*lanes 5–7*), or mock translation extract (*lanes 2*, *4*, and *8*), and 8 μM Tha4 antigen (Tha4sd; *lane 4*) for 10 min. The [^{3}H]Tha4 translation product was diluted with import buffer, as indicated *above* the panels, before being added to thylakoids. Mock translation extract was diluted 1:2 before being added to thylakoids. *A*, *in vitro* translated ^{3}H-labeled DT23 was added to the reaction mixtures, and transport was conducted at 15 °C in the light (~70 microeinsteins/m^2/s) for 15 min. *B*, *in vitro* translated ^{3}H-labeled DT17 was added to the reaction mixtures, and transport was conducted at 15 °C in the light (~70 microeinsteins/m^2/s) for 15 min. *A* and *B*, thylakoids were recovered by centrifugation and treated without (*upper panel*) or with (*lower panel*) thermolysin as described under "Experimental Procedures." *Lane 1* contains a 1:40 dilution of the precursor translation product. *Lanes 2–8* contain recovered thylakoids equivalent to ~3 μg of chlorophyll. Images of the fluorogram are shown. The migration of molecular mass markers (kDa) is shown on the *left side* of the panels.

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components (not shown). The amount of exogenous Tha4 recovered with membranes is obscured in the DT17 assays because of very close migration with mOE17 (Fig. 2*B*). However, the Tha4 is clearly visible in the DT23 assay (Fig. 2*A*). For this reason, DT23 was used as a substrate in most subsequent assays. Transport did not occur in assays treated with mock translation mixture and was minimal in the presence of the Tha4 antigen (Fig. 2, *lanes 3* and *4*). We occasionally observed a higher level of transport in the Tha4-complemented assays than in the mock- or PI IgG-treated assays, suggesting that Tha4 may be limiting in the transport reaction. However, this higher level of transport requires further study. In a separate experiment, we showed that exogenous Tha4 had no effect on transport of a Sec-pathway substrate, iOE33 (data not shown).

The Predicted Amphipathic Helix Is Important for Tha4 Function, but the Carboxyl Terminus is Largely Dispensable—Plant Tha4 proteins are highly conserved in the transmembrane and amphipathic helical domains but divergent in their carboxyl termini. Orthologous bacterial TatA proteins have similar conserved and divergent domains. A recent study of the *E. coli* Tat system demonstrated that the carboxyl terminus of TatA could be deleted without seriously impairing transport capability but that truncation of the amphipathic helix eliminated transport (9). As proof of the concept of our method, we prepared truncated Tha4 proteins and assayed them in the complementation assay. As seen in Fig. 3, removal of the carboxyl terminus up to the amphipathic helix (Tha4ΔC29) reduced but did not eliminate the complementation activity (Fig. 3*B*, *lanes 16–19*; compare with wild-type Tha4, *lanes 8–11*). However, further deletion, which removed the amphipathic helix, eliminated the ability of the truncated protein (Tha4ΔC54) to complement transport (Fig. 3*B*, *lanes 12–15*). Taken together with the fact that Tha4sd (Tha4 lacking the transmembrane domain) is nonfunctional, these results indicate that the essential regions of Tha4 are its amphipathic helix and transmembrane domain. The fact that these results are consistent with the results of *E. coli* TatA truncation analysis (9) supports the validity of our complementation assay procedure.

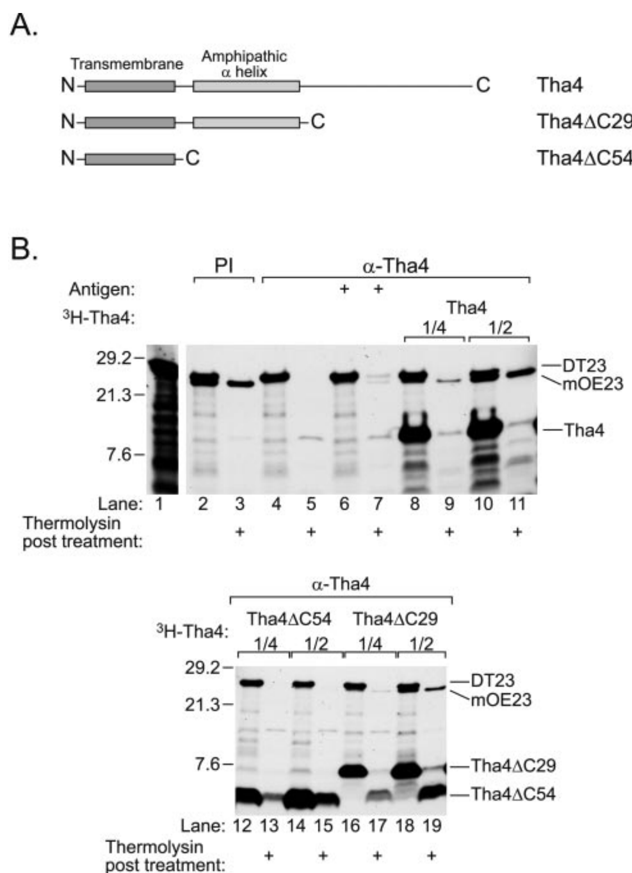

FIG. 3. **The carboxyl-terminal domain of Tha4 is not required for function, but the amphipathic helix is essential.** Stop codons were inserted in-frame into the Tha4 coding sequence resulting in truncations of 29 or 54 amino acids from the Tha4 carboxyl terminus (Tha4ΔC29 or Tha4ΔC54, respectively) as described under "Experimental Procedures." *A*, schematic diagram of the truncations. The resulting proteins were assayed for their ability to complement transport of DT23 at 15 °C. *B*, assays with thylakoids treated with PI IgG and protein A are shown in *lanes 2* and *3*; assays with thylakoids pretreated with α-Tha4 plus protein A in *lanes 4–19*. The antigen control is in *lanes 6–7*. [^{3}H]Tha4 (*lanes 8–11*), [^{3}H]Tha4ΔC54 (*lanes 12–15*), and [^{3}H]Tha4ΔC29 (*lanes 16–19*) were diluted with import buffer as indicated above the panels and described in the legend for Fig. 2. All other assays received mock translation extract diluted 1:2 (*lanes 2–7*). Transport was initiated by the addition of [^{3}H]DT23 and incubation in the light at 15 °C for 15 min. Thylakoids were recovered by centrifugation and treated with (+) or without thermolysin as depicted below the panels and described under "Experimental Procedures." *Lane 1* contains 1:40 dilution of the DT23 translation product. *Lanes 2–19* contain recovered thylakoids equivalent to ~3 μg of chlorophyll. Analysis was by electrophoresis through 16% Tris-Tricine-SDS gels and fluorography. The images shown are from the same fluorogram. Molecular mass markers (kDa) are shown on the *left side* of the panels.

A Chimeral Tha4:Hcf106 Protein Is Partially Active in Complementing Tha4 Deficiency—Tha4 is structurally similar to Hcf106 and moderately conserved in the transmembrane domain and amphipathic helix. For example, pea Tha4 and Hcf106 transmembrane domains are 65% identical, and pea Tha4 and Hcf106 amphipathic helical domains are 41% identical. *E. coli* TatA and TatB are conserved in these domains (17 and 30%, respectively) but much less so than Tha4 and Hcf106. Recently, it was shown that a chimeric protein containing the cytoplasmic domain of TatB and the transmembrane domain of TatA was able to partially substitute for a TatA deletion *in vivo*, whereas the complementary fusion was not active (9). As a second comparison between the thylakoid system and the *E. coli* system, we constructed similar chimeric proteins consisting of a fusion between the transmembrane domain of Tha4 and the stromal domain of Hcf106 (T6 construct) and a fusion between the transmembrane domain of Hcf106 and the stromal domain of Tha4 (6T construct). The 6T chimera complemented transport of α-Tha4-treated membranes (Fig. 4, *lanes 9* and *10*) although not as efficiently as wild-type Tha4 (*lanes 7* and *8*). The complementary fusion protein (T6) was unable to substitute for Tha4 (Fig. 4, *lanes 13* and *14*). Hcf106 showed no ability to complement Tha4 deficiency (Fig. 4, *lanes 11* and *12*). These results are interesting because they are reciprocal rather than analogous to those of the *E. coli* Tat system.

A Conserved Transmembrane Glutamate Is Required for Tha4 Function—One unique feature of Tha4 is an invariantly conserved transmembrane proline-glutamate. It has been suggested that the glutamate is essential for Tha4 function and possibly participates in sensing the pH gradient or transducing it into translocation work (3). To determine the importance of the transmembrane charge, the glutamate residue (Glu^{10}) was changed to aspartate (E10D), glutamine (E10Q), or alanine (E10A). In addition, to assess whether mutagenesis within the transmembrane domain was benign, a transmembrane leucine was changed to isoleucine (L20I), which is found in the same position in other plant Tha4 proteins (3). Fig. 5 shows the results of complementation assays with the different variants. The Tha4L20I variant was able to complement transport (Fig. 5, *lanes 13–16*) as effectively as wild-type Tha4 (*lanes 8–11*), indicating that mutagenesis of a residue not strictly conserved did not affect Tha4 activity. Replacing Glu^{10} with aspartate resulted in a minor amount of transport (Fig. 5, *lanes 17–20*),

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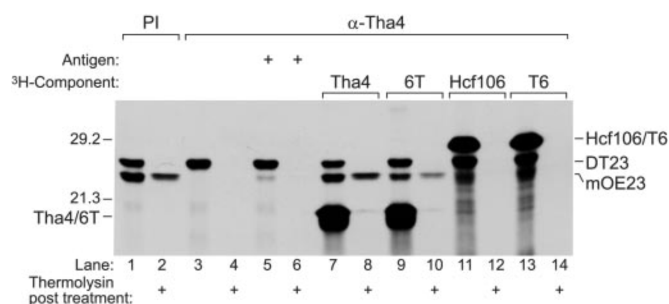
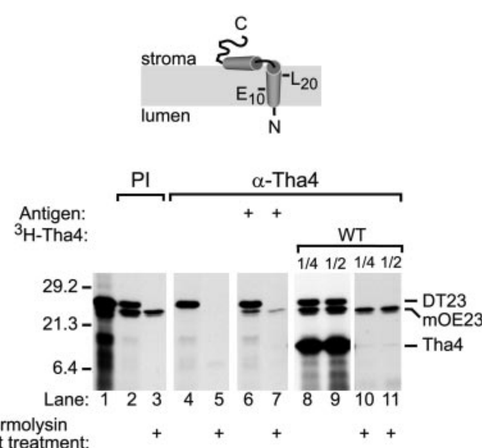


FIG. 4. **Chimeral Tha4/Hcf106 proteins indicate the importance of the amphipathic helix for Tha4 function.** The transmembrane domain of Hcf106 was fused to the stromal domain of Tha4 (*6T*), and the transmembrane domain of Tha4 was fused to the stromal domain of Hcf106 (*T6*). The resultant proteins were assayed for the ability to complement transport of DT23 at 15 °C. Thylakoids were pretreated with PI IgG and protein A (*lanes 1–2*) or with α-Tha4 IgG and protein A (*lanes 3–14*). Reactions were incubated with mock translation extract (*lanes 1–6*), with antigen (*lanes 5–6*), [^{3}H]Tha4 (*lanes 7–8*), [^{3}H]6T (*lanes 9–10*), [^{3}H]Hcf106 (*lanes 11–12*), or [^{3}H]T6 (*lanes 13–14*). Translation extracts were diluted 1:2 with IB. Transport was initiated by addition of [^{3}H]DT23 and incubation in the light at 15 °C for 15 min. Thylakoids were recovered by centrifugation and treated with (+) or without thermolysin, as shown below the panel and described under "Experimental Procedures." Samples were subjected to SDS-PAGE and fluorography. Lanes contain thylakoids equivalent to ~3 μg of chlorophyll. Molecular mass markers (kDa) are shown on the *left side* of the panels.

although this was similar to that of the competitive control (*lanes 6* and *7,* Tha4 antigen). The E10A (Fig. 5, *lanes 21–24*) and E10Q (*lanes 25–28*) variants showed no ability to promote precursor transport. We have also tested the *Arabidopsis* Tha4 and the Tha4 E10Q variant in this complementation assay. Essentially the same result was obtained. This is relevant because the *Arabidopsis* proteins are poorly recognized by our α-Tha4 IgGs.[3] In addition, complementation assays with maize *tha4* membranes also showed the E10Q variant to be inactive.[2] These data indicate that the transmembrane Glu[10] is required for Tha4 activity.

One potential trivial explanation for the failure of the glutamate variants to complement would be that they fail to integrate into the membrane. To examine this possibility, membranes recovered from integration assays were subjected to alkaline extractions and protease treatment (Fig. 6). Wild-type Tha4 is resistant to extraction with 0.2 M Na_2CO_3 (Fig. 6, *lane 4*) but is largely extracted by 0.1 M NaOH (*lane 3*).[2] In addition, protease treatment of integrated Tha4 produces an ~2.5–3-kDa degradation product presumed to represent the embedded transmembrane domain (Fig. 6, *lane 5*). All of the mutated Tha4s, containing a transmembrane charge (L20I, E10D), behaved similar to wild-type Tha4 in these assays (Fig. 6, compare *lanes 3–5* in each panel). Removal of the transmembrane charge (E10A, E10Q) increased stability in the membrane as evidenced by its increased resistance to extraction 0.1 N NaOH (Fig. 6, *lanes 3*). These results indicate that all of the mutated Tha4 proteins associate with the thylakoid membranes similar to WT Tha4, and thus the failure to integrate does not explain their failure to complement Tha4 activity.

Tha4 Variants Are Defective in Recruitment to the cpTatC-Hcf106 Complex—Mori and Cline (6) showed that Tha4 is recruited to the cpTatC-Hcf106 receptor complex in the presence of a twin arginine precursor protein (or signal peptide alone) and the pH gradient. Two possibilities for the failure of the transmembrane mutations E10D, E10Q, and E10A to restore transport are that they are either unable to assemble with cpTatC-Hcf106 to form the translocase or that, upon assembly,



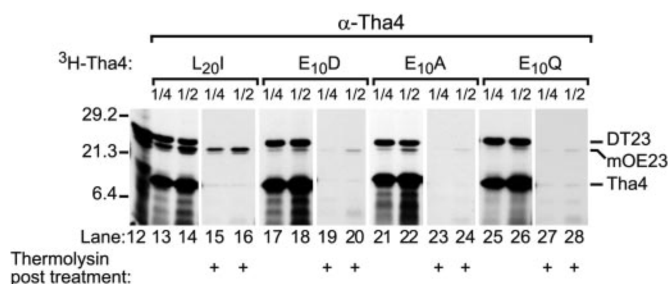


FIG. 5. **The transmembrane glutamate of Tha4 is critical for function.** Point mutations in the transmembrane domain of Tha4 (L20I, E10D, E10A, E10Q) were made as described under "Experimental Procedures" and shown in this diagram. The resultant proteins were assayed for their ability to complement transport of DT23 at 15 °C. Thylakoids were pretreated with PI IgG and protein A (*lanes 2* and *3*) or with α-Tha4 IgG and protein A (*lanes 4–28*). The antigen control is shown in *lanes 6* and *7*. [^{3}H]Tha4 (wild type (*WT*), *lanes 8–11*), [^{3}H]Tha4L20I (*lanes 13–16*), [^{3}H]Tha4E10D (*lanes 17–20*), [^{3}H]Tha4E10A (*lanes 21–24*), and [^{3}H]Tha4E10Q (*lanes 25–28*) were diluted with IB, as indicated above the panel, immediately prior to being added to thylakoids. All other assays received mock translation extract diluted at 1:2 (*lanes 2–7*). Transport was initiated by the addition of [^{3}H]DT23 and incubation in the light at 15 °C for 15 min. Thylakoids were recovered by centrifugation and treated with (+) or without thermolysin, as shown below the panel and described under "Experimental Procedures." Samples were analyzed by SDS-PAGE and fluorography. *Lanes 1* and *12* contain a 1:40 dilution of the DT23 translation product. Lanes contained recovered thylakoids equivalent to ~3 μg of chlorophyll. Molecular mass markers (kDa) are shown on the *left side* of the panels. Thermolysin-treated samples and untreated samples were electrophoresed on matching gels processed identically and in parallel. For ease of interpretation, lanes from corresponding samples were rearranged. Imaged panels represent the same exposure times.

they are unable to promote translocation. As an indirect approach to this question we examined the ability of the variants to interfere with transport by wild-type Tha4. When the mutated Tha4 proteins were integrated into untreated thylakoids they did not inhibit transport of precursor (not shown). In addition, when the mutated proteins were mixed with wild-type Tha4 and introduced to antibody-pretreated thylakoids, transport was essentially the same as that achieved with wild-type Tha4 alone (not shown). From this we conclude that transmembrane glutamate-variant Tha4 proteins do not exert a dominant interfering effect on wild-type Tha4 and thus are unlikely to interact with thylakoid Tat machinery.

To directly test for assembly of the Tha4 variants into the translocation complex, a chemical cross-linking and co-immunoprecipitation technique was used (6). Radiolabeled *in vitro* translated Tha4 and mutated Tha4 proteins were integrated into thylakoids (Fig. 7, *upper panel*). Recovered thylakoids were then incubated in the light with or without saturating

[3] C. Dabney-Smith and K. Cline, unpublished results.

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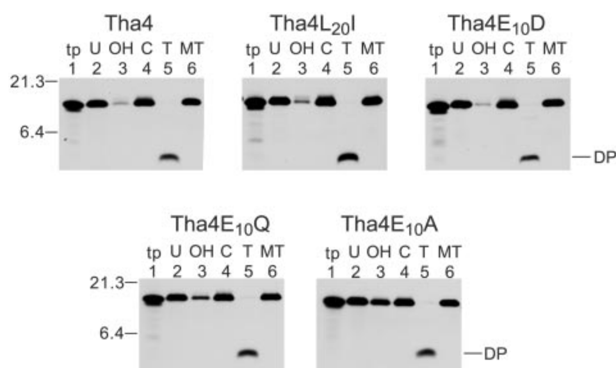

FIG. 6. **Decreased Tha4 activity correlates with increased stability in the membrane.** *In vitro* translated [^{3}H]Tha4 or mutated [^{3}H]Tha4 was integrated into thylakoids and then analyzed for its stability in the membrane. Chloroplast lysate was incubated with 5 mM ATP and [^{3}H]Tha4 component that had been diluted 1:6 with IB. Incubation occurred for 20 min at 25 °C in the light. Thylakoids were recovered by centrifugation, resuspended, divided into aliquots, and subjected to alkaline extraction with either 0.2 M Na_2CO_3 (*C*, *lane 4*) or 0.1 M NaOH (*OH*, *lane 3*) or untreated (*U*, *lane 2*) as described under "Experimental Procedures." Additional aliquots were treated with thermolysin (*T*, *lane 5*) or mock-treated (*MT*) for the same time period without protease (*lane 6*). Recovered thylakoids were analyzed by electrophoresis through 16% Tris-Tricine SDS-PAGE and fluorography. *Lane 1* contains 1:40 dilution of the respective Tha4 translation products (*tp*). Molecular mass markers (kDa) are shown on the *left side* of the panels. *DP*, degradation product (protected transmembrane domain).

amounts of unlabeled precursor protein DT23. The thiol-cleavable homobifunctional cross-linker dithiobis(succinimidyl propionate) was added to reaction mixtures for 5 min and then quenched with Tris-HCl. Recovered thylakoids were dissolved in SDS buffer and subjected to denaturing co-immunoprecipitation with anti-Hcf106 IgGs or with PI IgGs. Immunoprecipitates were then analyzed by SDS-PAGE in the presence of a reducing agent to cleave the cross-linker, and the gels were analyzed by fluorography. As seen in Fig. 7, wild-type Tha4 and Tha4L20I were co-immunoprecipitated with anti-Hcf106 in the presence of precursor (Fig. 7, *middle panel*, *lanes 2* and *5*) but not in its absence (*middle panel*, *lanes 8* and *11*). The glutamate variants were not co-immunoprecipitated under any conditions (Fig. 7, *middle panel*, *lanes 3*, *4*, *6*, *9*, *10*, and *12*), and no Tha4 proteins were immunoprecipitated by PI IgG (*lower panel*). As an additional control, thylakoids containing exogenous Tha4 were incubated with DT23 in the presence of nigericin to dissipate the pH gradient. Tha4 was not co-immunoprecipitated when the incubation was conducted without the ΔpH (Fig. 7, *middle panel*, *lane 13*).

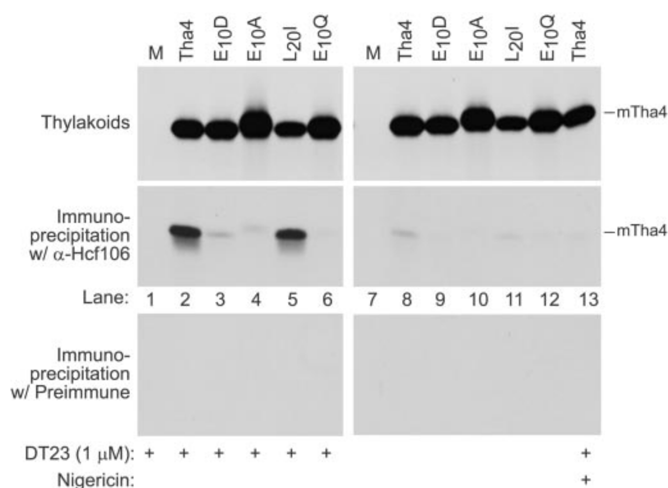

FIG. 7. **The conserved transmembrane glutamate of Tha4 is necessary for Tha4 recruitment to the TatC-Hcf106-precursor complex.** Thylakoids were incubated 5 min in the light at 25 °C to generate a pH gradient. Mock translation (*lanes 1* and *7*), [^{3}H]Tha4 (*lanes 2*, *8*, and *13*), [^{3}H]Tha4E10D (*lanes 3* and *9*), [^{3}H]Tha4E10A (*lanes 4* and 10), [^{3}H]Tha4L20I (*lanes 5* and *11*), and [^{3}H]Tha4E10Q (*lanes 6* and *12*) were diluted 1:2 with IB and preincubated with thylakoids for 10 min at 25 °C in the light. Where indicated, the reaction was supplemented during the preincubation (after 5 min) with 0.5 μM nigericin to dissipate the ΔpH (*lane 13*). Recruitment of Tha4 was initiated by the addition (+) of 1.0 μM of unlabeled DT23 as depicted below the panels (*lanes 1–6* and *13*) and incubation for 5 min at 25 °C in the light. Cross-linking was then initiated by the addition of 1 mM dithiobis(succinimidyl propionate) (final concentration). After 5 min at 25 °C in the light, cross-linking was quenched with 50 mM Tris-Cl, pH 8.0 (final concentration). Cross-linked thylakoids were recovered by centrifugation, dissolved with SDS, and subjected to denaturing immunoprecipitation with α-Hcf106 (*middle panel*) or PI (*lower panel*). Immunoprecipitated proteins, following solubilization from antibody-linked beads with SDS and urea, were released from cross-linked partners with β-mercaptoethanol and analyzed by SDS-PAGE and fluorography. An aliquot of cross-linked thylakoids was reduced with β-mercaptoethanol (5% final concentration) and subjected to SDS-PAGE and fluorography (*upper panel*) instead of immunoprecipitation. *Lanes* in the *upper panel* contained recovered thylakoids equivalent to ~3 μg of chlorophyll. The gel represented by the upper panel was exposed to film for 2 days, whereas the gels represented by the middle and lower panels were exposed to film for 10 days. Other than exposure time, all gels were processed in exactly the same manner.

DISCUSSION

Previously we have shown that translocation by the thylakoid Tat pathway occurs in at least two distinct steps, precursor binding to a 700-kDa cpTatC-Hcf106 complex and subsequent recruitment of Tha4 to that complex in the presence of a pH gradient to form the putative translocase (5, 6). The fact that assembly requires the pH gradient suggests that either the receptor complex or Tha4 (or both) undergoes some conformational change in the presence of the pH gradient that promotes assembly. It has been suggested that the transmembrane glutamate residues of Tha4 and/or Hcf106 could serve as sensors of the luminal pH to effect such a hypothetical conformational change (3). In addition, the requirement for recruitment of Tha4 for translocation to occur points to an important role for Tha4 in the translocation step. Current models suggest that Tha4 forms, at least in part, the translocation channel. To characterize the role Tha4 plays in translocation, we have developed biochemical complementation assays. We have shown elsewhere that exogenous Tha4 complements a *tha4* mutation in mutant maize thylakoids.[2] Here we have developed a complementation assay that uses wild-type pea thylakoid membranes in which endogenous Tha4 has been inactivated with antibodies directed against the stromally exposed regions of Tha4. Several lines of evidence argue that activity observed in our assay is due to the exogenously integrated Tha4 proteins rather than escaped endogenous Tha4. First, assay conditions were adjusted to minimize the dissociation of antibody and the associated release of endogenous Tha4. This included lowering assay temperatures to 10–15 °C and "clamping" the bound antibody with protein A. Under these conditions very little if any endogenous Tha4 is released. Second, each experiment included a control assay that was conducted in the presence of 8 μM α-Tha4 antigen, a concentration far in excess of the concentration of Tha4 used to complement the activity. Finally, Tha4 variants that are unaltered in their antigenic stromal domains, but contain substitutions of the transmembrane glutamate, were unable to complement activity.

As a first test of this complementation assay, we examined the ability of carboxyl-terminal truncations of Tha4 proteins to restore activity. Similar to previous findings with *E. coli* TatA (9), we found that deleting the nonconserved carboxyl-terminal tail had only a minor effect on activity, whereas deletion of the

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amphipathic helix eliminated activity. These results, along with the inability of the Tha4 stromal domain to complement activity, indicate that the amphipathic helix and transmembrane domains are the functionally relevant Tha4 domains. The role that the carboxyl tail might play in fine-tuning the transport process is not clear. However, the limited effect of its removal on transport suggests that modifying it with specific tags or cross-linking agents would have a minor effect on activity. Along these lines, we have found that a carboxyl-terminally FLAG-tagged *Arabidopsis* Tha4 is as active as wild-type pea or *Arabidopsis* Tha4 in the complementation assay.[3]

To assess the relative importance of the Tha4 transmembrane and amphipathic domains, chimeral proteins were constructed in which the stromal and transmembrane domains of Hcf106 and Tha4 were swapped. Importantly, only the fusion protein containing the Tha4 stromal domain showed any activity, suggesting that, in the thylakoid system at least, the amphipathic helix of Tha4 may have unique activity. The opposite result was reported with chimeral TatB-TatA proteins, *i.e.* only the fusion protein between the TatB cytoplasmic domain and the TatA transmembrane domain was able to complement a TatA deletion (9). This fusion protein was also capable of complementing a TatB deletion but not a TatA and TatB deletion. The extent to which this fusion protein restored transport is uncertain, as complementation was assessed by the ability of cells to grow in the presence of SDS, a previously observed correlation with an active Tat apparatus (9). The difference between our results and those in *E. coli* can be explained in part by the similarity of the Tha4 and Hcf106 transmembrane domains and the relative divergence of the TatA and TatB transmembrane domains. In particular, Tha4 and Hcf106 both possess conserved transmembrane glutamate residues, whereas a conserved transmembrane glutamate in TatB is absent from TatA. Rather, TatA proteins usually contain polar or basic residues in the comparable location (3).

The role of the Tha4 transmembrane glutamate has not been examined previously. Our results show that it is essential for activity. The structurally conserved glutamine was completely unable to functionally substitute for glutamate, and even the similarly charged aspartate was virtually unable to functionally substitute. This indicates that not only is an acidic residue important for activity, but that the length of the side chain is critical. At least two scenarios requiring the transmembrane glutamate can be envisioned. The first involves sensing the luminal pH through protonation, whereas the second utilizes potential salt bridge formation between the glutamate and a basic residue somewhere else.

If the transmembrane glutamate of Tha4 is involved in sensing the luminal pH, the side chain of the glutamate may become protonated upon acidification of the lumen, thus allowing Tha4 to assemble with the receptor complex. The exact mechanism by which protonation of Tha4 might stimulate assembly is not clear. Alkaline extraction assays reported here suggest an unstable or transient insertion of the Tha4 transmembrane domain. Thus protonation could serve to increase Tha4 stability in the membrane and at the same time reduce repulsive forces, allowing Tha4 to oligomerize and assemble the translocase channel. In this regard, we note that *E. coli* TatA, which lacks the transmembrane acidic residue, appears to associate constitutively, at least in part, with the TatB-TatC complex (18). However, this role for the transmembrane glutamate and the ΔpH seems less likely because the glutamine and alanine mutations, which are more resistant to alkaline extraction, are not functional. Therefore although protonation of the transmembrane glutamate may be important for Tha4 function, it probably is not because the protonation stabilizes Tha4 in the membrane.

Alternatively, the transmembrane glutamate of Tha4 may be critical in maintaining the translocation complex through formation of a salt bridge with basic residues on other components of the translocation complex. cpTatC, unlike its bacterial counterpart, contains a highly conserved transmembrane arginine that could be a potential partner. Support for the involvement of the transmembrane glutamate in salt bridge formation comes from our finding that none of the Tha4 variants with glutamate substitutions cross-linked to the cpTatC-Hcf106 complex in the presence of precursor and the pH gradient. Interestingly, a similar mutation of the conserved transmembrane glutamate of Hcf106 to glutamine prevented Hcf106 from associating with cpTatC even though the mutated protein easily integrates into the thylakoid membrane.[2] This raises the intriguing possibility that Tha4 and Hcf106 employ their transmembrane glutamate residues for interaction with the same or a similar assembly site on the translocase. Determining with which components of the cpTatC-Hcf106 complex Tha4 interacts would provide fundamental information for the mechanism of translocation.

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REFERENCES

1. Manting, E. H., and Driessen, A. J. (2000) *Mol. Microbiol.* **37,** 226–238
2. Schnell, D. J., and Hebert, D. N. (2003) *Cell* **112,** 491–505
3. Mori, H., and Cline, K. (2001) *Biochim. Biophys. Acta* **1541,** 80–90
4. Berks, B. C., Sargent, F., and Palmer, T. (2000) *Mol. Microbiol.* **35,** 260–274
5. Cline, K., and Mori, H. (2001) *J. Cell Biol.* **154,** 719–729
6. Mori, H., and Cline, K. (2002) *J. Cell Biol.* **157,** 205–210
7. Mori, H., Summer, E. J., and Cline, K. (2001) *FEBS Lett.* **501,** 65–68
8. Sargent, F., Gohlke, U., De Leeuw, E., Stanley, N. R., Palmer, T., Saibil, H. R., and Berks, B. C. (2001) *Eur. J. Biochem.* **268,** 3361–3367
9. Lee, P. A., Buchanan, G., Stanley, N. R., Berks, B. C., and Palmer, T. (2002) *J. Bacteriol* **184,** 5871–5879
10. Fincher, V. (2001) *Protein Translocation on the Delta pH-dependent Pathway of Chloroplasts.* Doctoral dissertation, University of Florida, Gainesville
11. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77,** 61–68
12. Cline, K., Henry, R., Li, C., and Yuan, J. (1993) *EMBO J.* **12,** 4105–4114
13. Cline, K. (1988) *Plant Physiol.* **86,** 1120–1126
14. Henry, R., Carrigan, M., McCaffery, M., Ma, X., and Cline, K. (1997) *J. Cell Biol.* **136,** 823–832
15. Mori, H., Summer, E. J., Ma, X., and Cline, K. (1999) *J. Cell Biol.* **146,** 45–56
16. Arnon, D. I. (1949) *Plant Physiol.* **24,** 1–15
17. Ma, X., and Cline, K. (2000) *J. Biol. Chem.* **275,** 10016–10022
18. Bolhuis, A., Mathers, J. E., Thomas, J. D., Barrett, C. M., and Robinson, C. (2001) *J. Biol. Chem.* **276,** 20213–20219

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