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Thylakoid ΔpH-dependent precursor proteins bind to a cpTatC–Hcf106 complex before Tha4-dependent transport

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The thylakoid ΔpH-dependent pathway transports folded proteins with twin arginine–containing signal peptides. Identified components of the machinery include cpTatC, Hcf106, and Tha4. The reaction occurs in two steps: precursor binding to the machinery, and transport across the membrane. Here, we show that a cpTatC–Hcf106 complex serves as receptor for specific binding of twin arginine–containing precursors. Antibodies to either Hcf106 or cpTatC, but not Tha4, inhibited precursor binding. Blue native gel electrophoresis and coimmunoprecipitation of digitonin-solubilized thylakoids showed that Hcf106 and cpTatC are members of an ~700-kD complex that lacks Tha4. Thylakoid-bound precursor proteins were also associated with an ~700-kD complex and were coimmunoprecipitated with antibodies to cpTatC or Hcf106. Chemical cross-linking revealed that precursors make direct contact with cpTatC and Hcf106 and confirmed that Tha4 is not associated with precursor, cpTatC, or Hcf106 in the membrane. Precursor binding to the cpTatC–Hcf106 complex required both the twin arginine and the hydrophobic core of the signal peptide. Precursors remained bound to the complex when Tha4 was sequestered by antibody, even in the presence of ΔpH. These results indicate that precursor binding to the cpTatC–Hcf106 complex constitutes the recognition event for this pathway and that subsequent participation by Tha4 leads to translocation.

Introduction

Nuclear-encoded thylakoid proteins are synthesized as precursors in the cytosol, imported into the chloroplast stroma, and then incorporated into thylakoids by one of four different pathways: the spontaneous, SRP, Sec, and ΔpH-dependent pathways (Keegstra and Cline, 1999). The ΔpH-dependent pathway and the homologous bacterial Tat pathway are recently discovered protein translocation systems with unique characteristics (Settles and Martienssen, 1998; Dalbey and Robinson, 1999; Keegstra and Cline, 1999; Berks et al., 2000). They use hydrophobic signal peptides similar to the Sec system (Henry et al., 1997), but differ in several important characteristics. The ΔpH-dependent pathway requires neither cis-soluble components nor NTP hydrolysis. Instead, transport is driven solely by the thylakoidal pH gradient. The ΔpH-dependent/Tat systems transport fully folded proteins (Clark and Theg, 1997; Hynds et al., 1998; Rodrigue et al., 1999), whereas Sec systems thread unfolded proteins across the bilayer (Arkowitz et al., 1993). Finally, ΔpH-dependent/Tat system signal peptides bear a conserved twin arginine (RR)* motif, which is usually required for efficient protein transport (Niviere et al., 1992; Chaddock et al., 1995; Henry et al., 1997; Stanley et al., 2000; Summer et al., 2000). The existence of this consensus sequence suggests that precursors specifically bind to a receptor component of the translocation machinery.

Three different components of the ΔpH-dependent and Tat systems have been identified: Hcf106 (Settles et al., 1997) and its bacterial orthologue TatB (Sargent et al., 1999); Tha4 (Mori et al., 1999; Walker et al., 1999) and its orthologue TatA (Sargent et al., 1998); cpTatC (Mori et al., 2001) and its ortholog TatC (Bogsch et al., 1998). Hcf106 and Tha4 have similar structures; they are anchored in the membrane by an NH_2-proximal transmembrane domain and expose a predicted amphipathic helix and an acidic COOH-terminal domain to the stroma. Hcf106 and Tha4 also possess significant sequence similarity in their NH_2-proximal regions, especially in the transmembrane domain (~65% identity), where a proline–glutamate motif is strictly

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*Abbreviations used in this paper: BN-PAGE, blue native PAGE; DSP, dithiobis (succinimidyl propionate); DTSSP, dithiobis (sulfosuccinimidyl propionate); RR, conserved twin arginine.

conserved. cpTatC is a membrane protein with six predicted transmembrane segments, exposing its NH_2 and COOH termini to the stroma (Mori et al., 2001). In the case of thylakoids, all three components directly participate in the transport process because antibody to any single component disables the pathway (Mori et al., 1999; Summer et al., 2000; Mori et al., 2001). Several models have been presented for the operation of these systems; each has suggested that a different component serves as precursor receptor, i.e., Hcf106/TatB and Tha4/TatA because of their receptor-like topology (Settles et al., 1997; Chanal et al., 1998), and TatC/cpTatC because it is the most conserved component among different species (Berks et al., 2000). However, evidence on this point has been lacking.

Previous studies showed that ΔpH-dependent pathway transport can be divided into two steps, ΔpH-independent binding of precursors to the membrane, and ΔpH-dependent translocation across the membrane (Ma and Cline, 2000, Musser and Theg, 2000a). Binding is productive, or on pathway, because >80% of bound precursor chases into the lumen upon reestablishment of the pH gradient (Ma and Cline, 2000). Furthermore, binding is specific as it can be competitively inhibited by ΔpH-dependent pathway precursors but not a Sec pathway precursor (Ma and Cline, 2000). Here, we have characterized the precursor binding site and determined essential features of precursors for binding. The combination of blue native PAGE (BN-PAGE), coimmunoprecipitation, and chemical cross-linking demonstrated that precursors specifically bind to an ~700-kD complex of cpTatC and Hcf106 and are in close contact with both components. Tha4 is absent from the precursor-bound and unbound cpTatC–Hcf106 complex. Nevertheless, Tha4 participation is required for progression of precursors beyond the cpTatC–Hcf106 bound state even in the presence of the pH gradient. These observations lead to a model for regulated assembly in which the cpTatC–Hcf106 complex serves as the core receptor complex for precursors, and subsequent recruitment of Tha4 reorganizes the components into a translocation channel.

Results

Antibodies to Hcf106 and cpTatC inhibit precursor binding to the thylakoid membrane

Several different precursors were previously shown to exhibit strong binding to thylakoids in the absence of the ΔpH (Ma and Cline, 2000). These include tOE17, a truncated form of the OE17 precursor, and two recombinant precursors called DT23 and DT17 that consist of a chimeric signal peptide fused to the mature OE23 and OE17 proteins. Importantly, all three precursors are highly efficient and specific substrates for ΔpH-dependent pathway transport (Henry et al., 1997; Ma and Cline, 2000).

To determine which components are involved in the binding step, antibodies to the pea components were preincubated with pea thylakoids, and the washed thylakoids were assayed for their ability to bind precursor. Anti-cpTatC IgG or anti-Hcf106 IgG inhibited binding (Fig. 1, lanes 4, 5, 7, and 8), whereas anti-Tha4 IgG (lanes 11, 12) and preimmune IgG (lane 3) had no effect on binding. In the experiment shown in Fig. 1, anti-cpTatC or anti-Hcf106 IgGs reduced DT23 binding to ~40% of control. In a similar experiment (data not shown), anti-cpTatC or anti-Hcf106 reduced tOE17 binding to ~25% of control. Inhibition of binding was suppressed by including antigen during the antibody preincubation step (Fig. 1, lanes 6 and 9), demonstrating the specificity of the inhibition. Although antibodies to Hcf106 and cpTatC inhibited binding, neither antibody eliminated binding, nor did a mixture of antibodies (Fig. 1, lane 10). Parallel assays for these experiments showed that Tha4 antibody treatments were sufficient to inhibit ≥85% of ΔpH-dependent pathway transport (data not shown). These results confirm those of Ma and Cline (2000) that Hcf106 is involved in precursor binding and that Tha4 is required for transport, but not for binding. They further implicate cpTatC in the binding step.

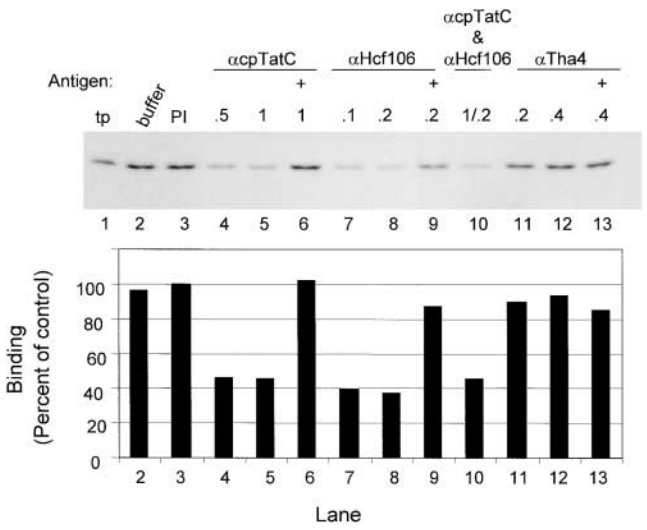

Figure 1. **Antibodies to Hcf106 and cpTatC inhibit precursor binding to thylakoids.** Pea thylakoids were preincubated with buffer (lane 2), preimmune (PI) IgG (lane 3), or two concentrations of anti-pea cpTatC IgG (lanes 4–6), anti-pea Hcf106 IgG (lanes 7–9), or anti-pea Tha4 IgG (lanes 11–13) in the absence or presence of 20 μM of the respective antigens as shown above the panel, or with a combination of 1 mg/ml anti-cpTatC and 0.2 mg/ml anti-Hcf106 (lane 10) (see Materials and methods). The concentrations chosen for IgG treatment (shown in mg/ml, top) were those previously determined to produce maximum inhibition of ΔpH-dependent transport and 50% of that concentration for each antibody. After washing, treated thylakoids were assayed for binding and transport (not shown) of radiolabled DT23. Thylakoids recovered from assays were analyzed by SDS-PAGE and fluorography. DT23 translation product (tp) is shown in lane 1. The amounts of bound DT23 are presented in the chart below the figure. All values are plotted relative to binding of the preimmune-treated thylakoids, which was 6% of the added precursor and is arbitrarily set at 100% for the bar chart.

cpTatC and Hcf106 are members of an ~700-kD complex that lacks Tha4

Among the possibilities suggested by the antibody inhibition studies is that Hcf106 and cpTatC are members of a complex that binds precursors. To examine the organization of ΔpH-dependent pathway components, detergent solubilized thyla-

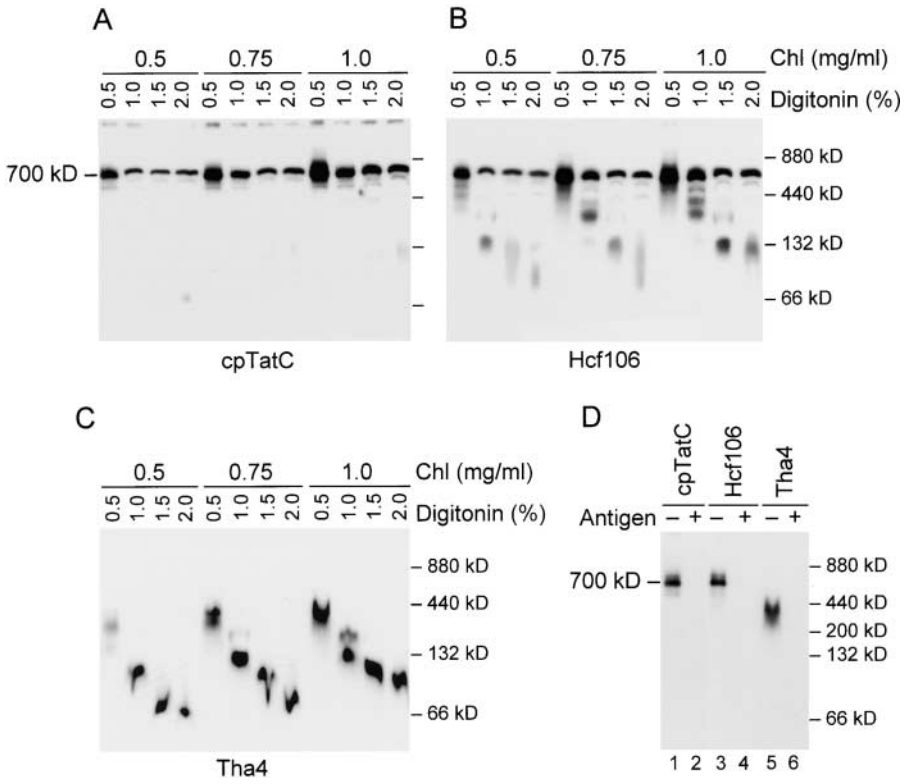

Figure 2. **Blue native gel analyses of components of the ΔpH-dependent protein transport pathway.** Pea thylakoids were solubilized with digitonin and subjected to BN-PAGE (Materials and methods). The final digitonin concentration (%) and the final thylakoid concentration (mg chlorophyll/ml) are designated (top). Proteins were detected by immunoblotting with anti-cpTatC (A), anti-Hcf106 (B), and anti-Tha4 (C). (D) Pea thylakoids were solubilized with 0.5% digitonin at 0.75 mg chlorophyll/ml. Proteins were immunodetected with anti-cpTatC (lanes 1 and 2), anti-Hcf106 (lanes 3 and 4), or anti-Tha4 (lanes 5 and 6) that was preincubated in the absence (lanes 1, 3, and 5) or presence (lane 2, 4, and 6) of the corresponding antigen. Molecular mass markers are ferritin (880 and 440 kD), β-amylase (200 kD), and bovine serum albumin (132 and 66 kD).

koids were subjected to BN-PAGE and immunoblotting. BN-PAGE is a high-resolution method for fractionating membrane protein complexes and simultaneously determining their molecular weights (Schägger and von Jagow, 1991). Several detergents were examined as solubilizing agents. Triton X-100 and dodecyl-maltoside proved too harsh, yielding a complex pattern of immunoreactive bands that varied with the detergent concentration. Importantly, banding patterns among cpTatC, Hcf106, and Tha4 did not overlap, and there was no association of components as determined by coimmunoprecipitation studies (data not shown). Digitonin, a milder detergent used to isolate complexes of protein translocation components (Görlich and Rapoport, 1993; Künkele et al., 1998), was ultimately used as it produced a simple and reproducible banding pattern (Fig. 2).

Anti-cpTatC consistently and exclusively decorated an ~700 kD band over a wide range of digitonin concentrations and detergent-to-protein ratios (Fig. 2 A). Anti-Hcf106 also reacted predominantly with an ~700-kD band, but additionally recognized several smaller species that varied in size with the detergent/protein ratio (Fig. 2 B). Significantly, the ~700-kD Hcf106 band was constant in size and relative abundance over a wide range of solubilization conditions, although the band observed with 0.5% digitonin was more diffuse and appears darker. Anti-Tha4 decorated a band that varied in size with detergent/protein ratio from ~400 kD to ~70 kD (Fig. 2 C). Tha4 species did not migrate at the same position as Hcf106 or cpTatC under the same conditions. Immunodecorations were suppressed by preincubating the antibodies with the corresponding antigens (Fig. 2 D), thereby demonstrating the specificity of the reactions. The fact that the 700-kD complex was unaffected by a wide range of digitonin concentrations suggests that it is a relatively stable complex. The fact that the Tha4 and smaller Hcf106 species varied in size with detergent concentrations suggests weaker or more transitory interactions and may reflect the presence of Tha4 and Hcf106 oligomers. These results suggest that cpTatC and Hcf106 are present in a large membrane complex that is devoid of Tha4.

This tentative conclusion was confirmed by coimmunoprecipitation analysis performed with digitonin-solubilized thylakoids under nondenaturing conditions (Fig. 3). All of the cpTatC was immunoprecipitated by either anti-cpTatC (lane 6) or anti-Hcf106 (lane 8). Hcf106 was coimmunoprecipitated with anti-cpTatC (lane 6), but a portion of the Hcf106 was found in the unbound fraction (lane 5). Tha4 was not coimmunoprecipitated with either anti-cpTatC or anti-Hcf106 (lanes 6 and 8). Likewise, anti-Tha4 immunoprecipitated only Tha4 (lane 10). As negative controls for this experiment, immunoprecipitation was conducted with antibodies to cpSecY (lanes 11 and 12) and to cpOxa1p (lanes 13 and 14), components of the thylakoid Sec and SRP translocation pathways, respectively (Mori et al., 1999; Moore et al., 2000). These antibodies did not coimmunoprecipitate any ΔpH-dependent pathway component, ruling out nonspecific associations. These results demonstrate that cpTatC and a portion of the Hcf106 form a stable ~700-kD complex. They further suggest that a portion of Hcf106 and all of Tha4 are present in separate pools in the membrane.

Precursors bind to the ~700-kD cpTatC–Hcf106 complex

As a first approach to identifying the precursor-binding site, radiolabeled tOE17 and DT23 were bound to thylakoids, and the recovered and solubilized thylakoids were analyzed by

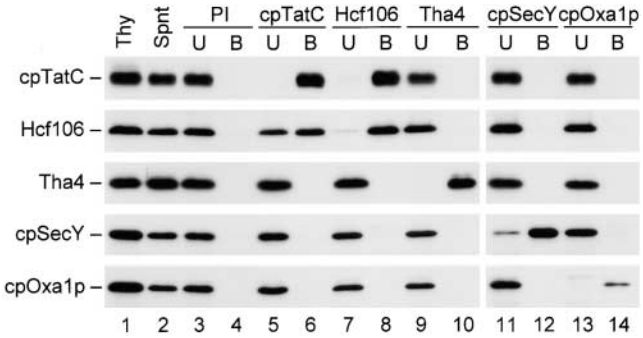

Figure 3. **Coimmunoprecipitation under nondenaturing conditions shows that cpTatC and Hcf106 are present in the same complex.** Pea thylakoids (lane 1) were solubilized with 0.5% digitonin at 0.75 mg chlorophyll/ml. After centrifugation to remove insoluble materials, the resulting supernatant (lane 2) was incubated with protein A–Sepharose to which preimmune (lanes 3 and 4), anti-cpTatC (lanes 5 and 6), anti-Hcf106 (lanes 7 and 8), anti-Tha4 (lanes 9 and 10), anti-cpSecY (lanes 11 and 12), or anti-cpOxa1p (lanes 13 and 14) IgG had been cross-linked. After 1 h at 4°C, proteins unbound (lanes 3, 5, 7, 9, 11, and 13) and bound (lanes 4, 6, 8, 10, 12, and 14) to IgG protein A–Sepharose were analyzed by SDS-PAGE and immunoblotting. Proteins bound to IgG protein A–Sepharose were equivalent to 5 μg chlorophyll of starting thylakoids; all other samples were equivalent to 2.5 μg chlorophyll of starting thylakoids. Antibodies used for the immunoprecipitations are designated (top). Antibodies used for immunoblotting are designated (left).

BN-PAGE and fluorography (Fig. 4 A). Both precursors migrated at ~700 kD on the blue native gel (Fig. 4 A, lanes 2 and 4). If bound precursors were chased into the lumen before solubilization, the radioactivity at ~700 kD was absent or reduced (Fig. 4 A, lanes 3 and 5). For example, all of the bound tOE17 was chased to the lumen, as evidenced by the disappearance of precursor and appearance of mature OE17 on the SDS-PAGE fluorogram (Fig. 4 B, lane 3). Correspondingly, there was no detected band at 700 kD on the blue native gel (Fig. 4 A, lane 3). In addition, appearance of radioactivity at ~700 kD depended on precursor interaction with intact thylakoids. When tOE17 and DT23 were combined with digitonin solubilization buffer or even solubilized thylakoids, they migrated as smears on the blue native gel (data not shown).

Precursor-containing ~700-kD bands possessed a slightly greater molecular weight than the cpTatC–Hcf106 complex (Fig. 4 A, compare lanes 2 and 1). As a marker for the cpTatC–Hcf106 complex, radiolabeled pHcf106 was imported into chloroplasts, and the recovered thylakoids were subjected to BN-PAGE (Fig. 4 A, lane 1). Hcf106 (as well as cpTatC) imported into chloroplasts assembles into the 700-kD cpTatC–Hcf106 complex (unpublished data). In this experiment, the free pool of Hcf106 migrated at ~300 kD (Fig. 4 A, lane 1). The absence of a distinct band of radiolabeled precursor at this location (Fig. 4 A, lanes 2 and 4) suggests that free Hcf106 does not bind precursors.

Electrophoresis of BN-PAGE lanes in a second dimension SDS polyacrylamide gel verified that radiolabel in the ~700-kD band was due to precursor (Fig. 4 C). DT23 appeared on the second dimension gel as a single spot at the relative position of the 700-kD BN-PAGE complex. tOE17 appeared as a spot at the ~700-kD position and also streaked down towards the bottom of the BN-PAGE lane, suggesting that some tOE17 dissociates from the complex during electrophoresis. In three experiments that were quantified, averages of 20% of the tOE17 and 70% of the DT23 applied to the blue native gel were recovered in the ~700-kD band.

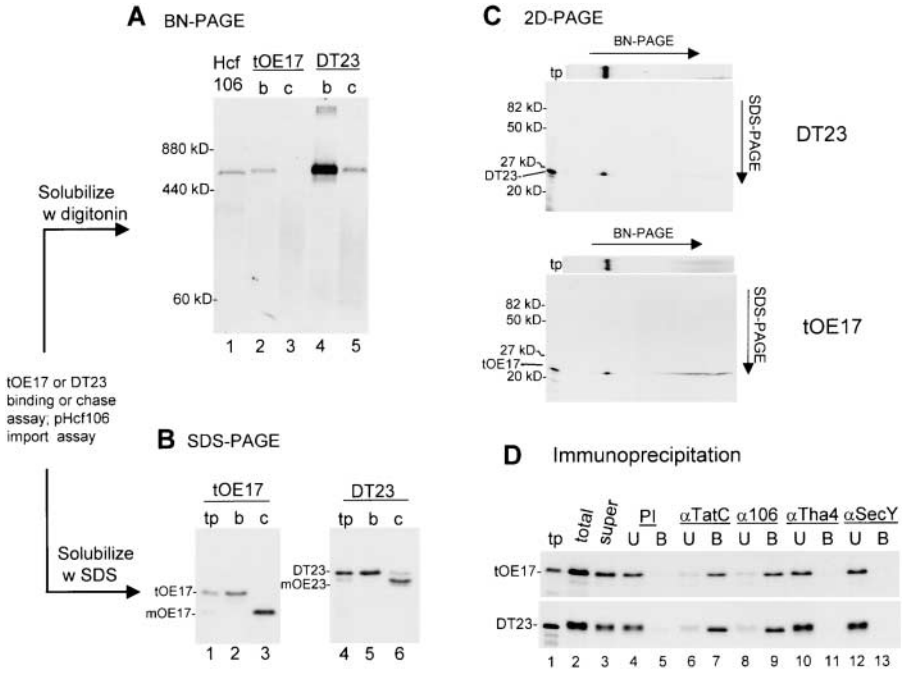

Figure 4. **Precursors bind to the ~700-kD cpTatC and Hcf106 complex.** (A) Pea thylakoids were assayed for binding (b; lanes 2 and 4) or binding and then transport chase (c; lanes 3 and 5) with in vitro translated tOE17 and DT23. Recovered thylakoids were solubilized with 1% digitonin, 20% glycerol, and import buffer and analyzed by BN-PAGE and fluorography (see Materials and methods). Radiolabled Hcf106 from an import assay (see text) was used as a marker for the cpTatC–Hcf106 complex (lane 1). (B) Recovered thylakoids (A) were also subjected to SDS-PAGE and fluorography. The precursors used for the assays (tp) are in lanes 1 and 4. (C) BN-PAGE lanes of bound DT23 and bound tOE17 were subjected to second dimension SDS-PAGE (Materials and methods). An aliquot of the translation product mixed with prestained molecular weight markers was run on the left side of the SDS-PAGE gels. (D) Recovered thylakoids from binding assays with in vitro translated tOE17 or DT23 were solubilized with 1% digitonin, 20% glycerol, 0.5 M aminocaproic acid, and 1/2 × import buffer (total; lane 2), and the soluble 200 *g* supernatant (super; lane 3) was subjected to immunoprecipitation with IgGs cross-linked to protein A–Sepharose as designated top (as in Materials and methods, except that recovered beads were washed with solubilization buffer, 0.5% digitonin). Bound (B) and unbound (U) proteins were adjusted to 1:1 stoichiometry with respect to the original thylakoid sample and analyzed by SDS-PAGE and fluorography. tp; an aliquot of translation product used for the assay.

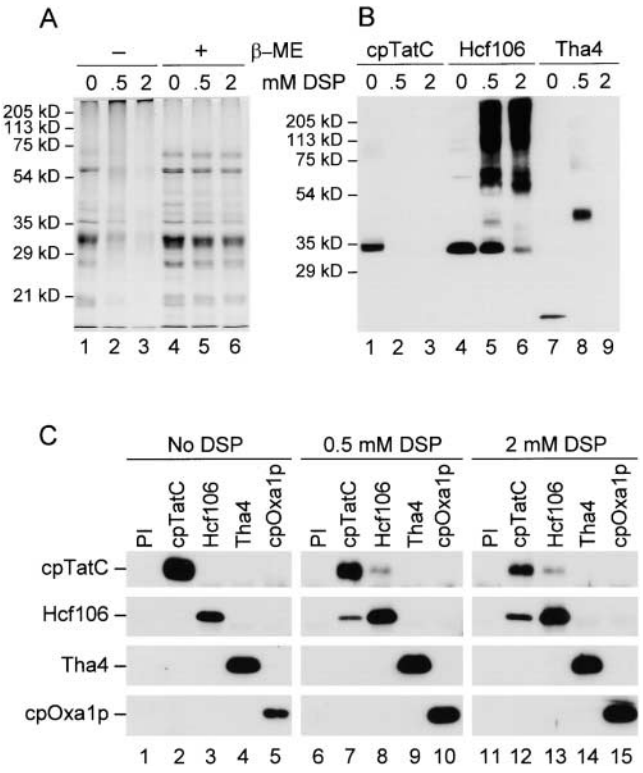

Figure 5. **Cross-linking of ΔpH-dependent pathway components in intact thylakoids.** Cross-linking experiments were conducted as described in Materials and methods. (A) Thylakoids were treated without (lanes 1 and 4) or with 0.5 mM (lanes 2 and 5) or 2.0 mM DSP (lanes 3 and 6). Thylakoid proteins were separated by SDS-PAGE and stained with Coomassie blue. Protein samples were not reduced (lanes 1–3) or reduced with β-mercaptoethanol (4–6) before SDS-PAGE. DSP concentrations are shown at the top of the figures. (B) Proteins were analyzed by nonreducing SDS-PAGE and immunoblotting with anti-cpTatC (lanes 1–3), anti-Hcf106 (lanes 4–6), or anti-Tha4 (lanes 7–9). (C) Cross-linked thylakoids were solubilized with SDS and subjected to immunoprecipitation with preimmune (lanes 1, 6, and 11), anti-cpTatC (lanes 2, 7, and 12), anti-Hcf106 (lanes 3, 8, and 13), anti-Tha4 (lanes 4, 9, and 14), or anti-cpOxa1p (lanes 5, 10, and 15). Bound proteins were eluted, reduced with β-mercaptoethanol, and analyzed by SDS-PAGE and immunoblotting. Antibodies used for the immunoprecipitations are designated (top); antibodies used for immunoblotting are designated (left).

Coimmunoprecipitation under nondenaturing conditions confirmed that bound precursor was in a complex with cpTatC and Hcf106. Digitonin solubilized precursor-bound thylakoids were subjected to immunoprecipitation and analyzed by SDS-PAGE and fluorography (Fig. 4 D). Either anti-Hcf106 IgG or anti-cpTatC IgG immunoprecipitated nearly all of the tOE17 and DT23 (lanes 6–9). In contrast, all detectable precursors were recovered in the unbound fraction when anti-Tha4 (lanes 10 and 11), anti-cpSecY (lanes 12 and 13), or preimmune IgGs (lanes 4 and 5) were used for immunoprecipitation. An immunoblot of the bound and unbound samples verified that Tha4 was quantitatively removed from the supernatant by the anti-Tha4 beads (data not shown). This data, combined with the antibody inhibition experiment (Fig. 1), indicates that the ~700-kD cpTatC–Hcf106 complex is the target for productive binding of precursors.

Chemical cross-linking of intact thylakoids confirms in situ associations of cpTatC and Hcf106, but not Tha4

Because detergent could disrupt labile interactions with Tha4, chemical cross-linking of intact thylakoids before and after precursor binding was undertaken to examine interactions that exist in the membrane. Conditions for cross-linking of thylakoids before precursor binding were designed to yield extensive cross-linking, such that interactions would be detected if present. Various cross-linkers were tested; homobifunctional amine-reactive cross-linkers proved effective for thylakoid proteins, and thiol-cleavable derivatives were used to facilitate identification of cross-linked partners. The membrane-permeable dithiobis (succinimidyl propionate) (DSP) was used in experiments shown in Fig. 5. Extensive cross-linking of thylakoid proteins was apparent by SDS-PAGE and Coomassie staining in the absence of reducing agents (Fig. 5 A, lanes 1–3). Reversibility of cross-linking was verified by treating samples with β-mercaptoethanol before SDS-PAGE (lanes 4–6). Immunoblotting confirmed that cpTatC, Hcf106, and Tha4 were extensively cross-linked into larger complexes, some of which must have failed to either migrate into the gels or transfer to the nitrocellulose membrane (Fig. 5 B, lanes 1–9).

To identify cross-linked partners, SDS-solubilized membranes were subjected to immunoprecipitation under nonreducing conditions, cross-links between immunoselected species were cleaved with β-mercaptoethanol, and immunoprecipitated proteins were identified by immunoblotting (Fig. 5 C). DSP at 0.5 and 2 mM cross-linked cpTatC to Hcf106 as evidenced by reciprocal coimmunoprecipitation (Fig. 5 C, lanes 7, 8, 12, and 13). In contrast, Tha4 cross-linked neither to cpTatC or Hcf106 (Fig. 5 C, lanes 7–9 and 12–14). Similar results were obtained with the membrane impermeable cross-linker dithiobis (sulfosuccinimidyl propionate) (DTSSP) (data not shown).

The specificity of interactions in these experiments was routinely monitored by immunoprecipitation of samples treated without cross-linker (Fig. 5 C, lanes 1–5) and by immunoprecipitation of cross-linked samples with preimmune IgG (lanes 6 and 11) or anti-cpOxa1p (lanes 10 and 15). In the experiment in Fig. 5, nonspecific cross-linking was not observed. We occasionally observed that a minor amount of Hcf106 ($\ll$1%) was immunoprecipitated with anti-Tha4, anti-cpOxa1p, and even preimmune IgGs. That these latter interactions represent nonspecific association is further supported by the fact that reciprocal interactions were not detected (data not shown). Together, the cross-linking results support conclusions of BN-PAGE and nondenaturing coimmunoprecipitation that before precursor binding, cpTatC and Hcf106 are members of the same membrane complex and that Tha4 is not present in this complex.

Chemical cross-linking of precursor-bound thylakoids demonstrates a direct interaction between precursor and both cpTatC and Hcf106

The primary objective for cross-linking of precursor-bound thylakoids was to identify components that directly interact with the precursor. The precursor DT17 (see below) was used as it yielded a high level of cross-linking products in prelimi-

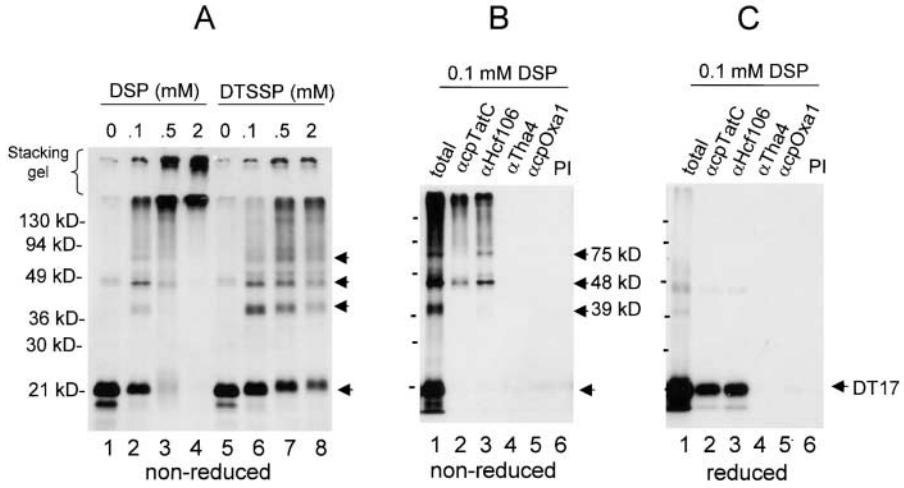

Figure 6. **Chemical cross-linking of precursor-bound thylakoids.** (A) In vitro translated DT17 was incubated with thylakoid membranes in a binding assay. The membranes were washed and treated with varying concentrations of DSP or DTSSP as described in Materials and methods. Concentrations of cross-linker (mM) are depicted at top. The membranes were then analyzed by SDS-PAGE and fluorography. (B) A 0.1-mM DSP-treated sample, equivalent to 0.33 mg total chlorophyll, was denatured with SDS and divided into six aliquots, five of which were subjected to immunoprecipitation under denaturing conditions with IgGs cross-linked to beads as designated above the panel. Immunoprecipitated samples were released with SDS and analyzed by SDS-PAGE and fluorography as described in Materials and methods. (C) A portion of each sample in B was treated with 2.5% mercaptoethanol to cleave the cross-linking agent and was then subjected to SDS-PAGE and fluorography. Each sample in B is equivalent to 6 μg chlorophyll, and each sample in C is equivalent to 4 μg chlorophyll of starting thylakoids.

nary experiments with DSP and DTSSP. A concentration series of each cross-linker identified amounts that produced distinctive bands representing putative 1:1 adducts between precursors and nearest neighbors (Fig. 6 A, lanes 2–4 and 6–8). Notable products were those at ~39, ~48, and ~75 kD. The relative amounts of these products varied with the cross-linker, but the band at ~48 kD was uniformly present. Because Hcf106 migrates on SDS gels at ~32 kD, cpTatC at ~33 kD, and DT17 at ~20 kD, the 48-kD band could conceivably represent a cpTatC-DT17 or Hcf106-DT17 cross-linking product. In addition to distinct bands, very large cross-linking products were also apparent (Fig. 6 A, lanes 2–4).

DSP at 0.1 mM was selected for immunoprecipitation analysis because it gave both distinct and very large cross-linking products. After cross-linking, thylakoids were denatured in 1% SDS and subjected to immunoprecipitation. Cross-linked DT17 was bound by anti-cpTatC (Fig. 6 B, lane 2) and anti-Hcf106 (Fig. 6 B, lane 3) IgGs, but not by anti-Tha4 (lane 4), anti-cpOxa1p (lane 5), or preimmune (lane 6) IgGs. The pattern of anti-Hcf106 and anti-cpTatC immunoprecipitated products was similar but not identical. Both antibodies immunoprecipitated an ~48-kD band, indicating that each component formed a 1:1 cross-linking product with DT17 (Fig. 6 B, lanes 2 and 3). Both antibodies also immunoprecipitated a range of higher molecular weight products. Neither antibody immunoprecipitated the 39-kD product, which may be a DT17 dimer. Treatment of immunoprecipitated cross-linking products with β-mercaptoethanol to cleave the cross-linker confirmed that Hcf106 and cpTatC were cross-linked to DT17 (Fig. 6 C, lanes 2 and 3), but that Tha4 was not (Fig. 6 C, lanes 4–6), even though some DT17 cross-linking products were very large. These results indicate that the precursor is in close contact with both Hcf106 and cpTatC. Similar results were obtained with DTSSP (data not shown). Combined with the nondenaturing immunoprecipitation data in Figs. 3 and 4 and the cross-linking data in Fig. 5, these results indicate that Tha4 is not a component of the cpTatC–Hcf106 complex either before or after binding of the precursor in the absence of a ΔpH.

Precursor binding to the cpTatC–Hcf106 complex requires both the RR and the hydrophobic core of the signal peptide

The above studies analyzed the nature of the precursor-binding site. The features of the precursors important for the interaction were determined by analyzing binding of precursors with altered signal peptides. Recovered thylakoids were subjected to BN-PAGE to assess the amount of precursor associated with the ~700-kD complex (Fig. 7 B) and by SDS-PAGE to monitor the amount of precursor that associated with thylakoids (Fig. 7 C). As expected, deletion of the signal peptide eliminated binding to the cpTatC–Hcf106 complex. Although tOE17 showed significant binding to the ~700-kD complex (Fig. 7 B, lane 9), mOE17 was not associated with the complex (lane 8). Similarly, the intermediate precursor iPftf also bound to the cpTatC–Hcf106 complex (lane 6); Pftf without its signal peptide (mPftf) did not (lane 7). iPftf is a membrane protein with an RR-containing signal peptide that is integrated into thylakoids by the ΔpH-dependent pathway (Summer et al., 2000).

Both the RR and the hydrophobic core of the signal peptide are required for efficient transport on the ΔpH-dependent pathway (Chaddock et al., 1995; Henry et al., 1997). To determine if either or both of these elements are required for specific binding, DT23 and DT17 with mutations in the relevant regions were prepared (Fig. 7 A). DT17, similar to DT23, exhibits strong productive binding to thylakoids (Ma and Cline, 2000) and to the cpTatC–Hcf106 complex (Fig. 7 B, compare lanes 3 and 10). Mutation of the consensus twin arginines of either precursor to twin lysines (Fig. 7 A, KK-DT23 and KK-DT17) renders them inactive for transport (unpublished data). Although both KK-DT17 and KK-DT23 exhibited some binding to thylakoids as seen in Fig. 7 C, neither precursor was associated with the cpTatC–Hcf106 complex (Fig. 7 B, lanes 4 and 11). Introduction of an aspartic acid residue into the hydrophobic core of the signal peptide, as in V11D-DT23 (Fig. 7 A), disables the precursor for transport (Henry et al., 1997). V11D-DT23 did

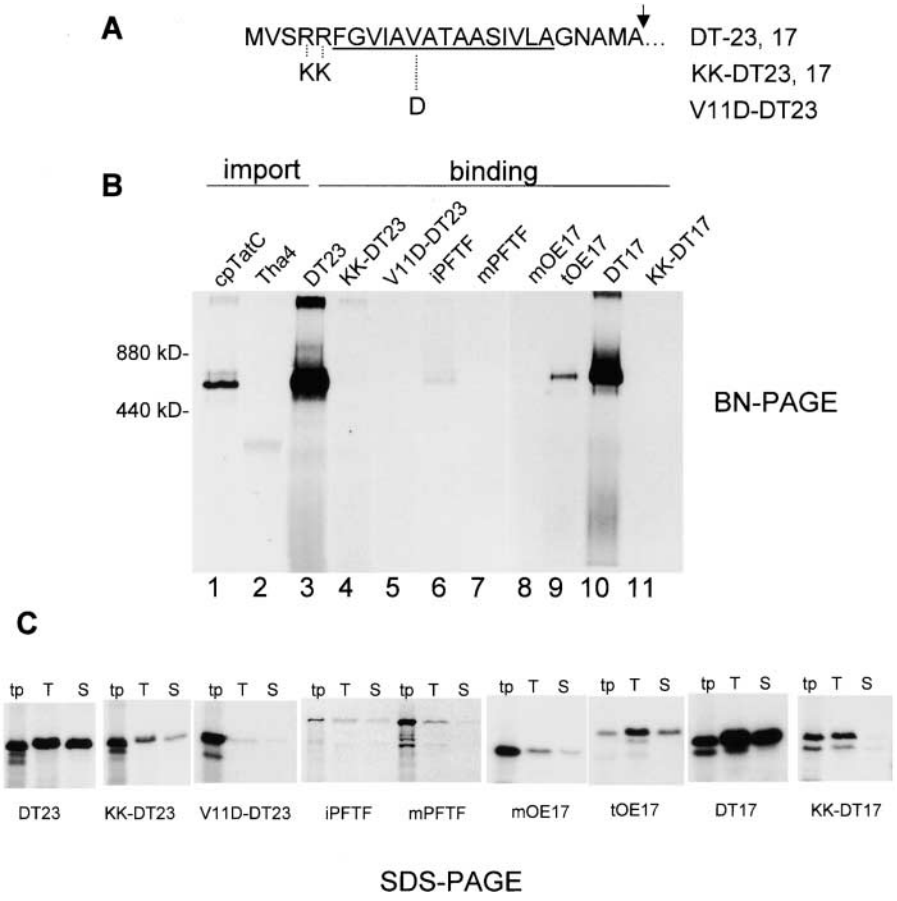

Figure 7. **Precursor binding to the cpTatC–Hcf106 complex requires both the RR and the hydrophobic core of the signal peptide.** In vitro translated proteins were incubated with thylakoids in binding assays. Recovered thylakoids were then analyzed by BN-PAGE and SDS-PAGE (Materials and methods). (A) The sequence of the DT signal peptide from the amino terminus to the thylakoidal processing protease cleavage site (arrow). The hydrophobic core is underlined. Mutations are shown as the substituted amino acids below the DT sequence and the resulting precursors designated as shown to the right. (B) Thylakoids from binding assays were solubilized with 1% digitonin, 20% glycerol, and import buffer and analyzed by BN-PAGE/fluorography. Chloroplast import of pcpTatC and pTha4 was conducted, and the recovered thylakoids were analyzed in adjacent lanes as markers for these components. The precursors used are designated (top). Each lane was loaded with sample equivalent to 8% of the assay, and gels were exposed to film for 7 d. (C) Aliquots of the translation products (tp) equivalent to 0.125% of that added to each assay, total solubilized thylakoids (T) equivalent to 3% of the assay, and 200,000 *g* supernatants (S; i.e., the BN-PAGE samples) equivalent to 3% of the assay were analyzed by SDS-PAGE and fluorography on identical gels in parallel, which were exposed to the same piece of film for 4 d. The precursors are designated (bottom).

not bind to the cpTatC–Hcf106 complex (Fig. 7 B, lane 5). These data indicate that productive binding of precursors to thylakoids represents the recognition event for this pathway. The participants of this recognition are the cpTatC–Hcf106 complex and the signal peptide of precursor proteins, of which both the twin arginine and the hydrophobic core are essential elements.

Precursors are not released from the ~700-kD complex unless Tha4 can participate in the transport reaction

Results presented here and previously (Mori et al., 1999; Ma and Cline, 2000) show that Tha4 is required for transport but not binding of precursor. To determine the fate of precursors in the presence of ΔpH when Tha4 is prevented from entering the reaction, thylakoid membranes were preincubated with anti-Tha4 IgG and then subjected to binding, chase, and transport reactions with DT23 (Fig. 8). Thylakoids recovered from assays were examined by SDS-PAGE/fluorography to assess the amount of precursor or mature protein associated with the membranes (Fig. 8 B) and by BN-PAGE/fluorography to assess specific binding (Fig. 8 A). As expected, antibody-treated membranes were as active as preimmune IgG–treated membranes in specifically binding DT23 in a standard binding assay on ice (Fig. 8, A and B, compare lanes 1 and 5). However, anti-Tha4–treated membranes were unable to transport bound precursor (Fig. 8 B, lane 6), which remained associated with the ~700-kD complex (Fig. 8 A, lane 6). In the control preimmune treated membranes, nearly all of the bound precursor was transported to the lumen, as assessed by the appearance of the mOE23 (Fig. 8 B, lane 2) and the absence of the ~700-kD band on BN-PAGE (Fig. 8 A, lane 2). When anti-Tha4–treated thylakoids were incubated at 25°C with freshly added DT23 in a protein transport assay, i.e., with a ΔpH, a substantially greater amount of precursor bound to the cpTatC–Hcf106 complex (Fig. 8, A and B, lane 7). Protease treatment of the membranes showed that the associated precursor was still exposed to the stromal face (data not shown). The pH gradient apparently contributed to the increased binding because considerably less precursor was bound in assays conducted with the protonophore nigericin (Fig. 8, A and B, lanes 8).

In a parallel assay with preimmune-treated membranes, a substantial amount of precursor was transported to the lumen, as judged by processing to mature size (Fig. 8 B, lane 3). However, even here a considerable amount of precursor was associated with recovered thylakoids (Fig. 8 B, lane 3) and the cpTatC–Hcf106 complex (Fig. 8 A, lane 3). The amount of precursor associated with membranes recovered from thylakoid protein transport assays can vary from the sizeable amount seen in Fig. 8, where a relatively high concentration of precursor was added, to very little, which we

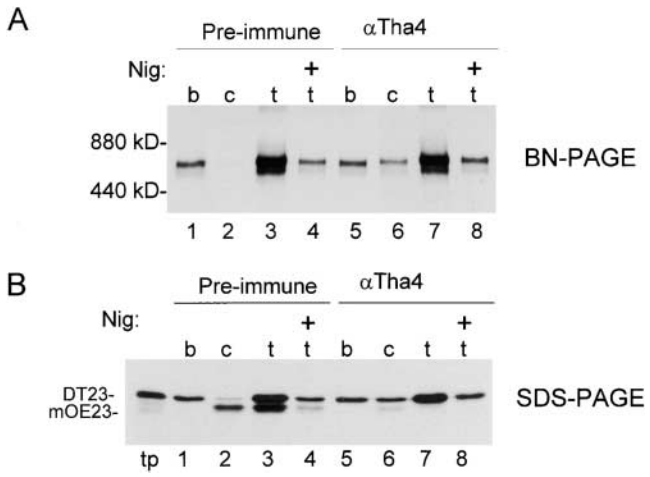

Figure 8. **Precursors remain bound to the cpTatC–Hcf106 complex under transport conditions unless Tha4 is free to participate.** Thylakoids were pretreated with 0.4 mg/ml of preimmune or anti-Tha4 IgGs, washed, and then incubated with in vitro–translated DT23 in a binding assay (b) or a binding and chase assay (c), or they were incubated with DT23 under transport conditions (t), i.e., 5 mM Mg-ATP, 5 mM dithiothreitol, ~500 μg stromal protein, and light at 25°C for 20 min, in the absence or presence of 0.4 μM nigericin as shown (top). Chase and transport assays were diluted 4.5- and 10-fold, respectively, with import buffer before removal from light and 25°C. (A) Recovered thylakoids were solubilized in 1% digitonin, 20% glycerol, and import buffer and analyzed by BN-PAGE and fluorography. Each lane contains sample equivalent to 6% of the assay. The minor band migrating just below the ~700-kD band is occasionally observed and presumably represents a breakdown product of the ~700-kD complex. It appears enhanced in overexposed lanes (3 and 7) but is present in the other lanes and in immunoblots (Fig. 3). (B) Recovered thylakoids (A) were analyzed by SDS-PAGE and fluorography. Lanes contain sample equivalent to 5% of the assay. DT23 translation product, equivalent to 0.25% of that added to each assay, is shown in the lane marked tp. (Lanes 1 and 5) Binding assays; (lanes 2 and 6) chase assays; (lanes 3, 4, 7, and 8) transport assays.

have observed in similar experiments with less added precursor (data not shown). The fact that the thylakoid-bound precursor is largely associated with the receptor complex (Fig. 8 A, lane 3) provides additional evidence that the limiting step in the transport reaction occurs after precursor binding (Ma and Cline, 2000; Musser and Theg, 2000a).

Discussion

Protein translocation is generally initiated by precursor binding to one or more components of the translocation machinery. In previous work, we showed that a subset of precursors binds to thylakoids in the absence of a ΔpH and is transported into the lumen when the ΔpH is imposed (Ma and Cline, 2000). Here, we provided evidence that a complex of cpTatC and Hcf106 is the target of precursor binding. In addition, we described the organization of known components both before and after precursor binding and tentatively identified the stages of the process in which different components participate.

Several lines of evidence support the conclusion that precursors bind to a cpTatC–Hcf106 complex. First, antibodies to Hcf106 and to cpTatC inhibited the binding of at least two different precursors, whereas antibodies to Tha4, the third known component, had no effect on binding (Fig. 1; Ma and Cline, 2000). Second, cpTatC and Hcf106 exist in thylakoids as an ~700-kD complex that is devoid of Tha4 (Figs. 2, 3, and 5). Upon detergent solubilization of precursor-bound thylakoids, precursors were associated with the cpTatC–Hcf106 complex as determined by BN-PAGE and coimmunoprecipitation analysis (Fig. 4). Finally, chemical cross-linking studies verified that precursors are in direct contact with both Hcf106 and cpTatC (Fig. 6), but not Tha4. These analyses also indicate that precursor binding per se did not alter the organization of components in the membrane. In particular, the precursor-bound complex contains cpTatC and Hcf106, but is still devoid of Tha4 (Figs. 4 and 6). In fact, an examination of component organization by BN-PAGE/immunoblotting subsequent to binding of saturating amounts of DT23 showed no significant changes in the banding pattern of any component, other than the expected slight shift in molecular mass of the 700-kD cpTatC and Hcf106 bands (unpublished data).

Analysis of precursor requirements for binding suggests characteristics of the binding site. The signal peptide appears to be the primary determinant of binding. Notably, precursor binding to the cpTatC–Hcf106 complex was strictly dependent on the twin arginine motif and the hydrophobic core of the signal peptide (Fig. 7), both of which are crucial factors for protein translocation on the ΔpH-dependent pathway (Henry et al., 1997). This suggests that the binding site consists of a hydrophobic pocket and acidic or polar residues to bind the arginine pair. Because the detergent-solubilized precursor cpTatC–Hcf106 complex is very stable, it is reasonable to suspect that the entire binding site is made up of protein domains of the complex. Several considerations suggest that the binding site resides either on cpTatC or is formed by the interaction of Hcf106 and cpTatC. First, bacterial Tat components also exist in a large ~600-kD complex (Bolhuis et al., 2000), which contains only TatB (Hcf106 orthologue) and TatC in 1:1 stoichiometry, and TatA (Tha4 orthologue) in substoichiometric amounts (Bolhuis et al., 2001). Similarly, we have partially purified an ~700-kD cpTatC–Hcf106–DT23 complex by affinity chromatography and found that cpTatC and Hcf106 are the major, if not only, thylakoid proteins present in the complex (unpublished data). Second, as precursors apparently fail to bind to the free pool of Hcf106 (Fig. 4), it is unlikely that Hcf106 possesses the entire binding site. Finally, the fact that bound precursor formed 1:1 cross-linking products with both Hcf106 and cpTatC demonstrates its proximity to each component (Fig. 6). Nevertheless, further analysis is required to identify the signal peptide–binding domain of the component(s). It will also be important to determine how many binding sites are present per ~700-kD complex, as it appears to be a multimer of cpTatC–Hcf106 heterodimers.

An important goal will be to elucidate the events that occur after precursor binding and establishment of the pH gradient. Several observations persuade us that Tha4 joins the large complex and that this leads to formation of an active translocon. First, results presented here (Fig. 8) and in Ma and Cline (2000) demonstrate that Tha4 functions after the binding step and that, when antibodies sequester Tha4, the

precursor cpTatC–Hcf106 remains intact even in the presence of a ΔpH. Second, kinetic analysis of precursor transport from the bound state indicates that a rate-limiting step occurs on the membrane after the pH gradient is formed (Ma and Cline, 2000; Musser and Theg, 2000a). Musser and Theg (2000b) additionally showed that proton transfer limits events on the membrane leading to translocation. This would be consistent with such a hypothetical ΔpH-triggered assembly process. Finally, the fact that the purified ~600-kD *Escherichia coli* Tat complex contains a small amount of TatA (Tha4 orthologue) in addition to the large amounts of TatC and TatB (Hcf106 orthologue) suggests that, under appropriate conditions, all three components can be present in one complex (Bolhuis et al., 2001). Analysis of the components in the process of protein translocation will be necessary to test this assembly model. This might be achieved by trapping a translocation intermediate. Berghöfer and Klösgen (1999) reported on a putative translocation intermediate of the ΔpH-dependent pathway, wherein the precursor protein appeared to be largely translocated into the lumen but still remained associated with the membrane. This intermediate migrated on BN-PAGE at ~560 and 620 kD, which is similar in size to the complex that we have seen. If these truly represent proteins in the process of translocation, an analysis of their composition will be valuable for determining the components present in a translocation apparatus.

The nature of the active translocon is currently unknown. Berks et al. (2000) speculate that multiples of TatB/Hcf106 and TatA/Tha4 assemble to form a flexible channel that accommodates protein substrates of varied size. Our recent isolation of a membrane-spanning protein substrate arrested in the process of translocation (unpublished data) supports the notion that a channel structure is involved, as does the recent imaging of overexpressed TatA and TatB as ring-like structures (Sargent et al., 2001). The 5–10-fold excess of Hcf106 and Tha4 over translocation sites (Mori et al., 1999, 2001) is consistent with a mechanism in which the cpTatC–Hcf106 complex forms a core complex that recruits channel components for the translocation step. One advantage of a dynamic structure for the ΔpH-dependent pathway would be to prevent premature formation of channels that would likely dissipate ion and pH gradients. Just as the studies reported here reveal insight into the precursor recognition step for this pathway, determining the nature of the active translocon will undoubtedly provide insight into the translocation step.

Materials and methods

Production of precursor proteins

Transcription plasmids for maize iOE17, tOE17, DT17, and pea iOE23 and DT23 are described in Henry et al. (1997); maize mOE17 and wheat iOE33 in Ma and Cline (2000); and pepper mPftf, in Summer et al. (2000). Pea pcpTatC and pHcf106 are as described (Mori et al., 2001), and pea pTha4 has been previously described in Mori et al. (1999). KK-DT23 and KK-DT17 are altered forms of DT23 and DT17 in which the twin arginine of the signal peptide was replaced with a twin lysine. KK-DT23 and KK-DT17 were constructed by a single round of PCR using DT23 and DT17 as templates, respectively. Codons to replace the twin arginines with twin lysines were incorporated in the forward primer, such that the translated proteins begin MVSKK. Pepper iPftf was constructed from pPftf (Summer et al., 2000) by a single round of PCR, such that the forward primer encodes an initiator methionine at the putative stromal processing site in the transit peptide. The resulting protein begins MASLQQR. The PCR products were cloned into pGEM 4Z in the SP6 orientation. V11D-DT23 is an altered form of DT23 in which aspartic acid replaced valine (residue 11) in the hydrophobic core of the signal peptide. V11D-DT23 was constructed by the SOE-PCR method (Horton et al., 1989) using DT23 as template and incorporating a GTC to GAC codon change into the overlap primers for the first round of PCR reactions. The SOE product was cloned into pGEM4Z in the SP6 direction. Capped RNA was transcribed by SP6 polymerase (Promega) and translated in a wheat germ system in the presence of [^{3}H]leucine (Cline, 1986). iPftf, mPftf, pcpTatC, pHcf106, and pTha4 were produced by coupled transcription and translation in an SP6/wheat germ TnT system (Promega) for 60 min at 30°C with [^{3}H]leucine according to manufacturer guidelines. Translation mixtures were transferred to ice and adjusted to import buffer (50 mM Hepes/KOH, pH 8, 0.33 M sorbitol) containing 30 mM unlabeled leucine. Translation extracts to be used for binding assays were depleted of NTPs with apyrase (grade VII; Sigma-aldrich) at 35 U/100 μl of translation mixture for ≥10 min on ice before use.

Isolation of chloroplasts and thylakoids and assay for import into chloroplasts and binding to thylakoid membranes

Chloroplasts and thylakoids were isolated from pea seedlings as described (Cline et al., 1993). Thylakoids were washed once with import buffer before use. Pretreatment of thylakoids with IgG was previously described (Mori et al., 1999). Chloroplast protein import assays were as described (Cline et al., 1993), except that incubations were for 20 min. Thylakoids were recovered from repurified chloroplasts by lysis and centrifugation and were washed with import buffer (Cline et al., 1993). Precursor binding assays were as described by Ma and Cline (2000). Typical assays contained radiolabeled precursor protein and thylakoids equivalent to 100 μg chlorophyll in a total volume of 300 μl of import buffer. After incubation on ice in darkness for 15 min, precursor-bound thylakoids were recovered by centrifugation, washed twice with import buffer, and transferred to a new microcentrifuge tube. Transport of precursor from the bound state (chase) was accomplished by resuspending precursor-bound thylakoids in import buffer, 5 mM $MgCl_2$, 5 mM Mg-ATP, 5 mM dithiothreitol, and ~500 μg stromal protein in a total volume of 300 μl and incubating in the light for 15 min at 25°C.

BN-PAGE

BN-PAGE was carried out as described (Schägger and von Jagow, 1991) with the following modifications. Washed thylakoids were suspended in resuspension buffer (20% [wt/vol] glycerol, 25 mM BisTris-HCl, pH 7.0) at 1.5 mg chlorophyll/ml or as otherwise indicated. An equal volume of resuspension buffer containing twice the final detergent concentration was added to the thylakoid suspension in a drop-wise manner. After incubation at 4°C for 1 h with end-over-end mixing, insoluble material (primarily appressed thylakoids) was removed by centrifugation at 200 *kg* for 20 min. The supernatant was combined with 1:10 volume of 5% Serva blue G (100 mM BisTris-HCl, pH 7.0, 0.5 M 6-amino-n-caproic acid, 30% glycerol) and applied to 0.75-mm-thick 5–13.5% acrylamide gradient gels in a Hoefer Mighty Small vertical electrophoresis unit connected to a cooling circulator. Electrophoresis was for a total of 3–5 h at 100–150 V and 2–4°C. The cathode buffer was exchanged with buffer lacking dye after the top 1/3–1/2 of the gel was covered with dye (~2 h). Gels to be analyzed by fluorography were treated with DMSO and PPO as described (Cline, 1986). Gels to be used for immunoblotting were incubated in 25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS for 10 min at room temperature and then blotted to PVDF membranes (Millipore) at 50 V for 45 min. Membranes were destained with 20% methanol and 7% acetic acid overnight and then immunodecorated. To verify the specificity of antibody reactions, antiserum was first incubated with antigen or 20 mM HEPES-KOH, pH 8.0, for 1 h on ice and centrifuged at 20 *kg* for 20 min to remove antigen–antibody complexes.

For two-dimensional analysis, excised BN-PAGE lanes were soaked in SDS sample buffer, 2.5% β-mercaptoethanol for 10 min and were layered onto 1-mm-thick 11.5% SDS polyacrylamide gels.

Coimmunoprecipitation under nondenaturing conditions

Washed thylakoids were solubilized with digitonin as described above, except that resuspension buffer also contained 150 mM NaCl, 0.05% BSA, and 1 mM PMSF, or as stated in the figure legends. The 200,000 *g* supernatant was mixed with a 60% slurry of protein A–Sepharose that had been cross-linked to IgG by dimethylpimelimidate at 3:1 (vol/vol), and the suspension was incubated for 1 h at 4°C with end-over-end mixing. The unbound proteins were recovered by centrifugation, and the beads were washed with resuspension buffer containing 0.5% digitonin and 150 mM NaCl, or as stated in the legend. Bound proteins were re-

covered by incubating the beads in 8 M urea, 5% SDS, 125 mM Tris-HCl, pH 6.8, for 1 h at room temperature followed by centrifugation.

Cross-linking and immunoprecipitation under denaturing conditions

Thylakoids or precursor-bound thylakoids, at 0.33 mg chlorophyll/ml in import buffer, were treated with DSP (Pierce Chemical Co.) from a stock solution in dimethyl sulfoxide or with DTSSP (Pierce Chemical Co.) from a stock solution in import buffer. Control treatments for DSP contained dimethyl sulfoxide. Reactions were quenched with Tris-glycine, pH 8.0, at a final concentration of 50 mM for 30 min on ice. Thylakoids were recovered by centrifugation, washed with import buffer containing 5 mM EDTA, and then solubilized with 1% SDS in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 10 min at 37°C at 0.5 mg chlorophyll/ml. Insoluble material was removed by centrifugation, and 50 μl of supernatant was combined with 1 ml of 1% BSA, 1% Triton X-100, 1 mM EDTA, TBS, and 10–20 μl packed volume of protein A–Sepharose that had been cross-linked to IgG. After incubation for 1 h at 4°C with end-over-end mixing, the beads were recovered by centrifugation, washed with 1% Triton X-100 and TBS, and then with TBS alone. Bound proteins were eluted from the beads with SDS sample buffer containing 8 M urea, but lacking β-mercaptoethanol, for 1 h at room temperature. β-mercaptoethanol was then added to aliquots of the eluates at a final concentration of 2.5% to cleave the cross-linker. Immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with horseradish peroxidase–conjugated anti–rabbit Fc as a secondary antibody. Radiolabeled proteins were analyzed by SDS-PAGE and fluorography.

Miscellaneous

Antibodies to pea Hcf106 and pea cpTatC are as described (Mori et al., 2001). Antibodies to pea SecY and pea Tha4 are described in Mori et al. (1999). For antibodies to cpOxa1p, the COOH-terminal domain (residues 305–442) was amplified by RT-PCR with pea mRNA based on the published sequence (EMBL/GenBank/DDBJ under accession no. Y12618), cloned into pETH3c, and overexpressed as an NH_2-terminal histidine–tagged fusion protein in *E. coli* BL21 (DE3) (Cline et al., 1993). The COOH-terminal peptide was purified from inclusion bodies with Ni-chelating Sepharose in the presence of 6 M urea. Antibodies were produced in rabbits by Cocalico Biological. Digitonin was purified as described (Mori et al., 1999). Protein was determined by BCA (Pierce Chemical Co.). Chlorophyll was determined according to Arnon (1949). Radiolabeled proteins were quantified by scintillation counting of proteins extracted from gel bands (Cline, 1986). For cpTatC analyses, all samples were dissolved in an SDS buffer containing 4 M urea at 25°C for 30–60 min to avoid aggregation. Immunoblots were developed by the ECL procedure (Pierce Chemical Co.).

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