Chloroplast TatC plays a direct role in thylakoid ΔpH-dependent protein transport

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Abstract The thylakoid ΔpH-dependent pathway transports folded proteins. Identified components include Hcf106 and Tha4. Orthologs of these proteins plus a membrane protein called TatC are essential for the homologous bacterial Tat system. Here we report identification of a chloroplast TatC (cpTatC). cpTatC is an integral thylakoid membrane protein as determined by in vitro chloroplast import and immunoblotting. Antibody to cpTatC specifically inhibited the thylakoid ΔpH-dependent pathway in vitro. cpTatC is present in about the same quantity as estimated translocation sites, whereas Hcf106 and Tha4 are present in 5–8-fold excess. These results are relevant to mechanistic models for this system. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Chloroplast protein transport; Tat; Twin arginine

1. Introduction

Nuclear-encoded thylakoid proteins are transported into thylakoids following import into the chloroplast stroma [1]. Thylakoid protein translocation systems evolved from prokaryotic protein export systems and are homologous to those present in extant bacteria. Thylakoids have two signal peptide-dependent translocation systems, the Sec and the ΔpH-dependent pathways. The ΔpH-dependent pathway has several unique characteristics. It requires neither *cis* soluble components nor NTP hydrolysis; instead, transport is driven solely by the pH gradient across the thylakoid membrane [2]. It has an essential twin arginine motif in the signal peptides of its substrate precursors. Finally, it can transport fully folded proteins [3,4] and thus differs from most protein translocation mechanisms, including the Sec pathway [4].

Genetic studies in maize identified the Hcf106 protein as a component of the ΔpH-dependent pathway [5,6]. Subsequent biochemical and genetic studies identified a second component called Tha4 [7,8]. Hcf106 and Tha4 are homologous proteins with similar structure; Each is anchored to thylakoids by an amino proximal transmembrane domain and exposes a predicted amphipathic helix and an acidic C-terminal domain to the stroma. In vitro studies demonstrate that Hcf106 and Tha4 are both required for transport by the ΔpH-dependent pathway [7].

A homologous pathway with similar properties has been described in *Escherichia coli* [9]. The *E. coli* system, called Tat, transports precursors with twin arginine-containing signal peptides [9] and appears to transport tightly folded proteins as large as ~7 nm in diameter (see [9], for discussion). Genetic studies show that TatA and TatB, the *E. coli* orthologs of Tha4 and Hcf106, respectively, are required for transport of Tat pathway substrates [10–12]. A gene for a polytopic membrane protein called TatC, which is co-transcribed with *tatA* and *tatB*, is also essential for Tat pathway transport [13].

The fact that TatC homologs are encoded in chloroplast genomes of the algae, *Odontella sinensis* and *Porphyra purpurea*, and the genome of the cyanobacterium *Synechocystis* PCC6803 [13] suggested that a TatC protein might be involved in chloroplast protein transport. We found TatC-homologous sequences in the *Arabidopsis* genomic database and cloned cDNAs for *Arabidopsis* [7]. We report that the encoded pea TatC is a chloroplast thylakoid integral membrane protein and is required in vitro for the ΔpH-dependent pathway, but not for Sec or SRP-dependent pathways. Transport of all five tested ΔpH-dependent substrates was inhibited by the antibody. This result combined with previous work indicates that three components, Hcf106, Tha4, and cpTatC all play essential and direct roles in protein transport by the ΔpH-dependent pathway. Their relative abundance in the membrane further suggests functions for the different components when considered in context with current models for the operation of the system.

2. Materials and methods

2.1. Isolation of cpTatC and Hcf106 cDNAs from pea

An internal fragment of pea TatC was amplified by RT-PCR (BRL Life Sciences) with *Taq* polymerase and primers corresponding to conserved regions of *Arabidopsis* TatC (accession number AAD33946) and algal TatC. Internal primers were designed from the sequence of the cloned fragment and the missing 3′ and 5′ sequences were obtained using the appropriate RACE kits according to the manufacturer's guidelines (BRL Life Sciences). Pea Hcf106 was obtained by the same strategy, except that degenerate primers corresponding to the conserved transmembrane domain and amphipathic helix regions of Hcf106 proteins were used to amplify its internal fragment. The entire coding regions of pea TatC and Hcf106 were amplified from cDNA and cloned into pGEM-4Z in the SP6 orientation. The sequences of cpTatC and Hcf106 from pea are available from GenBank/EMBL/DDBJ under accession numbers AF284759 (pea cpTatC) and AF284760 (pea Hcf106).

2.2. Antibody production

The predicted stromal domain (residues 109–261) of pea Hcf106 (psHcf106sd) was expressed in *E. coli* as an N-terminal histidine-

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tagged protein and was purified with Ni-chelating Sepharose as described [7]. The N-terminal soluble domain of pea cpTatC (residues 1–136) was expressed as an N-terminal GST fusion protein with a PreScission protease (Amersham Pharmacia) recognition site at the fusion junction. DNA encoding the fusion protein was synthesized by the SOE-PCR method [14] using pea cpTatC plasmid and pGEX-6 as templates for first round amplifications. The fusion protein was expressed in *E. coli*, purified with glutathione–Sepharose (Amersham Pharmacia), gel purified by SDS–PAGE, and the electro-eluted protein used for antibody production. To confirm that anti-cpTatC cross-reactivity is dependent on the cpTatC N-terminus rather than GST, we prepared a putative N-terminal domain peptide based on the predicted cleavage site of the pre-cpTatC transit peptide [15]. The sequence corresponding to amino acids 51 through 136 (called cpTatC-pep) was amplified by PCR and cloned into the *Bam*HI/*Eco*RI site of pGEX-2T. The GST–cpTatCpep fusion protein was expressed in *E. coli* and purified on glutathione–Sepharose, from which cpTatCpep was released with thrombin and used in antibody experiments as described in the figure legends. Antibodies were produced in rabbits by Cocalico Biological (Reamstown, PA, USA). IgGs were purified as described [7].

2.3. Chloroplast protein import and thylakoid protein transport assays

In vitro transcription plasmids for wheat iOE33, pea pLHCP, pea iOE23, and maize iOE17 have been described [7]. Capped transcripts were synthesized by SP6 polymerase and translated with a wheat germ extract in the presence of [^{3}H]leucine. Tritium-labeled cpTatC was produced by coupled transcription-translation using a wheat germ TNT system (Promega, Madison, WI, USA). Chloroplast protein import, recovery of intact chloroplasts, subfractionation into membranes and stroma, and treatment of the thylakoids with protease or 0.2 M sodium carbonate were as described [7]. Pretreatment of thylakoids with IgGs and subsequent protein transport or integration assays were conducted as previously described [7].

2.4. Miscellaneous

Protein was determined by BCA (Pierce) and chlorophyll according to Arnon [16]. Immunoblots were developed by the ECL procedure (Pierce). Endogenous proteins were quantified by SDS–PAGE and immunoblotting on nitrocellulose membranes of thylakoids in parallel with dilution series of cpTatCpep, Tha4sd or Hcf106sd standards. The protein content of standard solutions was determined by amino acid analysis conducted by the University of Florida Interdisciplinary Center for Biotechnology Research protein core facility. The density of scanned bands on X-ray film was determined using Alpha Imager software and protein quantities were estimated by comparison to standards in the linear response region of the film.

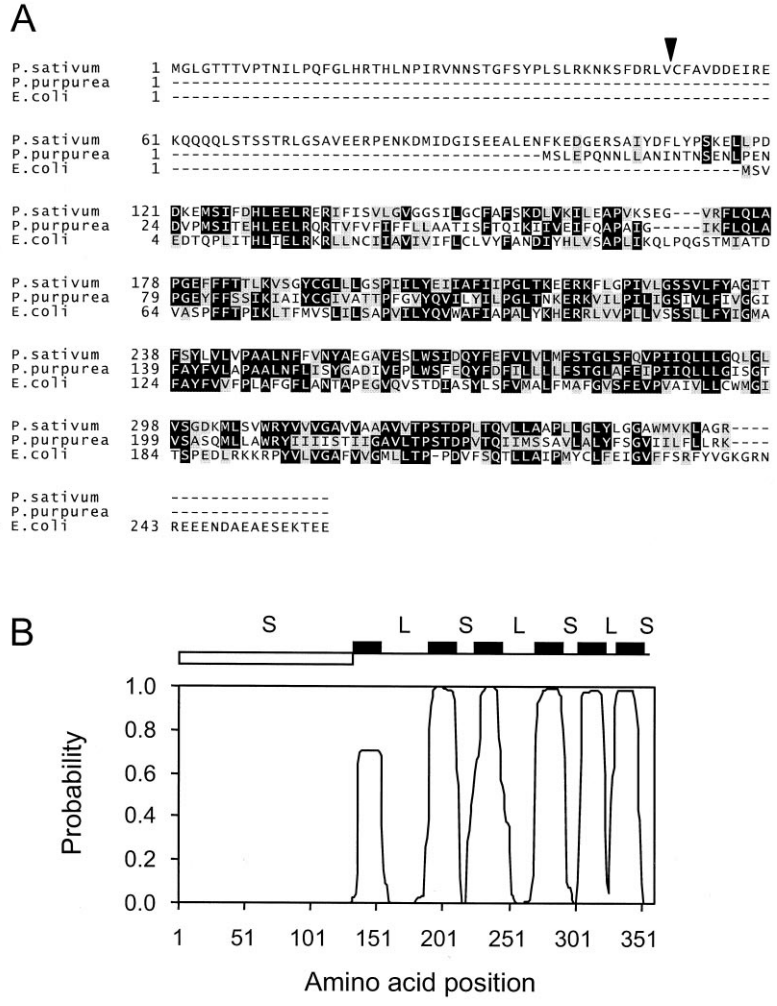

Fig. 1. Sequence alignment of TatC from plant, alga and bacteria and predicted topology of the pea chloroplast TatC. A: Amino acid sequences of TatC from *P. purpurea* (accession number P51264), *E. coli* (accession number P27857), and the TatC homolog from *Pisum sativum* were aligned by CLUSTAL W1.8 and shaded with BOXSHADE. The putative cleavage site of the pea chloroplast transit peptide is indicated by the arrowhead. B: Transmembrane domains of pea chloroplast TatC were predicted by TMHMM version 2.0 (www.cbs.dtu.dk/services/TMHMM-2.0/). The membrane topology is schematically outlined above the graph; closed box, transmembrane domain; S, stromal hydrophilic domain; L, lumenal hydrophilic domain; open box, N-terminal soluble domain of pea cpTatC used for antibody production.

3. Results and discussion

3.1. A pea TatC homolog is targeted to the chloroplast

A TatC homologous pea cDNA was isolated by a PCR-based strategy (Section 2). The pea cDNA encodes a 353-residue protein that is highly homologous to algal chloroplast and bacterial TatC throughout the six predicted transmembrane domains (Fig. 1). However, the encoded pea protein possesses an additional N-terminal sequence with characteristics of chloroplast transit peptides (Fig. 1A). Chloroplast targeting was examined with an in vitro protein import assay in which the pea cDNA was transcribed and translated in vitro and incubated with isolated pea chloroplasts (Fig. 2). Incubation of the ~37-kDa translation product (lane 1) with chloroplasts produced a smaller (~33-kDa) band (lane 2) that was resistant to protease treatment of the recovered chloroplasts (lane 3). This indicates that pea TatC was imported into the organelle and processed to mature size. Processed pea TatC was co-fractionated with thylakoids (lane 4, compare to lane 5) and was resistant to extraction by 0.2 M sodium carbonate (lane 8), indicating integral association with the bilayer. Treatment of thylakoids with protease partially degraded the imported protein, producing two protease-protected fragments with molecular masses of ~26 kDa and 23 kDa (lane 7). These results constitute one line of evidence that the pea TatC homolog is a chloroplast protein embedded in the thylakoid membrane.

3.2. Chloroplast TatC (cpTatC) is an integral thylakoid membrane protein with its N-terminus exposed to the stroma

cpTatC is predicted to expose both N- and C-termini on the stromal face of the thylakoid membrane (Fig. 1B). The predicted stromal processing site of cpTatC indicates that an ~85 N-terminal residue of the mature protein is exposed to the stromal surface of thylakoids. This is a much longer N-terminal soluble domain than those predicted for bacterial and algal TatC proteins (Fig. 1A). However, we verified the cleavage site by altering two residues in the predicted site. The resulting translation product was imported into chloroplasts, but was not processed to mature size (data not shown). We prepared an antibody to a peptide representing the predicted N-terminal soluble domain. Antibody to cpTatC reacted strongly with an ~33-kDa pea thylakoid protein (Fig. 3A, lane 1) in an antigen-reversible manner (lane 2) that was resistant to carbonate extraction (lane 6) and sensitive to protease treatment (lane 5). The smaller degradation products (see Fig. 2, lane 7) were not detected on the immunoblot. This confirms the predicted topology of cpTatC, i.e. that the N-terminus is exposed to the stromal face of the membrane.

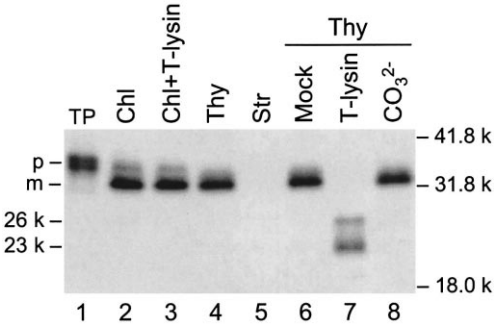

Fig. 2. Pea cpTatC import into isolated chloroplasts and localization to thylakoids. In vitro synthesized pea cpTatC was incubated with isolated chloroplasts for 15 min. Chloroplasts were repurified without (lane 2), or with (lane 3) thermolysin post-treatment. Repurified chloroplasts were fractionated into thylakoids (lane 4) and stroma (lane 5). Thylakoids, treated with import buffer (lane 6), thermolysin (lane 7), or 0.2 M Na_2CO_3 (lane 8), were recovered by centrifugation. Proteins were analyzed by SDS–PAGE and fluorography. Samples were dissolved in an SDS buffer, 4 M urea at 25°C for 60 min to avoid cpTatC aggregation. An aliquot of the translation product is shown in lane 1. The positions of precursor (p), mature (m) forms and degradation fragments (26 kDa and 23 kDa) of cpTatC are indicated at the left side of the figure.

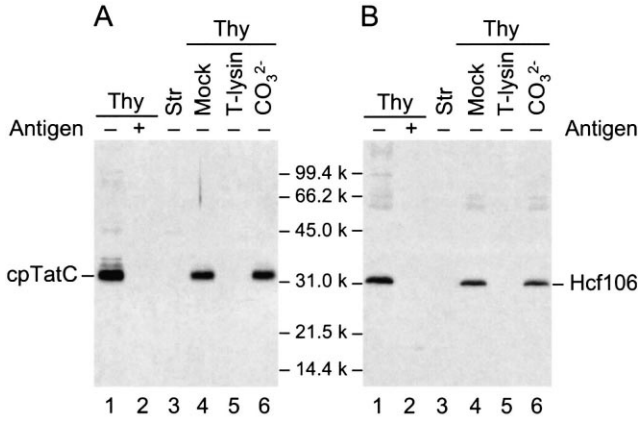

Fig. 3. Endogenous pea cpTatC is a integral thylakoid membrane protein. Thylakoids (lanes 1 and 2), stroma (lane 3), and thylakoids treated with import buffer (lane 4), thermolysin (lane 5) or 0.2 M Na_2CO_3 (lane 6) were fractionated by SDS–PAGE and analyzed by immunoblotting with antibodies against pea cpTatC (A) or pea Hcf106 (B). Antibody was preincubated with the corresponding antigen (lane 2) or buffer (lanes 1, 3–6) for 1 h at 4°C and then used for immunodecoration. All samples were equivalent to chloroplasts containing 2.5 μg chlorophyll.

For comparison, an immunoblot of pea Hcf106 is shown in Fig. 3B. The antibody was produced to the predicted amphipathic helix and carboxyl proximal acidic domain (Section 2). Pea Hcf106 migrated with an M_r of ~32 kDa, which is somewhat lower than the previously reported M_r of maize Hcf106 (~35 kDa). Both proteins migrate aberrantly on SDS–PAGE at about twice the M_r of their calculated size. Similar to maize Hcf106, the pea protein is a thylakoid integral membrane protein (lanes 1 and 6) with its C-terminus exposed to the stroma (lane 5).

The amounts of cpTatC, Hcf106, and Tha4 in thylakoids from 10-day-old pea plants were estimated by quantitative immunoblotting (Section 2) to be ~18 000 cpTatC molecules per chloroplast, ~95 000 Hcf106 molecules per chloroplast, and ~140 000 Tha4 molecules per chloroplast. The latter value is slightly higher than previously reported [7]. These values suggest that cpTatC is present at approximately the same molar concentration as the number of active translocation sites, estimated at 15 000 per chloroplast [17], and that Hcf106 and Tha4 are present at ~five and ~eight copies per translocation site, respectively.

3.3. Antibody to cpTatC inhibits ΔpH-dependent pathway transport

The potential role of cpTatC in protein transport was assessed by preincubating pea thylakoids with anti-cpTatC IgG

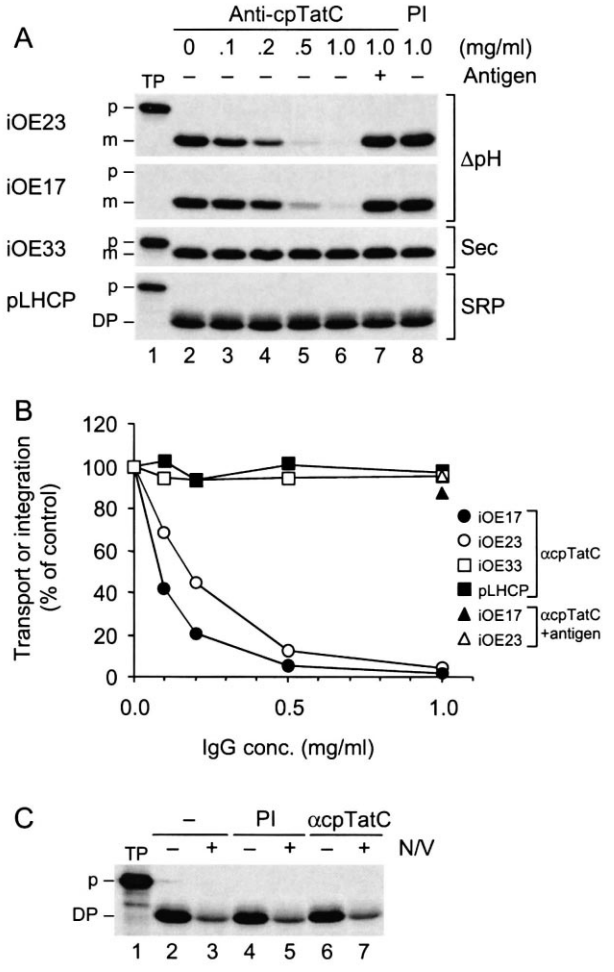

Fig. 4. Antibodies to pea cpTatC specifically inhibit ΔpH-dependent pathway protein transport. A: Pea thylakoids were preincubated with preimmune (PI) IgG or with increasing amounts of anti-pea cpTatC IgG in the absence or presence of 20 μM of the cpTatCpep antigen (see Section 2). After washing, the thylakoids were assayed for in vitro protein transport with precursors representing the three thylakoid protein transport pathways as designated to the right of the panels. Assay conditions and precursors used were as shown in the figure. Precursor (p), mature forms (m) and degradation products (DP) are indicated. B: Quantification of transport assay shown in panel A. Radiolabeled bands were extracted from the excised gel bands and quantified by scintillation counting. C: Pea thylakoids were pretreated with buffer (lanes 2 and 3), 1.0 mg/ml PI IgG (lanes 4 and 5), 1.0 mg/ml anti-pea cpTatC IgG (lane 6 and 7), washed and then assayed for pLHCP integration in the absence (lanes 2, 4 and 6) or presence (lanes 3, 5 and 7) of 0.5 μM nigericin and 1.0 μM valinomycin (N/V).

and assaying the resulting thylakoids for translocation of selected precursor proteins (Fig. 4A,B). Transport of ΔpH-dependent pathway precursors iOE17 and iOE23 was inhibited with increasing concentrations of anti-cpTatC IgG (Fig. 4A, lanes 1–6 and Fig. 4B). Anti-cpTatC IgG also inhibited transport of three other ΔpH-dependent pathway precursors: PSI-N, PSII-T (data not shown) and Pftf [18]. Transport inhibition was suppressed by including the cpTatC antigen during the IgG pretreatment step (lane 7). Anti-cpTatC had no effect on LHCP integration by the SRP pathway or iOE33 transport by the Sec pathway (lanes 2–6). Thus, anti-cpTatC specifically inhibited ΔpH-dependent pathway protein transport.

The ΔpH-dependent pathway absolutely requires the thylakoidal pH gradient [2,19]. To verify that antibody treatment did not impair the ΔpH, LHCP integration was assayed with IgG-pretreated thylakoids in the absence and presence of the ionophores nigericin and valinomycin (Fig. 4C). LHCP integration, which is stimulated by the pH gradient [2], was similarly inhibited by ionophores regardless of pretreatment with anti-cpTatC (lanes 6 and 7). This demonstrates that antibody to cpTatC inhibits the ΔpH-dependent pathway by a primary effect, rather than a secondary effect on the proton gradient. Antibody to pea Hcf106 similarly inhibited ΔpH pathway transport in an antigen-reversible manner and also was shown not to affect the pH gradient (data not shown).

In summary, our biochemical data demonstrate that a plant chloroplast TatC ortholog is required for protein transport of ΔpH-dependent pathway. Taken together with previous results [7], this demonstrates for the first time in any system, that Hcf106, Tha4 and cpTatC (or their bacterial orthologs) are all directly involved in protein transport. The relative amounts of the three components in the membrane compared to the estimated translocation sites favors a flexible channel model for the ΔpH-dependent system [9], where multiples of Tha4 and/or Hcf106 assemble around a core cpTatC/TatC to form the channel walls. Critical evaluation of this model awaits characterization of an active translocon.

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