In Vitro Reconstitution of Insertion and Processing of Cytochrome f in a Homologous Chloroplast Translation System*

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Using a homologous chloroplast translation system, we have reconstructed insertion and processing of the chloroplast-encoded thylakoid protein cytochrome f (pCytf). Cross-linking demonstrated that pCytf nascent chains when attached to the 70 S ribosome tightly interact with cpSecA, but this is strictly dependent on thylakoid membranes and a functional signal peptide. This indicates that cpSecA is only operative in pCytf biogenesis when it is bound to the membrane, most likely as part of the Sec translocon. No evidence for interaction between the 54-kDa subunit of the chloroplast signal recognition particle (cpSRP) and the pCytf nascent chain could be detected, suggesting that pCytf, in contrast to the polytopic D1 protein, does not require cpSRP for targeting. Insertion of pCytf occurred only co-translationally, resulting in processing and accumulation of both the processed signal peptide and the mature protein in the thylakoid. This co-translational membrane insertion and processing required a functional signal peptide and was inhibited by azide, demonstrating that cpSecA is essential for translocation of the soluble luminal domain. pCytf also associated post-translationally with thylakoids, but the soluble N-terminal domain could not be translocated into the lumen. This is the first study in which synthesis, targeting, and insertion of a chloroplast-encoded thylakoid membrane protein is reconstituted from exogenous transcripts and using the chloroplast translational machinery.

Chloroplasts in green algae and higher plants contain a heterogeneous thylakoid membrane system with four large protein complexes, comprising together at least 70 different proteins with many cofactors that are responsible for photosynthetic electron transport and ATP synthesis. About 50% of these proteins are encoded by the chloroplast genome, whereas the other half is encoded by the nuclear genome and is post-translationally imported into the chloroplast. To fulfill their function, the proteins have to be targeted to the thylakoid membrane and assembled into the respective multisubunit complexes.

The cytochrome b6f complex (Cytb6f) has 9 different subunits and several cofactors (1, 2), and its assembly has primarily been investigated through the use of mutant strains of the unicellular green algae Chlamydomonas reinhardtii. These experiments gave rise to the important conclusion that accumulation of the Cytb6f subunits is an assembly-mediated process (3). Pulse-labeling experiments show that pCytf, in the absence of its assembly partners Cytb6 or subunit IV, was synthesized at only 10% of its rate in the wild-type strain (4). An increased synthesis rate of pCytf was observed in strains lacking the C-terminal anchor, suggesting that the unassembled stromal C-terminal domain controlled the rate of pCytf translation by a negative feedback mechanism (5). The target for this autoregulation was postulated to be the 5’-untranslated leader (5’-UTL) of petA since assembly-dependent control of pCytf was lost when the petA promoter and the 5’-UTL were replaced by those of the atpA gene (6).

Cytf is a chloroplast-encoded protein bearing a classical N-terminal cleavable signal peptide (SP). The ATPase subunits CFoI and CFoIV also undergo N-terminal processing after insertion into the thylakoid membrane; however, those sequences do not resemble canonical luminal targeting sequences and the physiological relevance of this cleavage for CFo biogenesis remains unclear (7). After translocation of the pCytf N terminus, the SP is processed, and most of the mature protein is located on the luminal side of the thylakoid membrane. The afoform of the protein is converted to the holoform by covalent binding of the c-heme in the thylakoid lumen. Processing and heme ligation can occur independently (8).

As chloroplasts are of prokaryotic origin, the chloroplast transcriptional and translational machinery, as well as most proteins involved in protein targeting, insertion, and translocation show sequence and functional homologies to bacterial proteins. It has been shown previously that the leucine-rich signal peptide of pCytf can replace bacterial presequences conferring Sec-route specificity (9), i.e., the chloroplast SP is recognized in Escherichia coli by SecA. cpSecA is a 110-kDa ATP-hydrolyzing protein, which is located in the chloroplast stroma and at the thylakoid membrane (10). Genetic studies with the maize transposon mutant tha1, disrupted in the cpsecA gene, show that cpSecA is involved in pCytf membrane insertion, as well as in the post-translational translocation of the nuclear-encoded 33-kDa subunit of the oxygen-evolving complex and plastocyanin (11, 12). However, these in vivo studies do not address the mechanisms of interaction between precursor proteins and cpSecA.

Several polytopic chloroplast-encoded proteins, such as D1, D2, CP43, and CP47, are inserted co-translationally (7, 13, 14). However, based on transcript localization, the integral mem-

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1 The abbreviations used are: Cytb6f, cytochrome b6f complex; pCytf, precursor of cytochrome f; UTL, untranslated leader; SP, signal peptide; cpSRP, chloroplast signal recognition particle; RNCs, ribosome nascent chain complexes; BMH, bismaleimidothioxane; S-MBS, maleimidothiobenzoyl sulfosuccinimide ester; TM, transmembrane; PAGE, polyacrylamide gel electrophoresis; TPP, thylakoid-processing peptidase.
brane proteins CFpIII (with 2 putative TMs), subunit IV of the Cytb6f complex (with 3 putative TMs), and the two monotopic Cytb-559 subunits are likely to be synthesized on free ribosomes (15). In the case of pCytf, it has been shown that petA transcripts are localized at the thylakoid surface (15), suggesting co-translational targeting and insertion. Conflicting reports exist on the translation localization of several soluble stromal proteins. Translation of the large subunit of Rubisco has been reported to take place predominantly on free ribosomes (16, 17), whereas other groups (18, 19) observed synthesis on thylakoid membrane-bound ribosomes. This indicates that thylakoid-bound ribosomes do not necessarily translate integral membrane proteins and that free soluble ribosomes can possibly be engaged in the synthesis of bitopic and even polytopic thylakoid membrane proteins. It is thus important to devise an experimental system to address the relationship between targeting, assembly, and translation of the chloroplast-encoded membrane proteins.

In this study we have addressed the requirements of targeting and insertion of pCytf using a chloroplast initiation/translation system in which plasmid-derived transcripts can be faithfully translated (20–22). We show that an internally truncated form of pCytf is inserted in strict dependence of cpSecA, as well as a functional signal peptide. Importantly, the interaction between cpSecA and the signal peptide required the presence of thylakoid membranes, indicating that cpSecA functions at the membrane surface rather than as a soluble protein in the stroma. The results presented here show the first in vitro reconstitution of co-translational targeting and insertion of a chloroplast-encoded protein.

**EXPERIMENTAL PROCEDURES**

**Plant Growth, Isolation of Thylakoids, and Translation Experiments—** Pea plants (*Pisum sativum* L. var. de Grace) were grown for 6 to 8 days in a growth chamber with 12 h of artificial light per day. Intact thylakoids were isolated and further prepared for protein targeting essays as described in Ref. 21. The isolation of chloroplast translation experiments from pea chloroplasts was principally carried out as described in Ref. 20 with the modifications described in Ref. 21.

**DNA Constructs and mRNA Templates—** Plastid DNA was isolated according to Ref. 23. The petA gene was amplified with an 88 base pair 5′-UTL by polymerase chain reaction with the forward primer 5′-GGGGGAAGCTTGAATACGAGTAAATAGCACAGAATCATGGATA-GGG-3′ and the reverse primer 5′-GCCGCTCTAGACTAAAAATTCAT-GTGGGCTAGACTCTGC-3′ using pea plastid DNA as a template. After restriction with *Hin*/H11032, the product was ligated in pBluescript SKII (Stratagene) under the control of the *T7* promoter, resulting in pCytf. All modifications detailed below were carried out using the QuickChange site-directed mutagenesis kit (Stratagene). The point mutation pCytf/L215F/I268E was introduced in two rounds with the forward primer 5′-GCGGCTGTCCTGAGCTCATGATTCG-3′ and the reverse primer 5′-ATAGATCA-TGAGCAGCTAGAAATAGATC-3′ with pBSKpetA as a template, resulting in pCytf/L215F. This clone was used as a template for PCR with the forward primer 5′-CTCATGATCTAGAATATACCTGCGA-GCACC-3′ and the reverse primer 5′-GGGGGTGCTGGGATTATCATATCAT-3′ to obtain pCytf/L215F/I268E. pCytfΔf was obtained with the forward primer 5′-GGGGGGCCCATATGTTGTTTCTG-3′ and the reverse primer 5′-GGGGGGCCCATATTCTGTGTTTCTG-3′ with pBSKpetA as a template. This introduced an NdeI site encoding for histidine and methionine, between amino acids 90 and 286. Constructs pCytf/L215F/I268E and pCytfΔf/A35V were obtained using the forward primer 5′-CGGATTACCCATTATAATGATCAGCTAAC-3′ and the reverse primer 5′-ATGATCATGATCAGCTAAC-3′ with the forward primer 5′-CCTTATGATCAGCTAAC-3′ and the reverse primer 5′-GGGGGGCCCATATGTTGTTTCTG-3′, respectively, with pBSKpetA as a template. All constructs were verified by DNA sequencing. DNA templates and mRNAs were purified as described in Ref. 22.

**Co-translational Targeting and Insertion of pCytf**

The different pCytf constructs were translated from purified transcripts in the chloroplast translation system for 25–30 min at 28 °C. Targeting assays were performed in the light (50 μmol of photons m−2 s−1) for 25–30 min at 28 °C in the presence of nucleate-treated thylakoid membranes from pea at 200 μg of chlorophyll/ml, principally as described in Ref. 21. After targeting, thylakoids were collected by centrifugation (2 min at 10,000 × g) and resuspended in 500 mM KOAc in HMM buffer (21) unless stated differently. Trypsin treatment (10 μg/ml) was done for 20 min on ice with 200 μg of chlorophyll/ml in HMM buffer and stopped by the addition of trichloroacetic acid (10% final concentration). Ribosome nascent chain complexes (RNCs) were isolated on 500-μl sucrose cushions, and cross-linking using bismaleimidohexane (BMH) or m-maleimidobenzoyl-5'-2'-hydroxyxysulfosuccinimidyl ester (S-MBS) and immunoprecipitations of cross-linked products were carried out as described earlier (22).

During the course of this work we noticed that the amount of translation product and the accumulation of intermediate products showed some variation, depending on the 30 S preparation; this most likely reflects different levels of nuclease and protease activities as was clearly shown in Ref. 20.

**RESULTS**

**Expression of pCytf in Vitro and the Choice of 5′-UTL—** Northern blot and primer extension analysis showed that petA is co-transcribed with *trnQ-zfsa-psal-YPF10-petA*. This transcript is further processed leading to a monocistronic transcript for petA of 1.5 kbases with a 5′-UTL of ~90 bases (27, 28). To obtain expression of pCytf in the homologous chloroplast translation system, we used monocistronic transcripts of pCytf with 5′-UTLs of 88 bases since the 5′-UTL has clearly been shown to be involved in feedback regulation. This shorter transcript starts 30 base-pairs upstream of the ribosome binding site. A transcript with a longer 5′-UTL of 203 bases, which begins directly downstream of the *ycf10* stop codon, gave lower translation rates in the translation system (not shown). We thus used the physiologically correct 5′-UTL for all our experiments.

**pCytf Interacts with cpSecA—** To identify chloroplast components that interact with nascent chains of pCytf, stable RNCs were generated by translation of truncated pCytf transcripts of 96 and 134 amino acids without the stop codon (assigned pCytf/96 and pCytf/134, respectively; see Fig. 1). These stable RNCs were purified and incubated with the homobilfunctional cytochrome cross-linker BMH or the heterobifunctional cysteine cross-linker S-MBS. These cross-linkers were selected because the N terminus of pCytf contains 2 cysteines (at positions 56 and 59) and multiple lysine residues. For both translation products, a cross-link product of ~120 kDa appeared after incubation with S-MBS (Fig. 2, A and B). This band was only very weakly visible after cross-linking with BMH. The cross-linked product was precipitated with cpSecA-specific antiserum (Fig. 2, A and B). Immunoprecipitations with pSRP54-specific antiserum (Fig. 2B) did not reveal any cross-linked products.

**pCytf Can Be Translated in the Absence of Thylakoid Membranes—** But the Interaction of pCytf with cpSecA Is Thylakoid Membrane-dependent—The translation system used for pCytf was according to Hirose and Sugiu (20) contained thylakoids at a concentration of less than 1 ng of chlorophyll/μl, as indicated by Western blot analysis using polyclonal antisera against integral PSI core proteins and cpSecY (Fig. 3A). cpSecA is present in the chloroplast as a soluble protein, as well as a peripheral thylakoid membrane protein (10), but it is unknown if cpSecA...
can interact with its substrates as a soluble protein. We therefore investigated if the observed interaction of cpSecA with pCytf is membrane-dependent. Thylakoid membranes were removed from the translation extracts by additional centrifugation at different velocities prior to translation. At 20,000 rpm, thylakoid membranes accumulated as a green pellet, and the remaining supernatant was colorless. Western blot analysis of the pellet and an aliquot of the supernatant showed that the thylakoid membranes completely sedimented during this centrifugation (Fig. 3A). In contrast at least 75% of cpSecA and more than 95% of the 70 S ribosomes remained soluble in the supernatant after centrifugation (Fig. 3A). Importantly, the centrifugation at 20,000 rpm did not significantly affect the synthesis of pCytf/96 and other constructs, indicating that translation of pCytf in vitro does not require thylakoid membranes (Fig. 3B). Centrifugation of longer duration or with higher g values led to a much lower yield of translation products (Fig. 3B) due to proportional sedimentation of ribosomes as determined by Western blotting (Fig. 3A).

To show the membrane independence of pCytf/translation in a different way, translations were carried out in the presence of the detergent Triton X-100. Concentrations of Triton X-100 up to 0.5% had no influence on the translation efficiency of pCytf, as can be seen in Fig. 3B.

Translations with pCytf/96 were performed in the greenish and colorless stromal extract spun at 20,000 rpm. RNCs were then isolated and incubated with S-MBS, followed by immunoprecipitation with cpSecA antiserum. A cross-linked product of 120–125 kDa was observed when translations were carried out in the green membrane-containing extracts, and this product could be precipitated with cpSecA antiserum (Fig. 3C). On the other hand, only very low amounts (20–50-fold reduction) of cross-linked cpSecA were detected when translating in the thylakoid membrane free stromal extract. These results show that a stable interaction of pCytf and cpSecA requires the presence of thylakoid membranes.

The Interaction of pCytf with cpSecA Requires a Functional SP—pCytf has a classical N-terminal SP with a central hydrophobic core (Fig. 1). We therefore examined if the interaction of cpSecA with nascent chains of pCytf in the translation system was dependent on a functional SP (see Fig. 1). The hydrophobic core of the SP was disrupted by replacing isoleucine at position 21 and leucine at position 26 with two charged glutamic acid residues, leading to pCytf-L21E/I26E (see Fig. 1). Truncated 96-amino acid nascent chains of pCytf and pCytf-L21E/I26E were then synthesized and stable RNCs were isolated, incubated with S-MBS, and immunoprecipitated with cpSecA-specific antiserum. As shown in Fig. 4, the interaction between pCytf and cpSecA did absolutely require a functional SP, since no interaction was detected with pCytf-L21E/I26E.

Targeting, Insertion, and Processing of pCytf—To investigate the targeting and insertion of pCytf, we synthesized the wild-type precursor protein in the translation extract (Fig. 5A). However, multiple translation intermediates, premature termination, or degradation products also accumulated. Degradation is the most obvious explanation since the 30-kDa hydrophilic N-terminal domain is unlikely to be a tightly folded structure and will therefore be highly accessible to proteases. These shorter translation products make it difficult to diagnose cross-linking products, insertion, and processing. Therefore, a pCytf construct was engineered in which part of the internal soluble luminal domain (amino acids 91–285) was deleted. In this construct, named pCytfΔf, the SP, the heme-coordinating cysteines (Cys-56 and Cys-59), the TM (amino acids 286–305), and the C-terminal stromal extension (amino acids 306–320)
pellet (at 20,000, 30,000, or 40,000 rpm for 30 min in a TLA-100.3 rotor. The phosphorimager screen. Proteins were separated by gradient SDS-PAGE and exposed to a used for immunoprecipitations with cpSecA antiserum where indicated.

linking, proteins were solubilized with SDS and analyzed directly or extracted, obtained by a 30-min centrifugation at 20,000 rpm. Stable green extract and the colorless thylakoid membrane-free supernatants were used for translation of pCytf96. Lower panel, translation assays were kept for 30 min on ice in the presence of Triton X-100 at indicated concentrations, prior to the addition of mRNA. Translation products were separated on tricine gels and exposed to phosphorimager screens. C, pCytf96 was translated for 30 min at 28 °C in green or colorless extract, obtained by a 30-min centrifugation at 20,000 rpm. Stable RNCs were purified and incubated without or with S-MBS. After cross-linking, proteins were solubilized with SDS and analyzed directly or used for immunoprecipitations with cpSecA antiserum where indicated. Proteins were separated by gradient SDS-PAGE and exposed to a phosphorimager screen.

are still present, and the transcript ends with a termination stop codon (Fig. 1). Importantly, the C terminus, which has been implied in feedback regulation, is identical to wild-type Cytf. Translation of this shorter protein was successful and gave little accumulation of translation intermediates (Fig. 5A), making the interpretation of targeting and insertion assays more straightforward. Therefore, pCytf96 was used to investigate the targeting and insertion requirements.

cytf f was translated in the presence of nuclease-treated thylakoid membranes. Subsequently, the thylakoids were re-isolated, washed with different chaotropic agents, and the membranes were separated from soluble proteins by centrifugation. The soluble translation reaction (supernatant 1), the supernatant of the wash steps (supernatant 2), and the washed membrane pellet (pellet 2) were collected and analyzed by SDS-PAGE and autoradiography to monitor membrane association and integration (Fig. 5B). About 40% of the translation product was tightly membrane-associated or integrated into the membrane, as it could not be extracted by the chaotropic agents (Fig. 5B).

In the absence of thylakoid membranes in the translation reaction, less than 0.5% of translation product was sedimented in the presence of 0.1 M Na2CO3 (Fig. 5B).

Two radiolabeled products between 7 and 9 kDa and a 4.5-kDa product also accumulated in the thylakoid membrane pellets (Fig. 5B, pellet 2). Based on the molecular mass and further experimentation (see below and Fig. 5E), these membrane-integrated/translocated products were assigned as the processed translation product Cytf96 and the cleaved SP, as indicated (Fig. 5B). Incubation with chaotropic agents could remove neither the processed Cytf96 nor the cleaved SP. Only the treatment with 6 M urea extracted a small amount of the processed SP, most likely due to perforation of the thylakoid membrane (Fig. 5B, supernatant 2). The corresponding Coomassie-stained gel of the autoradiogram shows that the extraction procedure efficiently removed not only soluble stromal proteins (e.g., Rubisco), but also several peripheral thylakoid membrane proteins, such as CFIoβ (Fig. 5C).

A cross-section of the signal intensity from the autoradiogram in Fig. 5B shows a quantification of the membrane-associated thylakoid translation products, the processed Cytf96 and the cleaved SP (Fig. 5D). After correction for the difference in the number of methionines and cysteines in the three products (7, 5, and 2 methionines and cysteines in the pCytf96, the processed Cytf96, and SP, respectively), we calculated a molar ratio of 1.08:0.9 for the membrane-located Cytf96/Cytf96/SP. Thus processing was ~47% of the associated precursor translation product (0.9/1.98). The molar excess of SP over mature protein was expected, because proteolysis rates of unassembled proteins in the thylakoid membrane is much higher than unassembled proteins in the lumen (Ref. 29 and see “Discussion”).

The assignment of the SP and processed Cytf96 product was firmly established by point mutations in the cleavage site motif. This motif was changed from Ser-Asn-Ala to Ser-Asn-Val, leading to the construct pCytf96-A35V (Fig. 1). This amino acid substitution disrupts the semiconserved consensus sequence AXA that is recognized by the thylakoid-processing peptidase (TPP) (30). Therefore insertion and translocation of the mutant proteins should not lead to any processing, and the 4.5-kDa SP should not accumulate. As a control, the motif Ser-Asn-Ala was changed to Val-Asn-Ala in the construct pCytf96-S33V, which is a functional cleavage site, since the −1 position of the processing site remains an alanine. Both mutant and wild-type proteins were synthesized in the presence of thylakoids, and they all tightly associated with the membrane (Fig. 5E). However, SP and Cytf96 appeared only after translation of pCytf96 and...
pCyt\(\Delta F\)/S33V in the presence of thylakoids, whereas the proteins were not present with pCyt\(\Delta F\)/A35V (Fig. 5E). This completely confirms the assignment of SP and Cyt\(\Delta F\).

Since the TPP is located at the luminal side, processing can only occur after translocation of the SP through the membrane. The SP will accumulate on the luminal side of the membrane, and the mature protein is membrane-inserted. Thus, they should both be protected against exogenously added proteases. To test if the processed SP and mature proteins were indeed protected, the thylakoids were treated with trypsin, specifically cleaving the C-terminal of lysine and arginine residues (Fig. 5E). In the case of the wild-type construct and the pCyt\(\Delta F\)/S33V, the mature Cyt\(\Delta F\) as well as the cleaved SP were indeed protease-protected (Fig. 5E). We should note that the C-terminal hydrophilic domain of pCyt\(\Delta F\) has 3 lysines immediately C-terminal of the TM and that both the SP and the rest of the luminal domain have multiple lysines and arginines. pCyt\(\Delta F\) on the other hand was partially digested leading to a loss of intensity and accumulation of a 6.5-kDa degradation product (Fig. 5E, asterisk). The processing of pCyt\(\Delta F\) and pCyt\(\Delta F\)/S33V and the protease-insensitive accumulation of the Cyt\(\Delta F\) and processed SP in the thylakoids prove that the N terminus is correctly translocated across the thylakoid membrane. Complete cleavage of the short hydrophilic N terminus of the LHCP at the stromal side of the membrane by the trypsin treatment was visible on the corresponding Coomassie-stained gel and shows that the trypsin treatment was effective (Fig. 5E, lower panel).

Correct Insertion Requires a Functional SP and Is Inhibited
by Azide—In Fig. 4 we have shown that a functional SP is essential for the membrane-dependent interaction with cpSecA. These results, together with earlier observations in *E. coli* and *Chlamydomonas* (see the Introduction), strongly suggest that the SP and cpSecA are both required for membrane insertion and translocation. To obtain further support for these requirements, the hydrophobic core of the SP was disrupted by replacing two hydrophobic residues (Leu and Ile) with two charged residues (Glu) (see Fig. 1). This construct, assigned pCytΔf/L21E/I26E, was then translated in the presence of thylakoid membranes, and the membrane insertion was verified by re-isolation of the thylakoids and treatment with chaotropic agents (Fig. 6A). With the wild-type pCytΔf, accumulation of processed product and the cleaved SP were observed, as expected (Fig. 5A). In contrast, no processing of pCytΔf/L21E/I26E occurred, although a significant fraction of the translation product associated with the thylakoid membrane. These results indicate that pCytΔf/L21E/I26E can be targeted to thylakoid membranes, and that the N terminus cannot be processed by the luminal TPP. The most plausible explanation is that the N-terminal domain is not translocated into the lumen because a functional interaction with cpSecA is not possible, and without cpSecA assistance the N terminus cannot be translocated.

The strict SecA dependence for translocation of the N terminus is further demonstrated by a different approach, using azide. Azide is a well known inhibitor of bacterial and chloroplast SecA and acts through binding to the ATP binding domain (10, 31, 32). Addition of azide during translation of pCytΔf in the presence of thylakoid membranes completely prevented translocation of the N terminus, since neither the processed translation product nor the cleaved SP was detected in the isolated thylakoids (Fig. 6B).

*pCytΔf* Does Not Insert in Post-translational Assays—To investigate if pCytΔf could be post-translationally inserted in the chloroplast, translation was carried out in the absence of thylakoids (Fig. 7). After a 25-min translation, synthesis was stopped by the addition of the antibiotic lincomycin. Thylakoids were added to the translation mixture and incubated to allow protein targeting. As a control, translations were carried out in the presence of thylakoids. No processed translation product and no SP accumulated in the thylakoids in the post-translational assay, whereas both proteins were visible in the co-translational setup. The experiment clearly demonstrates that proper insertion into thylakoid membranes, *i.e.* a translocation of the N terminus followed by the cleavage of the SP in our *in vitro* reconstitution system is only possible when translation occurs in the presence of thylakoid membranes.

**FIG. 6.** Targeting of pCytΔf requires a functional SP and is sensitive to azide. A, translation of pCytΔf and pCytΔf/L21E/I26E was carried out in the presence of thylakoids. After translation, thylakoids were re-isolated, incubated for 10 min on ice in HMM buffer supplemented with 1 M NaCl, 0.1 M Na2CO3, or 6 M urea. The thylakoid membranes and supernatants were separated by centrifugation. B, pCytΔf was translated for 30 min in the presence of added thylakoid membranes and 0, 5, or 10 mM sodium azide (NaN3). Thylakoids were re-isolated, and incubated for 10 min on ice in HMM buffer containing 500 mM KOAc. A and B, samples were separated on tricine gels and exposed to phosphorimager screens. The full-length translation product, the processed mature protein, and the SP are indicated.

**FIG. 7.** pCytΔf is co-translationally inserted into the thylakoid membrane. pCytΔf was translated in the absence of thylakoids for 25 min at 28 °C, lincomycin (20 μM) was then added and translation continued for 5 min. Subsequently thylakoids were added and further incubated for 30 min at 28 °C (post-translational assay). Alternatively, pCytΔf was translated for 30 min at 28 °C in the presence of thylakoids (co-translational assay). After translation, thylakoid membranes were re-isolated, incubated for 10 min on ice with 500 mM KOAc in HMM buffer, and separated by centrifugation into thylakoids and wash buffer. Proteins were separated on tricine gels and exposed to phosphorimager screens. Incubation times are indicated.

**DISCUSSION**

**Why Reconstitute Targeting and Insertion of Chloroplast-encoded Membrane Proteins?**—The expression of chloroplast-encoded thylakoid membrane proteins is predominantly regulated at the translational level. Many issues remain to be resolved, such as the localization of translation, mechanisms of protein targeting, membrane insertion, and translocation and feedback regulation. Reconstitution in a homologous *in vitro* chloroplast translation system in which the process can be dissected into its different steps and components, and in which exogenous transcripts can be translated, are essential to unravel molecular mechanisms. The development of such a homologous chloroplast translation system (20–22) has formed the basis of the present reconstitution study.

In this study, we describe the reconstitution of targeting, insertion, and processing of pCytΔf, employing exogenous petA transcripts. We have chosen this protein for our experimental studies since (i) it has a classical cleavable signal, providing an excellent tool to evaluate targeting and membrane insertion and (ii) it has a long N-terminal domain, making in relatively easy to determine membrane insertion and topology by protease protection assays.

**Expression of pCyt in the Absence and Presence of Thylakoid Membranes**—Truncated versions and full-length pCyt could be expressed in the translation system both in the presence and absence of thylakoid membranes. Other membranes possibly present in the translation system, such as chloroplast envelope membranes or so-called low density membranes observed in *C. reinhardtii* (33), are not needed either since translations were unaffected by the addition of high concentration of detergent. This indicates that the soluble proteins within the chloroplast are sufficient for translational initiation and elongation, despite the observation that *in vitro* petA transcripts engaged in protein synthesis are located at the thylakoid membrane surface (15). Thus, translational regulators that are possibly tightly bound to (thylakoid) membranes are not required for translation *per se*, although they might be important to ensure that the translational product can effectively be assembled into...
the Cytb6f complex. In the case of COX2 and COX3, two mitochondrial-encoded membrane proteins in yeast, membrane-bound translational activators were not needed for translation but were needed to ensure assembly in their complex (34).

**Cytf Interacts with cpSecA in Strict Dependence of a Functional SP and Thylakoid Membranes**—To identify proteins that interact with nascent chains of pCytf emerging from the ribosomes, stable RNCs were generated, purified, and incubated with chemical cross-linkers. Predominantly one cross-linked somes, stable RNCs were generated, purified, and incubated in vivo the gene for cpSecA is disrupted (12). This is also in agreement thata1 the SP core region. Lowering the hydrophobicity of the pCyt translational SP and Thylakoid Membranes

![Co-translational Targeting and Insertion of pCytf](https://example.com/co-translational.png)

**Processing Is Strictly Dependent on a Functional SP and cpSecA**—To reconstitute the targeting and insertion of pCytf, we employed a pCytf construct, pCytfΔf, in which the 30-kDa luminal domain was shortened, but the SP, the TM, and the C terminus, as well as the 2 heme-coordinating cysteines all remained present. It is important to emphasize that both the transcript and the synthesized product contain the elements (the 5′-UTL and the C terminus) that have been shown to be important in translational regulation and feedback control in C. reinhardtii (6). This shortened construct was used since the full-length construct led to an accumulation of a larger number of translational intermediates or degradation products.

pCytfΔf could be targeted and inserted into the thylakoid membrane in the chloroplast translation system when supplemented with nuclease-treated thylakoid membranes. As was shown in Figs. 5 and 6, pCytfΔf insertion could be reconstituted in a highly specific manner as determined by the following observations. (i) The inserted protein was processed, and the mature protein and the processed SP accumulated in the thylakoids. The mature protein and the processed SP were protected against exogenous protease and different chaotropic agents could not extract them from the membrane. This illustrates that CytΔf was inserted with the correct topology and that the SP accumulated in the lumen. (ii) Processing could be blocked by a specific point mutation within the semiconserved cleavage site. Since the TPP is located at the luminal side of the thylakoid membrane, this further illustrates that the N terminus is translocated into the lumen. (iii) Lowering of the hydrophobicity of the SP prevented pCytfΔf insertion and processing. However pCytfΔf did associate with the membrane and was resistant to chaotropic agents, but the N terminus was not translocated across the membrane, and azide completely abolished processing, strongly indicating that the N-terminal translocation across the membrane is dependent on cpSecA, which is consistent with the cross-linking to cpSecA.

Processed CytfΔf was unlikely to be assembled into Cytb6f complexes in the insertion experiments. It has been well established that unassembled proteins or apoproteins in the stroma or in the thylakoid membranes are highly unstable (29, 42), whereas unassembled proteins accumulated in the lumen have a relatively long half-life (43). This explains the unequal stoichiometry between inserted processed CytfΔf and the luminal SP.

**Co- or Post-translational Insertion?**—In the experiments discussed in the previous sections, thylakoids were added at the start of translation. To determine whether insertion could be reconstituted in a post-translational assay, translation was carried out in the absence of membranes, after which thylakoids were added and further incubated with the translation products. The experiments showed that productive insertion was undetectable in the post-translational setup, since no processing took place. The negative results in the post-translational setup could be explained by an accumulation of Cytf in a translocation-incompetent form. However, we favor a co-translational insertion model since petA mRNA engaged in translation is located at the thylakoid surface (15) and also since Cytf translation is under feedback control of Cytf accumulated in the thylakoid membrane (in Chlamydomonas).

**Conclusions**—The data presented here show that synthesis, targeting, insertion, and processing of CytfΔf can be reconstituted *in vitro* using a homologous chloroplast translation system. Insertion of pCytf is a co-translational process and requires both a functional SP and cpSecA. Interactions between pCytf and CpSecA occur at the thylakoid membrane and not in the soluble stromal phase. pCytfΔf can be translated in the absence of thyla-
koid membranes, indicating that possible membrane translation activators are not required for translation.

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REFERENCES

1. Choquet, Y., and Vallon, O. (2000) Biochimie (Paris) 82, 615–634
2. Hamel, P., Olive, J., Pierre, Y., Wollman, F. A., and de Vitry, C. (2000) J. Biol. Chem. 275, 17072–17079
3. Wollman, F.-A. (1998) in The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas (Rochaix, J. D., Goldschmidt-Clermont, M., and Merchant, S., eds), pp. 459–476, Kluwer, Dordrecht
4. Kuras, R., and Wollman, F. A. (1994) EMBO J. 13, 1019–1027
5. Wollman, F.-A., and Joliot, P. (1995) Biochemistry 34, 7468–7475
6. Choquet, Y., Stern, D. B., Wostrikoff, K., Kuras, R., Girard-Bascou, J., and Wollman, F. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4380–4385
7. Wollman, F. A., Minai, L., and Nechushtai, R. (1999) Biochim Biophys Acta 1411, 21–85
8. Howe, G., and Merchant, S. (1994) J. Biol. Chem. 269, 5824–5832
9. Rothstein, S. J., Gatenby, A. A., Willey, D. L., and Gray, J. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7955–7959
10. Yuan, J., Henry, R., McCaffery, M., and Cline, K. (1994) Science 266, 796–798
11. Voelker, R., and Barkan, A. (1995) EMBO J. 14, 3905–3914
12. Voelker, R., Mendel-Hurtvig, J., and Barkan, A. (1997) Genetics 145, 467–478
13. Kim, J., Eichacker, L. A., Rudiger, W., and Mullet, J. E. (1994) Plant Physiol. 104, 907–916
14. van Wijk, K. J., Andersson, B., and Aro, E. M. (1996) J. Biol. Chem. 271, 9627–9636
15. Friemann, A., and Hachtel, W. (1988) Planta 175, 50–59
16. Minami, E., and Watanabe, A. (1984) Arch. Biochem. Biophys. 235, 562–570
17. Ellis, R. J. (1997) Biochim. Biophys. Acts 463, 183–215
18. Muhlbauer, S. K., and Eichacker, L. A. (1999) Eur. J. Biochem. 261, 784–788
19. Hattori, T., and Margulies, M. M. (1986) Arch Biochem. Biophys. 244, 630–640
20. Hirose, T., and Sugiura, M. (1990) EMBO J. 9, 1687–1695
21. Houwen, E., de Gier, J. W., and van Wijk, K. J. (1999) Plant Cell 11, 1553–1564
22. Nilsson, R., Brunner, J., Hoffman, N. E., and van Wijk, K. J. (1999) EMBO J. 18, 733–742
23. Lui, J.-W., and Rose, R. J. (1993) Plant Mol. Biol. Rep. 11, 48–53
24. Laemmll, U. K. (1970) Nature 227, 680–685
25. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
26. Forro, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) Biochim. Biophys. Acts 975, 384–394
27. Willey, D. L., and Gray, J. C. (1990) Plant Mol. Biol. 15, 347–356
28. Nagano, Y., Matsuno, R., and Sasaki, Y. (1991) Curr. Genet. 20, 431–436
29. Adam, Z. (2000) Biochimie (Paris) 82, 647–654
30. Bassham, D. C., Bartling, D., Mould, R. M., Dunbar, B., Weisbeek, P., Herrmann, R. G., and Robinson, C. (1991) J. Biol. Chem. 266, 23690–23610
31. Oliver, D. B., Cabelli, R. J., Dolan, K. M., and Jarosik, G. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8227–8231
32. Knott, T. G., and Robinson, C. (1994) J. Biol. Chem. 269, 7843–7846
33. Zerges, W., and Rochaix, J. D. (1998) J. Cell Biol. 140, 101–110
34. Sanchirico, M. E., Fox, T. D., and Mason, T. L. (1998) EMBO J. 17, 5796–5804
35. Nohara, T., Asai, T., Nakai, M., Sugiiura, M., and Endo, T. (1996) Biochem. Biophys. Res. Commun. 224, 474–478
36. Mould, R. M., Knight, J. S., Bogsch, E., and Gray, J. C. (1997) Plant J. 11, 1051–1058
37. Smith, T. A., and Kohorn, B. D. (1994) J. Cell Biol. 126, 365–374
38. High, S., Henry, R., Mould, H. M., Valenta, Q., Meacock, S., Cline, K., Gray, J. C., and Luirink, J. (1997) J. Biol. Chem. 272, 11622–11628
39. Haward, S. R., Napier, J. A., and Gray, J. C. (1997) Eur. J. Biochem. 248, 724–730
40. Keegstra, K., and Cline, K. (1999) Plant Cell 11, 557–570
41. Valenta, Q. A., Scotti, P. A., High, S., de Gier, J. W., von Heijne, G., Lentzen, G., Wintemeyer, W., Oudega, B., and Luirink, J. (1998) EMBO J. 17, 2504–2512
42. Halperin, T., and Adam, Z. (1998) Plant Mol. Biol. 30, 925–933
43. Hashimoto, A., Yamamoto, Y., and Thog, S. M. (1996) FEBS Lett. 391, 29–34