Alterations in the *Chlamydomonas* Plastocyanin Transit Peptide Have Distinct Effects on *in Vitro* Import and *in Vivo* Protein Accumulation*

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Nucleus-encoded chloroplast proteins that reside in the thylakoid lumen are synthesized as precursors with bipartite transit peptides that contain information for uptake and intra-chloroplast localization. We have begun to apply the superb molecular and genetic attributes of *Chlamydomonas* to study chloroplast protein import by creating a series of deletions in the transit peptide of plastocyanin and determining their effects on translocation into isolated *Chlamydomonas* chloroplasts. Most N-terminal mutations dramatically inhibited *in vitro* import, whereas replacement with a transit peptide from the γ-subunit of chloroplast ATPase restored uptake. Thus, the N-terminal region has stroma-targeting function. Deletions within the C-terminal portion of the transit peptide resulted in the appearance of import intermediates, suggesting that this region is required for lumen translocation and processing. Thus, despite its short length and predicted structural differences, the *Chlamydomonas* plastocyanin transit peptide has functional domains similar to those of vascular plants. Similar mutations have been analyzed *in vivo* by transforming altered genes into a mutant defective at the plastocyanin locus (K. L. Kindle, manuscript in preparation). Most mutations affected *in vitro* import more severely than plastocyanin accumulation *in vivo*. One exception was a deletion that removed residues 2–8, which nearly eliminated *in vivo* accumulation but had a modest effect *in vitro*. We suggest that this mutant precursor may not compete successfully with other proteins for the translocation pathway *in vivo*. Apparently, *in vivo* and *in vitro* analyses reveal different aspects of chloroplast protein biogenesis.

The majority of chloroplast proteins are encoded in the nucleus, synthesized in the cytosol as precursors, and directed to the organelle by a stroma-targeting domain within an N-terminal extension known as the transit peptide (TP).† Studies using vascular plant chloroplasts have shown that transport across the plastid envelope requires binding to specific receptors and translocation through a general import channel. A subset of imported proteins is integrated into or transported across the chloroplast thylakoid membrane, by one of at least four distinct mechanisms. For recent reviews on chloroplast protein import see Refs. 1–4.

The mechanism of routing proteins to the chloroplast envelope membranes and translocating them into the stroma appears to be unique, since aside from Hsp70s, none of the currently identified components is related to proteins associated with other membrane translocation pathways (1). Chloroplast protein import is initiated by specific contact between the stroma-targeting domain of the precursor and components of the import receptor, an interaction that requires GTP and ATP (5–9). This is followed by translocation across the envelope into the chloroplast stroma in a process that requires higher levels of ATP and may be assisted by chaperones (10–13). That the stroma-targeting domain is necessary and sufficient for sorting to the envelope and translocation into the stroma has been demonstrated both *in vivo* and *in vitro* (for numerous examples, see Ref. 14). Competition studies in which synthetic peptides or precursors were added to *in vitro* chloroplast protein import assays have demonstrated that a single pathway is used to translocate most precursors across the envelope (15–18). Although there is no primary amino acid sequence consensus in the stroma-targeting domains of transit peptides, they share certain features that are apparently important for efficient plastid targeting. Generally they are enriched in hydroxylated amino acid residues and deficient in acidic residues. They have an N-terminal region devoid of Gly, Pro, and charged residues; a middle region rich in Ser, Thr, Lys, and Arg; and a C-terminal region predicted to form a β-strand, which is terminated by the stromal processing consensus site (I/V/X 1/A/C)↓Δ, where ↓ indicates the cleavage site (19). Removal of the stroma-targeting domain by the stromal processing protease generates mature stromal proteins or intermediates that are subsequently targeted to other chloroplast locations (20, 21).

Lumen resident proteins contain bipartite transit peptides, which consist of an N-terminal stroma-targeting domain fused to a lumen-targeting domain. The lumen-targeting domain is required for translocation across the thylakoid membrane (for reviews see Refs. 1, 4, and 14) and contains a hydrophobic region similar to the signal sequences required for bacterial protein secretion (22). Transport into the lumen can be mediated by at least two mechanisms that are utilized by specific subsets of lumen resident proteins (18, 23). Two different pathways have also been described for integration of proteins into the thylakoid membrane (24–27). The function and evolution of these parallel targeting pathways into and across the thylakoid membrane remain to be elucidated.

Most chloroplast protein import studies have relied on *in vitro* assays in which isolated chloroplasts are incubated with proteins that have been synthesized *in vitro*. However, it is unlikely that the complexity of the *in vivo* milieu is fully repro-

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*The abbreviations used are: TP, transit peptide; PAGE, polyacrylamide gel electrophoresis; SSU, small subunit of ribulose bisphosphate carboxylase; PC, plastocyanin.*

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duced in vitro. In fact, very different results were obtained when two constructs in which the small subunit of ribulose-bisphosphate carboxylase (SSU) TP coding region was fused to that of neomycin phosphotransferase (NptII) were tested in vivo and in vitro (28, 29). Disparate results were also obtained when the plastocyanin TP was fused to β-lactamase (30). The interpretation of these results is complicated because they involved chimeric proteins, and different species were used for the in vitro and in vivo analyses.

Spinach or pea chloroplasts have generally been used for in vitro experiments because of their ease of handling and physiological longevity after isolation (31). Unfortunately, neither pea nor spinach is especially tractable for in vivo studies. To examine the role of the transit peptide in chloroplast protein import both in vitro and in vivo, using a native chloroplast protein in a homologous system, we have chosen to study plastocyanin (PC) import in the unicellular eukaryotic alga, Chlamydomonas reinhardtii.

The isolation of biochemically active chloroplasts from Chlamydomonas is not as well-documented as in pea or spinach. However, energy-requiring uptake of the SSU into these chloroplasts has been reported (32, 33). Chlamydomonas transit peptides are shorter than those in vascular plants, and the N-terminal region of the stroma-targeting domain, defined for vascular plant precursors as deficient in Gly, Pro, and charged residues, is either extremely short or missing. Furthermore, Chlamydomonas stroma-targeting domains have a central region with predicted α-helical secondary structure, a characteristic of mitochondrial presequences (34). Indeed, the Chlamydomonas SSU transit peptide had mitochondrial targeting function when expressed in yeast (35). These observations raise the question of whether uptake and processing of proteins by Chlamydomonas chloroplasts are analogous to these processes in vascular plants. Current data on this point are contradictory. For example, the specificity of the Chlamydomonas stromal processing peptidase has been reported to be the same as (36) and different from (37) the pea enzyme. Furthermore, although Chlamydomonas pre-SSU and pre-CF1-gamma were imported into spinach and pea chloroplasts, both incorrectly and correctly processed products were observed (37–39). On the other hand, a synthetic TP from CF1-gamma inhibited the uptake of pea pre-SSU into pea chloroplasts (40), suggesting that there must be functional similarities between the transit peptides.

To determine whether similar domains exist in Chlamydomonas transit peptides and those of vascular plants, we have examined in vitro import of plastocyanin (PC) precursors with a variety of deletions in the transit peptide. PC resides in the thylakoid lumen. The Chlamydomonas plastocyanin TP sequence has regions similar to those identified in the bipartite TPs of lumen resident proteins in vascular plants, although positively charged residues are not excluded from the N terminus of the stroma-targeting domain (41). The results presented below demonstrate that Chlamydomonas chloroplasts import PC in a transit peptide- and energy-requiring process. Furthermore, the Chlamydomonas PC transit peptide appears to contain functional domains similar to those of vascular plants.

We have compared the results of this in vitro analysis to those obtained in vivo by transforming genes with identical deletions in the coding region of the plastocyanin TP into a mutant defective at the PC structural gene locus. Although there are similarities in the results obtained with the two approaches, there are also several intriguing differences, which suggest that these complementary analyses may reveal different aspects of the chloroplast protein import pathway.

EXPERIMENTAL PROCEDURES

Subcloning of Plastocyanin cDNAs, in Vitro Transcription of RNA, and in Vitro Translation—A full-length PC cDNA clone (PC6-2) was obtained from Sabeena Merchant (UCLA; see Ref. 41). The downstream EcoRI site was removed by partially digesting the DNA with EcoRI, creating blunt ends with the Klenow fragment of DNA polymerase I, and religating the plasmid. EcoRI and XbaI digestion released the cDNA insert, which was subcloned into pGEM7Zf+ (Promega, Madison, WI) digested with the same enzymes. A series of deletion mutations were constructed in the genomic petE region as described in detail elsewhere. Briefly, an EcoRI site was introduced 60 base pairs upstream of the initiation codon, and Nhel sites were individually introduced at several locations encoding Ala-Ser in the transit peptide. A series of deletions within the transit peptide was constructed by combining EcoRI-Nhel fragments from appropriate pairs of plasmids. In addition, a deletion mutation removing residues 2–8 was made by site-directed mutagenesis. These deletions were subcloned into the cDNA context by the following procedure. A BbsI site in the first exon of the genomic clone is located 314 nucleotides downstream of the introduced EcoRI site. The EcoRI-BbsI fragments encompassing the transit peptide deletions were subcloned into the PC cDNA, replacing the EcoRI-BbsI fragment in the cDNA; this resulted in clones that are 31 base pairs longer than PC6-2 at the 5’ end, as shown in Fig. 1. Several chimeric genomic petE genes have been constructed previously by making translational fusions between the promoter, 5’-untranslated region, and 26- or 29 amino acid residues of the transit peptide-coding region, and 26- or 29 amino acid residues of the transit peptide-coding region of atpC to two different locations in the PC transit peptide. atpC encodes CF1-gamma, the γ-subunit of chloroplast ATPase. The chimeric transit peptide-coding regions were cloned into the petE cDNA context as EcoRI-BbsI fragments, as described above. Plasmid DNAs were isolated (42), linearized with XbaI, and transcribed with SP6 polymerase as described (43) except that the RNAs were resuspended in half the reported volume. These RNAs were translated with a wheat germ system as described previously (43) in the presence of [35S]methionine (Amersham Corp. SJ325).

Chlamydomonas Culture Conditions and Preparation of Chloroplasts—C. reinhardtii CC-406 (cw15mt) was obtained from the Chlamydomonas Genetics Center (Duke University). Chloroplasts were isolated using a modified method previously reported to yield a high fraction (90%) of intact chloroplasts (33, 44). Cells grown to different concentrations (1–5 × 10^6 cells/ml) under phototrophic or mixotrophic conditions yielded chloroplasts with similar import efficiencies. Therefore, cells were grown in liquid HSA medium (45) under 16 h room light:8-h dark cycles to a concentration of 3–4 × 10^6 cells/ml, collected

2 K. L. Kindle, manuscript in preparation.

3 K. L. Kindle and S. D. Lawrence, submitted for publication.
In Vitro Import of Plastocyanin in Chlamydomonas Chloroplasts

RESULTS

Characterization of Chlamydomonas in Vitro Chloroplast Protein Import—Since relatively few in vitro chloroplast protein import studies have been reported with Chlamydomonas chloroplasts, we first sought to establish that the system had characteristics expected of a bona fide in vitro import assay, i.e. that uptake of proteins by isolated chloroplasts is energy-dependent, requiring either white light or ATP, and that after uptake into the chloroplast the precursors are protected from added protease by the plastid envelope. We first characterized the uptake of the wild-type PC precursor into isolated Chlamydomonas chloroplasts in these respects.

Fig. 2A shows that white light or additional ATP was required for uptake and processing of plastocyanin (compare lanes 4–6). In the presence of 10 mM ATP and white light, Chlamydomonas chloroplasts converted about 14% of the input wild-type PC precursor to the mature size in a 15-min assay, and this polypeptide was resistant to thermolysin. Import was linear with time for 15–20 min (Fig. 2B). Approximately twice as much mature protein was produced when 10 mM ATP was added to an import reaction performed in white light, compared with white light alone (compare Fig. 2A, lanes 6 and 7). When import assays were conducted in foil-wrapped tubes without added ATP and plastids were treated with thermolysin following the import period, neither mature PC nor PC precursor was detected (Fig. 2B). This demonstrates that PC precursor was susceptible to thermolysin degradation and that there was insufficient ATP in the extract to support detectable import. However, protease-resistant precursor was detected in the light in the absence of added ATP, as shown in Fig. 2A, lane 6, and Fig. 2C, where it accounted for one-third of the total protease-protected plastocyanin species. When ATP was added to the assay, the amount of mature PC increased, and the amount of protease-protected PC precursor decreased (Fig. 2C).

In white light alone, the total amount of protease-protected PC species was about 75% as high as the maximal level, when additional ATP was included. Although the total amount of ATP above 1 mM did not further increase the total amount of protease-protected PC, the fraction that was mature increased slightly above 2.5 mM. This suggests that a first step in plastocyanin import, conversion to a protease-protected form, requires less ATP than subsequent steps for translocation into the thylakoid lumen and processing to the mature size.

by centrifugation at 2800 × g for 5 min, and washed in 50 ml of 20 mM Hepes/Na salt, pH 7.5 (Sigma). After centrifugation for 4 min at 2000 × g, cells were resuspended at a concentration of 2 × 10^6 cells/ml in breaking buffer (0.3 M sorbitol, 50 mM Hepes/Na salt, pH 7.5, 2 mM EDTA, 1 mM MgCl₂) plus 1% bovine serum albumin (fraction V from Sigma) and incubated on ice for 15 min. The algae were loaded into an ice-cold Kontes press, equilibrated for 3 min to 35 p.s.i. N₂, and lysed by rapid depressurization. Lysed cells (3 ml) were loaded onto Percoll step gradients made of 4 ml each of 45 and 70% Percoll in a 15-ml COREX tube (44). After 15 min centrifugation at 2000 × g in a swinging bucket rotor, chloroplasts were collected from the 45/70% Percoll interface, washed in 3 volumes of breaking buffer, and harvested by centrifugation for 3 min at 1400 × g. Generally, less dense cultures required higher breaking pressure to achieve maximal yields of chloroplasts. These preparations are enriched in chloroplasts, but not pure, since immunoblot analysis indicated substantial levels of the mitochondrial ATPase subunit F₁β and low molecular weight G proteins, which are localized to Golgi and flagellar membranes (Ref. 46; data not shown). Final yields averaged about 24%, so that a 2-liter culture produced plastids equaling about 3 mg of chlorophyll. Chloroplasts equivalent to 37.5 μg of chlorophyll were used in each assay, as described below; this allowed an average of 40 assays/liter of cultured cells.

In Vitro Import Assays—Chloroplasts were resuspended in import buffer (0.33 M sorbitol, 50 mM Hepes/KOH, pH 8.0) at 375 μg of chlorophyll/ml. Translation products were diluted 1:3 in import buffer containing 30 mM methionine. Equal amounts of [35S]methionine-labeled precursors were used for each import assay, as determined by quantifying bands from SDS-PAGE by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). Import assays were conducted at 24 °C in a volume of 150 μl, which included 25 μl of diluted translation products and chloroplasts equivalent to 37.5 μg of chlorophyll, with or without 10 mM Mg-ATP for 15 min, illuminated by white light unless otherwise indicated (43). Assays were also performed in the dark in foil-wrapped tubes without added ATP. After incubation, intact chloroplasts were resolated by centrifugation through a 35% Percoll cushion following treatment with 95 μg/ml thermolysin for 40 min at 4 °C (43), as indicated. Plastids were washed in import buffer, pelleted by centrifugation, and stored at -20 °C. Samples were resuspended in 18 μl of 10 mM EDTA, an equal volume of 2 × SDS-PAGE buffer was added, and they were then heated for 10 min at 67 °C. Aliquots of 7.5 μl were loaded into polyacrylamide gels (43), which were electrophoresed, dried, and exposed to PhosphorImager screens. In calculating import efficiency, the methionine content of the precursor (2 residues) and the fully processed mature protein (1 residue) was taken into account.

In Vitro Import of Plastocyanin in Chlamydomonas Chloroplasts requires white light or additional ATP. A, transcripts were synthesized in vitro and translated in a wheat germ translation system in the presence of [35S]methionine. Translation products were incubated with isolated Chlamydomonas chloroplasts, 10 mM ATP, and/or white light for 15 min, followed by treatment with or without thermolysin. Chloroplasts were resolated through Percoll cushions, and radioactive plastocyanin species were analyzed by SDS-PAGE in a 12.5% gel. T, 5% of input translation products; p, plastocyanin precursor; m, mature-sized plastocyanin. B, precursor was synthesized from the gPC cDNA and incubated with isolated chloroplasts in the presence of white light and 10 mM ATP for the indicated times. Import products were analyzed as in A. C, translation products were incubated for 15 min with Chlamydomonas chloroplasts in white light with varying amounts of added ATP, treated with thermolysin, and fractionated by SDS-PAGE in a 12.5% gel. The amount of 35S-labeled protein was quantified using a PhosphorImager as described under "Experimental Procedures."
Fig. 3. Deletions in the plastocyanin transit peptide result in lower amounts of processed plastocyanin in vitro. A, transcripts were synthesized in vitro and translated with a wheat germ extract in the presence of [35S]methionine. T, 1% of the input translation product; I, proteins produced after 15 min import into isolated Chlamydomonas chloroplasts in the presence of 10 mM ATP, followed by treatment with thermolysin and fractionation by SDS-PAGE in a 12.5% gel. The asterisk indicates proteins between the size of the precursor and mature plastocyanin that were found upon import. B, peptide sequence results are shown. The approximate location of the predicted stromal processing site is indicated by a star above the sequence (see text). and the hydrophobic region of the lumen targeting domain is also shown. Proteins imported in vitro were quantified as described under “Experimental Procedures.” A comparison between in vitro and in vivo results is shown. +++, ++, +, +, −, −, ±, detectable but <5%; 5–15%, 5–15%, and ±, detectable but <5%; −, not detectable. The asterisk denotes the presence of an intermediate-sized species upon import.

Effect of Transit Peptide Deletions on In Vitro Import—A series of deletions have been introduced into the petE gene, which resulted in in-frame deletions of 5–28 amino acid residues from the plastocyanin transit peptide. These deletions were subcloned into a cDNA context, so that they could be used to synthesize radioactively labeled precursors for in vitro chloroplast protein import assays. In vitro translation products from these clones yielded a small amount of an additional protein that migrated more slowly than SDS-PAGE. (Compare the translation products from the clone with the genomic 5′-untranslated region (gPC in Figs. 2 and 3A) to those from the cDNA clone (cPC in Fig. 4A).) Nonetheless, the precursor produced from wild-type gPC imported into isolated Chlamydomonas chloroplasts with an efficiency equivalent to the protein produced from the original cDNA (compare Figs. 2B and 4A).

Fig. 3 shows that most transit peptide deletions resulted in a dramatic reduction in the amount of PC produced compared to the wild type (cPC). The Δ13–19 precursor retained residual import activity, as demonstrated by the appearance of a small amount of mature PC. When the Δ30–37 precursor was incubated with Chlamydomonas chloroplasts, two to three smaller bands resulted, as shown in Fig. 4A, as well as protease-resistant precursor. The appearance of protease-resistant precursor suggests that import into the stroma may be impaired, and the accumulation of intermediates suggests that stromal processing may be aberrant and/or that lumen translocation may be inhibited. The lowest band may represent a small amount of mature PC, although it migrates very close to a band of unknown origin that sticks to the outside of chloroplasts in vitro and is present among translation products from this particular cDNA.

An import time course of the wild-type, Δ2–8, and Δ10–12 precursors is shown in Fig. 4. Although the overall uptake and processing of the Δ10–12 and wild-type precursors appear similar, the final amount of mature-sized protein was reduced by about half for the Δ10–12 precursor. The inhibition caused by this 3 amino acid deletion varied from one experiment to the next; mature PC produced from the Δ10–12 precursor ranged from 33 to 70% of the wild-type level. From the Δ2–8 cDNA construct, a low molecular weight translation product close to the size of mature PC was synthesized in vitro. However, this translation product did not seem to bind to the outside of the plastid or to be taken into the chloroplasts (Fig. 4). The time course of import of the Δ2–8 precursor did not change significantly for different mature PC produced after 15 min of import of the wild-type precursor was defined as 100%.

Substitution of the CFγ Transit Peptide for the Putative Stroma-targeting Domain of the Plastocyanin Transit Peptide—We next asked whether a portion of the transit peptide from W protein normally residing in the Chlamydomonas stroma could replace the N-terminal portion of the plastocyanin TP. If Chlamydomonas TPs have domains similar to those of vascular plants, it was expected that the N terminus of CFγ could replace the N terminus of the PC transit peptide and effect its import into isolated chloroplasts. The stromal processing protease site in Silene plastocyanin has been localized between Lys-41 and Ala-42 (20). A putative site for processing of Chlamydomonas PC by the stromal peptidase may occur
the plastocyanin TP at amino acid residue 20. As shown in Fig. 5, indicated by the name of the construct, so that in terminal to the presumed stromal processing site, at amino acid residues from CF1 plastocyanin, the addition of three amino acids from CF1 targeting domain whose function can be replaced by the N terminus of the PC TP is a stroma-targeting domain. This suggests that the N terminus of the PC TP is a stroma-targeting domain. We have also tested the import efficiency of PC into Chlamydomonas chloroplasts. Improvements in the chloroplast protein import assay reported that 5–15% of the SSU precursor was imported. We have also tested the import efficiency of the CF1-γ precursor and found that about 10% was taken up and processed.

**DISCUSSION**

We have demonstrated energy- and transit peptide-dependent import of a lumen resident protein into Chlamydomonas chloroplasts. The import efficiency of PC into Chlamydomonas chloroplasts averaged 14% of the input precursor. This is inefficient in comparison to import into pea chloroplasts, where, for example, Bauerle et al. (47) found that 34–50% of different PC precursors was imported. We have also tested the import efficiency of the CF1-γ precursor and found that about 10% was taken up and processed. Goldschmidt-Clermont et al. (33) reported that 5–15% of the SSU precursor was imported in vitro by Chlamydomonas chloroplasts. Improvements in the Chlamydomonas in vitro chloroplast protein import assay might increase our ability to detect low level import of mutant precursors.

Import of proteins into isolated pea chloroplasts has been divided into several steps that have different energy requirements. Initial binding of pre-SSU to the chloroplast requires low concentrations of GTP and ATP (50–100 μM), whereas translocation across the envelope is maximal with >1 mM ATP (10, 48, 49). Recently, Scott and Theg (50) have described a precursor-sized intermediate that accumulates at low ATP levels. This intermediate has apparently crossed the outer envelope membrane because it is protected from added protease and yet has not entered the stroma because its transit peptide has not been removed. The protease-protected precursor that we have observed may represent a similar translocation intermediate, since it was more abundant in the light without extra ATP. The ratio of mature to unprocessed, protease-protected precursor increased as more ATP was added, suggesting that low levels of ATP drive precursor uptake, but complete translocation into the lumen and processing into a mature-sized protein require more energy.

The lack of a discernible PC intermediate during in vitro import of precursors containing alterations in the transit peptide, the processed proteins were electrophoretically separated in adjacent wells, as shown in Fig. 5B. Proteins intermediate in size between the precursor and mature plastocyanin were produced with Δ30–37, γ29–20PC, and γ29–30PC. The protein produced from the γ29–30PC precursor migrated between either after Lys-22, if the processing site is similar to Silene PC, or perhaps after Ala-23, since the site LKAΔA is related to the stromal processing consensus site (V/I/LX/A/C)ΔA (19). As shown in Fig. 5C, two portions of the CF1-γ transit peptide, containing either 26 or 29 amino acid residues, were fused to the plastocyanin TP at two points, either N-terminal or C-terminal to the presumed stromal processing site, at amino acid residues 20 or 30, respectively. The sites of the fusions are indicated by the name of the construct, so that in γ26–20PC, 26 amino acid residues from CF1-γ were fused to the plastocyanin TP at amino acid residue 20. As shown in Fig. 5A, the γ26–20PC and γ29–20PC precursors were imported and processed to the mature size, although in the latter case, an intermediate species also accumulated. The Δ10–19 PC precursor, which showed no import activity in the in vitro assay, has a TP deletion that removes fewer amino acid residues than the 20 or 30 amino acid residues eliminated from the PC TP in the chimeric constructs. Together, these results indicate that the N-terminal 20 amino acid residues of the TP are necessary for import of PC into Chlamydomonas chloroplasts and that a portion of the CF1-γ TP is sufficient to restore this function. This suggests that the N terminus of the PC TP is a stroma-targeting domain whose function can be replaced by the N terminus of the CF1-γ TP.

Neither the γ26–30PC nor γ29–30PC chimeric precursors were imported and processed to the mature size upon incubation with Chlamydomonas chloroplasts. While import of the shortest precursor (γ26–30PC) did not result in any processed plastocyanin, the addition of three amino acids from CF1-γ (in the γ29–30PC precursor) allowed the production of a protein intermediate in size between the precursor and mature PC. Thus, the site of the fusion to the PC transit peptide is an important determinant of chimeric TP function.

To compare the sizes of the products created during in vitro import of precursors containing alterations in the transit peptide, the processed proteins were electrophoretically separated in adjacent wells, as shown in Fig. 5B. Proteins intermediate in size between the precursor and mature plastocyanin were produced with Δ30–37, γ29–20PC, and γ29–30PC. The protein produced from the γ29–30PC precursor migrated between the mature-sized and intermediate-sized proteins produced from the γ29–20PC precursor. The most prominent protease-resistant protein arising from the Δ30–37 precursor appears to migrate with the intermediate-sized protein found in γ29–20PC.

**Fig. 5.** The N-terminal portion of the CF1-γ transit peptide can replace most of the plastocyanin stroma-targeting domain. A, transcripts were synthesized in vitro, translated with a wheat germ extract in the presence of [35S]methionine, and imported into isolated Chlamydomonas chloroplasts as described in the legend to Fig. 3. T, 1% of the input translation products; I, proteins produced after a 15-min import reaction in the presence of 10 mM ATP, p, plastocyanin precursor; m, mature wild-type plastocyanin; *, intermediates produced from precursors containing mutant transit peptides. 1:5, the sample was diluted 1:5 relative to the other imported proteins. B, plastocyanin species produced following import of precursors with wild-type or mutant transit peptides into isolated Chlamydomonas chloroplasts. C, the transit peptide sequences of CF1-γ and PC are shown. Fusions of the first 26 or 29 amino acid residues of CF1 to PC at amino acid residue 20 or 30 of the transit peptide are also indicated. A comparison of in vitro and in vivo results is presented as described in Fig. 3. *, an intermediate-sized species was found; **, both intermediate and mature-sized species were observed.
import of the wild-type precursor is not too surprising. Using pea chloroplasts, the amount of PC intermediate that was detected depended on the import conditions and the precursor (47). It is possible that the Chlamydomonas stroma contains proteases that digest intermediates during post-import manipulations. However, intermediate-sized PC species that were presumably defective in lumen translocation were observed following import of Δ30–37 pre-PC and the γ29–20PC and γ29–30PC chimeric precursors. Therefore, it seems more likely that lumen translocation is so rapid in Chlamydomonas that wild-type intermediates are translocated and processed during post-import protease treatment and chloroplast isolation. It is clear from pulse-labeling experiments that PC precursor import proceeds through an intermediate in vivo (51).2 Perhaps treatments that rapidly stop the in vitro import reaction (for example HgCl₂) would be useful in Chlamydomonas as they have been in pea to identify pathway intermediates of proteins destined for the thylakoid (52, 53).

Our results show that the N-terminal half of the plastocyanin TP is required for import into Chlamydomonas chloroplasts, since no import was detected with the Δ10–19 precursor. There is no single part of the first 19 amino acid residues that is critical for stroma-targeting function, since individual deletions between residues 2–8, 10–12, and 13–19 all allowed at least residual import. This suggests that either there are redundant stroma-targeting elements or, perhaps more likely, that each part of this region contributes to the overall efficiency of chloroplast uptake. It should be noted, in contrast, that even short deletions at the extreme N terminus of the Silene pratensis ferredoxin TP eliminated import in pea chloroplasts (54). The reason that a similar deletion in the Δ2–8 precursor had a relatively small effect in the Chlamydomonas in vitro import assay is unknown but may relate to the lack of an uncharged N-terminal domain in the Chlamydomonas PC transit peptide. When the N-terminal 20 amino acid residues were replaced by regions of the CF₁-γ transit peptide, a mature-sized PC was produced upon import, demonstrating that PC stroma-targeting function can be replaced by the CF₁-γ transit peptide.

Deletion of part of the hydrophobic portion at the C-terminal end of the transit peptide in Δ30–37 led to accumulation of at least two intermediate-sized proteins, which were localized to the stroma in preliminary experiments (data not shown). These observations suggest that the Δ30–37 precursor is translocated across the chloroplast envelopes and processed in the stroma but that lumen translocation and processing by the thylakoid protease do not occur. Overall, the functional domains in the Chlamydomonas PC transit peptide therefore appear to be similar to those defined in vascular plant TPs (55).

The mutations described in this paper have also been tested in vivo, by transforming a series of mutant petE genes into a strain defective at the plastocyanin structural gene locus. 3 Fig. 3 shows a summary of the in vitro and in vivo results for the deletion mutants. Although these data were consistent in many cases, deletions generally had a more severe impact on in vitro chloroplast protein import than on accumulation in vivo. For example, although no import was detected with the Δ10–19 precursor in vitro, PC accumulated to about 15% of the wild-type level in transformants expressing this mutant gene. In vitro import may be sensitive to even subtle defects in envelope translocation, whereas envelope translocation may not normally be the rate-limiting step for PC accumulation. Indeed, envelope translocation is thought not to limit the targeting of lumen resident proteins during in vitro import into intact chloroplasts, since saturation of thylakoid transport can occur when large amounts of precursor are present (18). Envelope translocation may limit PC accumulation in vivo only when it is very severely impaired.

In contrast, the Δ2–8 precursor imported relatively well in vitro, but very little mature PC accumulated in vivo in transformants expressing the corresponding mutant gene at a high level. Since pulse-labeling experiments indicated that the Δ2–8 precursor was synthesized and relatively stable in these transformants, we suggest that it may be less efficient in binding to or translocation across the plastid envelope and therefore unable to compete with other wild-type precursors in vivo. Apparently there is a unique requirement for the extreme N terminus of the TP in vivo that is not reproduced by in vitro uptake into isolated chloroplasts.

C-terminal deletions in the PC transit peptide had a very severe effect on accumulation of PC species in vivo, whereas protease-protected PC intermediates were readily observed when the Δ30–37 precursor was imported in vitro. Since pulse-labeling experiments indicated that an intermediate species was produced in vivo, the protein is probably translocated across the envelope, but lumen translocation is blocked. In vivo, the intermediate is presumably too unstable to accumulate to a detectable level. We speculate that the effects of C-terminal transit peptide deletions are probably similar in vivo and in vitro, but the short duration of the in vitro assays allows easier detection of the intermediate species.

A comparison of the in vivo and in vitro analyses of the chimeric constructs is shown in Fig. 5C. Interestingly, mature PC accumulated to nearly the wild-type level when the γ26–20PC construct was expressed in vivo and to about half the wild-type level for the γ26–30PC and γ29–20PC constructs. 3 No intermediate species were observed for these constructs, suggesting that if stromal intermediates are generated in vivo, they are too unstable to accumulate. For the γ29–30PC construct, trace amounts of an intermediate species considerably smaller than the one observed in vitro accumulated. In vitro, the γ29–30PC precursor appeared to be imported and processed to an intermediate that probably lacks a functional lumen-targeting domain. It presumably fails to accumulate to a significant level in vivo because it remains in the stroma and is degraded. The γ26–30PC precursor imported poorly in vitro and remained unprocessed, yet substantial mature plastocyanin accumulated in transformants that expressed the mutant gene. We suggest that even if envelope translocation is inefficient in vivo, it is sufficient to support substantial accumulation of plastocyanin. The precursor may also remain unprocessed in vivo, but in any case, it is apparently competent for lumen translocation and thylakoid processing.

Clearly, the nature of in vitro and in vivo analysis of chloroplast import and targeting is quite different. An in vitro assay occurs over a period of minutes in isolated chloroplasts. The precursor has no competition from other precursors and is present in nonphysiological quantities. Chloroplast protein accumulation in transgenic organisms occurs over a period of hours to days and depends on many processes in addition to protein import, including transcription, translation, and, in the case of many chloroplast proteins, assembly with cofactors and other polypeptides, as well as turnover of the protein and/or complex. A small change in in vivo chloroplast protein import might not limit accumulation of the mature protein and therefore be undetectable. Moreover, mislocalized precursors and intermediates may be unstable in vivo and therefore not detected except in pulse-labeled cells (56, 57). 3 However, experiments examining cytosolic processes that regulate chloroplast protein import (58–60) and temporal or spatial coupling between synthesis and import should be more accessible in vivo. Moreover, differences between in vivo and in vitro results, such
as those noted here for the $\Delta 2-8$ deletion, may provide a window into the integration of the import pathway with cellular metabolism. The ability to compare in vitro and in vivo results in a homologous system provides a unique opportunity to study chloroplast protein import in a genetically tractable organism.

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