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Protein Import into and Sorting inside the Chloroplast Are Independent Processes

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Plastocyanin is a nuclear-encoded chloroplast thylakoid lumen protein that is synthesized in the cytoplasm with a large N-terminal extension (66 amino acids). Transport of plastocyanin involves two steps: import across the chloroplast envelope into the stroma, followed by transfer across the thylakoid membrane into the lumen. During transport the N-terminal extension is removed in two parts by two different processing proteases. In this study we examined the functions of the two cleaved parts, C1 and C2, in the transport pathway of plastocyanin. The results show that C1 mediates import into the chloroplast. C1 is sufficient to direct chloroplast import of mutant proteins that lack C2. It is also sufficient to direct import of a nonplastid protein and can be replaced functionally by the transit peptide of an imported stromal protein. C2 is a prerequisite for intraorganellar routing but is not required for chloroplast import. Deletions in C2 result in accumulation of intermediates in the stroma or on the outside of the thylakoids. The fact that C1 is functionally equivalent to a stromal-targeting transit peptide shows that plastocyanin is imported into the chloroplast by way of the same mechanism as stromal proteins, and that import into and routing inside the chloroplasts are independent processes.

INTRODUCTION

Chloroplasts import a large variety of proteins that are synthesized outside the chloroplast (Ellis, 1981; Schmidt and Mishkind, 1986). These imported proteins are synthesized in the cytoplasm as precursors with N-terminal extensions, referred to as transit peptides. Import takes place post-translationally after binding to a proteinaceous receptor (Cline et al., 1985), possibly by way of contact sites (Pain et al., 1988). Maturation of precursor proteins occurs by proteolytic processing during or shortly after transport across the chloroplast envelope (Robinson and Ellis, 1984). Inside the chloroplast, the imported polypeptides may assemble with other components, synthesized in either the chloroplast or the cytosol, into protein complexes (Schmidt and Mishkind, 1986). Chloroplasts contain different compartments. After crossing the chloroplast envelope, imported proteins arrive in the stroma, but many proteins must be routed further inside the chloroplast. Transport of these proteins requires a composite pathway with additional targeting and translocation events inside the organelle.

The information for chloroplast import is located in the transit peptide. Transit peptides are sufficient to direct chloroplast import of nonplastid proteins (Schreier et al., 1985; Van den Broeck et al., 1985; Lubben and Keegstra, 1986; Smeekens et al., 1987). Transit peptides of different proteins do not show significant homology as far as the primary sequence is concerned and can vary in length from 29 to approximately 100 amino acids. (For a recent compilation, see Keegstra and Olsen, 1989; Von Heijne et al., 1989.)

Plastocyanin (PC) is a nuclear encoded protein that functions in the thylakoid lumen as an electron carrier during photosynthesis. PC is synthesized in the cytoplasm as a precursor with a large N-terminal extension. In the case of *Silene pratensis*, the PC extension is 66 amino acids long (Smeekens et al., 1985a). Comparison with other chloroplast transit peptides showed that the PC extension can be divided into two parts, an N-terminal positively charged part that is rich in hydroxy amino acids and resembles transit sequences of stromal proteins, and a C-terminal hydrophobic part that resembles secretory signal peptides (Smeekens and Weisbeek, 1988). This division of the PC extension into two parts is also supported by experimental data since the extension is removed in two parts (Smeekens et al., 1986). The N-terminal part is cleaved during or just after the import across the chloroplast envelope into the stroma, releasing a processing intermediate in the stroma. The second part is cleaved off during translocation across the thylakoid membrane, releasing the mature protein into the thylakoid lumen. This two-step maturation sequence can be reconstituted in vitro by processing the precursor with a specific stromal processing protease, followed by processing with a specific thylakoidal processing protease (Hageman et al.,

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1986). On the basis of these data, a two-domain model has been proposed for the PC extension. In this model the first cleaved part of the extension, C1, mediates targeting of PC to the chloroplast and import into the stroma, and the second cleaved part, C2, is involved in subsequent routing to the thylakoid lumen (Hageman et al., 1986; Smeekens et al., 1986).

In this study we examined the functions of C1 and C2 using various mutant precursor proteins with deletions, insertions, or substitutions in either part. The results provided experimental evidence for the two-domain model. Moreover, the results showed that C1 and C2 function independently. The import behavior of the various constructs also provided information about the specific characteristics of C1 and C2.

RESULTS

C1 Is Required for Chloroplast Recognition and Import

The function of C1 was studied by making two deletion mutants, PCdel1-11 and PCdel1-37, lacking the first 11 and the first 37 amino acids, respectively, as illustrated in Figure 1A. The deletion of PCdel1-37 approximately corresponded to the entire first domain. PCdel1-37 is only a few amino acids larger than the stromal processing intermediate (Hageman et al., 1986). PCdel1-11 and PCdel1-37 were synthesized in vitro by translation of SP6-generated RNA in a wheat germ system in the presence of ^{35}S-methionine and tested in a chloroplast uptake experiment with isolated pea chloroplasts.

Using conditions that allowed efficient import of the wild-type PC precursor, PCdel1-11 and PCdel1-37 were not imported into chloroplasts, as shown in Figure 2. Under these conditions PCdel1-11 did not even bind to the chloroplast. PCdel1-37 only showed residual binding. These results show that at least the first 11 amino acids of C1 were essential for both the binding and import functions of the PC extension. The processing sites in PCdel1-11 were not affected by the deletion. In incubations with stromal and thylakoidal extracts, PCdel1-11 could be cleaved to the intermediate size and to the mature size by the stromal and the thylakoidal processing proteases, respectively. PCdel1-37 could only be processed at the mature processing site (data not shown).

In the translation mixes, faster migrating proteins were present in addition to precursor proteins (Figure 2, lanes 1). The size of the smaller proteins depends upon the transit peptide sequence of the full-size protein and corresponds well with the presence of methionine codons downstream from the initiator methionine codon. Most probably these smaller proteins result from internal initiations (see also Smeekens et al., 1986). As shown in Figure 2, these smaller proteins do not interact with chloroplasts.

C1 Can Be Replaced Functionally by the Ferredoxin Transit Peptide

The two-domain model proposes that C1 is involved in import into the chloroplast. To determine whether its only function is to direct import, hybrid proteins were con-

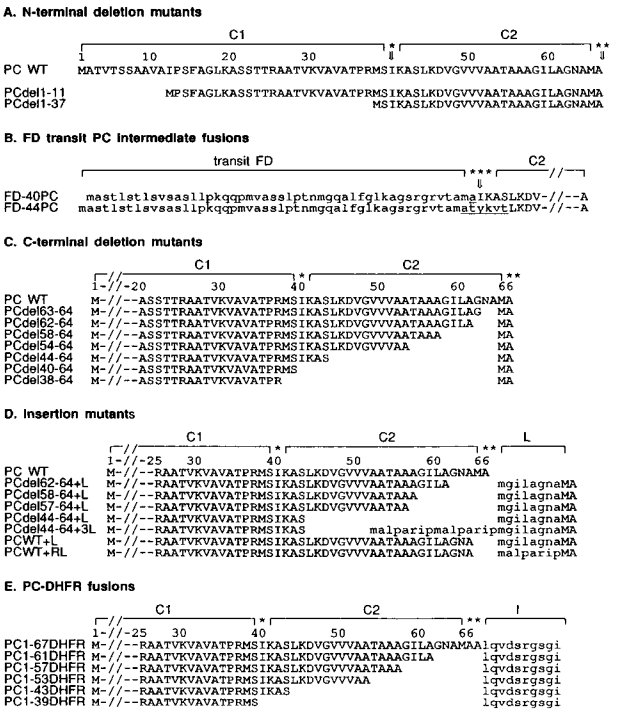

Figure 1. Amino Acid Sequence of the Extensions of the PC Constructs Used.

The amino acids are numbered from the native PC initiator methionine. The intermediate processing site (*) and the mature processing site (**) of the plastocyanin precursor are marked. (The place of the intermediate processing site is estimated from size comparison of the stromal processing intermediate with in vitro synthesized PCdel1-37 and internal initiation products of the PC precursor. It is most probably located left or right of the isoleucine residue at position 40.) C1, first cleaved part, and C2, second cleaved part of PC extension. All sequences should be read contiguously. Alignment of specific amino acids is only for convenience.

(A) N-terminal deletion mutants.

(B) Fusion proteins of the transit peptide of FD and the PC intermediate. The FD and PC amino acids are indicated in lower-case and upper-case letters, respectively. The FD mature processing site is marked (***). The amino acids from the FD mature protein are underlined.

(C) C-terminal deletion mutants of the PC extension.

(D) Insertion mutants. The amino acids that are coded for by the inserted linker sequence (L) are presented in lower-case letters.

(E) PC-DHFR fusion proteins. The amino acids that are coded for by the linker sequence (I) are presented in lower-case letters.

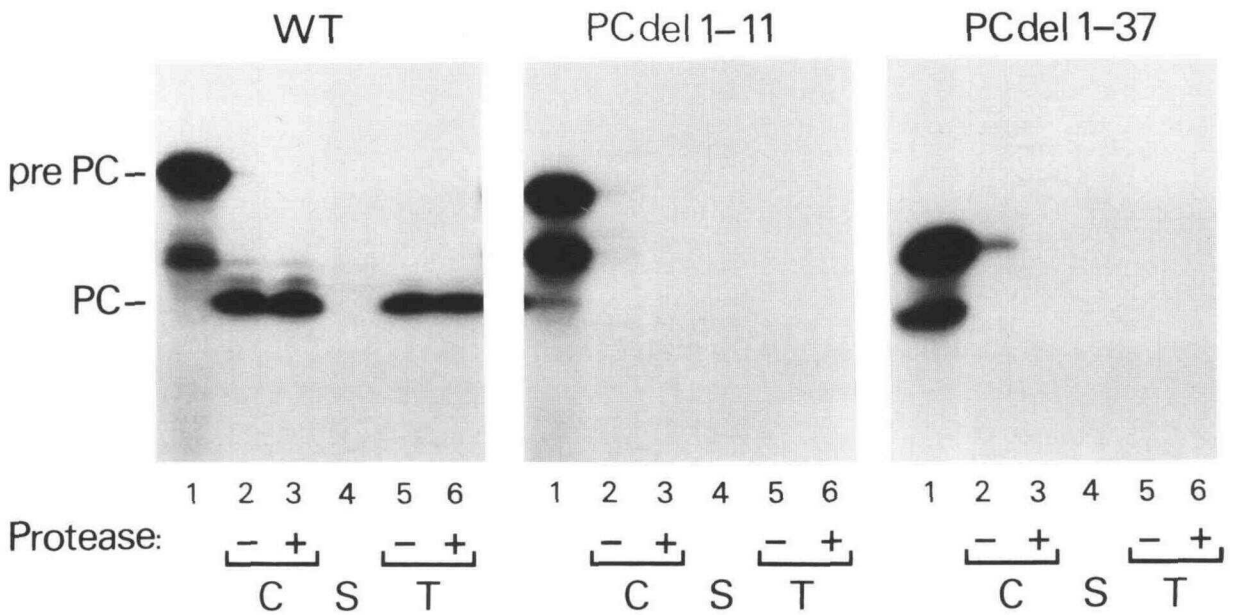

Figure 2. Import of N-Terminal Deletion Mutants into Isolated Pea Chloroplasts.

Chloroplasts were incubated in vitro with radiolabeled proteins. After incubation, chloroplasts were reisolated, fractionated, and analyzed by SDS-PAGE and fluorography. Lanes 1, in vitro translation products; lanes 2, chloroplasts before protease treatment; lanes 3, chloroplasts after protease treatment; lanes 4, stromal fraction; lanes 5, thylakoids before protease treatment; lanes 6, thylakoids after protease treatment. WT, wild type; C, chloroplast fraction; S, stromal fraction; T, thylakoid fraction.

structed in which C1 was replaced by the transit peptide of ferredoxin (FD), a stromal protein (Smeekens et al., 1985b). Two comparable hybrids were constructed with chimeric N-terminal extensions, FD-40PC and FD-44PC (Figure 1B). In FD-40PC the first 39 amino acids of the PC extension were replaced by the FD transit peptide plus the first amino acid of the FD mature protein. In FD-44PC the first 43 amino acids of the PC extension were replaced by the FD transit peptide plus the first 6 amino acids of the FD mature protein. The radiolabeled hybrid proteins were tested in a chloroplast uptake/fractionation experiment with isolated pea chloroplasts.

The effect of the replacement was examined by two criteria, the localization of imported proteins inside the chloroplast and the occurrence of a processing intermediate. The import results are shown in Figure 3A. FD-40PC and FD-44PC were imported into the chloroplast, processed to maturity, and routed correctly to the thylakoids. The mature proteins were rendered resistant to protease treatment of isolated thylakoids, indicating that they were localized inside the thylakoids. The occurrence of an intermediate processing product was assayed in a time course experiment. Figure 3B shows that in both cases a processing intermediate could be observed. The kinetics of appearance and disappearance of the processing intermediates of FD-40PC and FD-44PC were similar to that of wild-type PC (Figure 3B). These processing intermediates could also be generated by incubation of FD-40PC and FD-44PC with stromal extracts (data not shown). These results demonstrate that C1 can be replaced by the FD transit peptide without changing the import properties of the precursor protein. Quantification of the time-course experiment shows that compared with wild-type PC the import efficiency of FD-40PC and FD-44PC was 55% and 45%, respectively.

The mature processing products of FD-40PC and FD-44PC have the same size as the wild-type PC mature protein. In contrast, the intermediate processing products of FD-40PC and FD-44PC are a few amino acids larger than the wild-type PC intermediate (comparison not shown). Intermediate processing of the wild-type PC precursor is mediated by the stromal processing protease (Hageman et al., 1986). This peptidase is also responsible for maturation of imported stromal proteins such as the small subunit of ribulose-1,5-bisphosphate carboxylase and FD (Robinson and Ellis, 1984). In FD-40PC and FD-44PC the FD mature processing site remained intact, and was located a few amino acids upstream from the original PC intermediate processing site (Figure 1B). The size of the intermediate processing products suggests that in both cases the intermediate processing occurred at the FD processing site. These results show that import of FD-

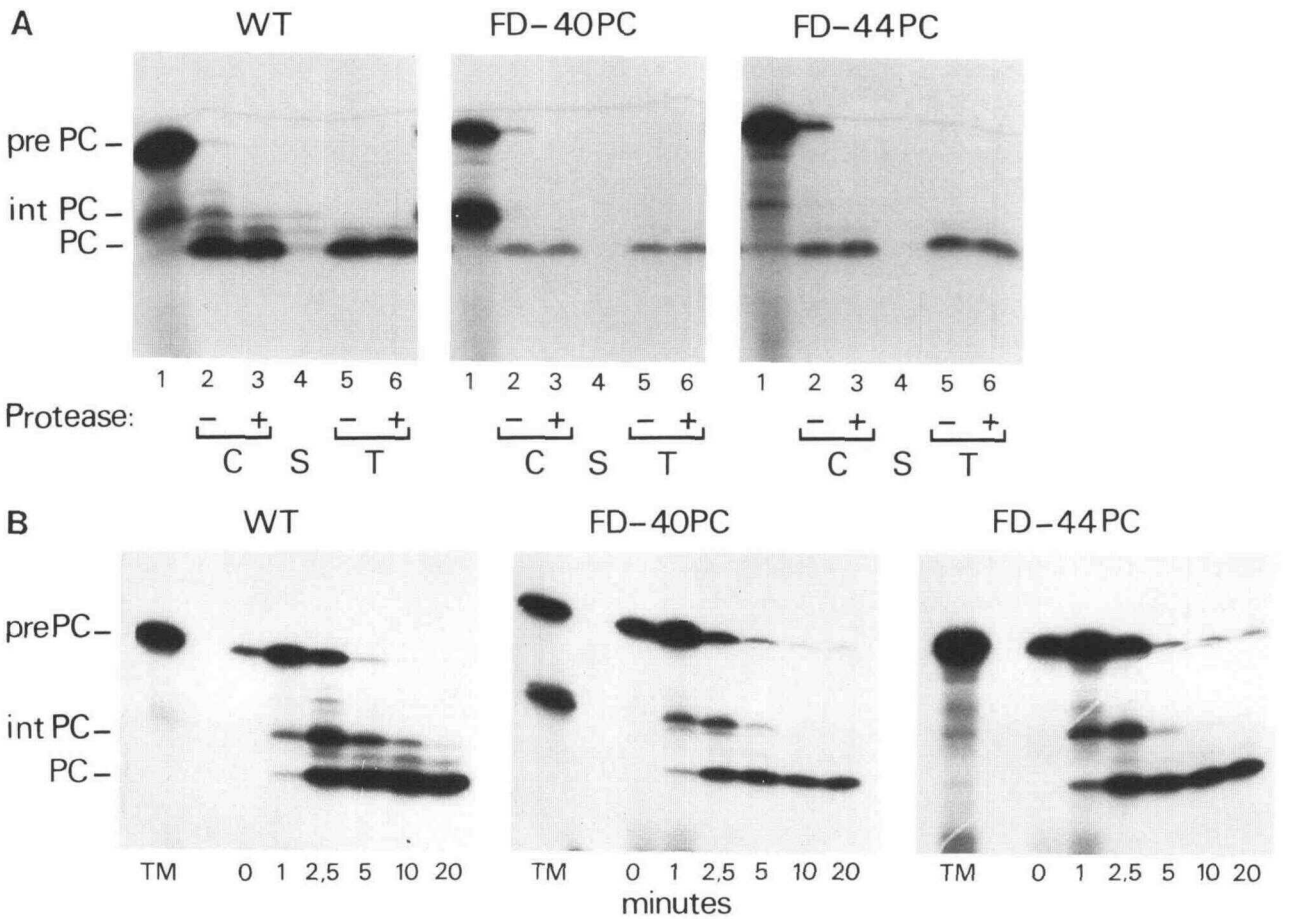

Figure 3. Import of FD Transit PC Intermediate Fusion Proteins into Isolated Pea Chloroplasts.

(A) Import/fractionation experiment. Legends are as in Figure 2.
(B) Time-course experiment.

40PC or FD-44PC into the chloroplast as well as processing is directed by the FD-specific part of the extension.

C2 Is Required for Intraorganellar Routing to the Thylakoid Lumen but not for Import into the Chloroplast

To analyze the role of C2, an ordered set of deletion mutants was constructed from which increasing portions of C2 were removed. All of these C-terminal deletions started at amino acid 64 and extended into the extension toward the N terminus (Figure 1C). The two smallest deletions, PCdel63-64 and PCdel62-64, lacked only a few amino acids in the vicinity of the mature processing site. PCdel58-64, PCdel54-64, and PCdel44-64 lacked 7, 11, and 21 amino acids, respectively. C2 was removed completely from PCdel40-64. In all of these deletion mutants, C1 remained intact. In PCdel38-64 the deletion extended into C1 and included the intermediate processing site. The mutant proteins were synthesized in vitro and tested in chloroplast uptake and binding experiments.

All deletion mutants in which C1 was left intact, PCdel63-64 up to PCdel40-64, were imported and processed to lower molecular weight forms, as shown in Figure 4A. The processed forms were rendered resistant to protease treatment of the isolated chloroplasts (Figure 4A, lanes 3). Only PCdel38-64, the protein in which C1 is truncated too, was not internalized. In the latter case only protease-sensitive precursor protein was found, which was bound to the outside of the chloroplasts. These results confirm the conclusion from the previous section that only C1 is required for chloroplast import, and C2 is not. However, none of the imported deletion mutants reached the thylakoid lumen. The two smallest deletion proteins, PCdel63-64 and PCdel62-64, cofractionated with the thylakoids

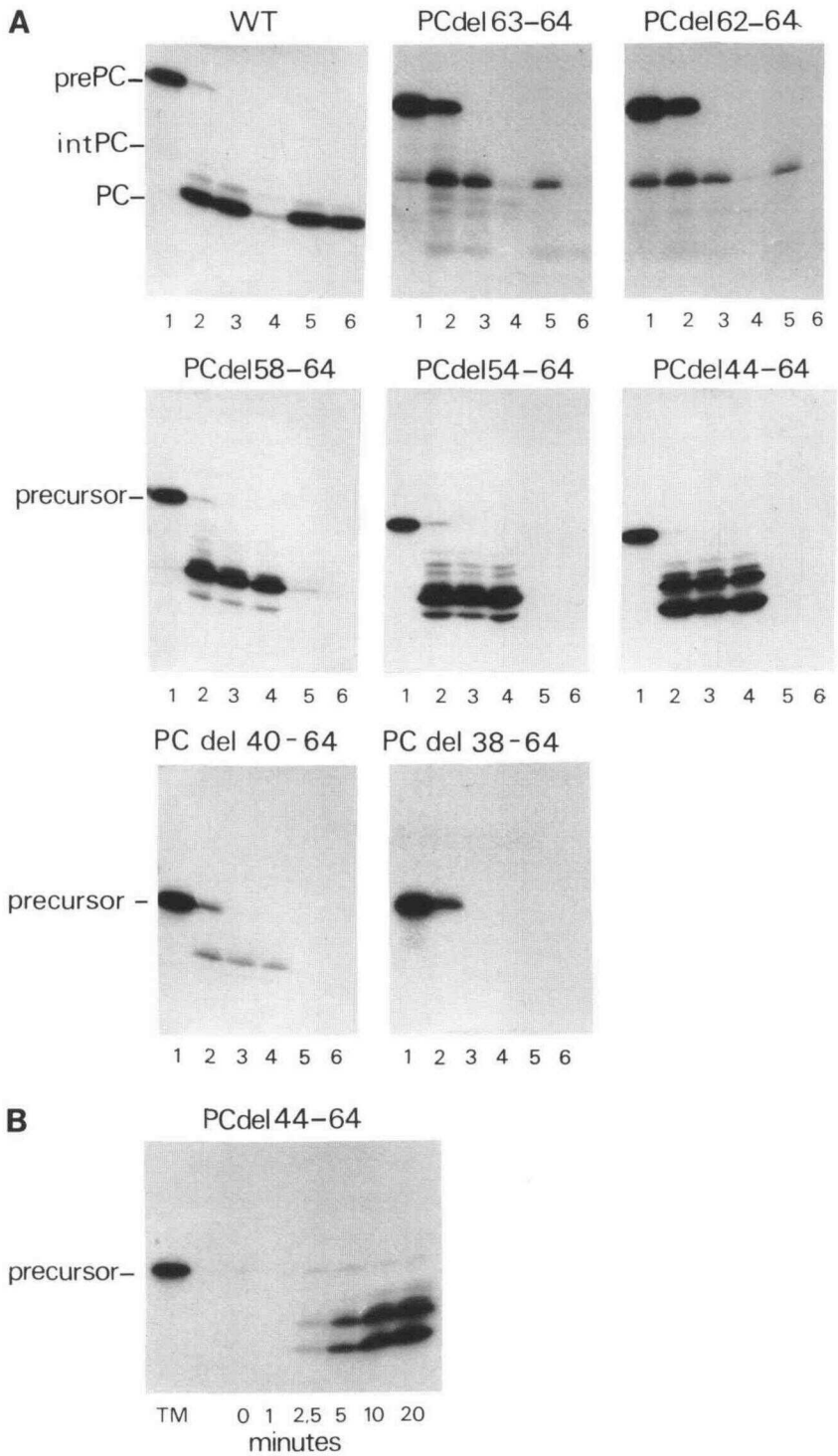

Figure 4. Import of C-Terminal Deletion Mutants into Isolated Pea Chloroplasts.

(A) Import/fractionation experiment. Legends are as in Figure 2.
(B) Time-course experiment with PCdel44-64.

(Figure 4A, lanes 5), but the processed forms were accessible to protease, indicating that the molecules were bound to the outside of the thylakoid membranes (Figure 4A, lanes 6). The mutant proteins that lacked 7 or more amino acids accumulated in the stroma. Obviously, C2 is essential for intraorganellar routing to the thylakoid lumen and at least the C-terminal end of C2 is involved in recognition of the thylakoid membrane.

All imported proteins containing deletions in C2 were processed to lower molecular weight intermediate forms (Figure 4). In the cases of PCdel63-64, PCdel62-64, PCdel58-64, and PCdel54-64, this intermediate form represents proteins that were processed at the PC intermediate processing site, as indicated by two observations. First, the size differences between these intermediate forms and the wild-type PC processing intermediate matched the size differences of the corresponding precursors. This indicates that only C1 was removed from all intermediate forms. Second, intermediate forms of the same size could be generated by incubation of the precursors with the stromal extracts (data not shown). None of the mutant proteins was processed to the mature size. This is consistent with the localization of the processing products in the stroma or on the stromal face of the thylakoid membranes. The thylakoidal processing protease is located inside the thylakoids (Hageman et al., 1986; Kirwin et al., 1987). However, spatial separation of the proteins and the peptidase might not be the only reason for the failure of maturation.

Upon import of PCdel44-64, two processed forms were found in the stroma. Comparison with the other deletion mutants shows that of these two the smallest processed form resulted from processing at the intermediate processing site. Therefore, the largest processed form represents processing at a site N-terminal from the intermediate processing site. Under normal conditions, processing at this internal processing site is not observed with wild-type PC or with the other deletion mutants mentioned above. This processing site appears to be a cryptic processing site that is exposed in PCdel44-64 as an effect of the deletion. When PCdel44-64 is incubated with stromal extracts, the cryptic processing site is favored over the intermediate processing site (data not shown). A time-course import experiment with PCdel44-64 shows that both processing products appeared with the same kinetics, indicating that there is no precursor-product relationship (Figure 4B). The processed form of PCdel40-64 also represented processing at an internal site, most probably the same cryptic processing site as in PCdel44-64.

Analysis of C1 shows that import was directed primarily by this part of the extension. Therefore, as far as binding to and import into the chloroplast are concerned, the deletions in C2 can be regarded as changes in the passenger parts of the proteins. For this reason it was of interest to determine the binding and import efficiencies of the C-terminal deletion mutants. The binding assay was performed in the presence of the ionophore nigericin to disable import (Cline et al., 1985). Figure 5 shows that all mutant proteins with an intact C1 region bound with approximately the same efficiency as wild-type PC. This confirms that C2 does not affect the initial interaction of the precursors with the chloroplasts. The only deletion mutant that bound with significantly reduced efficiency was PCdel38-64. However, in this mutant the C terminus of C1 was also affected by the deletion (Figure 1C). The reduction of binding efficiency indicates, therefore, that the C terminus is important for the binding function of C1, as was the N terminus. However, this reduction cannot account for the import block of

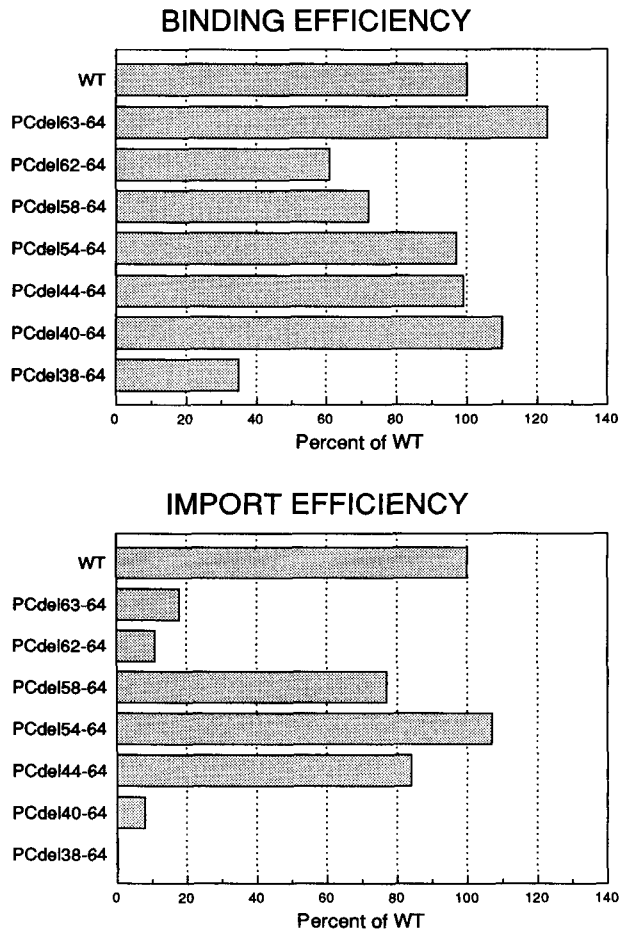

Figure 5. Binding and Import Efficiency of the C-Terminal Deletion Mutants.

For the binding experiment the chloroplasts were incubated in vitro with radiolabeled proteins in the presence of nigericin. After incubation, chloroplasts were reisolated and analyzed by SDS-PAGE and fluorography. The import efficiency is determined from the experiment shown in Figure 3. The bands were excised from the gel and the proteins were extracted with tissue solubilizer. The data refer to the number of molecules bound or imported per chloroplast, relative to wild-type PC.

PCdel38-64, suggesting that for membrane translocation additional parts are also involved.

Essentially, import efficiency was not influenced by the deletions in C2. The import efficiency of PCdel58-64, PCdel54-64, and PCdel44-64 were in the same range as wild-type PC (Figure 5), with two exceptions. The first exception is PCdel40-64, which lacks C2 entirely. Most probably PCdel40-64 also lacks the intermediate processing site. Although the binding efficiency of this mutant was not affected, the import efficiency was greatly reduced. Apparently, an intact processing site is important for efficient membrane translocation. The second exception is the greatly reduced import efficiency of PCdel63-64 and PCdel62-64. (Note that in Figure 4A the panels for PCdel63-64 and PCdel62-64 have a longer exposure.) This reduction cannot be explained by changes in C1 because in these mutant proteins this part is left intact. A possible explanation is that the three-dimensional structure of these mutant proteins is changed significantly by the deletions and that these changes affect the translocation process.

Function of the Processing Site Region in Thylakoid Targeting and Translocation

From the results with the C-terminal deletion mutants, it is clear that the region immediately in front of the mature processing site is essential for the function of C2. Deletion of 2 or 3 amino acids from this region, as in PCdel63-64 or PCdel62-64, completely blocked translocation across the membrane into the lumen, but the proteins were still targeted to the thylakoids and bound to the outside. When 7 amino acids were deleted, as in PCdel57-64, the interaction with the thylakoids was also disrupted, and the imported proteins accumulated in the stroma. To study the characteristics of this particular region in more detail, an oligonucleotide linker was synthesized that coded for amino acids 57 to 64. (MGILAGNA, the alanine residue at position 57, was substituted with a methionine residue.) This linker was inserted into the DNA sequence of the C-terminal deletion mutants at the site of the deletion (Figure 1D). The effect of the insertion was assayed in chloroplast import/fractionation experiments.

Insertion of this 8-amino acid segment into deletion mutant PCdel57-64 completely restored the deletion (Figure 1D). As a result, the routing of PCdel57-64+L to the lumen was reestablished (Figure 6A). PCdel57-64+L was imported, processed, and routed to the thylakoids where the mature size protein was resistant to thermolysin treatment. This confirmed that the deficiency in the original deletion mutant was the result of the deletion only.

The 8 amino acids were also inserted into PCdel58-64 and PCdel62-64. In the resulting mutant proteins, referred to as PCdel58-64+L and PCdel62-64+L, the insertion reconstructed the mature processing site region but also generated a duplication of 1 and 5 amino acids, respectively (Figure 1D). Nevertheless, for these proteins as well, the routing to the lumen was restored (Figure 6A). These results show that in C2 small duplications are tolerated. However, these results could also be explained by assuming that the inserted 8 amino acid segment is sufficient for targeting to the lumen or, alternatively, that proteins are routed to the thylakoid lumen when C2 has a minimum length. To exclude the first possibility, the linker was inserted into PCdel44-64, the mutant from which C2 was removed entirely. The original deletion mutant accumulated in the stroma (Figure 4A). The insertion did not restore the PC transport route, and PCdel44-64+L still accumulated in the stroma (Figure 6B). To exclude the second possibility, PCdel44-64+3L was constructed, which contained 3 linker segments coding for 24 amino acids. PCdel44-64 lacked 21 amino acids (Figure 1D). PCdel44-64+3L protein still accumulated in the stroma (Figure 6B). These results show that segment 57-64 does not contain all the necessary information for thylakoid routing and that the length of C2 is not the only determining factor for proper functioning.

The linker was also inserted in both orientations into the PC wild-type sequence, resulting in constructs PCWT+L and PCWT+RL (Figure 1D). In PCWT+L the insertion generated a duplication of 8 amino acids, but the protein was imported, processed, and routed correctly with wild-type efficiency (Figure 6C). The imported processed form was similar in size to the wild-type PC mature protein, indicating that PCWT+L was processed at the original mature processing site. Apparently, the PC transport system regarded PCWT+L as a precursor with an intact, mature protein and an extension that was 74 instead of 66 amino acids, and the system tolerated a duplication of 8 amino acids. In PCWT+RL the insertion added 8 unrelated amino acids to the PC precursor sequence. Nevertheless, PCWT+RL was imported, processed, and routed to the thylakoids, albeit with decreased efficiency (Figure 6C). The processed form of PCWT+RL that was located inside the thylakoids, resistant to thermolysin treatment, had a slightly higher molecular weight than the wild-type PC mature protein. This suggests that the processing of PCWT+RL occurred upstream from the original mature processing site, most probably 8 amino acids upstream where a mature processing site sequence (GILAGNAMA) was restored despite the reversed orientation of the inserted linker (Figure 1D). In the latter case, the transport system appeared to regard PCWT+RL as a precursor with an intact extension and an 8-amino-acid extended passenger protein.

The Plastocyanin Extension Does not Promote Efficient Transport of a Nonplastid Protein to the Chloroplast Thylakoid Lumen

The results demonstrate that C1 is responsible for chloroplast import and that C2 is essential for routing to the

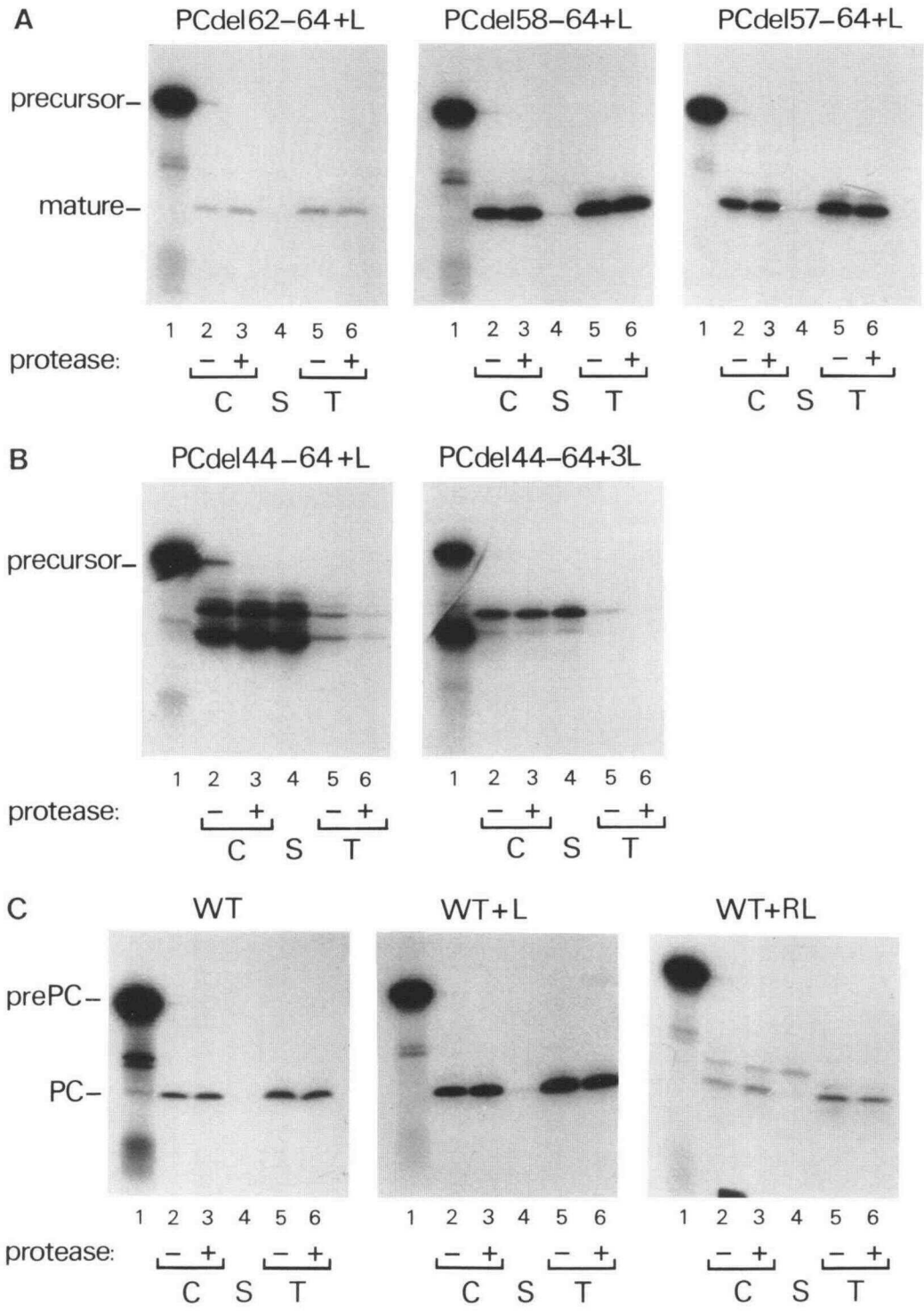

Figure 6. Import of the Insertion Mutants into Isolated Pea Chloroplasts.

(A) PCdel62-64+L, PCdel58-64+L, and PCdel57-64+L.
(B) PCdel44-64+L and PCdel44-64+3L.
(C) WT (pre-PC), WT+L, and WT+RL.
Legends are as in Figure 2. See text for details.

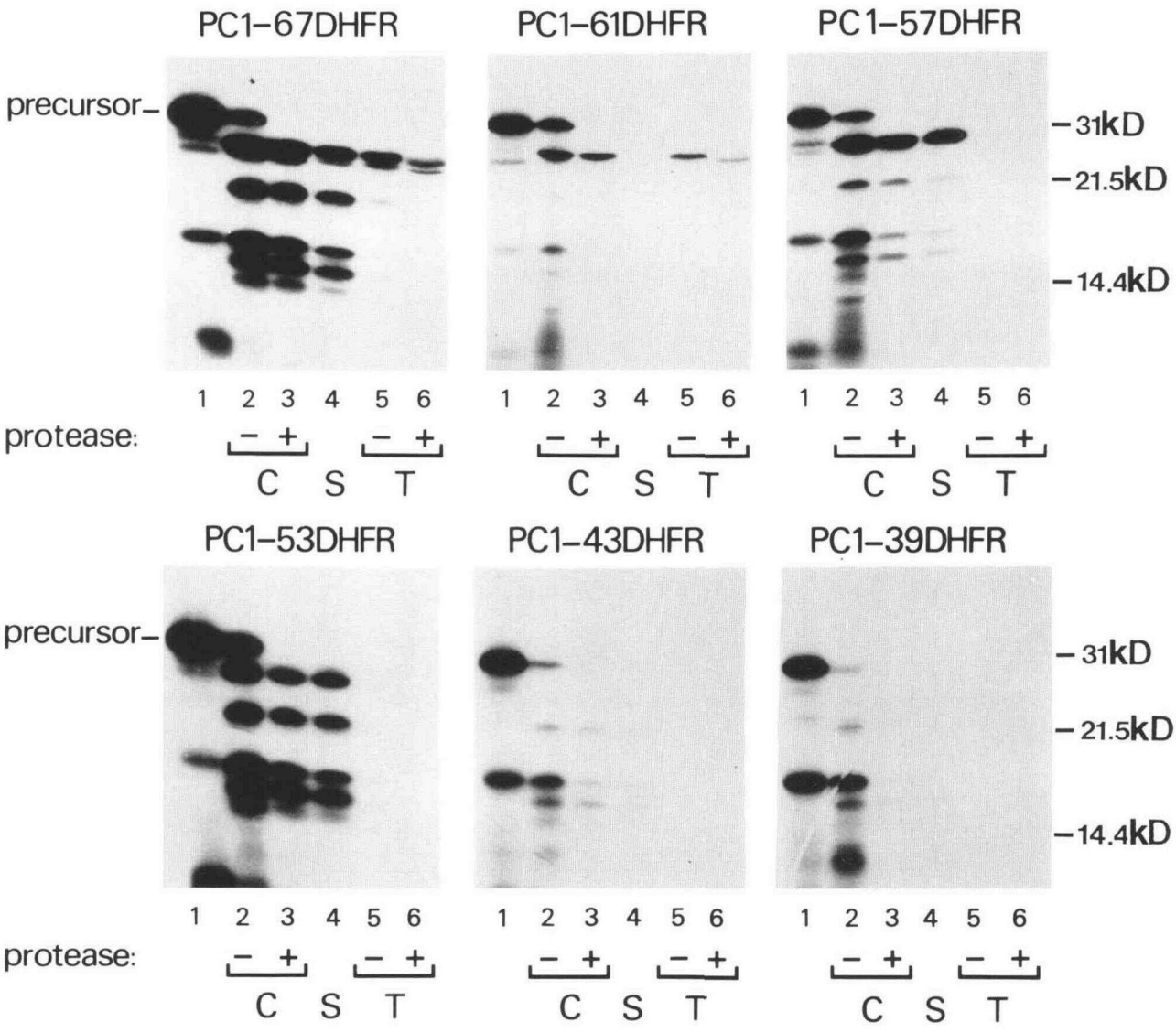

Figure 7. Import of PC-DHFR Fusion Proteins into Isolated Pea Chloroplasts.

Legends are as in Figure 2. See text for details.

thylakoid lumen. However, in these constructs sequences in the mature part of the protein also interact with the transport process. Therefore, fusion proteins were constructed with a nonchloroplast protein, mouse cytosolic dihydrofolate reductase (DHFR), as a passenger. To determine which part of the PC extension is minimally required for chloroplast import and thylakoid routing, a set of PC-DHFR fusion proteins was constructed with extensions of increasing length (Figure 1E). The smallest fusion protein of this set, PC1-39DHFR, contained only the first 39 amino acids of the PC extension and the largest fusion protein, PC1-67DHFR, contained the complete extension plus the first amino acid of the PC mature protein. Figure 7 shows the import results of these fusion proteins.

PC1-39DHFR was not imported into the chloroplasts. The precursor did not even bind to the chloroplasts. The addition of 4 more amino acids in PC1-43DHFR resulted in import with a very low efficiency. Efficient import was only observed when the extension was at least 53 amino acids long. The import efficiency reached approximately the level of the wild-type PC precursor in the case of PC1-53DHFR, PC1-57DHFR, and PC1-67DHFR. In this experiment, PC1-61DHFR was imported with a lower efficiency. The size of the smallest imported fusion protein suggests

that for chloroplast import a minimum of 43 amino acids, approximately the size of C1, is required. However, for efficient import, more amino acids are necessary. These data agree with the data for the C-terminal deletion mutants.

The processed forms of PC1-57DHFR, PC1-53DHFR, and PC1-43DHFR were all located exclusively in the stroma. Targeting to the thylakoids was only observed with PC1-67DHFR and PC1-61DHFR (Figure 7). However, for PC1-67DHFR, the largest part, and for PC1-61DHFR, all of the thylakoid-associated proteins were bound to the outside of the thylakoids, sensitive to protease treatment. Only for PC1-67DHFR was a small portion of the imported proteins internalized (lanes 5 versus lanes 6). These data show that 61 amino acids of the PC extension were sufficient for thylakoid targeting and binding of DHFR. However, in agreement with the data from the C-terminal deletion mutant PCdel62-64 (Figure 4A), these 61 amino acids were not enough for translocation to the lumen.

The imported PC-DHFR fusion proteins were processed during import into the chloroplast. PC1-67DHFR, PC1-57DHFR, and PC1-53DHFR characteristically and reproducibly yielded more than one processed form. The largest processed forms represented proteins that were processed at the intermediate processing site in the PC extension. These processed forms differed in size among the three fusion proteins and could also be generated by incubation with stromal extracts (data not shown). In contrast, the other three or four prominent processing products did not differ in size among the fusion proteins, indicating that they result from processing in the DHFR passenger part. These processing products were not observed when the fusion proteins were incubated with stromal or thylakoidal extracts. This suggests that this fragmentation was coupled with the translocation across the chloroplast envelope. It is not known which processing protease was responsible for this processing. In time-course experiments, all processed forms appeared in the same time interval, indicating that there was no precursor-product relationship between the different processing products. In import experiments with PC1-61DHFR, only the intermediate processed form was found inside the chloroplast and only a small amount of fragmentation product (Figure 7). This suggests that the vulnerability of the PC-DHFR fusion proteins for fragmentation products depends on the size of the PC specific extension or the transport route and localization of the imported proteins.

The processing pattern of PC1-43DHFR was significantly different from the other fusion proteins. The only internalized processed form for PC1-43DHFR was smaller than authentic DHFR. This processed form must have resulted from processing in the DHFR moiety of the fusion protein, despite the fact the PC1-43DHFR fusion protein contained the PC intermediate processing site. PC1-43DHFR was processed at the cryptic processing site that had been described for PCdel44-64 and PCdel40-64 (Figure 4A). As for PCdel40-64, the malfunctioning of the intermediate processing site might also be the cause of the low import efficiency of PC1-43DHFR. For PC1-67DHFR, the low efficiency of thylakoid translocation was not due to the lack of a functional mature processing site because PC1-67DHFR could be processed at this site by incubation with thylakoidal extracts (data not shown).

DISCUSSION

This work presents a detailed analysis of the characteristics of the bipartite transient extension of PC. On the basis of previous experiments, a two-domain model was proposed for the function of this extension in the two-step transport pathway of PC to the thylakoid lumen (Hageman et al., 1986; Smeekens et al., 1986). This model predicted that the first cleaved part of the extension, C1, would mediate import into the chloroplast across the chloroplast envelope and that the second cleaved part, C2, would be involved in subsequent routing to the thylakoid lumen across the thylakoid membrane. The results provided experimental evidence to support this two-domain model. Import of PC is primarily dependent on C1. Partial or complete deletion of C1 blocks import, whereas import occurs if regions of C2 are deleted. Furthermore, C1 is sufficient for import of a nonchloroplast protein. Thus, C1 is functionally equivalent to a stromal-targeting transit peptide. Routing from the stroma into the thylakoid lumen was demonstrated to require C2. Deletions in C2 resulted in accumulation of processing intermediates en route to the thylakoid lumen in the stroma or on the stromal face of the thylakoid membrane.

Both stages of the PC transport pathway, import and intraorganellar routing, can be divided into three distinct steps: binding to the target membrane (envelope and thylakoid membrane, respectively), translocation across the membrane, and processing by specific processing proteases. The results also provide information about the internal organization of functional domains in C1 and C2.

C1 contains the information for recognition of and import into the chloroplast. Deletion of the N-terminal 11 amino acids as in PCdel1-11 blocks binding to the chloroplast envelope and translocation across the envelope. However, membrane translocation is also inhibited when amino acids are removed from the C terminus of C1, as demonstrated by the import results of the C-terminal deletion mutants. In these mutant proteins an increasing portion of C2 is removed. Chloroplast import of the C-terminal deletion mutants is not inhibited unless the deletion extends into the C terminus of C1, as in PCdel38-64. These results show that both the N terminus and the C terminus are important for the function of C1. An essential role for both termini has also been found for the transit peptides of the small subunit of ribulose-1,5-bisphosphate carboxylase

and FD (Kuntz et al., 1986; Wasmann et al., 1986; Reiss et al., 1987; Smeekens et al., 1989), confirming that C1 is functionally equivalent to the transit peptide of stromal proteins. Although both termini are involved in import, the functions of the N and C termini are not homologous. The N terminus most probably is involved primarily in chloroplast recognition and the C terminus in recognition by the stromal processing protease. The N terminus, for example, is not involved in the recognition of the processing site since PCdel1-11 is processed correctly during incubation with stromal extracts. For efficient import of DHFR, C1 alone is not sufficient. The shortest PC-DHFR fusion protein that is imported with approximately the PC efficiency contains an extension of 53 amino acids, which is 12 amino acids longer than C1. This shows that sequences downstream from the intermediate processing site can be important in chloroplast import, as has been suggested previously (Wasmann et al., 1986; Smeekens et al., 1989).

C2 is essential for the targeting to and the translocation across the thylakoid membrane. It can be removed without blocking chloroplast import, but even small deletions in this part inhibit completion of the intraorganellar sorting process. When amino acids 63 and 64 at the C terminus of the second cleavable part are deleted, the mutant proteins accumulate on the stromal face of the thylakoid membranes. The latter association is also disrupted when amino acids 58 to 62 are removed. These data point to the importance of the 58 to 64 segment for thylakoid targeting and translocation. However, this segment by itself is not sufficient because insertion of this segment into deletion mutants that completely lack C2 does not restore the transport route. Interestingly, in contrast to the effect of even the smallest deletion, C2 does tolerate duplications of up to 8 amino acids.

One of the requirements for successful translocation across the thylakoid membrane appears to be the availability of a functional mature processing site. In the chloroplast import experiments, the proteins that are not transported to the lumen are not processed by the thylakoidal processing protease because this protease is located inside the thylakoids (Kirwin et al., 1987). However, these proteins, for example the C-terminal deletion mutants and the insertion mutants PCdel44-64+L and PCdel44-64+3L, are not processed at the mature processing site when they are incubated with thylakoidal extracts containing active thylakoidal processing protease (data not shown). On the other hand, all proteins that reach the lumen are processed during or just after translocation of the thylakoid membrane. This suggests that thylakoid membrane translocation and thylakoidal processing either are strongly related or depend on the same structural features.

Information on the structural requirements of C2 also comes from the FD transit-PC intermediate fusions. These fusion proteins most probably are processed at the internal FD mature processing site, resulting in processing intermediates that are a few amino acids larger than the wild-type PC intermediate. Despite this difference, these intermediates are routed correctly to the thylakoid lumen. This shows that the N terminus of C2 can be variable. The extra amino acids involved, however, are either neutral or positively charged and, therefore, are unlikely to change the characteristic of C2.

The combined action of C1 and C2 directs the PC mature protein to the thylakoid lumen. However, the results from the PC-DHFR fusion proteins indicate that the complete PC extension is not able to direct efficient transport of DHFR. All fusion proteins that contain C1 are imported into the chloroplast, and the two largest PC-DHFR fusion proteins bind to the thylakoids, but not even PC1-67DHFR, which contains the complete extension, is translocated to the lumen efficiently. It has been demonstrated recently that the extension of another lumenal protein, the 33-kD photosynthetic oxygen-evolving protein, is sufficient to target DHFR to the thylakoid lumen (Ko and Cashmore, 1989; Meadows et al., 1989). It has been found that the PC extension does not direct lumen transport of FD and superoxide dismutase (Smeekens et al., 1986, 1987). Therefore, this insufficience appears to be restricted to the PC extension. Apparently, in the case of PC, it cannot be excluded that for thylakoid membrane translocation additional information is present in the PC mature protein (De Boer et al., 1988). Alternatively, because of coevolution with the passenger protein, the PC extension might be exclusively suitable to direct transport of the PC mature protein. The extension is very vulnerable to change as even small deletions disrupt the proper transport of PC itself. The extension of *S. pratensis* PC can be replaced functionally by the extension of *Arabidopsis thaliana* PC (Vorst et al., 1988; J. Hageman, J. Dagelinckx, and O. Vorst, unpublished results), indicating that a possible coevolution between extension and passenger only affects differences between proteins, not between species.

The two-domain model makes no predictions about a possible interaction between C1 and C2. The results indicated that C1 and C2 function independently, as demonstrated by exchanging C1 with the transit peptide of FD. The FD transit peptide only contains information for chloroplast import. It can direct chloroplast import of PC when it is directly attached to the PC mature protein. However, in that case, PC is redirected to the stroma (Smeekens et al., 1986). When the FD transit is fused to the PC intermediate, as in FD-40PC and FD-44PC, the characteristics of PC transport, localization in the thylakoid lumen and two-step processing, are preserved. This finding supports the conclusion that C1 contains information for chloroplast import only. It is unlikely that sequences within C1 contribute to the function of C2 because such sequences will not be present within the FD transit peptide and the FD-transit-C2 chimeric extension would not have functioned properly without them. In addition, the fact that C1 is functionally equivalent to a stromal-targeting transit peptide implies that the PC intermediate is imported into the chloroplast

by way of the same route as stromal proteins and that the PC processing intermediate, when present in the stroma, contains all the information necessary for subsequent routing into the thylakoid lumen.

This existence of a common import route for proteins that function in different chloroplast compartments points to a common evolutionary origin. In the prokaryotic progenitor of the chloroplast, all proteins were synthesized in the stromal matrix. Plastocyanin in this progenitor must already have contained targeting information for transport to the thylakoid lumen, as does plastocyanin in the extant cyanobacteria. The bipartite structure of the PC extension of higher plant PC suggests that the genetic information for this thylakoid targeting signal was also transferred to the nucleus together with the coding information for the mature sequence. This means that C1 and C2 represent the added chloroplast import domain and the ancient thylakoid target domain, respectively. This model is supported by the comparison of the PC extension of higher plants with the extension of PC from cyanobacteria, which shows that cyanobacterial signal sequences closely resemble C2 (Van der Plas et al., 1989). The obvious advantage of this mechanism is that the chloroplast import step is simply added to the original thylakoid routing, and no completely new mechanism is necessary for transport to the thylakoid lumen (Smeekens et al., 1990).

The chloroplast thylakoid lumen proteins for which the primary sequences have been determined all show the same bipartite structure as the PC extension (Jansen et al., 1987; Mayfield et al., 1987; Tyagi et al., 1987). For one of these proteins, the 33-kD photosynthetic oxygen-evolving protein, experimental data are available that are consistent with the two-domain model (Ko and Cashmore, 1989). This protein also shows a two-step maturation sequence in in vitro processing experiments (James et al., 1989). These observations support the notion that the mechanism that is described here for PC is valid for all nuclear encoded lumenal proteins. Until now, the term transit peptide has been used for the entire transient extension of all chloroplast precursors. In view of the pathway described above, we propose to confine the phrase transit peptide to the first cleaved part of the extension of lumenal proteins. For the second cleaved part, which is essential for the routing to the thylakoid lumen, we propose the phrase thylakoid signal peptide. This means that lumenal precursors consist of three parts: a transit peptide, a thylakoid signal peptide, and a mature sequence.

The existence of a composite transport pathway consisting of a relatively recently evolved plastid-specific first step linking up to a more ancient routing step inside the plastid has also been described for mitochondria (Pfanner et al., 1988). As a biological phenomenon, linkage of transport mechanisms therefore seems not to be confined to chloroplasts. However, a composite pathway is not always reflected in a multipartite structure of the transient extension. This depends on the localization of the sequences that mediate the intraorganellar routing, which may be located in the mature sequence of the precursor protein. An example is the chlorophyll a/b binding protein (CAB), a nuclear encoded thylakoid membrane protein. The CAB precursor protein has an extension that is functionally equivalent to a stromal-targeting transit peptide (Hand et al., 1989). Integration into the thylakoid membrane is directed by noncleavable sequences within the mature protein (Kohorn and Tobin, 1989).

METHODS

Cloning Procedures

Restriction endonucleases, Bal31 exonuclease, DNA polymerase I large fragment (Klenow fragment), T4 DNA ligase, RNasin, and SP6 polymerase were obtained from Boehringer, Bethesda Research Laboratories, New England Biolabs, and Promega-Biotec and were used according to the manufacturers' directions. DNA manipulations and gel electrophoresis were performed essentially as described by Maniatis et al. (1982). Plasmid minipreps and maxipreps were performed using the boiling method (Holmes and Quigley, 1981) and the alkaline lysis method (Birnboim and Doly, 1979), respectively. All pEMBL8 (Dente et al., 1983) and pSP64 (Melton et al., 1984) constructs were cloned in *Escherichia coli* JM101 *rec*A, *hsd*S. Single-stranded pEMBL DNA was obtained by superinfecting plasmid containing bacteria with bacteriophage IR1 as described previously (Dente et al., 1983). If required, the constructions were confirmed by dideoxy sequencing (Sanger et al., 1980). Oligonucleotide-directed mutagenesis was performed using the gapped duplex method as described previously (Kramer et al., 1984) with the exception that single-stranded plasmid DNA from the pEMBL constructs was used as template. Vector plasmid pEMBL8 linearized with HindIII-EcoRI was used to form the duplex.

N-Terminal Deletions

The plastocyanin extension and mature sequences for all constructs were isolated from plasmid pPCS74, a pEMBL8 derivative that contains the entire coding sequence of the *Silene pratensis* PC precursor in the SmaI site (Smeekens et al., 1986). Plasmid pPCdel1-11 is derived from plasmid pPCS74 by oligonucleotide-directed mutagenesis. With the oligonucleotide used (5′-TGGCTGCAGGTCGACGGCATGCCGTCTTTCGCGGGCCT-3′), 51 nucleotides from the wild-type sequence, including the coding sequence for the initiator ATG codon and the first 11 amino acids, are removed and a new ATG codon (underlined) is introduced at position 12. This latter ATG codon serves as initiator codon for PCdel1-11, yielding a protein that is 11 amino acids shorter than the wild-type precursor.

In plasmid pPCdel1-37, the initiator codon of the wild-type sequence is removed by Bal31 exonuclease digestion. Plasmid pPCdel1-37 is derived from pPC2 by Bal31 exonuclease treatment in a manner similar to the construction of pPCS74 (Smeekens et al., 1986). In comparison with plasmid pPCS74, an additional 29

nucleotides are removed from the 5′ end of the cDNA in plasmid pPCdel1-37. Plasmid pPCdel1-37 lacks the coding sequence of the first 7 amino acids, including the initiator codon. Translation of PCdel1-37 initiates at the first ATG codon downstream at position 38. Plasmid pPCdel1-37 is identical to plasmid pPCS58, described in Hageman et al. (1986). To enable in vitro expression of the deletion mutants, the entire coding sequence of pPCdel1-11 and pPCdel1-37 was removed from the pEMBL vector by HindIII-EcoRI digestion and directionally cloned behind the SP6 promotor in pSP64.

FD Transit-PC Intermediate Fusions

For the construction of FD-40PC and FD-44PC, a BstEII and BalI restriction site, respectively, were introduced in the coding sequence of pPCS74 by oligonucleotide-directed mutagenesis. For this purpose two oligonucleotides were used. With oligonucleotide 88 (5′-CAATCAAGGTTACCCTTAAGG-3′), a BstEII restriction site was introduced that overlaps with the coding sequence of the alanine and serine residues at positions 42 and 43 of the wild-type sequence. Because of the introduction of this restriction site in plasmid pPCS74-88, the codons for these amino acid residues were replaced by a valine and a threonine codon, respectively, as illustrated in Figure 8. With oligonucleotide 89 (5′-CACCCCCAC-CATGGCCATCAAGGCC-3′), an NcoI and a BalI restriction site were introduced that overlap with the coding sequence of the arginine, methionine, and serine residues at positions 37, 38, and 39 of the wild-type sequence. Because of the introduction of these restriction sites in plasmid pPCS74-89, the arginine codon is replaced by a threonine codon and the serine codon is replaced by an alanine codon (Figure 8).

Plasmid pSPFD-40PC was constructed from pPCS74-89 and pSPFD22. Plasmid pSPFD22 contains the entire coding sequence for the *S. pratensis* FD precursor in the multiple cloning site of pSP64 (Smeekens et al., 1986). The coding region of the FD precursor contains a BalI and a BstEII restriction site in the neighborhood of the coding region of the mature processing site (Figure 8). The BalI-EcoRI fragment of pPCS74-89 containing the coding sequence of the PC mature and amino acids 40 to 66 of the extension was isolated from gel and ligated into plasmid pSPFD22 from which the coding region of the FD mature was removed by BalI-EcoRI digestion. In a similar manner pSPFD-44PC was constructed from pPCS74-88 and pSPFD22. The BstEII-EcoRI fragment of pPCS74-88 was isolated from gel and ligated into pSPFD22 digested with BstEII and EcoRI. Both plasmids, pSPFD-40PC and pSPFD-44PC, contain the SP6 promoter.

C-Terminal Deletions

The C-terminal deletions in the PC extension were introduced in plasmid pPCS74 using the same procedure as described previously for FD in Smeekens et al. (1989). Plasmid pPCS74 was linearized at the unique NcoI site that overlaps with the ATG codon that codes for the methionine at position 65. From this restriction site the coding sequence of the N-terminal extension was removed progressively by treatment with exonuclease Bal31. The frayed ends obtained by the Bal31 treatment were blunt ended by Klenow treatment. Because in this procedure the coding sequence of the mature is degraded too, it was removed by EcoRI digestion and replaced by an intact sequence. This intact sequence was isolated from pPCS74 as an NcoI-EcoRI fragment from which the NcoI sticky end was filled in by Klenow treatment. As a result, clones were obtained with a partially deleted coding sequence of the extension and an intact coding sequence of the mature protein. These clones were sequenced to determine the extent of the deletion. Plasmids in which the coding sequences appeared to be in frame were selected and were named pPCdelx-64 (where x stands for the number of the amino acid where the deletion in the extension starts). In all C-terminal deletion mutants except pPCdel63-64, the unique NcoI site from which the deletion starts is restored. This restored NcoI site, at the junction of the truncated PC extension coding sequence and the mature protein coding sequence, is used for the construction of the PC-DHFR fusion proteins described below. To enable in vitro expression of the deletion mutants, the entire coding sequence of these pPCdelx-64 plasmids was removed from the pEMBL vector by HindIII-EcoRI digestion and directionally cloned behind the SP6 promotor in pSP64, resulting in plasmids pSPPCdelx-64.

Insertion Mutants

For the construction of the insertion mutants, an oligonucleotide linker was used that was made up of two complementary synthetic oligonucleotides, 5′-CATGGGGATTCTCGCCGGCAACGC-3′ and 5′-CATGGCGTTGCCGGCGAGAATCCC-3′. Annealing of these

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PC (WT)   . ACC CCC AGG ATG TCA ATC AAG GCC TCC CTT AAG GAC GTC GGT GTC .
          . THR PRO ARG MET SER ILE LYS ALA SER LEU LYS ASP VAL GLY VAL .
            35                  40                  45

                        Bal I
PC74-89   . ACC CCC ACC ATG GCC ATC AAG GCC TCC CTT AAG GAC GTC GGT GTC .
          . THR PRO THR MET ALA ILE LYS ALA SER LEU LYS ASP VAL GLY VAL .
            35                  40                  45

                                    Bst EII
PC74-88   . ACC CCC AGG ATG TCA ATC AAG GTT ACC CTT AAG GAC GTC GGT GTC .
          . THR PRO ARG MET SER ILE LYS VAL THR LEU LYS ASP VAL GLY VAL .
            35                  40                  45


                        Bal I               BstEII
FD (WT)   . gtg act gca atg gcc aca tac aag gtt acc ttg att acc aag gag .
          . val thr ala met ala thr tyr lys val thr leu ile thr lys glu .
            45          48  49                      55

FD-40PC   . ... gtg act gca atg gCC ATC AAG GCC TCC CTT AAG GAC GTC GGT .
          . ... val thr ala met ala ILE LYS ALA SER LEU LYS ASP VAL GLY .
                45          48  49  40                  45

FD-44PC   . gtg act gca atg ggc aca tac aag GTT ACC CTT AAG GAC GTC GGT .
          . val thr ala met ala thr tyr lys val thr LEU LYS ASP VAL GLY .
            45          48  49                  54  44  45
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Figure 8. Nucleotide and Amino Acid Sequences of the Fusion Regions of the FD Transit-PC Intermediate Fusion Proteins and Cloning Intermediates.

The nucleotide and amino acid sequences of PC and FD are indicated in upper case and lower case, respectively. The amino acids for all protein fragments are numbered from the authentic initiator methionine of the original PC and FD proteins. The nucleotide changes and the resulting amino acid changes in pPC74-88 and pPC74-89 are italicized. The restriction sites used for cloning are underlined. In FD-44PC the amino acids valine and threonine at position 54 and 55 are actually coded for by codons that originate from the PC sequence. These codons are designated as FD amino acids because they exactly match with the wild-type FD amino acids at these positions.

two oligonucleotides yields a double-stranded linker of 20 bp with NcoI sticky ends on both sides. Inserted into the NcoI site of the PC constructs, in the correct orientation this linker provides the coding information for a stretch of 8 amino acids: MGILAGNAMA. This sequence is identical to the amino acids 57 to 64 of the PC wild-type sequence, except for an alanine-to-methionine substitution at position 57. In the reverse orientation this linker codes for the amino acid sequence MALPARIP. This linker was ligated into the unique NcoI site of the pPCdelx-64 plasmids described above, resulting in plasmids pPCdelx-64+L. The number of linkers that were inserted and the orientation were checked by sequencing. The coding sequence of the described plasmids was removed with HindIII-EcoRI digestion and transferred to pSP64.

PC-DHFR Fusion Proteins

The PC-DHFR constructs are derived from pPCSOD74, a pSP64 plasmid containing the PC extension coding sequence fused to the coding sequence of yeast mitochondrial superoxide dismutase (SOD) (Smeekens et al., 1987), and pDS5/2, a pDS5 plasmid containing the complete coding sequence of mouse cytosolic DHFR (Stueber et al., 1984). pPCSOD74 was used because in this plasmid a multiple cloning site is present between the PC extension coding sequence and the SOD coding sequence. For pSPPC1-67DHFR, the SOD sequence from pPCSOD74 is removed by BamHI-EcoRI digestion and replaced by the BamHI-EcoRI fragment from pDS5/2 containing the entire coding sequence of DHFR, plus 6 bp and a BamHI site in front of the ATG start codon and approximately 300 bp (including an NcoI site) from the pDS5 vector downstream from the TAA stop codon. In pSPPC1-67DHFR the DHFR sequence is in frame with the PC extension sequence. Because of the used cloning procedure, between the coding sequences for the PC extension and the DHFR mature protein a sequence of 30 nucleotides is present that codes for a "linker" segment of 10 amino acids (LQVDSRGSGI). The other PC-DHFR constructs are derived from pPC1-67DHFR and the pSPPCx-64 plasmids described above. The pSPPCx-64 plasmids were linearized at the unique NcoI restriction site located at the end of the coding sequence of the truncated extension. In this site the NcoI-NcoI fragment of pSPPC1-67DHFR was inserted that contains the entire DHFR coding sequence.

In Vitro Expression

SP6 transcripts were synthesized as described by Melton et al. (1984), with the exception that the DNase treatment was omitted and that cap analogue [G(5′)ppp(5′)G, Pharmacia LKB Biotechnology Inc.] was present during the synthesis reaction. Radiolabeled precursor protein was prepared by in vitro translation of the generated RNAs in a wheat germ system in the presence of ^{35}S-methionine (specific activity, 1100 Ci/mmol, Amersham International) as described (Smeekens et al., 1986).

Chloroplast Preparation

The chloroplast uptake experiments were performed essentially as described by Cline et al. (1985). Intact chloroplasts were isolated from 9-day-old to 12-day-old pea plants (cv Feltham First) using the Percoll gradient technique. A standard incubation mixture contained 200 μL of chloroplasts (equivalent to 200 μg of chlorophyll) and 1 to 3 $\times$ 10^6 dpm ^{35}S-labeled precursor protein. The mixture was incubated for 20 min in the light at 25°C. The time-course and fractionation experiments were performed as previously described (Smeekens et al., 1986). The in vitro processing experiments were performed with stromal and thylakoidal fractions as described (Hageman et al., 1986).

Electrophoretic Analysis

SDS-polyacrylamide gel electrophoresis (12.5%) was performed with the buffer system described by Laemmli (1970). After electrophoresis the gels were stained with Coomassie Brilliant Blue and prepared for fluorography with Amplify (Amersham). For quantification, bands were cut out, solubilized, and counted. The number of molecules was calculated as described (Cline et al., 1985).

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