Deletion Mutants of Chlorophyll *a/b* Binding Proteins Are Efficiently Imported into Chloroplasts but Do Not Integrate into Thylakoid Membranes[1]

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ABSTRACT

Chlorophyll *a/b* binding polypeptides (CABp) are integral thylakoid membrane proteins containing three membrane-spanning helices. We have created a series of mutations in tomato CABp to test whether individual membrane helices with hydrophilic flanking sequences, when fused to a transit peptide, can be imported into chloroplasts and correctly targeted to thylakoid membranes. All of the mutated precursors, including those with large C-terminal and internal deletions, were imported successfully, showing that these regions of the mature CABp are not required for import into chloroplasts. All mutants tested, containing either one or two membrane helices, were found primarily in the stroma and not in the thylakoids. The small amount of protein found associated with the thylakoids was largely resistant to alkali extraction but was sensitive to protease, unlike wild-type protein, which is resistant to both treatments. When incubated with thylakoids in the absence of stroma and/or ATP, a significant amount of wild-type protein assumes a form that is resistant to alkali extraction but is protease sensitive, like the imported deletion proteins. This form of the wild-type protein is not chased into a protease-resistant form by adding stroma and/or ATP. These results suggest that CABp can spontaneously associate with membranes as an aberrant species that is not an intermediate in the process of integration. The inability of the deletion forms of CABp to assume a protease-resistant conformation suggests that correct integration is afforded by elements within the entire protein that collectively contribute to the proper conformation of the protein. The ability of deletion mutants to associate with thylakoids in a nonphysiological way suggests that the study of such mutants may not be useful in elucidating thylakoid-targeting signals.

CABp[3] are the abundant, nuclear-encoded proteins that make up the LHC of PSI and PSII. Sequence analysis indicates

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[3] Abbreviations: CABp, Chl *a/b* binding protein; LHC, light-harvesting complex; RBCS, small subunit of ribulose-bisphosphate carboxylase; SH, 0.33 M sorbitol, 50 mM Hepes (pH 8.0); TE, 10 mM Tricine (pH 7.8), 2 mM EDTA; TGR, Tris-glycine resuspension buffer (12.6 mM Tris, 96 mM glycine, 5 mM EDTA), pH 8.0.

that each of the CAB proteins contain three membrane-spanning regions and two conserved domains flanking and including the first and third membrane-spanning α-helices (7). The analysis of predicted amino acid sequences and results of proteolytic mapping of LHC II CAB proteins have led to the proposal of a model describing the topology of CAB proteins within the chloroplast thylakoid membrane (9). This model predicts that LHC II CAB proteins are oriented with the N terminus in the stroma and the C terminus in the lumen. The recent elucidation of the three-dimensional structure of LHC II at 6 Å resolution confirms this predicted model (14).

CAB proteins are synthesized in the cytosol in a precursor form with a transit peptide at their N terminus. The transit peptide is necessary to target these proteins to the chloroplast and to initiate their translocation across the chloroplast envelope membranes (for review, see ref. 10). In the case of CAB proteins, it has been argued that the C terminus may facilitate transport because several C-terminally truncated CAB precursors failed to be imported into the chloroplast (3).

Following import into the chloroplast, the transit peptide is not required for targeting and integration of CAB proteins into the thylakoid membrane. Both Lamppa (17) and Hand et al. (8) found that a chimeric protein consisting of the mature part of CAB protein fused to the transit peptide of RBCSp, a stromal protein, was normally imported into isolated chloroplasts and integrated into the thylakoid membrane.

Cline (4) and Chitnis et al. (2) independently demonstrated that CAB polypeptide integrated into isolated thylakoids in the presence of stroma, Mg^{2+} and ATP. The assay exhibited physiological specificity because envelope membranes could not substitute for thylakoids. Viitanen et al. (26) used this *in vitro* assay to demonstrate that CABp lacking the transit peptide was able to integrate into isolated thylakoids. Although CAB polypeptides are hydrophobic, they are maintained in a soluble form that is competent for integration by complexing with a factor in the stroma (19, 21). CABp complexed to the stromal factor is still incapable of integration into the thylakoid unless additional stroma is supplied, suggesting that additional stromal factor(s) is required (19).

The aforementioned observations are consistent with the notion that signals for targeting to and integrating CAB polypeptides into the thylakoid membrane are found in the mature portion of the protein. Kohorn et al. (13) constructed a series of deletion mutants of CAB protein to define structural fea-

tures required for targeting and integration. The six deletions covered the entire mature protein. Mutants lacking the loop between the second and third membrane-spanning region or the third membrane-spanning region failed to be detected in the chloroplasts. Deletions in other regions did not prevent the protein from associating with the thylakoid, although these proteins had incorrect membrane topology and failed to assemble into LHC. Kohorn and Tobin (12) additionally found that, when the third membrane-spanning region was fused to the C terminus of RBCSp, the chimeric protein was associated with thylakoid membranes. Fusion of RBCSp to the first or second membrane-spanning regions did not result in this membrane association. Based on these experiments they proposed that the third membrane-spanning region may contain information required for targeting or integration of CABp into the thylakoid.

If the C terminus of CABp could direct RBCSp to the thylakoid, we reasoned that the individual helix and flanking sequence alone should integrate into the thylakoid. In the present study, we have constructed a series of CAB mutants containing a transit peptide fused to one or two membrane helices and flanking sequences from a CABp. We demonstrate that these proteins are very effectively imported into chloroplasts but the proteins are primarily found in the stroma. Some mutant protein remains with the thylakoid as a species that is not extracted by alkali treatment but is sensitive to protease. In this case, we say the protein has associated with the membrane. We can imagine a state where hydrophobic regions have looped into the membrane, but hydrophillic regions have not been translocated across, leaving the protein protease sensitive. In this case, we would say the protein has inserted into the membrane. Conceivably, this species may be an intermediate in the integration process. We reserve the term integration for the process by which the peptide becomes protease resistant.

To address the physiological relevance of the membrane-associated forms of the mutant CABp, we have also studied the intrinsic ability of wild-type CABp to integrate into different types of membranes. We found that wild-type protein, like the mutant proteins, can spontaneously associate with membranes to form a species resistant to alkali treatment but sensitive to protease. Evidence is presented to suggest that this species is not an intermediate in the integration process. From our observations, we conclude that the deletion mutants do not associate with the membrane in a physiological manner and hence do not reveal information about the structural features in CAB proteins that are required for targeting to the thylakoid. Our results support the notion that targeting to and integration of CABp into thylakoid membranes require complex interactions among different domains and at more than the primary structural level of the protein.

MATERIALS AND METHODS

Reagents

Thermolysin was from Boehringer, Percoll and cloning enzymes were provided by Pharmacia, ^{35}[S]methionine and dog microsomes were from Amersham, and SP6 Polymerase and RNasin were from Promega.

## Construction of Clones and in Vitro Expression of Proteins

Cab4BstEII was prepared from Cab4 (20) by site-directed mutagenesis using the oligo 5'-AAATTCGGTGACCCTG-TTTGGTTC-3' (15). It resulted in the creation of a BstEII site at nucleotide 434 and changed E_{127} to D and A_{128} to P. Cab4.23 was created by cutting cab4BstEII with ScaI and BstEII, filling in the sticky end with Klenow and 4dNTP, and religating the plasmid. Cab4.3 was created by cutting cab4 with ScaI and BglII, partially filling in the sticky end with Klenow and dATP and dGTP, blunt ending with S1 nuclease, and religating the plasmid. The sequence of both cab4.23 and cab4.3 were verified by dideoxy sequencing of the double-stranded plasmid (23). The template for 4.1p was prepared by linearizing cab4BstEII with BstEII, the template for 4.2p was prepared by linearizing cab4.23 with BglII, and the template for 4.12p was prepared by linearizing cab4 with BglII. The templates for 4p, 4.3p, 4.23p, and 3p were the unlinearized plasmids cab4, cab4.3, cab4.23, and cab3 (20). The plasmids were transcribed using SP6 Polymerase and translated in the presence of ^{35}[S]methionine using a wheat germ extract (18). A diagram of the constructs is shown in Figure 1. For CAB4.2p and 4.23p, amino acids corresponding to G_{126}, E_{127}, and A_{128} have been changed to C, D, and P. In CAB4.3p, the amino acid corresponding to I_{188} has been changed to F.

Import Experiments

Chloroplasts were isolated from 8- to 10-d-old peas (Pisum sativum) as described by Cline (4). Precursor proteins, 2 × 10^6 cpm, were incubated with isolated chloroplasts (300 μg Chl in 1 mL import buffer), treated with thermolysin, and reisolated through 40% Percoll as described by Cline (4). Thylakoids and stroma were prepared by breaking chloroplasts in 1 mL of TGR and centrifuging at 13,000g for 2 minutes. The stroma was collected, precipitated with 10% TCA, and resuspended in 20 μL of SDS solubilization buffer (0.2 M Tris/HCl [pH 6.8], 10% glycerol, 8% SDS, 20% β-mercaptoethanol, and 0.04% bromophenol blue). The thylakoid pellet was resuspended in 100 μL of TGR and divided into three 30-μL aliquots. One aliquot was untreated, the second was mixed with 30 μL of 0.2 N NaOH, and the third was mixed with 30 μL of 0.2 mg/mL of thermolysin. All three tubes were left on ice for 30 min, diluted with 1 mL of TGR, centrifuged as above, and resuspended in 20 μL of SDS solubilization buffer.

Integration Experiments

Chloroplasts were lysed by resuspension in 10 mM Hepes (pH 8.0), The suspension was centrifuged in a microfuge for 2 min. The thylakoid membrane pellet was separated from the supernatant and resuspended in SH to a Chl concentration of 2.5 to 3.0 mg/mL. The supernatant containing the stroma was centrifuged again at 75,000 rpm for 5 min in a TLA-100.3 rotor (Beckman) to remove any remaining thylakoid or envelope membranes. Integration reactions typically included thylakoid membranes (100 μg of Chl), stroma fraction (equivalent of 100 μg of Chl), 10 mM Mg·ATP, and 1 × 10^6 cpm of precursor. In some experiments, thylakoid membranes
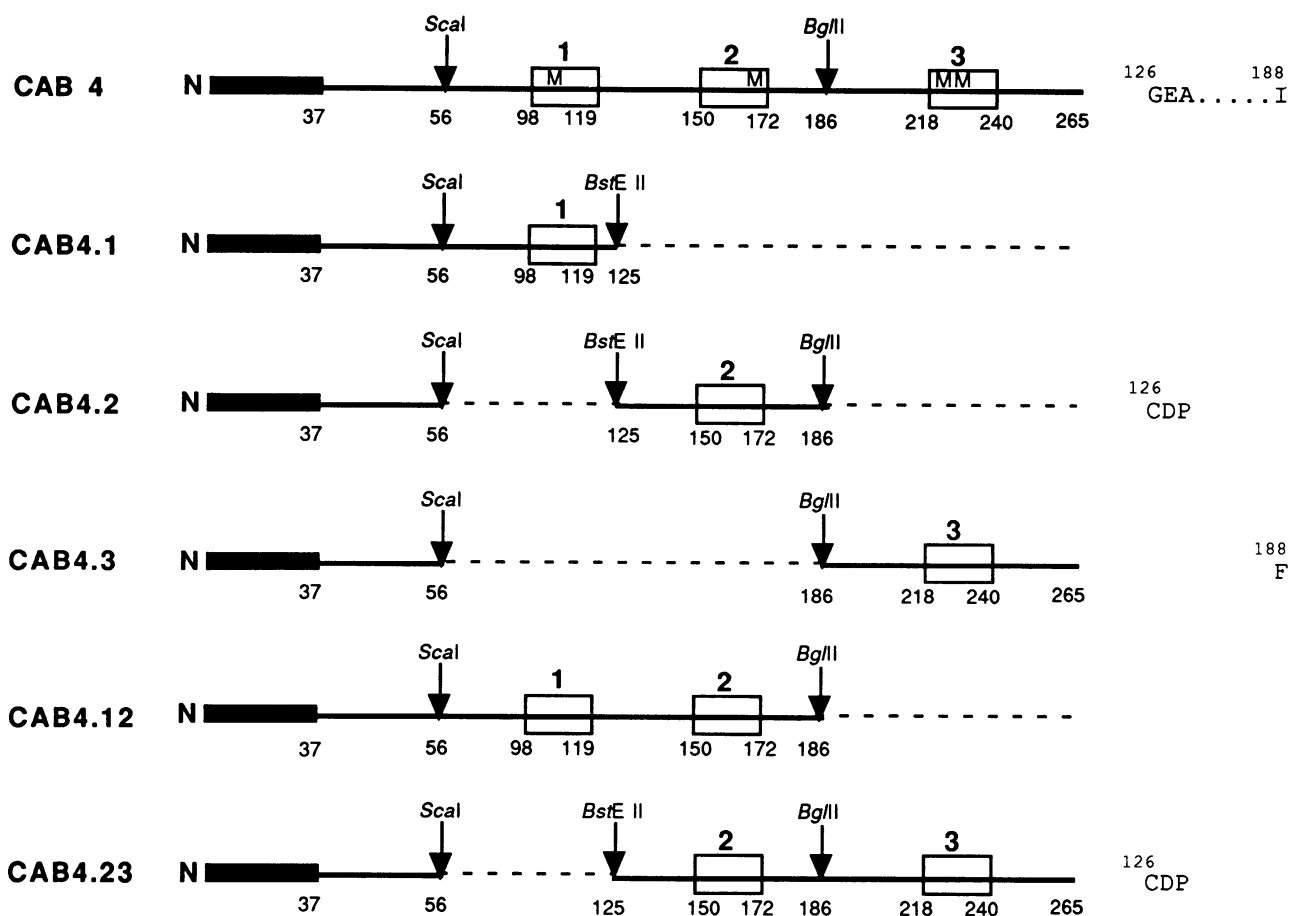
Figure 1. Diagram of cab4 gene constructs used to synthesize deletion proteins. The DNA sequence corresponding to the transit peptide is indicated by the solid box. The position of the membrane-spanning regions are indicated by the open boxes. Numbers refer to the amino acid sequence of CAB4p where the initiating methionine is 1. Another five methionines are at positions 37, 106, 168, 221, and 224. Relevant restriction sites are indicated by the arrows. The BstEII site at position 125 was created by oligo-directed mutagenesis. Deleted regions are indicated by dashed lines. Changes from the corresponding amino acids in the wild-type sequence are indicated on the right of the figure.

were substituted with either chloroplast envelopes (400 ng of carotenoids), dog microsomes (10 μL), or soybean lecithin liposomes (200 μg). Following integration reactions, thylakoid membranes were centrifuged as before, and the other types of membranes were centrifuged in a TLA-100.3 rotor at 75,000 rpm for 5 min. Membranes from either import or integration reactions, at a final Chl concentration of 0.5 mg/mL, were routinely treated with 0.1 N NaOH or 0.1 mg/mL of thermolysin for 30 min at 0°C. The membranes were then separated, washed in TGR, and solubilized in SDS-PAGE solubilization buffer.

Preparation of Envelopes and Liposomes

Chloroplast envelopes were isolated by a modification of a method described previously (11). Chloroplasts were resuspended in 0.6 M sucrose in a solution of 10 mM TE, to a final concentration of 3 mg Chl/mL. They were then frozen for 60 min, thawed, and diluted with two volumes of TE. This solution was centrifuged in a microfuge for 5 min. The supernatant was transferred to another tube, underlayered

with 0.2 mL of 1 M sucrose in TE and centrifuged at 75,000 rpm for 20 min in a TLA-100.3. The yellow band at the interface was removed, diluted with 10 mM Hepes (pH 8.0), and centrifuged again in the same rotor at the same speed for another 10 min. The pellet was resuspended in SH, spun down again as above, and resuspended in a minimal volume of SH to give a final carotenoid concentration of 20 ng/μL.

Liposomes were prepared from soybean lecithin (Sigma) by sonication, as previously described (1), at a final concentration of 10 μg/μL.

Gel Electrophoresis and Analysis

Solubilized membrane proteins were resolved by SDS-PAGE as described by Laemmli (16) or Schagger and von Jagow (24). Gels were routinely fluorographed with Autofluor (National Diagnostics) following the manufacturer's directions, scanned by a PhosphorImager instrument (Molecular Dynamics) for quantitation of radioactive bands, and exposed to X-ray film for documentation.

RESULTS

### C-Terminal Deletions Did Not Disrupt Import of CAB Precursors into the Chloroplast

Figure 1 illustrates the mutants constructed for this study. They were made from tomato *cab4,* a PSII gene (20). For CAB4.1 and CAB4.12, the protein was truncated after either the first or second membrane-spanning helices, respectively. CAB4.2, 4.3, and 4.23 contain the appropriate membrane-spanning helices and flanking regions fused to the CAB4 transit peptide. The precursor forms of these proteins are located in lanes marked P in Figure 2. Each protein was synthesized as one predominant band by a wheat germ extract.

When imported into chloroplasts, each of the proteins was processed into lower molecular mass forms. All proteins were imported into the chloroplast with a magnitude comparable to CAB4p (Table I). CAB4.1p, which lacks >50% of the C-terminus, was imported even more effectively than wild-type protein, demonstrating that the C terminus is not required for efficient import.

Deletion Mutants Are Found Primarily in the Stroma

Helix III, near the C terminus of CABp, has been suggested to contain information essential for thylakoid integration or targeting of the CABp (12, 13). We reexamined this point by

Figure 2. Import of CAB4p and deletion proteins into pea chloroplasts. *In vitro* translation products were imported into isolated pea chloroplasts and fractionated into stroma and thylakoids as described in "Materials and Methods." Samples in A to D were electrophoresed on a 14% acrylamide gel (24) and in E and F on a 15% acrylamide gel (16). Gels were soaked in Autofluor, dried, and fluorographed for 12 d (A, C, and D), 5 d (B), or 7 d (E and F). A, CAB4p; B, CAB4.1p; C, CAB4.2p; D, CAB4.3p; E, CAB4.12p; F, CAB4.23p. Lanes P, *In vitro* translation products of precursor proteins; lanes M, thylakoid membranes washed with buffer; lanes A, thylakoids washed with 0.1 N NaOH; lanes T, thylakoids treated with 0.1 mg/mL thermolysin; lanes S, stromal fraction. Molecular masses of the major bands are indicated to the left.
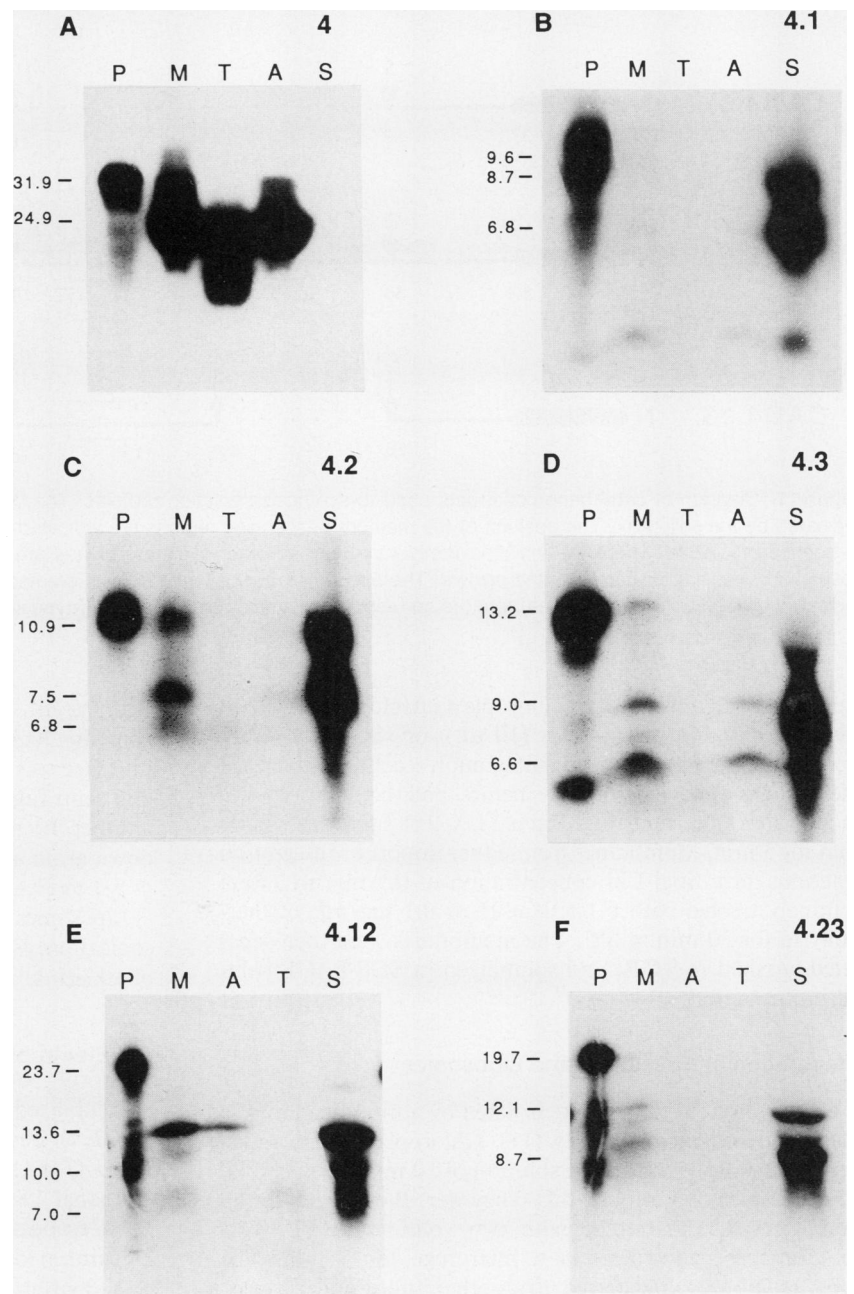
Table I. *Quantitation of Gels Used in Figure 2*

Gels were quantitated by radioanalytic imaging using a Molecular Dynamics Phosphorimager. Results are summarized from a single experiment; however, consistent results were obtained in at least five separate experiments.

Protein	Import Relative to CAB4[a]	% in Thylakoid[b]	% Alkali Resistant[c]	% Protease Resistant[d]
CAB4	1.00	96	96	75
CAB4.1	1.85	2.4	1.1	0.25
CAB4.2	0.66	10.2	4.5	1.1
CAB4.3	0.47	12.1	7.9	1.1
CAB4.12	0.76	16.2	9.6	4.4
CAB4.23	0.41	5.3	BB[e]	BB

[a] Sum of radioactivity in the buffer-washed thylakoid and stromal fraction expressed relative to CAB4.   [b] Ratio of [^{35}S]methionine in the buffer-washed thylakoids to total imported protein × 100.   [c] Ratio of [^{35}S]methionine in alkali-washed thylakoids to total imported protein × 100.   [d] Ratio of [^{35}S]methionine in protease-treated thylakoids to total imported protein × 100. All methionines are found in membrane-spanning helicies and should be quantitatively recovered in protease-protected fragments when proteins have integrated into the membrane.   [e] BB radioactivity was below background.

testing whether proteins containing only one or two of the three helices could be integrated into the thylakoid.
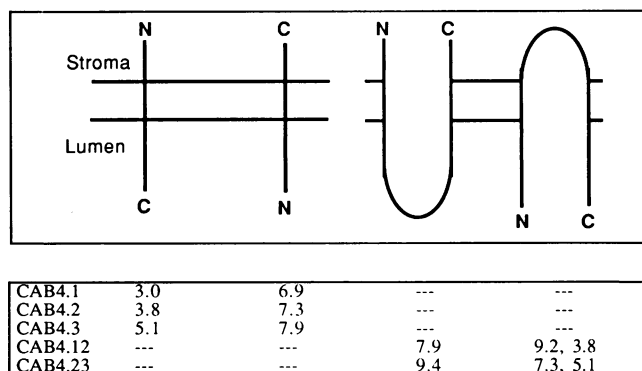
Figure 2 shows a fluorogram from a representative experiment with CAB4p and the five deletion proteins. Some of the lanes are overexposed to see the radioactivity in the membrane fraction. In the case of CAB4p imported into the chloroplast, >96% of the protein was found in the thylakoid membrane fraction as a single processed form with an apparent molecular mass of about 25 kD (Fig. 2A, lane M; Table I). Labeled protein found in the stroma was degraded to 6- to 7-kD species (data not shown). When thylakoids containing the radioactive CAB4p were washed with 0.1 M NaOH, none of the radioactivity was removed (Fig. 2A, lane A). Treatment of the membranes with thermolysin resulted in the formation of degradation products that were slightly smaller; combined, the bands contained approximately 75% of the radioactivity of the untreated thylakoid (Fig. 2A, lane T; Table I). CAB4p contained four Met residues, one in both the first and second membrane-spanning domains and two in the third (Fig. 1). The slight loss in radioactivity resulting from protease treatment was reproducible and suggested that a small population of the imported, membrane-associated wild-type protein was cleaved by protease.

In contrast, mutant proteins were found primarily in the stroma. The imported 4.1p yielded major processed forms of about 8.7, 6.8, and <3 kD, about 2.4% of which was associated with the membrane (Fig. 2B, lane M; Table I). Slightly <50% of the membrane-associated 4.1p was still present after the alkaline wash. Protease treatment digested the membrane-associated forms to <3-kD species and reduced the radioactivity to approximately 10% of the amount found in the buffer-washed thylakoids. CAB4.2p and 4.3p were also found primarily in the stroma (Fig. 2, C and D). When compared with 4.1p, slightly higher amounts, 4.5 and 7.9% of the imported protein, respectively, remained associated with the

alkali-washed thylakoids. A portion of imported 4.2p and 4.3p was not processed, possibly because the context of the normal processing site was altered in the construction of the fusion protein. Following import, 4.2p gave rise to two major processed forms of 7.5 and 6.8 kD, respectively. CAB 4.3p was processed down to a number of polypeptides; the major bands were located at 9 and 6.6 kD. In both cases, the thylakoid forms of these proteins were not nearly as susceptible to alkali extraction as they were to protease digestion. Both 4.2p and 4.3p were degraded by thermolysin to forms smaller than 7 kD, and these forms accounted for approximately 10% of the radioactivity originally present in thylakoids washed only with buffer.

Figure 2, E and F show the results of an import experiment with the proteins containing two membrane-spanning domains, 4.12p and 4.23p. Following import into chloroplasts, CAB4.12 was processed to a major 13.6-kD form and minor forms of 10 and 7 kD. The 13.6-kD band was somewhat resistant to extraction with alkali, suggesting that it was associated with the membrane, but it was degraded to a 7-kD species by protease treatment. Of the deletion proteins, 4.12p was associated with the membrane to the greatest extent; 9.6% of the imported protein was associated with alkali-washed thylakoids (Table I). 4.23p was processed to forms of 12.1 and 8.7 kD. The thylakoid form of 4.23p was largely washed out with alkali and was degraded by thermolysin treatment to polypeptides of <6 kD. Thus, 4.23p was even less effectively associated with the membrane than was 4.12p. As was noted for the proteins with one helix, most of 4.12p or 4.23p remained in the stroma.

Resistance to alkali extraction has traditionally been interpreted as evidence that a protein is interacting with the membrane. We do not know to what extent the hydrophobic portions of the protein have inserted into the membrane. The fact that they were largely sensitive to protease meant that a significant portion of the protein was exposed. Figure 3 shows diagrams of the conceivable membrane orientations of the

	N	C	N	C	
Stroma					
Lumen					
	C	N		N	C

CAB4.1	3.0	6.9	---	---
CAB4.2	3.8	7.3	---	---
CAB4.3	5.1	7.9	---	---
CAB4.12	---	---	7.9	9.2, 3.8
CAB4.23	---	---	9.4	7.3, 5.1

Figure 3. Predicted molecular masses of protease-protected fragments of integrated deletion proteins. The diagram represents integrated forms of proteins containing one or two membrane-spanning domains. The table below shows molecular masses in kilodaltons calculated assuming that the deletion proteins were integrated into the thylakoid in either orientation and the stromal domains were completely digested by protease.

CAB4 deletion proteins and their predicted molecular masses after protease treatment. In the cases of 4.2p, 4.3p, and 4.12p, in which some protease-resistant polypeptides were observed, the predicted protease-protected fragments are small enough to be consistent with the observed polypeptides. Hence, we cannot rule out the possibility that the resistance was afforded by protection from the membrane rather than an inherent property of the polypeptide.

## Cab Deletion Polypeptides Associate with Thylakoid Membranes in the Absence of Stroma

To test whether the deletion proteins had integrated into the thylakoids, we examined how they behaved in the in vitro integration assay developed by Cline (4) and Chitnis et al. (2). For wild-type protein, they have established that stroma and ATP are required for proper integration of CABp into the thylakoid. We tested whether stroma was required for the thylakoid association of 4.2p, 4.3p, and 4.12p and examined the resistance of these polypeptides to protease.

Figure 4 shows the results of a representative experiment. All three proteins associated with the thylakoid to the same extent in the presence or absence of stroma. Alkali treatment did not remove the proteins, indicating that the proteins had associated with the membrane (Fig. 4 cf. lanes M and A). Clearly, the mutant proteins did not require stroma to interact with the membrane. Protease treatment degraded each of the three polypeptides into lower molecular mass polypeptides (Fig. 4, lanes T). The pattern of these polypeptides on gels was the same whether or not the in vitro assays were conducted in the presence of stroma. Because stroma is required for integration of wild-type CAB, it is unlikely that the deletion proteins had integrated into the thylakoids either in the absence or presence of stroma. The protease resistance exhibited by the deletion proteins is most likely due to an inherent property of the polypeptides. This property of the polypeptides

most likely accounts for the small degree of protease resistance observed in the import experiments.

Association of CAB Protein with Different Membranes

Because the mutant proteins could associate with thylakoid membranes in an alkali-resistant form in the absence of stroma, we investigated whether the same was true for wild-type CABp. Our experiments were done with tomato CAB3p, a protein closely related to CAB4p but which integrates more efficiently into thylakoids in the in vitro assay. The in vitro assay for CABp integration is specific for thylakoids (2, 5). Therefore, we asked whether CABp would associate with other membranes in vitro. As a positive control, we incubated CAB3p with lysates and Mg-ATP. We compared the forms generated from this treatment with those generated from incubating CAB3p with chloroplast envelopes, dog pancreas microsomes, and liposomes prepared from soybean lecithin. No energy source or soluble chloroplast stromal factors were present in the reaction mixture. As shown in Figure 5 (lanes 1), CAB precursor was found associated with all membranes tested. Processing only occurred in the presence of stroma and ATP. Cline (4) had earlier reported that in the absence of ATP, 10% of the CABp associated with the thylakoid membrane was not extracted by alkali wash. We found that the amount of labeled protein not extracted from membranes by alkali treatment was 20% for thylakoids and envelopes, 50% for microsomes, and 80% for liposomes (Fig. 5, lanes 2), suggesting that CABp possesses an intrinsic capability to interact with the lipid phase. In each case, except lysates, the protein was sensitive to protease (Fig. 5, lanes 3), indicating that the major bulk of the membrane-associated protein was still exposed to the stroma. The same results were observed when Mg-ATP was also included (data not shown). For all membranes except thylakoids, CAB3p remained protease sensitive when stroma was included (data not shown). Thus,
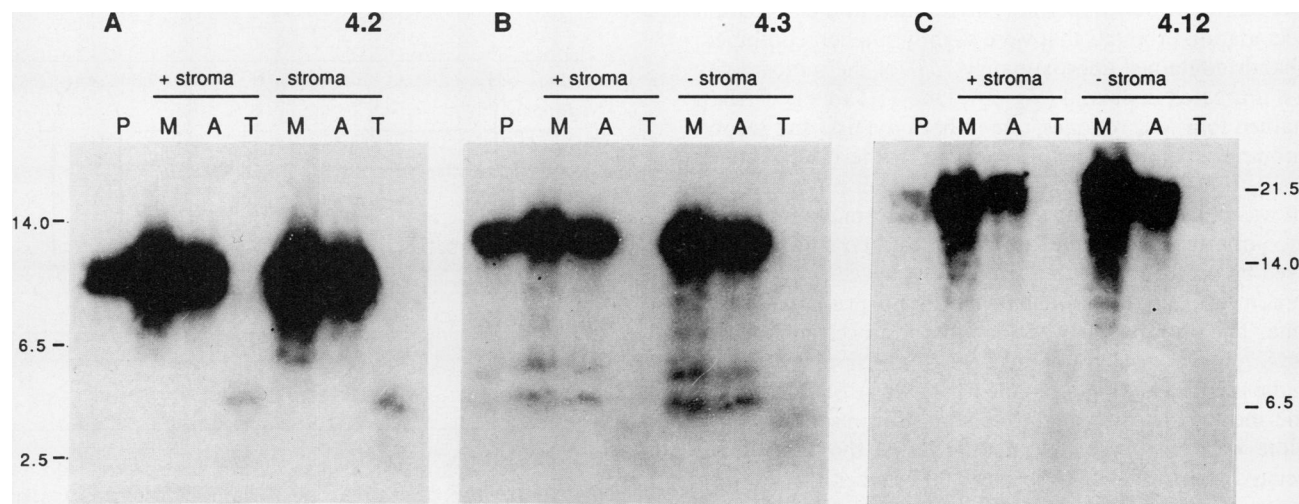
Figure 4. Insertion assay of deletion proteins with pea thylakoids in the presence and absence of stroma. In vitro translation products of deletion proteins were incubated with isolated thylakoids in the presence or absence of a stromal extract as described in "Materials and Methods." Samples were electrophoresed on a 14% acrylamide gel (24) and processed as described in Figure 2. Gels were exposed to film for 2 d. A, CAB4.2; B, CAB4.3; C, CAB4.12. Lanes are labeled as in Figure 2. Molecular mass markers for A and B are to the left and for C to the right.
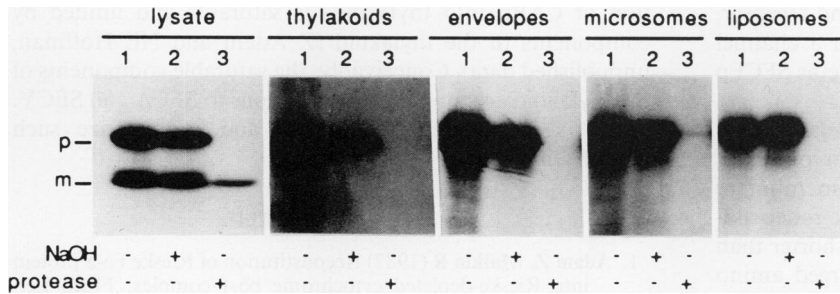
Figure 5. Insertion assay of CAB3p with various membranes. CAB3p synthesized *in vitro* was incubated with chloroplast lysates, thylakoids, chloroplast envelopes, dog microsomes, or liposomes. Only the lysates contained stroma and Mg-ATP. Samples were electrophoresed on a 13% acrylamide gel (16) and processed as described in Figure 2. Lane 1, Membranes were washed only in TGR; lane 2, membranes were incubated in alkali; lane 3, membranes were incubated with thermolysin. The precursor (p) and mature (m) forms of the protein are indicated to the left.

CABp, in the absence of stroma or ATP, will associate with an assortment of membranes and will not display an inherent preference for thylakoids. Correct integration only occurs in thylakoids and requires stroma and ATP.

One can envision a process in which the initial insertion into the membrane occurs spontaneously due to the hydrdophobic nature of CAB protein; only later stromal factor and ATP might play a role in translocating hydrophillic segments across the membrane. If the alkali-resistant form of CAB is an intermediate, we should be able to convert the alkali-resistant protease-sensitive form to a protease-resistant form by adding stroma and ATP to thylakoids containing membrane-associated CAB. To test this idea, thylakoid membranes were isolated from a reaction mixture lacking stroma, washed with 10 mM Hepes (pH 8.0), and resuspended in stroma and ATP. Following a 30-min incubation, no protease-resistant species could be observed (not shown), indicating that a membrane-associated protein cannot further integrate to a protease-resistant form. Collectively, these data indicate that CABp has an intrinsic ability to associate with membranes, but this is an irrelevant process and distinct from the mechanism that occurs *in vivo*.

DISCUSSION

C-terminal deletions, ranging from 79- to 140-amino acid residues, of CAB4p were imported into isolated chloroplasts with import efficiencies comparable to those of the wild type (Table I). These findings are in disagreement with a previous report (3) in which deletions of 27, 78, and 91 residues from the C terminus completely abolished the import ability of a CAB precursor from wheat. CAB4.12p is a close approximation of pΔ78 (3), and it is not clear what caused the discrepancy. CAB4.1p contains even a larger deletion, and it consistently imported better than wild type. Clearly, the requirement for the C terminus of CABp is not a general requirement for import. As expected from studies that show that the transit peptide is sufficient for import, large internal deletions did not abolish import, although the apparent import efficiency was reduced.

Although the deletions had a relatively small effect on import efficiency, these deletions eliminated the ability of the proteins to integrate into the thylakoids; <10% of the imported mutant proteins were found in the alkali-treated thylakoids. The minor association of mutant protein with the thylakoid was thought to be artifactual in nature for the following reasons: (a) the thylakoid forms of the deletion proteins were largely sensitive to protease; the protease resistance observed was likely due to an inherent property of the polypeptide and not from membrane protection. (b) CABp that had associated with the thylakoid could not be converted into a protease-resistant form, and therefore, the membrane-associated protein form is not likely to be an intermediate in the integration process. (c) Wild-type CABp can only integrate into thylakoids but can associate with all the membranes tested, *i.e.* thylakoids, envelopes, microsomes, and liposomes. Therefore, wild-type CAB can form a strong but biologically irrelevant association with each membrane tested.

Previously, it was argued that helix III in the C-terminal portion of CABp contains essential information for thylakoid targeting based on two types of observations: (a) Deletion of helix III prevented thylakoid association of a CAB protein or rendered it highly unstable in the chloroplast (3, 12, 13). (b) When fused with RBCSp, only helix III, but not helix I or II, caused the fusion protein to associate with thylakoids (12). Our results and interpretations are at odds with the previous observations. We found that all our deletion proteins were imported with high efficiency, and proteins were stable in the chloroplast. We also observed that CAB protein can interact with membranes in an artifactual manner. Resistance to proteolysis seems to be a reliable criterion for integration; however, resistance to alkali extraction fails to distinguish between polypeptides that have been correctly targeted but failed to integrate from those nonspecifically associated with the membrane. To meaningfully interpret experiments with deletion proteins and fusions, it is essential to demonstrate that these test proteins are interacting with the membrane in a productive way. Because we were unable to demonstrate that any of our deletion proteins were productively interacting with the thylakoids, our results cannot support the view that the C terminus has a special role in targeting or integration. Our results are consistent with the idea that multiple parts of the protein are involved in these processes.

Support for the endosymbiont hypothesis has spurred parallels to be drawn between the translocation of proteins through *E. coli* cytoplasmic membranes and the translocation of chloroplast proteins through the thylakoid membranes (25). In *E. coli*, protein translocation and integration into cytoplasmic membranes is largely dependent on the *Sec A, B, Y,* and *E* gene products (28). SECBp is a chaperonin that binds to the secretory protein and maintains it in a translocation-competent conformation. The SECBp-precursor complex as-

sociates with the peripheral membrane protein and receptor, SECAp, and the protein is translocated through a channel possibly composed of the integral membrane proteins SECEp and SECYp.

A small number of proteins such as M13 procoat protein (6), Pf3 coat protein (22), and a mutated form of leader peptidase (27) insert into *E. coli* cytoplasmic membranes spontaneously. von Heijne (27) concluded that Sec-independent translocation occurs for polypeptide regions shorter than 60 to 70 residues containing few positively charged amino acid residues. Could the structure of CABp allow spontaneous insertion? The regions of CABp presumably translocated across the thylakoid membrane are the first loop connecting the first and the second transmembrane helices and the C terminus. These regions are about 18 and 23 residues long, respectively, and contain not more than two positively charged residues. Therefore, based on the length and the charge distribution of the translocated regions, spontaneous insertion of CABp into the thylakoid membrane could be expected based on von Heijne's analysis. In fact, we did observe a spontaneous *in vitro* association of CAB protein with different types of membranes. However, the ability of CAB to associate with numerous membranes *in vitro* conflicts with its *in vivo* distribution solely in the thylakoids. Alkali extractability is not a sufficient criterion for distinguishing between protein that has embedded into the lipid bilayer and that is sticking to the membrane in an undefined way. Although we may not easily distinguish between these two possibilities, we may ask the more biologically relevant question as to whether the membrane-associated species is an intermediate in the integration process. We have attempted to do this by examining whether the membrane-associated species can be converted into a protease-resistant form upon addition of stroma and ATP. Because it could not, we believe that the membrane-associated form is an aberrant CAB species and not an intermediate in the process. Conceivably, the stroma factor minimizes this type of nonproductive membrane association and facilitates binding of CABp complex to a receptor in the thylakoid.

Experimental evidence establishing a parallel between the protein export machinery in *E. coli* with that in the thylakoids is indirect. The stromal factor that complexes with CABp and keeps it soluble and integration competent is a likely functional analog to SECB. The fact that CABp, in the presence of stroma, can only integrate into thylakoids and not other membranes, such as envelopes, indicates that factors responsible for the specificity reside in the thylakoid. Presumably, both thylakoids and envelopes contain distinct receptors that recognize unique features of thylakoid and envelope proteins, respectively. Consistent with the idea that protein machinery required for integration resides within the thylakoid, Cline (5) found that pretreatment of thylakoids with protease eliminates the ability of CABp to integrate. Washing thylakoids with $NaCO_3$ or 2 M NaBr did not inhibit CABp integration, implying that the protein machinery is tightly associated with the membrane (Z Adam, unpublished data). Treatment of thylakoids with 0.1 N NaOH did inhibit integration (Z Adam, unpublished data). However, this effect may be on the lipid environment or denaturation of protein rather than extracting protein from the membrane. Recently, we found that integra-

tion of CABp into thylakoids is saturable and limited by components in the thylakoid (Z Adam and NE Hoffman, unpublished data). Conceivably, the saturable components of the thylakoid are functionally analogous to SECA and SECY. Work is in progress to identify and characterize such components.

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