Signal recognition particles (SRPs) have been identified in organisms as diverse as mycoplasma and mammals; in several cases these SRPs have been shown to play a key role in protein targeting. In each case the recognition of appropriate targeting signals is mediated by SRP subunits related to the 54-kDa protein of mammalian SRP (SRP54). In this study we have characterized the specificity of 54CP, a chloroplast homologue of SRP54 which is located in the chloroplast stroma. We have used a nascent chain cross-linking approach to detect the interactions of 54CP with heterologous endoplasmic reticulum-targeting signals. 54CP functions as a bona fide signal recognition factor which can discriminate between functional and non-functional targeting signals. Using a range of authentic thylakoid precursor proteins we found that 54CP discriminates between thylakoid-targeting signals, interacting with only a subset of protein precursors. Thus, the light-harvesting chlorophyll a/b-binding protein, cytochrome f, and the Rieske FeS protein all showed strong cross-linking products with 54CP. In contrast, no cross-linking to the 23- and 33-kDa protein of the oxygen-evolving complex were detected. The selectivity of 54CP correlates with the hydrophobicity of the thylakoid-targeting signal and, in the case of light-harvesting chlorophyll a/b-binding protein, with previously determined transport/integration requirements. We propose that 54CP mediates the targeting of a specific subset of precursors to the thylakoid membrane, i.e. those with particularly hydrophobic signal sequences.
plication of these observations is that there may be specific signal-binding components that commit subsets of precursors to these different thylakoid-targeting/integration routes.

In this study we have used a cross-linking assay to establish that 54CP is a major signal-sequence-binding protein present in chloroplast stroma (cf. Ref. 6). Cross-linking analysis with authentic thylakoid precursors established that only a subset of these precursors interact with 54CP. The cross-linking efficiency was correlated to the hydrophobicity of the thylakoid-targeting signal suggesting that this feature is the crucial determinant which promotes 54CP binding. This proposal is supported by a dramatic increase in 54CP binding to a mutant carboxypeptidase Y signal sequence with increased hydrophobic character.

On the basis of these data we propose that 54CP interacts preferentially with thylakoid precursor proteins that have particularly hydrophobic signals. Hence, the SRP dependent targeting route may be particularly important for the delivery of integral membrane proteins to the thylakoid membrane.

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction enzymes were from New England Biolabs, Taq polymerase was from Boehringer Mannheim, and RNA polymerases were from Promega. The rNTPs and RNAsin were obtained from Pharmacia Biotech Inc. Cycloheximide and 7-methyl-guanosine 5’-monophosphate were from Sigma. [35S]Methionine was purchased from DuPont and cross-linking reagents were from Pierce and Prochem. The anti-P48 serum was raised against the purified protein (a gift from I. Sinning, EMBL, Heidelberg). Antibodies against mammalian SRP54 were provided by Mary-Jane Gething, I. Sinning, EMBL, Heidelberg, and N. Hoffman, Stanford University, respectively. The wild type and mutant CPY constructs were kindly provided by Mary-Jane Gething, University of Melbourne.

**Plasmid Constructs—**The PPL wild type and mutant constructs have been previously described (6). The coding region for the complete pea cytochrome f precursor was excised from pT7CP-35 (29) as a 1.5-kilobase Asp7181-SalI fragment, and inserted into the SalI site of pJ1101 (30) after ligation of SalI linkers (5’-CGTCGACG) to the end-filled fragment. This construct contains the SP6 promoter and the Ω translational enhancer from tobacco mosaic virus upstream of the cytochrome f coding region. The coding region of the mature pea Rieske protein was amplified by PCR from the full-length cDNA in pSP65 (31) and inserted into the SmaI site of pGEMZ3’-1 under the control of the SP6 promoter.

OE23 Met and OE33 Met are truncated constructs made from the pea iOE23 and wheat iOE33. They are designed to contain twin methionines at their COOH termini and thereby enhance labeling during cell-free translation. OE23 Met was PCR-amplified from iOE23 (32) in pGEMZ3’-1 under the control of the SP6 promoter. The PCR product contains the codons for the thylakoid-targeting domain and the first 58 amino acids of the mature protein plus two COOH-terminal methionines, it lacks a stop codon. The PCR product was inserted into the XhoI and HindIII sites of pGem 4Z under the control of the SP6 promoter. OE33 Met was amplified from wheat iOE33 (33) by PCR. The resulting PCR product contained codons for the lumen-targeting domain and the first 60 amino acids of the mature protein together with two additional methionines at the COOH terminus. No stop codons were present in the product and it was inserted into the SalI and HindIII sites of pGem 4Z under the control of the SP6 promoter.

The pLHCP coding region is the AB30 clone from pea (34) and was inserted into pSP64. The LHCP TM3-PC fusion protein was constructed using PCR splicing by overlap extension with pLHCP from pea and the plastocyanin precursor from Arabidopsis thaliana. The TM3 segment for pLHCP comprised amino acids 189–269 (the COOH terminus) while the plastocyanin segment contained the entire mature plastocyanin sequence plus the cleavage cassette AGNAMA from the lumen targeting domain. The spliced PCR product was ligated into HindIII and SalI sites of pGem 3Z under the SP6 promoter. All of the PCR-derived products were sequenced after subcloning, no alterations to the coding region were present in any of the constructs used for the subsequent cross-linking studies.

**Transcription Templates—**Templates for the transcription of truncated mRNAs were prepared either by using restriction sites within the coding region (naturally occurring and introduced) or using PCR (35). In the later case the PCR products were transcribed directly to avoid any selection of PCR-generated mutations. Plasmids encoding PPL and PPL-SSKO were linearized with PstI, cytochrome f, and TM3-PC constructs were linearized with EcoRI and the cDNA encoding the Rieske protein was linearized with AvaII. Both CFY constructs (36) were linearized with HinclI while the OE23 Met and OE33 Met constructs were linearized with HindIII. The LHCP-TM1 and LHCP-TM2 truncations were generated by PCR as described by Nilsson et al. (35). Transcription with T7 or SP6 polymerase was performed as described by the manufacturers (Promega).

**Cell Free Translation, Cross-linking, and Product Analysis—**Translation using a wheat germ lysate based system was performed as described previously (37) and mutant constructs were labeled by incorporation of [35S]Methionine. After translation for 15–20 min at 26 °C, samples were pulsed by the addition of 4 mM 7-methyl-guanosine 5’-monophosphate, incubated for a further 10 min and translation stopped by the addition of cycloheximide to 2 mM and placing the samples on ice. Ribosome bound nascent chains were then purified by centrifugation through a sucrose cushion (38). After purification, resuspended samples were incubated with one-fourth volume of crude stram extract (7.25 OD260/ml) prepared from pea seedlings (23) or with purified SRP for 10 min at 26 °C. The cross-linking reagents bis(sulfosuccinimidyl)suberate (BS3), EDC, BMH, or S-MBS, were then added to a final concentration of 1 mM, samples were incubated for a further 10 min and then the reaction quenched by the addition of one-tenth volume of 1 mM glycine, 500 mM NaCl, 50 mM Tris, and 100 mM EDTA as appropriate. Samples were then split into four equal fractions and either analyzed directly or after immunoprecipitation with antiserum specific for mammalian SRP54, Escherichia coli P48, or 54CP. Immunoprecipitation was in the absence of SDS and as described previously (39). All samples were analyzed by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gels.

**Quantitation and Estimation of Signal Sequence Hydrophobicity—**After SDS-polyacrylamide gel electrophoresis, gels were exposed to PhosphorImaging plates such that all the signals remained in the linear detection range. The plates were read using a Fujix BAS 2000 Bioimager and the resulting images were quantified using Fuji software. The amount of 54CP cross-linking product is expressed as a percentage of the total signal present as either total nascent chain (i.e. total total products) or cross-linking products). In many cases two total samples were run and the average value was then used; likewise the amounts of 54CP cross-linking product present after immunoprecipitation with anti-54CP and anti-P48 were also averaged.

The average signal sequence hydrophobicity was calculated using two scales for relative amino acid hydrophobicity, the von Heijne scale and the Kyte-Doolittle scale (both from Ref. 40). Functional signal sequences with hydrophobic stretches as short as seven amino acids have been identified (41). For this reason the values for the most hydrophobic stretch of six, seven, and eight contiguous amino acids were determined and these were then summed and averaged to give the hydrophobicity values shown in Table I. For the von Heijne scale the negative number represents the most hydrophobic sequence, while for the Kyte-Doolittle scale the largest number represents the most hydrophobic sequence. Such a simplified analysis does not take into account either the possible effects of flanking residues (i.e. those outside the calculated value), or the fact that the structure of the signal sequence may be important.

**RESULTS**

**Identification of a Signal Sequence-specific Binding Protein in Chloroplast Stroma—**We adapted an established cross-linking assay (6) to identify signal sequence-specific binding proteins present in chloroplast stroma. The previous approach utilized a UV activatable probe incorporated as a modified lysine residue during nascent chain biosynthesis. In the present analysis we initially investigated the cross-linking products obtained with different bifunctional cross-linking reagents (see also Ref. 22). A ribosome bound truncated preprolactin chain of 86 amino acids (PPL, see Fig. 1) was mixed with a normal extract from pea thylakoids and various bifunctional cross-linking reagents were added (Fig. 2, lanes 1–4). PPL86 was used since the NH₂-terminal ER-targeting signal is exposed from the ribosome and can be efficiently cross-linked to both mammalian and E. coli SRP (6). As a control, an engineered PPL construct (PPL-SSKO, see Fig. 1) with a mutated non-functional signal sequence (6) was analyzed in the same assay.
targeting signals (see Fig. 1). Cytochrome authentic thylakoid precursor proteins bearing true thylakoid-ER-targeting signals we analyzed the interaction of 54CP with were immunoprecipitated (data not shown). The different mobilities probably reflect cross-linking between different lysine residues within the nascent chain and 54CP. When PPL-SSKO was incubated with stromal factors. Cross-linking products of a similar size to those seen with cytochrome f were obtained (Fig. 3, lanes 1–4) or after the addition of S-MBS (lanes 1 and 5), BS3 (lanes 2 and 6), or BMH (lanes 3 and 7) to 1 mM each. The relative mobilities of molecular mass standards are indicated to the right of all figures in kDa. The arrowhead indicates a prominent 65-kDa cross-linking product.

Fig. 1. Outline of nascent polypeptides. The length of each truncated polypeptide is indicated in amino acids. The sequence of each targeting signal, together with the adjacent regions of polypeptide, is also illustrated. Sites of proteolytic processing are indicated by an arrowhead. Regions flanking the thylakoid-targeting signals are indicated by vertically hatched boxes, while for secretory protein precursors these regions are indicated by diagonally hatched boxes. Unless indicated by a vertically hatched box in front of the amino acid sequence, the sequence shown starts at the first residue of the nascent chain. Where the complete NH₂-terminal sequence is not given a number in italics illustrates the relative position of the first residue listed within the nascent chain. Asterisks indicate lysine residues from which BS3 mediated cross-linking can occur.

(Fig. 2, lanes 5–8). The addition of the bifunctional primary amine-specific reagent BS3 gave a strong 65-kDa cross-linking product with PPL but not with PPL-SSKO (Fig. 2, lane 2, arrowhead, cf. lane 6). Although less intense, a 65-kDa cross-linking product was also obtained with BMH (Fig. 2, lane 3). These 65-kDa cross-linking products were not observed in the absence of stroma (data not shown). Other cross-linking products were observed with both PPL and PPL-SSKO (e.g. the 26-kDa S-MBS-dependent product, Fig. 2, lanes 1 and 5) and were not further characterized.

54CP binds to functional ER targeting signals and to authentic thylakoid precursors—Immunoprecipitation of the BS3-dependent PPL cross-linking products obtained in the presence of stroma showed that the 65-kDa cross-linking product was efficiently immunoprecipitated with antisera specific for both 54CP (22) and for P48, the E. coli SRP54 homologue (Fig. 3, lanes 7 and 8). While the anti-P48 serum immunoprecipitated the 54CP cross-linking products as efficiently as the anti-54CP serum, antibodies specific for mammalian SRP54 did not recognize 54CP (Fig. 3, lane 6). A second weaker cross-linking product of 73 kDa was also immunoprecipitated under the conditions used (Fig. 3, lanes 7 and 8, asterisk). Both the 65- and 73-kDa products are still immunoprecipitated with anti-54CP serum after the samples have been denatured with SDS (data not shown). The different mobilities probably reflect cross-linking between different lysine residues within the nascent chain and 54CP. When PPL-SSKO was incubated with stroma and cross-linking induced with BS3, no 54CP adducts were immunoprecipitated (data not shown).

Having established that 54CP could bind to heterologous ER-targeting signals we analyzed the interaction of 54CP with authentic thylakoid precursor proteins bearing true thylakoid-targeting signals (see Fig. 1). Cytochrome f is a chloroplast genome-encoded protein with an NH₂-terminal cleavable thylakoid-targeting signal (42). Nascent cytochrome f showed a very similar cross-linking pattern to PPL with a major cross-linking product of 66 kDa and a weaker product of 74 kDa (Fig. 3, lane 2). Both products were recognized by antisera specific for 54CP and P48 (Fig. 3, lanes 10 and 11).

The Rieske FeS protein is a nuclear-encoded protein that is synthesized as a precursor with a cleavable chloroplast-targeting signal; the hydrophobic domain toward the NH₂-terminus of the Rieske protein functions as the thylakoid-targeting signal (43). A nascent Rieske polypeptide (Fig. 1) synthesized as a precursor with a cleavable chloroplast-targeting signal; the hydrophobic domain toward the NH₂-terminus of the Rieske protein functions as the thylakoid-targeting signal (43). A nascent Rieske polypeptide (Fig. 1) was also both substrates for mammalian SRP. The samples were split and further incubated in the absence of addition (lanes 4 and 8) or after the addition of S-MBS (lanes 1 and 5), BS3 (lanes 2 and 6), or BMH (lanes 3 and 7) to 1 mM each. The relative mobilities of molecular mass standards are indicated to the right of all figures in kDa. The arrowhead indicates a prominent 65-kDa cross-linking product.

In some cases a significant amount of nascent chain was co-immunoprecipitated with 54CP (e.g. Fig. 3, lanes 7, 8, 10, and 11; and Fig. 4, lanes 9 and 10). Such co-precipitation has been previously described with mammalian SRP54 (37) and presumably reflects the strength of the interaction between 54CP and particular protein precursors.
LHCP—The light-harvesting chlorophyll stable complex

GTP (22, 44). LHCP and 54CP have also been shown to form a

lakoids has been reconstituted and requires both 54CP and

the thylakoid membrane. LHCP integration into isolated thy-

plastocyanin coding region (TM3-PC) was produced

prepared (see Fig. 1). In addition, a chimera comprised of the

first and second transmembrane regions (LHCP-TM1/2) were

first transmembrane region alone (LHCP-TM1), or both the

first and second transmembrane regions have nearby acidic or basic amino acid res-

boxyl groups of adjacent polypeptides. All of the LHCP trans-

membrane regions have nearby acidic or basic amino acid res-

ides suitable for this approach (Fig. 1). EDC-mediated cross-

linking approach (Fig. 5) which acts to

promote a condensation reaction between free amino and car-

bonyl groups of adjacent polypeptides. For PPL and Rieske much

smaller amounts of peptide-tRNA species are observed (lanes 1, 3, and 5). The asterisk indicates a 73-kDa cross-linking product which is also

immunoprecipitated after denaturation with SDS (data not shown).

54CP Binds to the Third Transmembrane Domain of LHCP—The light-harvesting chlorophyll a/b-binding protein (LHCP) is, to date, the only thylakoid precursor that has been shown to require 54CP for its integration (22). LHCP is syn-

thesized as a precursor (pLHCP) in the cytosol, imported into

the chloroplast, and then integrated post-translationally into

the chloroplast, and then integrated post-translationally into

precursors to the thylakoid have been identified by biochemical ap-

proaches (15, 18). We determined whether the existing

classification of precursors could be correlated with 54CP bind-

ing. The 23-kDa protein of the oxygen-evolving complex (OE23) shows a ΔpH-dependent transport across the thylakoid mem-

brane (26, 27, 32) and has a twin-arginine motif characteristic of this pathway (28). In contrast, the transport of the 33-kDa

protein of the oxygen-evolving complex (OE33) is dependent

54CP cross-linking products while the third transmembrane

domain showed strong 54CP cross-linking adducts (Fig. 4, cf.

lanes 3, 6, and 7 with lanes 4, 9, and 10). This suggested that 54CP interacted specifically with the third transmembrane domain of 54CP.

In contrast to all the other signal sequences we had ana-

lyzed, the second transmembrane domain of LHCP has no

lysine residues anywhere near the stretch of hydrophobic amino acids (see Fig. 1). For this reason we also used a carbo-

diimide-based cross-linking approach (Fig. 5) which acts to

promote a condensation reaction between free amino and car-

bonyl groups of adjacent polypeptides. All of the LHCP trans-

membrane regions have nearby acidic or basic amino acid res-

ides suitable for this approach (Fig. 1). EDC-mediated cross-

linking of the three LHCP-derived nascent chains confirmed

that only the third transmembrane domain (TM3-PC) was

cross-linked to 54CP (Fig. 5, lanes 11 and 12, arrows). The efficiency of EDC-mediated cross-linking to TM3-PC was much

lower than that obtained with BS3. A similar reduction in

efficiency was seen when cross-linking products between PPL

and 54CP were generated with EDC (data not shown).

54CP Only Binds to a Subset of Thylakoid Precursors—Several distinct pathways for the targeting of protein precu-

sors to the thylakoid have been identified by biochemical ap-

proaches (15, 18). We determined whether the existing

classification of precursors could be correlated with 54CP bind-

ing. The 23-kDa protein of the oxygen-evolving complex (OE23) shows a ΔpH-dependent transport across the thylakoid mem-

brane (26, 27, 32) and has a twin-arginine motif characteristic of this pathway (28). In contrast, the transport of the 33-kDa

protein of the oxygen-evolving complex (OE33) is dependent

54CP-Thylakoid Precursor Protein Interactions

11625
upon ATP (33, 49) and chloroplast SecA (20, 21).

Analysis of OE23 showed no cross-linking of the nascent chain to 54CP following incubation with stromal extract (Fig. 6, lanes 2, 9, and 10). Likewise no evidence for any interaction between OE33 and 54CP was detected (Fig. 6, lanes 4, 15, and 16). When OE23 and OE33 were incubated with mammalian SRP some cross-linking to SRP54 was observed (Fig. 6, lanes 5 and 11). This may simply reflect the lack of specificity which is exhibited by purified mammalian SRP when incubated with purified nascent chains in the absence of the nascent chain-associated complex (see Ref. 50). Nevertheless, the cross-linking of OE23 and OE33 to mammalian SRP54 indicates that, were 54CP to be bound to these precursors, a cross-linking product could be formed. A novel, stromal extract-dependent cross-linking product with OE23 was observed (Fig. 6, lane 2, arrow). The apparent molecular mass of the cross-linking partner (i.e. after subtracting the contribution of the nascent chain) was about 70 kDa and the product was not immunoprecipitated by antisera recognizing 54CP. Both the identity of this cross-linking partner, and its significance, remain to be established.

54CP Interacts Preferentially with Hydrophobic Signal Sequences—The observation that 54CP interacts specifically with the third transmembrane domain of LHCP led us to compare the thylakoid and ER-targeting signals we had analyzed. This analysis (see Table I) showed that the efficiency with which 54CP was cross-linked to a nascent precursor could be correlated to the hydrophobicity of the signal sequence. Several different scales were used to calculate hydrophobicity and it soon became apparent that the scale developed by von Heijne (40) gave the best correlation with the cross-linking efficiency between a protein precursor and 54CP. In general, the more hydrophobic the signal sequence, the more efficient the 54CP cross-linking observed. The Kyte-Doolittle scale (see Ref. 40) also showed a similar trend but there were obvious anomalies, in particular the apparent lack of hydrophobicity in the third transmembrane region of LHCP.

To test the correlation between signal sequence hydrophobicity and 54CP cross-linking efficiency we compared two forms of the yeast protein carboxypeptidase Y in our assay (Fig. 7). Wild-type CPY has an amino-terminal ER-targeting signal sequence and, as expected, interacted efficiently with 54CP (Fig. 7, lanes 1–4 and 10). In contrast, CPY Mut, which has an ER-targeting signal from the yeast 40S ribosomal protein L16 (G. Tschiersch and H. B. Schmitz, unpublished results), showed no detectable interaction with 54CP (Fig. 7, lanes 6–9 and 11). This result is consistent with previous observations that a hydrophobic signal sequence is required for efficient recognition by mammalian SRP and subsequent targeting to the ER (see Ref. 49). The results of this comparison suggest that the hydrophobicity of a signal sequence is a major determinant of its efficiency in targeting a nascent chain to the ER.

TABLE I

| Nascent chain | % x-1 | Hydrophobicity |
|---------------|-------|---------------|
|               | v H   | K-D           |
| TM3-PC        |       |               |
| CPY-Mut       | 19.5  | -14.8         |
| PPL-WT        | 10.6  | -14.8         |
| Cyt F         | 7.1   | -13.4         |
| Rieske        | 4.2   | -11.7         |
| LHCP-TM1      | 1.1   | -11.5         |
| LHCP-TM2      | ND    | -11.4         |
| OE33          | <1    | -10.9         |
| OE23          | <1    | -10.7         |
| CPY-WT        | 1.5   | -10.0         |
| PPL-Mut       | <1    | -3.5          |

a ND, not determined.
54CP-Thylakoid Precursor Protein Interactions

which is functional in *Saccharomyces cerevisiae*, but not in mammalian cells (36). The introduction of two point mutations (Gly to Leu) into the CPY signal sequence increases its hydrophobicity and allows the precursor to be efficiently targeted and translocated in both mammalian cells and a rabbit reticulocyte translation system supplemented with canine pancreatic microsomes (36). These two CPY precursors were ideal for testing the effect of signal sequence hydrophobicity upon 54CP cross-linking efficiency. The mutant CPY has a very hydrophobic signal sequence while the wild-type protein does not (see Table I).

When the interaction of 54CP with wild-type CPY was analyzed it proved to be a very poor substrate for 54CP (Fig. 7, lanes 1–4 and 7). In contrast, the mutant CPY, differing only at two positions of the signal sequence, was an excellent substrate (Fig. 7, lanes 12 and 13). The very high efficiency of 54CP cross-linking to mutant CPY may reflect the presence of a tract of leucine residues present in a favorable context. When the amount of nascent chain-54CP cross-linking product was determined for each of the precursors used in this study, we observed a good correlation with the calculated hydrophobicity of the signal sequence (see Table I).

**DISCUSSION**

In this study we have identified 54CP as a major thylakoid signal-specific factor present in chloroplast stroma. Our initial analysis showed that 54CP can discriminate between functional and non-functional ER-targeting signals present on the model secretory protein PPL. Hence, 54CP behaves like a true signal sequence recognition factor. Other known SRPs are all ribonucleoprotein complexes comprising a minimum of an SRP54-like protein and a 7 S-like RNA. 54CP has been reported to have an apparent molecular mass of 200 kDa (15), suggesting that it may be part of a larger complex. However, this remains to be established.

The analysis of several authentic thylakoid precursor proteins showed that 54CP interacted only with a subset of the precursors. LHCP is the only thylakoid precursor for which direct evidence of 54CP-dependent targeting has been shown (22). We found that 54CP interacts strongly with LHCP, but only with the third transmembrane region. Analysis of 54CP binding to the individual transmembrane regions of LHCP using a native gel assay (cf. Ref. 22) also showed that the interaction was restricted to the third transmembrane region (data not shown). These data support the tentative proposal that the third transmembrane region of LHCP constitutes the thylakoid-targeting signal (47). All three of the transmembrane domains were found to be essential for the correct membrane integration of LHCP (48, 51).

The integration of LHCP into the thylakoid membrane is a post-translational event, and full-length LHCP has been shown to interact with 54CP in a ribosome-independent manner (22). We conclude that 54CP promotes LHCP integration by binding to the third transmembrane domain of the full-length protein and mediating its targeting to the thylakoid membrane. Thylakoid precursors which are encoded by nuclear genes, e.g. LHCP, are synthesized by cytosolic ribosomes and then transported across the chloroplast envelope. Thus, these precursors must be integrated into the thylakoid post-translationally.

Like LHCP, the Rieske FeS protein is also nuclear-encoded. When the precursor protein is imported into isolated chloroplasts, the protein is found associated with both Hsp60 and Hsp70 molecular chaperones (52) suggesting that stromal components may keep the imported the Rieske protein in a “translocation-competent” conformation. Our cross-linking analysis suggests there is also a significant interaction between 54CP and the mature Rieske protein. These data may simply reflect the ability of the Rieske protein to use multiple thylakoid-targeting routes. Alternatively, there may be sequential interactions between imported Rieske protein and various stromal components such as 54CP, Hsp60, and Hsp70. Indeed, the interactions of the Rieske protein with Hsp60 and Hsp70 were found to be time dependent and imported Rieske protein associated first with Hsp60 and then with Hsp70 (52). The use of a short nascent (i.e. ribosome bound) chain may have locked the Rieske polypeptide into a stable interaction with 54CP and prevented any subsequent interactions with other stromal components (see below).

Thylakoid precursors which are encoded by the chloroplast genome and synthesized in the stroma may preferentially interact with 54CP as ribosome-bound nascent polypeptides (i.e. co-translationally). An association of 54CP with nascent chains in vivo is supported by data showing that a fraction of 54CP co-sediments with 70 S chloroplast ribosomes (17). We have used cytochrome f as a representative chloroplast genome-encoded precursor and found it was efficiently cross-linked to 54CP. On this basis we propose that cytochrome f can utilize the 54CP-dependent pathway. This suggestion is further supported by the observation that the cytochrome f and LHCP-thylakoid integration pathways in *Chlamydomonas* share at least one, genetically defined, component (53) which is not required for OE33 transport. The hydrophobic core of the *Chlamydomonas* cytochrome f signal sequence was also found to be required for efficient thylakoid integration, again suggesting that cytochrome f is targeted to the thylakoid by 54CP.

---

2 S. Meacock and S. High, unpublished data.
The picture is complicated by the observation that, in maize, the tha 1 mutation interferes with both OE33 and cytochrome f targeting, but not with LHCP integration (19). This is consistent with OE33 transport (20, 21) and cytochrome f integration (54, 55) both being SecA-dependent. As with the Rieske protein, there may be sequential interactions between cytochrome f and different stromal factors (i.e. 54CP and chloroplast SecA); alternatively cytochrome f might use multiple thylakoid-targeting pathways. The ability of a single precursor protein to utilize multiple targeting pathways to the ER has recently been established in S. cerevisiae (13).

The presence of a functional SecA homologue in chloroplast stroma is well established and purified SecA and ATP are the only requirements for transport of in vitro synthesized OE33 across washed thylakoid membranes (21, 56). Consistent with these data is our finding that OE33 nascent chains show no significant cross-linking to 54CP; equally, no obvious adduct with SecA was observed in the total cross-linking products. In general, the use of short, ribosome-bound, nascent chains favors SRP binding when an appropriate signal sequence is present (cf. Refs. 6 and 57). Interactions between nascent polypeptides and other stromal components, for example, SecA, may require longer chains than those used in this study and no evidence of any interaction between short nascent polypeptides and E. coli SecA was obtained in a recent cross-linking study (57). In contrast to the other thylakoid proteins used in this study, the OE23 protein has been shown to be transported across the thylakoid membrane by a distinct, ΔP-dependent, route (26, 27, 32). As with OE33, we found no evidence of 54CP being cross-linked to a truncated OE23 polypeptide. OE23 was shown to be cross-linked to a 70-kDa stromal factor, this component remains to be identified.

Our analysis of several authentic thylakoid precursor proteins illustrates that 54CP interacts with only a subset of these precursors. The critical factor in determining 54CP binding appears to be the hydrophobicity of the signal sequence. The influence of hydrophobicity was underlined by the dramatic increase in 54CP cross-linking to CPY with a mutated signal sequence. Two point mutations in the signal sequence (36) have a profound effect on both the calculated hydrophobicity and the efficiency of 54CP cross-linking. A role for signal sequence components remains to be identified.

A profound effect on both the calculated hydrophobicity and the efficiency of 54CP cross-linking. A role for signal sequence components remains to be identified.

A profound effect on both the calculated hydrophobicity and the efficiency of 54CP cross-linking. A role for signal sequence components remains to be identified.

A profound effect on both the calculated hydrophobicity and the efficiency of 54CP cross-linking. A role for signal sequence components remains to be identified.

A profound effect on both the calculated hydrophobicity and the efficiency of 54CP cross-linking. A role for signal sequence components remains to be identified.