A novel precursor recognition element facilitates posttranslational binding to the signal recognition particle in chloroplasts

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Signal recognition particles (SRPs) in the cytosols of prokaryotes and eukaryotes are used to target proteins to cytoplasmic membranes and the endoplasmic reticulum, respectively. The mechanism of targeting relies on cotranslational SRP binding to hydrophobic signal sequences. An organellar SRP identified in chloroplasts (cpSRP) is unusual in that it functions posttranslationally to localize a subset of nuclear-encoded thylakoid proteins. In assays that reconstitute thylakoid integration of the light harvesting chlorophyll-binding protein (LHCP), stromal cpSRP binds LHCP posttranslationally to form a cpSRP/LHCP transit complex, which is believed to represent the LHCP form targeted to thylakoids. In this investigation, we have identified an 18-aa sequence motif in LHCP (L18) that, along with a hydrophobic domain, is required for transit complex formation. Fusion of L18 to the amino terminus of an endoplasmic reticulum-targeted protein, preprolactin, led to transit complex formation whereas wild-type preprolactin exhibited no ability to form a transit complex. In addition, a synthetic L18 peptide, which competed with LHCP for transit complex formation, caused a parallel inhibition of LHCP integration. Translocation of proteins by the thylakoid Sec and Delta pH transport systems was unaffected by the highest concentration of L18 peptide examined. Our data indicate that a motif contained in L18 functions in precursor recruitment to the posttranslational SRP pathway, one of at least four different thylakoid sorting pathways used by chloroplasts.

Signal recognition particle (SRP) and its receptor comprise essential components of a signal peptide-based protein targeting mechanism that is conserved across evolutionary boundaries (1–3). SRPs in the cytosols of eukaryotes and *Escherichia coli* target proteins cotranslationally to the endoplasmic reticulum and cytoplasmic membrane, respectively. Targeting is initiated as a result of SRP binding to the hydrophobic domain of amino-terminal signal peptides or signal anchors as they emerge from the ribosome. The entire ribosome/nascent polypeptide chain complex (RNC) then is piloted by SRP to an SRP receptor that functions at the membrane. GTP binding and hydrolysis by SRP and its receptor result in both the release of SRP from its receptor and the release of SRP from the RNC, whereupon the nascent chain enters a translocation pore that directs the translating polypeptide into or across the lipid bilayer.

An organellar SRP, which exhibits striking structural and functional differences from cytosolic SRPs, also has been identified in chloroplasts (4, 5). Chloroplast SRP (cpSRP) is a soluble ≈200-kDa stromal particle that contains an evolutionary conserved 54-kDa subunit (cpSRP54) as well as a unique 43-kDa polypeptide (cpSRP43) (6). Unlike cytosolic SRPs, an RNA moiety is conspicuously lacking in cpSRP. Biochemical and genetic evidence have demonstrated that cpSRP functions posttranslationally to localize a subset of nuclear-encoded thylakoid proteins belonging to the chlorophyll a/b-binding (cab) protein family (6–8). The activity exhibited by cpSRP is novel when compared with cytosolic SRPs, whose substrate binding is thought to be exclusively cotranslational. Neither eukaryotic SRPs nor *E. coli* SRPs bind full-length substrates or nascent polypeptides after their release from ribosomes (1–3).

The posttranslational function of cpSRP was first demonstrated for the pea *cab80* gene product (hereafter referred to as LHCP, light harvesting chlorophyll-binding protein) (7), which has been studied as a model to investigate intra-chloroplast sorting events (4, 5). LHCP is synthesized in the cytosol as a full-length precursor (pLHCP) and then imported into chloroplasts through the action of a cleavable amino-terminal transit peptide. Removal of the transit peptide by a stromal processing protease results in mature LHCP that, despite its hydrophobicity, is soluble in chloroplast stroma. Stromal LHCP is a soluble sorting intermediate that subsequently is integrated into thylakoid membranes. In assays that reconstitute transit complex formation and stroma-dependent integration into isolated thylakoids, stromal extract (SE) depleted of cpSRP neither supports transit complex formation nor LHCP integration into isolated thylakoids (7). Reconstitution of transit complex using purified components has established that transit complex is composed of LHCP, cpSRP54, and cpSRP43 (6). It is presumed that cpSRP is used to target LHCP as transit complex to a cpSRP receptor on the thylakoid membrane. This is consistent with findings that LHCP integration requires GTP hydrolysis (9, 10) and cpFtsY, a recently identified chloroplast SRP receptor homologue (10, 11).

Comparison of cpSRP and eukaryotic SRP to cotranslationally bind RNCs revealed that the substrate requirement for SRP binding is similar (12); signal sequence overall hydrophobicity stimulates SRP binding to RNCs. In these assays, the signal peptide of an endoplasmic reticulum-targeted protein (e.g. preprolactin, PPL) was efficiently crosslinked to the 54-kDa subunits of cpSRP and eukaroytic SRP. Moreover, both SRPs were able to discriminate between functional and nonfunctional signal peptides that differed only in signal peptide hydrophobicity. Despite the apparent similarities between SRPs in cotranslational assays, the rationale behind this study was the finding that full-length PPL does not form a transit complex with cpSRP. Why cpSRP is able to bind full-length LHCP is unclear. This novel interaction is likely to involve a unique recognition element(s) in LHCP that is used exclusively to promote posttranslational binding of LHCP to cpSRP. Hence, the recognition

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Abbreviations: cab, chlorophyll a/b binding; LHCP, light harvesting chlorophyll-binding protein; pLHCP, precursor to LHCP; SRP, signal recognition particle; cpSRP, chloroplast SRP; cpSRP54, 54-kDa subunit of cpSRP; cpSRP43, 43-kDa subunit of cpSRP; PPL, preprolactin; RNC, ribosome/nascent polypeptide chain complex; L18, 18-aa sequence motif in LHCP; SE, stromal extract; PC, plastocyanin; IB, import buffer; PCm, PC mature domain.

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element(s) in LHCP that mediates posttranslational binding to cpSRP may not be a hydrophobic signal anchor.

Using LHCP deletion constructs and fusion proteins to study the interaction between LHCP and cpSRP, we have identified an 18-aa segment of LHCP (L18) that is required for posttranslational, but not cotranslational, binding to cpSRP. Our data indicate that the L18 region contains a cpSRP-binding motif, which is necessary for precursor recruitment to the posttranslational SRP sorting pathway in chloroplasts, one of four different signal peptide-based sorting systems used to localize proteins to thylakoid membranes (4, 5).

Materials and Methods

Materials. With the exception of a synthetic peptide (see below), all reagents, enzymes, and standards were purchased commercially. Previously described plasmids were used for *in vitro* transcription/translation of pLHCP (psAB80XD/4) (13), tOE23 (14), iOE33 (15), and TM3-PC (12). However, the TM3-PC translation product, which codes for a fusion protein between the carboxyl-terminal third of pLHCP and the mature domain of plastocyanin (PCm), is designated here as $pLHCP_{189–269}$PCm to more clearly indicate the amino acids derived from pLHCP. Antibodies against LHCP and OE23 (23-kDa subunit of the oxygen evolving complex) were provided by Kenneth Cline, University of Florida, Gainesville. Antibody to cpSRP54 was a gift from Neil Hoffman, Stanford University, Stanford, CA. A custom peptide corresponding to the L18 region of pLHCP (VDPLYPGGSFDPLGLADD) was purchased from Research Genetics (Huntsville, AL). The L18 peptide, modified by acetylation of the amino terminus and amidation of the carboxyl terminus, was provided by the manufacturer at greater than 86% purity as determined by HPLC and MS and was used in the studies described herein without further purification.

Methods. *Construction of recombinant precursor proteins.* The coding sequence for bovine PPL in pGEM 4Z (16) was subcloned into pGEM 3Z (Promega) as an *Eco*RI/*Hin*dIII fragment, which placed the coding sequence in the SP6 direction. All other recombinant precursor proteins were constructed by PCR-based methods by using the above plasmids as templates and Pfu polymerase (Stratagene). PCR products containing restriction sites incorporated into the forward and/or reverse primers were digested and ligated into appropriately restricted pGEM 3Z or pGEM 4Z in the SP6 direction. One exception to this general strategy was the construction of a deletion clone, $pLHCP_{\Delta189–198}$, which codes for pLHCP lacking amino acids 189–198 (VDPLYPGGSF). Forward and reverse primers that bound immediately outside of the coding sequence for amino acids 189–198 were used to amplify the entire plasmid such that ligation of the PCR product resulted in a circular plasmid identical to psAB80XD/4, but lacking the appropriate 30 bases. DNA sequencing of all cloned constructs was performed to verify the fidelity of the PCR (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock).

Truncated precursors. Coding sequences for carboxyl-terminal truncations of pLHCP were constructed by using forward and reverse primers such that the translated proteins begin (MAASS) and end FGEAV ($pLHCP_{1–133}$), PLGEV ($pLHCP_{1–188}$), GGSFD ($pLHCP_{1–199}$), GLADD ($pLHCP_{1–206}$), EAFAE ($pLHCP_{1–212}$), LKVKE ($pLHCP_{1–217}$), or LKNGR ($pLHCP_{1–222}$). Forward and reverse primers were designed to introduce an initiator methionine codon and a stop codon, respectively. Amino terminal deletions of TM3-PC ($pLHCP_{189–269}$PCm, see above) were constructed by using the TM3-PC coding sequence as template, the reverse primer used to construct TM3-PC, and forward primers designed to introduce an initiator methionine codon such that the translated proteins begin MDPLGL ($pLHCP_{199–269}$PCm), MDPEAF ($pLHCP_{206–269}$PCm), MVKELK ($pLHCP_{215–269}$PCm), or MGRLAM ($pLHCP_{221–269}$PCm).

Chimeric precursors. Chimeric sequences for $pLHCP_{189–206}$PPL, $pLHCP_{189–222}$PPLh/m, and $pLHCP_{189–222}$PPLh*/m were constructed by overlap extension (17) using a forward primer for the LHCP coding regions that introduced an initiator methionine codon such that all fusion proteins begin MVDPLYPGG. The coding sequence for $pLHCP_{189–206}$PPL is an exact fusion between the coding sequences for amino acids 189–206 of pLHCP and full-length PPL lacking its initiator methionine. The coding sequence for $pLHCP_{189–222}$PPLh/m is an exact fusion between the coding sequences for amino acids 189–222 of pLHCP and PPL beginning at the PPL signal peptide hydrophobic domain such that in the translated protein, amino acid 222 of pLHCP is immediately followed by LLLLLVV. Translation of a similar construct, $pLHCP_{189–222}$PPLh*/m, results in a protein that lacks the first five residues of the PPL hydrophobic domain such that amino acid 222 of pLHCP is followed immediately by VVSNL (see Fig. 3 for additional sequence information of each chimeric protein).

Preparation of radiolabeled precursor, chloroplasts, lysates, thylakoids, and SE. Capped RNA for authentic, chimeric, and truncated precursors was produced by *in vitro* transcription of uncut plasmid using SP6 RNA polymerase; RNA was translated in a wheat germ system in the presence of [^{3}H] leucine (18). Translation products were diluted 3-fold and adjusted to import buffer (IB; 50 mM Hepes/KOH, pH 8.0, 0.33 M sorbitol), containing 30 mM unlabeled leucine. Intact chloroplasts were isolated from 9- to 10-day-old pea seedlings (Laxton's Progress) and resuspended in IB. Lysates and SE were prepared from isolated chloroplasts (14, 18). Chlorophyll was determined according to Arnon (19).

Assays for thylakoid protein transport, transit complex formation, and immunoretention of transit complex. Transport of radiolabeled precursors into or across thylakoids was conducted by using chloroplast lysate in 150-μl assays essentially as described (18) except that lysates (100 μl) containing 50 μg chlorophyll and 7.5 mM Mg-ATP were incubated for 10 min at 25°C with L18 synthetic peptide in IB (25 μl) or with an equal volume of IB alone. Assays were initiated by addition of diluted translation product (25 μl) and conducted for 30 min in a 25°C water bath. Recovered thylakoids were posttreated with thermolysin, which was terminated by adding an equal volume of 50 mM EDTA in IB, collected by centrifugation, and dissociated with 50 μl of SDS/PAGE sample buffer (14). Transit complex was formed in 30-μl assays by mixing 20 μl of SE (0.5 mg/ml chlorophyll equivalent) with 5 μl of 30 mM Mg-ATP and 5 μl of diluted translation product essentially as described (20). To examine the influence of L18 peptide, transit complex was formed in a similar manner except that translation products were added after a 10-min preincubation of SE with L18 peptide at 25°C. Assays then were placed on ice and prepared for native PAGE by the addition of 50% glycerol (5 μl) or mixed with antibodies for immunoretention assays. The latter were used to verify the identities of transit complexes in native gels. For immunoretention, 4 μl of IgG in IB was added to each 30-μl assay such that the final IgG concentration was 4.3 μg/μl. Samples then were incubated for 1.5 hr at 4°C before addition of 50% glycerol (5 μl) to prepare samples for native PAGE.

Analysis of samples. A portion of each assay (10 μl) for thylakoid transport/integration, transit complex formation, or immunoretention was analyzed by SDS/PAGE or native PAGE (20) as indicated above, followed by fluorography. Quantification was by scintillation counting of radiolabeled proteins extracted from excised gel bands (21).

Results

As a first step toward understanding the novel posttranslational interaction between cpSRP and LHCP, segments of pLHCP

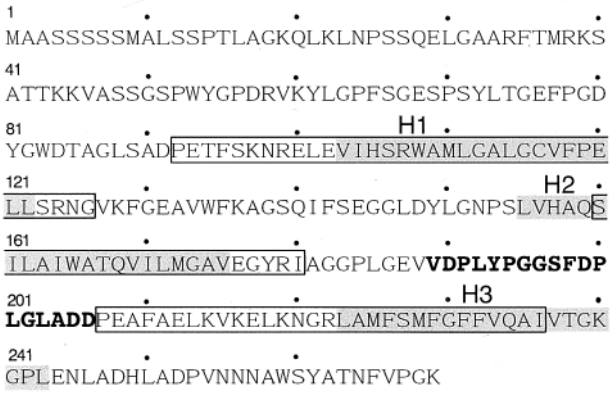

Fig. 1. The amino acid sequence of pLHCP is shown to illustrate the position of the three transmembrane α-helices (rectangles) predicted from the LHCP crystal structure (22). Hydrophobic H domains (H1, H2, and H3) predicted by the hydrophobicity analysis software TMPREDICT are represented by shadowed boxes. The 18-aa region, L18, required for posttranslational binding to cpSRP (this paper) is in bold.

were used to define the region(s) of LHCP necessary to form transit complex. For initial studies, three contiguous segments of pLHCP corresponding to amino acids 1–133, 134–188, and 189–269 were used as substrates in transit complex formation assays. Each segment of LHCP was designed to contain one of three hydrophobic domains identified by hydrophobicity analysis (see Fig. 1). The position of each hydrophobic domain correlates roughly with the position of the three transmembrane α-helices identified from the LHCP crystal structure (22).

Fig. 2 shows that incubation of radiolabeled pLHCP with SE reconstituted formation of a cpSRP/LHCP transit complex, which migrated with a R_f of 0.4 on a nondenaturing gel. In the absence of SE, LHCP remains in the sample well. Of the three LHCP segments similarly assayed, only $pLHCP_{189–269}$ formed transit complex (Fig. 2 *A–C*, an arrowhead denotes transit complex). The identity of these and other putative transit complexes observed in Figs. 2 and 3 was confirmed by anti-cpSRP54 immunoretention assays (see Fig. 4). Whereas $pLHCP_{189–269}$ maintained the ability to form transit complex when fused to the PCm, a similar chimeric protein missing residues 189–220 ($pLHCP_{221–269}$PCm) did not form transit complex (Fig. 2, compare *D* and *E*). Large C-terminal truncation deletions showed that the amino-terminal two-thirds of the LHCP molecule could form transit complex if it contained residues 189–222 (Fig. 2, compare *F* and *G*). Together, these results indicate that there is an essential element for transit complex formation that lies between residues 189 and 222 of pLHCP.

To better define the amino acid sequence necessary for posttranslational cpSRP binding, a series of smaller truncations from the amino terminus of $pLHCP_{189–269}$PCm (Fig. 2 *H–J*) and from the carboxyl terminus of pLHCP (Fig. 2 *K–N*) were assayed for their abilities to form transit complex. Fig. 2*H* shows that a 10-aa deletion from $pLHCP_{189–269}$PCm to produce $pLHCP_{199–269}$PCm resulted in the loss of transit complex formation (Fig. 2, compare *D* and *H*). Proteins with additional deletions from the amino terminus, $pLHCP_{206–269}$PCm and $pLHCP_{215–269}$PCm (Fig. 2 *I* and *J*, respectively), also were unable to form transit complex. In contrast, truncations from the carboxyl terminus of $pLHCP_{1–222}$ indicated that amino acids 207–222 were dispensable. Further truncations, however, resulted in loss of transit complex formation (Fig. 2, compare *F* to *K–N*). These data together define an 18-aa segment, L18, (amino acids 189–206 of pLHCP; represented in Fig. 2 by the shadowed box) as an essential element for transit complex formation. The position of an H domain amino- or carboxyl-proximal to L18 appears to have little influence on the efficiency of transit complex formation. For example, when $pLHCP_{189–269}$PCm and $pLHCP_{1–206}$ were compared in the same assay, ≈20% of the translation product for each construct was found in transit complex compared with ≈24% for pLHCP. One possible explanation is that the L18 motif is sufficient for transit complex formation.

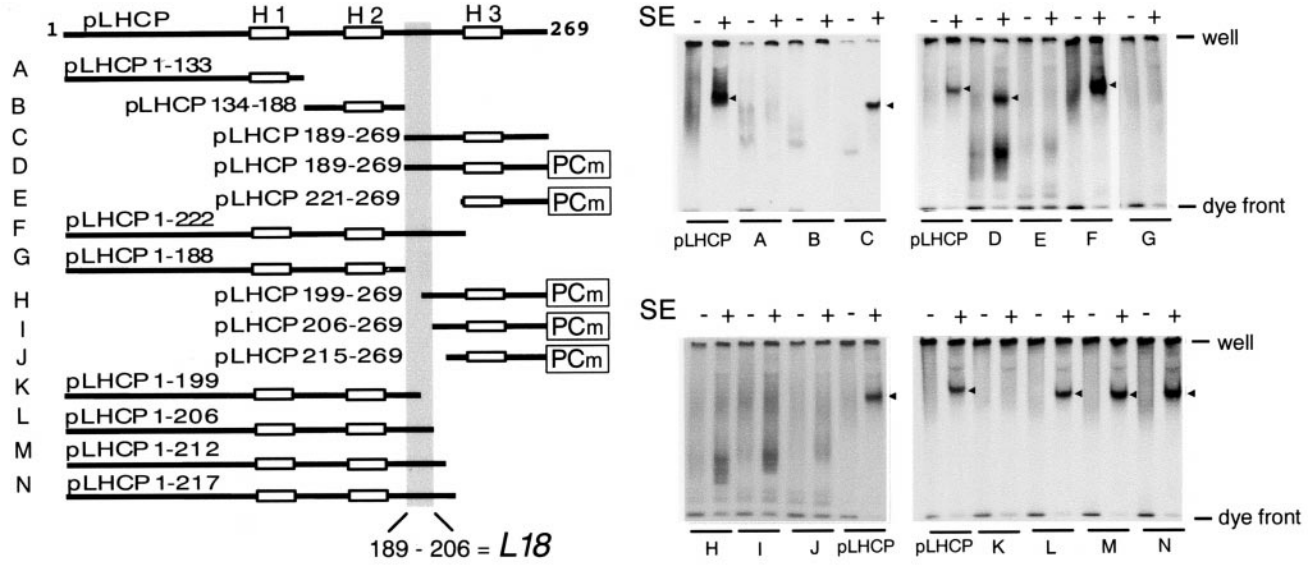

Fig. 2. An 18-aa region of LHCP is required for transit complex formation with cpSRP. Segments of *in vitro*-translated pLHCP alone or fused to the PCm were assayed for their abilities to form transit complex. Transit complex (denoted by arrowheads) was tentatively identified by using native PAGE to compare assays conducted in the presence or absence of SE and later verified by immunoretention assays (see *Materials and Methods*). The amino acid composition of each translation product (*A–N*), including the presence of H1, H2, or H3 (open rectangles, see legend to Fig. 1), is described relative to pLHCP next to the fluorograms. The 18 amino acids (L18) common to all segments that formed transit complex are indicated by the shadowed box and correspond to residues 189–206.

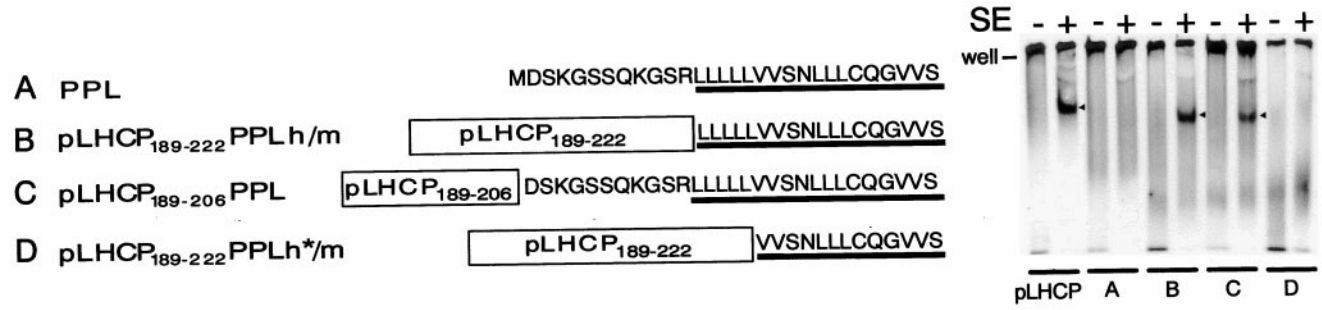

Fig. 3. The endoplasmic reticulum-targeted protein PPL binds cpSRP posttranslationally when fused behind an 18-aa region of LHCP. *In vitro*-translated PPL (*A*) or chimeric proteins with regions of LHCP fused at the amino terminus of PPL (*B–D*) were assayed for their abilities to form transit complex (arrowheads) as described in the legend to Fig. 2. The amino acid composition of the PPL signal sequence and the composition of signal sequences from chimeric proteins are shown to the left. The H domain for each construct is underlined. For chimeric proteins, the amino acids derived from LHCP (indicated in the rectangles) and from the PPL signal sequence are shown.

To determine whether L18 is both necessary and sufficient to promote posttranslational cpSRP binding, L18 was fused to PPL, an endoplasmic reticulum-targeted protein with a signal peptide that binds efficiently to cpSRP and eukaryotic SRP when presented cotranslationally as an 89-aa RNC (12). Fig. 3 shows that, unlike PPL RNCs, full-length PPL lacks the ability to bind cpSRP posttranslationally. However, fusion of L18 to the amino terminus of full-length PPL ($pLHCP_{189–206}PPL$) resulted in the formation of transit complex (Fig. 3*C*). Similar results were obtained with $pLHCP_{189–222}PPLh/m$, which also contains the L18 sequence at the amino terminus (Fig. 3*B*). Together, these data establish the necessity for L18 in posttranslational binding to cpSRP. However, the L18 region was not sufficient for transit complex formation because deletion of the first five amino acids in the H domain of $pLHCP_{189–222}PPLh/m$ (see $pLHCP_{189–222}PPLh^*/m$, Fig. 3*D*) led to a loss of transit complex formation (Fig. 3, compare *B* and *D*). Taken together, our results using PPL fusions demonstrated that transit complex formation requires two different recognition elements, an H domain that in cotranslational assays is necessary and sufficient for binding to cpSRP (12) and a novel element contained within L18 that is required exclusively for posttranslational interaction with cpSRP.

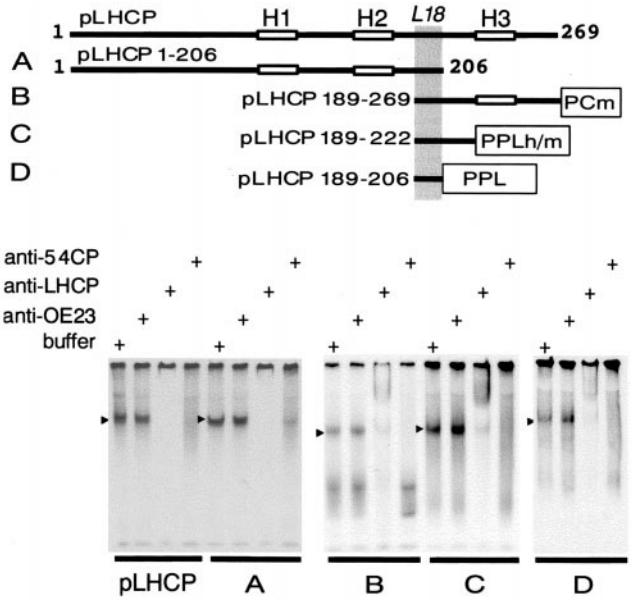

Fig. 4. Antibody to cpSRP54 specifically inhibits the migration of transit complex during native PAGE. The identities of the SE-dependent soluble species observed in Figs. 2 and 3 were confirmed as transit complex by using anti-cpSRP54 in immunoretention assays (see *Materials and Methods*). In short, transit complex assays were conducted with the radiolabeled proteins described above the fluorogram and then mixed with 4 μl of buffer or IgG directed against cpSRP54, OE23, or LHCP as indicated. After incubation, the assays were applied to native gels. The identity of transit complex (arrowheads) was assigned on the basis of its disappearance from native gels in the presence of anti-cpSRP54 IgG.

For each of the constructs examined in Figs. 2 and 3, immunoretention assays were performed to verify the identity of transit complex. Immunoretention assays rely on the ability of cpSRP54 antibody to bind transit complex after it is formed and retain it in the sample well of the native gel. Fig. 4 shows that when LHCP is the substrate, incubation of transit complex with anti-cpSRP54 or anti-LHCP IgG prevents the migration of transit complex from the sample well. Incubation with an equal amount of an irrelevant IgG (anti-OE23) had no influence on transit complex migration. For $pLHCP_{189–269}PCm$, where at least one additional soluble species is observed, anti-cpSRP54 prevented the migration of only the upper-most soluble species (Fig. 4*B*). Antibody to cpSRP54 had no influence on the faster migrating bands, indicating that these bands were not bound to cpSRP54. Only anti-LHCP prevented both transit complex and the faster migrating species from entering the gel. This finding demonstrated that the influence of IgG on transit complex migration is antibody specific and that immunoretention assays distinguish transit complex from unrelated soluble species. For substrates that exhibited a single stroma-dependent soluble species, a pattern of immunoretention similar to that observed for LHCP is seen (Fig. 4 *A*, *C*, and *D*). The presence of cpSRP43 in bands designated as transit complex was verified by immunoretention using antibody against cpSRP43 (data not shown). Together, our data verifies that the soluble species designated as transit complex in previous figures represented substrate bound to cpSRP.

The apparent requirement for the L18 sequence in promoting posttranslational interaction with cpSRP suggests that the L18 region may function as a cpSRP-binding domain. Hence, a synthetic peptide corresponding to L18 was examined for its ability to compete with LHCP for transit complex formation. Fig. 5*A* shows that when transit complex assays were conducted with radiolabeled pLHCP in the presence of increasing concentrations of L18 synthetic peptide, the formation of transit complex was inhibited severely in a peptide concentration-dependent manner. Consistent with a model for LHCP localization in which formation of transit complex is a necessary step for LHCP integration, we observed a parallel inhibition of LHCP integration into thylakoids when integration assays were conducted in the presence of L18 synthetic peptide. The specificity of the inhibition is demonstrated by the fact that OE23 and OE33

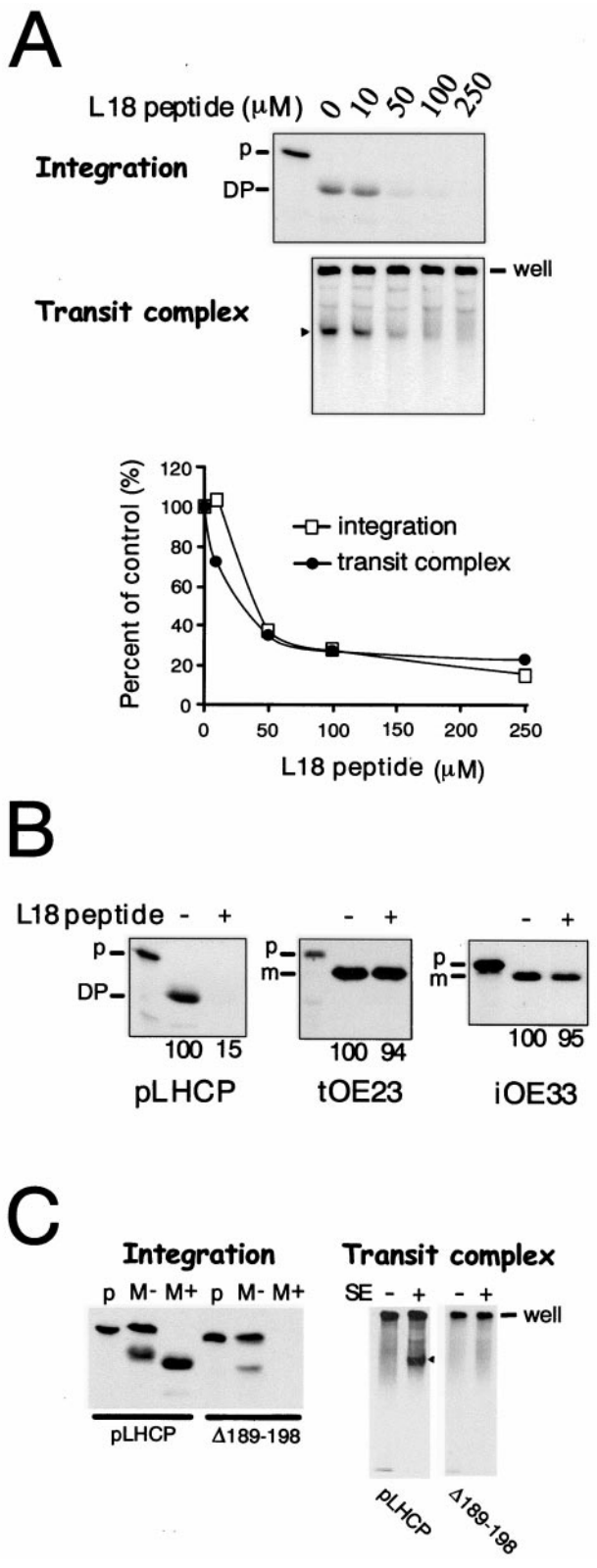

Fig. 5. Synthetic peptide corresponding to L18 competes with LHCP for binding to cpSRP, resulting in specific inhibition of LHCP integration into thylakoids. (*A*) Assays for transit complex formation and integration were conducted with radiolabeled pLHCP in the presence of increasing concentrations of a synthetic L18 peptide (VDPLYPGGSFDPLGLADD) as described in *Materials and Methods*. The final concentration of peptide in each assay is shown above the fluorograms. The radiolabeled precursor (p) represents 0.8% of the translation product in the assay. Lanes were loaded with equivalent amounts of recovered thylakoids representing 20% of each assay. An LHCP degradation product (DP) produced by protease posttreatment of thylakoids and indicative of integrated LHCP is shown. A graph showing the relative amount of pLHCP integrated into thylakoids or bound in transit complex (arrowhead) is based on quantification of radiolabel extracted from excised gel bands containing DP or transit complex (see *Materials and Methods*). (*B*) Assays for transport of radiolabeled tOE23 and iOE33 or integration of radiolabeled pLHCP were conducted in the presence (+) or absence (−) of 250 μM synthetic L18 peptide and analyzed as described in *A*. The relative percent transport for each protein is indicated below each lane and is based on quantification of DP or mature (m) forms of OE23 and OE33. (*C*) Assays for integration into thylakoids and for the formation of transit complex (arrowhead) were conducted as in *A* without L18 peptide by using radiolabeled pLHCP or $pLHCP_{\Delta 189-198}$ (Δ189–198). Thylakoid membranes recovered from each assay were washed in IB and analyzed directly (M−) or after protease posttreatment (M+). Lanes containing radiolabeled precursor (p) or membranes were loaded as in *A*. For transit complex, the fluorogram of $pLHCP_{\Delta 189-198}$ is from an exposure twice that of pLHCP.

precursors, substrates for the Delta pH and Sec translocation systems, respectively, transported efficiently in the presence of 250 μM L18 peptide whereas LHCP integration was inhibited by ≈85% (Fig. 5*B*). Further support that L18 functions as a cpSRP binding domain stems from the finding that LHCP with a deletion in the L18 region ($pLHCP_{\Delta 189-198}$) failed to form transit complex (Fig. 5*C*). Similar to the affect of conducting assays in the presence of L18 peptide, the inability of $pLHCP_{\Delta 189-198}$ to form transit complex correlated with its inability to integrate into thylakoids.

Discussion

In chloroplasts, four different signal peptide-based sorting systems are used to localize nuclear-encoded thylakoid proteins after their import into the chloroplast stroma (4, 5). One of these is an SRP-based system that functions in localizing LHCP. In this study, we investigated a novel posttranslational interaction between cpSRP and LHCP, an interaction that is necessary for LHCP integration into isolated thylakoid membranes and which leads to the formation of a cpSRP/LHCP transit complex in chloroplast stroma (6, 7). Taken together, our findings imply that an organellar SRP has evolved a unique posttranslational binding function that centers on its ability to interact with a novel recognition element contained in the L18 region of LHCP.

Several lines of evidence argue that the L18 region of LHCP functions as an SRP binding domain in transit complex formation. First, constructs missing as much as 70% of the LHCP molecule still formed a transit complex as long as the L18 region was intact (Fig. 2). In contrast, a 10-aa deletion in the L18 region of LHCP led to the complete loss of transit complex formation (Fig. 5*C*). Second, PPL, which binds cpSRP efficiently as a RNC in cotranslational assays (12), formed a transit complex only as an L18-PPL fusion protein. Full-length PPL alone did not form a transit complex (see Fig. 3). Third, synthetic peptide corresponding to L18 inhibited transit complex formation without affecting interactions critical to the localization of proteins transported by the thylakoid Sec and Delta pH pathways (Fig. 5). The specific inhibition of transit complex formation is indicative of competition between L18 peptide and LHCP, which must be at the level of cpSRP binding because no other stromal proteins are required for transit complex formation (6). The concentration of L18 peptide required to inhibit transit complex formation was considerably higher than the concentration of unlabeled LHCP (1–2 μM) necessary to compete for integration of radiolabeled LHCP (18). This difference may reflect the ability of LHCP at high levels to saturate a component of the localization pathway other than cpSRP. However, it is also possible that L18 peptide is degraded rapidly in SE or that the peptide binds cpSRP less efficiently without an adjacent H domain, therefore

requiring higher concentrations of peptide to compete for binding of LHCP to cpSRP.

It is expected that elements in L18 critical for precursor recognition by cpSRP will be conserved among proteins sorted by the posttranslational SRP targeting pathway. A BLAST search of the database for proteins with sequence similarity to L18 identified only proteins that belong to the cab protein family. Neither the Reiske FeS protein nor cytochrome *f* were identified despite the fact that both proteins crosslink efficiently to cpSRP54 in cotranslational assays (12). Hence, it is unlikely that chloroplast-synthesized proteins, e.g., cytochrome *f*, use the posttranslational SRP pathway for thylakoid localization. In comparing the sequences of cab proteins from a single species, e.g., tomato, certain members of this protein family exhibit a low level of sequence identity in the L18 region. This finding suggests that not all of the residues in the L18 sequence are required for binding to cpSRP. Alternatively, deviations in the L18 region of these cab proteins may be an indication that they do not require cpSRP for localization. The latter is consistent with the ability of mutants lacking cpSRP54 or cpSRP43 to still accumulate cab proteins, albeit at reduced levels (8, 23).

Whether the L18 region represents a binding domain for cpSRP54 or cpSRP43 is uncertain. However, phenotypic differences between *Arabidopsis* mutants that lack cpSRP54 (23, 24) or cpSRP43 (8, 24) suggest that L18 is a cpSRP43-binding motif. Accumulation of all thylakoid proteins is reduced in cpSRP54 mutants. In contrast, a cpSRP43 mutant exhibits a preferential reduction of cab proteins. This finding has led to the proposal that cpSRP43 function may be restricted to posttranslational localization of cab proteins, whereas cpSRP54 function is more general and includes cotranslational activity (8), e.g., targeting of the chloroplast-synthesized D1 protein (25). Because the L18 sequence appears to be restricted to cab proteins, mutations affecting the function of an L18 binding protein are predicted to closely match the cpSRP43 mutant phenotype.

It is inviting to speculate that L18 binding to cpSRP is used to regulate the affinity of cpSRP for hydrophobic sequences. This model is consistent with our observation that cpSRP only forms transit complex with precursors containing both L18 and a suitable H domain. In the absence of L18, a suitable H domain, e.g., the H domain of PPL, is not sufficient to support transit complex formation. It is expected that in the absence of such regulation, cpSRP would posttranslationally bind hydrophobic signal sequences that direct precursors to the thylakoid Sec or Delta pH transport systems, thereby resulting in mistargeting of these proteins. Using L18 binding to increase cpSRP affinity for H domains would insure that cpSRP interactions are restricted to hydrophobic sequences contained in LHCP, owing to their close proximity to L18.

It is undoubtedly the function of cpSRP54 to bind H domains in transit complex, similar to its role in cotranslational substrate binding (12) and similar to the role of cytosolic SRP54 homologues (2, 26). Binding of cpSRP to H domains is likely to contribute to the increased solubility exhibited by LHCP in transit complex (20). However, solubility appears not to be the factor limiting LHCP integration (27). One possibility is that binding of cpSRP to H domains preserves LHCP in a conformation suitable for integration. Our data show that more than one H domain in LHCP is capable of interacting posttranslationally with cpSRP. Yet, a recent report indicates that cpSRP is composed of two cpSRP43 subunits and only one cpSRP54 (10). This finding points to the possibility that cpSRP binds one preferred H domain to place LHCP in an integration competent structure. A clear understanding of transit complex structure is still forthcoming and is necessary to address this issue. Because L18 appears to facilitate posttranslational cpSRP interaction with H domains, identification of the L18 binding subunit of cpSRP should help elucidate the role of L18 in this novel posttranslational SRP localization mechanism.

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