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# Functional Characterization of Recombinant Chloroplast Signal Recognition Particle*

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**The signal recognition particle (SRP) is a ubiquitous system for the targeting of membrane and secreted proteins. The chloroplast SRP (cpSRP) is unique among SRPs in that it possesses no RNA and is functional in post-translational as well as co-translational targeting. We have expressed and purified the two components of the *Arabidopsis thaliana* chloroplast signal recognition particle (cpSRP) involved in post-translational transport: cpSRP54 and the chloroplast-specific protein, cpSRP43. Recombinant cpSRP supports the efficient *in vitro* insertion of pea preLhcb1 into isolated thylakoid membranes. Recombinant cpSRP is a stable heterodimer with a molecular mass of ~100 kDa as determined by analytical ultracentrifugation, gel filtration analysis, and dynamic light scattering. The interactions of the components of the recombinant heterodimer and pea preLhcb1 were probed using an immobilized peptide library (pepscan) approach. These data confirm two previously reported interactions with the L18 region and the third transmembrane helix of Lhcb1 and suggest that the interface of the cpSRP43 and cpSRP54 proteins is involved in substrate binding. Additionally, cpSRP components are shown to recognize peptides from the cleavable, N-terminal chloroplast transit peptide of preLhcb1. The interaction of cpSRP43 with cpSRP54 was probed in a similar experiment with a peptide library representing cpSPR54. The C terminus of cpSRP54 is essential for the formation of the stable cpSRP complex and cpSPR43 interacts with distinct regions of the M domain of cpSRP54.**

In higher plants photosynthesis is carried out within the chloroplast, starting with the initial capture of light energy by the light harvesting chlorophyll-binding proteins (LHCPs)[1] (1). Since these LHCPs are nuclear encoded and are also the most abundant proteins within the thylakoid membrane, there is a strong demand for the efficient transport and targeting of these proteins into the chloroplast and into the thylakoid membrane (for a recent review, see Ref. 2). The chloroplast signal recognition particle (cpSRP) has been identified as the major targeting factor involved in the post-translational targeting of Lhcb1 from the inner chloroplast membrane to the thylakoid membrane (3–7), and it is probable that many other LHCPs also utilize cpSRP.

Although the SRP system is conserved in all organisms, there are significant differences in its composition (8). All SRP systems described target their substrates as they are synthesized at the ribosome (co-translationally) through an interaction with a ribosome/nascent chain complex (9–11). The chloroplast SRP (cpSRP) differs from all other SRP systems in two respects. First, unlike SRPs from eukaryotes or prokaryotes, no RNA has so far been identified as part of cpSRP. Second, cpSRP is present within two pools in the chloroplast: a co-translationally active SRP54 homologue (cpSRP54), which associates with the chloroplast ribosome/nascent chain complex (3, 12) and a post-translationally active cpSRP, which has been shown to contain cpSRP54 and the novel SRP component, cpSRP43 (5, 6).

The co-translational and post-translational cpSRP pools may be distinguished by differing subunit compositions, as no cpSRP43 could be identified in cpSRP/ribosome/nascent chain complexes engaged in co-translational transport (12). cpSRP54 is unable to form a complex with the 4.5 S RNA from *Escherichia coli*, despite sharing 74% sequence identity with the SRP54 homolog (Ffh) from *E. coli* (13). cpSRP43 is also unable to form a functional transit complex with Ffh (13), making it unlikely that cpSRP43 is a direct functional protein substitution for the RNA of other SRPs. Rather, the presence or absence of cpSRP43 seems to determine the targeting activity of cpSRP54.

Although the LHCPs are highly hydrophobic, Lhcb1 is soluble in the stroma in a "transit complex," which has been shown to contain cpSRP54 and cpSRP43 (5, 6). These proteinaceous components maintain the substrate in an insertion competent form, but insertion of Lhcb1 requires the additional presence of the chloroplast SRP receptor homologue, cpFtsY, and GTP (14). Finally, cpSRP is again unusual in that it recognizes an inter-



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[1] The abbreviations used are: LHCP, light-harvesting chlorophyll *a/b*-binding protein; SRP, signal recognition particle; cpSRP, chloroplast signal recognition particle; cpSRP54, 54-kDa subunit of cpSRP; cpSRP43, 43-kDa subunit of cpSRP; (pre)Lhcb1, (precursor of) major light harvesting complex apoprotein of photosystem II; CTSP, chloroplast transit signal peptide; TM, transmembrane helix; Ffh, Fifty-four homologue; L18, 18-amino acid sequence motif of Lhcb1; L13, 13-amino acid sequence motif of Lhcb1; AMP-PNP, 5′-adenylylimidodiphosphate; GMP-PNP, 5′-guanylylimidodiphosphate; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PVDF, polyvinylidene difluoride; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

nal signal sequence within the LHCPs, rather than the usual N-terminal signal sequence recognized by the other SRP systems. Cross-linking experiments suggest this cpSRP targeting signal to be the highly hydrophobic third transmembrane helix (TM3) of LHCPs (15).

A hydrophilic peptide immediately N-terminal to TM3, known as L18, has been identified as a further targeting signal as the addition of this L18 peptide promotes the insertion of non-chloroplast membrane protein chimeras into thylakoid membranes (16). A glutathione *S*-transferase-L18 construct interacts with cpSRP43 in the absence of cpSRP54 *in vitro*, and cpSRP54 and cpSRP43 were proposed to interact independently with the substrate to form the transit complex (17). The molecular mass of this transit complex was determined to be around 120 kDa by non-denaturing gel analysis (18). However, more recent data indicated a molecular mass of ~200 kDa for the cpSRP43/cpSRP54/Lhcb1 transit complex (6). Although these data suggest a heterotetrameric form of the cpSRP43/54 complex, a heterotrimeric cpSRP consisting of two molecules of cpSRP43 and a single cpSRP54 with a mass of 129 kDa has been proposed (14). Additionally, it has been proposed that cpSRP43 is a dimer both in stroma and in recombinantly expressed material (14).

In this report, we have expressed and purified *Arabidopsis* cpSRP43 and cpSRP54 to homogeneity individually as well as in complex (cpSRP). Recombinant cpSRP is a heterodimer and cpSRP43 a monomer. In an *in vitro* insertion assay, using preLhcb1 from pea as a substrate, we show that recombinant cpSRP is active. We identify regions of interaction between cpSRP and preLhcb1 as well as between cpSRP43 and cpSRP54 using an immobilized peptide library.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of cpSRP and cpSRP Components*—A bicistronic plasmid coding for the mature forms of the cpSRP components was constructed from two plasmids coding for the mature cpSRP54 and cpSPR43 proteins (a kind gift of N. E. Hoffman), introducing a tobacco etch virus protease cleavage site C-terminal to the 6-His tag of cpSRP43. The forward and reverse primers for cpSRP43 were: 5′-GCTCTAGAAGGAGGGTCTCACATGGCCGCCGTACAAAGAAACTACG-3′ and 5′-GCGTTAACGCTAGCTCATTCATTCATTGGTTGTTG-3′, respectively. The forward and reverse primers for cpSRP54 were: 5′-GCTCTAGAAGGAGATATATACATGTTTGGTCAGTTGACTGGTGG-3′ and 5′-GCGGATCCGCTAGCTTAGTTACCAGAGCCGAAGCC-3′, respectively. The polymerase chain reaction products were digested with *Bsa*I and *Nhe*I (cpSRP43) or *Xba*I and *Bam*HI (cpSRP54) and ligated into the expression plasmid digested with *Nco*I and *Bam*HI. This plasmid was introduced into *E. coli* BL21(DE3) cells, which were then grown in LB medium prior to induction with isopropyl-1-thio-β-D-galactopyranoside. The identity of the recombinantly expressed cpSRP54 was confirmed by Western blot using a polyclonal antibody raised against cpSRP54 in rabbits (a gift from N. E. Hoffman). The cpSRP43 construct contains an N-terminal 6-His tag and was identified by an anti-6-His antibody (Qiagen). The expression products were further analyzed by matrix-assisted laser desorption ionization mass spectrometry.

Lysate from the expression was bound to Fast Flow Chelating Sepharose (Amersham Pharmacia Biotech) in 50 mM Tris, pH 8, 20 mM imidazole, pH 8, 10% (v/v) glycerol (buffer A). The column was washed, and cpSRP43/54 complex was eluted by a step gradient to 300 mM imidazole, pH 8. The complex can be dissociated on a MonoQ column (Amersham Pharmacia Biotech) to yield the individual proteins or further purified on a Sephadex-200 column (Amersham Pharmacia Biotech) equilibrated in 10 mM Tris, pH 7.5, 300 mM NaCl, 2 mM dithiothreitol (buffer B). Pure cpSRP complex elutes as a single peak. When the complex is reconstituted from individually purified cpSRP54 and cpSRP43 excess of either cpSRP component elutes as a separate single peak from the Sephadex-200 column.

A C-terminal deletion construct of cpSRP54 (cpSRP54Δ461), which lacks the C-terminal 26 amino acids, was made using standard polymerase chain reaction and restriction enzyme techniques and was expressed and purified on Fast Flow Chelating Sepharose as described above for the full-length construct.

*Molecular Mass of cpSRP Components*—Equilibrium ultracentrifugation techniques were used to determine the molecular mass of both the cpSRP complex and cpSRP43 in high and low salt conditions using a Beckman XL-A ultracentrifuge (Beckman; Ref. 19). The sample volumes were 120 μl, with the wavelength and protein concentrations chosen to allow data collection within the linear range of the detector over the height of the column (concentrations were typically 0.1–1 mg/ml), although, in all cases, the protein solution under investigation was prepared by dilution from a concentrated sample (≥10 mg/ml). Centrifugation speeds were chosen based on predictions from the Ultrascan II for Unix package (copyright 1998, 1999; UTHSCSA). The data were fitted to a single component system using the Beckman Ultrascan package.

*Dynamic Light Scattering Measurements of cpSRP and cpSRP Components*—A Dynapro (DYNAPRO-MS) dynamic light scattering device was used to test for aggregation of cpSRP complex and cpSRP components in all buffers used. This technique measures the average hydrodynamic radius of molecules in solution and is routinely used in crystallographic laboratories in order to test for conditions in which purified proteins are present in a single oligomerization state (monodisperse) or in a number of different oligomerization states (polydisperse) (for a review, see Ref. 20).

*Peptide Libraries of cpSRP54 and preLhcb1*—Immobilized peptide libraries representing cpSRP54 (15-mer peptides, five amino acids shift) and preLhcb1 (18-mer peptides, three amino acids shift) were synthesized with an ASP222 machine on activated cellulose membranes containing polyethylene glycol 600 amino spacers (Abimed, Langenfeld, Germany) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) amino acid derivatives (21). Experimental conditions are described elsewhere (22). Briefly, the membranes are incubated to equilibrium with cpSRP or individual components under different blocking conditions, washed, and decorated with either an anti-cpSRP54 and/or an anti-His antibody (for 6-His-cpSPR43). After washing the membranes were incubated with the appropriate secondary antibodies, washed again, and binding detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). In order to identify nonspecific interactions, two different washing buffers in independent experiments (10 mM Tris/HCl, pH 7.6, 150 mM 0.02% (v/v) Tween 20, with or without an additional 0.5% (w/v) milk powder) were used.

*Lhcb1 Insertion Assay*—The precursor of pea Lhcb1 (preLhcb1) was synthesized *in vitro* by transcription of the cDNA clone AB80, followed by translation in a wheat germ lysate system (Promega) in the presence of [$^{35}$S]methionine. The translation mixture was treated with puromycin and centrifuged to remove any aggregated material, as described by Thompson *et al*. (23). Thylakoid membranes and stromal extracts of pea chloroplasts were prepared as described previously (24).

After lysis of the chloroplasts in 10 mM Hepes-KOH, 5 mM $MgCl_2$, pH 8 (HM buffer), the membranes were washed twice in 82.5 mM sorbitol, 12.5 mM Hepes-KOH, pH 8, before being resuspended in HM buffer to a concentration of 1 mg/ml chlorophyll (all operations were carried out at 4 °C). All insertion assays contained the following components: washed thylakoid membranes equivalent to 25 μg of chlorophyll, 0.15 mM GTP, 5 μl of *in vitro* translated pLhcb1, and HM buffer to a final volume of 100 μl. For analysis of cpSRP function, insertion assays included 10 μl of purified cpSRP in 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM dithiothreitol (buffer B) (protein quantities given in figure legends). Stromal controls contained stromal extract equivalent to 110 μg of chlorophyll, supplemented with 10 μl of buffer B. Buffer controls contained 10 μl of buffer B.

Where appropriate, various inhibitors were added to the insertion assays: 2 units of apyrase (Sigma, type VI), 0.5 mM non-hydrolyzable ATP analogue AMP-PNP (pH 7), 0.5 mM non-hydrolyzable GTP analogue GMP-PNP (pH 7), 0.125 mM L18 peptide, or 0.125 mM L13 peptide. Assay samples were set up on ice and the incubation started with the addition of the *in vitro* translated preLhcb1. Samples were incubated in a water bath for 30 min at 26 °C, under 300 μmole photons $m^{-2}$ $s^{-1}$. After incubation the samples were diluted by the addition of 0.5 ml of ice-cold HM buffer, and the membranes were reisolated by centrifugation at 20,000 × *g* for 5 min at 4 °C.

The membranes were then extracted with 6.8 M urea, 20 mM Tricine-NaOH, pH 8, as described by Thompson *et al*. (23), to remove non-inserted preLhcb1. One half of each sample was then treated with 0.2 mg/ml trypsin (Sigma, type XIII) on ice for 30 min. The digestion was stopped by the addition of 0.5 mg/ml trypsin inhibitor (Sigma, type I-S) and 10 μg of bovine serum albumin, before reisolation of the membranes by centrifugation at 20,000 × *g* for 5 min at 4 °C. The proteolyzed membranes were finally resuspended in 15 μl of HM containing 10 μg of trypsin inhibitor, and 15 μl of protein sample buffer before

immediately heating the sample to 100 °C for 5 min. Non-proteolyzed membranes were resuspended in 15 $\mu$l of 20 mM Tricine-NaOH, pH 8, and 15 $\mu$l of protein sample buffer.

All samples were analyzed by SDS-polyacrylamide gel electrophoresis, followed by staining of the gels in Coomassie Brilliant Blue and fluorography. Synthetic peptides L18 (VDPLYPGGSFDPLGLADD) and L13 (YLGPFSGESPSYL) were purchased from Jerini Biotools (Berlin).

*Detection of cpSRP54, cpSRP43, and cpFtsY in Thylakoid Membranes and Stroma by Western Blot*—Rabbit antibodies against *Arabidopsis* cpFtsY and cpSRP54 were a kind gift of N. E. Hoffman; a chicken antibody against *Arabidopsis* cpSRP43 was a kind gift of L. Nussaume. Washed thylakoid membranes were prepared as described above. Additional aliquots of thylakoid membranes were washed once with 2 M potassium acetate, pH 8, before being washed twice with 82.5 mM sorbitol, 12.5 mM Hepes-KOH, pH 8. All membranes were finally resuspended in HM buffer and their respective chlorophyll concentrations determined. Samples of intact pea chloroplasts, stromal extract, and the various thylakoid membranes equivalent to 5 $\mu$g of chlorophyll, together with defined quantities of cpSRP complex, cpSRP54, and cpSRP43 were separated by SDS-polyacrylamide gel electrophoresis and the proteins transferred to PVDF membranes (Bio-Rad) using a semidry blotting apparatus.

The membranes were probed with 1:2000 dilution of the anti-cpFtsY antibody, followed by 1:5000 dilutions of the anti-cpSRP54 and anti-cpSRP43 antibodies, and were stripped and washed between each probing. Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

## RESULTS

*cpSRP Is a Stable Heterodimer*—Cross-linking, glutathione *S*-transferase binding, and gel filtration data published previously suggest that cpSRP43 is a dimer in solution (14). Based on gel filtration data, it had been proposed that cpSRP is most likely a heterotrimer containing one cpSRP54 and a dimer of cpSRP43 (14). To study cpSRP in more detail, we have co-expressed the cpSRP proteins cpSRP54 and cpSRP43 from *Arabidopsis* in milligram amounts. Both proteins have been purified to homogeneity as determined by SDS-PAGE analysis (Fig. 1*A*). Pure cpSRP and cpSRP43 elute from the Superdex-200 column with apparent molecular masses of approximately 120 and 53 kDa, respectively (Fig. 1*B*). cpSRP can be purified either directly from *E. coli* co-expressing cpSRP54 and cpSPR43, or it can be formed by mixing purified cpSRP54 and cpSRP43 prior to gel filtration. In both cases, no stable higher molecular mass complexes are obtained and the excess of either cpSRP component elutes as a monomer. Only a small fraction of cpSRP components elute in the void volume of the column, with apparent molecular masses in excess of 2 MDa (Fig. 1*B*). cpSRP43 and cpSRP54 co-elute from a gel filtration column equilibrated in 600 mM NaCl with the same elution profile as shown in Fig. 1*B*. However, at an even higher salt concentration of 1 M NaCl, cpSRP43 and cpSRP54 elute as two overlapping peaks, indicating that no cpSRP is formed (data not shown).

cpSRP and cpSRP43 were subjected to equilibrium ultracentrifugation at various salt concentrations in order to accurately determine their molecular masses. The molecular mass of the cpSRP complex after gel filtration was determined to be 98 kDa (S.D. of fit 5 kDa) in both 300 mM and 25 mM NaCl (data not shown), in good agreement with the molecular mass of 93 kDa predicted from the cDNA sequences of mature 6-His-cpSRP43 and cpSRP54. As both cpSRP54 and cpSRP43 are present in the sample, visualized from the Coomassie stain of SDS-PAGE (Fig. 1*A*), this clearly indicates a heterodimeric structure for cpSRP. The molecular mass of cpSRP43 in 100 mM NaCl was determined to be 43 kDa (S.D. of fit 2 kDa, data not shown), which is consistent with a monomer. Dynamic light scattering indicated a monodisperse (unaggregated) behavior for cpSRP43, cpSRP54, and cpSRP complex at concentrations in excess of 1 mg/ml in all buffer and salt conditions tested. No other additional higher molecular mass components could be identified in any of these experiments, and in all cases the concentration of the sample was greater than 0.1 mg/ml.

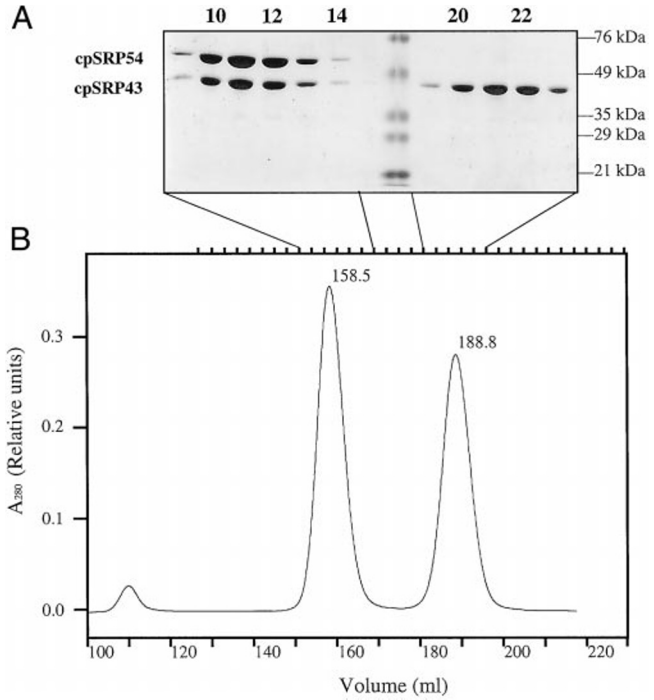


FIG. 1. **Purification of recombinant cpSRP.** *A*, SDS-PAGE analysis of cpSRP (*left*) and cpSRP43 (*right*). The bands corresponding to cpSRP components are marked. *Numbers* on *top* refer to fractions from the gel filtration chromatogram shown in *B*. Electrophoresis was carried out in a 12.5% polyacrylamide gel stained with Coomassie Brilliant Blue R-250. *B*, gel filtration elution profile of cpSRP reconstituted from individually purified cpSRP54 and cpSRP43. The Superdex-200 column was equilibrated as described under "Experimental Procedures." cpSRP54/43 complex elutes as a single peak (cpSRP) with excess cpSRP43 eluting as a separate single peak.

*Recombinant cpSRP Promotes Efficient Insertion of Lhcb1*—The activity of the recombinant cpSRP was tested using *in vitro* assays for the insertion of proteins into isolated thylakoid membranes. PreLhcb1 was used in these assays, as it is a known SRP substrate whose insertion is accompanied by the acquisition of pronounced resistance to trypsin proteolysis (6, 18). PreLhcb1 was incubated with thylakoids in the presence of insertion buffer, stromal extract (which contains cpSRP and so supports insertion) and the purified, heterodimeric cpSRP (Fig. 2*A*). In this experiment we also tested diagnostic inhibitors for their ability to inhibit insertion; these included the non-hydrolyzable analogues of GTP and ATP (GMP-PNP and AMP-PNP, respectively), the L18 peptide shown to inhibit preLhcb1 insertion, and an additional control peptide derived from the preLhcb1 sequence (termed L13).

Control assays carried out in the absence of any inhibitors (*lanes* marked *Cont*.) show that very little preLhcb1 inserts in the presence of buffer, whereas significant levels of protease-protected degradation product (*DP*) are observed using stromal extract. Importantly, the recombinant, dimeric cpSRP also supports efficient insertion, demonstrating that it is active. The activity of the cpSRP is completely inhibited in the presence of apyrase or the GTP analogue GMP-PNP, whereas AMP-PNP has no effect, confirming the GTPase activity of purified cpSRP. Interestingly, GMP-PNP also inhibits insertion using stromal extract, but in this case the inhibition is only partial. This can be understood on the basis that multiple rounds of cpSRP GTPase activity are needed for detectable levels of insertion.

The L18 peptide at 0.125 mM also inhibits the activity of the recombinant cpSRP. In this particular experiment, insertion

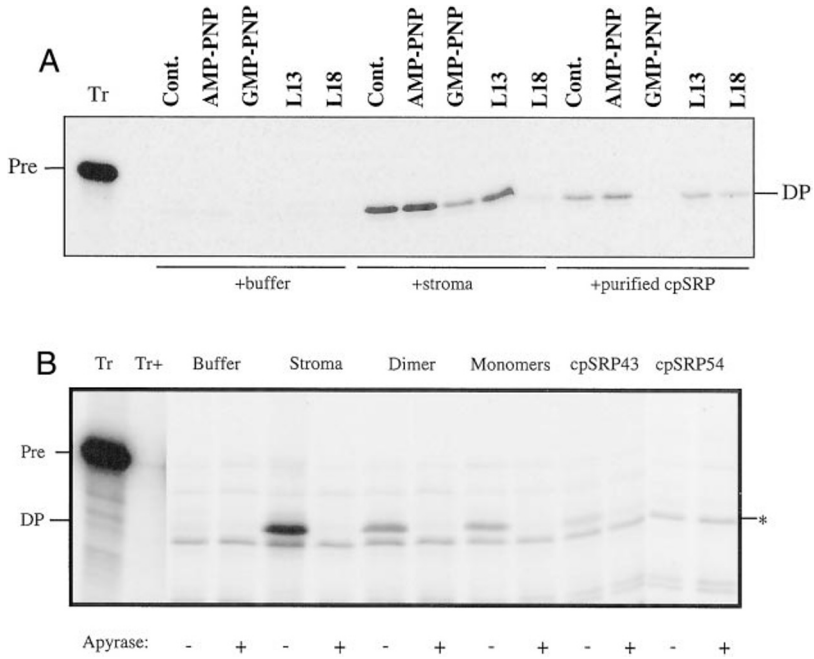


FIG. 2. **Recombinant cpSRP promotes insertion of preLhcb1.** *A*, *in vitro*-translated, radiolabeled pea preLhcb1 (*Pre*, *Tr*) was incubated with washed pea thylakoid membranes, as described under "Experimental Procedures," in the absence of additional cpSRP (+ *buffer*), in the presence of stromal extract (+ *stroma*), or in the presence of 10 $\mu$g purified cpSRP complex (+ *purified cpSRP*). Where indicated *above* the *lanes*, samples were also treated with 0.5 mM AMP-PNP, 0.5 mM GMP-PNP, 0.125 mM L13, 0.125 mM L18, or with no inhibitors (*Cont.*). After incubation, the thylakoid membranes were reisolated, extracted with 6.8 M urea, and then digested with 0.2 mg/ml trypsin, in order to measure the amount of Lhcb1 degradation product inserted in the membrane (*DP*). *B*, *in vitro* translated, radiolabeled pea preLhcb1 (*Pre*, *Tr*) was incubated with washed pea thylakoid membranes, as described under "Experimental Procedures," in the absence of additional cpSRP (*Buffer*), in the presence of stromal extract (*Stroma*), or 3 $\mu$g of purified cpSRP complex (*Dimer*), or a total of 1 $\mu$g of purified cpSRP monomers added separately (*Monomers*), or 0.6 $\mu$g of cpSRP43 alone (cpSRP43), or 0.3 $\mu$g of cpSRP54 alone (cpSRP54). Where indicated *below* the *lanes* (+, −), samples contained 2 units of apyrase or no apyrase. After the incubation, the membranes were reisolated, washed, and digested with 0.2 mg/ml trypsin, yielding a degradation product of preLhcb1 (*DP*), indicative of correct insertion. The band *below DP*, marked with an *asterisk* (*) is derived from non-inserted precursor protein stuck to the thylakoid membrane surface, and not removed by the membrane washes after the insertion assay. Samples in *A* and *B* were analyzed by SDS-PAGE and fluorography.

efficiency was reduced by about 50%, but near-complete inhibition has been observed in other experiments (data not shown). The L18 peptide also inhibits the ability of stromal extract to support insertion, as found previously (16). No effect is observed using the L13 peptide as a control.

Insertion assays using stromal extract, recombinant cpSRP, and the individual cpSRP subunits show that both components of cpSRP are required for insertion of preLhcb1 (Fig. 2*B*). The insertion observed with recombinant material is lower than that of stromal extract. No insertion is observed using purified cpSRP54 alone, and very little insertion is evident with cpSRP43 alone (this background insertion is probably due to contaminating cpSRP54 on the thylakoids; see below). These data indicate that the separate subunits can combine rapidly to form an active cpSRP complex during the timescale of the experiment (30 min). All of the insertion processes shown in this experiment are totally inhibited by pretreatment of the assay mixture with 2 units of apyrase, which hydrolyzes nucleoside triphosphates.

Further tests were carried out to compare the activity of recombinant cpSRP with that present in crude stromal extract and to investigate the basis for the cpSRP43 mediated low level insertion observed above. Fig. 3 (*A* and *B*) shows cpSRP43 and cpSRP54 immunoblots of stromal extract (*lane S*, equivalent to 5% of that present in the import assay) and the purified heterodimeric cpSRP (*lane D*), equivalent to 10% of that used in the insertion assay. The signals obtained in this and other experiments indicate that the amount of recombinant cpSRP used in these assays is within a factor of 5 to that of the stromal sample. Fig. 3*C* shows cpSRP43, cpSRP54, and cpFtsY immunoblots of stromal extract (*lanes S*), the thylakoids used in the insertion assays (*lanes T*) and thylakoids washed stringently using 2 M potassium acetate, which removes all extrinsic protein (*lanes Tac*). The cpSRP43 signal is evident in the stroma sample but completely absent from either thylakoid sample, demonstrating that cpSRP43 does not interact directly with the thylakoid membranes used in the insertion assays. In contrast, Fig. 3*C* demonstrates that some cpSRP54 is present associated with the thylakoids used in the insertion assays and this subunit is only totally removed by the more stringent acetate washing. These data serve to explain some of the findings using the individual cpSRP subunits (Fig. 2*B*). cpSRP43 alone was shown to support low level insertion of preLhcb1. cpSRP54 is present on the thylakoids, in low but significant amounts, and we propose that this endogenous cpSRP54 is able to bind to the recombinant cpSRP43 added to the insertion assays and thereby reconstitute an active cpSRP complex. It seems unlikely that cpSRP43 on its own can support insertion at any level, since it contains no identifiable nucleotide binding site and the insertion processes shown in Fig. 2*B* are all apyrase-sensitive. Taken together, these data strongly indicate that the cpSRP-dependent insertion of preLhcb1 is dependent on the presence of both subunits.

Although acetate washing of thylakoids is highly effective at removing cpSRP54, it is inappropriate in these experiments because it also removes any cpFtsY from the thylakoid membranes and this factor is also essential for the insertion of preLhcb1 (14). The *lower panel* of Fig. 3*C* confirms that the thylakoids in the assay (*lane T*) contain a faint signal in cpFtsY immunoblots, whereas acetate-washed thylakoids (*lane Tac*) display no signal at all. The vast majority of cpFtsY is in fact found in the stroma, which gives a much stronger signal (*lane*

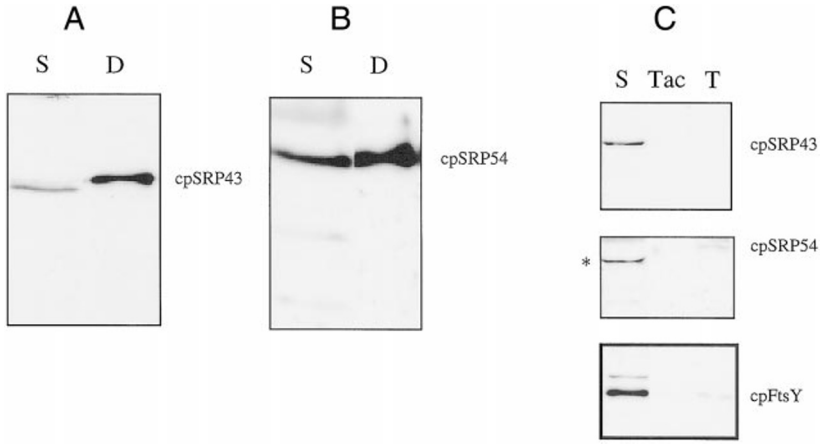


FIG. 3. **Western detection of cpSRP and cpFtsY in thylakoid membranes and stromal extract.** *A*, comparison of cpSRP43 present in stromal extract and recombinant cpSRP. Samples of stromal extract (*lane S*, equivalent to 5% of that present in all insertion assays) and dimeric cpSRP (*lane D*, 0.3 mg, equivalent to 10% of that present in the insertion assay of Fig. 2*B*) were separated by SDS-PAGE, transferred to PVDF membranes, and probed with anti-cpSRP43 antibodies. The position of stromal cpSRP43 is indicated. Recombinant cpSRP43 migrates more slowly than the corresponding pea protein, due to the presence of an N-terminal 6-His tag. *B*, comparison of cpSRP54 present in stromal extract and recombinant cpSRP. Samples of stromal extract (*lane S*) and dimeric cpSRP (*lane D*) were analyzed exactly as in Fig. 3*A*, but were probed with anti-cpSRP54 antibodies. The position of cpSRP54 is indicated. *C*, comparison of cpSRP and cpFtsY present in stromal extract and on washed thylakoid membranes. Samples of stromal extract (*lanes S*, equivalent to 5% of that present in all insertion assays), potassium acetate-washed thylakoid membranes (*lanes Tac*, equivalent to 5 mg of chlorophyll), and thylakoids prepared as for insertion assays (*lanes T*, equivalent to 5 mg of chlorophyll, 20% of that present in all insertion assays) were separated by SDS-PAGE, followed by transfer to PVDF membranes. The membranes were first probed with antibodies against cpSRP43 (*top panel*), and then the membranes were stripped and re-probed with antibodies against cpSRP54 (*middle panel*), followed by a final stripping and re-probing with antibodies against cpFtsY (*bottom panel*). The positions of the proteins are indicated to the *right* of the panels. In this experiment, the abundance of the large subunit of Rubisco in the stromal extract causes pea cpSRP54 to migrate further down the gel (marked *) in comparison to the corresponding protein on the thylakoid membrane.

*S*). This additional cpFtsY probably contributes to the higher overall insertion efficiency obtained using crude stromal extract and may explain why a slightly lower insertion efficiency is obtained using recombinant cpSRP, where only the residual thylakoid-associated cpFtsY is present.

*Interaction of cpSRP Components and PreLhcb1*—cpSRP54 has previously been shown by a cross-linking approach to bind preferentially to the hydrophobic TM3 of LHCPs (15). Binding assays (17) and fusion constructs (16) showed that cpSRP43 alone also interacts with the highly conserved L18 sequence located immediately N-terminal to TM3 and that the L18 sequence motif is involved in precursor recruitment to the post-translational cpSRP pathway. Although cpSRP43 was proposed to be a dimer (14), it was not clear whether the putative dimeric form is required for interaction with LHCPs.

In order to map the interactions between recombinant cpSRP and substrate components, we used an immobilized peptide library (pepscan) representing the sequence of preLhcb1 from pea (see Fig. 4*B*). 18-mer peptides representing Lhcb1, overlapping by 15 residues, were synthesized on a cellulose membrane, *i.e.* 85 peptides for the 269 residues of preLhcb1. Recombinant cpSRP or its components were incubated to equilibrium with the pepscan membrane under blocking conditions described elsewhere (22). Binding of cpSRP and components was detected by immunodecoration with anti-6-His or anti-cpSRP54 antibodies (Fig. 4*A*). Initially low concentrations of cpSRP components (5 $\mu$g/10 nM) and complex (5 $\mu$g/5 nM) were used, but cpSRP43 could only be detected at very low levels when compared with the signal obtained for cpSRP54 (*upper lanes*). Consequently, 20-fold higher concentrations of cpSRP components (100 $\mu$g/200 nM) and cpSRP complex (100 $\mu$g/100 nM) were used to probe the membranes (*lower lanes*), including an additional 0.5% (w/v) milk powder as blocking component. These two different conditions allow us to identify and discard nonspecific interactions that are not conserved across the two incubation conditions.

When the pepscan is incubated with low concentrations of cpSRP43 (5 nM), only rather weak signals with peptides from the chloroplast transit signal peptide (CTSP), TM2, and L18 can be detected (*upper lane*). Upon incubation with higher concentrations of cpSRP43 (*lower lane*), it can be seen that cpSRP43 interacts with peptides corresponding to the CTSP and L18 motif more strongly and that the signal from the TM2 is reduced (Fig. 4*A*). This indicates that cpSRP43 interacts preferentially with the L18, consistent with previous data (16, 17). cpSRP54 interacts with peptides from the CTSP and TM3 of preLhcb1. Only negligible amounts of cpSRP54 bind to peptides corresponding to the first and second transmembrane helices (TM1 and TM2) and to the L18 motif (Fig. 4*A*).

When probed with recombinant cpSRP, the CTSP, L18, and TM3 regions give the strongest signals, with the signal from the L18 peptides being significantly enhanced with respect to that obtained with a similar concentration of cpSRP54 alone. Peptides of the TM2 are again highlighted, but the relative strength of this signal is greatly reduced when an additional 0.5% (w/v) milk powder is included in the incubation and wash steps. The signals from the CTSP, L18, and TM3 are unaffected by this treatment.

The comparison of the signals obtained with the individual proteins (see above) with those of cpSRP suggests that L18 is recognized primarily by cpSRP43 and not by cpSRP54. Peptides from the L18 motif dominate the signal from the pepscan. We cannot directly compare the amounts of cpSRP components detected by the different antibodies, but the presence of cpSRP54 in cpSRP seems to enhance the interaction with L18. The spot (peptide) corresponding to the full L18 sequence gives a strong signal on the membranes probed with cpSRP. Peptides corresponding to regions of Lhcb1 N-terminal to the full L18 do not give a strong signal. However, peptides C-terminal to the full L18 do give a strong signal. Even when only a fraction of the L18 sequence is present in the peptide, with the remainder being from the TM3, the signal remains very strong (Fig. 4*A*). These data show that cpSRP binds strongly to peptides that contain contributions from both the L18 and TM3, but not to peptides that contain a part of L18 alone.

*Interaction of cpSRP54 and cpSRP43*—Structural informa-

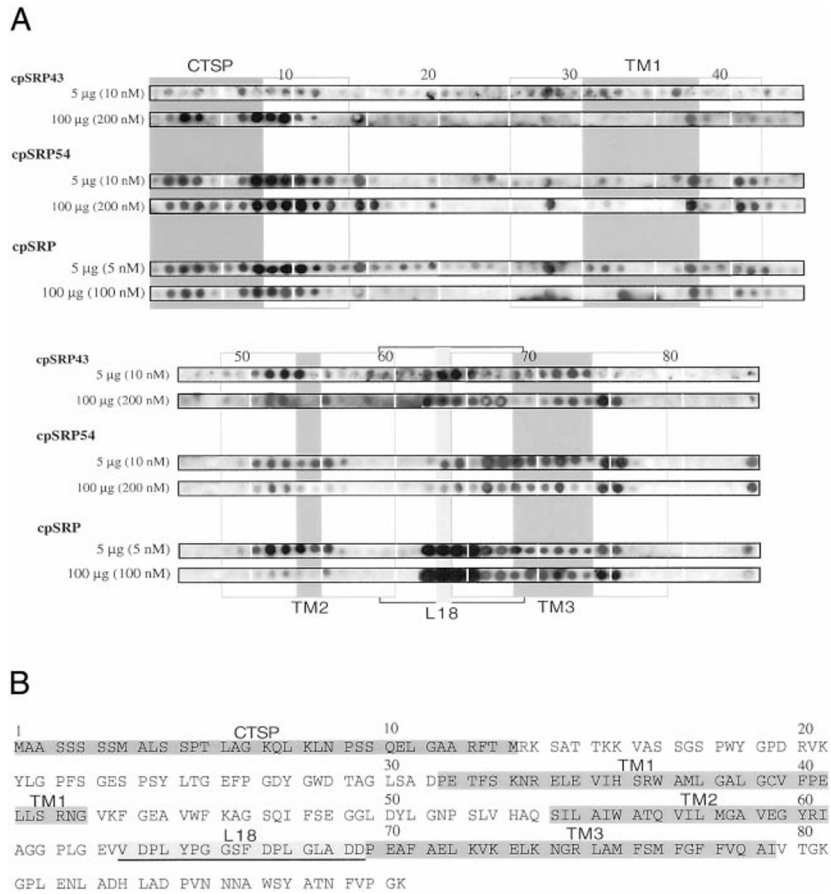


FIG. 4. **Mapping cpSRP substrate interactions using a pepscan of preLhcb1 from pea.** *A*, cpSRP, cpSRP54, and cpSPR43 were incubated with a peptide library (pepscan) derived from the preLhcb1 from pea (consisting of 85 18-mer peptides, shifted by three residues) using two different blocking conditions (see text for details). Bound proteins were detected by immunodecoration using anti-His or anti-cpSRP54 antibodies. The regions corresponding to the three transmembrane helices (TM1–3), the L18 motif, and CTSP are indicated. The *boxed* boundaries indicate peptides that contain at least two residues from these elements; the central part (*filled boxes*) represents peptides that contain only residues from CTSP, TM1–3, or L18, respectively. *B*, amino acid sequence of preLhcb1 from pea. The CTSP, TM1–3, and L18 motifs are indicated. The assignments for the transmembrane helices are taken from a structural model (48). The *numbering above* the sequence refers to the peptide in *A* which contains the following 18 residues.

tion is available for the individual domains of SRP54 homologues, *e.g.* the NG domain of SRP GTPases (25–27), the M domain (28, 29), and the M domain in complex with the RNA (30). We reasoned that the pepscan approach could give relevant information on the interaction of cpSRP54 and cpSRP43 when results from this technique are mapped on to models of the cpSRP54 NG and M domains derived from the crystal structures (modeled by the SWISS-MODEL server; Ref. 31). In cpSRP both proteins are folded, and one would expect that the heterodimer interface is formed by a number of exposed residues of both proteins. As a result, interactions between cpSRP43 and a peptide library representing cpSRP54 (and *vice versa*) could be reasonably anticipated to be rather weak.

We used a peptide library representing the sequence of cpSRP54 from *Arabidopsis* (Fig. 5*B*). The 15-mer peptides, overlapping by 10 residues, were synthesized on a cellulose membrane, *i.e.* 96 peptides for the 487 residues of cpSRP54. The experiment was performed under the same conditions as described above. When the pepscan of cpSRP54 is incubated with 0.1 mg (200 nM) cpSRP43 in the presence of 0.5% (w/v) milk powder, a larger number of interactions are highlighted (Fig. 5*A*). These peptides correspond to regions in the G domain neighboring the consensus elements for nucleotide binding (G elements I–V, Ref. 32) including the so-called switch regions and part of the I-box, an insertion specifically found in SRP-GTPases (25). The flexible linker between the G and M domains (33–35) and particular regions in the M domain also seem to contribute to the interaction. In particular, peptides of the C-terminal region of cpSRP54 appear to bind cpSRP43 strongly. cpSRP54 has a highly charged C-terminal extension when compared with the SRP54 homologue from *E. coli* (Ffh). Other regions of the M domain that seem to interact with cpSRP43 are part of the Ffh RNA binding site (RI and II) and also the signal peptide binding site. Since cpSRP43 is highly negatively charged, one could expect that all peptides with positively charged residues should give rise to a signal. However, this is not the case, as can be seen from Fig. 5. The interacting peptides were mapped onto the model of cpSRP54 and show that all peptides highlighted contribute to the molecular surface of cpSRP54 (data not shown). None of the peptides that give a signal consist only of buried amino acids. Since the regions that give a signal in this assay are rather spread out on the surface of cpSPR54, one would expect an elongated shape for cpSRP43. Analytical ultracentrifugation data indicate that cpSRP43 is indeed an elongated molecule with an axial ratio in excess of 7:1 (data not shown). This elongated shape is supported by the higher apparent molecular mass of cpSRP43, as derived from a gel filtration chromatography (see above).

The complementary experiment of mapping cpSRP43 interactions probed with cpSRP54 was also performed, and a distinct pattern of spots were highlighted (data not shown). Since structural models are only available for small domains of cpSRP43, they do not allow to produce a meaningful overall structural model for cpSRP43. Therefore, these data have to wait for a more detailed interpretation.

*C-terminal Region of cpSRP54 Is Essential for Stable cpSRP Complex Formation*—Since the C-terminal region of cpSRP54 gave a prominent signal in the pepscan, the relevance of this interaction for cpSRP was tested by a deletion construct of cpSRP54 lacking the C-terminal 26 residues (cpSRP54Δ461). cpSRP54Δ461 was highly expressed (assessed by SDS-PAGE and Western blot), but the protein did not co-purify with 6-His-tagged cpSRP43 on a nickel-chelating column as the wild type protein does (data not shown). This indicates that cpSRP54Δ461 is no longer able to form a stable complex with cpSRP43 and that the C-terminal region of cpSRP54 is essential for complex formation. The highly charged nature of the

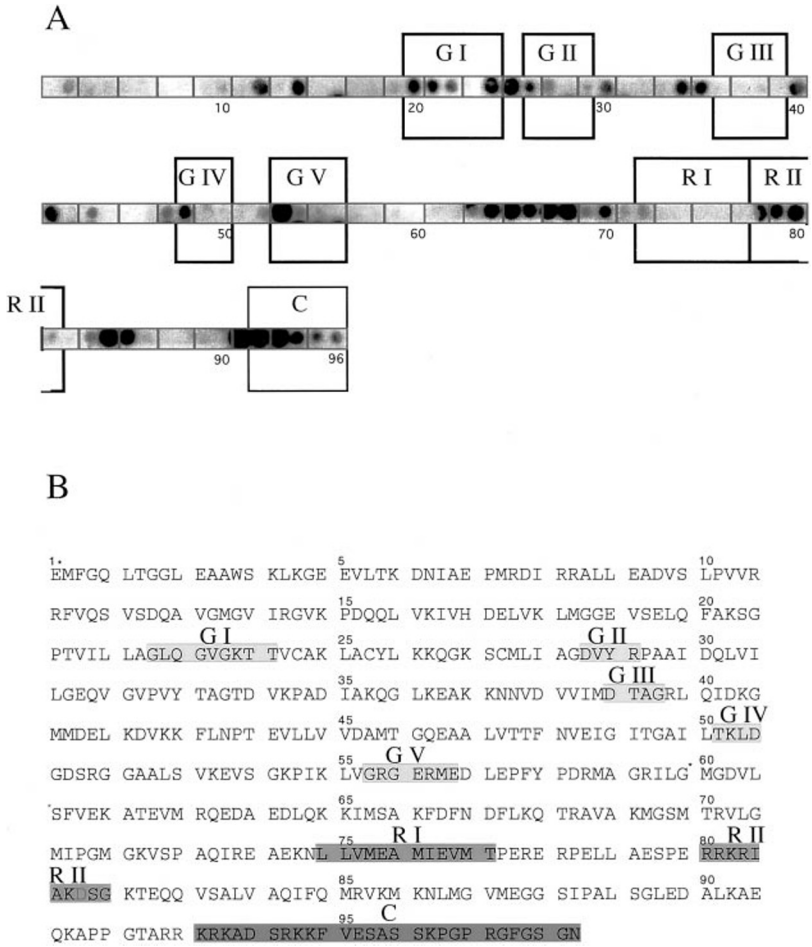


FIG. 5. **Mapping the interactions of cpSRP54 and cpSRP43.** *A*, 6-His-cpSRP43 was incubated with a peptide library derived from cpSRP54 from *A. thaliana* (see text for details). Regions corresponding to the consensus elements of nucleotide binding (*GI–V*) (32), signal peptide binding site (between *GV* and *RI*), RNA binding sites (*RI* and *RII*) (30), and the C-terminal extension (*C*) are indicated. Experimental conditions are described in the text and for Fig. 4*A*. *B*, amino acid sequence of cpSRP54 from *A. thaliana*. The assignments for the GI–V, RI, and RII regions are taken from sequence alignments against structural models (25–28, 30) and are indicated above the sequence. The *numbering above* the sequence refers to the peptide in *A* which contains the following 15 residues.

C-terminal amino acids may be a major contributor to the high salt stability of cpSRP.

## DISCUSSION

*Stoichiometry and Activity of Recombinant cpSRP*—cpSRP has been previously described as a heterotrimer, consisting of one molecule of cpSRP54 and a dimer of cpSRP43. This was based on the observation that *in vitro* translated cpSRP43 elutes from gel filtration columns with an apparent molecular mass of approximately 70 kDa (14). A cross-linking approach and regions in the protein that show homology to chromodomains further supported the idea of a dimeric form of cpSRP43 (14).

The detailed biochemical characterization described in this paper shows that recombinantly expressed cpSRP is a stable heterodimer consisting of one molecule of cpSRP54 and one molecule of cpSRP43 with a molecular mass of ~100 kDa. Reconstitution of cpSRP complex with either component in excess yielded no different species stable by gel filtration. An *in vitro* insertion assay using preLhcb1 as substrate showed that recombinant, heterodimeric cpSRP is biologically active as it promotes efficient insertion (Fig. 2). Both protein components are essential for activity since cpSRP43 or cpSRP54 alone are insufficient to support significant levels of insertion of preLhcb1 into thylakoid membranes. There is no indication from our experiments that cpSRP43 is able to form a dimer in solution under either high or low salt conditions, even at concentration levels in excess of 10 mg/ml (0.05 mM). However, preliminary data indicate that a truncated cpSRP43 construct (lacking the C-terminal chromodomain) forms a dimer that is stable by gel filtration and has a molecular mass of 60 kDa determined by analytical ultracentrifugation (data not shown). Recent publications on the structures of chromodomains show that they may exist as both monomers and dimers in nature (36, 37). For example, the NMR solution structure of a chromodomain from mouse modifier protein 1 is a monomer (36), whereas the chromo shadow domain Swi6 is a dimer in solution (37).

We have also shown that insertion of preLhcb1 can be efficiently competed with apyrase, GMP-PNP, or the L18 peptide. This indicates that recombinant cpSRP interacts with its major transport substrate in the same way as described previously and suggests that cpSRP follows the GTP-dependent insertion mechanism of other SRP systems. No GTP was detected by high performance liquid chromatography analysis of recombinant cpSRP (data not shown), which suggests that the binding affinities and kinetics of guanine nucleotides are similar for cpSRP as for other SRP GTPases, which are stable in the nucleotide-free form and have been characterized in detail (38, 39).

*Substrate Interaction of Recombinant cpSRP*—So far the pepscan method has been used predominantly to map antibody-antigen interactions (epitope mapping) (for a recent review, see Ref. 40) as well as chaperone-substrate interactions (*e.g.* Ref. 41). Only recently the pepscan approach was also used for mapping the interactions between proteins that interact in their folded states (42). We used the pepscan approach as a fast method for mapping the interactions of cpSRP and preLhcb1 since chloroplast proteins are transported across the chloroplast membranes in an unfolded state (reviewed in Ref. 43). We could show that cpSRP54 interacts strongly with the third transmembrane helix of preLhcb1, in agreement with previously published data (15). The clear preference of cpSRP54 for TM3 found in our experiments suggests that the length and hydrophobicity of the targeting sequence are the main discriminating factors for binding. The two other transmembrane helices (TM1 and TM2) are shorter

and less hydrophobic and give only a negligible signal, which can be further suppressed by modifying the blocking conditions. In bacterial SRPs the hydrophobicity of the signal peptide seems to serve as a main discriminating factor between the SRP and Sec pathways (44, 45).

Recombinant, monomeric cpSRP43 is shown to interact with preLhcb1 specifically via the L18 motif, confirming the observation of Tu *et al*. (17). Peptides containing the L18 and TM3 motifs in the peptide library are continuous, and the signal seems enhanced with cpSRP compared with the individual components. These results suggest that the L18 and TM3 motifs of Lhcb1 represent a continuous epitope for the recognition by cpSRP and that both cpSRP43 and cpSRP54 contribute to binding. Thus, cpSRP43 might serve in the post-translational targeting pathway to increase the selectivity of cpSRP for its target proteins.

LHCPs are synthesized in the cytosol as precursors with a hydrophilic CTSP N-terminal to the mature sequence. The CTSP is cleaved by a stromal metalloprotease, either during or after import across the chloroplast inner membrane (46). The CTSP region of preLhcb1 was identified as an additional region of interaction with cpSRP43 and cpSRP complex. Previously described deletion and mutagenesis experiments have shown that preLhcb1 constructs with a truncated CTSP are inefficiently inserted into the thylakoids when imported into intact chloroplasts (47). This defect is not found in the insertion of preLhcb1 into isolated thylakoid membranes. The interaction of CTSP and cpSRP highlighted by the pepscan results may play a role in the transfer of preLhcb1 from the translocation machinery of the chloroplast inner envelope to cpSRP.

*Complex Formation*—Although cpSRP has been studied by several groups, the manner of complex formation between cpSRP54 and cpSRP43 has not been analyzed in detail. The pepscan approach was used in order to map the interactions between cpSRP43 and cpSPR54 using a peptide library of cpSRP54. This approach was chosen since the availability of high resolution structures of SRP54 domains allows for modeling of cpSRP54, which enables a more substantial interpretation of the data. The first group of peptides highlighted on this experiments maps to regions of the G domain of SPR54 close to the G-elements and the I-box. These regions are known to be on the surface of the protein and to have functional relevance for the interaction with nucleotides or with regulatory proteins. Whether complex formation of cpSRP54 and cpSRP43 has consequences for nucleotide binding remains to be seen. Moreover, cpSRP43 also binds to regions that correspond to the proposed binding site of the signal peptide and the binding site of 4.5 S RNA in SRP54 as derived from the recent crystal structure of Batey *et al*. (30). cpSRP54 is unique among the SRP54 homologues in that a conserved glycine residue in the RNA binding box is replaced by an aspartate (Asp-403). Modeling of cpSRP54 based upon the recent crystal structure of the SRP54 M domain/RNA complex suggests that an aspartate in this region would introduce a negative charge in the immediate vicinity of the negatively charged RNA phosphate backbone. This would reduce the affinity of cpSRP for any potential chloroplast SRP RNA. Indeed, experiments to form a complex of cpSRP54 with 4.5 S RNA failed (13). The C-terminal region of cpSRP54 seems to interact strongly with cpSRP43. This region is not conserved within any of the other SRP GTPases. Deletion of the C-terminal 26 amino acids of cpSRP54 has shown this interaction to be crucial for the formation of a stable cpSRP heterodimer (see above). These results explain why other SRP54 homologs cannot form a complex with cpSRP43 as observed previously (13). Whether the positively charged C terminus also interacts specifically with ribosomal RNA remains to be examined. Evolutionary close relatives of cpSRP54 from *Synechocystis* sp. also possess a C-terminal extension, although with a smaller number of positively charged amino acids. If this region is mainly contributing to complex formation with cpSRP43, only cpSRP54 proteins from plant chloroplasts should have this extension.

Previous investigations of cpSRP-mediated targeting have been greatly hampered by the lack of large amounts of highly pure material. The availability of recombinant cpSRP provides the basis for a much more detailed biochemical characterization of cpSRP and transit complex components, which we have started here. The puzzling questions remain: Is cpSPR43 recruited only for the targeting of Lhcb1 and related proteins or does it have an additional role in protein import? Is cpSRP54 able to switch between the co- and post-translational targeting pathways depending on the availability of substrates?

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