A unique sequence motif in the 54 kD subunit of the chloroplast signal recognition particle mediates binding to the 43 kD subunit

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Running title: The cpSRP43-binding site of cpSRP54
SUMMARY

Chloroplasts contain a novel type of SRP (cpSRP) that consists of two proteins, cpSRP54 and cpSRP43. CpSRP is involved in the posttranslational targeting of the nuclear encoded light harvesting chlorophyll binding proteins (LHCPs) to the thylakoid membrane by forming a soluble cpSRP/LHCP transit complex in the stroma. Despite high sequence homology between chloroplast and cytosolic SRP54 proteins the 54 kD subunit of cpSRP is unique in its ability to bind cpSRP43. In this report we identified a 10 amino acid long segment of cpSRP54 that forms the cpSRP43-binding site. This segment is located at position 530-539 close to the C-terminus of cpSRP54. In addition, we demonstrate that arginine at position 537 is essential for binding cpSRP43 and that mutation of arginine 536 drastically reduced cpSRP43 binding. Mutations within the cpSRP43-binding site of cpSRP54 that reduced or completely abolished cpSRP complex formation did also inhibit transit complex formation and integration of LHCP into the thylakoid membrane reflecting the importance of these residues for LHCP targeting. Alignment studies revealed that the cpSRP43-binding site is conserved in chloroplast SRP54 proteins and is not present in any SRP54 subunit of cytosolic SRPs.
INTRODUCTION

The cytosolic signal recognition particle (SRP) is part of a ubiquitous protein targeting machinery that mediates the cotranslational insertion of membrane proteins into the endoplasmic reticulum of eukaryotes and the cytoplasmic membrane of prokaryotes. All known cytosolic SRPs are ribonucleoproteins and their minimal functional core is formed by an RNA component and a conserved ~54 kD protein (SRP54). SRP54 consists of an N-terminal NG-domain encoding a GTPase function and a C-terminal located M-domain, that binds to the signal sequence of the elongating substrate protein (1-3). Recently, it was demonstrated that chloroplasts contain an SRP that is involved in the posttranslational targeting of members of the nuclear encoded light harvesting chlorophyll binding protein family (LHCPs) to the thylakoid membrane (4-6). LHCPs form the peripheral antenna of photosystem I and II and comprise approximately one third of the thylakoid membrane proteins. Like all known cytosolic SRPs chloroplast SRP contains a 54 kD subunit (cpSRP54). Interestingly, in contrast to cytosolic SRPs chloroplast SRP does not contain a RNA, but rather a novel protein subunit of 43 kD (cpSRP43) (5, 6). Although cpSRP54 and bacterial SRP54 (Ffh) show high sequence similarity, the chloroplast protein is clearly distinguishable from Ffh since Ffh cannot bind to cpSRP43 (7). In the current model of LHCP targeting to the thylakoid membrane of higher plants the nuclear encoded LHCP is imported across the envelope membranes into the chloroplast stroma where, after cleavage of the transit peptide it is bound by cpSRP to form the soluble transit complex (6). Besides cpSRP (cpSRP54 and cpSRP43) a chloroplast homologue of the bacterial SRP receptor (cpFtsY) and GTP are required as soluble components for LHCP integration (8-10). Recently, the integral thylakoid membrane protein Alb3 was identified as the first transmembrane protein required for LHCP integration (11).
Several thylakoid membrane proteins (e.g. D1, D2, PSI-A, PSI-B) are encoded by the chloroplast genome and cotranslationally inserted into the thylakoid membrane. Recent reports describe that cpSRP54 is involved in the cotranslational targeting of D1 to the thylakoid membrane by binding to the first transmembrane domain of the elongating nascent chain of D1 (12, 13). Notably, no interaction of cpSRP43 with the D1 protein was detected (12). Further evidence for an involvement of cpSRP54 in cotranslational targeting of thylakoid membrane proteins came from the analysis of Arabidopsis mutants lacking functional cpSRP54. The young leaves of these plants showed a reduced level of the plastid encoded photosystem I and II reaction center proteins (14, 15). These results supported the earlier observation that the chloroplast stroma contains two different pools of cpSRP54. One pool is bound to cpSRP43 and active in transit complex formation with LHCP, whereas a second pool of cpSRP54 was found to be associated with 70 S ribosomes in absence of cpSRP43 (6, 16).

These findings raise the central question of how cpSRP54 is recruited for functioning in either the cpSRP43-dependent posttranslational or the cpSRP43-independent cotranslational cpSRP-pathway. To answer this question it is important to know the exact nature of protein-protein interactions occurring during the post- and cotranslational mode of action of cpSRP54. As a first step to solve this question and to identify specific sequence characteristics of cpSRP54 that makes cpSRP54 unique among the SRP54 subunits in its ability to bind cpSRP43, we aimed to characterize the binding site of cpSRP54 for cpSRP43. Recently, it was shown that cpSRP43 binds to the M-domain of cpSRP54 (17) and a report by Groves et al. (18) describes that the 26 C-terminal amino acids of cpSRP54 are essential for binding cpSRP43. Here, we show that a 10 amino acid long segment of cpSRP54 forms the cpSRP43-binding site and demonstrate that two amino acids within this region are crucial for binding cpSRP43. These residues are located close to the C-terminus of cpSRP54, but not within the last 26 amino acids.
acids. The cpSRP43-binding site identified in this report is conserved in all chloroplast SRP54 proteins and is not present in any SRP54 subunit of cytosolic SRPs.

**EXPERIMENTAL PROCEDURES**

**N- and C-terminal deletion constructs of cpSRP54 for the yeast two-hybrid system**

All cDNAs encoding the C-terminal M-domain (residues 371-564) of cpSRP54 (cpSRP54M) or various truncations of cpSRP54M were obtained by PCR amplification with Dynazyme (Biometra) using pAS2-cpSRP54 (17) as template. The following primer combinations were used to amplify the indicated constructs (Table 1): 1/7 to yield cpSRP54M, 2/7 to yield cpSRP54(485-564), 3/7 to yield cpSRP54(521-564), 4/7 to yield cpSRP54(530-564), 5/7 to yield cpSRP54(533-564), 6/7 to yield cpSRP54(535-564), 1/8 to yield cpSRP54(371-543), 1/9 to yield cpSRP54(371-541), 1/10 to yield cpSRP54(371-539), 1/11 to yield cpSRP54(371-538) and 1/12 to yield cpSRP54(371-537). All PCR-products were digested with the restriction enzymes *NcoI* and *BamH1* and cloned into the *NcoI*-*BamH1* site of the yeast two-hybrid bait plasmid pGBKT7 (BD Biosciences) encoding the Gal4 DNA binding domain. All constructs contained an additional c-Myc epitope tag. Plasmid pACT2-cpSRP43 (17) encoding the Gal4 activation domain as a fusion with mature cpSRP43 was used as prey plasmid. Each construct was confirmed by sequencing (Seqlab).

**Site-directed mutagenesis constructs for the yeast two-hybrid system**

All site-directed mutagenesis constructs were generated using the QuickChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The primer sets used for the mutant constructs are listed in Table 2. Plasmid pGBK7-cpSRP54M was used as template DNA to gain the single point mutation constructs cpSRP54M(R536G),
cpSRP54M(R537G), cpSRP54M(K538M), cpSRP54M(R539G), cpSRP54M(K540M) and the deletion constructs cpSRP54M(Δ531-539), cpSRP54M(Δ536-540) and cpSRP54M(Δ538-540). The double mutation constructs cpSRP54M(R537G, K538M), cpSRP54M(K538M, R539G), cpSRP54M(R539G, K540M) and cpSRP54M(K538M, K540M) were obtained by using the single point mutation constructs as template. The triple point mutation construct cpSRP54M(K538M, R539G, K540M) was obtained by using pGBKT7-cpSRP54M(R539G, K540M) as template and the quadruple point mutation constructs cpSRP54M(R537G, K538M, R539G, K540M) and cpSRP54M(R536G, K538M, R539G, K540M) by using pGBKT7-cpSRP54M(K538M, R539G, K540M) as template. The correct sequence of all constructs was verified by sequencing (Seqlab).

Yeast two-hybrid assay

The yeast two-hybrid assays were done as described in Jonas-Straube et al. (17), except for the following modifications. pGBKT7 constructs (see above) were used instead of pAS2 constructs as prey plasmids. For cpSRP54M constructs that showed only weak or no interaction with cpSRP43 in the yeast two-hybrid experiments, expression levels comparable with full-length cpSRP54M were verified by Western blot analysis using antibodies against the c-Myc epitope (BD Biosciences). Growth of the yeast cells on medium lacking leucine, tryptophan and histidine (-leu,-trp,-his) was classified in (++), (+) and (-), whereby (++) means that most colonies have a diameter of > 1,5 mm and (-) indicates normal background growth (whitish colonies < 0,6 mm). The filter lifts to measure β-galactosidase activity were incubated for at least 1.5 h to develop a blue color ((++): strong blue color; (+): medium blue color; (-): no blue color development). All pGBKT7-constructs were cotransformed with pACT2 in yeast cells and showed no self-activation of the reporter genes.
Plasmid construction for protein pull-down assays

The cDNAs coding for cpSRP54M, cpSRP54M(R536G), cpSRP54M(R537G),
cpSRP54M(K538M), cpSRP54M(R539G), cpSRP54M(K540M), cpSRP54M(R537G, K538M) and cpSRP54M(Δ536-540) were obtained by PCR amplification using primer combination 1/13 (Table 1) and the corresponding pGBK7-constructs described above as template. The PCR products were digested with NcoI and HindIII and cloned into the NcoI-HindIII site of the in vitro translation vector pGem4SS6.5 (16). The corresponding constructs encoding cpSRP54M(R536K), cpSRP54M(R537K), cpSRP54M(R537N),
cpSRP54M(R537Q) and cpSRP54M(R539K) were obtained by site-directed mutagenesis (see above) using the primer sets as listed in Table 2 and pGem4SS6.5-cpSRP54M as template DNA. Each construct was confirmed by sequencing. The translation vectors pSPUTK-GST-chaos encoding GST-cpSRP43 and pSPUTK-GST encoding GST were described in Tu et al. (10) and Jonas-Straube et al. (17).

Protein pull-down assays

GST-cpSRP43, GST and the indicated constructs of cpSRP54M were obtained by in vitro transcription and in vitro translation in a wheat germ extract (Promega). CpSRP54M and its mutagenesis constructs were labeled with [35S]-methionine. 15 µl of in vitro translated GST-cpSRP43 and equal pmoles of radiolabeled in vitro translated cpSRP54M or the indicated constructs were diluted with incubation buffer (20 mM Hepes-KOH pH 8.0, 50 mM KOAc, 10 mM MgCl2) in a total volume of 120 µl. Control reactions were performed with in vitro translated GST instead of GST-cpSRP43. All binding reactions contained equal amounts of wheat germ extract. The proteins were incubated at 25 °C for 20 min and the precipitation of the GST fusion proteins with glutathione-sepharose was performed as described in Tu et al.
The eluted samples were analysed on 15% acrylamide gels and detected by radioimaging on a Phosphorimager.

**Transit complex formation**

Transit complex formation was measured essentially as described (6) with the following modifications. 1.5 μl [35S]-labeled pLhcb1 translation product was incubated with 50 ng recombinant cpSRP43 (obtained as described in Tu et al. (10)) and equimolar amounts of *in vitro* translated cpSRP54M, cpSRP54M(R536G), cpSRP54M(R537G), cpSRP54M(K538M), cpSRP54M(R539G), cpSRP54M(K540M), cpSRP54M(R537G, K538M) or cpSRP54M(Δ536-540). Reactions were separated on 7% non-denaturing acrylamide gels and visualized on a phosphorimager.

**Plasmid construction for protein expression**

cDNA encoding cpSRP54M and cpSRP54M(Δ536-540) were obtained by PCR amplification using primer combination 1/14 and the corresponding pGBKT7-constructs as template. The PCR products were digested with *Nco*I and *Hind*III and cloned into the *Nco*I-*Hind*III site of the overexpression vector pET-29b(+) (Novagen). Each construct was confirmed by sequencing.

**Protein expression and FTIR spectroscopy**

Recombinant cpSRP54M and cpSRP54M(Δ536-540) were expressed from their corresponding pET-29b(+) constructs (see above) in the *E. coli* strain BL21(DE3). Cells were grown in LB medium containing 50 μg/ml ampicillin at 37 °C to an OD_{600} of 0.6 and expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Overexpressed protein was purified on Ni^{2+}-NTA agarose (Qiagen) under native conditions as
suggested by the manufacturer. Proteins were eluted from the Ni$^{2+}$-NTA agarose with 0.5 ml 50 mM NaH$_2$PO$_4$-NaOH pH 8.0, 300 mM NaCl and 250 mM imidazole. The buffer was changed to 50 mM Tris-DCl, pH 8.0 in D$_2$O and the protein samples were concentrated to 10-15 µg/µl using spin concentrators (Vivascience).

FTIR spectroscopy was carried out at 22°C on a Bruker IFS 88 spectrometer. For each spectrum, a 256-scan interferogram was collected at single beam mode with a 2 cm$^{-1}$ resolution and a 1 cm$^{-1}$ interval from the 2000 to 1000 cm$^{-1}$ region. Reference spectra were recorded under identical conditions with solute buffer only. Each measurement was repeated 3 times. The resultant protein absorbance spectrum was smoothed to 4 cm$^{-1}$ resolution with a fourier self deconvolution algorithm to reduce background. Signals originating from side chains were corrected using side chain absorbance bands according to Barth et al. (19) with an algorithm from Goormaghtigh et al. (20).
RESULTS

Previously, it was demonstrated that the interaction of cpSRP43 and cpSRP54 is mediated via the C-terminal located M-domain of cpSRP54 (cpSRP54M; residues 371-564) (17). In order to define more clearly the region of cpSRP54M that mediates binding to cpSRP43, serial deletions from either the amino- or the carboxyl-terminal end of cpSRP54M were cloned into the bait plasmid pGBK7. The interaction of cpSRP54M and the various deletion constructs with mature cpSRP43 was tested in the yeast two-hybrid system using pACT2-43 as prey plasmid (Table 3). No obvious change in binding intensity was observed between cpSRP43 and the N-terminal deletion constructs cpSRP54M(485-564), cpSRP54M(521-564) and cpSRP54M(530-564) in comparison with full-length cpSRP54M (Table 3). A weaker binding was detected between cpSRP43 and cpSRP54M(533-564) and no binding was observed for cpSRP54M(535-564). Yeast two-hybrid experiments testing the interaction of C-terminal deletion constructs of cpSRP54M with cpSRP43 demonstrated that the last 25 residues can be removed from the C-terminus without having an obvious influence on the binding intensity of the resulting construct cpSRP54M(371-539) to cpSRP43. However, further removal of two additional C-terminal residues, generating cpSRP54M(371-537), led to a complete loss of interaction with cpSRP43 (Table 3). Taken together, these results suggested that the region comprising residues 530-539 of cpSRP54 may form the cpSRP43-binding site. The amino acid sequence and the position of this putative cpSRP43-binding site within the M-domain of cpSRP54 is shown in Fig. 1.

To confirm this result we tested whether a synthetic peptide corresponding to the putative cpSRP43-binding site competes with cpSRP54M for the complex formation with cpSRP43 in in vitro pull-down experiments. Fig. 2 A shows that the binding of radiolabeled cpSRP54M to GST-cpSRP43 in the presence of increasing amounts of the synthetic peptide is progressively inhibited. In pull-down assays conducted in the presence of 7.5 µM synthetic peptide binding
of cpSRP54M to cpSRP43 was blocked completely, whereas the addition of an unrelated peptide did not lead to a reduction of complex formation (Fig. 2 A/B). Furthermore, we were able to corroborate the finding that residues 530-539 of cpSRP54 are involved in the formation of the cpSRP43-binding site by demonstrating that a deletion construct of cpSRP54M lacking this region is not able to bind cpSRP43 (Table 3).

A noticeable feature of the amino acid region of cpSRP54 comprising the cpSRP43-binding site is the presence of the positively charged pentapeptide (RRKRK) located at amino acid position 536-540. To determine whether this region is involved in binding of the highly negatively charged cpSRP43, we constructed cpSRP54(∆536-540) and tested its ability to bind cpSRP43 by yeast two-hybrid experiments and in vitro pulldown assays. As shown in Table 3 and Fig. 4, no interaction was observed between cpSRP54(∆536-540) and cpSRP43.

We proved that the removal of the positively charged pentapeptide did not lead to an overall structural rearrangement by performing FTIR spectroscopy of highly purified recombinant cpSRP54M and cpSRP54(∆536-540). Both proteins generated very similar spectra indicating that their secondary structure is almost identical (Fig. 3). This result, together with the above mentioned observation that a synthetic peptide containing the pentapetide RRKRK inhibits binding of cpSRP54 to cpSRP43, demonstrates clearly that residues within this motif are essential for the formation of the cpSRP43-binding site.

We next aimed to define the role of the individual amino acids within the pentapeptide RRKRK(536-540) in binding cpSRP43. Therefore, site-directed mutagenesis was used to exchange one or more of these positively charged residues into uncharged amino acids. The ability of the generated cpSRP54M mutants to bind cpSRP43 was initially tested in yeast two-hybrid experiments (Table 4). No significant change in binding intensity to cpSRP43 was observed when using the constructs cpSRP54M(K538M), cpSRP54M(R539G) or cpSRP54M(K540M). Even the simultaneous change of the positively charged residues K358,
R539 and K540 (cpSRP54M(K538M, R539G, K540M)) or the deletion of these amino acids (cpSRP54M(Δ538-540)) did not lead to a measurable loss of interaction with cpSRP43 in this system. However, the single mutation R537G caused a complete loss of binding of the corresponding construct cpSRP54M(R537G) to cpSRP43, since no β-galactosidase activity was detectable in the yeast two-hybrid system. Consistently, all other tested constructs containing the R537G mutation (cpSRP54M(R537G, K538M), cpSRP54M(R537G, K538M, R539G, K540M)) were also unable to interact with cpSRP43 (Table 4). Introduction of the mutation R536G reduced the binding intensity and no interaction with cpSRP43 was observed for the construct cpSRP54M(R536G, K538M, R539G, K540M). These results suggested that residues R537 and R536 of cpSRP54 are critical for the constitution of the cpSRP43-binding site, whereas K538, R539 and K540 are not or only slightly involved in binding cpSRP43.

To further support these results and to quantify them we measured the differences in binding of radiolabeled cpSRP54M or various constructs containing mutations within the RRKRK(536-540) motif to GST-cpSRP43 by in vitro pulldown experiments (Fig. 4 A). In accordance with the yeast two-hybrid experiments, results of the binding reactions show that cpSRP54M constructs containing the mutation R537G did not interact with cpSRP43 and that binding of cpSRP54M(R536G) to cpSRP43 was reduced on average by ~90 % compared to cpSRP54M. The mutations K540M and K538M did not influence binding significantly, whereas the conversion of R539 into G539 resulted in a considerable reduction of binding by ~45 %. This reduction value was not detected in the semiquantitative yeast two-hybrid system.

We next sought to analyse whether the positive charge or the specific structure of the arginine side chain at position 536, 537 or 539 is required for interaction of cpSRP54 with cpSRP43. Therefore, arginines at these positions were replaced individually with lysine and the interaction of the resulting constructs with cpSRP43 was tested by pull-down experiments. As shown in Fig. 4 B, cpSRP54M(R536K) and cpSRP54M(R537K) were not able to bind
A reduced binding efficiency was observed for cpSRP54(R539K). Hence, constructs containing the R/K mutations behaved in the same way as the R/G mutants, demonstrating that a positive charge at position 536, 537 or 539 is not sufficient to mediate interaction with cpSRP43. We then analysed whether the polar charged side chain of R537 can be functionally replaced by the polar side chains of glutamine or asparagine. As shown in Fig. 4 B no binding was observed between the constructs cpSRP54M(R537Q) or cpSRP54M(R537N) and cpSRP43. These results indicated that interaction of cpSRP43 with cpSRP54 specifically requires an arginine residue at position 537.

Previous experiments demonstrated that both subunits of cpSRP are required for the formation of the transit complex with LHCP (6). Therefore, mutations within cpSRP54 that abolish or diminish binding to cpSRP43 should also have a negative effect on transit complex formation, presumed that a complex formation between cpSRP43 and cpSRP54 is required for the interaction with LHCP. In order to test the function of R536, R537 and R539 of cpSRP54 in transit complex formation, radiolabeled LHCP was mixed with recombinant cpSRP43 and the indicated cpSRP54M constructs and assayed for transit complex formation. The transit complex represents a soluble form of LHCP and can be detected on non-denaturing gels. As shown in Fig. 5, those mutations (∆536-540, R537G, R537G/K538M, R536G and R539G), which reduced binding of cpSRP54 to cpSRP43, inhibited transit complex formation to approximately the same extent as binding to cpSRP43. As expected from the above results, the mutations K538M and K540M did not reduce the formation of transit complex.

To further examine the role of the cpSRP43-binding site of cpSRP54 in LHCP biogenesis, the integration of radiolabeled LHCP into thylakoid membranes was measured in the presence of recombinant cpSRP43, in vitro translated cpFtsY, GTP and in vitro translated cpSRP54 or the constructs cpSRP54(R537G), cpSRP54(R537K) and cpSRP54(R536K). Fig. 6 shows that the
integration activity in assays containing the mutated forms of cpSRP54 is strongly reduced in comparison to the assay containing cpSRP54. Taken together, these data demonstrate clearly, that those amino acids of cpSRP54, which are essential for the cpSRP complex formation, are also crucial for the transport and insertion of LHCP into the thylakoid membrane.
DISCUSSION

Chloroplast SRP involved in the posttranslational targeting of LHCP represents a specialized type of SRP, since it consists of the subunits cpSRP54 and cpSRP43 and lacks an RNA moiety. Interestingly, cpSRP54 is also involved in the cotranslational targeting of the chloroplast encoded D1 to the thylakoid membrane. Recently, some progress has been made to analyse the molecular nature of the interaction between cpSRP54 and cpSRP43. Yeast two-hybrid experiments in combination with in vitro pull down experiments demonstrated that the C-terminal M-domain of cpSRP54 mediates binding to cpSRP43 (17) and another report using a pepscan approach extended this observation by finding that the extreme C-terminal region of cpSRP54 interacts strongly with cpSRP43 (18). This report describes that the C-terminal 26 residues of cpSRP54 are essential for complex formation with cpSRP43. In the present study, we performed a detailed analysis of the cpSRP43-binding site of cpSRP54. We demonstrate, that the essential binding site is located within residues 530-539 of cpSRP54. Furthermore, we show that two residues R536 and R537 are crucial for binding cpSRP43. R539 is also involved in binding cpSRP43, but is not essential for this process. These data demonstrate that the essential amino acids mediating binding to cpSRP43 are located in the C-terminal region of cpSRP54. However, they are not located within the last 26 amino acids (539-564) of cpSRP54, explaining our initial finding that removal of these amino acids did not completely abolish binding to cpSRP43.

Based on the above results one would expect that the cpSRP43 binding region (residues 530-539), including the essential amino acids R536 and R537 of Arabidopsis cpSRP54, is conserved in all chloroplast SRP54 proteins. In Fig. 7 an alignment of the C-termini of all known chloroplast SRP54 proteins is shown. As expected, the double arginine motif is conserved in all sequences. In addition, in all species this motif is followed by two positively charged amino acids (KR or KK). Two other residues that are conserved throughout all
sequences (P532, G533) were identified. They are also located within the cpSRP43 binding region and current work is in progress to test whether these amino acids may also play an important role in binding cpSRP43. Alignment studies revealed that a region homologous to the cpSRP43-binding site is not present in any cytosolic SRP54. This is particularly noticeable in the case of the SRP54 homologue of *E. coli*, since chloroplast SRP54 differs from the cytosolic homologue of *E. coli* by a C-terminal extension containing the cpSRP43 binding site (Fig. 7). This finding explains our previous observation that bacterial SRP54 (Ffh) cannot form a complex with cpSRP43 (7).

In *E. coli* the Ffh protein is involved in the cotranslational transport of membrane proteins to the plasma membrane. During this process Ffh, that is bound to the bacterial SRP-RNA, interacts with the signal sequence of the nascent protein and also contacts the ribosome at the ribosomal subunit L23 that is located close to the nascent chain exit site (21-23). In plastids, cpSRP54 is a component of two fundamentally different mechanisms, since it is involved in post- and cotranslational targeting pathways. From these observation the question concerning the molecular details of switching between the post- and cotranslational mode of action of cpSRP54 arises. Provided that the cotranslational targeting mechanism in chloroplasts is similar to that in bacteria, the results from the present work show that the cpSRP43 binding site is located at a position of cpSRP54 that is apparently not required for the cotranslational pathway. However, since no RNA was identified yet as a component of the cotranslationally acting cpSRP, it might be possible that the cotranslational targeting mechanisms in chloroplasts and bacteria exhibit substantial differences. Therefore, more work is necessary to analyse the molecular details underlying the recruitment of cpSRP54 for functioning in the posttranslational targeting of LHCP and the cotranslational targeting of chloroplast encoded proteins to the thylakoid membrane.
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FIGURE LEGENDS:

**Fig. 1** Amino acid sequence of the M-domain of cpSRP54. The region required for binding to cpSRP43 is underlined (this work). Crucial amino acids within the cpSRP43 binding site are in bold (this work).

**Fig. 2** A synthetic peptide comprising the cpSRP43 binding site competes with cpSRP54M for binding cpSRP43. (A) *In vitro* pull-down assays were performed with 0.2 µg (0.03 µM final concentration) recombinant GST-cpSRP43 and 3 µl *in vitro* translated radiolabeled cpSRP54M (~ 0.06 µM final concentration) in the presence of increasing concentrations (0.5, 2.5, 7.5, 15 µM) of a synthetic peptide corresponding to residues 530-542 of cpSRP54 (APPGTARRKRKAD). A control reaction was performed with recombinant GST instead of GST-cpSRP43. (B) Pull-down assays were performed as described above in the presence of 7.5 µM of the specific peptide or 7.5 µM of the unrelated peptide REKGGEKVTPCPK. Experimental details are described in Experimental Procedures.

**Fig. 3** The amide-1 band of cpSRP54M (continuous) and cpSRP54M(Δ536-540) (dashed) obtained by FTIR spectroscopy. Curve fitting reveals only minor differences indicating that both proteins have an almost identical secondary structure. Experimental details are described in Experimental Procedures.

**Fig. 4** *In vitro* protein binding assays of cpSRP43 and cpSRP54M constructs containing various mutations within the cpSRP43 binding motif. (A) The mutations R537G or R536G in cpSRP54M abolish or drastically reduce binding of cpSRP43 to cpSRP54M. *In vitro* translated GST-cpSRP43 or GST as a control was incubated with equimolar concentrations of
the indicated radiolabeled site-directed mutagenesis constructs of cpSRP54M and recovered by precipitation using glutathione sepharose beads as described in Experimental Procedures. The average and standard deviation were calculated using data from three experiments. (B) R536 and R537 of cpSRP54 cannot be substituted by amino acids containing positively charged or polar side chains. In vitro translated GST-cpSRP43 was incubated with equimolar concentrations of the indicated radiolabeled site-directed mutagenesis constructs of cpSRP54M and recovered by precipitation using glutathione-sepharose beads as described in Experimental Procedures.

Fig. 5 Transit complex formation is completely abolished in the presence of cpSRP54M(R537G) and drastically reduced in the presence of cpSRP54M(R536G). In vitro translated radiolabeled pLHCP, recombinant cpSRP43 and in vitro translated cpSRP54M or the indicated cpSRP54M constructs were mixed and assayed for the formation of transit complex on non-denaturing gels by radioimaging as described in Experimental Procedures.

Fig. 6 Mutations within the cpSRP43-binding site of cpSRP54 result in a strong reduction of LHCP integration into the thylakoid membrane. Integration assays of [35S]-labeled pLHCP (TP) into isolated thylakoid membranes were performed as described in Tu et al. (10) using 1 µg recombinant cpSRP43, 0.15 mM GTP, 30 µl in vitro translated cpFtsY (translated in a rabbit reticulocytolytic lysate) and 20 µl in vitro translated cpSRP54 (lane 3) or equimolar amounts of the indicated cpSRP54 constructs (translated in a wheat germ extract) (lanes 5-7). A negative control reaction was performed in the absence of cpSRP54 (lane 4) and a positive control reaction was done by using stromal extract (SE) instead of cpSRP and cpFtsY (lane 1). After the incubation thylakoids were protease-treated and washed with 0.1 N NaOH to remove non-integrated pLHCP. The samples were analyzed by SDS-PAGE and radioimaging.
The translation vectors encoding the indicated cpSRP54 constructs were generated by site-directed mutagenesis using the cpSRP54 translation vector pAF1 (10) as template.

**Fig. 7** C-terminal section of an alignment of the *E. coli* SRP54 homologue and chloroplast SRP54 of various organisms. The SRP54 homologue of *E. coli* and the chloroplast SRP54 homologues of *Oryza sativum* (*Or. sat.*), *Zea mays* (*Z. m.*), *Lycopersicon esculentum* (*Lyc. esc.*), *Pisum sativum* (*Pis. sat.*), *Chlamydomonas reinhardtii* (*Chlamy.*), and *Arabidopsis thaliana* (*Ar. th.*) were aligned using Clustal W (version 1.8). Chloroplast SRP54 proteins distinguish from the *E. coli* SRP54 homologue by a C-terminal extension containing the cpSRP43 binding site. Conserved amino acids within this C-terminal extension are indicated by an asterix and are exclusively located within the cpSRP43 binding motif (underlined residues). Residues corresponding to R536 and R537 of cpSRP54 of *Arabidopsis* are indicated in bold.
TABLES

Table 1: Primers used for PCR amplification.
Restriction sites are underlined. A period indicates the first codon of the expressed protein. Stop codons within the reverse primers are italicized.

| forward primers: | reverse primers: |
|------------------|------------------|
| 1. cpSRP54M(Nco1) 5'-ctaaccaagtggagaatgggagatgtgctt-3' | 7. cpSRP54(BamH1) 5'-ttcggctctgtgtaactaaggagatcccaactaagctac-3' |
| 2. cpSRP54485(Nco1) 5'-ctaaccaagtggagaacacggtaagccac-3' | 8. cpSRP54543(BamH1) 5'-agagaaaggcagactcaagatccac-3' |
| 3. cpSRP54521(Nco1) 5'-ctaaccaagtggagacatgtaagccac-3' | 9. cpSRP54541(BamH1) 5'-agagaaaggcagactcaagatccac-3' |
| 4. cpSRP54530(Nco1) 5'-ctaaccaagtggagacatgtaagccac-3' | 10. cpSRP54539(BamH1) 5'-actgcaagagagagagagattccac-3' |
| 5. cpSRP54533(Nco1) 5'-ctaaccaagtggagacatgtaagccac-3' | 11. cpSRP54538(BamH1) 5'-ggacatgcaagagagagagattccac-3' |
| 6. cpSRP54535(Nco1) 5'-ctaaccaagtggagacatgtaagccac-3' | 12. cpSRP54537(BamH1) 5'-cttgcaagagagagagagattccac-3' |
|             | 13. cpSRP54(HindIII) 5'-tcggctctttgtaactaaggctctac-3' |
|             | 14. cpSRP54-OE(HindIII) 5'-ggctggctcgtaactaagctctac-3' |
Table 2: Primers used for PCR amplification of the site-directed mutagenesis constructs of cpSRP54M.

Nucleotides coding for the changed amino acids are underlined.

| Primer name | Primer sequence |
|-------------|-----------------|
| (∆531-539)  | 5’-gcagaacaaagctcagcagactcaag-3’ |
| (∆536-540)  | 5’-ccacctggaactgcagcagactcaagagaag-3’ |
| (R536G)     | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (R537G)     | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (K538M)     | 5’-gaactgcagaggatgaaaagcagactc-3’ |
| (R539G)     | 5’-ctgcaaggagagaagggcagac-3’ |
| (K540M)     | 5’-caaggagagagagagcagactcaagag-3’ |
| (R537G, K538M) | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (K538M, R539G) | 5’-gaactgcagaggatgaaaagcagactc-3’ |
| (R539G, K540M) | 5’-caaggagagagagaagcagactcaagag-3’ |
| (K538M, K540M) | 5’-gaactgcagaggatgaaaagcagactc-3’ |
| (R538-540)  | 5’-gaactgcagaggagaaaagcagactcaagag-3’ |
| (R536G, K538M, R539G, K540M) | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (R537G, K538M, R539G, K540M) | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (R536K)     | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (R537K)     | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (R537N)     | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (R537Q)     | 5’-ccctggaactgcagaggagaaaagcagac-3’ |
| (R539K)     | 5’-ctgcaaggagagagaaaagcagactcaag-3’ |
Table 3: Interaction of various deletion constructs of cpSRP54M with cpSRP43.

The indicated deletions of cpSRP54M were cloned into the yeast two-hybrid plasmid pGBKT7. Yeast strain Y190 was cotransformed with the combination of each pGBKT7-54M construct and pACT2-43 encoding mature cpSRP43. Activation of the –his- and lacZ-reporter was measured as described in Materials and Methods.

| pGBK7-constructs (bait) | pACT2-43 (prey) | –his growth | β-gal. activity |
|-------------------------|-----------------|-------------|----------------|
| 54M                     | ++              | ++          |                |
| 54M (485-564)           | ++              | ++          |                |
| 54M (521-564)           | ++              | ++          |                |
| 54M (530-564)           | +               | ++          |                |
| 54M (533-564)           | +               | +           |                |
| 54M (535-564)           | -               | -           |                |
| 54M (371-543)           | ++              | ++          |                |
| 54M (371-541)           | ++              | ++          |                |
| 54M (371-539)           | ++              | ++          |                |
| 54M (371-538)           | ++              | +           |                |
| 54M (371-537)           | +               | -           |                |
| 54M (Δ531-539)          | -               | -           |                |
| 54M (Δ536-540)          | -               | -           |                |
Table 4: Interaction of site-directed mutagenesis constructs of cpSRP54M with cpSRP43. Yeast strain Y190 was cotransformed with the combination of each pGBKT7-54M construct and pACT2-43 encoding mature cpSRP43. Activation of the $–\text{his}$- and lacZ-reporter was measured as described in Materials and Methods.

| pGBKT7-54M constructs (bait) | pACT2-43 (prey) | –his growth | β-gal. activity |
|-----------------------------|----------------|-------------|----------------|
| 54M                         |                | ++          | ++             |
| (R536G)                     |                | ++          | +              |
| (R537G)                     |                | +           | -              |
| (K538M)                     |                | ++          | ++             |
| (R539G)                     |                | ++          | ++             |
| (K540M)                     |                | ++          | ++             |
| (K538M, R539G)              |                | ++          | ++             |
| (R539G, K540M)              |                | ++          | ++             |
| (K538M, K540M)              |                | ++          | ++             |
| (K538M, R539G, K540M)       |                | ++          | ++             |
| (Δ538-540)                  |                | ++          | ++             |
| (R537G, K538M)              |                | +           | -              |
| (R537G, K538M, R539G, K540M) |                | +           | -              |
| (R536G, K538M, R539G, K540M) |                | +           | -              |
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
| Organism       | Sequence                        | Start | End |
|---------------|---------------------------------|-------|-----|
| E. coli       | PGFPGR---------------------------| 453   |     |
| Or. sat. (cp) | SLNADKEKAPPGTARRKKRHSKTRQ-------RELDAVPS------- |       |     |
| Z. m. (cp)    | SLKSEEKAPPGTARRKRNNNTSKQ-------RDLDAVLS-------- |       |     |
| Lyc. esc. (cp)| ALKSEQQQAPPGTARRKKRSEPRKQFA-----DSGSARPSRPGFGAKN-- | 541   |     |
| Pis. sat. (cp)| ALKANKKAPPGTARRKKKGLKLKRLFKGRSSKISLPAPRGFGSKN- |       |     |
| Chlamy. (cp)  | VASAGKKVAPGKVBRKKE----------------KEPLSKARGFGSSK | 549   |     |
| Ar. th. (cp)  | ALKAEQKAPPGTARRRKKADSRKKFV-----E3ASSKPGPRGFSGN-- | 564   | 541 |

Fig. 7
A unique sequence motif in the 54 kD subunit of the chloroplast signal recognition particle mediates binding to the 43 kD subunit
Silke Funke, Thomas Knechten, Julian Ollesch and Danja Schuenemann

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