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Assembly of Chloroplast Signal Recognition Particle involves Structural Rearrangement in cpSRP43

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Abstract

Signal recognition particle in chloroplasts (cpSRP) exhibits the unusual ability to bind and target full-length proteins to the thylakoid membrane. Unlike cytosolic SRPs in pro- and eukaryotes, cpSRP lacks an RNA moiety and functions as a heterodimer composed of a conserved 54 kD GTPase (cpSRP54) and an unique 43 kD subunit (cpSRP43). Assembly of the cpSRP heterodimer is a prerequisite for posttranslational targeting activities and takes place through interactions between chromatin modifier domain 2 (CD2) of cpSRP43 and a unique 10 amino acid region in cpSRP54 (cpSRP54_{pep}). We have used multidimensional NMR spectroscopy and other biophysical methods to examine assembly and structure of the cpSRP43-cpSRP54 interface. Our data show that CD2 of cpSRP43 binds to cpSRP54_{pep} in a 1:1 stoichiometry with an apparent K_d of ~ 1.06 μ M. Steady state fluorescence and Far UV circular dichroism data suggest that the CD2-cpSRP54_{pep} interaction causes significant conformational changes in both CD2 and the peptide. Comparison of the three-dimensional solution structures of CD2 alone and in complex with cpSRP54_{pep} shows that significant structural changes are induced in CD2 in order to establish a binding interface contributed mostly by residues in the N-terminal segment of CD2 (Phe5-Val10) and an arginine doublet (Arg536 and Arg537) in the cpSRP54 peptide. Taken together, our results provide new insights into the mechanism of cpSRP assembly and the structural forces that stabilize the functionally critical cpSRP43-cpSRP54 interaction.

Introduction

Light harvesting chlorophyll-binding proteins (LHCPs) are the most abundant proteins within the chloroplast thylakoid membrane. ¹ Studies to understand the localization of this nuclear encoded protein family have demonstrated that LHCPs are synthesized in the cytosol as full-length precursors and imported into the chloroplast stroma. Subsequent routing of LHCPs to the thylakoid requires GTP along with chloroplast homologues of signal recognition particle

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The atomic coordinates of the CD2-cpSRP54_{pep} complex have been deposited in the Protein Data Bank under accession number **2HUG**.

(SRP) and its receptor (cpSRP and cpFtsY, respectively).²⁻⁴ cpSRP binds to full-length LHCPs in the stroma to form a cpSRP-LHCP complex termed 'transit complex', which serves as the targeted form of LHCPs.^{5, 6} Binding of cpFtsY to the cpSRP-LHCP complex facilitates targeting of LHCP to Albino3 (Alb3), an integral thylakoid protein that functions in LHCP integration.^{7, 8, 9} In the absence of cpFtsY, transit complex accumulates in the stroma.¹⁰ The ability of cpSRP to bind and target full-length substrates is unusual in that cytosolic SRPs in pro- and eukaryotes function co-translationally. The posttranslational targeting activity of cpSRP is attributed to its unique subunit composition; cpSRP lacks an RNA moiety found in all cytosolic SRPs and functions as a heterodimer composed of a conserved 54 kD GTPase (cpSRP54; ref. ⁵) and a novel 43 kDa subunit (cpSRP43; ref. ³) that is required for LHCP binding and integration.¹¹

The cpSRP43 subunit consists of two types of structural domains. They include three Chromosome modifier Domains (CD1, CD2 and CD3) and four contiguous ankyrin-binding domains (Ank1, Ank2, Ank3 and Ank4).^{2, 12, 13} Whereas the Ank repeat serves to bind an 18 amino acid recognition motif conserved among LHCPs, CD1 functions at a step following transit complex formation and regulates GTP hydrolysis by cpSRP54/cpFtsY.¹¹⁻¹⁵ Funke et al (Ref. 17) through exhaustive mutational analysis identified that the ten amino acid segment (constituting the cpSRP54_{pep}, Fig. 1) in the cpSRP54 subunit solely contributes to the binding of cpSRP43. Mutations within the cpSRP43-binding segment (cpSRP54_{pep}) of cpSRP54 were not only shown to completely abolish the cpSRP complex formation, but also inhibit transit complex formation and integration of LHCP into the thylakoid membrane. Similarly, a thorough study using various deletion and site-specific mutants of cpSRP43 (Ref. 13) revealed that residues in CD2 alone (in the cpSRP43 subunit) contribute to the binding of the cpSRP54 subunit. Taken together, these results suggest that CD2 in cpSRP43 provides the binding interface for cpSRP54 through interactions with cpSRP54_{pep}. The primary objective of this study was to determine the three-dimensional solution structure of the CD2-cpSRP54_{pep} complex and understand the molecular forces that are responsible for the formation and stability of the cpSRP43-cpSRP54 structure. Our results show that CD2 of cpSRP43 must undergo a significant structural change in order to establish a binding interface with cpSRP54.

Results and Discussion

Binding affinity of CD2 to the cpSRP54 peptide

Isothermal titration calorimetry is a versatile technique that is routinely used to measure the thermodynamics, binding affinity and binding stoichiometry of protein-protein/ligand interactions.¹⁹ The sigmoidal isothermogram representing the titration of CD2 with cpSRP54_{pep} shows that binding stoichiometry between CD2 and cpSRP54_{pep} is 1:0.96 (Fig. 2). The CD2:cpSRP54_{pep} binary complex appears to be quite stable as it can be isolated as a 1:1 complex by size exclusion chromatography (Fig. S4). The interaction between CD2 and the cpSRP54_{pep} is exothermic and proceeds with evolution of heat ($\Delta H \sim -42$ kcal/mol). The apparent binding constant (K_d) defining the affinity of CD2 to the cpSRP54_{pep} is about 1.06 μ M. The binding affinity of CD2 to the intact cpSRP54 subunit was previously reported to be slightly higher ($K_d \sim 190$ nm, ref. ¹⁶) than observed for cpSRP54_{pep}. The higher binding of CD2 to intact cpSRP54 subunit may be attributed to the availability of binding pocket (in the cpSRP54 subunit) that sterically favors tight binding to CD2. On the other hand, the smaller size and higher flexibility of the unstructured (cpSRP54) peptide may not provide optimal steric constraints ("pocket/crevice") that promote strong binding with CD2. In general, protein-protein interactions are known to be relatively stronger than protein-peptide interactions.¹⁸

cpSRP54_{pep} induces conformational changes in CD2

CD2 contains two tryptophan (Trp29 and Trp37) residues partially buried in the hydrophobic core of the protein.^{13, 16} Since cpSRP54_{pep} lacks tryptophan residues, the global conformational changes possibly induced by the binding of cpSRP54_{pep} to CD2 can be reliably monitored by steady state fluorescence spectroscopy. A 15 nm blue shift (from 345 nm to 330 nm) is observed as the CD2 to cpSRP54_{pep} molar binding stoichiometry reaches 1:1, which is consistent with the binding stoichiometry observed by isothermal titration calorimetry (Fig. 3A). Importantly, in the near UV CD data the induced blue shift in the wavelength of emission maxima indicates a conformational change in CD2 leading to the movement of the tryptophan residues to a non-polar microenvironment. The difference(s) in the intensity and the fine splitting of the tryptophan signal also indicates a change in the microenvironment of the tryptophan residues in CD2 upon binding to cpSRP54_{pep} (Fig. 3C). Conformational changes in CD2 monitored by Far UV circular dichroism also suggest that cpSRP54_{pep} binding to CD2 stabilizes the disordered portions of the molecule (Fig. 3B). In addition, results of equilibrium thermal unfolding experiments (monitored by changes in tryptophan fluorescence) also indicate that the structure of CD2 is marginally stabilized upon binding to cpSRP54_{pep} (Fig. 3D).

Determination of the three-dimensional structure of the CD2-cpSRP54_{pep} complex

¹H-¹⁵N HSQC spectrum serves as a fingerprint of the protein conformation, and therefore the binding site(s) of ligands on target proteins can be conveniently mapped.²⁰ The ¹H-¹⁵N HSQC of CD2 is well-dispersed and all crosspeaks in the spectrum have been previously assigned.¹⁶ At a 1:1 molar ratio of CD2 to cpSRP54_{pep}, the ¹H-¹⁵N HSQC spectrum showed complete disappearance of many of the crosspeaks observed in the free state of CD2 with the simultaneous intensification of several new cross peaks (Fig. 4A). Therefore, the ¹⁵N chemical shift perturbation of residues in CD2 could not be unambiguously tracked. Significant perturbation (Fig. 4B) of a majority of crosspeaks in the ¹H-¹⁵N HSQC spectrum possibly indicates a conformational change in CD2 upon binding to cpSRP54_{pep}. The conformational change induced in CD2 due to the binding of CD2 is also obvious from the significant chemical shift perturbation of epsilon-delta NH resonances of both the tryptophan residues (Trp29 and Trp37) observed in the ¹H-¹⁵N HSQC spectra of CD2 in the presence and absence of cpSRP54_{pep} (Fig. 4C).

Complete assignment of resonances in the peptide-bound CD2 was accomplished using standard multiple dimensional heteronuclear NMR spectroscopy techniques (supplementary Fig. S1). Backbone and side-chain resonances of ¹³C, ¹⁵N-labeled CD2, in a complex with unlabeled cpSRP54_{pep}, were completed for all observable resonances. Ambiguities in assignment of certain residues such as Gln3, Leu26, Val37 and Val43 were resolved by selective ¹⁵N labeling. Interestingly, crosspeaks of residues (Gln3, Glu6 and Tyr7) which could not be located in the ¹H-¹⁵N HSQC spectrum of unbound CD2, appear in the spectrum corresponding to the cpSRP54_{pep} bound state.

Most of the NOE data used in the structure calculation were obtained from 3D ¹⁵N and ¹³C edited nuclear Overhauser effect spectroscopy (NOESY) spectra collected using ¹⁵N- and ¹³C labeled CD2 complexed with unlabeled cpSRP54_{pep}. 3D ¹⁵N-edited ¹⁴N-filtered NOESY and ¹³C-edited, ¹²C-filtered NOESY experiments provided intermolecular protein-peptide distance constraints between labeled CD2 and the unlabeled cpSRP54_{pep}.^{20, 21} The binding interface of the CD2-cpSRP54_{pep} complex is defined by 10 residues from CD2 (Val4, Phe5, Glu6, Tyr7, Ala8, Glu9, Val10, Glu36, Val38 and Glu45) and four residues from cpSRP54_{pep} (Ala535, Arg536, Arg537 and Glu539). Interestingly, we could not detect any intermolecular NOEs corresponding to Lys21 and Val27, which experienced prominent perturbation in the ¹H-¹⁵N HSQC spectrum obtained in the presence of the cpSRP54_{pep}.

Therefore, it appears that the marked ^{15}N chemical shift perturbation observed for these two residues are linked to conformational changes induced in CD2 by cpSRP54_{pep}. The ensemble of 20 lowest-energy structures of the CD2-cpSRP54 complex were well-defined. The root mean square deviations (RMSD) of the backbone secondary structure of CD2 and the backbone of cpSRP54_{pep} were 0.29 Å (residues, 4-57) and 1.1 Å (residues 4-12), respectively (supplementary Table-S3).

Structural changes induced in CD2 by the cpSRP54_{pep}

The three-dimensional solution structures for each of the three CDs in cpSRP43 have been determined.¹⁶ The structure of CD2 consists of triple stranded β -sheet and a C-terminal helix (Fig. 5A). Three-dimensional solution structure of CD2-cpSRP54_{pep} shows that CD2 undergoes a prominent conformational change upon binding to cpSRP54_{pep} (Fig. 5B). The beta-barrel type arrangement of the beta-strands in CD2 transforms to a flat planar triple-stranded beta-sheet structure when bound to cpSRP54_{pep} (Fig. 5B). The NOE observed between the amide proton of Val23 and Arg17 H α in the structure of CD2 (in the absence of cpSRP54_{pep}) is found missing in the ^1H - ^{15}N HSQC NOESY spectrum of the cpSRP54_{pep}-CD2 complex. However, two medium range NOEs between Phe5 and Lys19 (Phe5NH to beta proton of Lys19, and Phe5H α to Lys19H β) are observed in the ^1H - ^{15}N HSQC NOESY and ^1H - ^{13}C HSQC NOESY spectra of CD2 obtained in the presence of cpSRP54_{pep}. Interestingly, the corresponding NOEs are missing in the spectra of free CD2. The planar triple-stranded beta-sheet structure, observed in the presence of the peptide, is further stabilized by the extension of the beta-strand II formed upon new backbone interactions forged between Glu12NH and Val27CO, and the amide group of Ile13 with the carbonyl group of Tyr25.

One of the notable features of the three-dimensional structure of CD2 is the $\sim 62^\circ$ orientation of the C-terminal helix (residues, 45-57) with the plane of the beta sheet (Fig. 5C, ref. ¹⁶). In the presence of cpSRP54_{pep}, the angular orientation of the C-terminal helix, in relation to the plane of the beta-sheet, changes significantly from $\sim 62^\circ$ to $\sim 106^\circ$ (Fig. 5C & D). NOESY data on unbound CD2 revealed the presence of a few weak NOEs defining the direct long range interactions between residues located in the C-terminal helix and the rest of the CD2 molecule. The spatial orientation of the C-terminal helix in the unbound CD2 is primarily governed by two backbone hydrogen bonds involving residues located in the loop (Gly 40 NH and Val 43 NH, in Loop-IV, Fig. 5E) between β -strand III and the C-terminal helix. The two hydrogen bonds involving residues in Loop-IV produce a “knot”-like structure in Loop-IV. Binding of cpSRP54_{pep} to CD2 results in the disruption of the two hydrogen bonds in Loop-IV and consequently changes the spatial orientation of the C-terminal helix (Fig. 5D). The C-terminal helix (residues 44 to 57), in the cpSRP54_{pep} - CD2 complex, is oriented at an angle of about 106° to the plane of the triple-stranded β -sheet (Fig. 5D). A weak NOE is observed between NH of Gln3 (located in the N-terminal segment) and the H $^\delta$ of the aromatic ring proton of Tyr51 (located in the C-terminal helix). It appears that the C-terminal helix (in the cpSRP54_{pep}-CD2 complex) is held in position through a weak π -interaction between the amide group of Gln3 and the electron dense aromatic ring of Tyr51. The side-chain amide group is separated from the aromatic ring by a spatial distance of about 4.65 Å in the three-dimensional structure of the cpSRP54_{pep} - CD2 complex.

Significant differences can be observed in the spatial orientation of the N-terminal segment (residues 1 -10) in the three-dimensional structures of the unbound CD2 and in the cpSRP54_{pep} - CD2 complex (Fig. 5C & D). The N-terminal segment is quite flexible in the unbound CD2 structure. However, the spatial orientation of the N-terminal segment in the cpSRP54_{pep} - CD2 complex is drastically different from that observed in the structure of the unbound CD2 (Supplementary Table-S2). The N-terminal segment forms several new structural contacts with the residues in β strands I and II (residues 18 and 25 to 27, Fig. 5F and

Supplementary Table-S2). It appears that the new interactions forged by the N-terminal segment (in the cpSRP54_{pep} - CD2 complex) render it more rigid. In addition, ¹H-¹⁵N HSQC spectrum of CD2 in complex with the cpSRP54_{pep} shows several new crosspeaks corresponding to residues (Gln3, Glu6 and Tyr7) in the N-terminal segment which could not be observed due to high flexibility of the backbone (in the N-terminal segment) of the CD2 molecule in its free form.

Amide proton exchange experiments were monitored by the ¹H-¹⁵N HSQC spectra to examine the relative flexibility of residues in the N-terminal segment in unbound CD2 and in the cpSRP54_{pep}-CD2 complex. ¹H-¹⁵N HSQC spectrum acquired immediately after dissolution of CD2 in D₂O shows that more than 50% of the total number of amide proton crosspeaks are completely disappeared (data not shown). Almost all the amide proton crosspeaks corresponding to the N-terminal segment (residues 1-10) in the unbound CD2 disappeared in the ¹H-¹⁵N HSQC spectrum collected after 2000 minutes of exchange in D₂O. In marked contrast, the amide protons of most of the residues in the N-terminal segment in the cpSRP54_{pep}-CD2 complex are protected from exchange (Fig. 6A and 6B). For example, 75% of the crosspeak intensity of the amide proton of Phe5 (located in the N-terminal segment) in the cpSRP54_{pep}-CD2 complex is protected from exchange even after 5000 minutes (Fig. 6C). Interestingly, the amide proton of Phe5 is completely exchanged in the unbound CD2. {¹H}-¹⁵N heteronuclear NOE measurements were performed to understand the relative flexibility of the backbone in CD2 in the absence and presence of cpSRP54_{pep}. With the exception of Tyr57, which exhibits a negative NOE, all other residues in free CD2 show positive NOE values (Fig. 6D). The steady state {¹H} - ¹⁵N NOE values of most residues increase significantly on binding to cpSRP54_{pep} suggesting an overall decrease in the backbone flexibility in CD2. The average {¹H} - ¹⁵N NOE value of residues in the N-terminal segment (residues 4 to 12) increases from 0.701 ± 0.022 in CD2 to 0.887 ± 0.031 in the CD2-cpSRP54_{pep} complex. These results are consistent with the hydrogen-deuterium exchange data which show that residues in the N-terminal segment in CD2 are significantly more protected to deuterium exchange upon binding to cpSRP54_{pep}.

cpSRP54_{pep} contact sites on CD2

Far UV circular dichroism data clearly indicate that cpSRP54_{pep} is a disordered coil in aqueous solution (Fig. 3B). The cpSRP54_{pep} appears to bind CD2 in an extended conformation (Fig. 5B). The peptide is aligned parallel to the N-terminal segment of CD2. Intermolecular NOEs reveal that three consecutive residues in the cpSRP54_{pep}, Ala535, Arg536 and Arg537, show strong interaction with residues in CD2. Interestingly, the double arginine motif (Arg536-Arg537) is well-conserved in all known chloroplast cpSRP54 proteins.²² Mutational studies of Funke *et al.* (2005) demonstrated that Arg536 and Arg537 are indispensable for the cpSRP54-cpSRP43 interaction. Our data is consistent with these observations as Arg536 and Arg537 in the cpSRP54_{pep} have extensive interactions with residues located between Phe5 and Val10 in the N-terminal segment of CD2. A strong intermolecular NOE observed between the γH of Arg536 (in the cpSRP54_{pep}) and the γH of Glu6 (in CD2) supports the interaction between the positively charged guanido group of Arg536 and the negatively charged side chain of Glu6 (Fig. 7A & Fig. 7B). Intermolecular NOE data show that Arg537 is involved in several strong short-range interactions with residues in CD2 such as, Glu6, Tyr7, Ala8 and Glu9. The backbone of the amide proton of Arg536 in cpSRP54_{pep} shows a weak NOE with the side chain γH of Glu9 suggesting a salt bridge type of interaction between these two residues. In addition, the positively charged side-chain of Arg539 appears to interact with the negatively charged side-chain carboxyl group of Glu45 located in the C-terminal helix of the CD2 molecule (Fig. 7A). Interestingly, Arg539Gly mutation was shown to decrease the binding affinity between cpSRP54 and cpSRP43 by about 45%.¹⁷

Structural studies presented here suggest the involvement of multiple residues in cpSRP43 in maintaining an interaction with cpSRP54. In order to further examine the role of these residues, GST-fused residue replacement and deletion constructs of cpSRP43 were examined for their ability to interact with the M-domain of cpSRP54. Results of co-precipitation assays examining the ability of equimolar amounts of modified, GST-fused cpSRP43 constructs to co-precipitate the M-domain of cpSRP54 demonstrate that alteration of negatively charged glutamate at positions 6 and 9 to positively charged Lys or Arg strongly inhibits the ability of cpSRP43 to interact with the M-domain of cpSRP54. Similarly, replacement of Ala8 with Gln, Lys or Arg also greatly diminishes the ability of the cpSRP43 construct to co-precipitate M-domain. Mutations at positions 6 and 9 had a greater impact on the ability of cpSRP43 to co-precipitate cpSRP54-M-domain than mutations made at position 10, suggesting differences in the relative strength of the interaction. As predicted by the structure, a double mutation of E6 and E9 to Ala as well as deletion of residues 6-9 (EYAE) also showed dramatic decreases in their ability to co-precipitate the M-domain of cpSRP54 (Fig. 8). Taken together, this information supports the structural identification of these residues as being important in the interaction of cpSRP43 with cpSRP54.

Formation of cpSRP43-cpSRP54 heterodimer is important for LHCP localization on to the thylakoid membrane

Unlike cpSRP43, cpSRP54 in the chloroplast occurs in two distinct pools, either in a ribosome-bound or cpSRP43-bound fraction. The ribosomal bound form retains the ability to co-translationally bind signal peptides of thylakoid proteins synthesized in the chloroplast. However, the post-translational localization of nuclear encoded LHCPs by cpSRP54 requires that it be in cpSRP54-cpSRP43 heterodimer. It is observed that the ribosomal pool of cpSRP54 only gains transit complex-forming activity in the presence of cpSRP43.² These observations combined with the absence of 'free' cpSRP43 in the stroma suggests that the concentration of cpSRP43 in the stroma acts to control the partitioning of cpSRP54 between the co-translational and post-translational mode of targeting proteins in the chloroplast. Therefore, understanding the structural forces that govern the formation of the cpSRP43-cpSRP54 complex is extremely important in unraveling the mechanism(s) underlying cpSRP mediated post-translational protein targeting.

Results of this study clearly show that CD2 serves as the primary binding and recognition site for cpSRP54. Initial interaction of CD2 with the M domain of cpSRP54 triggers conformational changes in both cpSRP43 and cpSRP54 which consequently promote the formation of secondary interaction sites that likely stabilize the cpSRP43-cpSRP54 complex. This idea is supported by a recent study which shows that the CD2-cpSRP54 interaction is marginally weaker than the cpSRP43-cpSRP54 interaction.²³ Conformational changes in both cpSRP43 and cpSRP54 upon their interaction appear to be essential for the post-translational integration of LHCP. Although cpSRP54 alone does not bind significant levels of LHCP, formation of the cpSRP43-cpSRP54 complex appears to generate an interface for interaction between cpSRP54 and a hydrophobic domain in LHCP that has previously been demonstrated to be required for transit complex formation.¹⁵ We are currently working on the three-dimensional solution structure of the minimal cpSRP43 unit (Ank-CD2) capable of supporting transit complex formation in a ternary complex with the M-domain of cpSRP54 and the L18 region of LHCP in order to understand the structural forces stabilizing the cpSRP targeting complex.

Recent *in vivo* studies have shown that in the absence of cpSRP54, cpSRP43 alone is able to support LHCP integration by an alternative pathway that no longer requires cpFtsY.²⁴ However, the severe defect in LHCP accumulation along with an accumulation of transit complex when only cpFtsY is absent indicates that the primary pathway for LHCP localization requires cpSRP54-cpSRP43.^{10, 24} Hence, the physiological significance of this alternative

pathway is currently unknown. Nevertheless, the identification of the alternative pathway underscores the significance of cpSRP43-cpSRP54 heterodimer formation since in its absence, cpSRP54 would be restricted to co-translational activities and cpSRP43 function would be independent of both cpSRP54 and cpFtsY. It will be important to understand whether cpSRP43 alone, which our studies show is structurally distinct from the cpSRP54-bound form, interacts with LHCP in a distinct or similar manner when it must function in the absence of the canonical SRP targeting components.

Materials and Methods

Expression and purification of CD2 and the cpSRP54_{pep}

Escherichia coli BL21 (DE3) cells were transformed with the expression vector pGEX 4T2 encoding the cDNA sequence of CD2 or cpSRP54_{pep} from *Arabidopsis thaliana* and grown at 37 °C in diluted 1-liter cultures of Luria broth containing 100 µg/ml ampicillin (Amersco). Protein expression was induced at an A₆₀₀ of 0.50 with 1 mM IPTG, and the cells were incubated for an additional 4 h. Cultures were then harvested by centrifugation. Cell pellets were then resuspended in 25 ml of resuspension buffer (20 mM phosphate buffer, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (Sigma)) and lysed by sonication. The expression of the GST fusion proteins (GST-CD2, GST-cpSRP54_{pep}) was checked by SDS-PAGE stained with Coomassie Blue.

GST-fused CD2 and cpSRP54_{pep} were purified from bacterial lysates by affinity chromatography using Glutathione Sepharose™ (GE Healthcare). GST-fused CD2 and cpSRP54_{pep} were eluted with 10 mM Tris buffer (pH 8.0) containing 10 mM reduced glutathione (Sigma). The affinity tag (GST) was removed by cleavage with thrombin (Sigma), and the CD2 and cpSRP54_{pep} were purified on a Glutathione Sepharose™ column using 20 mM phosphate buffer (pH 7.2) containing 150 mM NaCl. The authenticity of the purified samples was checked by SDS-PAGE and electrospray ionization mass spectroscopy. The concentration of the CD2 was measured from its molar extinction ($\epsilon = 2.594 \text{ M}^{-1} \text{ cm}^{-1}$) coefficient at 280 nm.

Preparation of Isotope (¹⁵N or ¹⁵N/¹³C)-enriched CD Samples

Uniformly ¹⁵N-labeled CD2 and cpSRP54_{pep} were prepared by growing *E. coli* in M9 medium containing 0.5 g/liter ¹⁵NH₄Cl (Cambridge Isotope Labs, ¹⁵N >98%) as the sole source of nitrogen (pH 6.8). ¹³C and ¹⁵N doubly labeled CD2 and cpSRP54_{pep} samples were prepared in M9 medium containing 4 g/liter [¹³C]glucose (Cambridge Isotope Labs, ¹³C >98%) and 0.5 g of ¹⁵NH₄Cl as the sole sources of carbon and nitrogen, respectively. The expression and purification protocols for the isotope-labeled CD samples were the same as those used for the unlabeled samples.

NMR spectroscopy

NMR experiments were performed at 25 °C on Bruker Avance DMX-500 and Bruker Avance 700 MHz spectrometers equipped with four frequency channels and 5 mm triple resonance cryogenic probes. NMR spectra were recorded on samples in 150 mM NaCl, 20 mM sodium phosphate (pH 6.5) and 10% D₂O. Resonance assignments for CD2 in the cpSRP54_{pep} bound form were accomplished using a variety of standard triple resonance experiments. Assignments on the cpSRP54_{pep} in its free and CD2 bound forms were accomplished using similar experimental strategies on a double labeled (¹³C/¹⁵N) sample. NOEs were assigned using ¹⁵N-separated (200 ms mixing time) and ¹³C-separated NOESY spectra, as well as a ¹³C/¹⁵N X-filtered NOESY spectrum on ¹³C/¹⁵N CD2 in complex with unlabeled cpSRP54_{pep}.²¹

Isothermal titration calorimetry measurements

Binding of CD2 to the cpSRP54_{pep} was analyzed by measuring heat change during the titration of cpSRP54_{pep} with CD2 using a VP-ITC titration microcalorimeter (MicroCal Inc., Northampton, MA). All protein and ligand (cpSRP54_{pep}) solutions were degassed under vacuum and equilibrated at 25 °C prior to titration. The sample cell (1.4 mL) contained 0.1 mM CD2 dissolved in PBS buffer (pH 6.5). The reference cell contained water. Upon equilibration, 1 mM cpSRP54_{pep} was injected in 35×6 µl aliquots using the injection rate of 240 second intervals between each injection to allow the sample to return to the baseline. The resulting titration curves were corrected for the CD2-free buffer control and analyzed using the software supplied by Microcal, Inc.

Steady state fluorescence

All fluorescence measurements were made using Hitachi spectrofluorimeter. The excitation and emission slit widths used were 5 nm and 10 nm, respectively. CD2 (50 µM) dissolved in 20 mM phosphate buffer, containing 150 mM NaCl was progressively titrated with cpSRP54_{pep} dissolved in the same buffer. Emission spectra were acquired (at various protein to peptide ratios) between 350 nm and 450 nm using an excitation wavelength of 280 nm. Necessary blank corrections were made in all spectra.

Circular Dichroism

Far UV and Near UV circular dichroism spectra of CD2 and cpSRP54_{pep} were acquired on a Jasco J-720 spectropolarimeter using 1 cm and 0.1 mm quartz cells, respectively. The concentrations of CD2 and cpSRP54_{pep} used were 100 µM and 500 µM, respectively. All solutions were prepared in 20 mM phosphate buffer (pH 6.5), containing 150 mM NaCl.

Protein binding assays

M-domain binding assays were performed essentially as reported in Goforth *et al.*¹³ Briefly, 300 pmol of each GST-fused cpSRP43 deletion or point mutation construct was incubated with 20 µl of S³⁵ labeled, *in vitro* translated cpSRP54-M-domain and 70 µl of 50% Glutathione Sepharose™ slurry. Samples were allowed to mix end-over-end for 30 min at 25°C, and then washed as described previously utilizing Handee™ Spin Columns (Pierce). Proteins were eluted in 50 µl of 20 mM glutathione in 100 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. Eluted proteins were separated by 12.5% SDS-PAGE and visualized directly by staining with Coomassie Blue (GST-fusion) and phosphorimaging (cpSRP54-M-domain).

Structure Calculation

Structures were calculated using ARIA 1.2 and CNS 1.1.²⁵ 3D structure of CD2 bound to the cpSRP54_{pep} was calculated based on the observed intramolecular NOE restraints. The structure of the binary complex of CD2 and the cpSRP54_{pep} was calculated starting from the structure of CD2 and an extended template for the peptide. The lowest energy conformer from the extended calculation was used as the starting structure for incorporating the cpSRP54_{pep} into the structure calculation. A total of 885 experimental NMR constraints were used for the CD2-cpSRP54_{pep} complex structure, 830 of which were NOE based distance constraints, 35 are from experimental dihedral angle constraints and 20 are hydrogen bond constraints. Intermolecular NOEs were added with a weight of >1 to ensure that they were not altered during the course of the ARIA calculation. Initially, 50 structures (out of a total of 200 structures) which showed no NOE violations greater than 0.3 Å and no dihedral angle violations greater than 5 ° (at a threshold NOE value of 0.3 Å). RDC constraints (to be discussed later) were added at this stage and an ensemble of 20 lowest-energy structures were calculated. The structures were analyzed using MOLMOL²⁶ and assessed using PROCHECK analysis.²⁷

Residual dipolar couplings (RDC) were measured by the strain-induced gel alignment procedure (refs. 28, 29) using a 4% (w/v) polyacrylamide gel with an inner diameter of 6 mm for CD2 and CD2_{pep}. The gel containing sample was stretched into the NMR tube using the in house built funnel-like device described by Bax.³⁰ RDC values were determined from the difference between one-bond ¹⁵N-¹H couplings measured in the absence and presence of alignment media using a combination of doublet-separated sensitivity-enhanced IPAP-HSQC spectra.³¹ Initial estimates for the axial component (Da) and the rhombicity (R) were obtained from the unrefined 50 structures using PALES (Fig. S5).³² The tensor parameters were obtained by best-fitting of the RDC value (D^{ij}) to the equation,

$$D^{ij}(\theta, \Phi) = Da^{ij} \left[(3\cos^2\theta - 1) + \frac{3}{2} R \sin^2\theta \cos(2\Phi) \right],$$

by using the unrefined CD2 or CD2/cpSRP54_{pep} complex structure as input structural model, where Da^{ij} is the axial component of the alignment tensor, R is the rhombicity, and θ and Φ are the polar angles of the vector ij with respect to the tensor principal axis.

{¹H}-¹⁵N heteronuclear NOE measurements

{¹H}-¹⁵N heteronuclear NOE data (3 sets) were acquired at 25 °C using Bruker DMX 700 MHz NMR.³³ 32 scans were collected per t_1 point. {¹H}-¹⁵N steady state NOEs were obtained by recording spectra with and without a proton presaturation (6 secs) applied before the start of the ¹H-¹⁵N HSQC experiment. NOE spectra were acquired in an interleaved manner, in which each individual FID was collected with and without proton presaturation. {¹H}-¹⁵N NOEs were determined from the ratios of the integrated peak volumes with and without presaturation. The standard deviation of the NOE was determined from the root mean square value of the background noise in the spectra.

Hydrogen-deuterium exchange (H/D)

CD2 and cpSRP54_{pep}-CD2 complex were individually lyophilized (~0.5 mM in 0.5 ml buffer containing 20 mM phosphate, 150 mM NaCl, pH 6.5) and dissolved in 0.5 ml D₂O, immediately before data collection. ¹H-¹⁵N HSQC at different time points was collected on Bruker 700 MHz spectrometer. ¹H-¹⁵N HSQC spectra were collected continuously for every 40 min for 4 days, every 1 h on the 5th day. Amide proton delays were followed by measuring the crosspeak volumes in ¹H-¹⁵N HSQC spectra.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A	531	541
	A PPGTARRKRK ADS	

B	
GSQVFEYAEV DEIVEKRGKG KDVEYLVRWK GGDCEWVKG VHVAEDVAKD YEDGLEY	

Fig. 1.
Amino acid sequence of, **A**-cpSRP54_{pep} and **B**-CD2.

Fig. 2.

Panel-A: Isothermogram representing the titration of CD2 with cpSRP54_{pep} at 25°C. The top panel shows the raw data for the titration. The bottom panel shows the integrated data after subtraction of the heat of dilution. The solid line in the bottom panel represents the best-fit curve to the data, using the one-independent site model from Microcal Origin.

Fig. 3.

Panel-A: Change(s) in the intrinsic tryptophan emission intensity (in CD2) upon binding to cpSRP54_{pep}. Inset figure depicts the emission spectra of CD2 obtained at various CD2 to cpSRP54_{pep} ratio. The titration was carried out in 20 mM phosphate buffer (pH 6.5) containing 150 mM NaCl. The concentration of CD2 used was 50 μ M. Appropriate background corrections were made in all spectra.

Panel-B: Far UV circular dichroism spectrum of **a**- CD2, **b**-CD2 in the presence of the cpSRP54_{pep} at a molar ratio of 1:1, **c**- cpSRP54 peptide and, **d**- cpSRP54 peptide in the presence of 1: 1 molar ratio of CD2. Curves “c” and “d” represent the difference spectrum obtained after subtracting the spectral contributions of the cpSRP54 peptide and CD2, respectively. Samples were prepared in PBS buffer (pH 6.5). All spectra were obtained at 25°C and represent an average of 10 scans. Appropriate background corrections were made in all spectra. The final concentrations of both CD2 and cpSRP_{pep} were 100 μ M. 1 cm and 0.01 cm pathlength quartz cells were used for acquiring spectra in the Near UV (250 nm to 320 nm) and Far UV (200 nm to 250 nm) regions, respectively. **Panel-C:** Near UV circular dichroism spectra of CD2 (open circle) and of CD2 in the presence of cpSRP54_{pep} at a 1:1 ratio (closed circle). **Panel-D:** Equilibrium thermal unfolding of CD2 in the presence (open circle) and absence (closed circle) of cpSRP54_{pep}. Thermal unfolding was monitored by the change in the emission intensity (at 338 nm). Fluorescence spectra were acquired with an excitation wavelength of 280 nm. The concentration of protein used was 10 μ M. Samples for the thermal unfolding experiment were prepared in 20 mM phosphate buffer (pH 6.5) containing 150 mM NaCl.

Fig. 4.

Panel-A: ^1H - ^{15}N HSQC spectra of CD2 in the presence (green) and absence (red) of cpSRP54_{pep} (red). CD2 and cpSRP54_{pep} were used at approximately 0.5 mM. NMR samples were prepared in 20 mM phosphate buffer (pH 6.5) containing 150 mM NaCl and 10% D₂O. **Panel-B:-** Weighted average (of ^{15}N and ^1H) chemical shift perturbation ($\Delta\delta = \sqrt{(\delta\text{H})^2 + 0.2(\delta^{15}\text{N})^2}$) of residues in CD2 upon complex formation with cpSRP54_{pep} at a molar ratio of 1:1). **Panel-C:** Portion of the ^1H - ^{15}N HSQC spectra of CD2 depicting the perturbation of the epsilon-delta NH resonances of Trp29 and Trp37 in CD2, in the presence and absence of cpSRP54_{pep}.

Fig. 5. MOLMOL representation of the ensemble of structures of **A-** CD2, **B-** CD2 binary complex of CD2 and cpSRP54_{pep}. The beta-sheets, C-terminal helix, unstructured portions in CD2 and the cpSRP54_{pep} are shown in, red, green, blue and gold, respectively. **C & D-** Orientation of the C-terminal helix in CD2 (A) and the cpSRP54_{pep} - CD2 complex (B). The C-terminal helix in CD2 is oriented at an angle of 62° to the plane of the triple stranded beta-sheet. Panels **C & D** depict the orientation of the C-terminal helix changes by about 40° in the structure of the cpSRP54_{pep} - complex. Panels **E & F** show the Comparison of the hydrogen bonds involving residues in Loop-IV in CD2 (E) and the cpSRP54_{pep} - CD2 complex (F). Binding of cpSRP54_{pep} results in the disruption of hydrogen bonds involving residues in Loop-IV, resulting in a shift in the orientation of the C-terminal helix.

Fig. 6.

A portion of ^1H - ^{15}N HSQC spectra of (A) CD2 and (B) cpSRP54_{pep}-CD2 complex depicting the exchange in D_2O . (C) The amide proton of Phe5, Ala8 in CD2 are exchanged rapidly in D_2O . Phe5 and Ala8 are significantly protected in the CD2-cpSRP54_{pep} binary complex even after 5000 minutes of initiation of deuterium exchange. D- ^1H - ^{15}N NOE of residues in CD2 in the presence (open bars) and absence (filled bars) of cpSRP54_{pep}.

Fig. 7.

Panel-A: Surface of CD2 depicting the cpSRP54_{ppe} binding sites (yellow). The backbone of cpSRP54_{ppe} is depicted in grey. Portion of the backbone of the cpSRP54_{ppe} containing residues (Ala535, Arg536 and Arg537) involved in binding with CD2 is shown in green.

Panel-B: Selected ¹³C-¹H strips from the ¹³C-edited/¹²C-filtered 3D NOESY spectrum identifying NOEs at the interface of CD2-cpSRP54_{ppe} complex. The spectrum was acquired using a sample prepared by mixing unlabeled cpSRP54_{ppe} and ¹³C/¹⁵N-enriched CD2.

Fig. 8. cpSRP43/M-domain interaction sites. Equimolar concentrations of each GST-fused cpSRP43 construct were incubated with *in vitro* translated cpSRP54-M-domain and then recovered with Glutathione Sepharose™ beads and eluted with 20 mM glutathione. The eluates containing each GST fusion and co-purifying cpSRP54-M-domain were resolved by 12.5% gel SDS-PAGE and stained with Coomassie Blue to quantify GST-cpSRP43 constructs. Co-precipitating cpSRP54-M-domain was visualized by phosphorimaging for quantification. The percentage of cpSRP54-M-domain co-precipitated relative to that co-purified for GST-cpSRP43 is indicated. Adjustments were made for the slight variations in the amount of GST-construct recovered. The average and standard deviation were calculated using data from three experiments.