Regulation of the GTPase Cycle in Post-translational Signal Recognition Particle-based Protein Targeting Involves cpSRP43*

The chloroplast signal recognition particle consists of a conserved 54-kDa GTPase and a novel 43-kDa chromodomain protein (cpSRP43) that together bind light-harvesting chlorophyll a/b-binding protein (LHCP) to form a soluble targeting complex that is subsequently directed to the thylakoid membrane. Homology-based modeling of cpSRP43 indicates the presence of two previously identified chromodomains along with a third N-terminal chromodomain. Chromodomain deletion constructs were used to examine the role of each chromodomain in mediating distinct steps in the LHCP localization mechanism. The C-terminal chromodomain is completely dispensable for LHCP targeting/integration in vitro. The central chromodomain is essential for both targeting complex formation and integration because of its role in binding the M domain of cpSRP54. The N-terminal chromodomain (CD1) is unnecessary for targeting complex formation but is required for integration. This correlates with the ability of CD1 along with the ankyrin repeat region of cpSRP43 to regulate the GTPase cycle of the cpSRP-receptor complex.

Signal recognition particle (SRP) and its receptor are components of a ubiquitous mechanism for cotranslational protein targeting to the endoplasmic reticulum in eukaryotes and to the cytosolic membrane of prokaryotes (for review see Refs. 1 and 2). An SRP-like protein targeting pathway in chloroplasts mediates the post-translational localization of a family of light-harvesting chlorophyll a/b-binding proteins (LHCPs) to the thylakoid membrane. LHCPs are nuclear encoded thylakoid proteins that are synthesized in the cytoplasm and post-translationally imported into the chloroplast. Following import into the chloroplast stroma, LHCPs bind chloroplast SRP (cpSRP) to form a soluble targeting complex (for review see Refs. 3 and 4). cpSRP contains a conserved 54-kDa GTPase (cpSRP54) (5, 6) but is structurally unique from cytosolic SRPs in that it lacks an RNA moiety (7) and contains a novel 43-kDa protein (cpSRP43) (8, 9). The cpSRP-LHCP targeting complex, termed transit complex, is directed to a thylakoid translocone containing Albino3 (ALB3) (10, 11) by a mechanism that requires cpFlsY (11–14), a homolog of pro- and eukaryotic SRP receptors, FlsY and SRa, respectively. Consistent with the GTPase activity of cpFlsY and cpSRP54, GTP is required for LHCP integration (6). It is noteworthy that GTP is not required for transit complex formation (14, 15). Rather, binding of GTP by cpSRP54 and cpFlsY appears to take place upon their interaction at the thylakoid membrane (11), which closely resembles GTP binding by SRP54 and its receptor in protein targeting to the ER (16, 17).

The unique ability of cpSRP to bind substrates post-translationally is attributed to cpSRP43, which binds both cpSRP54 and a charged 18-amino acid element in LHCPs (L18) to bring about the formation of transit complex (18, 19). In the absence of L18 binding, the transit complex fails to form, and LHCPs do not properly integrate into the thylakoid membrane (18). These adaptor functions associated with cpSRP43 are likely mediated by the putative ankyrin (Ank) domains and chromodomains (chromosome organization modifier) present throughout the protein (see Fig. 1 for domain organization) (9). Ank domains are repetitive ~33-amino acid motifs that together form a characteristic and stable structure with surfaces that are tailored for many different macromolecular interactions (for review see Ref. 20). Chromodomains (CDs) are a family of highly conserved ~44-amino acid motifs first discovered in the Drosophila Polycomb gene silencing protein (21). CD-containing proteins have been found in diverse eukaryotic organisms ranging from Schizosaccharomyces pombe to humans, where CDs mediate protein-protein interactions within the nucleus (for review see Ref. 22).

Yeast two-hybrid studies have been used to examine the protein interaction targets of Ank and CDs in cpSRP43 (23). Results from this work suggest that the first domain of a four-Ank domain repeat binds to the L18 motif of LHCPs, whereas the third and fourth Ank domains appear to support homodimerization of cpSRP43. However, the relevance of homodimerization domains is not clear because cpSRP appears to function as a cpSRP43/cpSRP54 heterodimer (7). In the same two-hybrid study, binding to cpSRP54 was attributed to the combined effects of the two CDs at the C terminus of cpSRP43. Whether CD-mediated binding of cpSRP43 to cpSRP54 is needed for any step of the LHCP localization mechanism is not currently known.

Recent progress in the production of functional recombinant cpSRP (7) and cpFlsY (11, 14) has provided the tools to examine the relationship between protein interaction activities and the role of these activities in mediating distinct steps in the LHCP...
localization mechanism. In this context, we have examined CD-specific mutants of cpSRP43 in assays that reconstitute cpSRP formation, cpSRP-LHCP targeting complex formation, and LHCP integration into isolated thylakoids. Our analyses extend to a previously unexamined third CD near the N terminus identified here by structural modeling. Results of these studies also led us to examine the influence of CD mutants on cpSRP targeting of cpSRP45 and cpSRP54 to SRx (FhII and PscY in bacteria) reciprocity stimulates GTP hydrolysis by each protein in cotranslational SRP targeting (24), an activity promoted by the 50 S ribosomal subunit in targeting to the ER (17). Most importantly, our data suggest that each CD functions at distinct steps in the LHCP targeting/insertion mechanism and that in post-translational targeting, cpSRP43 may functionally replace the ribosome as an activator of the cpSRP54/pFtsY GTPase cycle.

EXPERIMENTAL PROCEDURES

All reagents, enzymes, and standards were purchased commercially. A peptide corresponding to the L18 region of pLHCP (VDPYLPFGS-FDPLGLASS) was described previously (18). Precursor LHCP template L18-14 was used to express GST-cpSRP43 from which mature cpSRP43 was obtained following proteolytic removal of the protein preproteolysis (L18-PPL) was described previously (18). Primers for DNA amplification using PCR were purchased from Integrated DNA Technologies. PCR amplifications were performed with Phusion DNA polymerase (Stratagene), and PCR products were restricted with enzymes purchased from New England Biolabs. All cloned sequences were verified by DNA sequencing (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock).

Cloning, Expression, and Purification of Recombinant Proteins—Recombinant, purified cpSRP54-his and Trx-cpFtsY were produced and isolated as described previously (11, 14). Mature cpSRP43 in pGEX-6P-2 (14) was used to express GST-cpSRP43 from which mature cpSRP43 was obtained following proteolytic removal of the GST fusion partner. GST-cpSRP43 from the soluble bacterial fraction was initially purified over glutathione-Sepharose (Amersham Biociences) and further purified by anion exchange chromatography using a Resource Q column (Amersham Biociences) with bis-Tris, pH 6, and elution with a linear gradient of KCl followed by desalting into 10 mM HEPES-KOH, pH 8.0, 10 mM MgCl2 (HKM) prior to use. For the production of cpSRP43/pFtsY and GST-cpSRP43 and GST-cpSRP43-pFtsY, BirAase was used in the region. The coding sequence for mcpSRP43 that begins AAVQRN and lacks the C-terminal CD3 along with an exact forward primer that binds at the beginning of the Anc region such that the protein begins YETP and ends GLEYE. All deletion and domain expression constructs were transformed into Escherichia coli strain BL21 Star (Invitrogen) for isopropyl-1-thio-β-D-galactopyranoside-induced protein expression. GST-cpSRP43D1, GST-cpSRP43D2, GST-cpSRP43CD3, and GST-cpSRP43-pCD2 were purified as described above for GST-cpSRP43. Cleaved constructs were produced and purified as described for cpSRP43.

Coding sequences for cpSRP43 domains CD1, CD2, and CD3 were amplified by PCR using forward primers corresponding to the coding sequences of the desired amino acids indicated in Fig. 1. A BamHI site was incorporated at the 5′ end of the forward primer to allow ligation of the restricted PCR products into pGEX-4T-2 (Amer-
sham Biociences) restricted with BamHI and Smal. CD1, CD2, and CD3 expression constructs were transformed into E. coli strain BL21 Star for isopropyl-1-thio-β-D-galactopyranoside-induced protein expression. Following bacterial lysis, expressed proteins were affinity-purified by using glutathione-Sepharose affinity chromatography followed by purification by anion exchange chromatography before being desalted into HKM buffer.

The nucleotide sequence coding for cpSRP54 lacking the C-terminal 26 amino acids was amplified by using an exact forward primer that introduced a KpnI site and a reverse primer that bound immediately following changes. Thirty picomoles of recombinant cpSRP43 or appropriate deletion construct and an equimolar amount of recombinant cpSRP54-his or cpSRP54CD3 were added to the 50 S ribosomal subunit in target (SE) and added to 50 f 35S-labeled pLHCP translation product containing 1.7 fmol of LHCP diluted 1:3 in HK buffer (10 mM HEPES-KOH, pH 8.0). Each assay was brought to 30 °C for 10 min of sample was analyzed by 6% nondenaturing PAGE and imaged on a Typhoon PhosphorImager (Amersham Biociences) to distinguish cpSRP43-LHCP transit complex from aggregated LHCP. For assays in which L18 synthetic peptide was used to compete with HKP and final concentrations of 0.2 mM GTP and 5 mM ATP and incubated for 30 min at 25 °C the addition of 5 μl of cold 50% glycerol. Control assays were conducted as above but included either no protein components or 20 μl of SE. For each assay, 10 μl of sample was analyzed by 6% nondenaturing PAGE and imaged on a Typhoon PhosphorImager (Amersham Biociences) to distinguish cpSRP54-LHCP transit complex from aggregated LHCP. For assays in which L18 synthetic peptide was used to compete with HKP and final concentrations of 0.2 mM GTP and 5 mM ATP and incubated for 30 min at 25 °C for 10 min of sample was analyzed by 6% nondenaturing PAGE and imaged on a Typhoon PhosphorImager (Amersham Biociences) to distinguish cpSRP54-LHCP transit complex from aggregated LHCP. For assays in which L18 synthetic peptide was used to compete with HKP and final concentrations of 0.2 mM GTP and 5 mM ATP and incubated for 30 min at 25 °C for 10 min of sample was analyzed by 6% nondenaturing PAGE and imaged on a Typhoon PhosphorImager (Amersham Biociences) to distinguish cpSRP54-LHCP transit complex from aggregated LHCP. For assays in which L18 synthetic peptide was used to compete with HKP and final concentrations of 0.2 mM GTP and 5 mM ATP and incubated for 30 min at 25 °C.
by centrifugation and protease treatment with thermolysis (28), the thylakoid membranes were solubilized with 50 μl of SDS-PAGE solubilization buffer and analyzed on 12.5% SDS-PAGE by loading 10 μl of each assay per lane. Integrated LHCP, as noted by the formation of a correctly sized degradation product, was visualized by PhosphorImaging.

**Protein Binding Assays**—cpSRP43-his and cpSRP3A26–54-his binding assays were performed by combining 100 pmol of each GST-fused cpSRP43 deletion or domain expression construct with 100 pmol of cpSRP43-his or cpSRP3A26–54-his and 70 μl of a 50% glutathione-Sepharose slurry in 10 ml HK, 50 mM potassium acetate, and 10 mM MgCl₂, pH 8.0, in a final volume of 270 μl. Samples were allowed to mix end-over-end for 1 h at 4 °C and then were washed as described (19) by utilizing PSU 6.5-mm centrifuge filters (Whatman). Proteins were eluted in 50 μl of 40 mM glutathione in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. Eluted proteins were separated by 12.5% SDS-PAGE and visualized directly by staining with Coomassie Blue. Interactions with cpHCP, L18-PPL, cpSRP54-M domain, and cpSRP54-NG domain were performed by incubation of 100 pmol of each GST fusion protein with 20 μl of 850 nm. Throughout the duration of the assay, the amount of GTP hydrolyzed increased linearly. Furthermore, a standard curve of inorganic phosphate was linear between 2 and 75 nmol of Pi and was used to determine the amount of Pi released in each assay. A substrate organic phosphate was linear between 2 and 75 nmol of Pi and was used to determine the amount of Pi released in each assay.

**Assay of GTPase Activity**—Recombinant cpSRP43 and deletion constructs were assayed for GTPase activity in the presence and absence of 2 mM GTP in 10 mM HEPES, pH 8.0, and pyrophosphate. Assays containing 150 pmol of each indicated protein component and 2 μM GTP in 10 μl HEPEs, pH 8.0, and 10 mM MgCl₂ were incubated at 30 °C for 1 h. After incubation SDSA was added to a final concentration of 6% to denature protein components and prevent subsequent GTPase activity. The addition of ascorbic acid and ammonium molybdate (to 6 and 1%, respectively) was followed by 40% (Fig. 4) of 50% glutathione in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. Eluted proteins were separated by 12.5% SDS-PAGE and visualized directly by staining with Coomassie Blue. Interactions with cpHCP, L18-PPL, cpSRP54-M domain, and cpSRP54-NG domain were performed by incubation of 100 pmol of each GST fusion protein with 20 μl of 850 nm. Throughout the duration of the assay, the amount of GTP hydrolyzed increased linearly. Furthermore, a standard curve of inorganic phosphate was linear between 2 and 75 nmol of Pi and was used to determine the amount of Pi released in each assay. A substrate organic phosphate was linear between 2 and 75 nmol of Pi and was used to determine the amount of Pi released in each assay.

**Domain Analysis Indicates the Presence of Three Chromodomains in cpSRP43**—The unusual function of a chromodomain protein (see Fig. 1 for domain organization) in protein targeting led us to investigate the role of individual CDs at distinct steps of the LHCP localization pathway. We first subjected the cpSRP43 sequence to SMART (Simple Modular Architecture Research Tool) analysis (30), which indicated the presence of a repeating ankyrin domain region and two CDs at the C-terminal region of cpSRP43 as reported previously (23). In addition, an uncharacterized third CD was predicted at the C-terminus of cpSRP43. An alignment of the CDs (termed CD1, CD2, and CD3) of cpSRP43 revealed a high level of similarity between all three domains and a CD from a well characterized mouse heterochromatin-associating protein, mouse modifier protein 1 (mM-HP1β) (Fig. 2A).

Fig. 2 shows the core structure of each CD present in cpSRP43, each derived using the homology building program Swiss model (31) and the coordinates from a determined three-dimensional structure of a known chromatin binding domain from mouse modifier protein 1 (1AP0) (32). The models suggest that the most highly conserved residues found among the three predicted chromodomains likely stabilize the β-sheet structure. The amino acids of the β-sheet core exhibit a strikingly invariant conformation, reflecting the canonical CD conformation that has been observed in other systems. For example, the positions of the Tyr and Trp residues indicated by asterisks are identical to those important for function in HP1 of Drosophila and mouse (33). However, the third conserved residue necessary for histone binding in HP1 domains, Phe, is changed to a Gly or Met in the CDs of cpSRP43 which may influence target specificity. The most variable region among the CDs is the C-terminal α-helix, the function of which is not well characterized in other CD-containing proteins. The structural features of the models are reasonable in terms of what is known about protein structure in general, i.e. a well packed core is formed by hydrophobic residues and surface residues are primarily hydrophilic. Furthermore, main chain conformations are in acceptable regions where bond angles and lengths as well as planarity are all acceptable using PROCHECK (34). Overall, the models indicate reasonable conformations that adhere to known chromdomain structures. As such, these models argue strongly for the presence of a previously unidentified CD, CD1, near the N terminus of cpSRP43.

We have used these models to construct plasmids that either express cpSRP43 deletions lacking individual CDs or express only the individual CDs (refer to Fig. 1). The proteins were expressed in E. coli as GST fusion proteins, purified to at least 85% homogeneity (Fig. 3), and were used in assays that reconstitute distinct steps of the LHCP localization process. **Chromodomains Exhibit Distinct Functions in Two Stages of LHCP Localization**—In order to examine the functional role of CDs in post-translational cpSRP-based protein targeting, purified CD deletion constructs of cpSRP43 were mixed with recombinant cpSRP43-his, radiolabeled LHCP, and assayed for transit complex formation. A soluble cpSRP-LHCP transit complex migrates as a single band when subjected to nondenaturing PAGE (Fig. 4) (15, 35). Whereas deletion of either CD1 or CD3 had little or no effect on the efficiency of transit complex formation (Fig. 4A, compare lanes 2 and 4 with lane 1), deletion of both the first and third CDs typically reduced the efficiency by 40–50% (Fig. 4A, compare lane 5 with lane 1). Notably, deletion of CD2 nearly eliminated transit complex formation (Fig. 4A, lane 3). Together, these data indicate that the central CD of cpSRP43 is critical for transit complex formation. In addition, it appears that neither the N-terminal nor the C-
terminal CDs play any significant individual role in the formation of transit complex, although simultaneous removal of both, leaving only the Ank-CD2 region of cpSRP43, decreases the efficiency of transit complex formation.

In order to confirm that the interaction between the Ank1-CD2 region of cpSRP43, LHCP, and cpSRP54 occurs in a manner fundamentally similar to cpSRP, we sought to determine the binding specificity of this core region for the L18 motif in LHCP, a charged region of LHCP that binds cpSRP43 (19) and is required for transit complex formation (18). Separate transit complex assays were conducted with full-length cpSRP43 and cpSRP-A1-CD2 in the presence of increasing concentrations of L18 synthetic peptide, which competes with LHCP for binding endogenous cpSRP (18). Although the transit complex forming activity of cpSRP-A1-CD2 is somewhat lower overall, the specificity of the protein for L18 mirrors that of the full-length protein as judged by the ability of the L18 peptide to compete with LHCP for binding to cpSRP (Fig. 4C). These findings indicate that the interactions within the Ank1-CD2 core region of cpSRP43 are necessary and sufficient for formation of a soluble cpSRP-LHCP transit complex.

To examine further the role of CDs in LHCP integration, full-length cpSRP43 or each of the CD deletion constructs was incubated with recombinant cpSRP54-his before addition of radiolabeled LHCP, purified recombinant cpFtsY, and salt-washed thylakoids. Because salt-washed thylakoids lack appreciable levels of cpFtsY, integration of LHCP requires the addition of both recombinant cpSRP and cpFtsY (14). Fig. 4B shows that deletion of CD3 had no deleterious influence on integration whereas deletion of CD2 abolished integration, effects that mirror the influence of these CD deletions on transit complex formation (Fig. 4A). However, cpSRP43CD1, which supported transit complex formation, exhibited a severe inability to support integration. The integration activity in assays containing cpSRP43CD1 was typically reduced 70–90% relative to assays containing cpSRP43. Likewise, integration with cpSRP-A1-CD2 was greatly reduced. Taken together, these data indicate that CD1 functions downstream from transit complex formation at a step that is critical for efficient LHCP integration into thylakoids.

Defining a Functional cpSRP43/cpSRP54 Interface—The results in Fig. 4 suggest that CD3 is not required for functional interactions with either soluble or membrane proteins required for LHCP integration into isolated thylakoids. In contrast, protein interactions mediated by CD2 appear necessary at all stages of the localization mechanism. Previous results from yeast two-hybrid assays indicated a critical role for both CD2 and CD3 in binding cpSRP54 (23); hence, we employed the use of GST fusion proteins to examine CD interactions with cpSRP54-his in protein binding assays. GST fused to CD deletion constructs or fused to individual CDs were incubated with cpSRP54-his and re-purified using glutathione-Sepharose™ beads. The proteins were then eluted from the beads with buffer containing glutathione (see “Experimental Procedures”).
Eluted proteins were separated by SDS-PAGE and visualized directly by staining with Coomassie Blue. Fig. 5, A and B, shows that cpSRP54-his bound to CD2 alone and to deletion constructs that contain CD2. Neither CD1 nor CD3, whether present in the cpSRP43CD2 deletion construct or as individual domains, showed any affinity for cpSRP54-his (Fig. 5, A and B). When equimolar amounts of GST-cpSRP43 and GST-CD2 are combined with cpSRP54-his, equivalent amounts of cpSRP54-his are copurified (Fig. 5B, top panel), suggesting that CD2 is solely responsible for any interaction with cpSRP54-his. These experiments were repeated using stromal extract as a source of cpSRP54 with identical results, further demonstrating that CD2 alone is responsible for interactions with cpSRP54 rather than the combination of CD2 and CD3 as has been suggested previously. In order to examine the possibility that loss of cpSRP54 binding is attributable to misfolding of the cpSRP43 constructs that lack CD2, we examined their ability to bind L18, the cpSRP43-binding motif in LHCP. When the same coprecipitation assays were performed with L18-PPL (18), no constructs that lack CD2, we examined their ability to bind cpSRP54 binding is attributable to misfolding of the cpSRP43 construct tested showed any interaction with the NG domain of cpSRP54 above background levels. Taken together, these data indicate that cpSRP54 and cpSRP43 must be in a heterodimer mediated by the CD2/M domain interaction to form transit complex and support LHCP integration.

It is noteworthy that removal of the C-terminal 26 amino acids from cpSRP54, described previously as essential for cpSRP43 binding (7), did not eliminate interaction of cpSRP54 with GST-cpSRP43. The remaining interaction activity (40%; Fig. 6A, compare 2nd and 4th lanes) supported integration of LHCP at levels similar to full-length cpSRP54 (Fig. 6B, compare 2nd and 3rd lanes).

cpSRP43 Regulates the GTPase Activity of cpSRP54/cpFtsY—Because of the role of GTP binding and hydrolysis by SRP54 and SR (FtsY in E. coli) in modulating events at the target membrane, we explored the possibility that CD1 function is associated with the GTPase cycle, which occurs during targeting/integration events. In protein targeting to the ER, protein interactions between membrane and soluble components are regulated through GTP binding and hydrolysis to ensure that the substrate is released only in the presence of an open translocase (36). It has been shown recently that interactions between the ribosome and SRP as well as between SRβ and the ribosome are involved in the regulation of GTP hydrolysis (17, 36). However, the cpSRP system lacks a ribosomal

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* R. L. Goforth and R. L. Henry unpublished data.
component, and no SRβ sequence homolog has been identified. Therefore, it seems likely that proteins unique to the cpSRP system may act to replace the regulatory function of the ribosome and/or SRβ.

To examine the possibility that cpSRP43 may play a role in regulating GTPase activity of cpSRP54 and cpFtsY, we utilized a colorimetric assay that measures release of inorganic phosphate by GTP hydrolysis (29), a method made possible by the availability of chemical quantities of purified cpSRP targeting components (see Fig. 3). Comparison of the amounts of inorganic phosphate generated by equimolar amounts of constituent proteins indicates that little GTP is hydrolyzed when any single protein component (e.g. cpSRP43, cpSRP54-his, and Trx-cpFtsY) is present (Fig. 7A, lanes 4, 8, 12, 16, 20, 23, and 24). When cpSRP54-his and Trx-cpFtsY are both present, GTP hydrolysis is slightly more than what would be expected from additive GTP hydrolysis (Fig. 7A, compare lane 22 with lanes 23 and 24). Notably, when cpSRP43 is added, GTP hydrolysis is increased (Fig. 7A, compare lane 1 with lane 22), suggesting that cpSRP43 may play a role in the regulation of GTP hydrolysis by cpSRP54 and cpFtsY. Further support for such a role is provided by results of GTP hydrolysis assays conducted with CD deletions in place of cpSRP43. Removal of either the second or the third CD (Fig. 7A, compare lanes 9 and 13 with lane 1) had little effect on the overall amount of P_i generated when compared with full-length cpSRP43. However, GTP hydrolysis by cpSRP54-his/Trx-cpFtsY was greatly increased by removal of CD1 (Fig. 7A, compare lane 5 with lanes 1 and 22), demonstrating that cpSRP43 has the ability to increase GTP hydrolysis by cpSRP54-his/Trx-cpFtsY 4-fold in the absence of CD1. Similarly, the addition of cpSRP-A1-CD2, which lacks both CD1 and CD3, to cpSRP54 and cpFtsY also resulted in a dramatic increase in GTP hydrolysis as compared to the addition of cpSRP43 (Fig. 7A, compare lane 17 with lanes 1 and 22). The increased rate of hydrolysis observed when either cpSRP43 CD1 or cpSRP-A1-CD2 is added to cpSRP54/Trx-cpFtsY was specific for GTP. In equivalent assays where GTP was replaced with ATP, only low levels of nucleotide hydrolysis were observed. The greatest level of inorganic phosphate released from ATP hydrolysis for any mixture of proteins did not exceed 2.2 nmol.2

To examine whether stimulation of cpSRP54/cpFtsY GTP hydrolysis activity by CD1 deletion constructs (cpSRPΔCD1 or cpSRP-A1-CD2) stems from CD2 alone or involves the Ank repeat region of cpSRP43, GTP hydrolysis by cpSRP54/cpFtsY was measured in the absence or presence of individual chromodomains. GTP hydrolysis by cpSRP54-his in combination with Trx-cpFtsY was unaffected by addition of CD1, CD2, or CD3 (Fig. 7B). Taken together, these data suggest that the requirement for CD1 in LHCP integration may stem from its participation in events that regulate GTP binding and/or hydrolysis. Moreover, our data are consistent with a model where CD1 acts to negatively modulate the GTPase stimulatory activity of an Ank repeat region in cpSRP43.

**DISCUSSION**

In this report, we have used recombinant cpSRP to understand the role of cpSRP43 chromodomains in the LHCP targeting/insertion mechanism. We have combined functional assays...
and interaction studies to understand the functional relevance of chromodomain interactions within the LHCP targeting/insertion pathway. Our studies address the role of two previously identified chromodomains (CD2 and CD3) as well as the role of a chromodomain identified by sequence alignment and structural modeling (CD1). In this context, our work establishes the importance of chromodomain interactions in the formation of a cpSRP-LHCP transit complex, which serves as the soluble form of LHCP targeted to the membrane. Of the three CDs, only CD2 is required for transit complex formation and for binding of cpSRP43 to cpSRP54. Together, these data show for the first time that cpSRP43 must be in a heterodimer with cpSRP54 in order to support transit complex formation. It is noteworthy that a previous structural model of cpSRP, based largely on results from a yeast two-hybrid approach, supports a structural model in which CD2 and CD3 of cpSRP43 create a single binding site for cpSRP54 (23). However, CD3 alone shows no ability to bind cpSRP54, and deletion of CD3 has no impact on cpSRP43 binding to cpSRP54 (Fig. 4). Hence, our data support a different structural model of cpSRP in which CD3 plays little or no role in cpSRP54-cpSRP43 heterodimer formation. Furthermore, CD3 was found to be dispensable for both transit complex formation and LHCP integration suggesting that its evolutionary preservation may be related to LHCP localization steps not examined by our assays and which precede transit complex formation in the stroma, e.g. import across the envelope membranes mediated by TIC and TOC transporters (reviewed in Refs. 37 and 38). In this context, we are currently examining Arabidopsis mutants for the ability of cpSRP43/CD3 to compensate for the absence of cpSRP43 expression.

It has been suggested that the cpSRP43 binding region of cpSRP54 lies within the methionine-rich C-terminal M domain (23), and peptide scanning of cpSRP54 indicated that the interaction is contained within the C-terminal 26 amino acids of this domain (7). In contrast, we show that removal of the C-terminal 26 amino acids from recombinant cpSRP54 reduces binding to cpSRP43 by 60% (Fig. 6A) but does not eliminate the interaction, suggesting that additional interaction sites within the cpSRP54 M domain participate in binding to cpSRP43. The remaining ability of cpSRPΔC26-54-his to bind cpSRP43 is sufficient for SRP targeting/integration activities; deletion of this region from cpSRP54 did not inhibit LHCP integration (see Fig. 6B). This may point to stabilization of the cpSRP43/cpSRP54 interaction by association with LHCP, which is known to interact with both SRP components (18, 19, 35). Additionally, we cannot exclude the possibility that once transit complex is formed, other regions of cpSRP54 and/or cpSRP43 interact that do not function in SRP heterodimerization per se.

In contrast to transit complex formation, LHCP insertion into the thylakoid membrane requires both CD2 and a previously unexplored chromodomain, CD1. Loss of LHCP integration would be expected by using a CD2 deletion of cpSRP43; CD2 is required for transit complex formation, which is a prerequisite for LHCP integration. However, LHCP integration is nearly lost in the absence of CD1, whereas transit complex formation appears unhindered. This demonstrates for the first time that cpSRP43 functions downstream from the formation of a cpSRP-LHCP transit complex and supports a much expanded role for cpSRP43 that extends beyond its im-

![Graph A](image)

**Fig. 7.** cpSRP43 stimulates GTPase activity of cpSRP54/55FtsY. The effect of cpSRP43 chromodomain deletions (A) or individual chromodomains (B) on the GTPase activity of cpSRP54/55FtsY was measured in assays containing 150 pmol of each indicated component and 2 mM GTP as described under "Experimental Procedures." GTPase activity resulting in release of inorganic phosphate was determined according to Ref. 29 by using known phosphate standards (see "Experimental Procedures"). The average and S.D. were calculated using data from three experiments.

![Graph B](image)
portance in allowing cpSRP to bind full-length substrates. The absence of CD1 interaction with other components in transit complex, along with a requirement for CD1 to promote efficient integration, suggests the possibility that CD1 may be interacting with an integral membrane protein necessary for integration of LHCPs. However, the recently defined functional interactions between cpSRP, FtsY, and the ALB3 translocase demonstrate that cpSRP43 is not required for functional association of these components (11). Therefore, it is not surprising that deletion of CD1 has no impact on the ability to form this complex at the membrane. The fact that removal of CD1 from cpSRP43 increases by 4-fold the rate at which GTP is hydrolyzed by cpSRP/cpFtsY alone (Fig. 7) suggests an alternative explanation. It now seems likely that cpSRP43 coordinates activities at the membrane by regulating the GTPase cycle of cpSRP/cpFtsY. Specifically, CD1 appears to act as a negative regulator because its removal leads to increased rates of GTP hydrolysis. By this same view, a region of cpSRP43 outside of CD1 appears to act as a positive regulator of GTP hydrolysis because removal of CD1 leads to elevated GTPase activity of cpSRP/cpFtsY beyond the level of stimulation seen when full-length cpSRP43 is added. It is noteworthy that the amount of GTP hydrolysis is low in all cases relative to the amount of recombinant protein present in the assays. This may indicate that additional factors are required for high (catalytic) rates of GTP hydrolysis in stroma.

The ankyrin repeat region of cpSRP43 is part of the mechanism by which cpSRP is able to regulate the GTPase cycle of cpSRP/cpFtsY and LHCP release from cpSRP to ALB3. The ankyrin repeat and CD1, which we hypothesize are used to regulate the timing of GTP binding/hydrolysis by cpSRP/cpFtsY and LHCP release from cpSRP to ALB3.

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