

cpSRP43 Is a Novel Chaperone Specific for Light-harvesting Chlorophyll *a,b*-binding Proteins*[§]

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The biosynthesis of most membrane proteins is directly coupled to membrane insertion, and therefore, molecular chaperones are not required. The light-harvesting chlorophyll *a,b*-binding proteins (LHCPs) present a prominent exception as they are synthesized in the cytoplasm, and after import into the chloroplast, they are targeted and inserted into the thylakoid membrane. Upon arrival in the stroma, LHCPs form a soluble transit complex with the chloroplast signal recognition particle (cpSRP) consisting of an SRP54 homolog and the unique cpSRP43 composed of three chromodomains and four ankyrin repeats. Here we describe that cpSRP43 alone prevents aggregation of LHCP by formation of a complex with nanomolar affinity, whereas cpSRP54 is not required for this chaperone activity. Other stromal chaperones like trigger factor cannot replace cpSRP43, which implies that LHCPs require a specific chaperone. Although cpSRP43 does not have an ATPase activity, it can dissolve aggregates of LHCPs similar to chaperones of the Hsp104/ClpB family. We show that the LHCP-cpSRP43 interaction is predominantly hydrophobic but strictly depends on an intact DPLG motif between the second and third transmembrane region. The cpSRP43 ankyrin repeats that provide the binding site for the DPLG motif are sufficient for the chaperone function, whereas the chromodomains are dispensable. Taken together, we define cpSRP43 as a highly specific chaperone for LHCPs in addition to its established function as a targeting factor for this family of membrane proteins.

Many proteins fold spontaneously *in vitro* in agreement with Anfinsen's pioneering studies that showed that the information for protein folding resides in the primary structure (55). However, *in vivo* folding of soluble proteins often requires molecular chaperones to efficiently reach the native functional state (1). The folding of proteins can start during their synthesis assisted by ribosome-associated chaperones that act on the nascent polypeptide chain (2). Most membrane proteins utilize the ribosome-bound signal recognition particle (SRP)² for

co-translational targeting to the plasma membrane in prokaryotes and the endoplasmic reticulum in eukaryotes, which allows coupling membrane protein biosynthesis directly to membrane insertion (3–5). In this way, aggregation and misfolding of membrane proteins in the cytoplasm are prevented. Nuclear encoded membrane proteins destined for organelles, *e.g.* mitochondria or chloroplasts, however, are excluded from the “classical” co-translational targeting and insertion mechanisms. Recently an endoplasmic reticulum- and Golgi-mediated protein targeting pathway to the chloroplast for soluble, glycosylated proteins was described (6–8). However, it is not known at present whether this pathway is also used to target chloroplast membrane proteins.

Prominent examples of post-translationally targeted membrane proteins are the light-harvesting chlorophyll *a,b*-binding proteins (LHCPs) that serve as antenna proteins in photosynthesis and represent one of the most abundant membrane proteins on earth. After synthesis in the cytoplasm, they are imported across the two chloroplast envelope membranes utilizing the TOC/TIC import machinery (9, 10). Upon arrival in the stroma, LHCPs are sequestered into a soluble, so-called transit complex with the chloroplast signal recognition particle (cpSRP), consisting of cpSRP54 and a novel protein component, cpSRP43 (11). LHCPs contain three transmembrane helices (TM1–3) to which carotenoids and chlorophylls are attached upon thylakoid insertion to form the light-harvesting complexes (LHCs) that are active mainly as trimers (12, 13). The mechanisms of LHCP folding *in vitro* have been analyzed extensively (14–16), and unfolded LHCP was shown to be sufficient for chloroplast import but not for thylakoid insertion (17). The hydrophobic TMs present a particular challenge as they are prone for aggregation and have to be kept in a conformation competent for subsequent membrane insertion and folding into a functional structure. This calls for a molecular chaperone that prevents LHCPs from aggregation in the stroma and allows membrane insertion. In early studies, a stromal factor was shown to be absolutely required for targeting and membrane insertion (17), but the factor remained unknown. Although in mitochondria insertion of the nuclear encoded multispinning membrane protein Oxa1 into the inner envelope was shown to require Hsp70 (18), neither Hsp70 nor Hsp60 supported thylakoid insertion of LHCP in chloroplasts (17, 19). In the meantime, cpSRP43 was identified as a novel component of cpSRP (11). It consists of protein-protein interaction domains, three chromodomains, and four ankyrin

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² The abbreviations used are: SRP, signal recognition particle; cpSRP, signal recognition particle; LHC, light-harvesting complex; LHCP, light-harvesting chlorophyll *a,b*-binding protein; CBB, Coomassie Brilliant Blue R; TM, transmembrane helix; CD, chromodomain; GAPDH, glyceraldehyde-3-

phosphate dehydrogenase; GdmCl, guanidinium chloride; DTT, dithiothreitol; TF, trigger factor; Rbc, Rubisco; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

cpSRP43 Is a Novel Chaperone

repeats (20, 21) and interacts with a conserved region in LHCPs, the L18 region (L18) between TM2 and TM3 (22). L18 is an internal targeting signal that allows LHCPs to interact with cpSRP43 in a highly specific manner, unlike the canonical interaction of signal sequences with SRP, which is not sequence-specific (3, 4). We have previously shown that a DPLG motif in L18 is strictly required for the interaction and wraps around a tyrosine hook in the ankyrin repeats of cpSRP43 (21). The tyrosine seems to mimic the lutein headgroup of the carotenoid and to allow cofactor attachment upon LHCP membrane insertion (21). The stoichiometry of the transit complex is not known, but in *in vitro* thylakoid insertion assays, both cpSRP43 and cpSRP54 are required for LHCP insertion, whereas *in vivo*, cpSRP43 alone seems sufficient when cpSRP54 and the SRP receptor cpFtsY are both knocked out (23). This points to a central role for cpSRP43, with the C-terminal chromodomains (CD2–3) interacting with cpSRP54 (24, 25) and the C-terminal tail of the membrane insertase Alb3 (26), whereas the ankyrin repeats bind the DPLG motif of LHCPs (18).

Here we show that cpSRP43 is a novel molecular chaperone required for LHCP membrane insertion. cpSRP43 alone is able to form a soluble complex with LHCP and thereby is sufficient to prevent aggregation. The chaperone function resides in the ankyrin repeats of cpSRP43 and strictly requires the conserved DPLG motif in LHCPs. The role of cpSRP43 cannot be taken over by other stromal chaperones.

EXPERIMENTAL PROCEDURES

Cloning, Protein Production, Isolation of Inclusion Bodies, and Protein Purification—The different LHCP constructs (LHCP, LHCP-L201K, LHCP Δ TM3, and LHCP Δ L18 Δ TM3) were cloned into pET21d using NcoI and XhoI restriction sites leading to a C-terminal His tag. LHCP and its mutants were produced in BL21(DE3) cells. Protein production was induced by 1 mM isopropyl-1-thio- β -D-galactopyranoside at an A_{600} of 0.6. After 3 h at 37 °C, the cells were harvested and stored at –80 °C. Cells were resuspended in lysis buffer (100 mM HEPES/NaOH, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.02% 1-thioglycerol, pH 7.5). The cells were lysed by two passes through an M-110L Microfluidizer (Microfluidics). After cell lysis, DNase I was added, and the solution was stirred on ice for 15 min. Insoluble material was collected by centrifugation (20 min, 8000 \times g). The obtained pellet was washed three times in washing buffer containing 1 mg/ml sodium deoxycholic acid, 1 mM EDTA, 10 mM 2-mercaptoethanol, pH 8.0. Subsequently the inclusion bodies were washed three times with 20 mM HEPES/NaOH, 10 mM 2-mercaptoethanol, pH 7.5. The collected inclusion bodies were stored at –80 °C until use. For purification, the inclusion bodies were solubilized in buffer A (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, pH 8.0) for 1 h at 20 °C, and insoluble material was removed by centrifugation (30 min, 30,000 \times g). The supernatant was applied onto a 1-ml His-Trap FF column (GE Healthcare). The column was washed with buffer A containing 100 mM imidazole. The protein was eluted with buffer A containing 300 mM imidazole. After elution, the protein was exchanged into buffer A using a PD-10 desalting column (GE Healthcare). Purification of cpSRP43

(21), cpSRP54 (27), Alb3CT (26), and cpFtsY (28) was performed as described.

Refolding Assay—Purified denatured LHCP in buffer A was diluted 40-fold to a final concentration of 5 μ M in the absence or presence of proteins (all 10 μ M) (cpSRP43, cpSRP54, cpSRP, cpFtsY, or Alb3CT) or in the presence of 0.5% SDS at 20 °C. After incubation for 15 min at 20 °C, the soluble and insoluble fractions were separated by centrifugation (15 min, 12,000 \times g) and analyzed by SDS-PAGE on NuPAGE 4–12% polyacrylamide gels (Invitrogen) with subsequent Coomassie Brilliant Blue R (CBB) (Sigma) staining.

Light-scattering Assays—As a control for trigger factor activity, the ability to prevent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) aggregation was analyzed. 125 μ M GAPDH from rabbit muscle (Sigma) was denatured in 100 mM potassium phosphate, 1 mM EDTA, 3 M GdmCl, 5 mM DTT, pH 7.5 overnight at 4 °C. Aggregation was initiated by 50-fold dilution of denatured GAPDH into buffer without GdmCl (100 mM potassium phosphate, 1 mM EDTA, 3 M GdmCl, 5 mM DTT, pH 7.5) under constant stirring at 20 °C. Protein aggregation was monitored with right angle light-scattering measurement at 650 nm using a Jasco FP 6500 spectrofluorometer in the presence or absence of 2.5 μ M trigger factor.

Prevention of LHCP Aggregation—12 μ M LHCP 100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, pH 8.0 was diluted 100-fold into 100 mM potassium phosphate, 1 mM EDTA, 5 mM DTT, pH 7.5 under constant stirring at 20 °C. Protein aggregation was monitored with right angle light-scattering measurement at 650 nm using a Jasco FP 6500 spectrofluorometer in the presence or absence of 0.13 μ M cpSRP43 or 0.13 μ M trigger factor.

LHCP Disaggregation Assay—LHCP and LHCP-L201K (2 μ M) was at 20 °C for 1 h in 100 mM potassium phosphate, 1 mM EDTA, 5 mM DTT, pH 7.5, and diluted to 0.67 μ M in the absence or presence of 0.67 μ M cpSRP43. LHCP disaggregation was determined by monitoring right angle light-scattering at 650 nm using a Jasco FP 6500 spectrofluorometer.

Native PAGE—Clear native PAGE was performed according to Laemmli (29) with the modification that SDS was omitted.

Pulldown Assay—400 μ M denatured LHCP in buffer A was diluted 40-fold into buffer without urea containing 10 μ M non-tagged cpSRP43 at 20 °C. After incubation for 15 min at 20 °C, the sample was centrifuged (15 min, 12,000 \times g), and the supernatant was mixed with 20 μ l of nickel-nitrilotriacetic acid agarose followed by 1 h of incubation at 4 °C. The beads were washed three times with 500 μ l of low salt buffer (150 mM NaCl) or high salt buffer (1 M NaCl), and bound proteins were eluted with buffer including 300 mM imidazole. Proteins were detected by SDS-PAGE on NuPAGE 4–12% polyacrylamide gels (Invitrogen) with subsequent CBB (Sigma) staining.

RESULTS

cpSRP43 Is a Chaperone for LHCPs—cpSRP43 (Fig. 1A) and cpSRP54 form a stable heterodimer (27) and participate in the formation of a soluble transit complex with LHCPs (11). Because only little is known about the interactions in the transit complex, we first asked which cpSRP component is required to keep LHCPs in solution. All components of cpSRP were tested

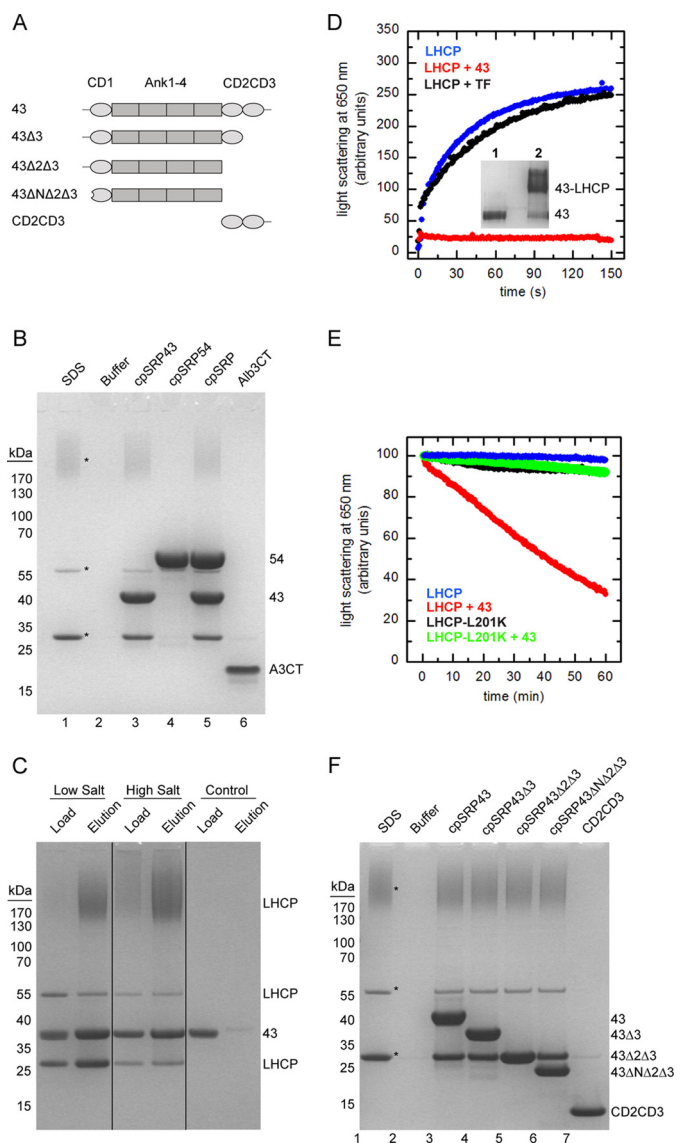


FIGURE 1. cpSRP43 is an LHCP-specific chaperone. *A*, schematic representation of cpSRP43 variants used in this study. The domain organization of cpSRP43 with chromodomains (CD1–3) (light gray ovals) and ankyrin repeats (Ank1–4) (dark gray rectangles) is shown. *B*, cpSRP43 alone is able to keep LHCP in solution. Denatured LHCP (8 M urea) was diluted 40-fold in the absence or presence of the proteins indicated above each lane or in the presence of 0.5% SDS and divided into supernatant and pellet by centrifugation followed by SDS-PAGE/CBB staining analysis. Only the supernatant fraction is depicted. Bands are labeled on the right side of the gel as follows: cpSRP54 with 54, cpSRP43 with 43, and the C-terminal domain of Alb3 with A3CT. The three characteristic bands for LHCP representing monomer, dimer, and higher oligomers are marked with an asterisk. *C*, the LHCP-cpSRP43 interaction is predominantly hydrophobic. LHCP and cpSRP43 form a stable, salt-resistant complex. His-tagged LHCP (marked with an asterisk) was refolded in the presence of non-tagged cpSRP43 (labeled 43). Complex formation was analyzed by pull-down experiments under low salt (150 mM NaCl) or high salt (1 M NaCl) conditions. No binding of cpSRP43 was observed in the absence of LHCP (Control). *D*, cpSRP43 but not trigger factor prevents the aggregation of LHCP. Aggregation of LHCP is indicated by the increase in right angle light scattering at 650 nm after 100-fold dilution of denatured LHCP (final concentration 0.12 μ M) and was monitored in the absence (blue line) or presence of 0.13 μ M cpSRP43 (red line) or 0.13 μ M trigger factor (black line). The inset shows the analysis of cpSRP43 (lane 1) and the cpSRP43-LHCP complex (lane 2) by clear native PAGE followed by CBB staining. Formation of the cpSRP43-LHCP complex is indicated by a band shift to lower mobility. *E*, solubilization of aggregated LHCP and LHCP-L201K mutant was monitored in the absence or presence of cpSRP43. The turbidity of aggregated LHCP or LHCP-L201K was set to 100%, and disaggregation was monitored by the decrease in right angle light scattering at 650 nm. Blue line and red line, LHCP

in a chaperone assay. LHCP expressed in *Escherichia coli* forms inclusion bodies that were isolated and solubilized by urea and purified under denaturing conditions by Ni²⁺ affinity purification. The purity of LHCP (and variants) was analyzed by SDS-PAGE/CBB staining (supplemental Fig. S1A). In the presence of SDS, LHCP migrates as a monomer, dimer, and higher oligomer (Fig. 1B, lane 1). cpSRP54, cpSRP43, cpFtsY (see supplemental Fig. S1B), and even the C-terminal tail of Alb3, which we have recently shown to recruit cpSRP43 to the thylakoid membrane (23), have been tested for their ability to keep LHCPs in solution after dilution of denatured LHCP into buffer without detergent or urea. Fig. 1B shows the analysis of the supernatant fractions by SDS-PAGE/CBB staining. In the presence of cpFtsY, cpSRP54, or Alb3CT, LHCP aggregated and could not be detected in the supernatant (Fig. 1B, lanes 4 and 6). Only in the presence of cpSRP43 or cpSRP (cpSRP54/cpSRP43), LHCP was kept in solution (Fig. 1B, lanes 3 and 5). cpSRP54 does not seem to contribute to the chaperone function of cpSRP43 as it does not increase the fraction of soluble LHCP in our assay (Fig. 1B, lane 5).

The cpSRP43-LHCP Interaction Is Predominantly Hydrophobic—We have shown that LHCPs are kept in solution by cpSRP43 alone. We now wanted to analyze whether both proteins can also be copurified in a pull-down experiment. Indeed, untagged cpSRP43 copurifies with His-tagged LHCP (Fig. 1C, left). cpSRP43 did not bind unspecifically to the beads (Fig. 1C, right). The structure of cpSRP43 revealed two strikingly different surfaces. While one exposes two hydrophobic grooves, the other one is highly negatively charged (21). To investigate whether the latter one is involved in LHCP binding, we tested whether the interaction is salt-sensitive. The complex can be copurified even at salt concentrations of 1 M sodium chloride, indicating that the interaction is predominantly hydrophobic (Fig. 1C, middle). The transmembrane regions of LHCP seem to contribute significantly to the interaction with cpSRP43.

Trigger Factor Is Not Able to Prevent LHCP Aggregation—Molecular chaperones bind to hydrophobic regions in their substrate proteins and form stoichiometric complexes; thereby protein aggregation is prevented. Protein aggregation can be directly monitored using light scattering at 650 nm (30). Upon 50-fold dilution in aqueous buffer, we observed rapid aggregation of LHCP (Fig. 1D, blue line). However, the presence of stoichiometric amounts of cpSRP43 efficiently prevented aggregation of LHCP as light scattering at 650 nm did not increase (Fig. 1D, red line). The formation of a soluble complex is supported by native PAGE (Fig. 1D, inset). Next we asked whether LHCPs could be kept in a soluble state by other classi-

in the absence (blue line) or presence (red line) of cpSRP43. Black line and green line, LHCP-L201K in the absence (black line) or presence of cpSRP43 (green line). Only in the presence of cpSRP43 does disaggregation occur. *F*, the cpSRP43 ankyrin repeats carry the chaperone function. Denatured LHCP (8 M urea) was diluted 40-fold in the absence or presence of the different cpSRP43 variants (indicated above each lane) or in the presence of 0.5% SDS. The samples were divided into supernatant and pellet by centrifugation followed by SDS-PAGE/CBB staining. Only the supernatant fraction is depicted. Bands are labeled on the right using the abbreviations given in panel A. The three characteristic bands for LHCP representing monomer, dimer, and higher oligomers are marked with an asterisk.

cpSRP43 Is a Novel Chaperone

cal chaperones with a broad substrate repertoire or whether cpSRP43 is specifically required. Because trigger factor (TF) is a major chaperone in bacteria that interacts with hydrophobic regions (31) and a homolog is present in chloroplasts (32), we tested TF from *E. coli* in our light-scattering assay (*black line*). The activity of recombinant TF was confirmed using GAPDH as substrate (33). Stoichiometric amounts of TF efficiently suppressed aggregation of GAPDH (*supplemental Fig. S2*). TF, however, did not protect LHCP from aggregation (Fig. 1D, *black line*), indicating that it cannot act as a chaperone for LHCP. The specific requirement for cpSRP43 fits previous observations that neither Hsp70 nor Hsp60 (17, 19) is able to act as chaperone for LHCP. Taken together, our data show that cpSRP43 is an LHCP-specific chaperone that cannot be replaced by other chaperones in the chloroplast stroma.

cpSRP43 Can Dissolve LHCP Aggregates—Chaperones of the Hsp104/ClpB family were shown to resolubilize aggregated proteins under ATP consumption (34, 35). To test whether cpSRP43 can also interact with aggregated LHCP and solubilize the aggregates, we performed a disaggregation assay monitoring the decrease in light scattering at 650 nm. Indeed, cpSRP43 efficiently solubilized aggregated LHCP (Fig. 1E, *red line*). Interestingly, when LHCP aggregation was allowed to proceed for more than 10 h, cpSRP43 could not solubilize aggregated LHCP, indicating that the aggregates mature over time and reach a state from which disaggregation is no longer possible. We have previously shown that an L201K mutation in the DPLG motif of L18 (Fig. 2A) abolishes the interaction between L18 and cpSRP43 and thereby prohibits LHCP insertion in an *in vitro* thylakoid import assay (21). We therefore asked whether the interaction with the DPLG motif is also necessary for the disaggregation of LHCP. cpSRP43 could not solubilize aggregates formed by the LHCP-L201K mutant (Fig. 1E, *green line*), which clearly shows that disaggregation by cpSRP43 is a highly specific process that strictly depends on the presence of an intact DPLG motif in LHCPs.

The cpSRP43 Ankyrin Repeats Are Sufficient for the Chaperone Function—cpSRP43 has a modular structure with three chromodomains and four ankyrin repeats, and cpSRP43 alone seems sufficient to act as an LHCP chaperone. Because the DPLG motif that is crucial for the recognition by cpSRP43 binds to the ankyrin repeats (21), we asked whether the chromodomains are required for the chaperone function. We used truncation variants of cpSRP43 (Fig. 1A), which were previously used to characterize the interaction with Alb3 (26) and lack CD3 or both CD2 and CD3, and performed the same assay as described above. Deletion variants with one or both C-terminal chromodomains missing (cpSRP43 Δ 3, cpSRP43 Δ 2 Δ 3) were still able to bind similar amounts of LHCP as the full-length protein (Fig. 1F, lanes 4, 5, and 6). A CD2CD3 construct was not able to bind and keep LHCP in solution (Fig. 1F, lane 7). CD2 and CD3 do therefore not contribute to the chaperone function. In the cpSRP43 structure, CD1 is connected to the first ankyrin repeat by a continuous α -helix, and deletion of CD1 resulted unstable (insoluble) protein. Therefore, an N-terminal variant lacking the N-terminal extension of cpSRP43 and the first tyrosine participating in the aromatic cage of CD1

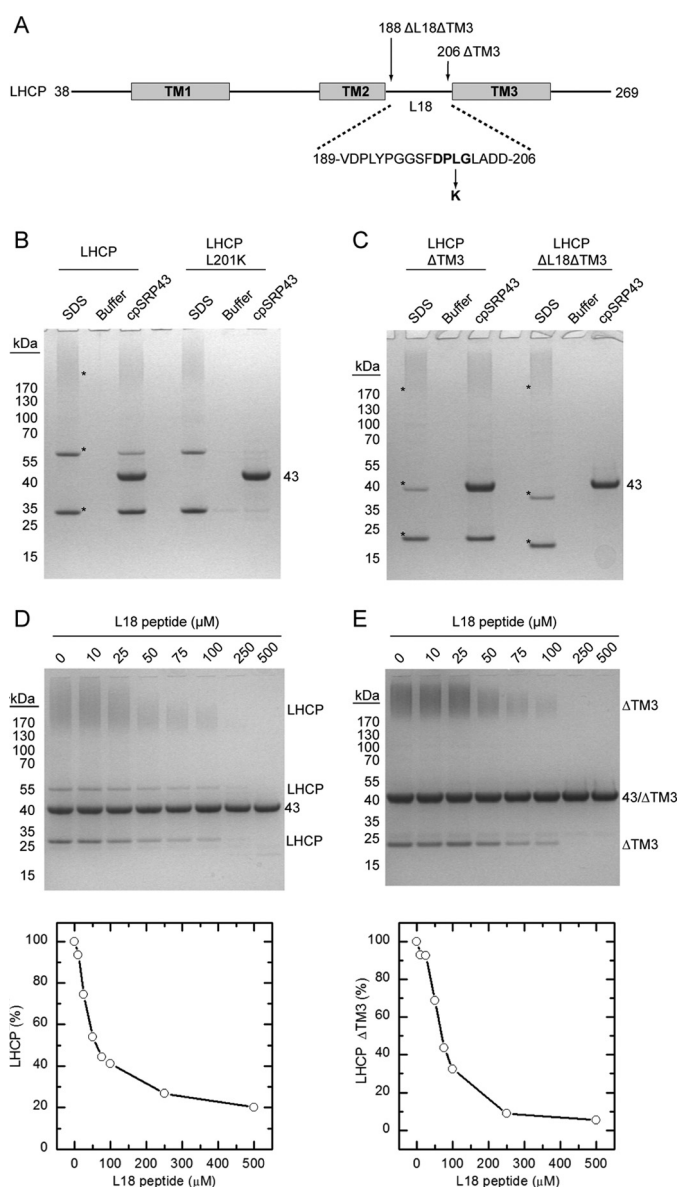


FIGURE 2. The LHCP L18 region is essential for interaction with cpSRP43. A, schematic representation of the LHCP topology with three transmembrane helices (TM1–3). The sequence of the L18 peptide is given from the major LHCP (Lhcb1 *P. sativum*), and the DPLG motif is highlighted in bold. The residue numbers for the truncation constructs used are indicated by an arrow. B and C, the L18 region is strictly required for cpSRP43-LHCP complex formation. Denatured LHCP and LHCP-L201K (B) and LHCP Δ TM3 or LHCP Δ L18 Δ TM3 (C) were diluted 40-fold into buffer in the presence or absence of 0.5% SDS or cpSRP43. The samples are divided into supernatant and pellet by centrifugation followed by SDS-PAGE/CBB staining. Only the supernatant fraction is depicted. Bands corresponding to LHCP variants are labeled with an asterisk, and the band corresponding to cpSRP43 is labeled on the right with 43. Note: The dimer of LHCP Δ TM3 comigrates with cpSRP43. D and E, L18 competes with LHCP for binding to cpSRP43. Formation of the cpSRP43-LHCP complex (D) and the cpSRP43-LHCP Δ TM3 (E) complex was analyzed in the presence of increasing amounts of L18 peptide (0–500 μ M). The samples were prepared as in panel B. Bands are labeled as follows: cpSRP43 with 43, LHCP with LHCP, LHCP Δ TM3 with Δ TM3. Note: In E, the dimer of LHCP Δ TM3 comigrates with cpSRP43. For quantification of bound LHCP, the lowest band representing LHCP monomer was analyzed by ImageJ (54), and the band intensity was plotted against L18 concentration.

(cpSRP43 Δ N Δ 2 Δ 3) was used. It binds similar amounts of LHCP as full-length cpSRP43 (Fig. 1F, lane 6). Taken together, the chromodomains are not required, whereas the

four ankyrin repeats (Ank1–4) harbor the chaperone function.

LHCP-TM3 Does Not Contribute to cpSRP43 Binding—Having analyzed the contribution of cpSRP43 domains to the chaperone function, we next set out to study which regions of LHCP contribute to the interaction (Fig. 2A). Mutated and truncated LHCP variants were tested for their ability to bind cpSRP43. The disaggregation assay (Fig. 1E) indicated that the DPLG motif is essential for the specific recognition of LHCPs by its chaperone cpSRP43. When the LHCP-L201K mutant was used in our chaperone assay, the interaction with cpSRP43 is abolished (Fig. 2B). We next investigated the effect of C-terminal truncations by removing TM3 (LHCP Δ TM3) or L18TM3 (LHCP Δ L18 Δ TM3) from LHCP (Fig. 2C). Deletion of TM3 still allowed complex formation with cpSRP43; however, deletion of L18TM3 abolished the interaction completely. Taken together, these data show that in the absence of an intact DPLG motif, the hydrophobic interactions (Fig. 1C) are not sufficient for binding to cpSRP43.

We previously determined a K_d of around 1 μ M for the L18-cpSRP43 complex using isothermal titration calorimetry (21). However, isothermal titration calorimetry cannot be applied to determine the K_d of the LHCP-cpSRP43 complex as LHCP is not soluble in the absence of cpSRP43. Therefore, we used an indirect method. LHCP was refolded in the presence of constant amounts of cpSRP43 but now in the presence of increasing amounts of L18 (Fig. 2D). L18 competed with LHCP for binding to cpSRP43, but very high concentrations were needed to completely displace LHCP from the LHCP-cpSRP43 complex. At an L18 concentration of around 60 μ M, 50% of LHCP is displaced, indicating that cpSRP43 binds LHCP with higher affinity than L18. With the K_D of 1 μ M for the cpSRP43-L18 complex in the absence of LHCP and a K_D of 60 μ M in the presence of LHCP, a K_i for LHCP of around 170 nM can be calculated, assuming competitive binding of L18 and LHCP to cpSRP43 (36). This supports the contribution of the hydrophobic TMs to the interaction. In the refolding assay, however, removal of TM3 did not apparently influence cpSRP43-LHCP complex formation (Fig. 2C). To analyze these findings in more detail, we performed the competition assay with LHCP Δ TM3 (Fig. 2E). To our surprise, removal of TM3 even seems to slightly enhance the interaction with cpSRP43—as higher amounts of L18 are required to displace LHCP Δ TM3 when compared with LHCP. However, the observed difference is within the error of the experiment and may not be significant.

DISCUSSION

Previous experiments implicated that both cpSRP43 and cpSRP54 are required for the formation of a soluble transit complex with LHCP. Complex formation could not be observed when one of the subunits of cpSRP was missing (24, 25). In addition, in cpSRP43 knock-out plants, the level of LHCP in the thylakoids was significantly reduced (20). It is, however, unclear how the remaining LHCP is targeted and inserted in the absence of cpSRP43. We now show that cpSRP43 alone is sufficient to form a soluble complex with LHCP and to prevent aggregation. This supports a recent idea that cpSRP43 alone fulfills all requirements for targeting of

LHCP to the thylakoid membrane (23). The ankyrin repeats alone are sufficient for the chaperone function of cpSRP43, whereas the chromodomains CD2 and CD3 allow a direct interaction with the C-terminal domain of Alb3 at the thylakoid membrane (23).

The thylakoids in chloroplasts host the complete photosynthetic machinery and comprise an additional compartment separate from the inner envelope. Therefore, lateral release of thylakoid membrane proteins upon import, as shown in mitochondria for members of the solute carrier family or single spanning membrane proteins of the inner membrane (37, 38), is not possible, and targeting systems from the inner envelope to the thylakoids are required. In contrast to mitochondria, all classical bacterial targeting systems are present in chloroplasts (4). For most nuclear encoded membrane proteins destined for the thylakoids, neither the targeting pathway nor the components have been identified. Many of them are believed to insert spontaneously, but their transit through the aqueous environment of the stroma is still enigmatic. LHCPs are a remarkable exception as all components required for targeting and insertion have been reported. They are the only integral membrane proteins for which a specific chaperone has been identified so far.

The specific recognition of LHCPs by cpSRP43 relies on the presence of the DPLG motif (21). It is conserved in all LHCPs (21) and also in fucoxanthin chlorophyll *a/c*-binding proteins of diatoms (39) that are also targeted by cpSRP. In LHCPs, the DPLG motif is part of a conserved interaction site for carotenoids (40), which are crucial for folding (41). The LHCP-L201K mutation, exemplarily shown for the major LHCP from *Pisum sativum* (Lhcb1), is sufficient to abolish the LHCP-cpSRP43 interaction (21) and thereby prohibits the chaperone function of cpSRP43. We noted previously that a conserved tyrosine in cpSRP43 (Tyr-204) in the crystal structure of the cpSRP43-L18 complex superimposes with the carotenoid headgroup in LHC (21) and concluded that the interactions in the transit complex might be a prerequisite for subsequent cofactor attachment upon membrane insertion. The current study suggests that the DPLG motif already plays a crucial role at an earlier stage as it ensures the formation of a soluble complex of LHCP with cpSRP43. We have shown that the hydrophobic TMs of LHCP contribute significantly to the interaction. The structure of cpSRP43 revealed two strikingly different surfaces. Although one is highly negatively charged, the other one exposes two hydrophobic grooves. Although one of them binds L18 (21), it is not clear at present how the three hydrophobic transmembrane helices are accommodated. Recognition of hydrophobic regions in substrate proteins is the common principle of molecular chaperones (1). However, in the absence of a functional DPLG motif in LHCPs, the hydrophobic regions are not sufficient for complex formation. In addition, TM3 seems not to interact with cpSRP43, which agrees with previous experiments (27, 42), which suggested that TM3 interacts preferentially with cpSRP54. However, cpSRP54 did not show chaperone activity for LHCP and did not enhance the efficiency to form a soluble complex together with cpSRP43 in our experiments. Thus cpSRP54 seems to play a regulatory role in the transit complex and also allows the use of the downstream components of the cpSRP system for post-translational targeting of LHCPs. The

cpSRP43 Is a Novel Chaperone

highly symmetric SRP54-FtsY GTPase complex of the cytosolic SRP system (43, 44) is conserved in chloroplasts (3, 45). cpSRP54 might thereby enable the coordinated release of LHCP from the transit complex at the thylakoid membrane. We could imagine that cpSRP43 as part of cpSRP binds to unfolded LHCPs as they emerge through the TIC translocation pore to assure that these proteins do not aggregate in the stroma.

Interestingly, cpSRP43 is also able to dissolve aggregated LHCP. All chaperones known so far that can dissolve aggregates require ATP hydrolysis. They first remove polypeptides from the aggregates, which results in the release of unfolded polypeptides that can subsequently fold into their native structure alone or with the assistance of other chaperones (46). While ClpB actively dissolves aggregates, cpSRP43 might rather shift the equilibrium between aggregated and soluble LHCP by complex formation. The physiological relevance of the disaggregation function of cpSRP43 is not clear at present because aggregated LHCP similar to inclusion bodies found in the cytoplasm of *E. coli* have not been reported in the stroma. However, the ability to dissolve aggregates might be advantageous if LHCP import exceeds the capacity of cpSRP43 to form the transit complex.

Chaperones are mostly considered as proteins that assist other proteins in folding, have a broad substrate specificity, and consume ATP. However, more and more examples have been reported that describe specific chaperones that act in the assembly/disassembly of macromolecular complexes and that do not have an ATPase activity. The term molecular chaperone was in fact invented to describe the function of proteins that assist the assembly of folded subunits into oligomeric structures, with nucleosome assembly as the first example (47, 48). Other examples include the pili assembly of Gram-negative bacteria via the chaperone-usher pathway (49, 50), the delivery of flagellar building blocks to a type III secretion system in flagella biogenesis (51), and the stromal form I Rubisco (Rbc), which is the most abundant soluble protein on earth (52). Rbc is an oligomer built up from eight large and eight small subunits. To assemble into a functional enzyme, the large subunit requires a specific chaperone, RbcX2. Like cpSRP43, RbcX2 is a highly specific chaperone that recognizes a defined motif in RbcL (53). Taken together, specific chaperones might be used either when oligomeric assembly is tightly coupled to subunit folding or when the assembly of multisubunit complexes from individual components must take place in a highly orchestrated manner. In this respect, LHCPs present a unique problem as they are integral membrane proteins, and the assembly of functional LHC requires the attachment of cofactors and the formation of trimers upon membrane insertion. cpSRP43 prevents aggregation in the stroma and allows delivery to the membrane insertase at the target membrane. The modular architecture of cpSRP43 is designed for its dual function as targeting factor and as a highly specific chaperone for LHCPs. Whether cpSRP43 can act as a chaperone for other chloroplast proteins remains to be seen.

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