Interaction Studies between the Chloroplast Signal Recognition Particle Subunit cpSRP43 and the Full-length Translocase Alb3 Reveal a Membrane-embedded Binding Region in Alb3 Protein* [5]

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Posttranslational targeting of the light-harvesting chlorophyll a,b-binding proteins depends on the function of the chloroplast signal recognition particle, its receptor cpFtsY, and the translocase Alb3. The thylakoid membrane protein Alb3 of Arabidopsis chloroplasts belongs to the evolutionarily conserved YidC/Oxa1/Alb3 protein family; the members of this family facilitate the insertion, folding, and assembly of membrane proteins in bacteria, mitochondria, and chloroplasts. Here, we analyzed the interaction sites of full-length Alb3 with the cpSRP pathway component cpSRP43 by using in vitro and in vivo studies. Bimolecular fluorescence complementation and Alb3 proteoliposome studies showed that the interaction of cpSRP43 is dependent on a binding domain in the C terminus of Alb3 as well as an additional membrane-embedded binding site in the fifth transmembrane domain (TMD5) of Alb3. The C-terminal binding domain was mapped to residues 374–388, and the binding domain within TMD5 was mapped to residues 314–318 located close to the luminal end of TMD5. A direct binding between cpSRP43 and these binding motifs was shown by pepspot analysis. Further studies using blue-native gel electrophoresis revealed that full-length Alb3 is able to form dimers. This finding and the identification of a membrane-embedded cpSRP43 binding site in Alb3 support a model in which cpSRP43 inserts into a dimeric Alb3 translocation pore during cpSRP-dependent delivery of light-harvesting chlorophyll a,b-binding proteins.

The YidC/Oxa1/Alb3 protein family includes universally conserved proteins that can be found in the Escherichia coli cytoplasmic membrane (YidC), the inner membrane of mitochondria (Oxa1), or in the thylakoid membrane of chloroplasts (Alb3) (1–3). These family members mediate the insertion, folding, and assembly of membrane proteins which mostly belong to the energy-transducing complexes of the photosynthetic or respiratory electron transport chain. The YidC/Oxa1/Alb3 family members share a hydrophobic core region of five transmembrane domains. In contrast to Alb3 and Oxa1, E. coli YidC contains an additional transmembrane segment and a large periplasmic domain at its N terminus. In bacteria the majority of nascent inner membrane proteins are bound by the signal recognition particle (SRP)3 and inserted cotranslationally into the plasma membrane via the SecY protein-conducting channel in the plasma membrane (4). YidC has been identified as an accessory component of the bacterial Sec translocase (5, 6) where it is involved in the lateral release, folding, or assembly of membrane proteins. A Sec-independent insertase function of YidC has also been demonstrated (3, 7). Likewise, mitochondrial Oxa1 functions independently of the SRP/Sec machinery because mitochondria do not contain any SRP or Sec components (8).

In chloroplasts, the major thylakoid membrane proteins belonging to the light-harvesting chlorophyll a,b-binding protein (LHCP) family are posttranslationally inserted into the membrane by a cpSRP/Alb3-dependent, Sec-independent mechanism (9). After the import of the nuclear encoded LHCPs across the chloroplast envelope membranes into the stroma they are bound by cpSRP to form the soluble transit complex. CpSRP consists of a conserved 54-kDa GTPase (cpSRP54) and a 43-kDa subunit (cpSRP43) that is unique to chloroplasts (10–13). With the participation of the partly membrane-bound GTPase, cpFtsY, the transit complex is targeted to the translocase Alb3 (14), which is required for LHCP insertion (15). CpSRP43 is composed almost completely of protein-protein interaction domains, containing three chromodomains (CD1–CD3) and four ankyrin repeats. While CD2 is required for complex formation with cpSRP54 (16, 17), the ankyrin repeats bind the conserved internal L18 motif in the LHCPs (18–21). In addition to binding its cargo protein, cpSRP43 acts as a specific chaperone and prevents aggregation of the hydrophobic LHCPs (22, 23). A direct interaction between cpSRP43 and Alb3 was demonstrated recently, and cpSRP43 was suggested to play a central role in the docking of the cpSRP-LHCP-cpFtsY complex.
complex to the translocase Alb3 (24–26). Data to define the Alb3 binding motif in cpSRP43 are controversial because the second chromodomain (25) and the ankyrin repeat region (26) were described to provide the primary binding region for Alb3. Studies to map the cpSRP43 binding site in Alb3 demonstrated that the soluble, stroma-exposed C terminus of Alb3 is sufficient for the interaction with cpSRP43 in vitro. The Alb3 C-terminal domain is intrinsically disordered and folds into an α-helix containing a conformation upon binding of cpSRP43. Two conserved, positively charged motifs within the C terminus that are essential for this interaction were identified (25). However, bimolecular fluorescence complementation (BiFC) experiments investigating the interaction between cpSRP43-nYFP and Alb4/Alb3-cYFP fusion constructs using Arabidopsis protoplasts indicated that a larger fragment comprising TMD5 and the soluble C terminus of Alb3 is required for efficient complex formation in vivo (24). Alb4 is a thylakoid membrane paralog of Alb3 but is not required for LHCP biogenesis (27) and does not interact with cpSRP43 (24, 25).

The aim of this study was to analyze the role of the stroma-exposed C terminus and TMD5 of Alb3 for cpSRP43 binding using full-length proteins in a lipid environment. Therefore, we extended the BiFC experiments and analyzed the interaction between recombinant cpSRP43 and proteoliposomes containing various Alb3 constructs. In addition, we performed pepscan analysis and in vitro pulldown assays to map the cpSRP43 binding site in Alb3. Our data indicate that both TMD5 and the C terminus contain direct binding sites for cpSRP43. The identification of a membrane-embedded binding site that is located close to the luminal end of TMD5 leads to a model whereby cpSRP43 is recruited to Alb3 via its C terminus and might partially immerse into an Alb3 pore during the LHCP insertion.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of Proteins—The recombinant proteins His-cpSRP43, His-cpSRP54, and the His-cpSRP complex as well as the Alb3 in vitro translation products were produced as described previously (24).

The coding sequence for the mature form of Arabidopsis thaliana Alb3 (residues 55–462) was synthesized in an optimized form for E. coli codon usage by GenScript according to the manufacturer’s suggestions. Using NdeI and XhoI restriction sites, the Alb3 coding sequence was cloned into the pET29b(+) plasmid (Novagen) resulting in a C-terminal His-tag. The pET29b(+)–Alb3-His plasmid was transferred into E. coli “Walker” C43 (DE3) (28). Cells were grown in LB medium at 37 °C until an A600 of 0.6–0.8 was reached, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for an additional 4 h at 37 °C. Cells were harvested by centrifugation, resuspended in 10 ml of resuspension buffer (50 mM Tris-HCl, pH 8.0, 20% (v/v) glycerol) per g fresh weight of the bacterial pellet for subsequent disruption by sonication. The suspension was cleared of debris by centrifugation (30 min, 10,000 × g, 4 °C). The membranes were collected from the supernatant (45 min, 150,000 × g, 4 °C) and resuspended in solubilization buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20% (v/v) glycerol, 20 mM imidazole) to a final protein concentration of 2–5 mg/ml. Dodecyl-β-D-maltoside (DDM) was added to a final concentration of 1% (w/v), and the membranes were solubilized by gentle rotation for 1 h at 4 °C. The solubilized suspension was diluted with solubilization buffer to a final detergent concentration of 0.25% (w/v). Unsoluble material was separated from solubilized proteins by centrifugation (45 min, 150,000 × g, 4 °C). The supernatant was incubated for 2 h with Ni-NTA agarose (Qiagen), followed by two subsequent washing steps (washing buffer 1: 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole, 0.015% (w/v) DDM; and washing buffer 2: same as washing buffer 1 but with 2% (v/v) glycerol), and the elution of Ni-NTA bound Alb3-His (elution buffer: 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.015% (w/v) DDM). Sequences encoding mutated Alb3-His proteins (Alb3-His (LVFKF314–318AVTKL); Alb3-His(Δ374–388) and Alb3-His(LVFKF314–318AVTKL, Δ374–388)) were generated via the QuikChange® XL Site-directed Mutagenesis method according to the manufacturer’s instructions (Stratagene) with the pET29b(+)–Alb3-His plasmid as the template and the overlap PCR technique. For subsequent cloning into pET29b(+) the restriction sites NdeI and XhoI were used. Mutated proteins were produced and purified as described above.

Construction of Split YFP Plasmids and Arabidopsis Proteoliposome Transfection—The constructs pSpyne-43 and pSpyce-Alb3 were described previously (24). The indicated pSpyce-Alb4/Alb3 fusion constructs were generated using the overlap PCR technique and cloned into the BamHI/Sall site of pUC-Spyce (29). The indicated mutagenesis constructs were generated using the QuikChange® XL Site-directed Mutagenesis kit (Stratagene) according to the manufacturer’s protocol and pSpyce-Alb3 as a template. All constructs were verified by sequencing. Protoplast transfection and immunodetection of YFP fusion proteins were performed according to (30).

Pepscan Analysis—A peptide library comprising amino acids 299–462 of Alb3 was ordered from JPT Peptide Technologies GmbH, Berlin. The peptide membrane (51 15-mer peptides, overlapping by 12 amino acids) was incubated in ethanol and washed with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5). Blocking of the membrane was performed in 3% (w/v) bovine serum albumin (BSA) dissolved in TBS buffer containing 0.3% (v/v) Tween 20 (TBST) for 3 h at room temperature. After washing with TBS containing 0.05% (v/v) Tween 20, the peptide membrane was incubated with 5 μg/ml His-cpSRP43 in 3% (w/v) BSA dissolved in TBST for 2 h at room temperature and overnight at 4 °C. After washing with TBST, bound His-cpSRP43 was detected by immunoblotting using PVDF membranes and an antibody against cpSRP43.

Pulldown Analysis—Recombinant His-cpSRP43 (5 μg) or His-cpSRP54 (5 μg) was incubated with 10 μl of in vitro translated Alb3(299–462) (1:30 dilution) or equal amounts of Alb3 deletion constructs in 100 μl of 50 mM HEPES-NaOH, pH 8.0, and 10 mM MgCl2 for 30 min at room temperature. Proteins were incubated with Ni-NTA resin for 30 min at room temperature and washed with 50 mM HEPES-NaOH, pH 8.0, 300 mM NaCl, 1 mM DTT, and 20 mM imidazole. Bound proteins were eluted with 250 mM imidazole in a volume of 30 μl, and aliquots of 15 μl were used for SDS-PAGE. Coelution of Alb3 in vitro
Binding of cpSRP43 to the Translocase Alb3

Identification of a Membrane-embedded cpSRP43 Interaction Site in Alb3 by BiFC Using Arabidopsis Protoplasts—In a previous study, we observed that the stromal C terminus of Alb3 is required but not sufficient to mediate an interaction between Alb3-cYFP and cpSRP43-nYFP in vivo in protoplasts. Using different Alb4/Alb3-cYFP fusion constructs, our data indicated that, in addition to the Alb3 C terminus (residues 343–462), the presence of a region comprising TMD5 of Alb3 is necessary for cpSRP43-nYFP binding (residues 314–342) (24). The predicted TMD5 of Alb3 and the location of residues 314–342 are presented in Fig. 1. An alignment of residues 314–342 and the corresponding region of Alb4 shows a very high sequence identity with only two nonconservative amino acid exchanges close to the luminal side (positions 314 and 316 in Alb3) (Fig. 1A). Fig. 1B shows that the leucine at position 314 (Leu-314) or phenylalanine at position 316 (Phe-316) is invariant, and Phe-316 (colored in light blue) is highly conserved among the Alb3 proteins, but both are absent in Alb4. Invariant and highly conserved residues present in both Alb3 and Alb4 are boxed in dark and light gray, respectively. These residues are not specifically conserved in either Alb3 or Alb4. Numbering of the residues corresponds to the A. thaliana Alb3 sequence.

FIGURE 1. Prediction and alignment of TMD5 of Alb3 and Alb4 from A. thaliana. A, the regions of TMD5 from Alb3 and Alb4 were predicted by ARAMEMNON. Sequence alignment was performed using ClustalW2. The green region represents the consensus prediction of the different programs. The maximal predicted region of the transmembrane domains is indicated by light green boxes. The region of Alb3 required for cpSRP43 binding in vivo (residues 314–462) (24) is shown by a black line. Nonconservative amino acid exchanges in TMD5 within this binding region are marked in red. The positions of the amino acids of Alb3 and Alb4 are given by numbers. Symbols display the degree of conservation: identical residues (star), conserved substitution (colon), semiconserved substitution (dot). B, TMD5 of Alb3 and Alb4 from various organisms are compared. Amino acid Leu-314 (colored in blue) is invariant, and Phe-316 (colored in light blue) is highly conserved among the Alb3 proteins, but both are absent in Alb4. Invariant and highly conserved residues present in both Alb3 and Alb4 are boxed in dark and light gray, respectively. These residues are not specifically conserved in either Alb3 or Alb4. Numbering of the residues corresponds to the A. thaliana Alb3 sequence.

RESULTS

Identification of a Membrane-embedded cpSRP43 Interaction Site in Alb3 by BiFC Using Arabidopsis Protoplasts—In a previous study, we observed that the stromal C terminus of Alb3 is required but not sufficient to mediate an interaction between Alb3-cYFP and cpSRP43-nYFP in vivo in protoplasts. Using different Alb4/Alb3-cYFP fusion constructs, our data indicated that, in addition to the Alb3 C terminus (residues 343–462), the presence of a region comprising TMD5 of Alb3 is necessary for cpSRP43-nYFP binding (residues 314–342) (24). The predicted TMD5 of Alb3 and the location of residues 314–342 are presented in Fig. 1. An alignment of residues 314–342 and the corresponding region of Alb4 shows a very high sequence identity with only two nonconservative amino acid exchanges close to the luminal side (positions 314 and 316 in Alb3) (Fig. 1A). Fig. 1B shows that the leucine at position 314 (Leu-314) or phenylalanine at position 316 (Phe-316) is invariant, and Phe-316 (colored in light blue) is highly conserved among the Alb3 proteins, but both are absent in Alb4. Invariant and highly conserved residues present in both Alb3 and Alb4 are boxed in dark and light gray, respectively. These residues are not specifically conserved in either Alb3 or Alb4. Numbering of the residues corresponds to the A. thaliana Alb3 sequence.

Binding Studies with Alb3-His Proteoliposomes—The binding of His-cpSRP43, His-cpSRP54, or the His-cpSRP complex to wild-type or mutant Alb3-His proteoliposomes was analyzed by sucrose density centrifugation. Samples of 5 μg of His-cpSRP43 or His-cpSRP54 or 10 μg of the preformed His-cpSRP complex were incubated with 500 μl of Alb3-His proteoliposomes for 30 min with gentle rotation at room temperature and then loaded onto the top of a discontinuous sucrose density gradient (2 ml of 40% (w/v) sucrose, 1.5 ml of 20% (w/v) sucrose, 1.5 ml of 15% (w/v) sucrose, 3 ml of 10% (w/v) sucrose, 3 ml of 5% (w/v) sucrose; each solved in liposome buffer). The proteoliposomes migrated into the gradients and formed a distinct band at a mean sucrose density of 30% (w/v) as determined using a refractometer. The cofractionation of His-cpSRP43, His-cpSRP54, or the His-cpSRP complex with wild-type or mutant Alb3-His proteoliposomes was determined by SDS-PAGE and Western blot analysis.
Binding of cpSRP43 to the Translocase Alb3

A YFP chlorophyll overlay

cpSRP43-nYFP Alb4(1–300)-Alb3(314–462)-cYFP

cpSRP43-nYFP Alb4(1–302)-Alb3(319–462)-cYFP

B Alb3-cYFP

Pellet Sup > α-HA

Alb4(1–300)-Alb3(314–462)-cYFP
Alb4(1–302)-Alb3(319–462)-cYFP
control

FIGURE 2. Analysis of the interaction between cpSRP43 and Alb4/Alb3 fusion constructs using BiFC. A, Arabidopsis mesophyll protoplasts were transiently transformed with two plasmids encoding cpSRP43-nYFP and the Alb4/Alb3-cYFP fusion constructs Alb4(1–300)Alb3(314–462) and Alb4(1–302)Alb3(319–462). All control reactions using a combination of the indicated constructs and control plasmids encoding a chloroplast transit sequence fusions to nYFP or cYFP were negative (data not shown). B, protoplasts transfected with Alb3-cYFP, Alb4(1–300)Alb3(314–462), and Alb4(1–302)Alb3(319–462) were lysed and centrifuged, and the supernatant (sup) was precipitated with TCA. The membrane pellet was washed with 0.2 m NaOH and centrifuged. Western blot analysis with the pellet and the supernatant of Alb3(314–462) was demonstrated as control. For the control, untransfected protoplasts were used.

TABLE 1 Analyses of the interaction between Alb3 mutant constructs and cpSRP43 using BiFC

| Mutations in TMD5 | Interaction with cpSRP43-nYFP |
|-------------------|-----------------------------|
| Alb3(D314–317)    | Weaker                      |
| Alb3(L314–315)    | Weaker                      |
| Alb3(L314A)       | Weaker                      |
| Alb3(F316T)       | Positive                    |
| Alb3(L313Q,L314A) | Weaker                      |

| Mutations in the C terminus | Interaction with cpSRP43-nYFP |
|-----------------------------|-----------------------------|
| Alb3(Δ374–388)              | Weaker                      |
| Alb3(Δ389–415)              | Positive                    |
| Alb3(1–442)                 | Positive                    |
| Alb3(1–415)                 | Positive                    |
| Alb3(1–366)                 | Negative                    |
| Alb3(1–359)                 | Negative                    |

The indicated mutant Alb3-cYFP constructs were cotransformed with the cpSRP43-nYFP construct into protoplasts from A. thaliana as described in Fig. 2A.

constructs, and those Alb3-cYFP mutant constructs, which resulted in no or weak fluorescence, were demonstrated by immunodetection of these constructs in the NaOH-washed membrane fraction prepared from transformed protoplasts (Fig. 2B and supplemental Fig. S2). Taken together, our data indicate that the Alb3 region 314LVFKF318 is important for cpSRP43 binding and point to a special role of Leu-314 in this binding process. However, the mutation of the 314LVFKF318 region in a construct in which the complete N-terminal part of Alb3 (up to residue 318) is replaced with Alb4 (see Alb4(1–302)Alb3(319–462) above) resulted in a more drastic reduction of cpSRP43 binding than the mutation of this region in full-length Alb3. This finding suggests that the N-terminal part of Alb3 including the two stromal loops might contribute to cpSRP43 binding. This could either occur directly by presenting additional binding sites for cpSRP43 or indirectly by binding other proteins that might stabilize cpSRP43 binding in vivo.

Mapping the cpSRP43 Interaction Site in the Soluble C Terminus of Alb3 by BiFC and in Vitro Pulldown Assays—While this study was in progress, it was shown that two conserved, positively charged motifs, named motif II (residues 369–378) and motif IV (residues 451–460), in the recombinant C terminus of Alb3 are essential for the interaction between Alb3 and cpSRP43 (25). Here, we describe the analysis of the cpSRP43 binding site in the C terminus of Alb3 using full-length Alb3 and the in vivo BiFC assay. As shown in Table 1, the constructs Alb3(1–442)-cYFP and Alb3(1–415)-cYFP lacking the C-terminal 20 and 47 amino acids, respectively, interacted with cpSRP43-nYFP in chloroplasts, whereby the strength of the YFP fluorescence was similar to samples expressing full-length Alb3-cYFP. However, further removal of the C terminus generating the constructs Alb3(1–366)-cYFP and Alb3(1–359)-cYFP led to a complete loss of cpSRP43-nYFP binding. These data indicated that residues 367–415 are important for efficient interaction with cpSRP43. Further experiments to narrow down the cpSRP43 binding motif showed that the deletion of residues 389–415 within Alb3-cYFP did not affect cpSRP43-nYFP binding, whereas coexpression of a Alb3-cYFP construct lacking residues 374–388 and cpSRP43-nYFP induced only a weak fluorescence signal of 30% compared with wild-type Alb3-cYFP (Table 1 and supplemental Table S1) (for the intention to use Alb3(Δ374–388) see also below). Expression of Alb3(Δ374–388)-cYFP in protoplasts was demonstrated as described above (supplemental Fig. S2).

Previously, we reported that His-cpSRP43 binds in vitro translated Alb3(299–462), whereas no interaction was observed using His-cpSRP54 (24). To confirm the importance of residues 374–388 within the stroma-exposed C terminus of Alb3 for cpSRP43 binding, the pulldown assays were extended using various mutation constructs of in vitro translated Alb3(299–462). Fig. 3 shows that Alb3(299–462/Δ389–415) copurified with His-cpSRP43 to an extent similar to Alb3(299–462), whereas no binding was detected using Alb3(299–462/Δ374–388). Deletion of the region 299–319 (Alb3(320–462))
Mapping the cpSRP43 Interaction Sites in Alb3 Using a Pepscanapproach—To demonstrate that the regions 314–318 and 374–388 of Alb3 provide direct binding sites for cpSRP43, we mapped the interaction using an immobilized peptide library comprising the Alb3 amino acids 299–462. The peptide library contained 51 15-mer peptides, overlapping by 12 residues. Bound protein was detected by immunoblotting using an antibody against cpSRP43. Bound spots were detected for peptides corresponding to spots 1–4 (residues 305–328) of Alb3. The LVFKFL motif is present in each of the four peptides and marked in red. C, schematic representation of Alb3 topology. The cpSRP43 binding sites in the stromal C terminus and the membrane-embedded region of Alb3 are indicated.

and the C-terminal 20 residues (Alb3(320–442)) from the translation product did not result in a detectable loss of coprecipitation with His-cpSRP43. In summary, our in vivo and in vitro results both suggest that the Alb3 residues 374–388 function as an important cpSRP43 binding site on the soluble C terminus. Furthermore, our data showed that the region 314–318 is not required for cpSRP43 binding in pulldown analysis, which supports previous findings that the soluble C terminus of Alb3 is sufficient to mediate interaction with cpSRP43 in vitro (25, 26).

Binding of cpSRP43 to the Translocase Alb3
led to a complete digestion, a protease-protected fragment of ~35 kDa was detected in samples containing Alb3 proteoliposomes. This fragment was only detectable using an antibody directed against the first stromal loop region (Fig. 6A, lane 8), but not using an antibody directed against the soluble C terminus of Alb3 (Fig. 6A, lane 4). This finding indicated that the ~35-kDa fragment lacks the soluble C terminus. Hence, the C terminus must have been directed outward from the liposome during trypsin digestion to enable accessibility for trypsin. Furthermore, the observed molecular mass of the protease-protected fragment corresponds to the calculated molecular mass of 33 kDa of an Alb3 construct without the soluble C terminus. The generation of a 30-kDa Alb3 degradation product was also described for protease-treated thylakoid membranes (26). If Alb3 is oriented in the inverted direction with its C terminus exposed to the interior, the generation of other fragments of ~20 kDa or 40 kDa detectable with both antibodies was expected. However, such degradation products were not observed. In conclusion, the results showed that most if not all Alb3-His was oriented with its C terminus exposed to the exterior and its N terminus located in the interior of the liposomes.

To verify that Alb3-His in the proteoliposome sample is completely integrated into the vesicles, the proteoliposomes were subjected to sucrose density centrifugation, whereby the gradient contained 5% (w/v) to 40% (w/v) sucrose (nonintegrated Alb3 should aggregate and sediment during ultracentrifugation). In Fig. 6C, it is shown that the proteoliposomes formed a distinct lipid band at ~30% (w/v) sucrose. Western blotting of the gradient fractions demonstrated an entire cofractionation of Alb3-His with this lipid band (Fig. 6C).

Binding Studies of Recombinant cpSRP Proteins with Alb3 Proteoliposomes—To analyze whether the Alb3 proteoliposomes can be used to map the Alb3-cpSRP43 binding interface, we initially tested the ability of the Alb3 proteoliposomes to bind cpSRP43. To this end, recombinant His-cpSRP43, His-cpSRP54, or a preformed His-cpSRP complex was incubated with Alb3 proteoliposomes followed by a sucrose density centrifugation. His-cpSRP43 as well as the His-cpSRP complex cofractionated with the Alb3 proteoliposomes, whereas His-cpSRP54 did not (Fig. 7A). Unbound cpSRP components were detected in the upper fractions of the gradients (data not shown). These data demonstrated the functionality of reconstituted Alb3 with respect to its ability specifically to bind the cpSRP subunit cpSRP43.

To confirm the above described cpSRP43 binding sites in Alb3, especially the one within the fifth transmembrane domain, Alb3-His constructs containing mutations in the binding regions were generated and reconstituted into proteoliposomes. In Alb3-His(LVFKF314–318AVTKL), the binding motif 314LVFKF318 in TMD5 was replaced by the correspond-
ing 298AVTKL302 region of Alb4 by changing three residues: L314A, F316T, and F318L. Furthermore, the Alb3-His(H9004374–388) mutant, lacking amino acids 374–388 of the binding site within the soluble C terminus, was generated. The third mutant, Alb3-His(LVFKF314–318AVTKL, H9004374–388) comprised both mutations. The mutant Alb3-His proteins were expressed and purified as well as reconstituted into proteoliposomes according to the same procedures used for the wild-type Alb3-His protein. Trypsin treatment of the proteoliposomes containing the mutated Alb3 proteins generated the same digestion pattern as observed for wild-type Alb3 proteoliposomes, and therefore mutated proteins were integrated in the same direction with their C termini exposed to the outsides of the liposomes (data not shown).

Binding of His-cpSRP43 to the Alb3-His constructs reconstituted into proteoliposomes was analyzed as described above. The results showed that His-cpSRP43 cofractionated with wild-type Alb3-His proteoliposomes (Fig. 7Bi), but no significant cofractionation of His-cpSRP43 was observed with Alb3-His(LVFKF314–318AVTKL), Alb3-His(H374–388) or Alb3-His(LVFKF314–318AVTKL, H374–388) proteoliposomes (Fig. 7Bi, ii–iv). In some experiments, we observed background signals of cpSRP43 also in those fractions containing the Alb3 proteoliposomes. However, quantification analyses using the Scion Image software revealed a clear correlation between Alb3-His and His-cpSRP43 signals only if both cpSRP43 binding sites in Alb3 were unaffected (data not shown). Notably, the mutation of the binding motif in TMD5 had a severe effect on cpSRP43 binding, whereas deletion of this binding motif in the BiFC experiments was less pronounced. Therefore, it seems likely that other proteins that are absent in the liposome system enhance cpSRP43 binding to the Alb3 C terminus in vivo. In summary, our data show a clear Alb3-cpSRP43 binding using recombinant purified full-length proteins in a lipid environment similar to the lipid composition of thylakoid membranes. Furthermore, both cpSRP43 binding sites in Alb3 were unaffected (data not shown). Notably, the mutation of the binding motif in TMD5 had a severe effect on cpSRP43 binding, whereas deletion of this binding motif in the BiFC experiments was less pronounced. Therefore, it seems likely that other proteins that are absent in the liposome system enhance cpSRP43 binding to the Alb3 C terminus in vivo. In summary, our data show a clear Alb3-cpSRP43 binding using recombinant purified full-length proteins in a lipid environment similar to the lipid composition of thylakoid membranes. Furthermore, both cpSRP43 binding sites in Alb3, one within TMD5 comprising amino acids 314–318 and one within the soluble C terminus comprising amino acids 374–388, were confirmed.

DISCUSSION

Recent studies demonstrated a direct interaction between cpSRP43 and the translocase Alb3, and a model whereby cpSRP43 links the cpSRP pathway components to the translo-
Binding of cpSRP43 to the Translocase Alb3

case was suggested (24–26). To elucidate the molecular mechanisms of the LHCP targeting and insertion process, a detailed analysis of the interaction between cpSRP43 and Alb3 is important. Here, we identified two regions in Alb3 that are involved in cpSRP43 binding. The region 374–388 is part of the stroma-exposed C terminus, whereas residues 314–318 are located in TMD5 close to the luminal side of the thylakoid membrane. In the course of these investigations, it has been reported that two conserved motifs within the stromal C terminus of Alb3 are essential for cpSRP43 binding: the AKRS motif at position 375–378 and the SKRS motif that is located at the extreme C terminus (residues 453–456) (25). Because the AKRS motif is present in the binding region 374–388 that was identified in this study, our data support the importance of this binding motif for cpSRP43 binding. However, our data do not point to a major role of the extreme C terminus of Alb3 in cpSRP43 binding. While deletion of the AKRS motif led to a significant reduction or complete loss of cpSRP43 binding in the BiFC or pulldown experiments, no influence of the removal of the extreme Alb3 C terminus on cpSRP43 binding was detected. Furthermore, the pepspot analysis did not indicate an interaction between cpSRP43 and peptides corresponding to the extreme C terminus of Alb3. Overall, our data indicate that the region containing the AKRS motif represents the major cpSRP43 binding site in the Alb3 C terminus. However, because we did not measure the Alb3-cpSRP43 interaction in a quantitative manner, we cannot rule out that other regions within the soluble C terminus might contribute to binding to some extent.

Previously, we reported that the efficient interaction between cpSRP43-nYFP and Alb3-cyFP in vivo requires the presence of both the stroma-exposed C terminus and the TMD5 of Alb3 (24). Here, we analyzed the function of TMD5 for cpSRP43 binding in more detail, and our data indicate that TMD5 is directly involved in cpSRP43 binding. Notably, the binding interface within TMD5 was mapped to the luminal side-facing end of the transmembrane domain. Because this binding site is concealed within the membrane, our data suggest that the initial recruitment of cpSRP43 is mediated by the stromal C terminus and that the membrane-embedded binding site becomes accessible during the LHCP insertion process. Consequently, it is conceivable to conclude that (i) binding of cpSRP43 to the C terminus triggers a pore-forming conformational change of Alb3 and that (ii) cpSRP43 inserts partially into the pore to contact the second binding site. We speculate that this mechanism might lead to a partial coinsertion of the cargo protein LHCP into the translocation channel. A model of this mechanism is presented in Fig. 8 and discussed below.

Several studies show the possibility that YidC or Oxa1 forms dimers or oligomers (33–35). Recently, it was suggested that dimers of YidC and Oxa1 form insertion pores, whereby dimerization is stabilized by a direct interaction of the YidC/Oxa1 C terminus with the translating ribosomes in the cotranslational targeting pathway (36). In this study, the ability of Alb3 to form dimers was demonstrated by BN-PAGE. It is conceivable that in the posttranslational cpSRP-dependent transport mechanism, cpSRP43 might fulfill a role analogous to the ribosome because it is involved in the delivery of LHCP to the translocon Alb3 and binds to its C terminus. Remarkably, dimeric YidC or Oxa1 seems to possess structures basically similar to the monomeric SecYEG translocase and might share the same principle of channel opening (36). Depending on the targeting pathway, current models propose that the association of either the ribosome (cotranslational pathway) or SecY (posttranslational pathway) to cytosolic domains of SecY induces conformational changes resulting in channel opening (37). In cotranslational insertion of membrane proteins, proteins are inserted into the channel during chain elongation and released into the membrane through a lateral gate in the translocon (38). In the SecA-dependent posttranslational translocation of secretory proteins, SecA functions as an ATP-dependent motor protein and pushes the preprotein through the translocation channel by moving up and down inside the cytoplasmic funnel of SecY (4, 39, 40). A membrane-buried SecA binding site in SecY, whose accessibility requires a substantial conformational change of the translocon, was described by van der Sluis et al. (41).

Currently, the molecular mechanism of posttranslational LHCP insertion into the thylakoid membrane and its underlying driving force are largely unknown. It is known that GTP hydrolysis by the GTPases cpSRP54 and cpFtsY is required for LHCP integration in vitro and that ATP stimulates this process if it is present in combination with GTP (42, 43). However, in an analogy to the bacterial SRP system, it is unlikely that GTP hydrolysis drives the actual insertion process but rather regulates the interaction of the GTPases with each other and with the translocase. Accordingly, recent data indicate that GTP hydrolysis triggers the release of cpSRP and cpFtsY from Alb3 (24). The role of ATP in LHCP insertion remains puzzling because no ATP-binding protein involved in this process has yet been identified. Therefore, the driving force for LHCP insertion remains largely unknown. However, our data support the importance of this binding motif for cpSRP43 binding. The region 374–388 is part of the stroma-exposed C terminus as well as in TMD5. Therefore, cpSRP43 is postulated to immerse into the Alb3 pore. A coinsertion of the LHCP can be hypothesized because a binding region for LHCP has been localized to a region involving TMD5 (24). The precise functions of cpSRP54 and cpFtsY in the docking process remain to be clarified.

binding of cpSRP43 to the Translocase Alb3
binding might be similar to the one that drives the SRP/Sec-independent interaction of proteins by the YidC pathway in bacteria, which is independent of nucleotide triphosphate hydrolysis. Regarding this pathway, it has been proposed that the binding of the substrate to YidC drives the insertion by stabilizing a membrane-inserted configuration and the subsequent release of the substrate into the lipid bilayer (3). In contrast to the membrane protein insertion by the YidC pathway, LHCP insertion by Alb3 is cpSRP-dependent. Our data indicate that cpSRP43 is able to insert into a pore formed by Alb3. Because the cpSRP43 binding motif in TMD5 of Alb3 is hydrophobic, this finding might explain our previous observation of a salt-resistant interaction between Alb3 and cpSRP43 (24). Similar to the SecA-dependent translocation of secretory proteins, we speculate that the movement of cpSRP43 into the pore might support the insertion of hydrophobic segments of LHCP. In analogy to the model of the YidC pathway, the binding of LHCP segments to Alb3 might trigger the folding and subsequent release of LHCP into the thylakoid membrane. A direct interaction between LHCP and Alb3 was recently observed by BiFC experiments in vivo (24). The observation of a cpSRP43 binding site in TMD5 of Alb3 suggests that this domain contributes to pore formation. However, Kohler et al. suggested that YidC TMD2 and TMD3 form the core of the dimer and are part of the insertion pore (36).

In summary, our data suggest that the C terminus as well as the TMD5 of Alb3 provide binding regions for cpSRP43. The identification of the membrane-embedded binding site leads to a model indicating that cpSRP43 might insert into an Alb3 pore. To support this model further, it will be important to analyze the influence of cpSRP43 on Alb3 dimerization and pore formation and to identify which region of cpSRP43 contacts which binding site in Alb3.

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