

Functional interaction of chloroplast SRP/FtsY with the ALB3 translocase in thylakoids: substrate not required

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Integration of thylakoid proteins by the chloroplast signal recognition particle (cpSRP) posttranslational transport pathway requires the cpSRP, an SRP receptor homologue (cpFtsY), and the membrane protein ALB3. Similarly, *Escherichia coli* uses an SRP and FtsY to cotranslationally target membrane proteins to the SecYEG translocase, which contains an ALB3 homologue, YidC. In neither system are the interactions between soluble and membrane components well understood. We show that complexes containing cpSRP, cpFtsY, and ALB3 can be precipitated using affinity tags on cpSRP or cpFtsY. Stabilization of this

complex with GMP-PNP specifically blocks subsequent integration of substrate (light harvesting chl a/b-binding protein [LHCP]), indicating that the complex occupies functional ALB3 translocation sites. Surprisingly, neither substrate nor cpSRP43, a component of cpSRP, was necessary to form a complex with ALB3. Complexes also contained cpSecY, but its removal did not inhibit ALB3 function. Furthermore, antibody bound to ALB3 prevented ALB3 association with cpSRP and cpFtsY and inhibited LHCP integration suggesting that a complex containing cpSRP, cpFtsY, and ALB3 must form for proper LHCP integration.

Introduction

Cellular compartmentalization relies on the ability of protein targeting and translocation systems to correctly and efficiently move proteins from their site of synthesis into or across a membrane to their functional location within the cell. Protein sorting of nuclear-encoded thylakoid proteins uses two sequential routing systems. After synthesis of full-length precursors in the cytosol, these proteins are targeted to the general import machinery in the chloroplast envelope membranes by a cleavable transit peptide that is removed in the stroma by a processing protease (for review see Jarvis and Soll, 2002). Once in the stroma, imported proteins enter one of four different transport pathways that are used to target proteins to the thylakoid membrane for translocation into the lumen or integration into the bilayer (for review see Keegstra and Cline, 1999). Each of the transport pathways appears dedicated to the localization of a specific subset of thylakoid proteins and is distinguishable by pathway-specific

protein components and energy requirements for transport into or across the membrane.

One of these, the spontaneous pathway, which is responsible for the integration of membrane proteins such as Elip2 into the thylakoid membrane, appears to lack proteinaceous and energetic requirements (Kim et al., 1999). The chloroplast twin-arginine translocation (cpTAT) pathway depends on a trans-thylakoidal pH gradient to supply the energy needed to transport substrates, including the luminal 17-kD subunit of the oxygen-evolving complex (OE17; Cline et al., 1992). Although no soluble protein components have been reported for this pathway, a membrane translocase containing Tha4, Hcf106, and cpTatC, is required (Mori and Cline, 2001). The chloroplast Sec (cpSec) pathway, homologous to the bacterial and ER secretory (Sec) pathways, utilizes cpSecA, cpSecY, and cpSecE to transport a subset of luminal proteins including the 33-kD subunit of the oxygen-evolving complex (OE33) in an ATP-dependent reaction (Mori and

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Abbreviations used in this paper: chl, chlorophyll; cpSRP, chloroplast signal recognition particle; cpTAT, chloroplast twin-arginine translocation; GMP-PNP, 5'-guanylyl-imidodiphosphate trisodium salt; LHCP, light harvesting chl a/b-binding protein; OE, oxygen-evolving complex; SE, stromal extract; Sec, secretory; SPDP, *N*-succinimidyl 3-[2-pyridyldithio]-propionate.

Cline, 2001). Based on homology to the bacterial Sec system, it is expected that cpSecY and cpSecE form a proteinaceous pore through which proteins are translocated (Muller et al., 2001). Homology between the translocase components cpSecY/E, bacterial SecY/E and Sec61 α/γ in the ER membrane provides strong support for a common evolutionary history of these three translocation systems.

A chloroplast signal recognition particle (cpSRP) also functions in protein localization to the thylakoid (for review see Eichacker and Henry, 2001). Homologous SRPs in the cytosol of pro- and eukaryotes function exclusively to cotranslationally target proteins to the cytoplasmic and ER membranes, respectively (Walter and Johnson, 1994; Rapoport et al., 1996). cpSRP is unique in that it functions posttranslationally (Li et al., 1995) to transport a family of light-harvesting chlorophyll (chl) *a/b*-binding integral membrane proteins, the LHCs. The most studied of these is light harvesting chl *a/b*-binding protein (LHCP), the *lhcb1* gene product. During or after import into the chloroplast, LHCP is bound by cpSRP, a heterodimer composed of an evolutionarily conserved 54-kD subunit (cpSRP54) and a unique 43-kD subunit (cpSRP43; Schuenemann et al., 1998; Groves et al., 2001). LHCP integration also requires cpFtsY, a homologue of the bacterial SRP receptor, FtsY, and the SR α subunit of the SRP receptor in the ER (Kogata et al., 1999; Tu et al., 1999). Like FtsY in *Escherichia coli* (Zelazny et al., 1997), it is anticipated that cpFtsY functions at the thylakoid membrane as a cpSRP receptor during LHCP targeting. Consistent with the fact that both cpSRP54 and cpFtsY are GTPases, GTP is required for LHCP integration into isolated thylakoids (Hoffman and Franklin, 1994). Recently, we have shown that the purified recombinant proteins, cpSRP and cpFtsY, along with GTP, are necessary and sufficient for in vitro integration of LHCP into isolated thylakoid membranes (Yuan et al., 2002).

Though the soluble protein requirements for LHCP integration are well established, a detailed understanding of the membrane components is lacking. In an earlier study, we showed that the integral thylakoid protein, ALB3, functions in the LHCP integration mechanism (Moore et al., 2000). Antibodies bound to ALB3 were able to prevent LHCP integration without affecting transport by the cpSec or cpTAT pathways. Conversely, antibodies bound to cpSec or cpTAT translocase components had no effect on LHCP integration, but inhibited transport by the cpSec and cpTAT pathways specifically. These results implicate ALB3 as a necessary component of the LHCP integration machinery and are supported by recent genetic studies in *Chlamydomonas reinhardtii* (Bellafiore et al., 2002). The results also imply that the translocase used by LHCP is distinct from those used by other pathways. However, these findings do not rule out the possibility that ALB3 functions with the cpSec translocase in cotranslational integration of chloroplast synthesized proteins, a scenario suggested by results of cotranslational integration studies in *E. coli* (for reviews see Luirink et al., 2001; Chen et al., 2002).

In bacteria, the ALB3 homologue YidC appears to act in two functionally separate pools (Stuart and Neupert, 2000). One pool is associated with the Sec translocase (Scotti et al., 2000) and functions during cotranslational integration to

interact with transmembrane segments that have exited the SecYE pore (Beck et al., 2001). YidC's association with SecYEG appears to stem from its ability to interact with a SecDFYajC complex (Nouwen and Driessen, 2002). The second pool of YidC operates in the absence of a functional Sec translocase to integrate a distinct subset of membrane proteins (Samuelson et al., 2000). Although sequence homologues of SecDF and YajC are absent in *Arabidopsis thaliana*, the fact that cpSecY and ALB3 can be cross-linked in thylakoid membranes (Klostermann et al., 2002) suggests that ALB3, like YidC, may also have two distinct functions. ALB3 associated with cpSecYE may serve in cotranslational integration activities, whereas a second pool of ALB3, functionally independent of cpSecYE, mediates LHCP integration posttranslationally. In this context, it is unclear whether cpSRP or cpFtsY play a direct role in delivering LHCP to ALB3, or simply act to target LHCP to the membrane in a conformation suitable to promote LHCP interaction with ALB3. A direct role would be supported by the ability of cpSRP or cpFtsY to associate with ALB3.

Currently, nothing is known about how cpSRP and cpFtsY interact with each other and/or with membrane proteins that function in LHCP integration. Here, we have used recombinant cpSRP and cpFtsY to explore these interactions. Our results show that cpSRP and cpFtsY interact with and target to the ALB3 translocase. Using 5'-guanylyl-imidodiphosphate trisodium salt (GMP-PNP), we can stabilize the association between cpSRP and cpFtsY at the membrane and show that they form a complex containing ALB3 and cpSecY. The complex occupies functional ALB3 translocation sites, demonstrated by decreased LHCP integration into thylakoids where this complex was stabilized before integration assays, whereas cpSecY function is not interrupted. Furthermore, we show that treatments of thylakoid membranes with anti-ALB3 serum are able to inhibit the association of a cpSRP–cpFtsY complex with ALB3, which correlates with the antibody treatment's inhibitory effect on LHCP integration. Antibody against cpSecY, together with anti-rabbit IgG, removes cpSecY from complexes containing cpSRP, cpFtsY, and ALB3 without inhibiting ALB3 activity, indicating that cpSecY is likely not part of the functional complex. Interestingly, neither cpSRP43, nor LHCP, is required to form a complex with ALB3, suggesting that cpSRP43 functions to link the substrate to the true targeting components, cpSRP54 and cpFtsY, which form the targeting/translocation interface with ALB3.

Results

cpSRP and cpFtsY form a membrane-bound complex containing ALB3

To facilitate studies of the protein–protein interactions between the soluble and membrane components used by the cpSRP protein transport pathway, we produced recombinant cpSRP and cpFtsY, each with unique affinity tags. The affinity-tagged proteins are active in reconstituting integration and the tags enable these proteins to be combined with nucleotides and/or thylakoid membranes and then repurified with tag-specific resins to identify copurifying proteins. Because the SRP and its receptor in the ER interact via

SRP54 and SR α , and remain associated in the presence of the nonhydrolyzable GTP analogue, GMP-PNP (Connolly et al., 1991), we tested the effect of various guanine or adenine nucleotides for their ability to promote the formation of a stable cpSRP–cpFtsY complex at the membrane (Fig. 1). After incubating cpSRP, cpFtsY, salt-washed thylakoids, and the indicated nucleotides, membranes were washed, solubilized with maltoside, and mixed with S-protein agarose beads to precipitate the Trx-tagged cpFtsY and all associated proteins. In each assay, similar amounts of cpSRP and cpFtsY bound to the thylakoid membrane before solubilization (Fig. 1 B). However, the quantity of cpSRP coprecipitated with cpFtsY was elevated four- to fivefold in the presence of GMP-PNP relative to assays conducted with no nucleotide, GTP, GDP, ATP, or AMP-PNP (Fig. 1 A).

By closely examining the eluate from these complex formation and precipitation assays, we observed that ALB3 also precipitated with cpFtsY (Fig. 1 A). The quantity of ALB3 associated with cpFtsY was independent of added nucleotide, as well as the amount of coprecipitating cpSRP. To address the specificity of the coprecipitation assays, thylakoids were titrated with increasing amounts of cpSRP and cpFtsY in the presence of 0.5 mM GMP-PNP. After washing the membranes to remove unbound proteins, thylakoids were solubilized and incubated with S-protein agarose to re-isolate cpFtsY as before. Fig. 2 shows that as the amount of cpFtsY precipitated is increased, the quantity of cpSRP and ALB3 coprecipitated is also increased. As a negative control, antibody to LHCP, which makes up to 50% of the total thylakoid membrane protein content, was used to determine its presence or absence in the precipitated complexes. Unlike

ALB3, the amount of coprecipitated LHCP remained the same over the entire range of the titration, clearly demonstrating the specificity of the coprecipitation assay. Previous studies of thylakoid protein translocation suggest that ALB3 functions independently of the cpSec translocase (Moore et al., 2000; Cline and Mori, 2001). Yet, studies of the ALB3 homologue in *E. coli*, YidC, support a model wherein YidC functions both as a component of the Sec translocase during cotranslational integration of polytopic membrane proteins (Scotti et al., 2000; Chen et al., 2002) and Sec independently for insertion of a subset of membrane proteins (Samuelson et al., 2000). Therefore, we also screened eluted proteins that coprecipitate with cpFtsY for the presence of cpSecY. As shown in Fig. 2, cpSecY is also present in membrane complexes containing cpFtsY, and the amount coprecipitated increases concomitantly with the rising levels of recombinant protein. Because the signal from precipitated cpSecY is weak at the lower end of the titration, most of the remaining experiments were conducted using the ratio of 10 μ g of each recombinant protein to thylakoids equal to 75 μ g chl.

Because S-protein agarose precipitates all of the membrane-associated cpFtsY, these assays do not address the possibility that cpFtsY is in different pools: one associated with cpSRP and one with ALB3 and/or cpSecY. To analyze the component structure of the complexes formed at the membrane, salt-washed thylakoids were mixed with cpSRP, cpFtsY, or both, in the presence of GMP-PNP to form complexes on the membranes (Fig. 3 C). Membranes were then washed with buffer, solubilized, and precipitated with S-protein agarose as before (Fig. 3 A), or with anti-FLAG IgG and protein G agarose to precipitate the FLAG-tagged cpSRP43 (Fig. 3 B). Western blots of the coprecipitating proteins indicate that cpFtsY alone associates with ALB3, but the association is enhanced more than sixfold by the addition of cpSRP (Fig. 3 A, compare lanes 3 and 4). A minor amount of cpSecY also precipitated with cpFtsY alone, however, the quantity was also increased by the inclusion of cpSRP in the preincubation. The difference in the amount of coprecipitating membrane proteins correlates with a reduction in the

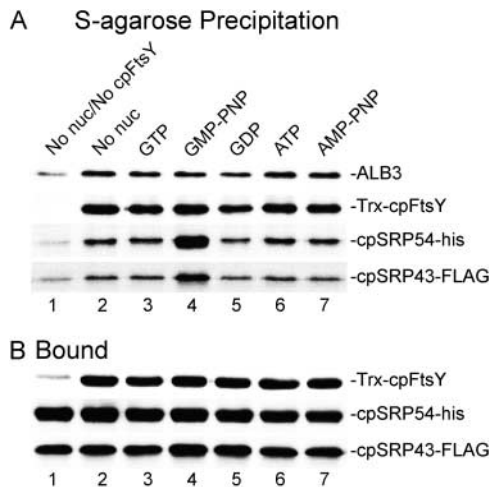


Figure 1. GMP-PNP is required to form a stable complex between cpSRP and cpFtsY. (A) Salt-washed thylakoids equal to 150 μ g were incubated with 4 μ g cpSRP-FLAG and with (lanes 2–7) or without (lane 1) 2 μ g Trx-cpFtsY in the presence of the nucleotide indicated at 0.5 mM final concentration (No nuc, no nucleotide added). Treated thylakoids were buffer washed, solubilized in maltoside, and mixed with S-protein agarose to precipitate Trx-tagged cpFtsY and all coprecipitating proteins. Western blots of the precipitates were probed to identify the presence of the proteins indicated to the right. (B) Thylakoids with bound recombinant proteins (see Materials and methods) were Western blotted to show relative amounts of soluble protein bound to the membranes.

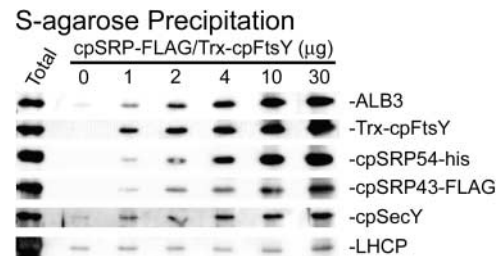
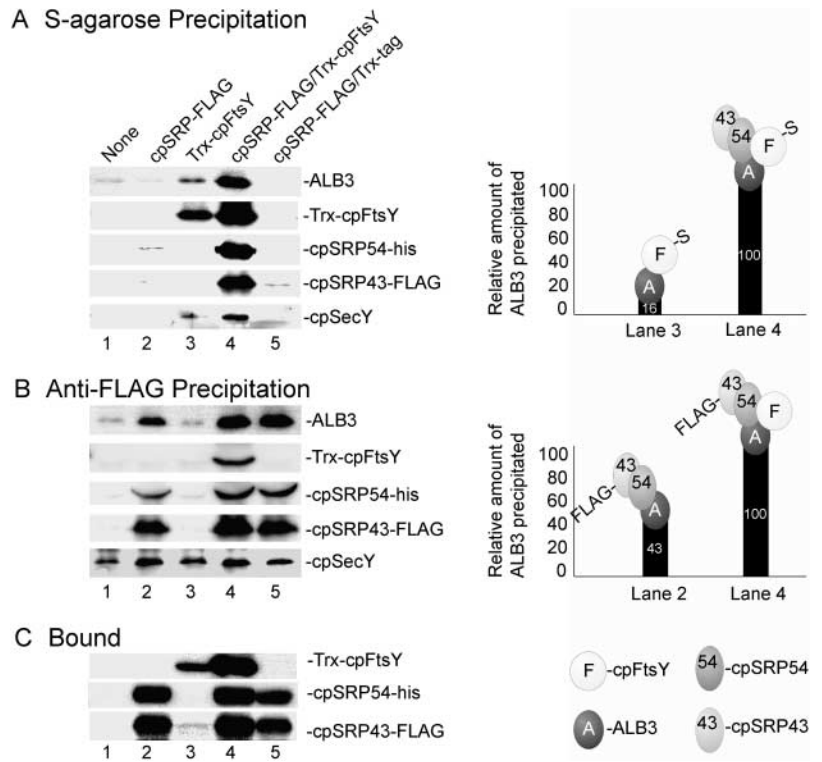


Figure 2. ALB3 and cpSecY specifically interact with cpFtsY and cpSRP. Salt-washed thylakoids containing 75 μ g chl were incubated with increasing amounts of cpSRP and cpFtsY in the presence of 0.5 mM GMP-PNP. After washing, the membranes were solubilized and used for precipitation assays with S-protein agarose. Western blots of the eluates are shown probed for the proteins indicated to the right. Numbers above the blots represent the amount of each protein added (e.g., cpSRP54-his, cpSRP43-FLAG, and Trx-cpFtsY). The first lane (Total) contains thylakoid membranes with bound cpSRP-FLAG and Trx-cpFtsY for sizing.

Figure 3. cpSRP and cpFtsY individually form a complex with membrane proteins at the targeting/translocation interface. Complex formation and precipitation assays (see Materials and methods), were performed with salt-washed thylakoids containing 125 μ g chl and included 34 μ g cpSRP-FLAG, 17 μ g Trx-cpFtsY, or 17 μ g Trx-tag as indicated above the first panel (A) as well as 0.5 mM GMP-PNP. After solubilization, coprecipitating proteins were Western blotted and probed to identify the proteins listed on the right of each panel. Illustrations at the far right indicate the percentage of coprecipitating ALB3 relative to the amount in lane 4 where cpSRP and cpFtsY are both included. The background level of coprecipitating ALB3 in lane 1 (A and B) was set to zero. (A) Precipitations using S-protein agarose, (B) precipitations using anti-FLAG IgG and protein G agarose, and (C) total membrane-bound protein before precipitations.



amount of cpFtsY bound to the membrane in the absence of cpSRP (Fig. 3 C), and therefore, the amount precipitated (Fig. 3 A). Reciprocally, cpSRP alone precipitates ALB3, but the amount of cpSRP associated with ALB3 is increased more than twofold by the presence of cpFtsY (Fig. 3 B, compare lanes 2 and 4). Though the background levels of anti-FLAG precipitated cpSecY were high, it is interesting to note that the relative amounts of cpSecY coprecipitated with cpSRP or cpSRP and cpFtsY were unchanged. This suggests that cpSRP alone has affinity for cpSecY, which agrees with previous evidence showing that cpSRP54 is involved in targeting proteins to cpSecY during cotranslational translocation (Zhang and Aro, 2002). Antibody to Tha4, a cpTAT pathway translocase component, was also used to probe eluates, but did not indicate its presence in the complex (unpublished data).

Precipitation assays were also conducted using thylakoids pretreated with cpSRP, cpFtsY, GMP-PNP, and overexpressed, purified LHCP. The presence of substrate did not have any significant effect on the formation of the membrane complex(es) (unpublished data). However, only a small percentage of urea-solubilized LHCP forms a complex with cpSRP, as shown by native gels, during in vitro complex formation assays (Yuan et al., 2002). Therefore, we do not rule out the possibility that the presence of LHCP may affect the efficiency of complex formation at the membrane.

Further evidence that cpSRP, cpFtsY, and ALB3 exist in a single complex comes from cross-linking data. The heterobifunctional cross-linker *N*-succinimidyl 3-[2-pyridyldithio]propionate (SPDP) reacts with amino and sulfhydryl groups and is cleavable with reducing agents. When thylakoids loaded with the cpSRP–cpFtsY complex are treated with 0.1 mM SPDP, a large portion of ALB3, cpSRP54, cpSRP43, cpFtsY, and cpSecY appears in very large complexes on non-

reducing Western blots (unpublished data). To determine if these proteins were all cross-linked in a single complex, cross-linked thylakoids were solubilized in SDS, diluted with buffered Triton X-100, and mixed with anti-FLAG IgG and protein G agarose to repurify cpSRP43-FLAG and any cross-linking adducts under denaturing conditions. Precipitated samples were then treated with β -mercaptoethanol to cleave cross-links, thereby allowing proteins to migrate as monomers during SDS-PAGE. As shown in Fig. 4, ALB3, cpSRP54, and cpFtsY were all coprecipitated with cpSRP43-FLAG in the presence of cross-linker. Furthermore, all four components were found in a single stained band, which was excised from a 5% polyacrylamide gel and treated with reducing agent (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200307067/DC1>). Individual components were then separated by SDS-PAGE and identified by Western blotting. Both cpSecY and Tha4 were remarkably absent from the cross-linked complex suggesting that cpSecY is either not properly placed to cross-link to other complex components or is only loosely associated with the other protein components. It is noteworthy that cpSRP54 also coprecipitates with cpSRP43-FLAG in the absence of cross-linker. This suggests that cpSRP43 and cpSRP54 may be linked via a disulfide bond when in a complex on the thylakoids. Alternatively, cpSRP54 and cpSRP43 may refold upon addition of Triton to SDS-solubilized membranes allowing them to reunite. We are currently investigating these possibilities.

A complex of cpSRP–cpFtsY occupies functional integration sites on thylakoid membranes

Thus far, the data support a model where ALB3, cpSecY, and/or an unidentified protein associated with either ALB3,

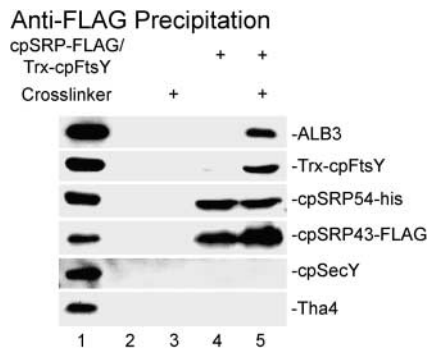


Figure 4. Cross-linked cpSRP, cpFtsY, and ALB3 are precipitated under denaturing conditions with tagged cpSRP43. Complexes of cpSRP-FLAG and Trx-cpFtsY were formed on salt-washed thylakoids (lanes 4 and 5) in the presence of GMP-PNP. Thylakoids were treated with SPDP (lanes 3 and 5) or DMSO (lanes 2 and 4). After quenching, the membranes were solubilized in SDS and diluted with Triton X-100 to lower the SDS concentration. Anti-FLAG IgG and protein G agarose were used to precipitate proteins cross-linked either directly or indirectly to cpSRP43-FLAG. Coprecipitating proteins were eluted with SDS solubilization buffer containing β -mercaptoethanol to cleave the cross-linker. Western blots of the coprecipitating proteins were probed with the antibody indicated on the right. Lane 1 contains proteins from thylakoids with cpSRP-FLAG and Trx-cpFtsY bound.

cpSecY, or both, serves as a membrane target for cpSRP and cpFtsY, the soluble components of the cpSRP posttranslational targeting pathway. To test the physiological relevance of the GMP-PNP-bound cpSRP–cpFtsY complex, we asked whether this complex was immobilized on functional translocation sites and, thus, able to hinder subsequent integration of LHCP. Because neither mammalian SRP54 nor SR α binds GMP-PNP in a stable manner until an SRP–receptor complex is formed (Rapiejko and Gilmore, 1997), we hypothesized that both cpSRP and cpFtsY would be needed to bind GMP-PNP and form a stable complex capable of blocking translocase function. Therefore, isolated cpSRP and/or cpFtsY were mixed with salt-washed thylakoid membranes in the presence or absence of GMP-PNP. After washing to remove unbound recombinant proteins, *in vitro* transport assays were conducted with treated thylakoids. Stromal extract (SE) was used as a source of active cpSRP and cpFtsY because the recombinant material bound to thylakoids was incubated with GMP-PNP. This likely prevents dissociation of the cpSRP–cpFtsY complex, and, therefore, inhibits its ability to function in repeated integration activities, as in bacterial (Miller et al., 1994) and eukaryotic (Connolly et al., 1991) SRP systems. SE also provides soluble proteins required for the integration of other pathway substrates (e.g., cpSecA is required for OE33 transport). As shown in Fig. 5, transport of the cpSRP pathway substrate, LHCP, was dramatically reduced (>70%) only when both cpSRP and cpFtsY were present together with GMP-PNP during the thylakoid pretreatment. When bound individually or in the absence of GMP-PNP, cpSRP and cpFtsY increase or do not hinder LHCP integration, likely because they bind GMP-PNP in an exchangeable manner or not at all, and were, therefore, functional during transport assays.

Transport of the cpSec pathway substrate, OE33, was only slightly affected by the cpSRP/cpFtsY/GMP-PNP treatment, which agrees with previous evidence that suggests that the cpSec transport machinery is functionally distinct from that required for LHCP integration (Mori et al., 1999; Moore et al., 2000). Also, neither the cpTAT nor the spontaneous pathways were negatively affected by any treatment. Assays conducted with less cpSRP and cpFtsY during the preincubation (6 μ g of each component per 75 μ g chl) resulted in a \sim 50% loss of LHCP integration activity, whereas the cpSec pathway was not affected at all (unpublished data). This confirms that the cpSRP/cpFtsY/GMP-PNP thylakoid pretreatment inhibits LHCP integration in a pathway specific manner. Together, the results in Figs. 2–5 indicate that a stable membrane complex between cpSRP and cpFtsY occupies saturable integration sites that contain functional ALB3. Though cpSecY is coprecipitated with cpSRP, we cannot rule out that it may do so only because it functions with cpSRP during cotranslational transport or with another component also found in the cpSRP–cpFtsY–ALB3 complex.

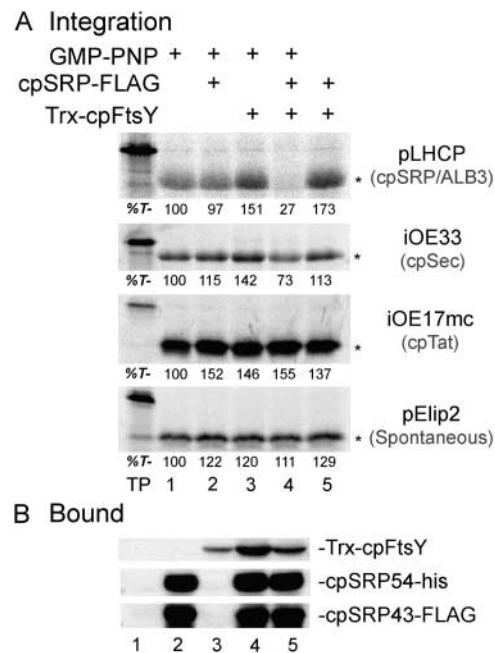


Figure 5. LHCP integration is inhibited by cpSRP and cpFtsY bound to thylakoid membranes with GMP-PNP. (A) Salt-washed thylakoids (equal to 125 μ g chl) were mixed with 34 μ g cpSRP, 17 μ g Trx-cpFtsY, and 1 mM GMP-PNP as shown above the top panel. After washing with buffer, the treated thylakoids were used for *in vitro* transport assays by adding the radiolabeled substrates indicated to the right and SE as a source of required soluble proteins. Transport pathways used by the substrates are indicated in parentheses. Phosphorimages of thylakoids after transport assays are shown with numbers beneath representing the percentage of transport (%T) relative to that in lane 1. Asterisks indicate the correctly integrated protease-resistant fragments of LHCP and Elip2, or the properly transported mature OE33 and OE17. TP is a lane of translation product. (B) After initial treatment with cpSRP, cpFtsY, and GMP-PNP, buffer-washed thylakoids were Western blotted and probed to identify the membrane bound proteins as indicated.

Complexes lacking cpSRP43 associate with ALB3 and cpSecY and block LHCP integration

Because cpSRP is able to associate with ALB3 and cpSecY (Fig. 3 B), individual cpSRP subunits along with cpFtsY were tested alone or in different combinations for their ability to form a complex with the known membrane proteins. Precipitations with anti-FLAG IgG and protein G agarose did not show a significant association between cpSRP43 and ALB3 (unpublished data). Attempts to examine binding of cpSRP54 to membrane components proved unsuccessful due to the inability of metal affinity resin or anti-cpSRP54 antibodies to efficiently precipitate the membrane-bound hexahistidine-tagged cpSRP54. However, using S-protein agarose, we found that the increased association of cpFtsY with ALB3 obtained in the presence of cpSRP (Fig. 3 A) is due to the cpSRP54 subunit (Fig. 6 A). Although cpFtsY alone has affinity for ALB3, addition of cpSRP43 did not increase, but decreased the amount of coprecipitating ALB3. In contrast, inclusion of either cpSRP or cpSRP54 alone resulted in elevated quantities of coprecipitating ALB3 and also coprecipitation of cpSecY (Fig. 6 A, compare lane 5 with lanes 8 and 9). Together, these data indicate that maximal stabilization of cpFtsY with ALB3 requires cpSRP54.

To confirm that cpFtsY and cpSRP54 are stabilized by GMP-PNP in a complex with integration competent ALB3, we examined the ability of individual components and pairs to inhibit subsequent LHCP integration. As before, components were bound to the thylakoids in the presence of GMP-PNP. After a wash step, the treated thylakoids were used for *in vitro* transport assays with SE providing fresh cpSRP and cpFtsY (Fig. 6 B). Again, when cpSRP54, either as a monomer or as the cpSRP heterodimer, was included with cpFtsY during the thylakoid pretreatment, both were able to associate in a stable complex with ALB3 that also led to the inhibition of subsequent LHCP integration. The cpSec pathway was only slightly affected by these treatments, possibly due to the sequestration of some cpSecY by cpSRP54. Hence, cpSRP43 appears to play no role in the targeting by cpSRP and cpFtsY to ALB3 and/or cpSecY in the membrane. However, the possibility remains that cpSRP43, in addition to its role in binding LHCP, may be required for events at the membrane necessary for LHCP integration.

Antibody inhibition of LHCP integration correlates with the loss of cpSRP and cpFtsY binding to ALB3

In an earlier report, we demonstrated that antibodies bound to ALB3 inhibited the subsequent *in vitro* integration of LHCP (Moore et al., 2000). To better understand these results in the context of the protein-protein interaction data presented above, we examined the influence of ALB3 and cpSecY antibodies on the formation of complexes between cpSRP, cpFtsY, ALB3, and cpSecY (Fig. 7). Salt-washed membranes were first treated with various rabbit-produced antibodies, washed, incubated again in the presence or absence of anti-rabbit IgG, and then split into two aliquots. One was used to verify the effect of antibody treatment on integration of the ALB3 substrate, LHCP, or the cpSec substrate, OE33. As shown previously, only immune antibody against ALB3 was able to inhibit LHCP integration, whereas

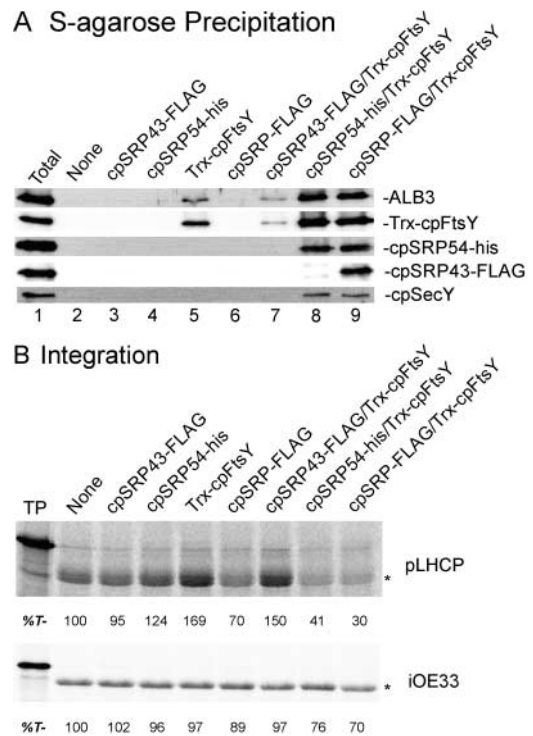


Figure 6. cpSRP43 is not required to coprecipitate ALB3 or cpSecY or to inhibit LHCP integration. (A) Assays were performed as in Fig. 3 and contained 34 μ g cpSRP-FLAG, 17 μ g cpSRP43-FLAG, 16 μ g cpSRP54-his, or 17 μ g Trx-cpFtsY as indicated. Western blots of proteins coprecipitating with Trx-cpFtsY on S-protein agarose beads were probed for the proteins listed to the right. Lane 1 (Total) contains thylakoid membrane proteins with bound cpSRP-FLAG and Trx-cpFtsY. (B) Assays were performed as in Fig. 5 by mixing 6 μ g cpSRP43-FLAG, 8 μ g cpSRP54-his, 15 μ g cpSRP-FLAG, and 4 μ g Trx-cpFtsY as specified above the top panel with salt-washed thylakoids containing 150 μ g chl in the presence of 1 mM GMP-PNP. Washed thylakoids were used for transport assays with the radio-labeled substrate indicated to the right. Phosphorimages are shown with numbers beneath which represent the amount of transport (%T) relative to that into thylakoids with no protein added during the pretreatment (None).

anti-cpSecY serum inhibited only OE33 transport (Fig. 7 A). Also, the presence of anti-rabbit antibody does not appear to inhibit the function of ALB3 or cpSecY, demonstrated by transport of both LHCP and OE33 into thylakoids treated with preimmune antibody followed by anti-rabbit IgG. The second aliquot of antibody-treated thylakoids was used to perform complex formation assays by mixing with GMP-PNP, cpSRP, and cpFtsY. After removal of unbound recombinant proteins, thylakoids were solubilized and cpFtsY was repurified along with associated proteins using S-protein agarose. Although immune antibodies had no influence on the amount of precipitated targeting components, thylakoid treatment with anti-ALB3 severely inhibited cpFtsY association with ALB3 (Fig. 7 B, lanes 2 and 5), further establishing the specificity of cpFtsY-ALB3 association. In contrast, formation of a cpFtsY-cpSRP-cpSecY complex at the membrane was unaffected by anti-cpSecY alone. However, by adding anti-rabbit IgG to bind the anti-cpSecY on the thylakoids, cpSecY was prevented from interacting with the cpSRP-cpFtsY-ALB3 complex without affecting LHCP in-

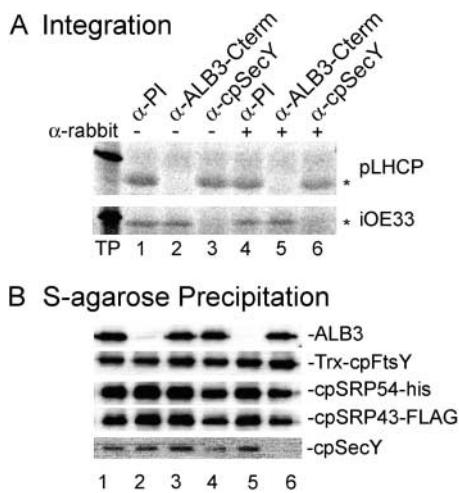


Figure 7. Anti-ALB3 serum inhibits both LHCP integration and binding of a cpSRP-cpFtsY complex to ALB3. Thylakoids were incubated with serum against proteins shown above the first panel (PI, preimmune serum; ALB3-Cterm, the soluble COOH-terminal tail of ALB3), washed, and incubated in the presence (+) or absence (-) of anti-rabbit IgG. (A) After washing, the treated thylakoids were used in transport assays containing radiolabeled substrate protein as indicated to the right of each phosphorimage. Asterisks indicate the correctly integrated protease-resistant fragment of LHCP or the properly transported mature OE33. (B) A separate aliquot of treated thylakoids was used in complex formation assays containing cpSRP-FLAG, Trx-cpFtsY, and GMP-PNP and precipitated with S-protein agarose. Western blots of coprecipitating proteins were probed for the protein shown to the right.

tegration. These data indicate that whereas cpSecY may be present in a complex with ALB3, its presence is not needed for posttranslational ALB3 function. In no case was the association between cpFtsY and cpSRP affected, signifying that these proteins do not require ALB3 or cpSecY to associate. Together, with data shown above, these results suggest that the direct association of a cpSRP-cpFtsY complex with ALB3, or with an unidentified ALB3-associated protein, is a required event for LHCP integration.

Discussion

Here, we have examined the protein-protein interactions that occur at the thylakoid membrane between the known soluble and membrane components of the posttranslational cpSRP protein transport system in chloroplasts. Our data demonstrate that cpSRP and cpFtsY form a stable complex at the membrane in the presence of a nonhydrolyzable GTP analogue. These results solidify the hypothesis that cpSRP54 and cpFtsY, like the ER SRP54 and SR α , require GTP hydrolysis to be released from each other once bound at the membrane (Connolly et al., 1991). Importantly, this cpSRP-cpFtsY complex associates with available membrane translocase components. Both ALB3 and cpSecY were found to coprecipitate with cpFtsY either by direct association or through the presence of cpSRP. Both were also found in a maltoside-solubilized complex with cpSRP alone. We show in Fig. 3 that cpSRP and cpFtsY can individually bind to the thylakoid membrane, but when present together, the amount of cpFtsY remaining associated with the membrane

is significantly increased. This increased amount of membrane-bound cpFtsY leads to a more efficient association between cpSRP and ALB3. However, the association between cpSRP and cpSecY does not appear to be influenced by the presence of cpFtsY, suggesting that cpSRP, specifically cpSRP54, has affinity for cpSecY, but cpFtsY may not. The association between cpSRP and cpSecY does not come as a surprise because cpSRP54 is known to function during targeting of chloroplast-encoded proteins to cpSecY for co-translational translocation (Zhang and Aro, 2002).

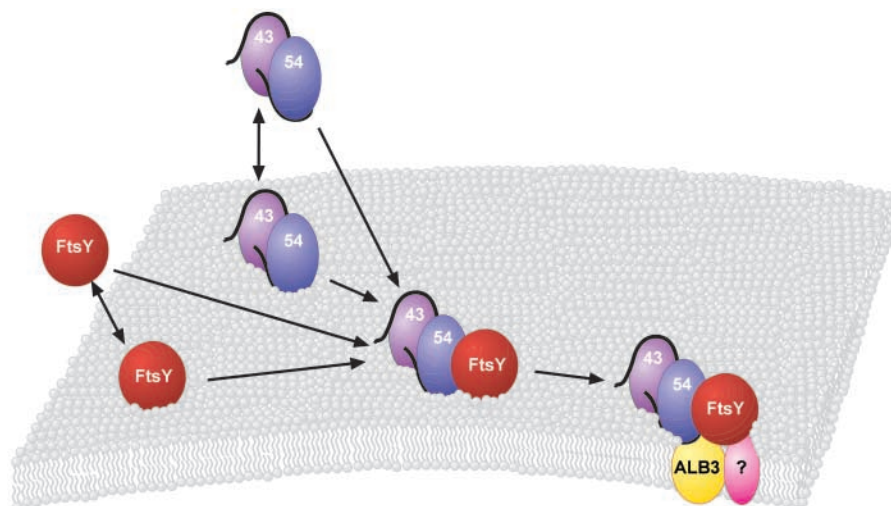
An association between ALB3 and cpSecY was recently reported by the finding that the two proteins form a cross-linking adduct (Klostermann et al., 2002). The mitochondrial homologue of ALB3, Oxa1p, does not have an available SecY homologue to associate with and is thought to form homooligomeric complexes in the inner membrane (Nargang et al., 2002). However, the ALB3 homologue in *E. coli*, YidC, can be found in SecY-associated and nonassociated pools (Scotti et al., 2000; Nouwen and Driessen, 2002); and YidC functions in both a Sec-dependent and -independent manner (Samuelson et al., 2000). So far, only ALB3 is known to be necessary for posttranslational translocation of LHCs. Co-translationally integrated thylakoid membrane proteins that appear to use both cpSRP54 and cpSecY, e.g., D1 of photosystem II (Eichacker and Henry, 2001; Nilsson and van Wijk, 2002; Zhang and Aro, 2002), may also require ALB3, but this has yet to be demonstrated.

The data presented here also demonstrate that the GMP-PNP-bound complex of cpSRP-cpFtsY occupies functional ALB3 integration sites as evidenced by the ability of the complex to inhibit LHCP integration. The inhibition was pathway specific, not inhibiting any of the other three post-translational protein targeting pathways. The fact that post-translational cpSecY function was not abolished by cpSRP and cpFtsY binding provides additional support for the hypothesis that ALB3 and cpSecY function independently during posttranslational transport. Further experiments showed that a complex lacking cpSRP43, but containing cpSRP54 and cpFtsY, was similarly able to associate with ALB3 and inhibit LHCP integration. Therefore, we hypothesize that cpSRP43 is not required for targeting to ALB3, but functions as a bridge between the substrate molecule and the actual targeting components, cpSRP54 and cpFtsY.

It has been proposed that cpSRP acts as a chaperone during posttranslational targeting to maintain hydrophobic substrates in an integration competent form, and once at the membrane, the substrate is recognized by the translocase and consequently released for integration (Eichacker and Henry, 2001). The observation that the substrate LHCP was unnecessary for complex formation between cpSRP, cpFtsY and ALB3 argues that cpSRP-bound full-length (posttranslational) substrate molecules will be directed to ALB3, or an associated protein, because of the affinity of cpSRP and cpFtsY for the ALB3 translocase. We hypothesize that the formation of a complex between cpSRP-cpFtsY and ALB3 is a necessary step in the integration mechanism. Further support for this hypothesis stems from our finding that anti-ALB3 antibodies, which inhibit LHCP integration, also prevent formation of a complex containing cpSRP, cpFtsY, and ALB3. Using antibody against cpSecY, coupled with anti-

Figure 8. Model for cpSRP/cpFtsY targeting to the ALB3 translocase.

cpFtsY (FtsY, red) is shown located both in the stroma and at the thylakoid membrane. cpSRP, composed of cpSRP54 (54, blue) and cpSRP43 (43, purple), with bound LHCP substrate (black line) binds to the membrane and forms a four-subunit complex with cpFtsY. This complex may form after all proteins are bound to the membrane, or the components may come together as they bind, but the order of events leading up to formation of a four-component complex is unclear. With both cpFtsY and cpSRP54 in the GTP-bound state, this large complex slides along the membrane until it reaches an open translocase containing ALB3 (ALB3, yellow) and possible unknown components (? , pink). Here, the substrate is released for integration, and GTP is hydrolyzed to release cpSRP and cpFtsY for further rounds of targeting.



rabbit IgG, we were able to eliminate cpSecY from the cpSRP–cpFtsY–ALB3 complex. Under the same conditions, ALB3 was still active and LHCP was properly integrated, strongly suggesting once more that cpSecY is not involved in ALB3-dependent LHCP integration. However, we cannot completely rule out the possibility that treatment with anti-rabbit IgG inhibits cpSecY coprecipitation yet still allows cpSecY to function with ALB3. Based on data presented in this report and homology with bacterial translocation pathways, we propose that ALB3 and cpSecY act separately during posttranslational transport activities, but complexes between the two may form in the thylakoid membrane for cotranslational translocation purposes as found in *E. coli*. Therefore, cpSecY may be found in cpSRP–cpFtsY–ALB3 complexes due to its interactions with both cpSRP54 and ALB3 during cotranslational transport activities.

Interestingly, cpSRP and cpFtsY form a complex at the membrane, even when ALB3 is made unavailable by bound antibody. This leads us to hypothesize that the formation of a cpSRP–cpFtsY complex at the membrane is a step in the targeting mechanism that precedes interaction with ALB3. We anticipate that the ordered assembly of this complex must take place for efficient integration of substrates.

In view of the data shown here, we propose the following model for cpSRP-based targeting to the ALB3 translocase in thylakoids (Fig. 8). Chloroplast FtsY is found both in the stroma and at the thylakoid membrane. Chloroplast SRP arrives at the membrane loaded with substrate and in a guanine nucleotide-free form (Yuan et al., 2002). cpFtsY interacts with cpSRP to promote GTP binding by both cpSRP54 and cpFtsY, which stabilizes the subunits together on the membrane. In the absence of accessible ALB3, the complex containing cpSRP, cpFtsY, and LHCP remains associated with the membrane until the ALB3 translocase is available. There, the substrate is released to ALB3 and/or possible unknown translocase components for integration, and GTP hydrolysis liberates the cpSRP and cpFtsY for a successive round of targeting.

Questions for future investigations

Results from these studies have identified two distinct cpSRP–cpFtsY complexes at the membrane: one associated with ALB3 and one formed when association with ALB3 is blocked by ALB3 antibodies. These findings raise several questions regarding the mechanism by which these two complexes are formed. Are thylakoid proteins required for the formation of the cpSRP–cpFtsY membrane complex, which lacks ALB3, or does the cpSRP–cpFtsY complex form in the stroma and then associate with the membrane? Does ALB3 directly interact with cpSRP–cpFtsY or is there an ALB3-associated protein that provides the binding site for a cpSRP–cpFtsY complex at the membrane? Additional proteins in either of these two complexes may be critical for regulating LHCP release in the absence of available ALB3, much like the role of SR β in SRP-based targeting to the ER (Fulga et al., 2001). Comparisons between SRP targeting systems in bacteria and thylakoids suggest that the ALB3 homologue, YidC, may similarly interact with SRP and/or FtsY. Work in our lab and with others is currently directed toward answering these questions.

Materials and methods

All reagents and enzymes used were purchased commercially. Plasmids described previously were used for in vitro transcription/translation of pLHCP (psAB80XD/4; Cline et al., 1989), iOE33 (Hulford et al., 1994), and pElip2 (Kim et al., 1999). The clone for OE17 was a gift from S. Theg (University of California, Davis, CA). It contains the coding sequence for maize OE17 preceded by the lumen targeting domain, but lacks the chloroplast signal peptide. At the COOH terminus, four methionines and a cysteine were added for radiolabeling purposes. Radiolabeled precursors (Moore et al., 2000) and chloroplast materials (Yuan et al., 2002) were prepared as described previously. Antibodies used for inhibition assays and probing Western blots have also been described previously: anti-cpSecY, anti-Tha4 (Mori et al., 1999), and anti-LHCP, which were provided by K. Cline (University of Florida, Gainesville, FL) and anti-GST-ALB3-Cterm (Woolhead et al., 2001). Recombinant, purified proteins were produced and isolated as described previously for cpSRP54-his and Trx-cpFtsY (Yuan et al., 2002). The thioredoxin tag (Trx-tag) was expressed from the empty vector pET-32b (Novagen), and purified using metal affinity resin.

Antibody production against cpSRP54 and cpSRP43

Metal affinity purified mature cpSRP54 from *A. thaliana* was expressed with a His₆-tag at the COOH terminus and used as antigen for antibody production. Similarly purified mature cpSRP43 from *A. thaliana* was expressed with an NH₂-terminal His₆-tag and used as antigen. Both antibodies were prepared in rabbits (Cocalico Biologicals).

Construction, expression, and purification of affinity-tagged cpSRP43

The nucleotide sequence coding for the mature region of cpSRP43, beginning AAVQRN, was subcloned from pGEX-6P-2 (Yuan et al., 2002) into pGEX-4T-2 (Amersham Biosciences) using BamHI and SmaI sites to create pGEX-4T-m43. This clone contains two residues, which differ from the published sequence (GenBank/EMBL/DDBJ accession no. AAD01509): K140R and R192L. The mature cpSRP43 was then PCR amplified from pGEX-4T-m43 using a forward sequencing primer, which amplified a BamHI site at the 5' end. The reverse primer added all but the last residue of the FLAG antigenic sequence to the 3' end of the cpSRP43 coding sequence. The PCR product was inserted into pGEX-4T-2 using the BamHI and SmaI restriction sites to make pGEX-4T-m43-FLAG. Sequencing of the plasmid indicated that the amino acid sequence DYKDDDDGSTRAAAS was added to the COOH terminus of the translated protein.

BL21 Star (Invitrogen) cells harboring pGEX-4T-m43-FLAG were grown to mid-log phase and induced with 0.3 mM IPTG for 2 h for expression of GST-m43-FLAG. Glutathione Sepharose™ fast flow (Amersham Biosciences) was used for initial purification. After overnight treatment with thrombin and desalting, cleaved GST was removed by a second pass over Glutathione Sepharose™. To complete the process, anion exchange was used and proteins were eluted with a linear KCl gradient in 10 mM Hepes-KOH, pH 8.0, and 10 mM MgCl₂.

Chloroplast SRP used for assays was made by combining equimolar amounts of isolated cpSRP54-his and m43-FLAG and incubating overnight at 4°C. Further purification by gel filtration using a HiLoad 26/60 Superdex 75 (Amersham Biosciences) with 10 mM Hepes-KOH, pH 8.0, and 10 mM MgCl₂ buffer yielded cpSRP-FLAG.

Complex formation and precipitation assays

Complexes between thylakoid membrane proteins and cpSRP-FLAG and Trx-cpFtsY were formed by incubating indicated components (i.e., salt-washed thylakoids, nucleotide, and purified proteins) at 25°C for 30 min. Membranes were recovered by centrifugation and washed with 50 mM Hepes-KOH, pH 8.0, and 0.33 M sorbitol (IB) plus 10 mM MgCl₂ (IBM). Thylakoids equal to 25 µg chl were removed and resuspended in 250 µl SDS solubilization buffer for subsequent examination of bound recombinant proteins. For precipitation assays, membranes equal to 50 µg chl were solubilized in 50 µl IB containing 1% *n*-dodecyl β-D-maltoside and 1.5% BSA for 10 min followed by centrifugation at 70,000 *g* for 12 min to pellet insoluble material. The soluble portion was added to 50 µl S-protein agarose (Novagen) as a 50% slurry in IB or 10 µl anti-FLAG M2 (Sigma-Aldrich) and 50 µl protein G agarose (Sigma-Aldrich) as a 50% slurry in IB and incubated for 30 min at RT with gentle mixing. Afterward, the agarose beads were washed three times with 0.1% *n*-dodecyl β-D-maltoside in IB, resuspended in IB and transferred to new tubes. Coprecipitating proteins were eluted with 100 µl SDS solubilization buffer.

Cross-linking and denatured precipitation assays

Salt-washed thylakoids containing 350 µg chl and 0.5 mM GMP-PNP were incubated with IBM or 7 µg cpSRP-FLAG and 3.5 µg Trx-cpFtsY as indicated in the Fig. 4 legend to form complexes on the membrane. Afterward, membranes were washed with IBM, a total bound protein sample was removed (50 µg chl) and the remaining thylakoids were aliquoted for cross-linking. Pre-treated thylakoids containing 150 µg chl were incubated in 333 µl IBM containing DMSO or 0.1 mM SPDP (Pierce Chemical Co.) in DMSO for 30 min at 25°C. The cross-linker was quenched by the addition of Tris, pH 8.0 to a final concentration of 3 mM. Membranes were washed with IB containing 3% BSA and subsequently resuspended in 2% SDS at 1 mg/ml chl. After 30 min at 25°C, insoluble material was pelleted. The soluble fraction was then mixed with 10 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, and 1% BSA so that the final SDS concentration was 0.03%. 5 µl anti-FLAG IgG and protein G agarose were added and the solution was mixed overnight at 4°C. Pelleted agarose was washed twice in 10 mM Tris, pH 8, 150 mM NaCl, 0.2% Triton X-100, and once with buffer lacking detergent. Precipitated proteins were eluted with 100 µl SDS solubilization buffer containing β-mercaptoethanol and heated at 70°C for 12 min.

Transport inhibition assays

Complexes of proteins on the thylakoid membranes were formed using salt-washed thylakoids, 1 mM GMP-PNP, and protein components as indicated in Figs. 5 A and 6 B legends. Afterward, membranes were washed with IBM, a total bound protein sample was removed and the remaining thylakoids were aliquoted for integration assays. Pre-treated thylakoids containing 25 µg chl were incubated with 1 mM ATP, 0.1 mM GTP, SE (equal to 100 µg chl), and 12.5 µl radiolabeled translation product for 15 min at 25°C. Thylakoids were recovered by centrifugation and treated with thermolysin.

Antibody inhibition assays

Salt-washed thylakoids containing 250 µg chl were incubated with 75 µl of the indicated antisera as described previously (Mori et al., 1999; Moore et al., 2000) for 1 h on ice. Pelleted membranes were washed with IBM, divided in half, and resuspended in 125 µl 3% BSA in IB. 50 µl of 3 mg/ml anti-rabbit IgG (Sigma-Aldrich) was added to one of each pair and both brought to a final volume of 750 µl with 10 mM Hepes-KOH, pH 8. After 30 min at 4°C with light mixing, membranes were again pelleted, washed with IBM, and aliquoted for individual assay procedures. Treated thylakoids containing 25 µg chl were used for transport assays as described previously (Mori et al., 1999; Moore et al., 2000). The remaining 75 µg chl from sera-treated thylakoids were used for complex formation and precipitation assays. These membranes were incubated with 0.5 mM GMP-PNP, 20 µg cpSRP-FLAG, and 10 µg Trx-FtsY in a final volume of 450 µl. Bound and precipitated samples were obtained as described earlier (Complex formation and presentation assays).

Analysis of samples

After integration assays, pelleted thylakoids were resuspended in 10 µl 20 mM EDTA and 15 µl 2× SDS solubilization buffer. After heating, proteins from each sample (10 µl) were separated by SDS-PAGE and analyzed by phosphorimaging using a Typhoon 8600 and IQ Solutions software (Molecular Dynamics). Thylakoid membranes with bound recombinant proteins and precipitation samples (10 µl) were separated on 12.5% SDS-polyacrylamide gels, blotted, and probed according to standard Western blotting methods. Secondary antibodies conjugated to horseradish peroxidase were used and detected by ECL. Images were recorded using a Fujifilm LAS-1000 Plus and individual spot intensities were quantified with Science Lab 98 for Windows software (Fujifilm).

Online supplemental material

Salt-washed thylakoids containing 15 mg chl were mixed with 0.1 mM GMP-PNP, 300 µg cpSRP-FLAG, and 150 µg Trx-cpFtsY in a final volume of 45 ml. After incubation at 25°C for 30 min, membranes were washed with IBM and resuspended in 30 ml IBM. SPDP was added to a final concentration of 0.1 mM and the solution was incubated for 30 min at RT. The cross-linker was quenched by the addition of 1 M Tris, pH 8.0 to a final concentration of 50 mM. After a wash with IBM, membranes were treated as above (Cross-linking and denatured precipitation assays) for solubilization in SDS and dilution with buffered Triton X-100. 500 µl of a 50% slurry of anti-FLAG M2 Affinity Gel (Sigma-Aldrich) was added to the solution and incubated overnight before reisolated by centrifugation. The resin was washed as described earlier (Cross-linking and denatured precipitation assays) and coprecipitating proteins were eluted by incubation for 1 h with SDS solubilization buffer lacking β-mercaptoethanol, but containing 8 M urea. A sample of the eluted proteins was electrophoresed on a 5% SDS-polyacrylamide gel and stained; a sample was also run on an identical unstained gel. A gel piece corresponding to the stained band was excised from the unstained gel and treated with DTT. Proteins from segments of the treated gel slice were analyzed on a 12.5% polyacrylamide gel and Western blotted. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200307067/DC1>.

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References

- Beck, K., G. Eisner, D. Trescher, R.E. Dalbey, J. Brunner, and M. Muller. 2001. YidC, an assembly site for polytopic *Escherichia coli* membrane proteins located in immediate proximity to the SecYE translocon and lipids. *EMBO Rep.* 2:709–714.
- Bellaïf, S., P. Ferris, H. Naver, V. Gohre, and J.D. Rochaix. 2002. Loss of Albino3 leads to the specific depletion of the light-harvesting system. *Plant Cell.* 14:2303–2314.
- Chen, M., K. Xie, F. Jiang, L. Yi, and R.E. Dalbey. 2002. YidC, a newly defined evolutionarily conserved protein, mediates membrane protein assembly in bacteria. *Biol. Chem.* 383:1565–1572.
- Cline, K., and H. Mori. 2001. Thylakoid Δ pH-dependent precursor proteins bind to a cpTatC-Hcf106 complex before Tha4-dependent transport. *J. Cell Biol.* 154:719–729.
- Cline, K., D.R. Fulsom, and P.V. Viitanen. 1989. An imported thylakoid protein accumulates in the stroma when insertion into thylakoids is inhibited. *J. Biol. Chem.* 264:14225–14232.
- Cline, K., W.F. Ettinger, and S.M. Theg. 1992. Protein-specific energy requirements for protein transport across or into thylakoid membranes. Two luminal proteins are transported in the absence of ATP. *J. Biol. Chem.* 267:2688–2696.
- Connolly, T., P.J. Rapiejko, and R. Gilmore. 1991. Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor. *Science.* 252:1171–1173.
- Eichacker, L.A., and R. Henry. 2001. Function of a chloroplast SRP in thylakoid protein export. *Biochim. Biophys. Acta.* 1541:120–134.
- Fulga, T.A., I. Sinning, B. Dobberstein, and M.R. Pool. 2001. SRbeta coordinates signal sequence release from SRP with ribosome binding to the translocon. *EMBO J.* 20:2338–2347.
- Groves, M.R., A. Mant, A. Kuhn, J. Koch, S. Dubel, C. Robinson, and I. Sinning. 2001. Functional characterization of recombinant chloroplast signal recognition particle. *J. Biol. Chem.* 276:27778–27786.
- Hoffman, N.E., and A.E. Franklin. 1994. Evidence for a stromal GTP requirement for the integration of a chlorophyll a/b-binding polypeptide into thylakoid membranes. *Plant Physiol.* 105:295–304.
- Hulford, A., L. Hazell, R.M. Mould, and C. Robinson. 1994. Two distinct mechanisms for the translocation of proteins across the thylakoid membrane, one requiring the presence of a stromal protein factor and nucleotide triphosphates. *J. Biol. Chem.* 269:3251–3256.
- Jarvis, P., and J. Soll. 2002. Toc, tic, and chloroplast protein import. *Biochim. Biophys. Acta.* 1590:177–189.
- Keegstra, K., and K. Cline. 1999. Protein import and routing systems of chloroplasts. *Plant Cell.* 11:557–570.
- Kim, S.J., S. Jansson, N.E. Hoffman, C. Robinson, and A. Mant. 1999. Distinct “assisted” and “spontaneous” mechanisms for the insertion of polytopic chlorophyll-binding proteins into the thylakoid membrane. *J. Biol. Chem.* 274:4715–4721.
- Klostermann, E., I. Droste Gen Helling, J.P. Carde, and D. Schuenemann. 2002. The thylakoid membrane protein ALB3 associates with the cpSecY-translocon in *Arabidopsis thaliana*. *Biochem. J.* 368:777–781.
- Kogata, N., K. Nishio, T. Hirohashi, S. Kikuchi, and M. Nakai. 1999. Involvement of a chloroplast homologue of the signal recognition particle receptor protein, FtsY, in protein targeting to thylakoids. *FEBS Lett.* 447:329–333.
- Li, X., R. Henry, J. Yuan, K. Cline, and N.E. Hoffman. 1995. A chloroplast homologue of the signal recognition particle subunit SRP54 is involved in the posttranslational integration of a protein into thylakoid membranes. *Proc. Natl. Acad. Sci. USA.* 92:3789–3793.
- Luirink, J., T. Samuelsson, and J.W. de Gier. 2001. YidC/Oxa1p/Alb3: evolutionarily conserved mediators of membrane protein assembly. *FEBS Lett.* 501:1–5.
- Miller, J.D., H.D. Bernstein, and P. Walter. 1994. Interaction of *E. coli* Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor. *Nature.* 367:657–659.
- Moore, M., M.S. Harrison, E.C. Peterson, and R. Henry. 2000. Chloroplast Oxa1p homolog albino3 is required for post-translational integration of the light harvesting chlorophyll-binding protein into thylakoid membranes. *J. Biol. Chem.* 275:1529–1532.
- Mori, H., and K. Cline. 2001. Post-translational protein translocation into thylakoids by the Sec and Δ pH-dependent pathways. *Biochim. Biophys. Acta.* 1541:80–90.
- Mori, H., E.J. Summer, X. Ma, and K. Cline. 1999. Component specificity for the thylakoidal Sec and Δ pH-dependent protein transport pathways. *J. Cell Biol.* 146:45–56.
- Muller, M., H.G. Koch, K. Beck, and U. Schafer. 2001. Protein traffic in bacteria: multiple routes from the ribosome to and across the membrane. *Prog. Nucleic Acid Res. Mol. Biol.* 66:107–157.
- Nargang, F.E., M. Preuss, W. Neupert, and J.M. Herrmann. 2002. The Oxa1 protein forms a homooligomeric complex and is an essential part of the mitochondrial export translocase in *Neurospora crassa*. *J. Biol. Chem.* 277:12846–12853.
- Nilsson, R., and K.J. van Wijk. 2002. Transient interaction of cpSRP54 with elongating nascent chains of the chloroplast-encoded D1 protein; “cpSRP54 caught in the act”. *FEBS Lett.* 524:127–133.
- Nouwen, N., and A.J. Triessen. 2002. SecDFyajC forms a heterotetrameric complex with YidC. *Mol. Microbiol.* 44:1397–1405.
- Rapiejko, P.J., and R. Gilmore. 1997. Empty site forms of the SRP54 and SR alpha GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum. *Cell.* 89:703–713.
- Rapoport, T.A., B. Jungnickel, and U. Kutay. 1996. Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* 65:271–303.
- Samuelson, J.C., M. Chen, F. Jiang, I. Moller, M. Wiedmann, A. Kuhn, G.J. Phillips, and R.E. Dalbey. 2000. YidC mediates membrane protein insertion in bacteria. *Nature.* 406:637–641.
- Schuenemann, D., S. Gupta, F. Persello-Cartiaux, V.V. Klimyuk, J.D.G. Jones, L. Nussaume, and N.E. Hoffman. 1998. A novel signal recognition particle targets light-harvesting proteins to the thylakoid membranes. *Proc. Natl. Acad. Sci. USA.* 95:10312–10316.
- Scotti, P.A., M.L. Urbanus, J. Brunner, J.W. de Gier, G. von Heijne, C. van der Does, A.J. Triessen, B. Oudega, and J. Luirink. 2000. YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. *EMBO J.* 19:542–549.
- Stuart, R.A., and W. Neupert. 2000. Making membranes in bacteria. *Nature.* 406:575, 577.
- Tu, C.J., D. Schuenemann, and N.E. Hoffman. 1999. Chloroplast FtsY, chloroplast signal recognition particle, and GTP are required to reconstitute the soluble phase of light-harvesting chlorophyll protein transport into thylakoid membranes. *J. Biol. Chem.* 274:27219–27224.
- Walter, P., and A.E. Johnson. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 10:87–119.
- Woolhead, C.A., S.J. Thompson, M. Moore, C. Tissier, A. Mant, A. Rodger, R. Henry, and C. Robinson. 2001. Distinct Albino3-dependent and -independent pathways for thylakoid membrane protein insertion. *J. Biol. Chem.* 276:40841–40846.
- Yuan, J., A. Kight, R.L. Goforth, M. Moore, E.C. Peterson, J. Sakon, and R. Henry. 2002. ATP stimulates signal recognition particle (SRP)/FtsY-supported protein integration in chloroplasts. *J. Biol. Chem.* 277:32400–32404.
- Zelazny, A., A. Seluanov, A. Cooper, and E. Bibi. 1997. The NG domain of the prokaryotic signal recognition particle receptor, FtsY, is fully functional when fused to an unrelated integral membrane polypeptide. *Proc. Natl. Acad. Sci. USA.* 94:6025–6029.
- Zhang, L., and E.M. Aro. 2002. Synthesis, membrane insertion and assembly of the chloroplast-encoded D1 protein into photosystem II. *FEBS Lett.* 512:13–18.