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The Membrane Interface of Chloroplast Signal Recognition Particle-Dependent Protein Targeting

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THE MEMBRANE INTERFACE OF CHLOROPLAST SIGNAL RECOGNITION PARTICLE-DEPENDENT PROTEIN TARGETING

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By

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ABSTRACT

A novel signal recognition particle (SRP) found in the chloroplast (cpSRP) works in combination with the cpSRP receptor, cpFtsY, to facilitate the post-translational targeting of a family of nuclear-encoded thylakoid proteins to the Alb3 translocase in thylakoid membranes. Work here focused on understanding events at the membrane that take place to ensure targeting of the cpSRP-dependent substrate to Alb3. Specifically, we sought to understand the structural and functional role of membrane binding by cpFtsY, a protein that exhibits the ability to partition between the membrane (thylakoid) and soluble (stroma) phase during protein targeting. We also sought to understand whether a novel SRP subunit (cpSRP43) in chloroplasts is involved in targeting events at the membrane beyond its role in substrate binding. Lastly, we chose to examine the possible association of Alb3 with chlorophyll (Chl) biosynthetic enzymes, which provide Chl ligands to SRP-targeted protein substrates.

Our data show that cpFtsY houses a membrane-binding motif whose activity is linked to the SRP GTPase cycle. This membrane-binding motif is necessary and sufficient for binding thylakoid membranes and appears to be conserved among prokaryotic and organellar FtsY homologues. Interestingly, the removal or mutation of key residues in this region of cpFtsY results in a higher basal rate of GTP hydrolysis in solution. Furthermore, these changes correspond to a loss of lipid-induced hydrolysis stimulation, suggesting that the membrane binding region houses a negative regulator of hydrolysis is naturally switched off by a membrane-induced conformational shift.

Using recombinant cpSRP43 and a construct corresponding to the soluble C-terminal extension of Alb3 (Alb3-Cterm), we show that cpSRP43 contributes to the

specificity for the targeting reaction by interacting with the C-terminal region of Alb3. Furthermore, a peptide corresponding to the C-terminal region of Alb3 stimulates cpSRP GTP hydrolysis only in the presence of cpSRP43. These results suggest that cpSRP43 mediates key targeting events at the thylakoid membrane, such as release of the targeting complex from Alb3. Furthermore, these data support a model in which cpSRP43 functions as a translocon 'sensing' component critical for membrane-associated steps in the post-translational cpSRP-dependent targeting pathway.

Lastly, our results suggest that Alb3-dependent LHCP insertion is linked to the final stages of Chl biosynthesis. Indeed, we have identified two pools of Alb3: one that is associated with SRP targeting components and one that is associated with a late-stage chlorophyll biosynthesis enzyme (geranylgeranyl reductase). This data provides the first evidence that Chl biosynthesis enzymes are in complex with Alb3, supporting the hypothesis that the final stages of Chl biosynthesis are coordinated with the assembly of proteins that require Alb3 for assembly.

This dissertation is approved for recommendation to the Graduate Council.

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ABBREVIATIONS

ΔpH – pH gradient across a membrane

ΔΨ – electrical gradient across a membrane

Alb3 – Albino3 protein

Alb3-50aa – 50 amino acid peptide from stromal facing loop of Alb3

ATP – adenosine triphosphate

BSA – bovine serum albumin

CD – circular dichroism

cDNA – complementary DNA

Chl or chl – chlorophyll

cpSec – chloroplast secretory

cpSRP – chloroplast signal recognition particle

cpSRP43 – 43 kDa subunit of the cpSRP

cpSRP54 – 54 kDa subunit of the cpSRP

cpFtsY – chloroplast FtsY homologue (cpSRP receptor)

cpSecA – chloroplast SecA

cpTat – chloroplast twin-arginine translocation

cpTatC – cpTat subunit C

DNA – deoxyribonucleic acid

ER – endoplasmic reticulum

Ffh – fifty-four homologue

FtsY – SRα homologue in bacteria

GDP – guanosine diphosphate

GGR – geranylgeranyl reductase

GMP-PNP – 5'-guanyl-imidodiphosphate trisodium salt

GST – glutathione S-transferase

GTP – guanosine triphosphate

Hcf106 – cpTat translocon subunit homologous to bacterial TatB subunit

HKM – 10 mM HEPES-KOH pH 8, 10 mM $MgCl_2$

IB – import buffer, 50 mM HEPES-KOH pH 8, 0.33 M sorbitol

IBM – IB, 10mM $MgCl_2$

IgG – immunoglobulin G

ITC – isothermal titration calorimetry

K_d – dissociation constant

kDa – kiloDalton

LHC – light-harvesting complex

LHCP – light-harvesting chlorophyll-binding protein

mal – n-Dodecyl β-D-Maltoside

mant – 2'(3')-*O*-(*N*-Methylanthraniloyl)

mantGTP – GTP substituted with mant group at 2'(3')-*O* position of the ribosyl residue

min – minute

m – mature form

NOE – nuclear overhauser effect

NOESY – nuclear overhouser effect spectroscopy

NMR – nuclear magnetic resonance

OE17 – 17 kDa component of the oxygen evolving complex

OE23 – 23 kDa component of the oxygen evolving complex

OE33 – 33 kDa component of the oxygen evolving complex

p, i, and t – precursor, intermediate, and truncated precursor forms

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PI – preimmune

PMF – proton motive force

PsaG, PsaK – photosystem I reaction center proteins G, K

PsbX, PsbS, PsbW, PsbY – photosystem II reaction center proteins X, S, W, Y

PT – protease-treated

PVDF – polyvinylidene fluoride

RNA – ribonucleic acid

RNC – ribosome nascent chain complex

RT-PCR – reverse transcription PCR

S – Svedburg unit

SE – stromal extract

Sec – secretory

SecA – cytosolic chaperone in Sec pathway

SecB – cytosolic chaperone in Sec pathway

SecYEG – Y, E, G subunits of the bacterial Sec translocon

Sec61αγβ – α, γ, β subunits of the eukaryotic Sec translocon

SecGDFyajC – G, D, F, yajC subunits of the bacterial Sec translocon

SDS – sodium dodecylsulfate

SDS-PAGE – SDS-polyacrylamide gel electrophoresis

SR – SRP receptor

SRα, SRβ – α and β subunits of the SR

SRP – signal recognition particle

SRP9, SRP14, SRP19, SRP54, SRP68, SRP72 – 9, 14, 19, 54, 68, and 72 kDa subunits of the mammalian SRP

SW – salt-washed

Tat – twin-arginine translocation

TatA, TatB, TatC – A, B, C subunits of the bacterial Tat translocon

Tic – translocase at the inner membrane of the chloroplast

Tha4 – cpTat translocon subunit homologous to bacterial TatA

Toc – translocase at the outer membrane of the chloroplast

TM – transmembrane domain

TP – translation product

Trx-tag – thioredoxin tag

UV – ultraviolet

WT – wild-type

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INTRODUCTION

INTRODUCTION

Although the vast majority of proteins produced in a eukaryotic cell are encoded by nuclear DNA and synthesized in the cytosol, many proteins must be localized from the cytosol to the cellular compartment where they function. Protein routing relies on molecular machinery for targeting and membrane insertion or translocation. Proteins targeted to the inner membrane compartments of organelles, such as chloroplasts and mitochondria, must frequently rely on the function of a second level of routing found within the organelle. Work conducted here focuses on the function of a chloroplast signal recognition particle (cpSRP) and Albino3 (Alb3)-dependent targeting/translocation pathway.

Generally, a single targeting system may be responsible for routing a host of different proteins; hence localization information resides as a distinct type of targeting sequence contained in the targeted protein. There are common mechanistic themes to protein routing: soluble proteins bind a targeting sequence within a substrate, assist the substrate to its target membrane via an interaction with a membrane-localized receptor protein, and finally interact with protein translocation machinery to release the substrate for either translocation into or across the membrane. Variations on this theme found among different targeting pathways indicate the specialization of each system to accommodate specific transport requirements. The best studied routing systems function in bacteria and rely on signal sequences to promote transport from the bacterial cytosol into or across the cytoplasmic membrane.

PROTEIN TARGETING WITHIN THE CHLOROPLAST

There is much evidence that chloroplasts originated from an endosymbiotic event with cyanobacteria (for reviews see (Fulgosi *et al.*, 2004; Hormann *et al.*, 2007)). The chloroplast genome is circular, resembling that of bacteria, and contains only 128 genes, most of which are integral membrane components of photosystems and electron transport complexes, with the few remaining involved in synthesis of these chloroplast-encoded proteins. Though chloroplasts still contain a functional genome, the majority of chloroplast proteins are now encoded by nuclear DNA (Heazlewood *et al.*, 2005). This development has led to the necessity for protein targeting pathways that are capable of directing proteins from the cytosol into chloroplasts. From the chloroplast stroma, nuclear-encoded proteins may be directed to photosynthetic thylakoid membranes where polypeptides are integrated into the bilayer or transported across the bilayer into the thylakoid lumen. Protein targeting pathways in the chloroplast are homologous to signal peptide-based targeting pathways utilized in prokaryotic and eukaryotic cells, indicating that the pathways have likely evolved from a common prokaryotic ancestor (for reviews see (Fulgosi *et al.*, 2004; Jekely, 2006)). As such, a large portion of our understanding concerning homologous targeting systems in the chloroplast have been resolved by means of combined findings from chloroplast, bacterial, and mammalian systems.

Four distinct protein targeting pathways have been described in chloroplasts (see Figure 1.1). Disregarding spontaneous insertion, for which neither proteinaceous nor energetic requirements have been found, translocation into or across thylakoid membranes is catalyzed by pathways utilizing the Sec translocon, Tat translocon, or Alb3 translocase, as well as soluble components such as the signal recognition particle.

Several excellent reviews describe the protein translocation machinery found in chloroplasts (Agarraberes and Dice, 2001; Robinson *et al.*, 2001; Cline, 2003; Fulgosi *et al.*, 2004). These protein targeting pathways are categorized based on the protein and energetic requirements for translocation and are briefly described below.

Spontaneous Insertion

Some thylakoid proteins insert into the thylakoid membrane without any detectable requirement for proteins used in known targeting pathways, nucleotides, or other energetic components, such as the proton motive force provided by a trans-thylakoidal pH difference (ΔpH) or electrical potential (Δψ) (for reviews see(Robinson *et al.*, 2001; Cline, 2003)). Examples of these 'spontaneously-inserting' proteins include photosystem II proteins PsbS, PsbX, PsbW, and PsbY (Woolhead *et al.*, 2001). Thylakoid proteins that spontaneously insert generally contain one or two very hydrophobic transmembrane (TM) regions with short lumenal domains. It is thought that the interaction of TM regions with the bilayer provides sufficient energy to drive the lumenal domains across the bilayer. Stromal-facing portions of the proteins may play a role in orchestrating the proper conformation for insertion as well. For PsaG, a spontaneously-inserting photosystem I protein with two transmembrane-spanning regions and a positively-charged stromal loop, insertion is dependent upon the charge distribution of the stromal loop (Zygadlo *et al.*, 2006). Though it is possible that the spontaneous insertion pathway is mediated by proteinaceous factors that have not yet been indentified, evidence such as the unassisted insertion of PsaK, a photosystem I protein homologous to

PsaG, into isolated thylakoids and artificial liposomes suggests this is not the case (Mant *et al.*, 2001) (C. Robinson, unpublished as seen in Zydaglo et al., 2006).

Twin Arginine Translocation

The majority of thylakoid lumen proteins are transported across the thylakoid membrane by the twin arginine translocation (Tat) system (Peltier *et al.*, 2002; Fulgosi *et al.*, 2004). Also found in the cytoplasmic membranes of some archaea, at least one animal, and many bacteria, the Tat translocon is specialized for translocating sizeable (up to at least 132 kDa) fully-folded proteins without compromising membrane integrity or impermeability (Bogsch *et al.*, 1998; Lee *et al.*, 2006; Sargent, 2007). This feat requires coordinated efforts of three essential proteins: Tha4, Hcf106, and cpTatC in chloroplasts (TatA, TatB, and TatC in bacteria).

Tat components work together in large membrane complexes to accomplish the general steps of protein translocation (see Figure 1.2). Upon emerging from the ribosome, the preprotein is diverted from other pathways such as those involving the Sec translocon due to characteristics of the Tat signal sequence and mature protein. A prerequisite for translocation of Tat substrates seems to be the acquisition of native tertiary structure (DeLisa *et al.*, 2003). After folding, any additional subunits and cofactors are added. Folded protein interacts with a large signal recognition module composed of equimolar amounts of Hcf106 and cpTatC. A flexible proteinaceous pore is formed by oligomers of Tha4 proteins. Binding of substrate to the Hcf106/cpTatC supercomplex induces a conformational change, exposing a Tha4-binding site that results in formation of the complete and functional Tha4/Hcf106/cpTatC translocon. Following

translocation through the Tha4 pore, the signal sequence is removed and the mature protein is released to the thylakoid lumen. To accommodate a wide range of substrates, it is thought that smaller Tat complexes come together to form pores that match the size of the particular substrate being transported.

Though the general steps of protein translocation appear to be conserved in Tat translocation, the Tat pathway has distinct differences from the other protein localization pathways. Foremost, all known essential Tat components are membrane-localized (see Figure 1.2). CpTatC, the most highly-conserved component, is predicted to have six membrane-spanning TM domains, while the others contain only a single TM domain. Secondly, none of the Tat proteins contain nucleotide hydrolysis activity. Hence, Tat translocation is not nucleotide-dependent. Instead a proton motive force is necessary for Tat translocation. The ΔpH of the target membrane has been traditionally thought to be required as it is for reconstitution of transport *in vitro*; however, data from the chloroplast Tat pathway (cpTat) suggests that it is the Δψ rather than a trans-thylakoidal ΔpH that is necessary for substrate transport *in vivo* (Alder and Theg, 2003; Di Cola *et al.*, 2005). Further study is warranted to describe at what stages a proton motive force is required for Tat transport.

As in other targeting pathways, the presence of a signal peptide is necessary to initiate Tat localization. Tat pathway substrates are synthesized with N-terminal signal peptides containing the conserved 'twin arginine' sequence motif (SRRxFLK). Tat signal sequences include a polar N-terminal region that varies in length, a hydrophobic region of 12-20 residues, and a C-terminal region that frequently contains basic residues. The Tat signal sequence has been described as a 'Sec avoidance' signal, due to the fact

that modifying this motif may turn the protein into a substrate for the Sec-dependent pathway (for review see (Agarraberes and Dice, 2001)). In comparison to Sec signal peptides, Tat signal peptides, although very similar, tend to be slightly longer and less hydrophobic. Interestingly, the avoidance signal does not come from the invariant twin arginine motif because mutagenesis of the invariant twin arginine motif blocks cpTat translocation but does not necessarily result in cpSec targeting as the twin arginine is compatible with the cpSec system (Chaddock *et al.*, 1995; Henry *et al.*, 1997; Halbig *et al.*, 1999). Instead, Sec avoidance has been attributed to the charge distribution of the C-terminal region of the signal sequence; removal of a conserved lysine in this region of a cpTat signal sequence has been shown to result in cpSec translocation (Bogsch *et al.*, 1997; Henry *et al.*, 1997). Recent analysis of predicted Tat signal peptides confirmed that *Escherichia coli* Tat selectivity is housed in the C-terminal region of the peptide; a positive net charge (at least +2) results in Tat specificity while a negative net charge (-1 or lower) results in indiscriminant targeting with both Sec and Tat (Tullman-Ercek *et al.*, 2007). The fact that several proteins have been shown to be localized by both the Tat and Sec pathways *in vitro,* and thus have similarities in targeting specificity, supports the idea that some functional redundancy is beneficial.

Sec-Dependent Targeting

The chloroplast sec<u></u>retory (Sec) pathway translocates soluble thylakoid lumen proteins and integral thylakoid membrane proteins (for review see (Cline, 2003)). Similarly, homologous bacterial and eukaryotic Sec pathways are responsible for the translocation/insertion of many membrane and soluble proteins across the plasma

membrane or endoplasmic reticulum respectively (for reviews see (Osborne *et al.*, 2005; Bibi, 2007)). All Sec-dependent substrates are threaded through the hydrophilic interior of the Sec channel in an unfolded manner. The core of the Sec translocon consists of an oligomer of heterotrimeric integral membrane proteins, identified as SecYEG and Sec61αγβ in bacteria and eukaryotes respectively (see Figure 1.3). For the chloroplast Sec translocon, only homologues to SecY and SecE have been identified. CpSecY (SecY/Sec61α) and cpSecE (SecE/Sec61γ) are minimally required for formation of a functional translocon; they appear to form large ring structures composed of 3-4 subunits each. In bacteria, maximal rates of translocation are supported with additional integral membrane proteins: SecG, SecD, SecF, and SecyajC. Chloroplast homologues to these proteins have not been identified, therefore chloroplast Sec-dependent targeting appears to function with the minimum required components.

The Sec translocon is utilized for both post-translational and co-translational transport and can work in different translocation modes depending upon the organism and which soluble components are involved. Generally, a Sec-dependent substrate contains a signal peptide with three characteristic regions: a positively-charged amino acid at the N-terminus, a highly-hydrophobic segment, and a polar region containing a signal peptidase cleavage site. Whether a Sec-dependent substrate is routed in a co-translational or post-translational manner is also determined by characteristics of the signal sequence; co-translationally targeted substrates bear a signal sequence with helical structure in the hydrophobic segment (Adams *et al.*, 2002). If the signal sequence has sufficient hydrophobicity and helicity, the substrate is recognized by a soluble factor known as the signal recognition particle (SRP). Cytosolic SRPs usually function in a co-translational

targeting mechanism, bringing a ribosome bearing an appropriate nascent chain signal to the target membrane. In targeting to the endoplasmic reticulum, translation is paused during SRP-dependent targeting and resumed upon interaction with the translocon. The co-translational mode utilizes the translating ribosome as an energy source for translocation. In the second mode, post-translational translocation, the substrate is bound by cytosolic chaperones SecA or SecB. Though SecA association with substrates has been thought to be a membrane event, SecA has been shown to interact with Sec signal peptides in either an aqueous or membrane environment, indicating the possibility that SecA also plays a role in substrate transport (Wang *et al.*, 2000). This chaperone binding likely serves to keep the targeted protein in a soluble state until translocation can take place. Importantly, the majority of thylakoid proteins are imported into chloroplasts as fully-synthesized substrates. Hence, chloroplast Sec-dependent translocation is primarily post-translational.

Post-translational targeting to the cpSec translocon requires cpSecA, ATP, and a proton motive force (see Figure 1.3) (for reviews see (Eichler and Duong, 2004; Osborne *et al.*, 2005; Bibi, 2007)). CpSecA, the motor protein that drives protein translocation, is an ATPase capable of partitioning between the thylakoids and stroma by means of an interaction with acidic lipids and the cpSecYE-containing translocon. In bacteria, a fully-translated SecA-dependent substrate is maintained in a translocation-competent state by association with a tetramer of SecB. Acting as a molecular chaperone, SecB brings the substrate to the Sec translocon via an affinity for a SecA homodimer. A chloroplast homologue of SecB has not been identified, suggesting again the frugality of chloroplast targeting. Upon substrate interaction, homodimeric SecA binds ATP and undergoes

conformational changes that drive 20-30 amino acids (~2.5 kDa) of the substrate through the SecYE pore. Recently, it has been shown that the hourglass-shaped Sec translocon pore does not bind the substrate, but instead simply provides friction to keep the substrate from moving backwards during translocation (Erlandson *et al.*, 2008). As ATP hydrolysis causes dissociation of SecA from both the membrane and substrate, another SecA dimer quickly takes its place. Several cycles of SecA insertion/release are needed for a single protein to be translocated.

In *E. coli*, YidC has also been found to associate with the bacterial Sec translocon. YidC is a member of the YidC/Oxa1/Alb3 (bacteria/mitochondria/chloroplast) family of polytopic membrane proteins that assist the transition of transmembrane portions of translocating membrane proteins into bilayers. The association of YidC and the Sec translocon is a powerful combination with the ability to translocate soluble portions across and insert transmembrane regions into a bilayer. Recently, a novel pathway requiring both the Sec translocon and YidC was described for the insertion of a subunit of cytochrome o oxidase (du Plessis *et al.*, 2006). Because YidC depleted cells exhibit large-scale losses in biogenesis of respiratory chain complexes, it has been suggested that this novel pathway is utilized by many key players in these complexes (van der Laan *et al.*, 2003). Similarly, Alb3, a thylakoid homologue of YidC, appears to function alone to insert/assemble post-translationally transported substrates and in conjunction with cpSecY to insert/assemble co-translationally transported substrates. In contrast, the Secαγβ complex appears to translocate proteins without the assistance of a YidC homologue as none have been identified in eukaryotes (excluding chloroplasts and mitochondria).

Signal Recognition Particle-Dependent Targeting

The signal recognition particle (SRP) pathway is a protein targeting system responsible for delivering integral membrane proteins and secretory proteins to the appropriate translocons (see Figure 1.4). Remarkably, SRP-dependent targeting is conserved in all kingdoms of life. The most well studied examples of SRP-dependent targeting include proteins that are co-translationally targeted to the endoplasmic reticulum in eukaryotes and to the plasma membrane in prokaryotes (for reviews see (Keenan *et al.*, 2001; Doudna and Batey, 2004; Egea *et al.*, 2005; Pool, 2005; Bibi, 2007). Unlike cytosolic SRPs that must first interact with a ribosome to interact with signal peptides, the chloroplast SRP is novel in the sense that it can function in the absence of a ribosome to bind and post-translationally target proteins to a destination membrane. Hence, cpSRP is structurally and functionally specialized for post-translational protein targeting, however the general targeting steps are similar to that of prokaryotic or mammalian SRP.

Prokaryotic and mammalian SRP bring targeted proteins to the Sec translocon in a co-translational manner. If an emerging nascent chain signal sequence is sufficiently hydrophobic and helical, the substrate is recognized by ribosome-bound SRP. In eukaryotes, it has been shown that binding of the SRP to the ribosome-nascent chain (RNC) complex pauses translation. The RNC-SRP complex is guided to the target membrane via SRP's affinity for an SRP receptor (SR) protein at the membrane. The SR and SRP contain homologous GTPase domains and form a heterodimer with an extensive interaction face that spans their GTPase domains and places their bound nucleotides

across from each other. At the membrane, the RNC-SRP/SR complex interacts with the Sec translocon. In endoplasmic reticulum targeting, upon transfer of the substrate to the translocon, translation resumes. The translating ribosome provides the driving force for pushing the substrate through the pore. Finally, the SRP and SR simultaneously hydrolyze GTP, breaking the supercomplex apart for another round of targeting.

Though the core SRP components and the mechanism of GTP-dependent targeting are highly conserved, the system ranges in complexity (see Figure 1.5). Several reviews are available regarding the structure, function, and evolution of the SRP components (Lutcke, 1995; Agarraberes and Dice, 2001; Keenan *et al.*, 2001; Nagai *et al.*, 2003; Doudna and Batey, 2004; Pool, 2005). In the mammalian system, the SRP consists of one RNA molecule (7SL RNA) and six proteins named according to their apparent molecular weight, SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72. The corresponding SR consists of two proteins, SRα and SRβ, both containing GTPase activity. SRα and SRP54 are evolutionarily related and share N and G (GTPase) domains with similar structure. In archaea, the SRP contains an RNA molecule (7S RNA) and two proteins, SRP19 and SRP54. Archaea SR is simplified to an SRα homologue called FtsY. Similarly, SRP systems found in eubacteria such as *E. coli* contain a shorter RNA molecule (4.5S RNA), a homologue of SRP54 known as Ffh (fifty-four homologue), and FtsY, the SRα homologue.

The chloroplast SRP (cpSRP) system consists of the conserved SRP54 (cpSRP54) and SRα (cpFtsY) homologues and an approximately 43 kDa protein (cpSRP43) not found in any other SRP system. Co-translational targeting of chloroplast-synthesized proteins is independent of cpSRP43, but requires cpSRP54 and cpFtsY. The translocon

utilized during co-translational cpSRP targeting is not well-defined. In contrast, post-translational protein targeting to an Alb3 translocase requires both SRP subunits and cpFtsY (Tzvetkova-Chevolleau *et al.*, 2007). The model substrate for post-translational cpSRP-dependent protein targeting is the gene product of *lhcb1*, a light-harvesting chlorophyll-binding protein (LHCP), which we commonly refer to as LHCP. Together cpSRP43 and cpSRP54 hold LHCP in transit in an integration-competent state by interacting with an 18-amino acid motif located between the second and third transmembrane domains of LHCP and a hydrophobic domain, respectively.

Lhca and *Lhcb* designate genes corresponding to the LHCPs of photosystems I and II, respectively (Jansson *et al.*, 1992). Six *Lhca* and six *Lhcb* gene families have been described to date (reviewed in (Jensen *et al.*, 2007; van Amerongen and Croce, 2008)). Though Lhcb1 has been the object of most *in vitro* studies, all of the Lhca and Lhcb members in *Arabidopsis thaliana* are nuclear-encoded and contain sequence homologous (50-83% identity) to the 18 amino acid SRP-binding region in *Pisum sativum* Lhcb1 (L18) (Cline, 2003; Jensen *et al.*, 2007). Due to the conservation of the SRP-binding region, it is probable that like Lhcb1, LHCP homologues are also localized to thylakoids by the cpSRP-dependent targeting pathway. In agreement with this data, treatment of thylakoids using Alb3 antibody diminishes the integration of at least Lhcb1, Lhcb4.1, and Lhcb5 (Moore *et al.*, 2000; Woolhead *et al.*, 2001)

Presumably, the pathway steps of cpSRP targeting are similar those of cytosolic SRPs, yet the absence of a ribosome and presence of a unique subunit lend themselves to distinct differences. Current research findings support the following brief model of cpSRP-dependent protein targeting of post-translationally targeted substrates. After the

precursor form of LHCP is imported into the chloroplast, a stromal processing peptidase removes the chloroplast targeting peptide. CpSRP recognizes and binds to cpSRP-binding motif in mature LHCP forming a transient intermediate termed transit complex. This transit complex subsequently interacts with the SRP receptor (cpFtsY) on the thylakoid membrane prior to interaction with the translocase, Alb3. Upon interaction of cpSRP, cpFtsY, and Alb3, the protein substrate is most likely transferred to Alb3, although an LHCP-Alb3 interaction has never been demonstrated. Simultaneous GTP hydrolysis by cpSRP54 and cpFtsY releases the protein components for subsequent rounds of targeting.

Many questions remain concerning the orchestration and timing of cpSRP-dependent targeting events that take place at the membrane. How does the SRP receptor protein interact with thylakoid membranes? In eukaryotes, the SR is composed of two proteins, SRα and SRβ. SRα is held at the endoplasmic reticulum by its association with SRβ, an integral membrane component. For the bacterial SRα homologue, FtsY, no SRβ counterpart has been identified. Instead, FtsY is capable of partitioning on and off the target membrane, thought to be due to a large N-terminal acidic domain (~200 residues in length) that interacts with phospholipid head groups. CpFtsY, in comparison to FtsY, contains a much shorter, less acidic N-terminal region (~20 residues in length), yet also exhibits the ability to partition on and off thylakoid membranes. Hence, the identification and characterization of cpFtsY's membrane binding region is likely to reveal a core structural requirement for this activity.

Secondly, which cpSRP components facilitate interaction with the Alb3 translocon? Functional association with Alb3 must reside in the soluble targeting

components since LHCP is not required for association of Alb3 with cpSRP and cpFtsY (Moore *et al.*, 2003).

Finally, GTP hydrolysis between the cpSRP GTPases, cpSRP54 and cpFtsY, must be finely-controlled for execution only at the appropriate time in the targeting process. Premature GTP hydrolysis between the SRP/SR GTPases must be prevented to ensure productive transfer of substrate to the translocation channel. (For a review of the regulation of GTP hydrolysis during SRP targeting, see Chapter V.) As such, perhaps the most interesting (and most complex) question that remains is what components regulate the membrane-associated steps of the GTPase cycle for the cpSRP GTPases, cpSRP54 and cpFtsY?

Even more mystery surrounds the steps following LHCP delivery to the membrane. Following cpSRP-mediated routing, LHCP is presumably inserted into thylakoids as a monomer, undergoes ligand (chlorophyll) attachment, and is assembled into trimers that function in light harvesting. Alb3 is necessary for LHCP insertion and has been implicated in the folding/assembly process of chloroplast-synthesized reaction center-binding proteins. Because LHCP stability is dependent upon chlorophyll biosynthesis, and vice versa, it has long been proposed that chlorophyll attachment occurs during LHCP insertion and assembly. Does LHCP insertion/assembly take place in conjunction with chlorophyll biosynthesis and attachment? If so, we may expect to find late-stage chlorophyll biosynthesis enzymes associated with complexes containing Alb3.

Several questions remain concerning the events that must take place for SRP-dependent targeting at the membrane interface. How are the SRP components localized to the target membrane? Which components interact with the translocon? How is GTP

hydrolysis regulated so that it only occurs at the right step in the targeting cycle? Which components and interactions trigger the hydrolysis of GTP by the SRP GTPases? Is LHCP insertion dependent on other thylakoid-associated proteins, such as Chl biosynthesis enzymes? In answering the preceding questions, work presented in this thesis provides an understanding of events that take place at the membrane interface merging SRP-dependent protein targeting to Alb3-dependent insertion and assembly.

The studies described in **chapter II** confirm that cpFtsY, like bacterial FtsY, houses a membrane binding region whose activity is linked to the SRP GTPase cycle. We identified an amphipathic helix located at the N-terminus of the mature cpFtsY protein that is necessary and sufficient for binding thylakoid membranes. When fused to a soluble protein, the membrane binding region stably tethers the attached protein to thylakoids. Interestingly, the removal or mutation of key residues in this region of cpFtsY results in a higher basal rate of GTP hydrolysis in solution. Furthermore, these changes correspond to a loss of lipid-induced hydrolysis stimulation. We propose that the membrane binding region houses a negative regulator of hydrolysis that becomes naturally switched off by a membrane-induced conformational shift.

In **chapter III**, we show that cpSRP43 contributes to the specificity for the targeting reaction by interacting with the C-terminal region of Alb3. Furthermore, a peptide corresponding to the C-terminal region of Alb3 stimulates cpSRP GTP hydrolysis only in the presence of cpSRP43. These results suggest that cpSRP43 mediates Alb3-dependent stimulation of hydrolysis between cpSRP54 and cpFtsY.

The experiments in **chapter IV** suggest that Alb3-dependent LHCP insertion is linked to the final stages of Chl biosynthesis. Indeed, we have identified two pools of

Alb3: one that is associated with SRP targeting components and one that is associated with a late-stage chlorophyll biosynthesis enzyme (geranylgeranyl reductase). We believe this is indicative of a switch in activity from LHCP localization and insertion involving SRP targeting components to LHCP folding and assembly correlating with the late stages of Chl biosynthesis.

Chapter V contains a summary of the findings presented here in light of current research concerning SRP protein targeting systems. Subsequently, a current cpSRP-targeting model is presented with a discussion of the questions that remain to be addressed.

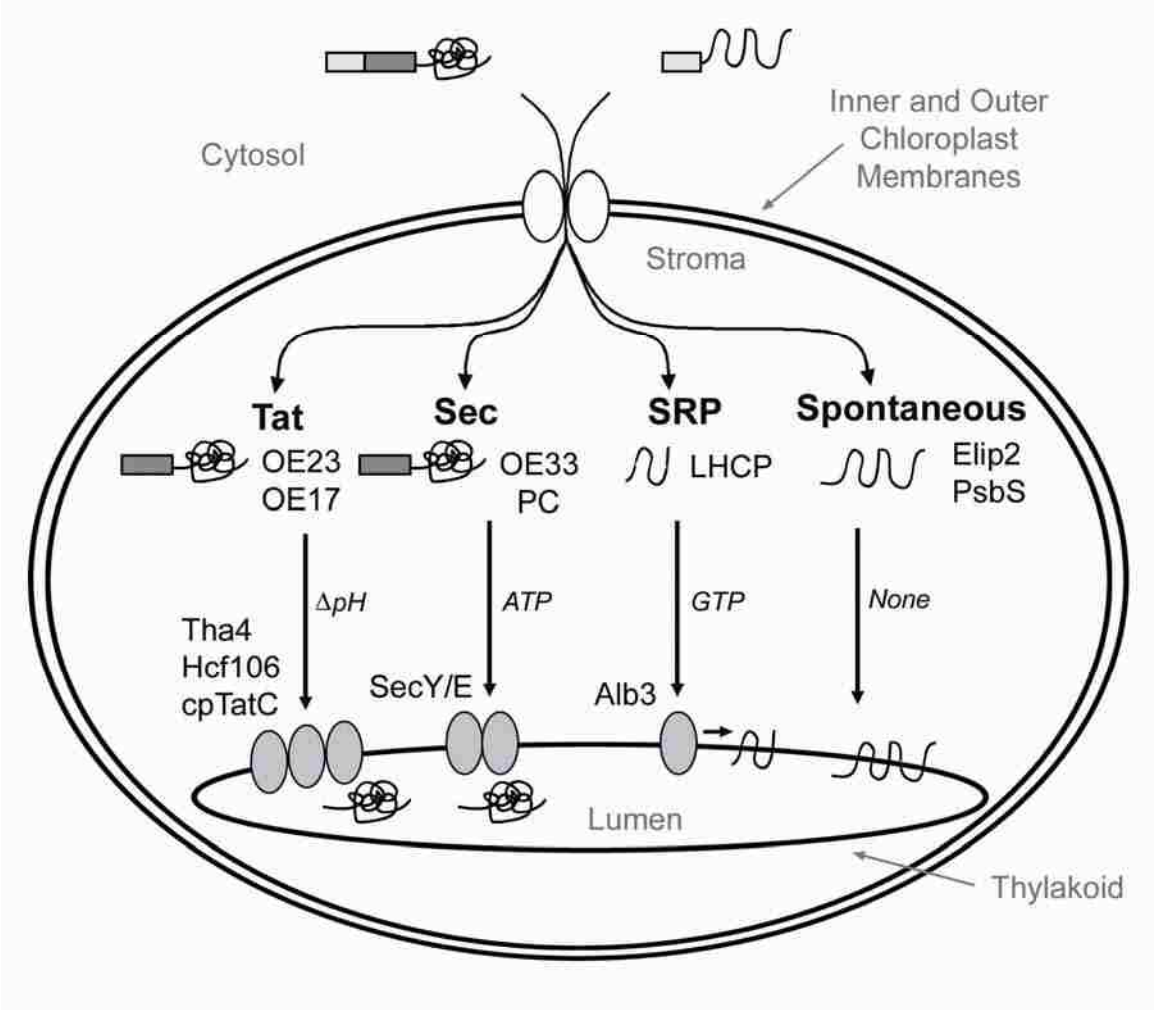

Figure 1.1. Model for nuclear-encoded thylakoid protein localization.

Precursor proteins are shown as synthesized in the cytosol with an N-terminal chloroplast targeting sequence (light gray rectangle) followed by a lumen targeting domain (dark gray rectangle) on proteins destined for the thylakoid lumen. Once imported into the chloroplast through the translocase of the outer and inner membranes or Toc/Tic (white ovals), proteins either spontaneously insert into thylakoids or are localized by one of three targeting/translocation pathways: cpTat, cpSec, or cpSRP. Pathway substrates are indicated by labels near the model proteins. Energetic requirements for transport are shown in italic letters beside the arrow. The membrane translocase used by each pathway is shown in gray and labeled with required membrane components.

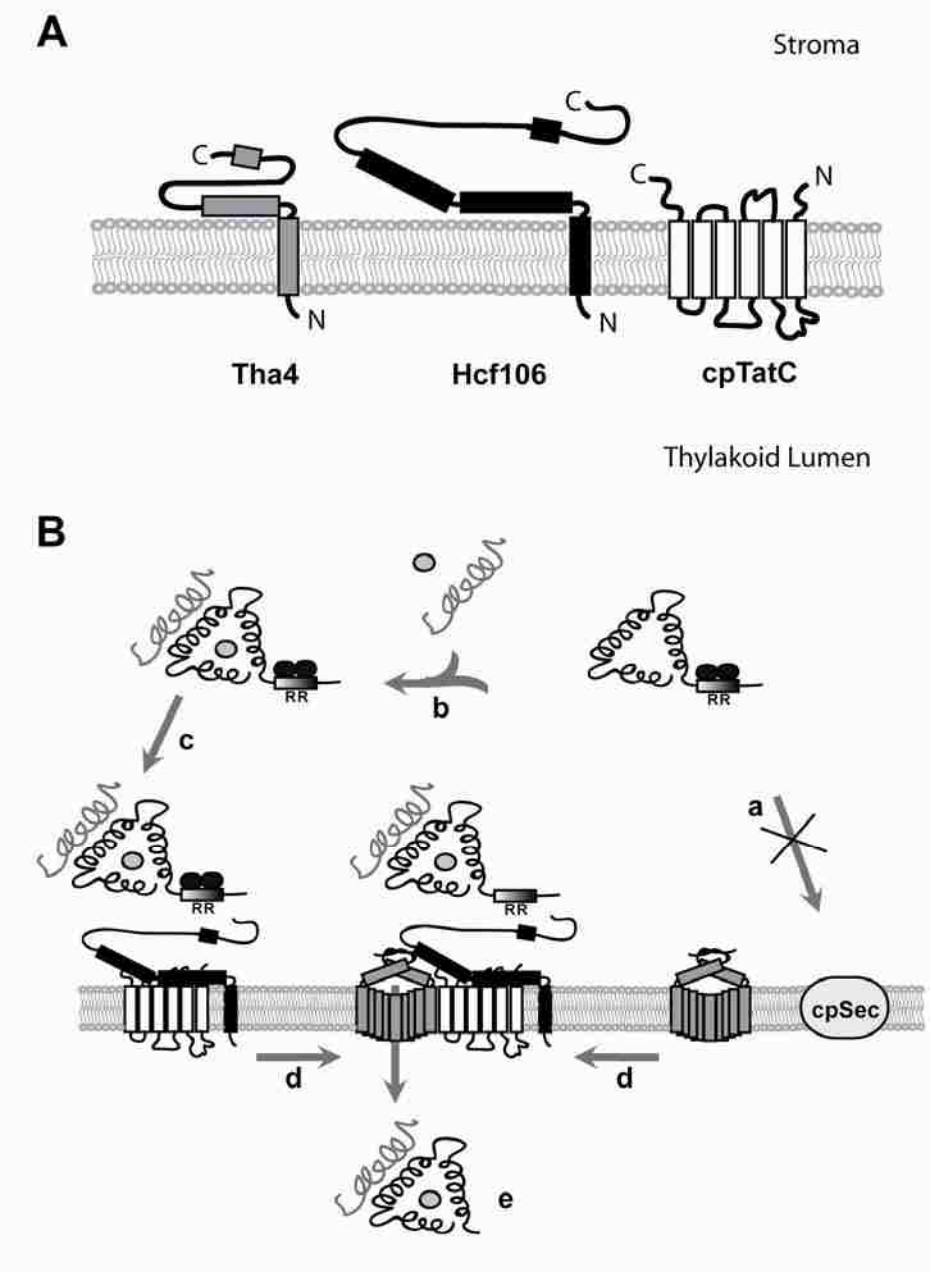

Figure 1.2. Model of Tat targeting and translocation.

A) The predicted structure and topology of the chloroplast and *E. coli* Tat components. Predicted helical regions are shown in boxes. **B**) (*a*) Upon emerging from the ribosome, the binding of Tat-specific chaperones (black circles) and/or characteristics of the Tat signal sequence and mature protein divert the preprotein from other pathways such as the Sec-dependent pathway. (*b*) After folding, any additional subunits and cofactors are added. (*c*) Folded protein is targeted to the Hcf106/cpTatC receptor complex. (*d*) An active translocation channel is formed by the addition of a Tha4/Tha9 homooligomeric complex to the Hcf106/cpTatC substrate complex. (*e*) Following translocation through a pore consisting mainly of Tha4/Tha9, the signal sequence is removed and the mature protein is released to the thylakoid lumen. These figures adapted from Lee et al., 2006.

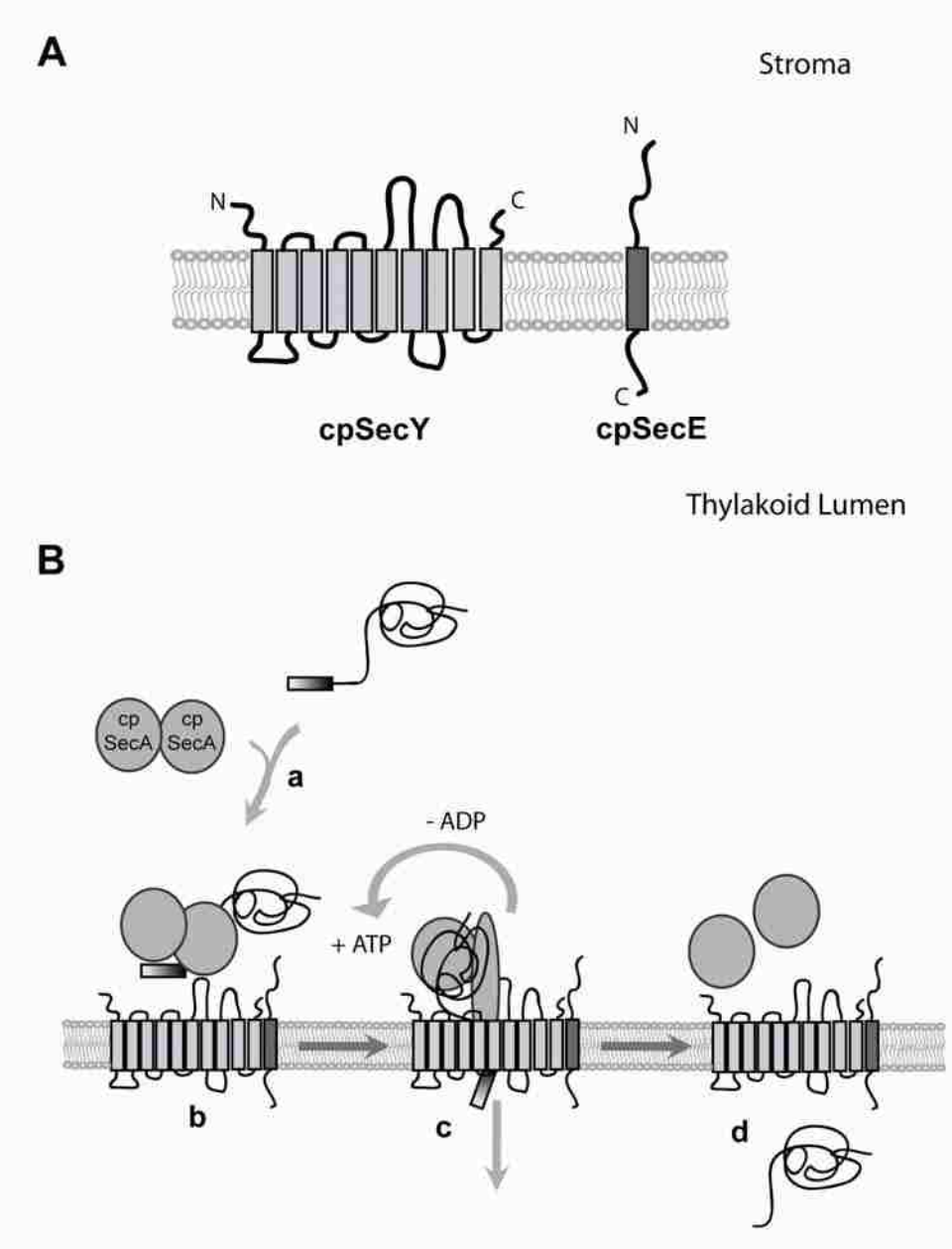

Figure 1.3. Model of Sec-dependent targeting and translocation.

A) The predicted structure and topology of the chloroplast Sec membrane components. Predicted transmembrane regions are shown in boxes. **B**) (*a*) Characteristics of the Sec signal sequence and mature protein route the preprotein to the Sec-dependent pathway. (*b*) CpSecA binds the substrate and (*c*) ATP, driving a portion of the substrate through the Sec translocon. As cpSecA hydrolyzes ATP, the substrate is released and cpSecA dissociates from the translocon. (*d*) CpSecA molecules repeat steps *b* and *c* in succession until the substrate is translocated across the membrane.

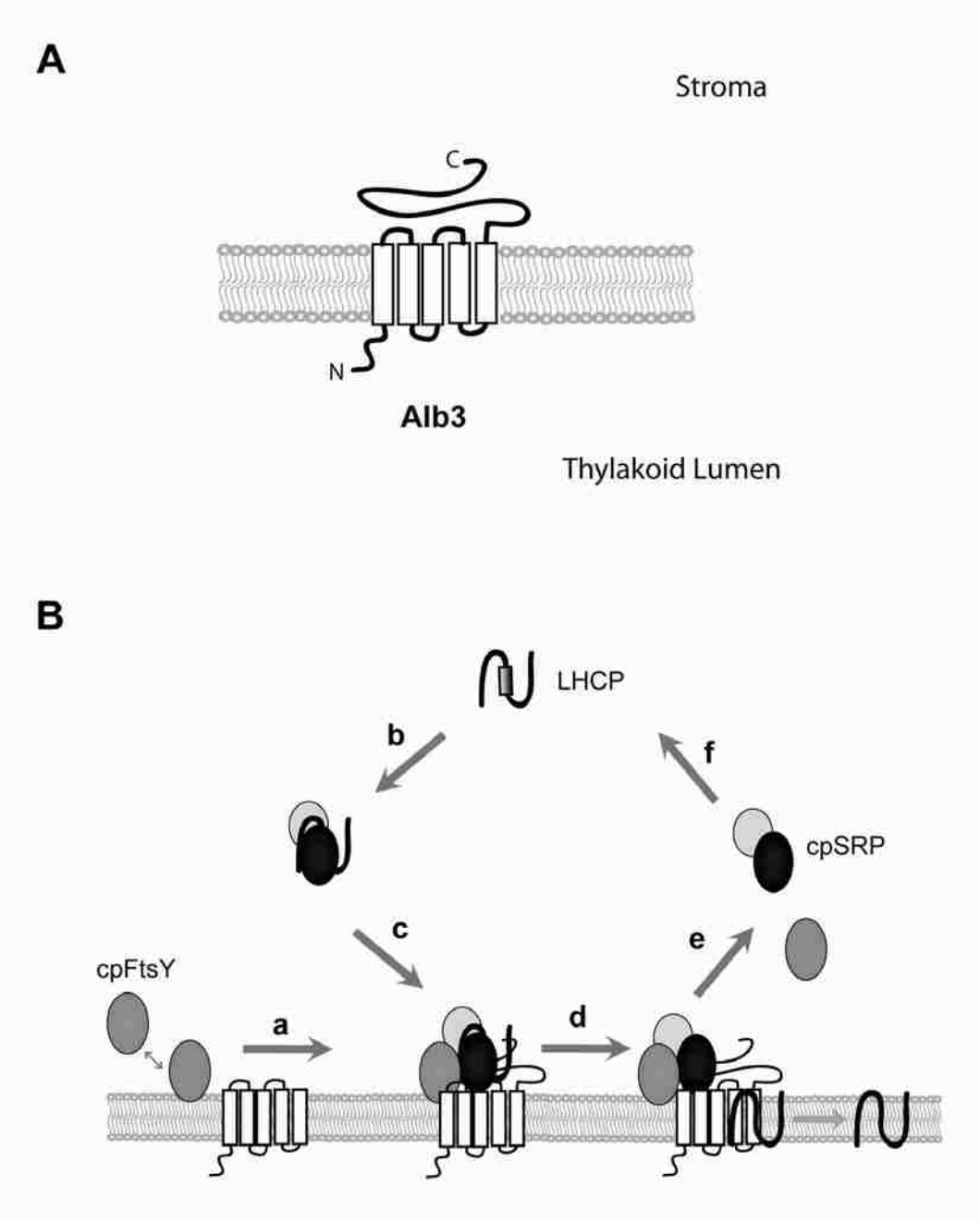

Figure 1.4. Model of cpSRP-dependent targeting and translocation.

A) The predicted structure and topology of the chloroplast SRP membrane translocase, Alb3. Predicted transmembrane regions are shown in boxes. **B**) (*a*) CpSRP recognizes and binds a signal sequence (gradient box) in the mature sequence of LHCP. (*b*) The cpSRP receptor, cpFtsY partitions onto the thylakoid membrane. (*c*) The cpSRP is brought to the thylakoid membrane, and subsequently Alb3, via its affinity for cpFtsY. (*d*) LHCP is released to Alb3 and (*e*) cpSRP and cpFtsY hydrolyze GTP (*f*) breaking the complex apart for another cycle.

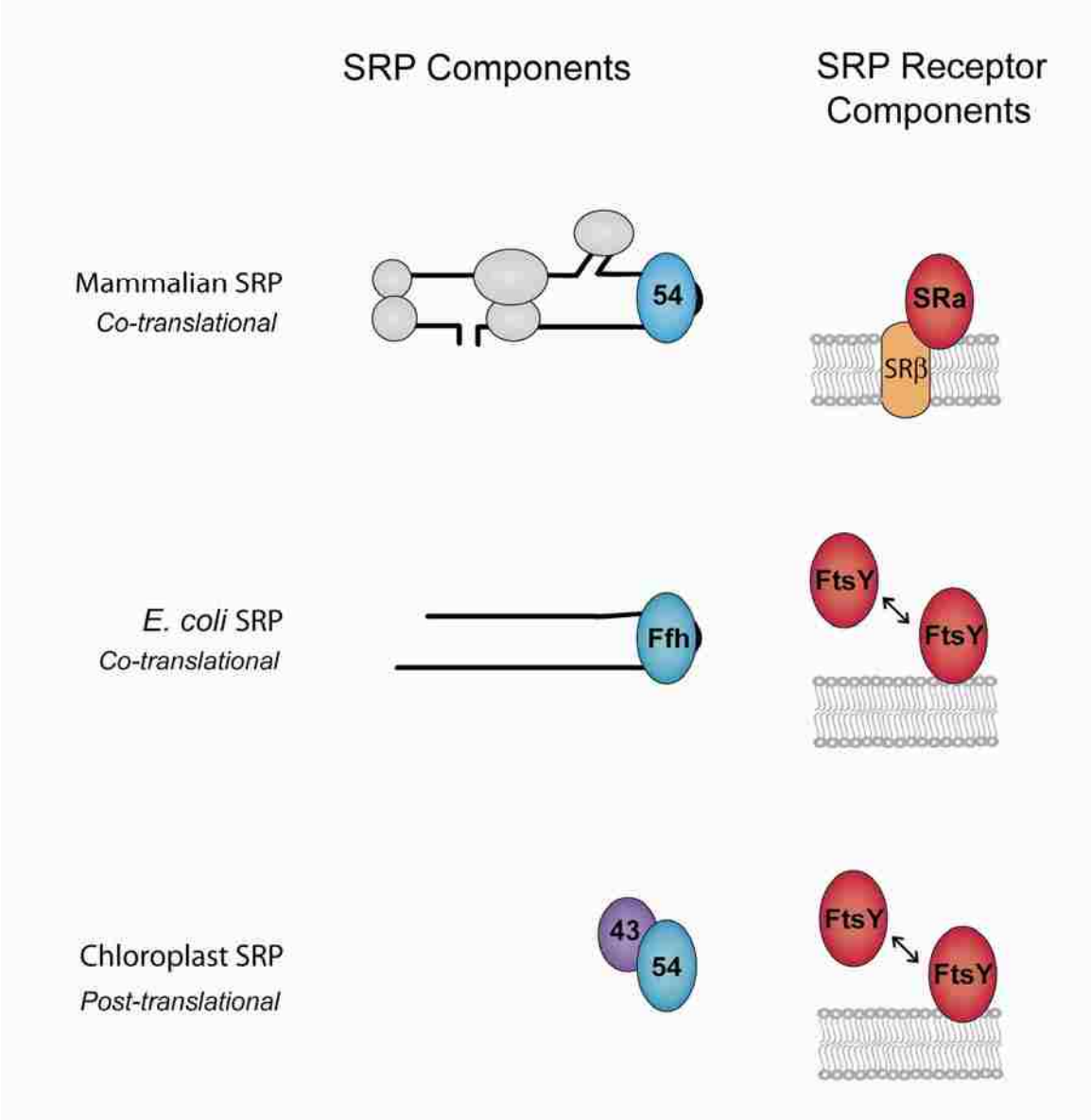

Figure 1.5. Comparison of SRP and SRP receptor composition from eukaryotes, bacteria, and chloroplasts.

SRP RNA moieties are depicted as black lines. SRP polypeptides are indicated by gray or black shaded ovals. Conserved SRP subunits are identified by abbreviated names: eukaryotic SRP54, 54; bacterial fifty-four homologue, Ffh; and chloroplast SRP54, 54. The unique 43 kDa subunit of the cpSRP is also indicated by the label 43. Conserved SRP receptor subunits are also identified by abbreviated names: eukaryotic SRα/SRβ, SRα/SRβ; bacterial FtsY, FtsY; and chloroplast FtsY, FtsY.

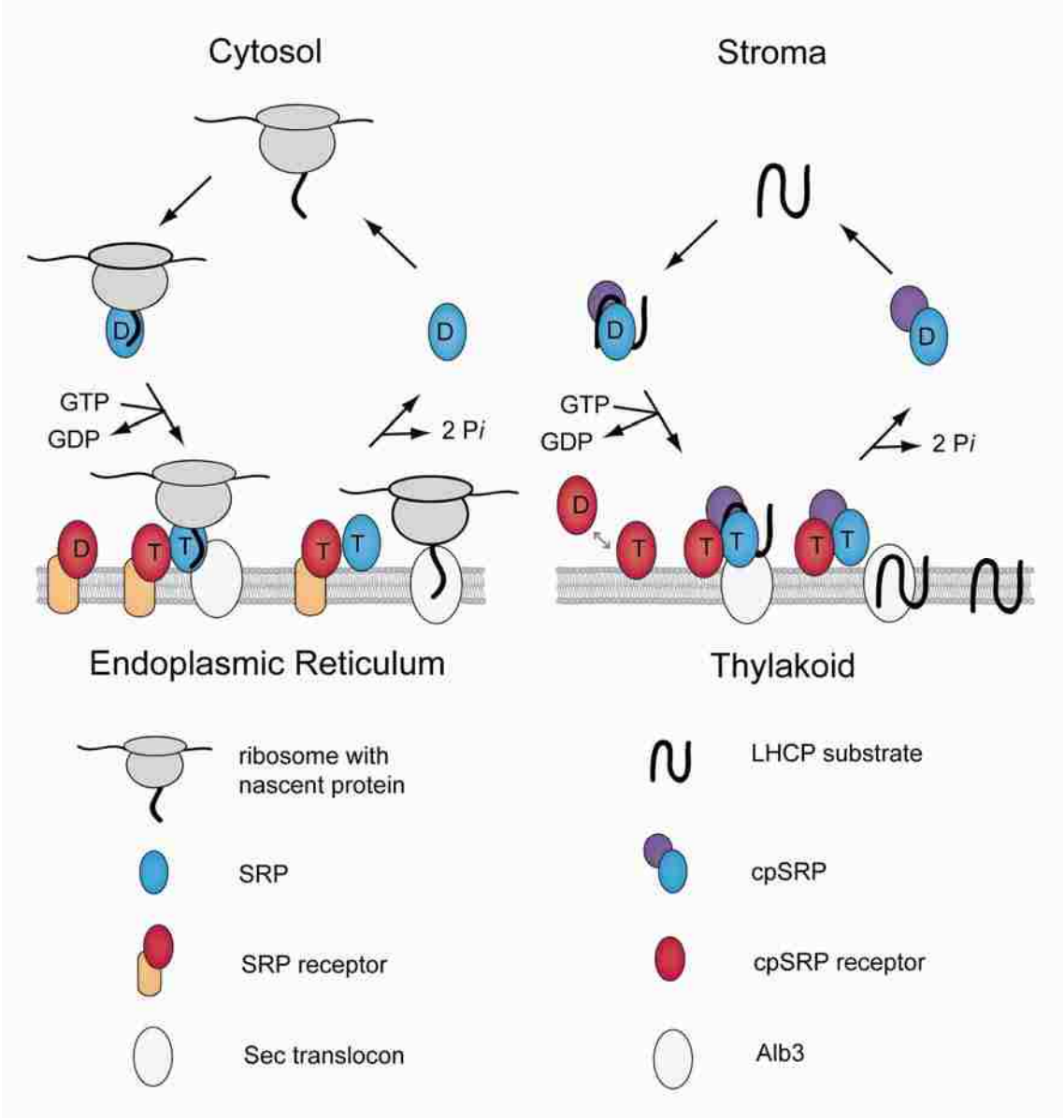

Figure 1.6. Comparison of mammalian and chloroplast SRP-dependent protein targeting.

The model on the left illustrates details of mammalian SRP-dependent protein targeting to the endoplasmic reticulum. The model on the right illustrates details of chloroplast SRP-dependent protein targeting to thylakoid membranes. Nucleotides bound to the SRP GTPases are shown as GTP (T) or GDP (D). In the mammalian system, SRP binds the targeted substrate as a nascent chain emerging from a ribosome, whereas, the chloroplast SRP (cpSRP) recognizes a fully-translated polypeptide (LHCP). The targeted substrate is delivered to the target membrane as SRP interacts with its receptor. Upon arrival to the membrane, SRP releases its cargo to the membrane translocon. Once the substrate is released, both SRP and its receptor hydrolyze GTP, releasing the components to be recycled.

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II

MEMBRANE PARTITIONING AND ACTIVITY OF CPFTSY RELIES ON A CONSERVED MEMBRANE-BINDING MOTIF

Parts of this research accepted for publication as:

Marty, N, D Rajalingam, AD Kight, NE Lewis, D Fologea, TKS Kumar, RL Henry, and RL Goforth. (2009) The membrane-binding motif of chloroplast signal recognition particle receptor (cpFtsY) regulates GTPase activity. ***J. Biol. Chem.***

SUMMARY

Prokaryotic and eukaryotic signal recognition particles (SRPs) differ in the ability of the receptor to partition between the membrane and soluble phase during protein targeting. However the regulation of the SRP particle receptor partitioning and the conformation of the membrane-bound state remain unclear. Using the chloroplast SRP receptor, we have identified a small N-terminal region responsible for stabilizing a membrane interaction critical to the targeting reaction. Functional studies of this region reveal that it is both necessary and sufficient for binding the target membrane. Furthermore, NMR and CD structural studies of this region and a similar region in the *E. coli* SRP receptor reveal a conformational change in secondary structure that takes place upon lipid binding. These studies suggest a conserved mechanism for both membrane binding and the intramolecular communication that regulates SRP receptor functions at the membrane.

INTRODUCTION

Proper compartmentalization of proteins relies on the ability of protein localization pathways to transport proteins efficiently from their site of synthesis to their site of function. Signal recognition particle (SRP) and its receptor function in every kingdom of life to target proteins to the endoplasmic reticulum (eukaryotes), cytoplasmic membrane (prokaryotes), and thylakoid membrane (chloroplasts) (Pool, 2005). The targeting function of SRP relies on a conserved 54 kDa SRP subunit (SRP54; Ffh in *E. coli*, cpSRP54 in chloroplasts) as well as a conserved SRP receptor (SRα; FtsY in *E. coli*, cpFtsY in chloroplasts). Both SRP54 and its receptor are GTPases and GTP binding by both proteins in eukaryotes and prokaryotes enables interaction of the SRP-ribosome nascent chain complex with SRα at the membrane. GTP binding and hydrolysis by both SRP54 and SRα coordinates substrate release from SRP to the translocon and release of SRP from SRα. In chloroplasts, cpFtsY functions along with a unique SRP (cpSRP) to post-translationally target nuclear-encoded proteins to thylakoid membranes (Henry *et al.*, 2007). Light-harvesting chlorophyll a/b-binding proteins (LHCPs) imported into the chloroplast stroma are bound by cpSRP to form a soluble targeting complex, which directs the LHCP substrate to the thylakoid membrane translocon Albino3 (Alb3) in a GTP- and cpFtsY-dependent manner (Moore *et al.*, 2003; Asakura *et al.*, 2004). While many general steps of SRP protein targeting seem largely conserved across evolutionary boundaries, the nature and dynamics of the receptor appear to have diverged.

In eukaryotic systems, SRα is peripherally bound to the membrane through association with the integral membrane subunit SRβ. In contrast, no chloroplast nor bacterial homologue of SRβ has been identified. CpFtsY and *E. coli* FtsY (EcFtsY) are

found partitioned between the membrane and the stroma or cytosol respectively, via a mechanism that is not well understood. The membrane binding capacity of EcFtsY serves to stimulate GTPase activity and appears critical in that only membrane-associated EcFtsY supports the release of nascent chains from SRP to the translocon (Valent *et al.*, 1998; de Leeuw *et al.*, 2000). However, the partitioning activity is not strictly required since EcFtsY tethered to the membrane is functional *in vivo* (Zelazny *et al.*, 1997). Given the conserved nature of partitioning among bacterial and chloroplast SRP receptors, partitioning may play an as of yet unidentified role in protein targeting by SRP. Nevertheless, differences in lipid composition between bacterial and thylakoid membranes make it interesting to speculate that there are mechanistic differences in membrane partitioning.

CpFtsY, like many prokaryotic FtsY homologues (e.g. *Thermus aquaticus*), lacks the N-terminal acidic A domain implicated in EcFtsY membrane binding (Samuelsson and Zwieb, 1999). Sequence alignment reveals that the residues of cpFtsY N-terminal to the NG domain are not conserved among SRP receptor proteins, with the exception of a double Phe motif commonly found in bacterial SRP receptors. Although the NG GTPase domain of EcFtsY ($EcFtsY_{NG}$) fails to support protein targeting, addition of the last A domain residue, Phe196 of a conserved double Phe motif ($EcFtsY_{NG+1}$), restores protein targeting *in vivo* (Eitan and Bibi, 2004). *In vitro* studies also show that $EcFtsY_{NG+1}$ retains the capacity to bind membranes and support integration of SRP-dependent substrates, though at significantly reduced levels compared to full-length EcFtsY (Angelini *et al.*, 2006). For cpFtsY, the necessity and functional role(s) of partitioning between a thylakoid bound and soluble phase, as well as the role of N-terminal residues

in these functions, remains unknown. In addition, both the conformational state of membrane-bound cpFtsY and EcFtsY as well as the mechanism responsible for controlling membrane partitioning and altered GTPase activity remain unclear. Due to the gain of function exhibited by $EcFtsY_{NG+1}$, we hypothesized that this conserved double Phe motif is necessary to support membrane binding and corresponding functions not only in *E. coli* FtsY, but also in FtsY homologues, including cpFtsY.

To examine the functional role of the N-terminal region of cpFtsY, we have utilized deletion and point mutants in assays that reconstitute cpFtsY activities, including the cpSRP-dependent integration of LHCP. We have also determined the three-dimensional solution structure of a cpFtsY N-terminal peptide in order to understand the structural determinants critical for interaction of cpFtsY with a lipid bilayer. Together, our data indicate that an N-terminal membrane-binding motif flanked by several conserved residues is both necessary and sufficient for thylakoid membrane binding and critical for proper LHCP targeting. Moreover, this region appears to contain a structural switch that modulates the ability of cpFtsY to partition to thylakoid membranes and function in the cpSRP targeting pathway. Liposome-induced structural changes within the cpFtsY N-terminal peptide, as well as in a peptide corresponding to an aligned region within *E. coli* FtsY, suggest that the structural switch mechanism is conserved among SRP receptor homologues. Furthermore, these lipid-induced structural changes may constitute a conserved mechanism for regulating bacterial SRP receptor functions unique to the membrane-bound state.

MATERIALS AND METHODS

All reagents and enzymes used were purchased commercially. All primers were from Integrated DNA Technologies. The plasmid used for *in vitro* transcription and translation of pLHCP (psAB80XD/4) has been described (Cline *et al.*, 1989)). Recombinant purified cpSRP43, cpFtsY, and cpSRP54 were prepared as described with the exception of a new restriction site (XhoI) for cpFtsY (Yuan *et al.*, 2002; Goforth *et al.*, 2004; Jaru-Ampornpan *et al.*, 2007).

Construction of cpFtsY and cpSRP43 Clones

Forward and reverse primers were designed to match the mature coding sequence of *A. thaliana* cpFtsY starting with the predicted mature sequence CSAGPSGF and to include KpnI and XbaI sites, respectively, for ligation into pGEM-4Z. The forward primer also included extra bases cacg at the 5' end which encode a Kozak sequence (cacgatgg) when added to the atg of the initiator methionine. The resulting PCR fragment was restricted with KpnI and XbaI, then ligated into similarly-restricted pGEM-4Z to create the plasmid cpFtsY-pGEM-4Z. The same process was utilized with appropriately-designed forward primers to create the following deletion (Δ) and residue-replacement mutants of the mature form of cpFtsY: Δ41-43, Δ41-46, Δ41-49, Δ41-52, Δ41-56, Δ41-47 (or $cpFtsY_{NG+2}$), Δ41-48 (or $cpFtsY_{NG+1}$), F48A, F49A, F48A/F49A, F48G, F48V, F48L, F48E, F48Q, F48K, F48Y, and F48W. CpFtsY-F48A was subcloned out of pGEM-4Z using KpnI and HindIII and inserted into pET-32b expression vector (Novagen) using KpnI and XbaI. This plasmid was transformed into BL21* (Invitrogen) and used for expression of cpFtsY-F48A.

The chimeric sequence for Tha4TM-cpFtsY is an exact fusion of the mature *Pisum sativum* Tha4 transmembrane and hinge region with the mature *A. thaliana* cpFtsY that was constructed by overlap extension (Horton et al., 1989). Forward and reverse primers were designed to match residues of the transmembrane and hinge region of *P. sativum* Tha4 beginning with AFFGLG and ending with VFGPKK. The forward primer also included a 5' BamHI site and the extra bases cacg at the 5' end which encode a Kozak sequence (cacgatgg) when added to the atg of the initiator methionine of the precursor sequence. Forward and reverse primers were designed to match residues of the mature coding sequence of *A. thaliana* cpFtsY beginning CSAGPS and including a 3' HindIII site. PCR fragments were spliced by overlap extension, restricted with BamHI and HindIII, and then ligated into similarly-restricted pGEM-4Z to create the plasmid Tha4TM-cpFtsY. This plasmid was used for *in vitro* transcription/translation of Tha4TM-cpFtsY. The same process was utilized with appropriately-designed forward primers to create Tha4TM-F48A, and Tha4TM-F48A/F49A.

CpSRP43 transcription/translation clone was designed using forward and reverse primers to match the mature predicted sequence of *A. thaliana* cpSRP43 beginning with AAVQRNYE and including a Kozak sequence, and BamHI and XhoI restriction sites for insertion into similarly-restricted pGEM-7Z. The PCR fragment obtained was restricted with BamHI and XhoI, ligated into similarly-restricted pGEM-7Z and used for *in vitro* transcription/translation of cpSRP43.

The chimeric sequence for cpFtsY$_{39\text{-}56}$-cp43 was constructed by overlap extension (Horton et al., 1989) using forward and reverse primers for the mature *A. thaliana* cpFtsY construct including residues 41-56 (CSAGPSGFFTRLGRLI) and introducing a KpnI site

and a Kozak sequence (acgatgg, MA_{39-40}). Forward and reverse primers were also designed to match the mature coding sequence of *A. thaliana* cpSRP43 and introduce an EcoRI site. Amplified $cpFtsY_{39-56}$ and cpSRP43 DNA were then spliced by overlap extension using forward and reverse primers designed to fuse exactly the $cpFtsY_{39-56}$, a small linker region VFGPKK, and cpSRP43. The PCR fragment obtained was restricted with KpnI and EcoRI, ligated into similarly-restricted pGEM-4Z and used for *in vitro* transcription/translation of $cpFtsY_{39-56}$-cp43. Likewise, $EcFtsY_{186-204}$-cp43 constructs are exact fusions *E. coli* FtsY residues 186-204 (EQEKPTKEGFFARLKRSLL), a linker (VFGPKK), and the predicted mature sequence of *A. thaliana* cpSRP43.

The chimeric sequence for $cpFtsY_{39-56}$-RubSS was constructed by overlap extension (Horton et al., 1989) using forward and reverse primers for the mature *A. thaliana* cpFtsY construct including residues 41-56 (CSAGPSGFFTRLGRLI) and introducing a KpnI site and a Kozak sequence (acgatgg, MA_{39-40}). Forward and reverse primers were also designed to match the mature coding sequence of *Pisum sativum* Rubisco Small Subunit (RubSS) and introduce an EcoRI site. Amplified $cpFtsY_{39-56}$ and RubSS DNA were then spliced by overlap extension using forward and reverse primers designed to fuse exactly the $cpFtsY_{39-56}$, a small linker region VFGPKK, and RubSS. The PCR fragment obtained was restricted with KpnI and EcoRI, ligated into similarly-restricted pGEM-4Z and used for *in vitro* transcription/translation of $cpFtsY_{39-56}$-RubSS. RubSS transcription/translation clone was designed using forward and reverse primers to match a small linker region VFGPKK and the mature predicted sequence of *Pisum sativum* Rubisco Small Subunit beginning with QVWPPI. A Kozak sequence and BamHI and EcoRI restriction sites were added for cloning into pGEM-3Z.

All cloned sequences were verified by DNA sequencing (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR).

Preparation of Chloroplast Materials and Radiolabeled Proteins

Intact chloroplasts were isolated from 10-12 day old pea seedlings (*P. sativum* cv. Laxton's Progress) and used to prepare thylakoids and stroma as described (Cline *et al.*, 1993). Chlorophyll (Chl) content was determined according to (Arnon, 1949). Thylakoids were isolated from lysed chloroplasts by centrifugation and SW two times with 1M potassium acetate in import buffer (IB; 50 mM Hepes-KOH, pH 8.0, 0.33 M sorbitol) and two times with IB with 10 mM $MgCl_2$ (IBM) prior to use. For protease treatment, SW thylakoids were diluted to 0.5 mg/ml Chl in IB with 0.2 mg/ml thermolysin and 1 mM $CaCl_2$, incubated for 40-60 min, combined with EDTA in IB to 20 mM EDTA, and applied to a 7.5% Percoll™ (GE Healthcare) gradient in IB containing 10 mM EDTA. Pellets were washed once with IB containing 10 mM EDTA and twice with IBM. Protease-treated thylakoids were resuspended at 1 mg/ml Chl in IBM.

In vitro transcribed capped RNA was translated in the presence of [^{35}S] methionine (Met) using a wheat germ system to produce radiolabeled proteins (Cline *et al.*, 1993). Constructs were labeled with ratios of labeled and unlabeled Met such that equal [^{35}S] signal represented equimolar protein. Constructs were quantified by comparing the [^{35}S] signal from a given protein band as analyzed by SDS-PAGE and phosphorimaging. Equimolar amounts of proteins were added to each experiment. Precursor LHCP translation products (TP) were diluted twofold with 30 mM unlabeled Met in IB.

Protein Integration Assays

Integration assays included SW thylakoids (equal to 50 µg Chl) in IBM, 5 mM ATP, 1 mM GTP, 12.5 µL radiolabeled pLHCP TP, and stromal extract (equivalent to 50 µg Chl) or 25 µl radiolabeled cpFtsY TP and recombinant cpSRP43 and cpSRP54. Stromal extract, containing cpSRP and cpFtsY, was used as a positive control. IB was used to bring the final volume to 150 µl. The mixtures were incubated at 25°C for 30 min with light. Membranes were collected by centrifugation at 3200 x *g* for 6 min and protease treated with thermolysin. Protease-treated membranes were solubilized in SDS buffer and heated. Amounts equivalent to 10 µg Chl per assay were analyzed by SDS-PAGE and phosphorimaging.

Assays for Determining Membrane Binding/Partitioning

Partitioning assays included thylakoids (equal to 75 µg Chl) in IBM and radiolabeled TP. Reactions were incubated for 30 min in light at 25°C. Thylakoids were centrifuged at 3200 x *g* for 6 min, washed in 1 ml IBM, and transferred to clean tubes. Thylakoids were then pelleted, solubilized in SDS buffer, and heated. Amounts equivalent to 7.5 µg Chl per sample were analyzed by SDS-PAGE and phosphorimaging.

To determine the approximate percentage of total *P. sativum* cpFtsY partitioned to thylakoids, chloroplasts (equal to 100 µg Chl) were lysed in 10 mM Hepes, pH 8 (with KOH), 10 mM $MgCl_2$ (HKM) at a final concentration of 1 mg Chl/ml. Chloroplast, thylakoid membrane, and stromal samples were separated by centrifugation, solublized in

SDS buffer, heated, and analyzed by 12.5% SDS-PAGE. Separated samples were transferred to Biotrace™ polyvinylidene fluoride membrane (Life Sciences) and incubated with rabbit anti-*A. thailiana* cpFtsY polyclonal antibodies (Moore *et al.*, 2003). Horseradish peroxidase-labeled immunoglobulin G from mouse (Southern Biotech) was used as a secondary antibody. Proteins reacting with antibodies were revealed by incubation with SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

CpFtsY Membrane Binding Saturation Assays

SW or SW and protease-treated thylakoids (equal to 50 µg Chl) were incubated with 0, 1, 2, 4, 8, 16, 32, or 64 µg cpFtsY in a final volume of 100 µl 1x IBM. Thylakoids were re-isolated, washed, resuspended to a final volume of 50 µl and 5 µl of each sample was analyzed by SDS-PAGE. Separated samples were transferred to Biotrace™ polyvinylidene fluoride membrane (Life Sciences) and incubated with rabbit anti-*A. thaliana* cpFtsY polyclonal antibodies (Moore *et al.*, 2003). Horseradish peroxidase-labeled mouse IgG (Southern Biotech) was used as secondary antibody. Proteins reacting with antibodies were revealed by incubation with SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

CpFtsY Cloning and Antisera Production

Precursor cpFtsY (pcpFtsY) sequence was amplified from *A. thailiana* RNA by RT-PCR using Thermoscript RT (Gibco) and ligated into SmaI restricted pGEM-4Z in the SP6 direction to create plasmid pcpFtsY4Z. The sequence was deposited to Genbank database (Accession # AF120112). The coding fragment for pcpFtsY was cut from

pcpFtsY with KpnI and HindIII and ligated into the corresponding restriction sites of pBAD (Invitrogen) forming pcpFtsYhis. This plasmid was then transformed into *E. coli* strain TOP10 (Invitrogen). The protein was expressed and purified on Talon™ Superflow™ affinity resin (Invitrogen) and used to generate rabbit polyclonal antibodies (Cocalico Biologicals).

MantGTP Binding Assays

MantGTP was purchased from Molecular Probes (Invitrogen). Binding of mantGTP to mature cpFtsY or F48A was monitored by fluorescence measurements using excitation and emission wavelengths of 355 nm and 448 nm, respectively. Fluorescence emission spectra were recorded at 25 ± 2°C in BD Falcon Microtest 384-well black/clear plates on a Molecular Devices SpectraMax GeminiXS spectrofluorimeter upon excitation at 355 nm. Proteins at a final concentration of 5 μM in HKM, 13% (v/v) glycerol, and 27 mM KCl were incubated in the presence or absence of 150 μM GTP at 25°C for 20 min prior to mixing with 0.5 μM mantGTP. Each reaction was aliquoted into three wells and the resulting spectral emission relative fluorescence units were averaged at each wavelength for a single experiment.

Imaging Acquisition

SDS-PAGE gels were imaged using a Typhoon 8600 (GE Healthcare) and analyzed with IQ Solutions Software (Molecular Dynamics). Western blots were imaged using a FluorChem™ 8900 (Alpha Innotech) and analyzed with the corresponding AlphaEase® FC StandAlone Software.

Isothermal Titration Calorimetry

Binding of GMP-PNP/GDP to cpFtsY/F48A was analyzed by measuring heat change during titration of nucleotide into a protein solution using a VP-ITC titration microcalorimeter (MicroCal Inc.). All solutions were degassed under vacuum and equilibrated at 25°C prior to titration. The sample cell (1.4 ml) contained 0.1 mM protein in 10 mM Tris buffer (pH 7.0), 50 mM KCl. The reference cell contained MilliQ water. Upon equilibration, 5 mM nucleotide was injected in 20 × 6 µl aliquots using the default injection rate. Titration curves were corrected for protein-free buffer and analyzed using Origin ITC software (MicroCal Inc).

Circular Dichroism (CD)

CD spectra were recorded on a Jasco J-715 spectropolarimeter. Spectra were acquired at 0.1 nm interval and scan speed of 10nm/min. All far-UV CD spectra were acquired using a sandwich quartz cell of 1 mM pathlength. Spectra were averaged over 10 scans and corrected for background absorption.

Nuclear Magnetic Resonance (NMR) Structural Studies

All NMR spectra were acquired at 25°C on a Bruker AVANCE DMX-500 MHz spectrometer, equipped with a 5 mm triple resonance cryoprobe. NMR samples (~ 1 mM concentration) were prepared both in 90% H_2O + 10 % D_2O (pH 7.0) containing 100 mM NaCl and in DMSO-d_6. 2D ^1H TOCSY and NOESY (Wuthrich, 1986) data were acquired with 2048 data points in the f2 dimension and 512 increments in the f1 dimension over a

spectral width corresponding to 12 ppm. 2D ^{1}H TOCSY data were acquired with mixing times of 60 ms and 75 ms. NOE based distance restraints were derived from 2D ^{1}H NOESY data obtained with various mixing times (200, 250, 300, and 350 ms). All NMR spectra were processed using XWIN-NMR and Sparky software (Goddard and Kneller, 1997). The backbone dihedral angle restraints derived from $^{3}JNH_{\alpha}H$ coupling constants and the $\chi 1$ dihedral angles derived from the TOCSY data were used as additional constraints for the structure calculation (Wang *et al.*, 1997).

Distance restraints were derived from the NOESY spectrum of the peptides. NOE cross peak intensities were measured and converted into distance. Structure calculation was performed using ARIA-CNS (1.2 version) (Linge *et al.*, 2001). Several cycles of ARIA were performed using standard protocols by varying the chemical shift tolerance between 0.04 ppm and 0.01 ppm. Assignments and violations were analyzed after each cycle. An ensemble of 12 structures was chosen (from a pool of 50 structures) on the basis of lowest energy terms associated with violation of experimentally derived constraints. The ensemble of the best overlapping structures (with least RMSD) of peptides was viewed using MOLMOL (Koradi *et al.*, 1996).

Preparation of Liposomes

Soybean total extract (Avanti Polar Lipids) lipids were dissolved at 100 mg/ml in chloroform, dried under nitrogen, and vacuum desiccated overnight. Lipid pellets were resuspended to10 mg/ml (13mM) in either 100 mM HEPES, 10 mM KCl, 1 mM EDTA or 50 mM KCl (pH 7.0 KOH). The lipid solution was subjected to 15 sec sonication/15 sec rest cycles for 2 min. Liposomes were clarified by centrifugation at 11,700 x *g* for 10

min and stored at 4°C for up to 1 month. Liposomes were sized (Avanti Mini-Extruder) by passing through polycarbonate filters 7 times. Brominated lipids were obtained by bromine addition to the unsaturated carbons of the soybean PC fatty acyl chain as described (Carney *et al.*, 2006). The brominated lipid mixture was extruded through 80 nm polycarbonate membranes and homogenized via freeze/thaw cycles.

Fluorescence quenching was measured using a Spectramax Gemini XS Spectrofluorometer (Molecular Devices) set for maximum sensitivity and 282 nm excitation/330 nm emission wavelengths. 10 μg protein in 50 μL HKM and 0-50 μL liposomes were mixed, equilibrated for 20 min at 25°C, and the fluorescence measured. For each concentration, six measurements of five separate samples were acquired. Fluorescence quenching was estimated as the normalized value of $(F_0-F)/ F_0$ where F_0 is the average fluorescence of the samples without liposomes and F the average fluorescence for each concentration.

Sequence Alignments

Sequence alignments of *A. thaliana* chloroplast, *E. coli*, *Thermotoga maritima*, and *T. aquaticus* FtsYs were performed using ClustalW (Chenna *et al.*, 2003). Sequences were input in FASTA format and ClustalW was run using default settings. Alignment files were viewed using Jalview v2.0 (Clamp *et al.*, 2004).

Organeller cpFtsYs were obtained by searching for short, nearly exact matches using protein-protein BLAST (Altschul *et al.*, 1997). Residues 41-366 of *A. thaliana* cpFtsY were blasted against Eukaryota with a word size of two and otherwise default settings. A non-redundant set of six chloroplast FtsY sequences was obtained and

aligned for a consensus sequence using ClustalW as described. A prokaryotic FtsY consensus was obtained by blasting the same cpFtsY sequence against bacteria with 500 descriptions. Sequences were shortened to contain only the NG domain plus 25 N-terminal residues. Resulting sequences were reduced to a non-redundant set of 375 and aligned using ClustalW. The percentage of each residue represented in an alignment column represents the total number of appearances of an amino acid divided by the total number of residues in that column.

GTPase Assays

GTPase activity assays were conducted at 22°C and contained 100 nM cpFtsY or F48A, 0.5 μM [α-32P]GTP (400 Ci/mmol), and liposomes in final volume of 5 μl buffer (50 mM Hepes, pH 8.0, 150 mM potassium acetate, 10 mM potassium chloride, 2 mM magnesium acetate, 0.01% octaethyleneglycol mono-N-dodecyl ether (C12 E8), and 2 mM DTT). Aliquots were removed at frequent time points and spotted onto PEI-cellulose thin layer plates as in (Connolly and Gilmore, 1993).

RESULTS

The N-terminal region of mature cpFtsY is necessary for LHCP integration and thylakoid membrane binding.

To understand whether the cpFtsY N-terminus is functionally important in targeting of LHCP by cpSRP, cpFtsY was replaced with N-terminal deletion mutants in assays that reconstitute LHCP integration into isolated thylakoids (Figure 2.1). Proper integration of LHCP results in a protease-resistant degradation product (DP), as seen in Figure 2.2. Deletion of cpFtsY residues 41-46 had little effect on LHCP integration, whereas further deletions (Δ41-49, Δ41-52, and Δ41-56) decreased integration by ~90% relative to cpFtsY.

To address whether the integration defect associated with the cpFtsY N-terminal deletions is related to a loss in membrane partitioning competency, salt-washed (SW) thylakoids were incubated with radiolabeled cpFtsY N-terminal deletion constructs and repurified to remove unbound protein. Deletion of the first six residues (Δ41-43 and Δ41-46) reduced membrane binding to 40-50% of that observed for cpFtsY (Figure 2.3). Further N-terminal deletions (Δ41-49, Δ41-52, and Δ41-56) reduced membrane binding to only 13% of that seen for cpFtsY, correlating with the precipitous drop in LHCP integration observed for the same cpFtsY deletions (Figures 2.2 and 2.3).

Phe48 and Phe49 are required for efficient thylakoid membrane binding and LHCP integration.

CpFtsY$_{NG+1}$ and cpFtsY$_{NG+2}$—consisting of the cpFtsY NG domain (residues 50-366) and Phe49 (+1) or Phe48 and Phe49 (+2) respectively (Figure 2.1)—were examined

for their ability to support LHCP integration and bind thylakoids. Though cpFtsY$_{NG+2}$ binds membranes with ~50% lower efficiency than cpFtsY, this construct supports significant (~90% relative to cpFtsY) LHCP integration *in vitro* (Figures 2.4A and B). CpFtsY$_{NG+1}$ associates with thylakoids with 25% the efficiency of cpFtsY and exhibits integration efficiency comparable to that found in assays conducted without added cpFtsY. These data imply that cpFtsY's N-terminus plays an active role in thylakoid binding and that membrane binding retained by cpFtsY$_{NG+1}$ is not productive in terms of supporting targeting events at the membrane.

CpFtsY constructs with Phe48, Phe49, or both replaced with alanine (F48A, F49A, or F48A/F49A) were examined for LHCP integration and membrane binding defects. Strikingly, the F48A mutation reduces LHCP integration efficiency by nearly 80%, while F49A exhibits a 40% decrease in integration efficiency (Figure 2.5A). Results using the double mutant, F48A/F49A closely resemble those obtained with F48A. Thylakoid binding with F48A, F49A, and F48A/F49A mutations is reduced by ~75%, 60%, and 75%, respectively (Figure 2.5B).

Alanine substitution of Phe48 does not affect nucleotide binding.

Mature cpFtsY is primarily composed of the GTPase active NG domain. To ensure that the F48A mutation did not induce large global structural changes, we used the fluorescent GTP analogue mantGTP to assess the structural integrity and GTP binding ability of the F48A mutant (Jagath *et al.*, 1998). As shown in Figure 2.6, the relative fluorescence intensity increases from the basal emission spectra of mantGTP alone when cpFtsY or F48A is added to the reaction, indicating that both of these proteins bind

mantGTP. This binding is specific seeing as pre-incubation with GTP competes with mantGTP and blocks the characteristic increase in fluorescence. Notably, the emission spectra for cpFtsY and F48A with mantGTP bound are nearly identical, suggesting similar binding affinities. Retention of GTP binding suggests that the global structure of F48A is intact, with minimal structural differences between cpFtsY and F48A.

Isothermal titration calorimetry (ITC) was also used to compare the binding affinities of cpFtsY and F48A for both GMP-PNP and GDP (Figure 2.7). Interaction of GMP-PNP with cpFtsY and F48A is exothermic and proceeds with changes in enthalpy of -3.4 kcal.mol^{-1} and -3.3 kcal.mol^{-1}, respectively. The number of binding sites (n) for GMP-PNP on cpFtsY and F48A are estimated to be 0.98 ± 0.01 and 0.96 ± 0.01, respectively. GMP-PNP binds to cpFtsY and F48A with similar affinity (K_d ~1.4μM), which is in agreement with previous studies (Jaru-Ampornpan *et al.*, 2007). The binding affinity of GDP (K_d ~1.2 μM) for cpFtsY and F48A is similar to that exhibited by GMP-PNP. Taken together, these results suggest that the global structure of F48A is intact with minimal structural differences between cpFtsY and F48A.

Liposomes stimulate basal hydrolysis of cpFtsY but not F48A.

The presence of liposomes has been shown to stimulate GTP hydrolysis in full-length EcFtsY, but not a construct lacking the A domain (de Leeuw *et al*., 2000). Furthermore, the A domain of EcFtsY has been implicated as a repressor of GTP hydrolysis in the absence of a lipid bilayer because its removal results in higher basal GTPase activity in solution (de Leeuw *et al.*, 2000). In agreement with *E. coli* FtsY data, Figure 2.8 shows that liposomes stimulate basal GTP hydrolysis by cpFtsY but not F48A.

Importantly, F48A exhibits a GTP hydrolysis rate that is four times greater than cpFtsY in the absence of liposomes and does not respond to a rise in liposome concentration (Figure 2.8). Taken together, these data indicate that F48 is part of a distinct structural, lipid responsive, domain that represses GTP hydrolysis when in solution, thereby limiting futile GTP hydrolysis by cpFtsY when not engaged in protein targeting activities at the membrane.

CpFtsY thylakoid interaction is not saturatable or protease-sensitive.

In contrast to SecY/FtsY interaction in the bacterial system (Angelini *et al.*, 2006), no proteinaceous thylakoid component has been identified to provide a binding site for cpFtsY to the thylakoid membrane. Neither protease treatment of SW thylakoids nor pre-treatment of the thylakoid membranes with antisera for SecY or Albino3 prevents cpFtsY from partitioning to the thylakoid membrane (Moore *et al.*, 2003). To determine the saturation amount for cpFtsY thylakoid association, SW thylakoids or protease-treated (PT) thylakoids were incubated with 0-64 μg purified cpFtsY. Thylakoids were then buffer-washed and reisolated. As shown in Figure 2.9, cpFtsY association with thylakoids increases with the amount of cpFtsY added. CpFtsY thylakoid binding is not saturated even using 64 μg purified cpFtsY/ 50 μl 2X SW or PT thylakoids. Taken together, these results suggest that cpFtsY is able to bind thylakoids through interaction with the lipid bilayer.

The N-terminus of cpFtsY partially inserts into the lipid bilayer during membrane association.

To determine whether membrane binding in cpFtsY is affected by the F48A mutation, soybean liposomes containing brominated acyl chains were used to examine the interaction of cpFtsY or the F48A mutant with lipid bilayers. Bromine quenching of cpFtsY Trp fluorescence served as an indicator of protein-bilayer interactions (Carney *et al.*, 2006). As shown in Figure 2.10, cpFtsY Trp fluorescence quenching increases with the amount of brominated lipid in the assay. One of two Trp residues in cpFtsY, Trp88 is positioned spatially closer to the putative lipid binding site in one of the N domain helices. Since quenching requires that the protein be in close proximity to the brominated acyl chains, these data indicate that cpFtsY partially inserts into the bilayer. In contrast, brominated lipids exhibit a greatly reduced ability to quench Trp fluorescence of the F48A mutant, indicating impairment in lipid binding of F48A which mirrors the loss of thylakoid binding.

F48A mutation is complemented by N-terminal fusion of a spontaneously-inserting transmembrane domain.

To differentiate between a reduction in membrane binding and other potential causes of decreased integration efficiency, we fused the transmembrane portion of *P. sativum* Tha4 (PsTha4), to the N-termini of mature cpFtsY, F48A, and F48A/F49A. PsTha4 is a spontaneously-inserting thylakoid membrane component of the twin arginine translocase protein targeting pathway (Dabney-Smith *et al.*, 2006). Tha4TM-cpFtsY exhibits a 25% increase in LHCP integration as compared to cpFtsY (Figure 2.11). Furthermore, fusion of Tha4TM to F48A and F48A/F49A completely restores their ability to support LHCP integration. It should be noted that fusion of PsTha4

transmembrane domain (residues 87-111) restored membrane binding to cpFtsY constructs, F48A (Tha4TM-F48A) and F48A/F49A (Tha4TM-F48A/F49A) (Figure 2.12). These data strongly suggest that F48A is incapable of supporting LHCP integration due to a loss of thylakoid binding capacity that can be overcome by fusing a transmembrane domain to the N-terminus.

The N-terminus of cpFtsY is necessary and sufficient to promote thylakoid binding.

CpFtsY (residues 39-56) and the analogous region in EcFtsY (residues 186-204) were fused to the N-terminus of the soluble protein, cpSRP43 (cpFtsY$_{39-56}$-cp43) to investigate whether these residues can function independently of the NG domain in promoting thylakoid localization. CpSRP43 (cp43) exhibits low background binding to protease-treated thylakoid membranes (Figure 2.13), whereas cpFtsY$_{39-56}$-cp43 stably associates with membranes at a level of more than ten-fold that of cp43 alone. Similarly, cpFtsY$_{39-56}$ fused to the mature small subunit of Rubisco (RubSS) leads to a nearly three-fold increase of thylakoid binding (unpublished data). Membrane localization of cp43 fused to the cpFtsY membrane-binding region is severely reduced by alanine replacement of F48, F49, or F48/F49, which reflects similar reductions in membrane localization of full-length cpFtsY point mutants (Figures 2.11 and 2.5B). Importantly, fusion of the analogous region from EcFtsY (residues 186-204) to cp43 resulted in a 6-fold increase in membrane binding of cp43. Alanine replacement of either F195 or F196 in the EcFtsY region resulted in complete loss cp43 localization to thylakoid membranes. These data demonstrate that the N-terminal residues 39-56 of cpFtsY or the analogous region of

EcFtsY are sufficient for tethering unrelated proteins to thylakoid membranes and do not require the NG domain to promote protein binding to thylakoids.

Determination of cpFtsY N-terminal peptide structures reveals potential membrane-interaction domains.

Although multiple crystal structures of FtsY homologues have been published, the local conformation of N-terminal A domain regions including the double Phe motif remains uncertain (Egea et al., 2004; Focia et al., 2004; Freymann et al., 1997; Gawronski-Salerno et al., 2007; Montoya et al., 1997). In this context, we determined the three-dimensional solution structures of cpFtsY$_{39\text{-}56}$ and cpFtsY$_{39\text{-}56}$(F48A) peptides using multidimensional NMR techniques (Figure 2.14, Panel I, A). CpFtsY$_{39\text{-}56}$ peptide is mostly unstructured. However, a segment comprising residues Phe48 to Leu52, assumes an α-helical conformation (Figure 2.14, Panel I, B). Helical conformation in this segment of cpFtsY$_{39\text{-}56}$ is supported by the presence of several i to i + 4 NOEs in the 2D ^{1}H NOESY spectrum. The root mean square deviation of the backbone heavy atoms structured helical segment (residues 48-52) is 0.22 ± 0.03 Å.

Several NOEs between the γH of Arg51 and the ring protons of Phe48 strongly suggest a side-chain interaction between the aromatic ring of Phe48 and the positively charged guanido group of Arg51 (Figure 2.14, Panel I, B). This interaction decreases the freedom of the aromatic ring of Phe48 and provides a microenvironment conducive to the development of a hydrophobic core consisting of Phe48, Phe49 and Leu52 and Leu55. The positively-charged guanido groups of Arg51 and Arg54 together with the hydrophobic core generate a local amphipathic structure (Figure 2.14, Panel I, C).

Furthermore, helix predictions place Lys59 and Lys61 on the same face as Arg51 and Arg54, likely extending the amphipathic helix (Jayasinghe *et al.*, 2006).

The three-dimensional solution structure of cpFtsY$_{39-56}$(F48A) shows i to i +3 interactions (characterizing a 3_{10} helix) between the backbone atoms of residues spanning Ala48 to Arg51 (Figure 2.14, Panel II, A and B). The side-chain interaction observed between residues 48 and 51, which is crucial for the packing of the hydrophobic core in cpFtsY$_{39-56}$, is missing in cpFtsY$_{39-56}$(F48A) (Figure 2.14, Panel II, C). Comparison of the three-dimensional solution structures of the WT and F48A cpFtsY peptides suggests that the prominent projection of the hydrophobic side chain at position 48 and the unique asymmetric distribution of residues at the N-terminus may be crucial for interaction with the membrane.

Circular dichroism reveals liposome-induced structural changes in cpFtsY and EcFtsY peptides.

To examine whether the interaction of cpFtsY with the thylakoid membrane could involve structural rearrangements, the backbone conformations of cpFtsY$_{39-56}$ and cpFtsY$_{39-56}$(F48A) were examined in the presence of soybean liposomes using far-UV circular dichroism (CD). Surprisingly, presence of the α-helical segment is not reflected in the far-UV CD spectrum of the cpFtsY$_{39-56}$ peptide. The CD spectrum of cpFtsY$_{39-56}$ shows negative ellipticity centered around 232 nm, but the 208 nm and 222 nm bands characteristic of the α-helical conformation are not present (Figure 2.15, A, Line A). Such anomalies in the CD spectra have been attributed to the contribution(s) of the aromatic side chains to the absorption in the far-UV region (Viguera and Serrano, 1995;

Sreerama *et al.*, 1999). The combined absorption effects of the Phe doublet appear to dominate and mask the far-UV CD signal(s) typical of the α-helices. This is obvious from the CD spectrum of cpFtsY$_{39-56}$(F48A), which shows the signature α-helix bands at 211 nm and 222 nm (Figure 2.15, A, Line B). The CD spectrum for EcFtsY$_{186-204}$ is nearly identical to that for cpFtsY$_{39-56}$ peptide, as it also contains two Phe residues (Figure 2.15, B, Line C).

The CD spectra of the WT peptides in the presence of 50 μM soybean liposomes are significantly different from those obtained in the absence of liposomes (Figure 2.15, A and B, compare Lines A to A' and C to C'). The spectra for both cpFtsY$_{39-56}$ and EcFtsY$_{186-204}$ show prominent negative bands centered at ~224 nm, suggesting that portions of these peptides in liposomes assume a β-turn type of structure (Figure 2.15, A and B, Lines A' and C'). Hence, liposome interaction of both cpFtsY$_{39-56}$ and EcFtsY$_{186-204}$ induces a conformational switch from helix to a β-turn type of structure. Induction of the structural change requires a higher concentration of liposomes for cpFtsY$_{39-56}$(F48A), EcFtsY$_{186-204}$(F195A), and EcFtsY$_{186-204}$(F196A) as compared to cpFtsY$_{39-56}$ or EcFtsY$_{186-204}$, suggesting that liposomes have a weaker influence on these alanine replacements (Figure 2.15, C). Apparent K_d values, calculated from molar ellipticity changes at 208 nm as a function of liposome concentration, are 130nM for cpFtsY and 200nM for F48A (Figure 2.16). Taken together, these data suggest that the regions in cpFtsY and EcFtsY containing the double Phe motif respond to lipid bilayers by changing local backbone conformation.

Point substitutions of Phe48 reveal structural requirements.

To examine characteristics of Phe48 important for function, we produced residue-replacement mutants using amino acids differing in side-chain length, charge, polarity, aromaticity, and secondary structure propensity (A, G, V, L, E, Q, K, Y, and W). LHCP integration assays performed with each mutant (Figure 2.17A) reveal that small, nonpolar side chain replacements (F48A and F48G) and polar side chain replacements (F48E and F48Q) result in severe integration defects. Larger, nonpolar side chain replacements (F48L and F48V) exhibit integration efficiencies closer to cpFtsY—98% and 72%, respectively. Valine appears to be the smallest residue that can serve as a functional replacement for Phe48. Residue replacements containing aromatic rings (F48W and F48Y) also maintain high levels of integration (104% and 85% of cpFtsY, respectively). Thylakoid binding capabilities of each mutant mirror LHCP integration efficiency (Figure 2.17B). Only F48L, F48W, F48V, and F48Y maintain sufficient membrane binding to support LHCP integration. Sequence alignments reveal a high degree of conservation of hydrophobic residues at the same positions in bacterial and chloroplast FtsYs (Figure 2.17). Residues frequently found in alignment with the conserved double Phe motif include Trp, Leu, and Val, all of which are functional replacements for cpFtsY Phe48 (Figure 2.17A and B). Alignment of 375 bacterial FtsYs revealed a strong conservation of two Leu residues and three positively-charged residues in positions compatible with the formation of an amphipathic helix in this region (see Figure 2.18). In comparison, this pattern of residues is not conserved in eukaryotic SRα homologues, perhaps owing to the presence of the integral membrane receptor SRβ.

To examine whether the conserved positively-charged residues (R51, R54) and hydrophobic residues (L52, I56) are also critical for cpFtsY function, we used the

following point mutants in integration and membrane partition assays: R51A/R54A, L52A, L52Q, and I56A. As shown in Figure 2.19, alteration of any of these conserved residues decreases integration efficiency by at least 70%. Likewise, mutation of any of the hydrophobic residues (L52, I56, F48, F49) to alanine or a charged amino acid decreases membrane binding by 40-75% (Figure 2.20). The double mutant R51A/R54A exhibits an appreciable loss of both membrane binding (~60% loss) and LHCP integration. This data suggests that the conserved positively-charged residues R51 and R54 play a role in membrane partitioning, although the extent to which each residue is involved remains to be explored.

DISCUSSION

In eukaryotic systems the SRP receptor SRα associates with the endoplasmic reticulum through interaction with the integral membrane protein SRβ. Though no bacterial or organellar homologue of SRβ has been identified, the SRα homologues, *E. coli* FtsY (EcFtsY) and chloroplast FtsY (cpFtsY), partition between membrane bound and soluble phases. Previous studies have shown membrane association is critical for EcFtsY function, yet the mechanism of productive membrane binding for prokaryotic and organellar SRP receptors during protein targeting remains uncertain. Our results demonstrate that cpFtsY must interact with the thylakoid membrane for proper function. Furthermore, this binding takes place through a conserved amphipathic helix that is both necessary and sufficient for interaction with the thylakoid membrane.

In cpFtsY, the $_{NG+2}$ position Phe (Phe48) of the conserved double Phe motif is an essential component for functional binding of cpFtsY to thylakoids. Although $EcFtsY_{NG+1}$ appears sufficient *in vivo* to maintain cell viability (Eitan and Bibi, 2004), *in vitro* results indicate a significant reduction in the ability of this construct to support integration of SRP-dependent substrates (Angelini *et al.*, 2006). It seems probable that $EcFtsY_{NG+2}$ in *in vitro* experiments would correlate more closely with results for $cpFtsY_{NG+2}$. Regardless, both Phe residues in EcFtsY likely contribute to membrane binding since removal of Phe195 or Phe196 in proteins directed to the membrane by fusion of EcFtsY residues 186-204 lose this function (Figure 2.10). Furthermore, *E. coli* FtsY peptide structural data suggest Phe195 and Phe196 play roles in membrane interaction comparable to cpFtsY's Phe48 and Phe49. Our results strongly suggest that

conserved regions in both cpFtsY and EcFtsY play a critical role in functional association with target membranes.

Although multiple crystal structures of FtsY homologues have been published, the local conformation of the double Phe motif remains uncertain (Gawronski-Salerno and Freymann, 2007). The three-dimensional structure of $cpFtsY_{39-56}$ shows that Phe48 is located in a hydrophobic core lined alongside positively-charged residues. The aromatic ring of Phe48 projects out of the core, and its rotational freedom is restricted by interaction with the positively charged guanidino group of Arg51. It appears that the phenyl ring of Phe48, together with the asymmetric distribution of the hydrophobic core and the positively-charged residues Arg51 and Arg54 provide a microenvironment conducive to membrane interaction. The aromatic ring of Phe48, or a suitably large aromatic or aliphatic replacement, is seemingly necessary for a crucial association between cpFtsY and the thylakoid membrane. The other non-polar residues in the hydrophobic core, along with the nearby charged residues, may support or stabilize partial insertion of this region into the membrane. Evidence presented here supports a model in which membrane insertion results in a local backbone conformational change (helix to β-turn) in the N-terminal segment of cpFtsY. That this backbone change is functionally relevant for targeting events is consistent with mutational data concerning the residues able to functionally replace Phe48 (Figure 2.17A and B). Furthermore, the *E. coli* FtsY peptide structural data suggest that Phe195 and Phe196 play roles in membrane interaction comparable to cpFtsY's Phe48 and Phe49. These results strongly suggest that conserved regions in both cpFtsY and *E. coli* FtsY undergo lipid-induced

conformational changes as a result of membrane association by employing a similar lipid binding mechanism.

A model emerges for membrane association of cpFtsY with thylakoid membranes whereby the initial interaction takes place between the N-terminus and the lipid bilayer via an amphipathic helix containing the double Phe motif. The projection of the non-polar phenyl ring of Phe48 appears to be vital for the recognition and stable association of cpFtsY with the membrane. The efficiency of membrane integration for cpFtsY F48Y is marginally reduced as compared to that of WT cpFtsY or the cpFtsY F48W mutant, likely owing to the polar nature of the tyrosine phenolic group. Amino acids with shorter hydrophobic side-chains (e.g. Ala, Gly) at position 48 may be buried in the hydrophobic core and therefore unable to access the membrane. Similarly, substitution of Phe48 with a charged group (Asp, Glu, Lys, Arg) does not energetically favor interaction with non-polar membrane regions. In addition to Phe48, the other residues in the compact core of hydrophobic residues (including Phe49 and Leu52) may provide additional interaction sites for cpFtsY on the thylakoid membrane. Far-UV CD data clearly show that the membrane interaction of the cpFtsY N-terminal residues is accompanied by a dramatic α-helix to a β-turn conformational switch (Figure 2.13, A, Lines A and A'). The drastic structural change in the backbone may help stabilize cpFtsY at the membrane. In any case, the correlation of integration and thylakoid binding defects with a reduced lipid-induced change in conformation, combined with the conserved nature of this structural motif at a position immediately preceding the NG domain in prokaryotic SRP receptors, suggests that the lipid-induced conformational change in the cpFtsY N-terminus from

helix to β-hairpin serves as a functional switch to communicate a membrane-bound state and induce or enhance associated activities.

It is noteworthy that the N-terminus of cpFtsY appears to provide little specificity for thylakoid lipids, but rather exhibits a more generic lipid binding activity. Thylakoid membranes and soybean total extract liposomes have vastly different lipid compositions, with very few, if any, lipids in common, yet cpFtsY is capable of interacting with both thylakoid membranes and soybean liposomes by a mechanism that is sensitive to Phe mutation (Figures 2.4B, 2.10, and 2.8B). Given that the lipid composition of the thylakoid and inner envelope is quite similar (Douce and Joyard, 1996), it would be expected that cpFtsY is able to bind both the thylakoid and inner envelope. Membrane specificity for the targeting mechanism is therefore likely to stem from interaction of cpSRP, cpFtsy, or targeting substrates with proteins that reside at the target membrane. The ability of mammalian SRα to interact with its integral membrane partner SRβ provides membrane specificity for SRP-based targeting by ensuring that SRα is localized to the endoplasmic reticulum. However, an SRβ homologue is absent in chloroplasts and prokaryotes and there is only a single membrane target in prokaryotes. We hypothesize that membrane specificity in chloroplasts is provided by necessary interactions between the membrane translocon Alb3 and cpSRP components. Although cpFtsY shows little affinity for Alb3, a complex composed of cpSRP and cpFtsY specifically co-precipitates with Alb3 in the presence of GMP-PNP (Moore *et al.*, 2003). In addition, cpSRP43 alone exhibits the ability to bind Alb3 (Tzvetkova-Chevolleau *et al.*, 2007). In this context, it will be important to determine the membrane distribution of Alb3 and the required interactions between cpSRP and Alb3.

Lipid binding by the N-terminus of cpFtsY appears to play a key role in the SRP targeting cycle by influencing the GTP hydrolyzing activity of the adjacent NG domain. FtsY mutants defective in GTP hydrolysis (or stabilized with non-hydrolysable GMP-PNP) have been found to have a stronger association with membranes (Angelini *et al.*, 2006). Importantly, mutations to the lipid binding region (e.g. F48A) uncouple membrane binding of cpFtsY from increased GTP hydrolysis; GTP hydrolysis is elevated in the F48A mutant without the need for lipids (Figure 2.8A). In this context, fusion of a membrane anchor to F48A, which restores its ability to support LHCP integration, further supports the need for cpFtsY membrane binding to be coupled with elevated cpFtsY GTP hydrolysis activity (Figure 2.9A). The proposed cpFtsY lipid-responsive motif is supported by previous work demonstrating that, in *E. coli*, FtsY binding to anionic phospholipids results in a reduced α-helical content, increased β-sheet content, which corresponds with stimulated GTPase activity (de Leeuw *et al.*, 2000). Because at least a portion of the A domain is necessary for liposomes to stimulate GTPase activity, and the basal GTPase activity of the $EcFtsY_{NG}$ construct is double that of full-length EcFtsY in solution, it has been suggested that the A domain acts as a repressor of GTP hydrolysis in the absence of membrane binding. Furthermore, a crystal structure of *T. aquaticus* FtsY indicates the N-terminal helix containing the double Phe motif must be displaced for the formation of a stable heterodimeric complex with the SRP54 homologue, Ffh (Gawronski-Salerno and Freymann, 2007). This suggests that FtsY membrane association must occur and adjust the position of the N-terminal helix prior to formation of a complex with SRP54. By structurally linking lipid binding to the ability of the SRP receptor to both bind SRP and hydrolyze GTP, futile hydrolysis in the absence of target

membranes would be minimized. We speculate that during membrane association, the N-terminus of cpFtsY shifts and may serve as a membrane sensor for the GTPase domain.

Concluding Remarks

We have investigated the functional requirement and role of residues N-terminal to the NG domain of cpFtsY in cpSRP protein targeting. Our data demonstrate that the N-terminus of mature cpFtsY is critical in the cpSRP-based targeting mechanism, owing predominantly to an interaction with the membrane. Specifically, an amphipathic helix flanked by a conserved double Phe motif (residues 48 and 49) is indispensable for cpFtsY binding to thylakoids and efficient promotion of LHCP integration. Notable results of this research include the development of an 18-amino acid sequence (consisting of cpFtsY residues 39-56) that as a fusion is capable of tethering unrelated proteins to lipid bilayers. Structural studies of peptides of corresponding regions within *E. coli* FtsY and the N-terminus of cpFtsY reveal a conformational switch from amphipathic α-helix to β-hairpin induced by the presence of lipid bilayers. This switch mechanism appears important for stabilizing cpFtsY in a functional manner at the thylakoid membrane and requires the conserved double Phe motif. It is plausible that the switch enables the N-terminus of cpFtsY to communicate its membrane association to the NG GTPase domain. Furthermore, it is attractive to envision that this structural switch serves as a universal mechanism for functional membrane association in prokaryotic SRP-based protein targeting as a whole.

Confirmatory Findings

During revision of this manuscript, two relevant papers were published demonstrating results similar to our findings (Parlitz *et al*., 2007; Bahari *et al*., 2007). A resolved structure of $EcFtsY_{NG+1}$ suggests that the region containing Phe196 is α-helical in nature and the amphipathic nature of this region plays a critical role in membrane association (Parlitz *et al.*, 2007). In the second pertinent paper, liposomes were shown to stimulate GTP hydrolysis rates of SRP with $EcFtsY_{NG+1}$, but not with $EcFtsY_{NG,}$ (Bahari *et al.*, 2007) supporting the idea that the A domain in its entirety is not strictly required. It is interesting to note that Bahari *et al* did not find a higher basal hydrolysis rate for $EcFtsY_{NG}$ as compared to either EcFtsY or $EcFtsY_{NG+1}$, though this may be attributable to the presence of SRP. In contrast, cpFtsY F48A shows a higher basal GTP hydrolysis rate as compared to wild type cpFtsY (Figure 2.8).

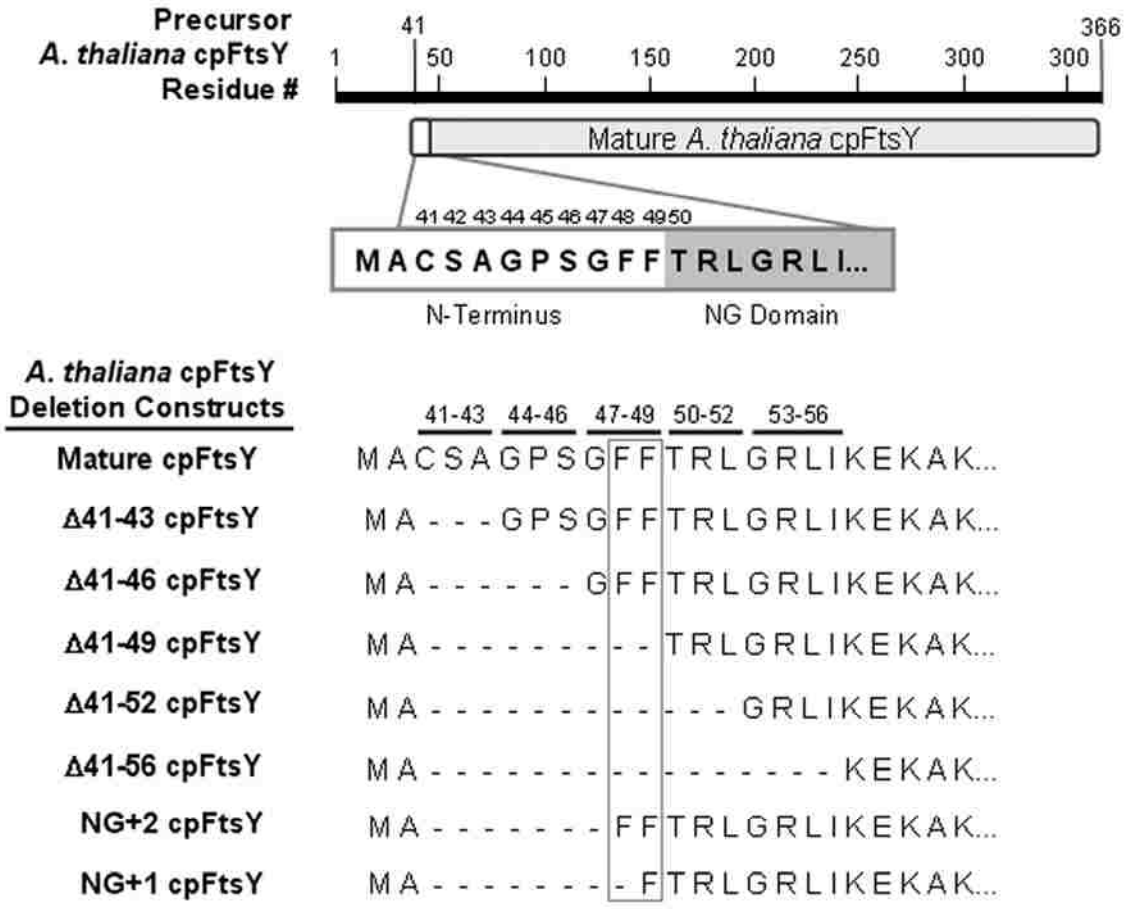

Figure 2.1. Schematic representation of cpFtsY N-terminal deletions.

N-terminal deletions of mature cpFtsY (residues 41 to 366). The conserved NG domain is indicated by shading. N-terminal residues Met and Ala (MA) are added for translation initiation in the recombinant cpFtsY constructs which lack a chloroplast transit peptide (residues 1-40).

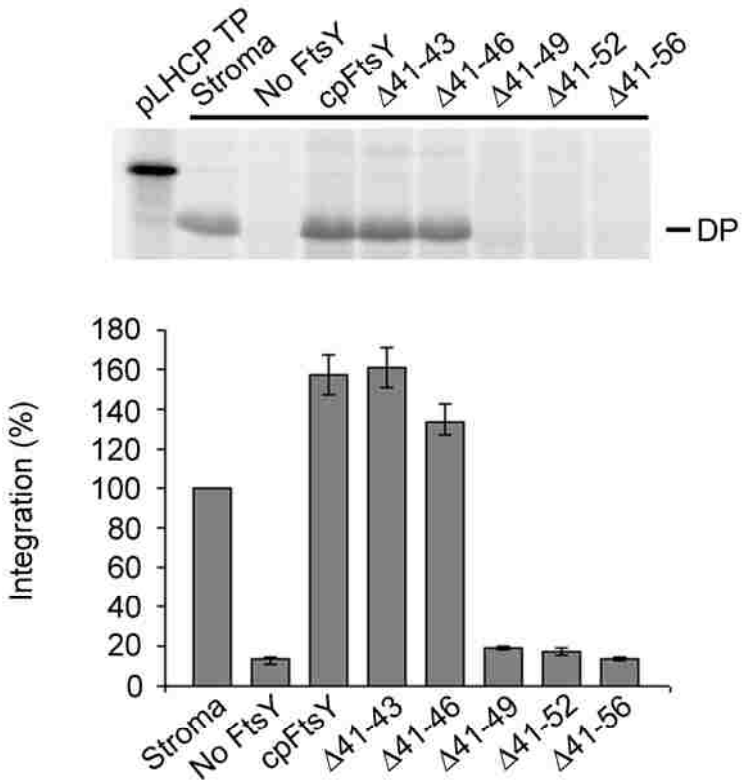

Figure 2.2. CpFtsY residues 47-49 (GFF) are required for LHCP integration.

Integration of LHCP was reconstituted with SW thylakoids using stromal extract or recombinant proteins, and equimolar amounts of *in vitro* translated cpFtsY construct as indicated. Correctly integrated LHCP migrates as a protease-resistant degradation product (DP). A lane of pLHCP translation product (TP) is shown for comparison. LHCP integration was calculated from a minimum of three separate experiments and is shown relative to the level of integration observed for stroma.

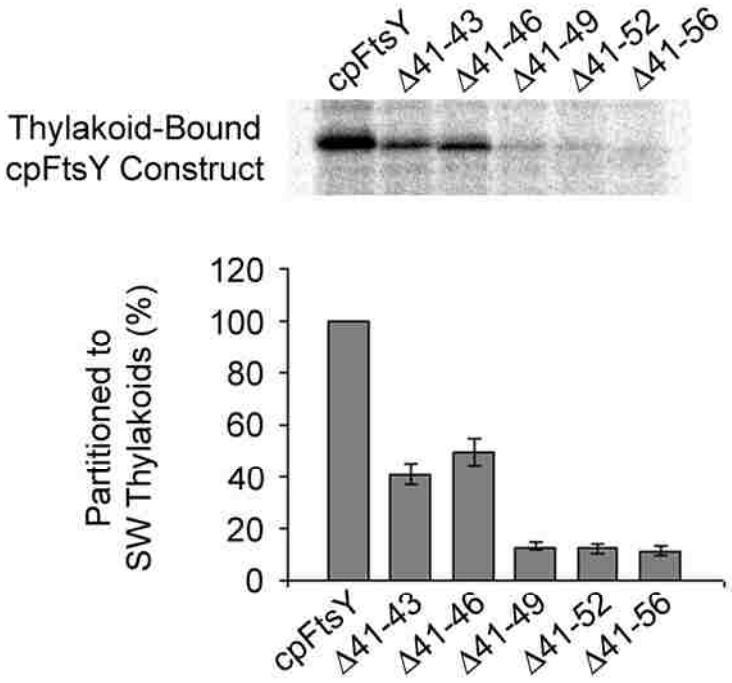

Figure 2.3. CpFtsY residues 47-49 (GFF) are required for efficient thylakoid partitioning.

Membrane binding of radiolabeled cpFtsY construct as indicated was examined by incubation with SW thylakoids. Thylakoids were re-isolated, washed and analyzed by SDS-PAGE and phosphorimaging. The level of each membrane-bound cpFtsY construct was calculated from three separate experiments and is shown relative to bound cpFtsY.

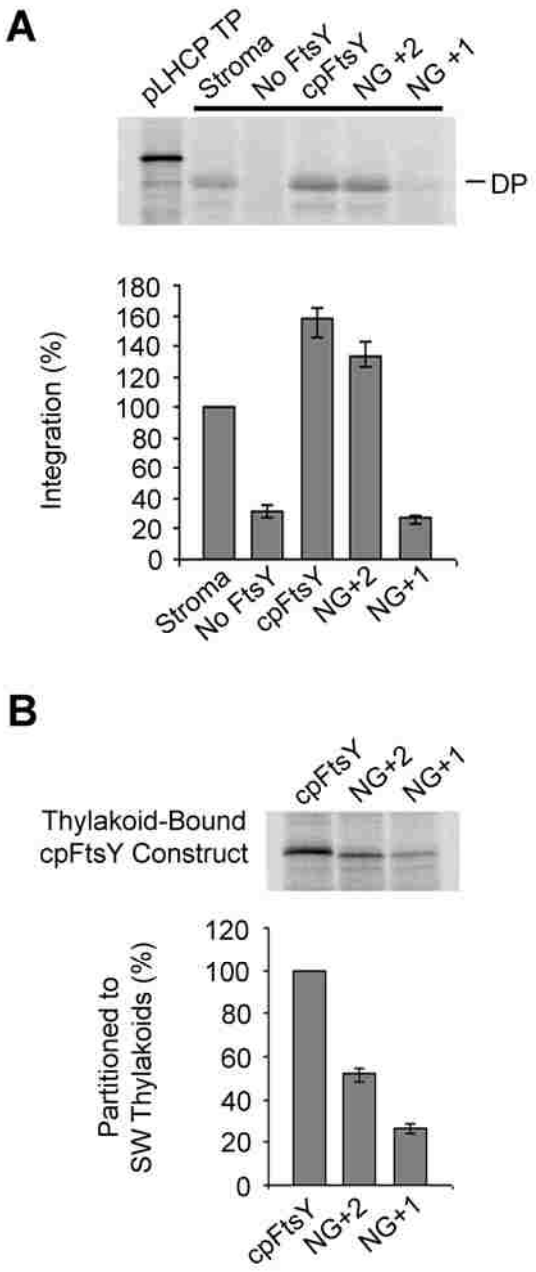

Figure 2.4. CpFtsY$_{NG+2}$ functions in LHCP integration and thylakoid partitioning.

(**A**) Integration of radiolabeled LHCP was reconstituted as described in Figure 2.2. LHCP integration efficiency was calculated from three separate experiments and is presented relative to integration observed in the presence of stroma. (**B**) Membrane binding of each radiolabeled cpFtsY construct indicated was examined by incubation with SW thylakoids as described in Figure 2.3. The level of each cpFtsY construct bound to membranes was calculated from three separate experiments and is shown relative to bound cpFtsY.

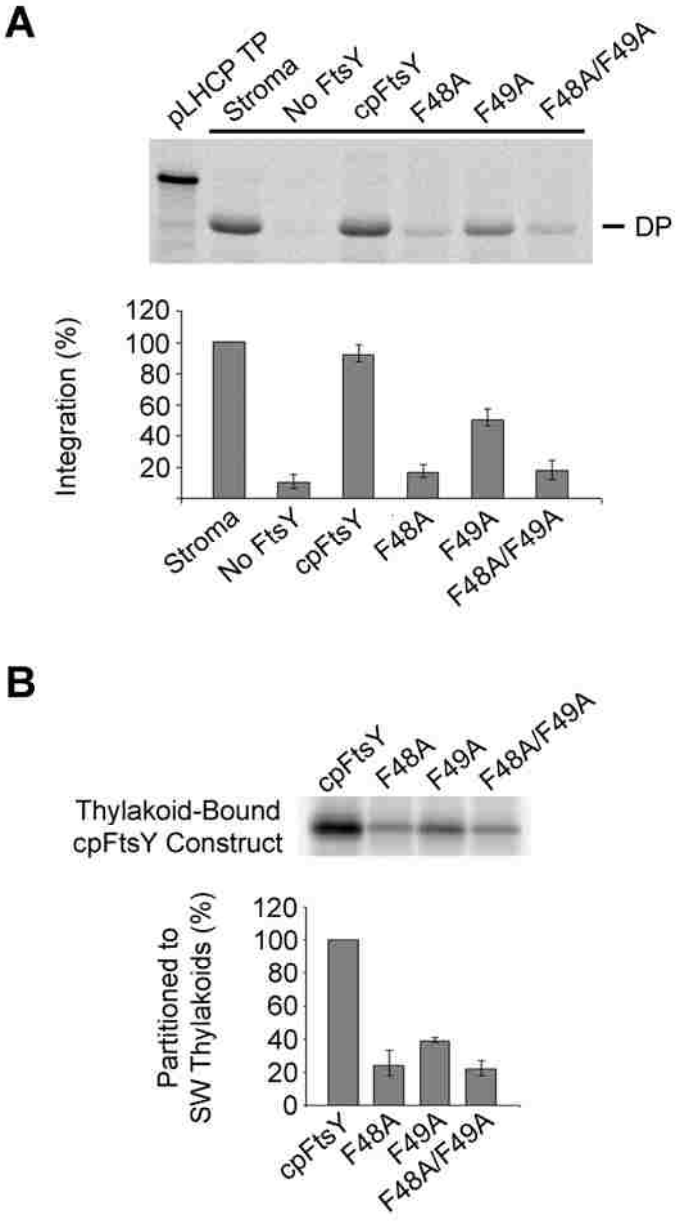

Figure 2.5. CpFtsY Phe48 plays a critical role in LHCP integration and thylakoid partitioning.

(**A**) Integration of radiolabeled LHCP was reconstituted as described in Figure 2.2. LHCP integration efficiency was calculated from three separate experiments and is presented relative to integration observed in the presence of stroma. (**B**) Membrane binding of each radiolabeled cpFtsY construct indicated was examined by incubation with SW thylakoids as described in Figure 2.3. The level of each cpFtsY construct bound to membranes was calculated from three separate experiments and is shown relative to bound cpFtsY.

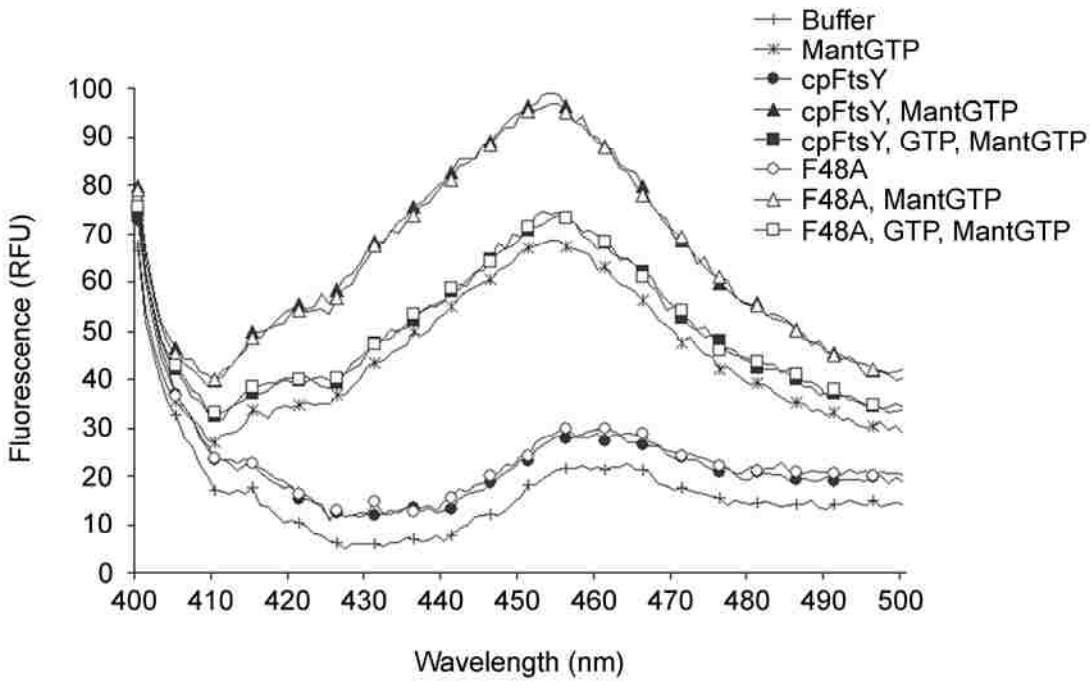

Figure 2.6. Alanine substitution of Phe48 does not affect nucleotide binding activity.

Fluorescence emission spectra of purified cpFtsY or cpFtsY-F48A protein (5 μM) was examined alone or in the presence of either 150 μM GTP, 0.5 μM mantGTP, or both. Each sample was examined for fluorescence emission between 400-500 nm using an excitation wavelength of 355 nm. Emission spectra of samples lacking protein (buffer alone and mantGTP alone) are shown for reference.

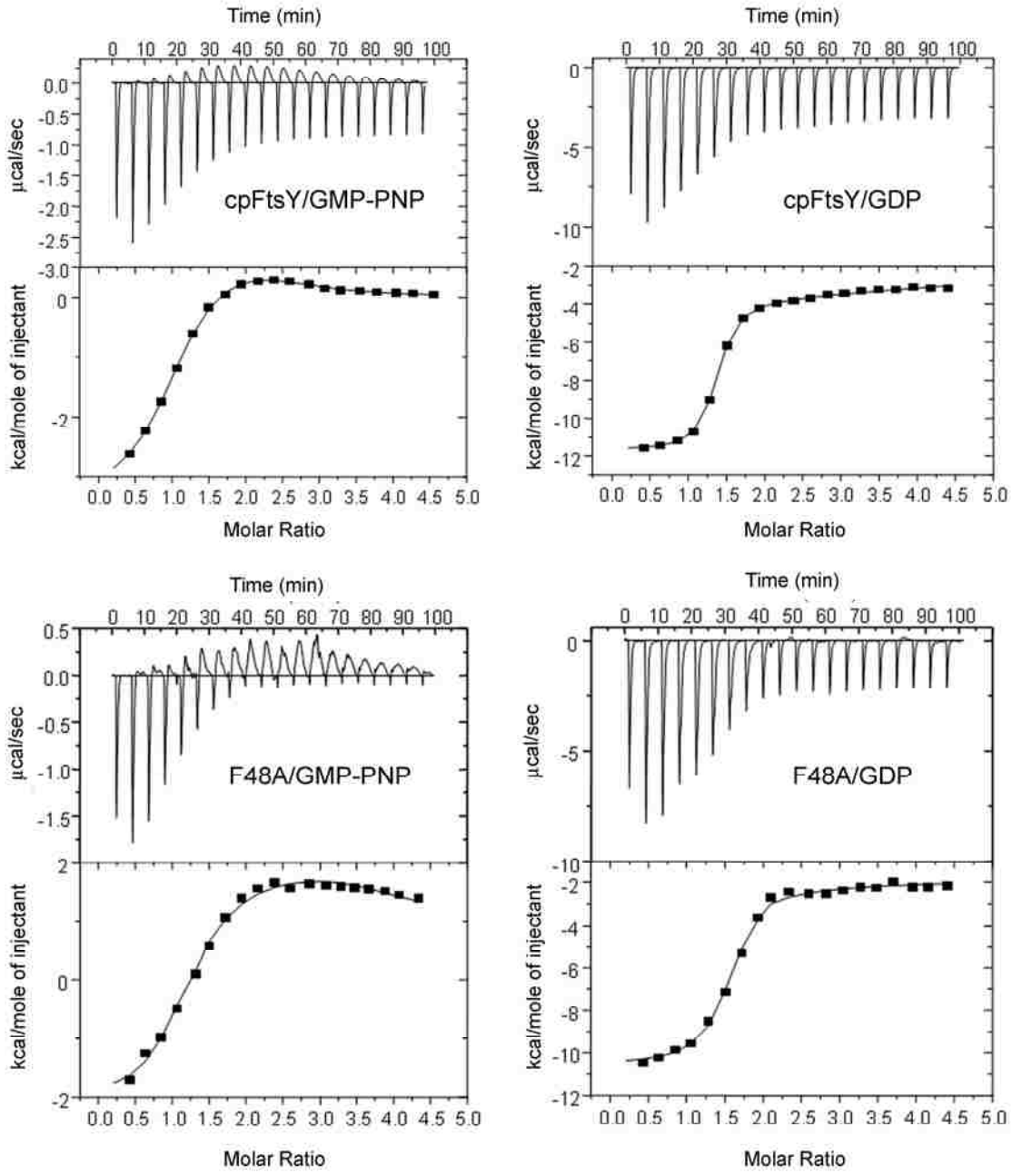

Figure 2.7. Alanine substitution of Phe48 does not affect affinity for nucleotides.

ITC curve showing binding of GMP-PNP or GDP with cpFtsY or F48A at 25°C. The upper and lower panels show the raw and integrated data, respectively, of the titration of the protein with nucleotide as indicated. The solid line in the bottom panels represents the best-fit curve of the data (Microcal Origin). Background corrections were made in all spectra. These experiments were performed by Dakshinamurthy Rajalingam (Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR).

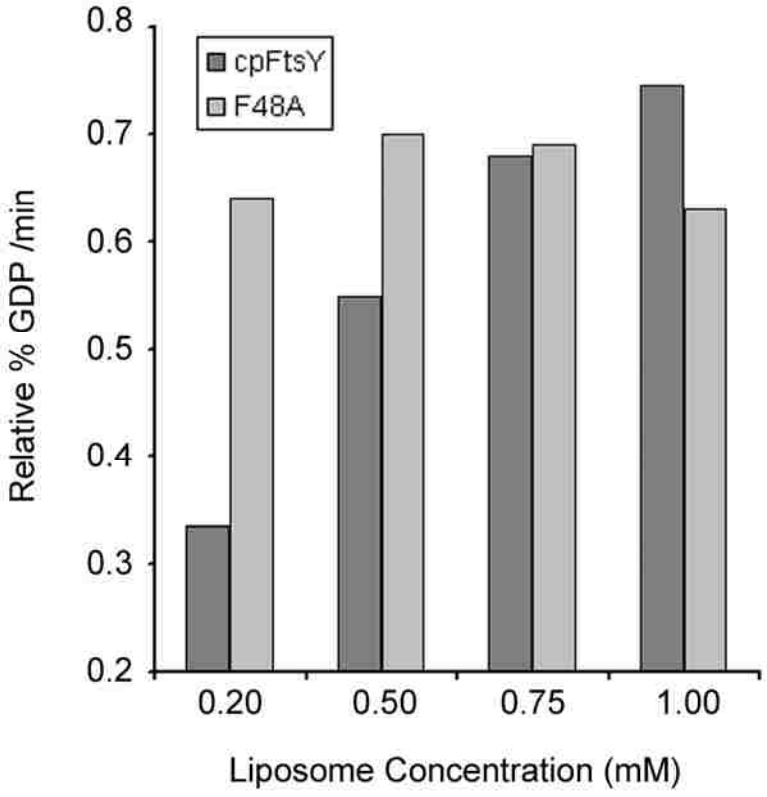

Figure 2.8. GTP hydrolysis by cpFtsY, but not F48A, is stimulated by liposomes.

GTPase assays containing 100nM cpFtsY (dark) or F48A (light) and 0.5 μM GTP in the presence of soybean liposomes as indicated. Activity levels shown are the average of a minimum of two separate experiments. Variation between independent assays of equivalent conditions was less than 15% in all cases. These experiments were performed by Robyn Goforth (Department of Biology, University of Arkansas, Fayetteville, AR).

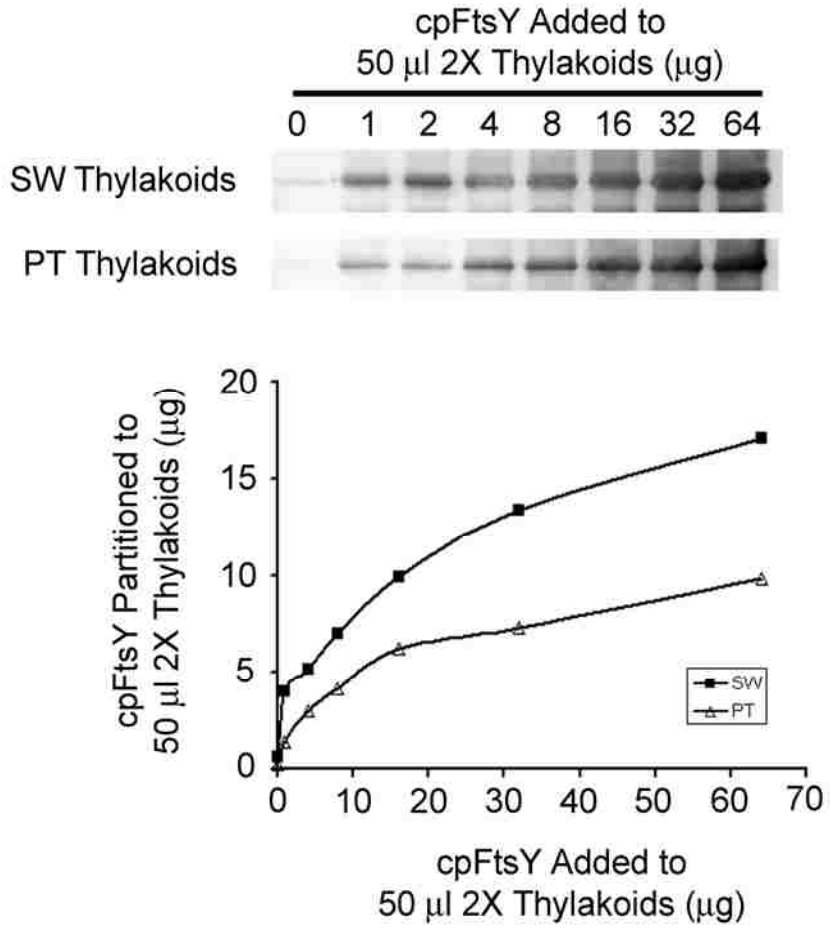

Figure 2.9. CpFtsY binds to thylakoids in a non-saturatable manner.

CpFtsY membrane binding was reconstituted using salt-washed (SW) or protease-treated (PT) thylakoid membranes. Thylakoids were re-isolated, washed, and analyzed by SDS-PAGE and Western Blotting. Blots were probed with antisera for cpFtsY.

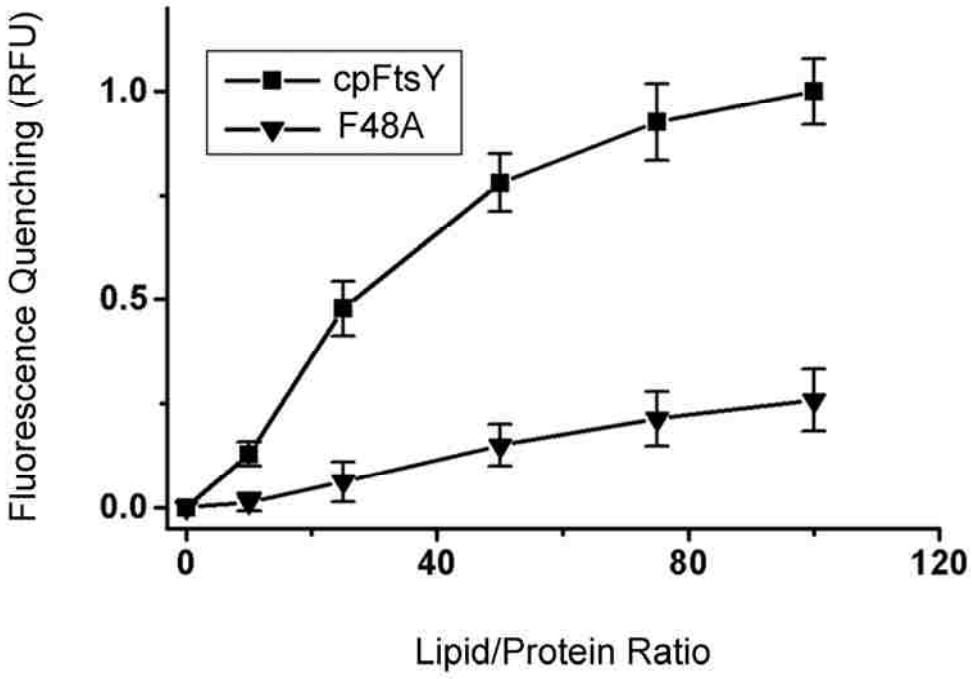

Figure 2.10. CpFtsY partially inserts into lipid bilayers.

Liposome binding estimated from fluorescence quenching suggests that cpFtsY (solid squares) has a higher binding affinity for the lipid membrane than F48A (solid down triangles). Fluorescence quenching, which requires close proximity of Trp and the brominated acyl chain, suggests partial insertion of the protein into the bilayer. Fluorescence quenching was calculated from three separate experiments. These experiments were performed by Daniel Fologea (Department of Biology, University of Arkansas, Fayetteville, AR).

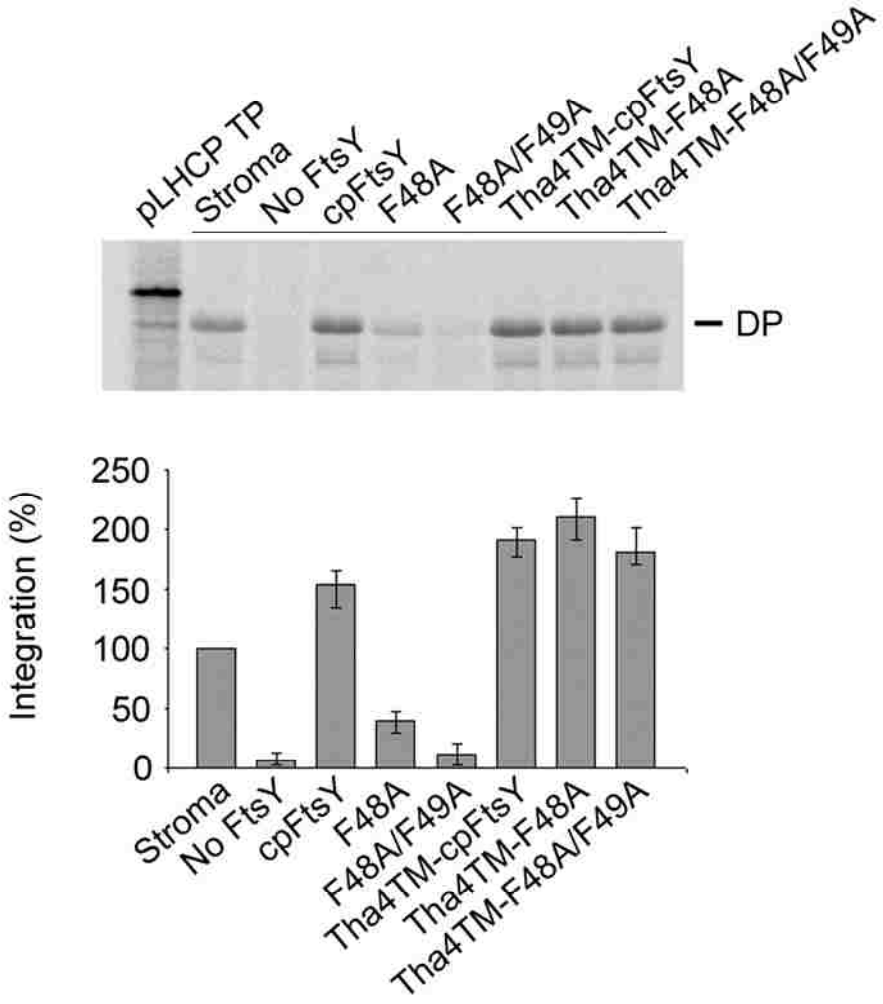

Figure 2.11. Fusion of membrane-tethering region restores targeting function in F48A mutant.

Integration of radiolabeled LHCP was reconstituted as described in Figure 2.2. Integration efficiency was calculated from three separate experiments and is presented relative to integration observed in the presence of stroma.

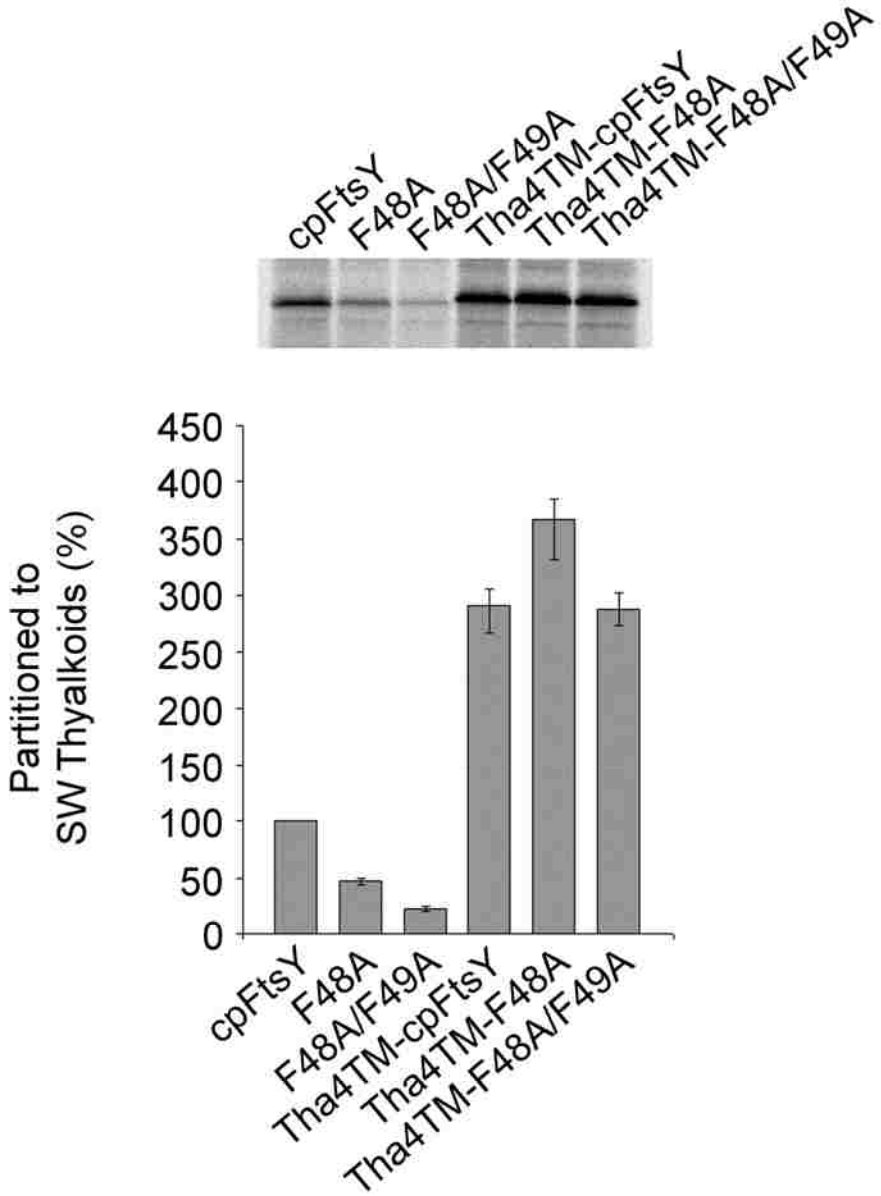

Figure 2.12. Fusion of membrane-tethering region restores thylakoid binding capacity in F48A mutant.

Membrane binding of radiolabeled cpFtsY constructs was examined by incubation with SW thylakoids as described in Figure 2.3. The level of each cpFtsY construct bound to membranes was calculated from three separate experiments and is shown relative to bound cpFtsY.

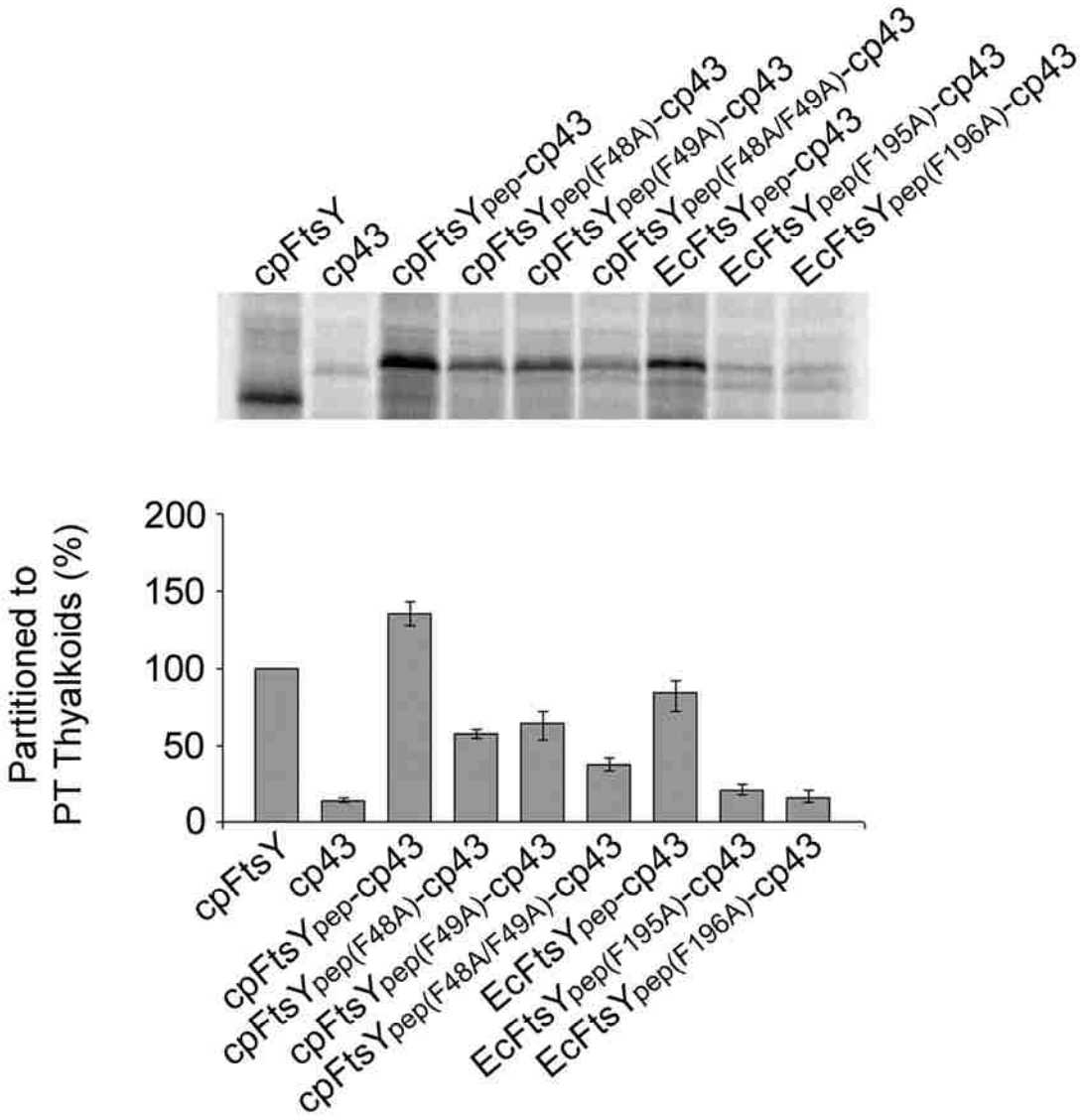

Figure 2.13. The membrane active N-terminus of cpFtsY is necessary and sufficient for targeting proteins to the thylakoid membrane.

Membrane binding of equimolar, radiolabeled cpFtsY, cp43, and chimeric constructs of either cpFtsY$_{39\text{-}56}$ (cpFtsY$_{pep}$) or EcFtsY$_{186\text{-}204}$ (EcFtsY$_{pep}$) with cp43 was examined by incubation with protease-treated thylakoids. Thylakoids were re-isolated, washed, and examined by SDS-PAGE and phosphorimaging. The level of each construct was calculated from three separate experiments and is shown relative to bound cpFtsY.

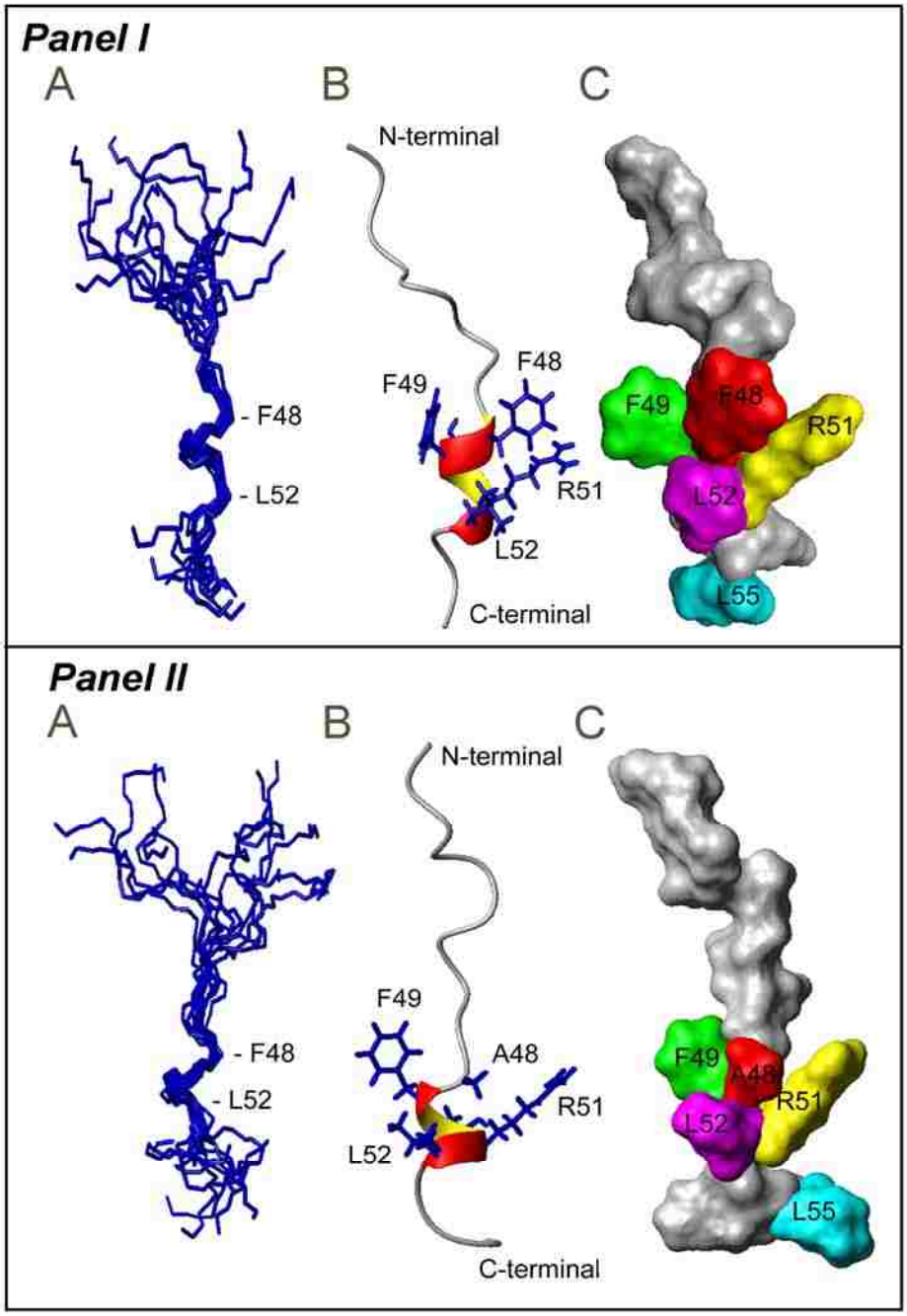

Figure 2.14. NMR structure studies of cpFtsY peptides.

Panel I - cpFtsY$_{39-56}$ peptide, Panel II - cpFtsY$_{39-56}$(F48A) peptide: from left to right: ensemble of 12 lowest energy structures; ribbon diagram depicting the backbone fold; depiction of the distribution of hydrophobic residues. These experiments were performed by Dakshinamurthy Rajalingam (Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR).

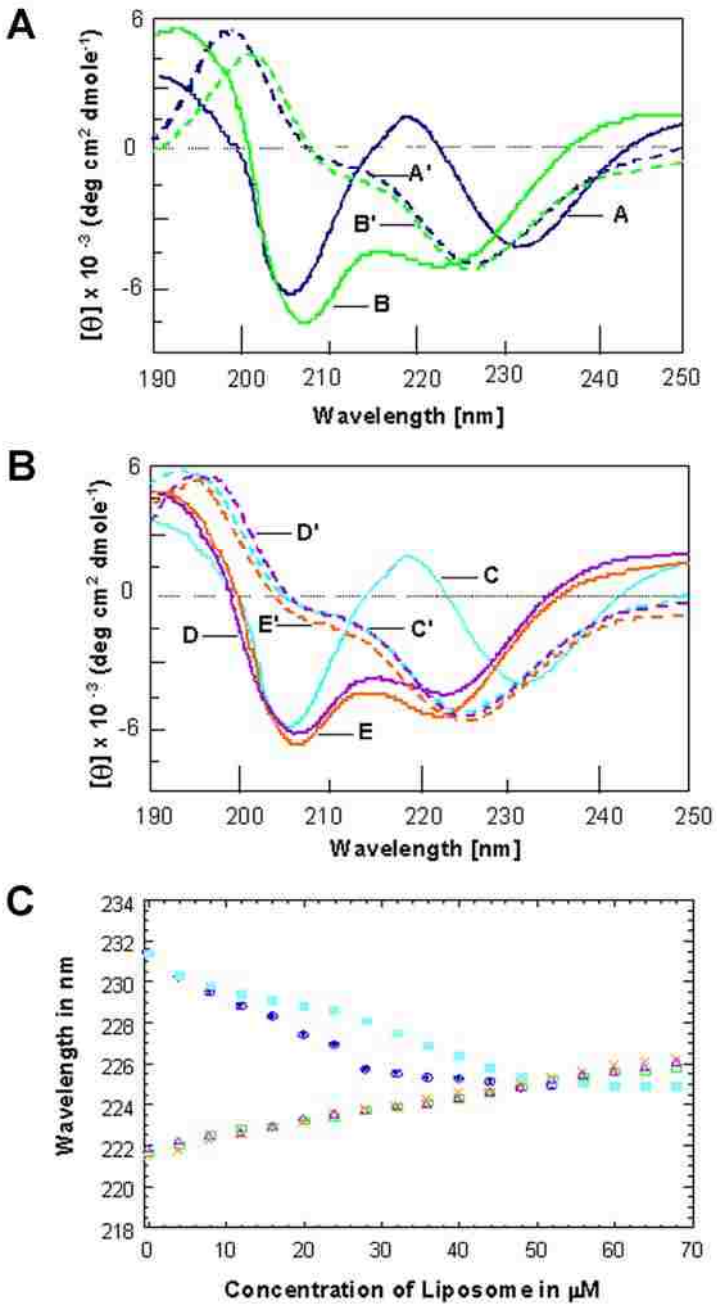

Figure 2.15. CD structural studies of cpFtsY and EcFtsY peptides.

Far UV CD spectra of cpFtsY$_{39\text{-}56}$ (A, blue, solid circles); cpFtsY$_{39\text{-}56}$(F48A) (B, green, open square); EcFtsY$_{186\text{-}204}$ (C, cyan, solid square); EcFtsY$_{186\text{-}204}$(F195A) (D, purple, open triangle); EcFtsY$_{186\text{-}204}$(F196A) (E, orange, cross). A' thru E' labels indicate the corresponding spectra in the presence of liposomes. The lowermost graph shows the shift in secondary structure as a function of liposome concentration. These experiments were performed by Dakshinamurthy Rajalingam (Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR).

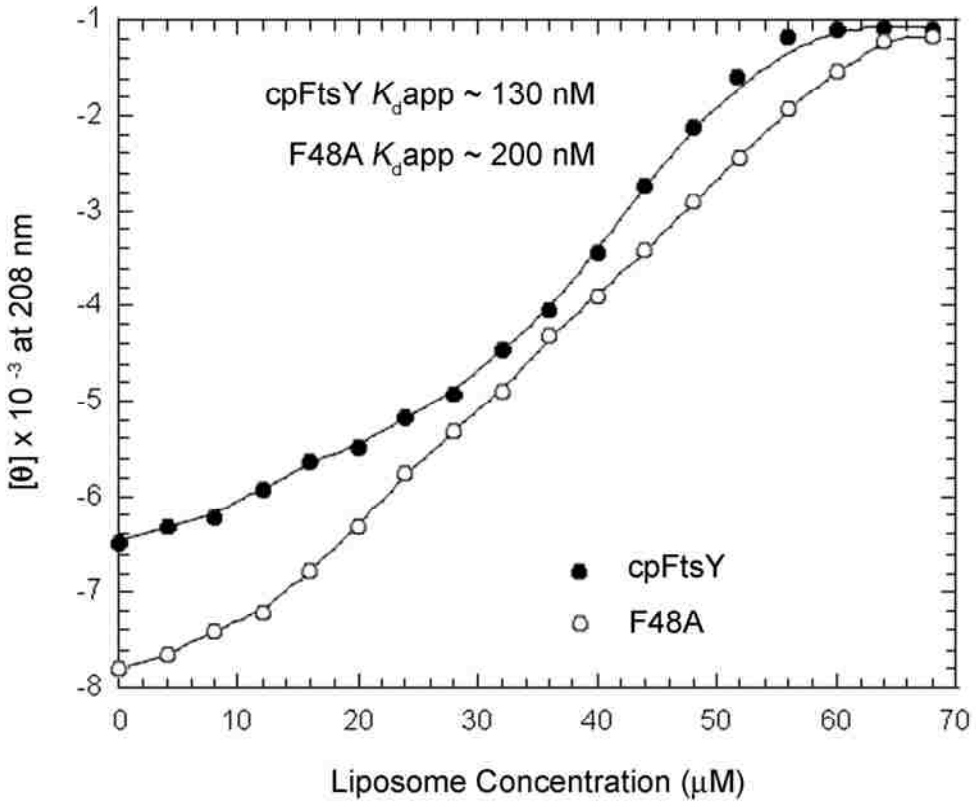

Figure 2.16. A**pparent K_d values for interaction of cpFtsY and F48A with liposomes.**

Molar ellipticity changes at 208nm for cpFtsY (closed circle) and F48A (open circle) are shown as a function of liposome concentration. The solid line represents the best-fit curve of the experimental data generated using Microcal Origin. Appropriate background corrections were made in all spectra. These experiments were performed by Dakshinamurthy Rajalingam (Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR).

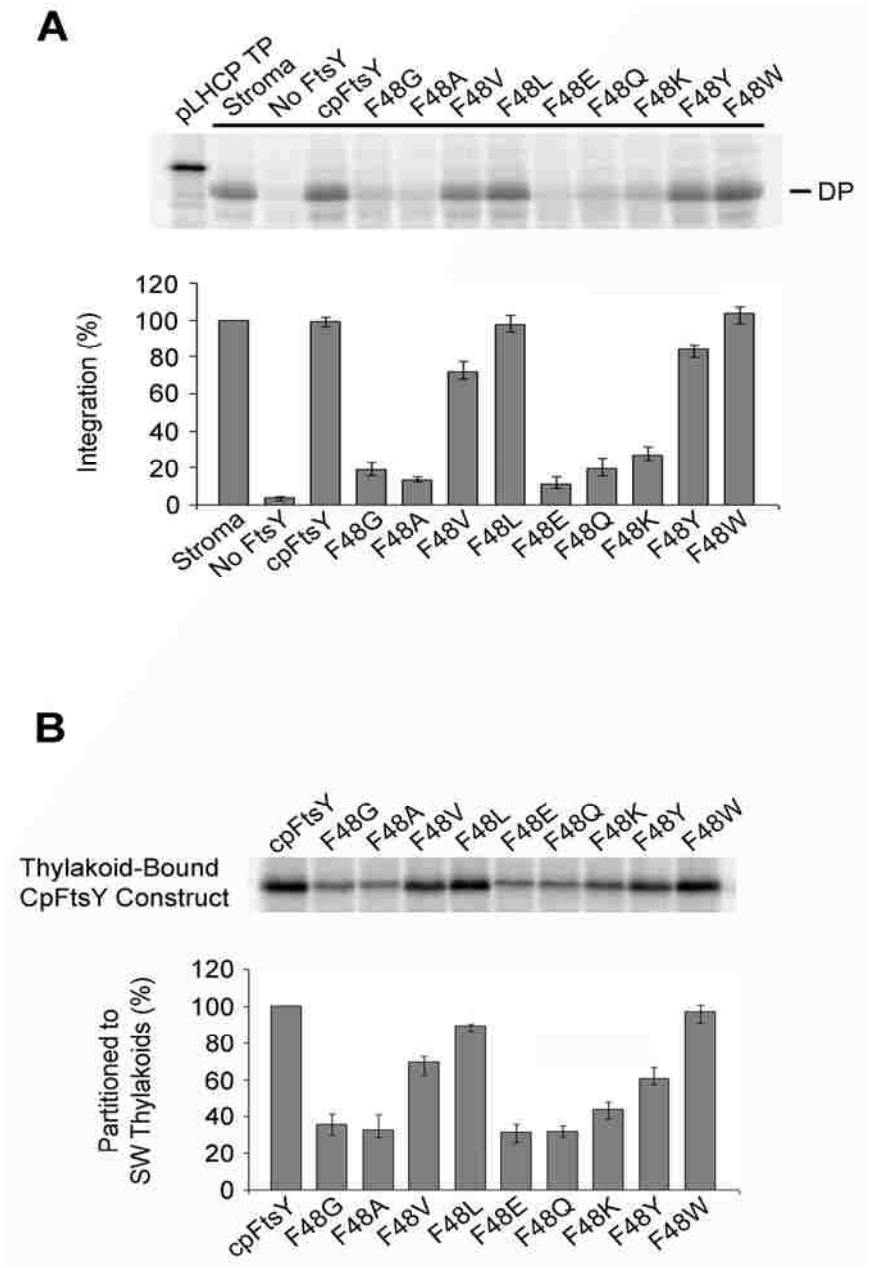

Figure 2.17. CpFtsY N-terminal residue replacement studies.

(**A**) Integration of radiolabeled LHCP was reconstituted as described in Figure 2.2. Integration efficiency was calculated from three separate experiments and is presented relative to integration observed in the presence of stroma. (**B**) Membrane binding of each radiolabeled cpFtsY construct was examined by incubation with SW thylakoids as described in Figure 2.3. The level of each cpFtsY construct bound to membranes was calculated from three separate experiments and is shown relative to bound cpFtsY.

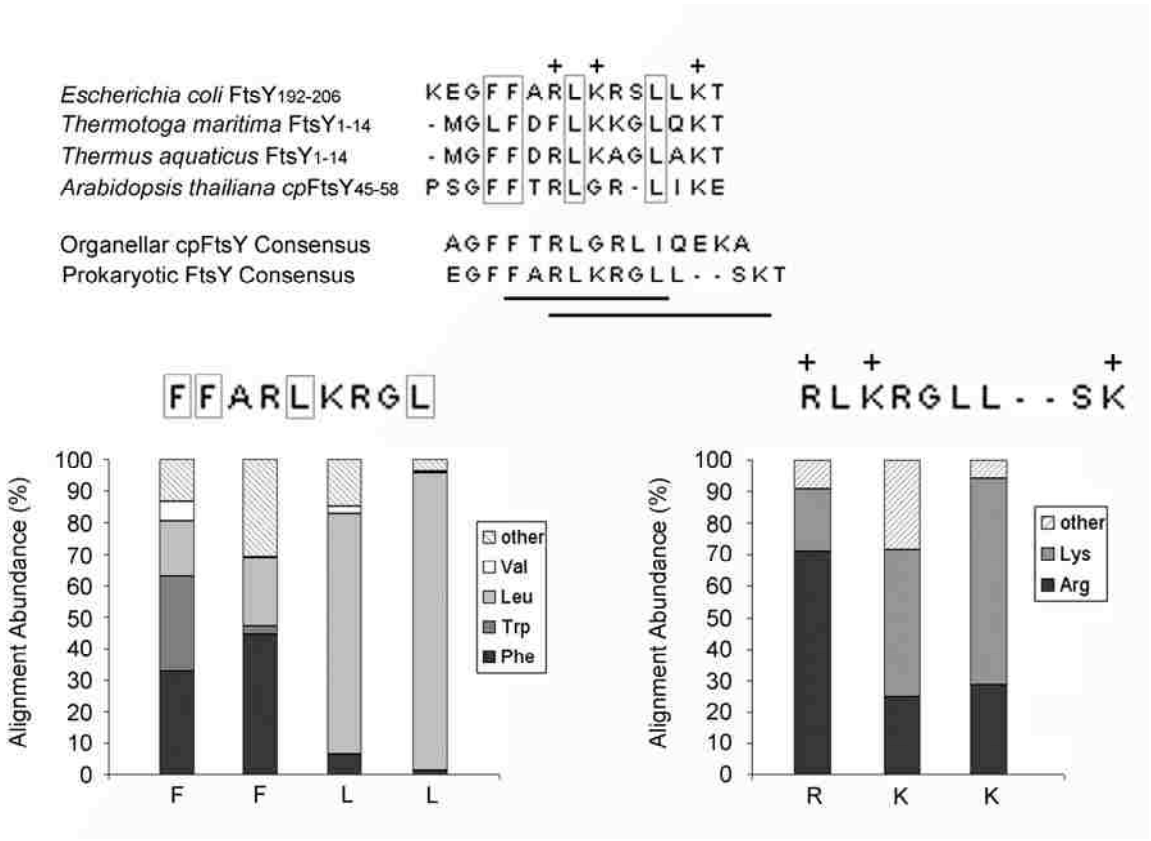

Figure 2.18. Alignment of conserved cpFtsY N-terminal residues.

The *A. thaliana* cpFtsY double Phe region was aligned with the corresponding regions of *E. coli*, *T. maritima*, and *T. aquaticus* FtsYs using ClustalW. Hydrophobic and positively-charged residues thought to be important for lipid binding are indicated by gray squares and +, respectively. ClustalW was used to generate consensus sequences for prokaryotic and organellar FtsYs. The bottom graphs indicate the relative abundance of each hydrophobic or positively-charged residue at the position indicated.

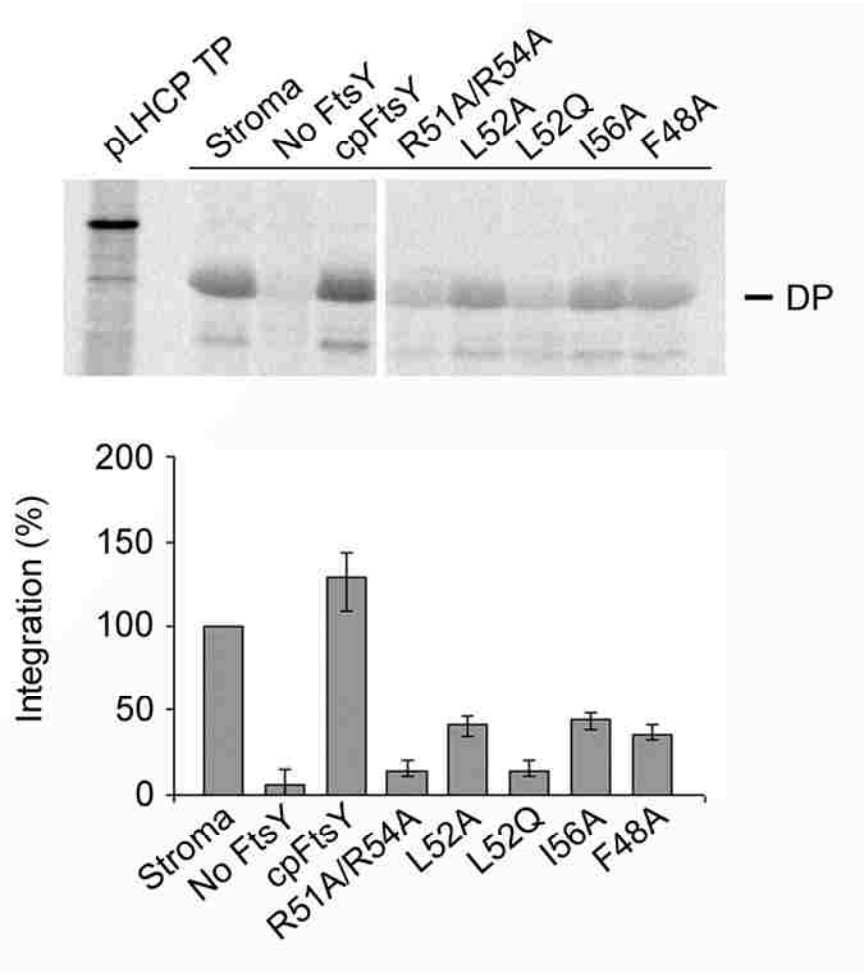

Figure 2.19. Mutation of cpFtsY conserved residues in membrane-binding region affect LHCP targeting.

Integration of radiolabeled LHCP was reconstituted as described in Figure 2.2. Integration efficiency was calculated from three separate experiments and is presented relative to integration observed in the presence of stroma.

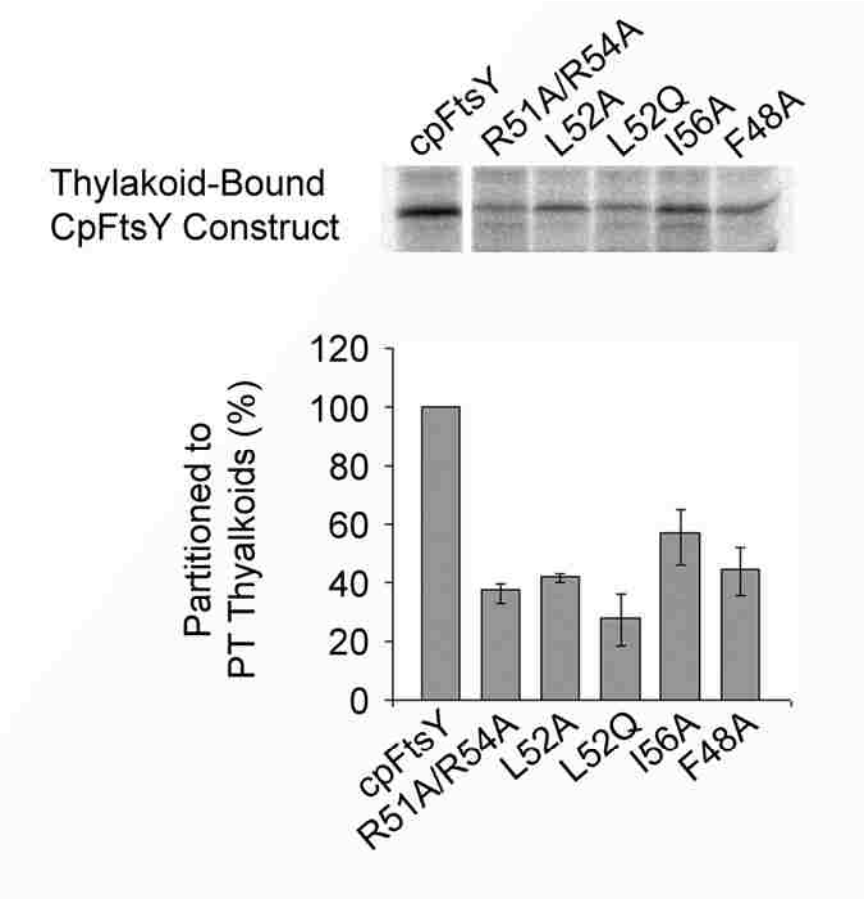

Figure 2.20. Mutation of cpFtsY conserved residues in membrane-binding region affect thylakoid binding.

Membrane binding of each radiolabeled cpFtsY construct was examined by incubation with SW thylakoids as described in Figure 2.3. The level of each cpFtsY construct bound to membranes was calculated from three separate experiments and is shown relative to bound cpFtsY.

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III

CPSRP43 MEDIATES ALB3 REGULATION OF CPSRP TARGETING COMPONENTS

SUMMARY

The chloroplast signal recognition particle (cpSRP) and its receptor, chloroplast FtsY (cpFtsY) form a membrane complex with Alb3 during a cpSRP/Alb3-dependent post-translational targeting cycle. However, the mechanism for cpSRP/Alb3 interaction is not known. Using recombinant purified cpSRP43 and a construct corresponding to the soluble C-terminal extension of Alb3 (Alb3-Cterm), we have demonstrated a direct interaction between cpSRP43 and the C-terminus of Alb3. To explore the importance of interaction between cpSRP43 and Alb3, we have utilized the Alb3-Cterm peptide in assays that reconstitute cpSRP targeting activities. The Alb3-Cterm peptide is able to compete for membrane complex formation with Alb3 and reduce transit complex stability *in vitro*, suggesting that an interaction between cpSRP and Alb3-Cterm is necessary for promoting distinct membrane events. Furthermore, Alb3-Cterm peptide is able to stimulate GTP hydrolysis between cpSRP54 and cpFtsY only in the presence of cpSRP43, indicating that cpSRP43 facilitates a response to Alb3 in cpSRP54 and cpFtsY. Results that demonstrate that stimulation of GTP hydrolysis by Alb3 C-terminus is dependent upon the presence of cpSRP43 suggest that cpSRP43 mediates key targeting events at the thylakoid membrane, such as release of the targeting complex from Alb3. Furthermore, these data support a model in which cpSRP43 functions as a translocon 'sensing' component critical for membrane-associated steps in the post-translational cpSRP-dependent targeting pathway.

INTRODUCTION

The inner membranes of mitochondria and chloroplast thylakoid membranes are densely populated with protein complexes vital to electron transport. For both, their biogenesis requires specialized protein sorting and integration systems, which localize nuclear-encoded as well organelle-encoded proteins to the target membrane. Consistent with the prokaryotic origin of both organelles, Oxa1p in the mitochondrial inner membrane and Albino3 in the thylakoid membrane are integral membrane proteins that belong to a family of protein insertases that also includes YidC in bacteria (Luirink *et al.*, 2001; Yen *et al.*, 2001; Stuart, 2002; Kuhn *et al.*, 2003; Dalbey and Chen, 2004; Yi and Dalbey, 2005).

Alb3 (Albino3) was the most recently identified member of the YidC/Oxa1/Alb3 family (Sundberg *et al.*, 1997). The Alb3 insertase is located in the thylakoid membrane and appears to be present in two pools, one associated with chloroplast SecY (cpSecY) and another pool independent of cpSecY. With SecY, Alb3 is responsible for the assembly of chloroplast-encoded Photosystem II proteins, such as D1 (Kuhn *et al.*, 2003; Ossenbuhl *et al.*, 2004). Alb3 also works in conjunction with a post-translational chloroplast signal recognition particle (cpSRP) targeting system to integrate a family of nuclear-encoded light-harvesting chlorophyll-binding proteins (LHCPs) into the thylakoid membrane where they are assembled with chlorophyll to form light harvesting complexes (Moore *et al.*, 2000).

Though YidC/Oxa1/Alb3 homologues can vary in length quite dramatically (225-795 residues), all share a hydrophobic core region of about 200 residues (Yen *et al.*, 2001). Unexceptional in regards to sequence homology, the hydrophobic core region has

a conserved structure with five or six transmembrane segments broken up by hydrophilic loops (see Figure 3.1). As shown in Figure 3.1, both Oxa1p and Alb3 proteins have five transmembrane domains with similar topology. The N-termini of Oxa1p and Alb3 face into the mitochondrial intermembrane space and thylakoid lumen while the C-termini face into the mitochondrial matrix and chloroplast stroma, respectively. YidC contains a sixth transmembrane segment (required for its membrane insertion), such that both N- and C-termini face in to the cytoplasm (Saaf *et al.*, 1998). Complementation studies with chimeric fusions of the conserved core of either yeast Oxa1p or chloroplast Alb3 with the non-essential targeting region of YidC demonstrated that the core regions of both Oxa1p and Alb3 could functionally replace the core of YidC to insert membrane proteins integrated via a "YidC only" pathway (Jiang *et al.*, 2002; van Bloois *et al.*, 2005). Similarly, a chimera of YidC fused with the C-terminal ribosome-binding domain of Oxa1p has been useful in demonstrating that the core region of YidC can functionally replace the core region of Oxa1p (Preuss *et al.*, 2005). These experimental results suggest that the core regions of YidC/Oxa1/Alb3 homologues are interchangeable and house the capacity for assisting membrane protein transition into adjacent bilayers, while the hydrophilic extensions are specialized for each particular system (Preuss *et al.*, 2005; van Bloois *et al.*, 2005).

Certain hydrophilic loops are responsible for interacting with translating ribosomes or targeting machinery, conceivably increasing the efficiency of the integration reaction. For example, the hydrophilic C-terminal extension of Oxa1p forms an α-helical domain essential for interacting with the ribosome during co-translational integration (Jia *et al.*, 2003; Szyrach *et al.*, 2003). Not surprisingly, this α-helical C-

terminal extension does not appear to be required for post-translational integration events (Szyrach *et al.*, 2003). Like Oxa1p, Alb3 contains a hydrophilic C-terminal extension. Due to the fact that the cpSRP targeting machinery can be stabilized in complex with Alb3 without targeting substrate, it is thought that Alb3 interacts with cpSRP directly rather than through a substrate-mediated event (Moore *et al.*, 2003). Binding of Alb3's C-terminus (Alb3-Cterm) using Alb3-Cterm specific antisera inhibits LHCP integration and prevents Alb3 interaction with cpSRP (Moore *et al.*, 2003), suggesting that interactions with Alb3-Cterm are directly involved in the targeting reaction.

CpSRP is composed of a highly conserved 54 kDa protein (cpSRP54) that serves as the core SRP molecule as well as a 43 kDa protein (cpSRP43) unique to chloroplasts (Schuenemann, 2004). CpSRP works in combination with a membrane-associated SRP receptor protein (cpFtsY) and Alb3 to integrate LHCPs into the thylakoid membrane. LHCPs are synthesized with an N-terminal chloroplast targeting peptide, which is removed by a stromal processing peptidase soon after chloroplast import. After conversion from precursor to mature protein, LHCP is thought to be bound by the cpSRP, forming a soluble transit complex capable of transporting LHCP to the thylakoid membrane in an integration-competent state. On the thylakoid membrane, cpSRP/LHCP along with cpFtsY forms a complex with Alb3. Though the intermediate steps are not very well understood, LHCP must be transferred from cpSRP to Alb3. Presumably after LHCP release from SRP, cpSRP54 and cpFtsY hydrolyze GTP in a reciprocal fashion, releasing the cpSRP and cpFtsY for subsequent rounds of targeting.

The cpSRP-dependent targeting reaction is novel in that it functions post-translationally, targeting fully-synthesized substrates. All other known SRP targeting

systems utilize the translating ribosome as a regulator of GTP hydrolysis and protein-protein interactions (e.g. with SRP54 and FtsY homologues) (Bacher *et al.*, 1996; Mandon *et al.*, 2003). The evolutionary acquisition of cpSRP43 appears critical for post-translational targeting of LHCPs. CpSRP43 has been shown to bind the substrate, interact with cpSRP54, and regulate GTP hydrolysis between cpSRP54 and cpFtsY, all seemingly critical roles. Moreover, we have recently demonstrated that affinity-tagged cpSRP43 is able to specifically coprecipitate Alb3 from isolated thylakoid membranes (Tzvetkova-Chevolleau *et al.*, 2007).

To explore the importance of interaction between cpSRP43 and Alb3, we have utilized a recombinant construct corresponding to the soluble C-terminal region of Alb3 in assays that reconstitute cpSRP targeting activities, including the cpSRP-dependent targeting of LHCP. Our results indicate that cpSRP43 directly interacts with Alb3 via the hydrophilic C-terminal region of Alb3. Furthermore, a soluble construct corresponding to the C-terminus of Alb3 is able to stimulate GTP hydrolysis between cpSRP54 and cpFtsY only in the presence of cpSRP43, indicating that cpSRP43 facilitates a response to Alb3 in cpSRP54 and cpFtsY. Results that demonstrate that stimulation of GTP hydrolysis by Alb3 C-terminus is dependent upon the presence of cpSRP43 suggest that cpSRP43 mediates key targeting events at the thylakoid membrane, such as release of the targeting complex from Alb3. Furthermore, these data support a model in which cpSRP43 functions as a translocon 'sensing' component critical for membrane-associated steps in the post-translational cpSRP-dependent targeting pathway.

MATERIALS AND METHODS

All reagents and enzymes used were purchased commercially. All primers were from Integrated DNA Technologies. Plasmids described previously were used for *in vitro* transcription and translation of cpSRP54 (Schuenemann *et al.*, 1999), pLHCP (Cline *et al.*, 1989), iOE33 (Hulford *et al.*, 1994), iOE17mc (Moore *et al.*, 2003), and pElip2 (Kim *et al.*, 1999). Recombinant purified cpSRP43, cpSRP43-his, cpFtsY, and cpSRP54 were prepared as described with the exception of a new restriction site (XhoI) for cpFtsY (Yuan *et al.*, 2002; Moore *et al.*, 2003; Goforth *et al.*, 2004; Jaru-Ampornpan *et al.*, 2007; Tzvetkova-Chevolleau *et al.*, 2007). A peptide corresponding to the cpSRP43 binding site in LHCP, L18 has been previously described (DeLille *et al.*, 2000). Antibodies to the following proteins have also been described: Alb3-Cterm (Woolhead *et al.*, 2001), Alb3-50aa (Moore *et al.*, 2000), cpSecY (Mori *et al.*, 1999), cpSRP43 (Moore *et al.*, 2003), and cpSRP54 (Moore *et al.*, 2003). Those against cpSecY were a generous gift from Dr. Kenneth Cline, University of Florida, Gainesville. All cloned sequences were verified by DNA sequencing (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR).

Construction of His-Alb3-Cterm Clone

A cDNA clone for PPF-1 (defined as Alb3 in *Pisum sativum*) was obtained by reverse transcription-polymerase chain reaction (PCR) using total RNA from *Pisum sativum*. Forward and reverse primers (Integrated DNA Technologies) matching the sequence for PPF1 (Accession #Y12618) were designed to include EcoRI and XbaI sites, respectively, for ligation into pGEM-4Z (Promega). The coding sequence for PPF1-

Cterm, a 124-amino acid segment of PPF1 beginning at NNVLSTA and ending at SKRKPVA, was amplified by PCR from PPF-1-pGEM-4Z. The resulting PCR fragment was restricted with BamH1 and XbaI, then ligated into similarly-restricted pGEM-4Z to produce the plasmid Alb3-Cterm-pGEM-4Z. Alb3-Cterm-pGEM-4Z was restricted with BamHI and SalI and the resulting PPF1-Cterm was inserted in-frame behind the coding sequence for glutathione S-transferase (GST) in pGEX-6P-2 (GE Healthcare) to produce the plasmid Alb3-Cterm-pGEX-6P2. Alb3-Cterm-pGEX-6P-2 was restricted with BamHI and XhoI and the resulting Alb3-Cterm was inserted in-frame behind the coding sequence for a 6-histidine tag in pET-32a (Novagen) to produce the plasmid Alb3-Cterm-pET-32a. Forward and reverse primers were designed to match the beginning and ending of the Alb3-Cterm (described above) and to include SphI and HindIII sites, respectively, for ligation into pQE-80L (Qiagen). The forward primer also included a 2 amino acid linker (SA), a Flag Tag, and a Thrombin cleavage site. The resulting PCR fragment was restricted with SphI and HindIII, then ligated into similarly-restricted pQE-80L to create the plasmid Alb3-Cterm-pQE-80L. This plasmid was transformed into BL21* (Invitrogen) and used for expression of His-Alb3-Cterm.

Construction of cpSRP43 and cpFtsY Clones

Forward and reverse primers were designed to match the mature coding sequence of *A. thaliana* cpFtsY starting with the predicted mature sequence CSAGPSGF and to include KpnI and XbaI sites, respectively, for ligation into pGEM-4Z. The forward primer also included extra bases cacg at the 5' end which encode a Kozak sequence (cacgatgg) when added to the atg of the initiator methionine. The resulting PCR

fragment was restricted with KpnI and XbaI, then ligated into similarly-restricted pGEM-4Z to create the plasmid cpFtsY-pGEM-4Z. This plasmid was used for *in vitro* transcription/translation of cpFtsY.

CpSRP43 transcription/translation clone was designed using forward and reverse primers to match the mature predicted sequence of *A. thaliana* cpSRP43 beginning with AAVQRNYE and including a Kozak sequence, and BamHI and XhoI restriction sites for insertion into similarly-restricted pGEM-7Z. The PCR fragment obtained was restricted with BamHI and XhoI, ligated into similarly-restricted pGEM-7Z and used for *in vitro* transcription/translation of cpSRP43.

Preparation of Chloroplasts and Radiolabeled Precursors

Intact chloroplasts were isolated from 10-12 day old pea seedlings (*P. sativum* cv. Laxton's Progress) and used to prepare thylakoids and stroma as described (Cline *et al.*, 1993). Chlorophyll (Chl) content was determined according to (Arnon, 1949). Thylakoids were isolated from lysed chloroplasts by centrifugation and SW two times with 1M potassium acetate in import buffer (IB; 50 mM Hepes-KOH, pH 8.0, 0.33 M sorbitol) and two times with IB with 10 mM $MgCl_2$ (IBM) prior to use. For protease treatment, SW thylakoids were diluted to 0.5 mg/ml Chl in IB with 0.2 mg/ml thermolysin and 1 mM $CaCl_2$, incubated for 40-60 min, combined with EDTA in IB to 20 mM EDTA, and applied to a 7.5% Percoll™ (GE Healthcare) gradient in IB containing 10 mM EDTA. Pellets were washed once with IB containing 10 mM EDTA and twice with IBM. Protease-treated thylakoids were resuspended at 1 mg/ml Chl in IBM.

In vitro transcribed capped RNA was translated in the presence of [^{35}S] methionine (Chu *et al.*, 2004) using a wheat germ system to produce radiolabeled proteins (Cline *et al.*, 1993). Precursor LHCP translation products (TP) were diluted twofold with 30 mM unlabeled Met in IB. CpSRP43, cpSRP54, and cpFtsY constructs were labeled with ratios of labeled and unlabeled Met such that equal [^{35}S] signal represented equimolar protein. Constructs were quantified by comparing the [^{35}S] signal from a given protein band as analyzed by SDS-PAGE and phosphorimaging. Equimolar amounts of proteins were added to each experiment.

Assays for Determining Thylakoid Binding

Thylakoid binding assays included SW or PT thylakoids (equal to 75 µg Chl) in IBM and radiolabeled cpSRP43, cpSRP54, or cpFtsY. Reactions were incubated for 30 min in light at 25°C. Thylakoids were centrifuged at 3200 x *g* for 6 min, washed in 1 ml IBM, and transferred to clean tubes. Thylakoids were then pelleted, solubilized in SDS buffer, and heated. Amounts equivalent to 7.5 µg Chl per sample were analyzed by SDS-PAGE and phosphorimaging.

Protein Binding Assays

CpSRP43/His-Alb3-Cterm binding assays were performed by incubating 300 pmol of GST-fused cpSRP43 with 1500 pmol of His-Alb3-Cterm for 15 min at 25°C and adding 25 µl of a 50% glutathione Sepharose slurry in 10 mM HK, 50 mM potassium acetate, and 10 mM $MgCl_2$, pH 8.0, in a final volume of 100 µl. Samples were allowed to mix end-over-end for 30 min at 4 °C and then transferred to a 0.8 ml Centrifuge Column

(Pierce) and washed three times with 0.75 ml 20 mM HK, 300 mM KCl, 10 mM $MgCl_2$, 2% Tween 20, three times with 0.75 ml 0.1% Mal in IB, and three times with 10 mM HK, 10 mM $MgCl_2$. Coprecipitating proteins were eluted in 50 µl of SDS-PAGE solubilization buffer. Eluted proteins were separated by 12.5% SDS-PAGE and visualized by staining with Coomassie Blue.

Isothermal Titration Calorimetry

Binding of His-Alb3-Cterm to cpSRP43 was analyzed by measuring heat change during titration of nucleotide into a protein solution using a VP-ITC titration microcalorimeter (MicroCal Inc.). All solutions were degassed under vacuum and equilibrated at 25°C prior to titration. The sample cell (1.4 ml) contained 0.072 mM His-Alb3-Cterm in PBS buffer, pH 5.5. The reference cell contained MilliQ water. Upon equilibration, 0.72 mM cpSRP43 was injected in 50 × 6 µl aliquots using the injection rate of 300 sec intervals between each injection to allow the sample to return to the baseline. Titration curves were corrected for protein-free buffer and analyzed using Origin ITC software (MicroCal Inc).

Complex Formation and Precipitation Assays

For complex formation in the presence of His-Alb3-Cterm, both SW thylakoids and the protein constructs for each assay were separately preincubated with 500 µg His-Alb3-Cterm in IBM (final volume of 75 µl and 425 µl, respectively) and then combined as follows. Complexes between thylakoid membrane proteins and His-cpSRP54, His-Trx-cpFtsY, and cpSRP43 were formed by incubating 10 µg indicated purified proteins

with 0.5 mM GMP-PNP and SW thylakoids equal to 75 µg Chl at 25°C for 30 min in light. Membranes were recovered by centrifugation and washed with 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 (Mal) 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 and incubated for 30 min at RT with gentle mixing. Afterward, the resin mixture was transferred to a 0.8 ml Centrifuge Column (Pierce) and washed four times with 0.5 ml 0.1% Mal in IB and one time with 0.5 ml IB. Coprecipitating proteins were eluted in 100 µl SDS solubilization buffer.

Transit Complex Formation Assays

Transit complex was formed in 30 µl assays by mixing 5 µl of stromal extract (SE) (equivalent to 10 µg Chl) or 100 pmol of cpSRP43 and 50 pmol of cpSRP54 and 0-3200 pmol of His-Alb3-Cterm peptide as indicated with 5 µl of diluted translation product similar to assays described previously (Payan and Cline, 1991; DeLille *et al.*, 2000). Assays were incubated at for 30 min at 25°C, then cooled on ice and prepared for native PAGE by the addition of 5 µl 50% glycerol.

Analysis of Samples

A portion of each sample (10 µl) from each assay was analyzed by SDS-PAGE (or native PAGE as indicated) followed by Western blotting or phosphorimaging. An

exception to this is the saturation data from Figure 3.2 for which 5 µl was analyzed. Molecular Dynamics image analysis software (Image Quant) was used for quantification of integration assays from phosphorimages obtained using a Typhoon 8600. For Western blots, separated samples were transferred to Biotrace™ polyvinylidene fluoride membrane (Life Sciences) and incubated with rabbit polyclonal antibodies. Horseradish peroxidase-labeled mouse IgG (Southern Biotech) was used as secondary antibody. Proteins reacting with antibodies were revealed by incubation with SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Western blots were imaged using an Alpha Innotech Fluorchem™ IS-8900 using chemiluminescent detection. AlphaEase FC Stand Alone software (Alpha Innotech) was used for quantification of Fluorchem™ IS-8900 images. SDS-Page Standards (Invitrogen) were used to calculate molecular weights (MagicMark™ XP Western Standard for Western blots; Benchmark™ Protein Ladder for Coomassie-stained gels). Protein concentrations were estimated by Coomassie Blue staining of purified proteins along with protein standards.

GTPase Assays

Recombinant cpSRP54 and cpFtsY were assayed for GTPase activity alone or in the presence of L18 peptide, recombinant cpSRP43, and/or His-Alb3-Cterm as described (Gonzalez-Romo *et al.*, 1992; Goforth *et al.*, 2004). GTPase activity was measured in solution by determining the amount of inorganic phosphate released by GTP hydrolysis. Assays containing 150 pmol of cpSRP43, cpSRP54, cpFtsY as indicated, the indicated number of pmol of His-Alb3-Cterm, 750 pmol L18 peptide as indicated, and 2 mM GTP in 10 mM Hepes, pH 8, and 10 mM $MgCl_2$ were incubated at 30°C for 1 h. After

incubation SDS was added to a final concentration of 6% to denature protein components and prevent subsequent GTPase activity. The addition of ascorbic acid and ammonium molybdate (to 6% and 1%, respectively) was followed by a 5 min incubation, and subsequently each assay was brought to 1% sodium citrate, sodium (meta)arsenite, and acetic acid for a final volume of 1.05 ml. The absorbance of each sample was then measured at 850 nm. Throughout the duration of the experiment the amount of GTP hydrolyzed increased linearly. Furthermore, a standard curve of inorganic phosphate (P_i) was linear from 2 to 75 nmol P_i and was used to determine the amount of P_i released in each assay. A substrate control that lacked protein components and a zero time control with the protein denatured by the addition of 6% SDS prior to the addition of GTP varied from 0.0 to 1.6 nmol of P_i between experiments and were used to correct for nonspecific hydrolysis and background hydrolysis for each assay.

RESULTS

CpSRP43 binds to thylakoids in a protease-sensitive manner.

CpFtsY exhibits the ability to partition between the stroma and the thylakoid membranes and, for that reason, is thought to be, at least partially, responsible for the thylakoid localization of the cpSRP targeting complex (Tu *et al.*, 1999). However, it has been demonstrated that cpSRP43 and cpSRP54 can also associate together on thylakoid membranes in the absence of cpFtsY (Moore *et al.*, 2003). To determine whether a proteinaceous binding site is required for cpSRP component binding, we compared the association of radiolabeled cpSRP43, cpSRP54, and cpFtsY to salt-washed (SW) or protease-treated (PT) thylakoids. After incubation, thylakoids were reisolated by centrifugation, buffer-washed, and solubilized. Constructs were labeled such that equal isotope label represents equimolar amounts. As shown in Figure 3.2A, cpSRP43, cpSRP54, and cpFtsY bind SW thylakoid membranes appreciably. While both cpFtsY and cpSRP54 interact efficiently with both SW and PT thylakoids, protease-treatment reduces thylakoid association of cpSRP43 by ~80% (Figure 3.2A, lanes 1-3 for SW and PT thylakoids). The considerable reduction in cpSRP43 thylakoid association to PT thylakoids suggests that cpSRP43 binding to thylakoids is largely dependent upon a protease-sensitive binding site on thylakoid membranes. CpSRP43 thylakoid association is restored partially by the presence of cpSRP54, suggesting that a cpSRP43/54 complex is able to bind thylakoid membrane via interactions with the bilayer (Figure 3.2A, compare lanes 3 and 6 with lanes 4 and 7). Together our observations suggest that cpSRP43 (in the absence of cpSRP54) binds to thylakoids via a protease-sensitive thylakoid protein that is partially or fully exposed to the stroma.

Protease treatment of thylakoids results in the removal of the soluble C-terminal region of Alb3 (*P. sativum* PPF1); protease-treated Alb3 has an apparent molecular weight of 30 kDa and can be detected by antisera to a protease-inaccessible 50aa loop (Figure 3.3), but not by antibody against the C-terminus. We have recently shown that, in the absence of cpSRP54 and cpFtsY, cpSRP43 copurifies Alb3 from thylakoid membranes (Tzvetkova-Chevolleau *et al.*, 2007). This, along with the protease-sensitive binding of cpSRP43 to thylakoids, has led us to examine the role of cpSRP43 in cpSRP/Alb3 binding.

Alb3 is specifically copurified from thylakoids using cpSRP43.

We have previously published that cpSRP54 and cpFtsY, in the absence of cpSRP43, are capable of forming a complex with Alb3 (Moore et al., 2003), raising the question of whether the presence of cpSRP43 is stimulatory for cpSRP/cpFtsY complex formation with Alb3. To investigate the relative strength of interactions between cpSRP components and Alb3, we have utilized His-tagged cpSRP43, cpSRP54, and cpFtsY constructs in copurification assays. The affinity-tagged proteins are active in reconstituting integration and can be used with thylakoid membranes to examine their ability to interact with Alb3. After incubating the indicated His-tagged constructs with SW thylakoids in the presence or absence of GMP-PNP, membranes were solubilized with maltoside and mixed with Talon™ metal affinity resin to repurify His-tagged constructs and all associated proteins. Samples were probed for His-tagged constructs and coprecipitating Alb3. As shown in Figure 3.4, assays containing cpSRP43 coprecipitate ~15% of the available Alb3. In comparison, assays containing cpSRP54 or

cpFtsY coprecipitate ~5% or less of the available Alb3, which is only slightly above background binding to the resin alone. Similar amounts of each added His-tagged construct were copurified indicating that changes in the amount of copurified Alb3 are not due to inaccessible His-tags. The distinct capability of cpSRP43 to copurify Alb3 advocates for cpSRP43 functioning as the bridge that connects cpSRP to Alb3.

CpSRP43 interacts with His-Alb3-Cterm.

The results from Figure 3.2 suggest that cpSRP43 interacts with the thylakoid membrane via a protease-sensitive binding site. Since we have shown that cpSRP43 specifically copurifies Alb3 from thylakoid membranes, it is probable that cpSRP43 binds to the ~13 kDa, soluble, C-terminus of Alb3 that faces the stroma. To determine whether this is the case, we produced recombinant His-Alb3-Cterm for use in cpSRP43 binding assays. Glutathione-S-Transferase (GST) or cpSRP43-GST were incubated with His-Alb3-Cterm and repurified using Glutathione-Sepharose™ resin. The proteins were then eluted from the resin using buffer containing glutathione. Eluted proteins were separated by SDS-PAGE and visualized directly by staining with Coomassie Blue. Figure 3.5 shows that cpSRP43-GST specifically coprecipitates His-Alb3-Cterm (apparent MW ~20 kDa).

Isothermal titration calorimetry (ITC) was also used to verify an interaction between cpSRP43 and His-Alb3-Cterm (Figure 3.6). Interaction of His-Alb3-Cterm with cpSRP43 proceeds with a 1:1 stoichiometry. CpSRP43 exhibits a high binding affinity for His-Alb3-Cterm (K_d ~65 nM). Taken together, these results indicate that cpSRP43 likely interacts directly with the soluble C-terminus of Alb3.

His-Alb3-Cterm inhibits the formation of transit complex.

Assuming that the 1) C-terminus of Alb3 is able to interact with cpSRP43 during the targeting reaction and 2) cpSRP43 binding to the C-terminus of Alb3 is required for LHCP integration then His-Alb3-Cterm should be able to compete with endogenous Alb3 thereby limiting LHCP integration. On the contrary, we found that physiologically-relevant concentrations of His-Alb3-Cterm are not able to inhibit cpSRP-dependent LHCP targeting and integration *in vitro* (data not shown). This raises the question of whether the C-terminus of Alb3 is able to interact with cpSRP43 that is engaged in transit complex (cpSRP43/LHCP/cpSRP54) or in a cpFtsY-associated complex (cpSRP43/LHCP/cpSRP54/cpFtsY). Figure 3.7 shows that incubation of radiolabeled pLHCP with stromal extract (SE) or recombinant purified cpSRP43 and cpSRP54 reconstitutes formation of a cpSRP/LHCP transit complex, which clearly migrates into a nondenaturing gel. In the absence of SE, pLHCP remains in the sample well (data not shown) as previously documented (DeLille *et al.*, 2000). In the presence of His-Alb3-Cterm, pLHCP remains in the sample well, suggesting that His-Alb3-Cterm either inhibits the formation of transit complex or affects the stability of transit complex. Interestingly, a transit complex formed with purified cpSRP43/54 appears to migrate a shorter distance into the nondenaturing gel. We continue to examine this experiment to determine the cause of the apparent migration shift. Regardless, it is clear that the His-Alb3-Cterm is capable of affecting transit complex and thus, we expect that it is able to interact with cpSRP43 in transit complex.

His-Alb3-Cterm competes with Alb3 for binding cpSRP membrane complex.

If an interaction between cpSRP and the C-terminus of Alb3 is critical for cpSRP/cpFtsY/Alb3 membrane complex formation then His-Alb3-Cterm should be able to compete with endogenous Alb3 thereby inhibiting the formation of cpSRP/cpFtsY membrane complex with Alb3. To investigate whether His-Alb3-Cterm can compete with Alb3 for binding to a cpSRP/cpFtsY membrane complex, we isolated a stabilized complex containing soluble cpSRP components and Alb3 in the presence or absence of His-Alb3-Cterm. After incubating cpSRP, S-tagged cpFtsY, salt-washed thylakoids, GMP-PNP, and His-Alb3-Cterm, membranes were washed, solubilized with maltoside, and mixed with S-protein agarose resin to precipitate S-tagged cpFtsY and all associated proteins. This complex was probed for coprecipitating Alb3. As expected, Alb3 is coprecipitated with S-tagged cpFtsY in the presence of cpSRP components, cpSRP43 and cpSRP54 (Figure 3.8A). In the presence of His-Alb3-Cterm, cpSRP43/cpSRP54/cpFtsY form a stable (albeit slightly reduced) complex on thylakoids. The amount of Alb3 copurified with cpSRP43/cpSRP54/cpFtsY is reduced by ~74% in the presence of His-Alb3-Cterm (Figure 3.8A and C). In comparison, a cpSRP54/cpFtsY complex lacking cpSRP43 copurifies ~30% less Alb3 in the presence of His-Alb3-Cterm. These results suggest that His-Alb3-Cterm is able to compete for Alb3 in formation of a cpSRP43/cpSRP54/cpFtsY/Alb3 membrane complex.

His-Alb3-Cterm stimulates GTP hydrolysis between cpSRP GTPases in a cpSRP43-dependent manner.

GTP binding and hydrolysis by cpSRP54/cpFtsY is critical in the proper integration of LHCP into the thylakoid membrane. Furthermore, cpSRP43 has been shown to play an important role in regulation of GTP hydrolysis by cpSRP54 and cpFtsY (Goforth *et al.*, 2004). Given that the timing of substrate release is critical and that cpSRP43 interacts directly with Alb3, it seems plausible that the presence of Alb3 may also affect cpSRP GTP hydrolysis rates. To examine a possible influence of Alb3 on the GTP hydrolysis activity between cpSRP54 and cpFtsY, we utilized a colorimetric assay that measures the release of inorganic phosphate by GTP hydrolysis as described previously (Gonzalez-Romo *et al.*, 1992; Goforth *et al.*, 2004). Comparison of the amounts of inorganic phosphate generated by equimolar amounts of constituent proteins indicates that less than 1 nmol of GTP is hydrolyzed when any single protein is present (data not shown). When cpSRP54 and cpFtsY are both present, hydrolysis is above additive background levels (28.5 nmol GTP) (Figure 3.9). In the presence of cpSRP43, GTP hydrolysis between cpSRP54 and cpFtsY is stimulated in a linear fashion with increasing amounts of His-Alb3-Cterm. The presence of a peptide corresponding to the 18-aa cpSRP43 binding motif in LHCP (DeLille *et al.*, 2000) appears to decrease GTP hydrolysis by ~25% and results in a reduction in the GTPase stimulation caused by the addition of cpSRP43 and His-Alb3-Cterm. This observation could reflect a requirement for release of LHCP from the cpSRP targeting complex prior to GTP hydrolysis. Taken together, these observations suggest that cpSRP43 is required for the GTPase activity stimulation between cpSRP54 and cpFtsY in response to the presence of Alb3, but not necessarily the interaction between cpSRP54/cpFtsY and Alb3.

DISCUSSION

Membrane events that occur during the routing of nuclear-encoded thylakoid proteins via the cpSRP-dependent pathway are not well understood. However, it has been clearly established that integration of imported thylakoid proteins by the cpSRP-dependent transport pathway requires the formation of a membrane complex containing cpSRP with bound substrate, cpFtsY, and Alb3. We have previously demonstrated that cpSRP and cpFtsY form of a stable complex with Alb3 (Moore *et al.*, 2003), yet the mechanism for interaction with Alb3 was not well defined.

In this report, we examined a possible protein-protein interaction that occurs at the thylakoid membrane between cpSRP43 and Alb3. Our data demonstrates that cpSRP43 interacts directly with Alb3 via a hydrophilic C-terminal extension facing the stroma. Furthermore, a soluble construct corresponding to the C-terminus of Alb3 (His-Alb3-Cterm) inhibits cpSRP complex formation with endogenous Alb3 *in vitro*, suggesting that protein-protein interactions involving the C-terminus of Alb3 are critical to the targeting reaction. One such interaction may involve regulation of GTP hydrolysis at the thylakoid membrane, as His-Alb3-Cterm stimulates GTP hydrolysis between cpSRP54 and cpFtsY only in the presence of cpSRP43. CpSRP43, therefore, appears to function not only as a regulator of key targeting steps such as GTP hydrolysis but also as a mediator linking the translocon, substrate, and SRP GTPases. Taken together, these results support a model in which cpSRP43 functions as a translocon 'sensing' component critical for membrane-associated steps in the post-translational cpSRP-dependent targeting pathway.

In co-translational SRP-dependent routing pathways, the SRP/SR-bound ribosome interacts directly with the translocase (Halic *et al.*, 2006), yet a ribosome is absent in the post-translational cpSRP-dependent targeting reaction. Instead, a unique subunit of cpSRP, cpSRP43, has been implicated as a functional replacement for the ribosome, as well as the SRP RNA, in the novel post-translational cpSRP routing pathway (Goforth *et al.*, 2004; Stengel *et al.*, 2008). Reminiscent of the ribosome-binding domain contained with in the hydrophilic C-terminal extension of Oxa1p, the C-terminus of Alb3 appears to house a cpSRP43-binding domain. Although, cpSRP54 and cpFtsY can form a stable complex with Alb3 in the absence of cpSRP43, when comparing similar amounts of the repurified His-tagged cpSRP43, cpSRP54, and cpFtsY, it is clear that, as an individual component, cpSRP43 has the strongest interaction with Alb3 (Figure 3.4). This finding does not, however, rule out the possibility that the C-terminus of Alb3 also interacts directly with cpSRP54 or cpFtsY alone or in complex together. It will be interesting to determine the necessary characteristics of Alb3's cpSRP43 binding domain and whether that region (or surrounding segments) is critical for other protein-protein interactions, such as with cpSRP54 or cpFtsY.

Although previously published results indicated that the amount of Alb3 in a GMP-PNP-stabilized cpSRP54/cpFtsY complex was not increased by the addition of cpSRP43 (Moore *et al.*, 2003), our results indicate that cpSRP43 is stimulatory for the cpSRP/cpFtsY/Alb3 membrane complex (Figure 3.4. and 3.8). The results shown in Figure 3.4 likely differ because our coprecipitation was not limited to the thylakoid bound fraction of affinity-tagged constructs and, more importantly, did not select for proteins interacting with cpFtsY. Moore *et al* selected for cpFtsY using an S-tag, which

would not precipitate cpSRP43 or cpSRP43/cpSRP54 bound to Alb3 in the absence of cpFtsY. The results shown in Figure 3.8 likely differ due to changes we have made in cpSRP43 storage buffers. Unfortunately, cpSRP43 was less stable, and hence less active, in the cpSRP43 storage buffer utilized in Moore *et al (Moore et al., 2003)*.

In regards to whether Alb3 interacts with cpSRP54 or cpFtsY via its C-terminal domain, we have shown evidence that it is the C-terminus of Alb3 that is critical for the formation of stable cpSRP43/cpSRP54/cpFtsY/Alb3 or cpSRP54/cpFtsY/Alb3 membrane complex. However, removal of the Alb3-Cterm region only appears to affect membrane binding of cpSRP43, supporting our hypothesis that cpSRP54 and cpFtsY do not depend on interactions with membrane proteins for thylakoid binding. The increased thylakoid binding exhibited by cpSRP43 and cpSRP54 in the presence of excess His-Alb3-Cterm construct was an unexpected observation (Figure 3.8B, compare lanes 3 and 4 with 7 and 8). However, this may be explained by the observation that His-Alb3-Cterm associates with thylakoid membranes. If His-Alb3-Cterm is capable of interacting with cpSRP43 and thylakoid membranes simultaneously, the membrane-associated His-Alb3-Cterm could provide additional binding sites for cpSRP43 on thylakoids. Consequently, increased thylakoid association of cpSRP54 in the presence of His-Alb3-Cterm and cpSRP43 may be explained the possibility of cpSRP54 interacting with thylakoid-associated cpSRP43.

It is puzzling that the His-Alb3-Cterm construct appears to influence cpSRP components in transit complex formation/stability, formation of a membrane complex with Alb3, and GTP hydrolysis, yet does not inhibit LHCP integration *in vitro*. Work to understand this discrepancy continues. We anticipate that cpSRP/Alb3-Cterm

interactions are dependent upon several factors reflective of particular steps in the targeting cycle. Whether the His-Alb3-Cterm construct affect transit complex migration into a nondenaturing gel (Figure 3.7) is due to inhibition of formation of transit complex or the release of pLHCP will also be interesting to determine.

An additional note-worthy observation from our findings is that the presence of the L18 peptide is somewhat inhibitory to GTP hydrolysis between cpSRP54 and cpFtsY (Figure 3.9). This is consistent with previous data from the mammalian SRP system showing that signal peptides inhibit GTP hydrolysis in the absence of an available translocon (Miller and Walter, 1993; Rapiejko and Gilmore, 1997). It is interesting to speculate that upon release of LHCP from the cpSRP, cpSRP43 may be in a conformation conducive to mediate Alb3-Cterm stimulation of GTP hydrolysis between cpSRP54 and cpFtsY. Further investigations of the dynamic relationships of cpSRP43 with its binding partners (i.e. LHCP, cpSRP54, cpFtsY, and Alb3) are required to validate this hypothesis.

Considering the data shown here and the current model for GTPase regulation of cytosolic SRPs (Shan *et al.*, 2004), we propose the following model for cpSRP GTPase regulation at the thylakoid membrane. Step 1, interactions with thylakoid membranes prime cpFtsY for binding cpSRP54 and GTP and interactions with cpSRP43/LHCP prime cpSRP54 for binding GTP. Step 2, the GTP-bound cpSRP43/LHCP/cpSRP54 complex associates with GTP-bound cpFtsY on thylakoid membranes. Step 3, the membrane-associated complex is directed to Alb3 via an interaction between cpSRP43 and the C-terminus of Alb3. Step 4, cpSRP binding to the C-terminus of Alb3 stimulates LHCP release from cpSRP. LHCP, which acts as a negative regulator of hydrolysis, is

released from cpSRP to Alb3 for insertion into thylakoids. Step 5, in the absence of LHCP, interactions with thylakoid membranes, cpSRP43, and Alb3 trigger reciprocal stimulation of GTP hydrolysis between cpSRP54 and cpFtsY. Step 6, GTP hydrolysis leads to dissociation of cpSRP43/54 and cpFtsY components from Alb3 and the thylakoid membrane.

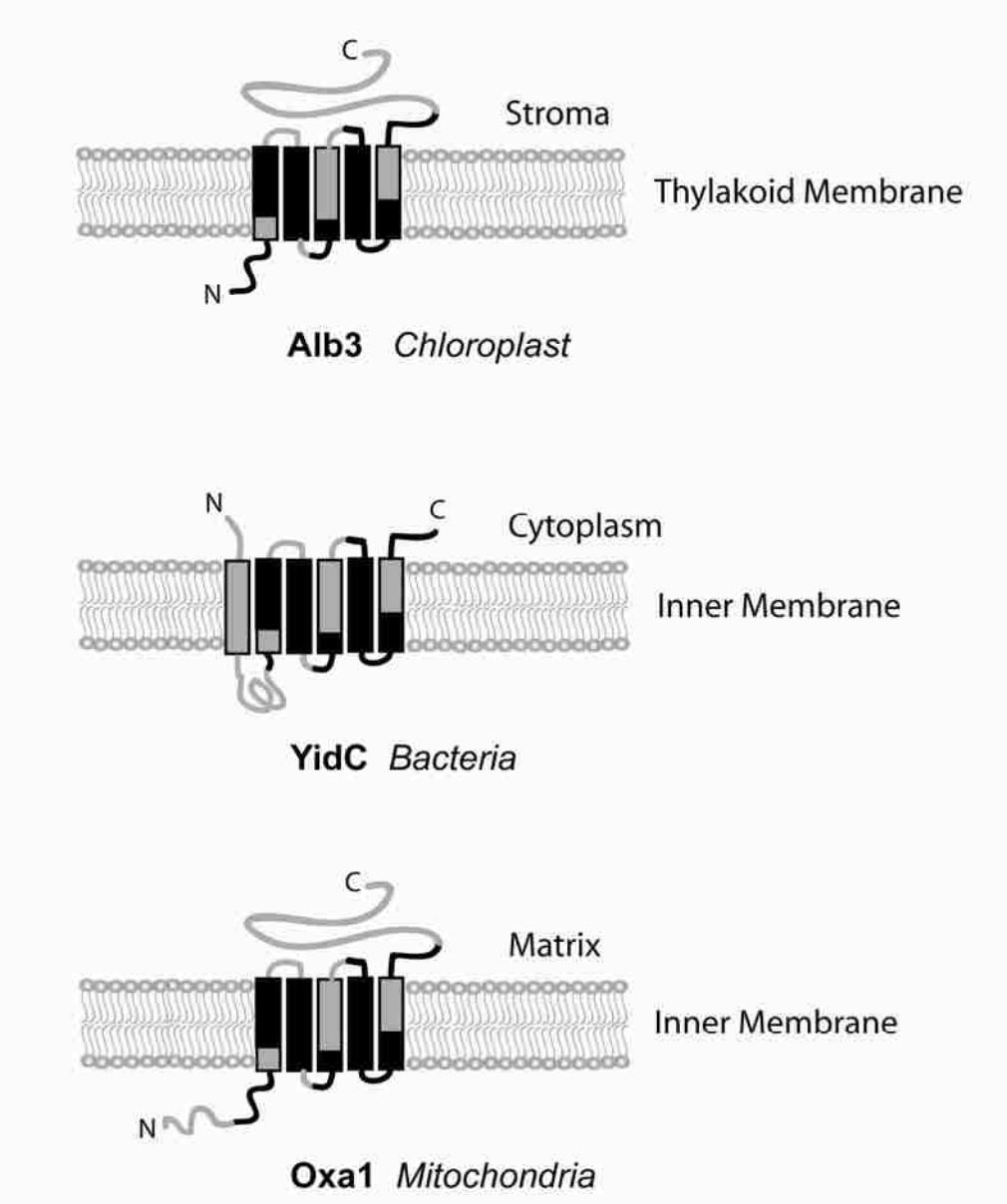

Figure 3.1. Schematic representation of the membrane topology of the YidC/Oxa1/Alb3 family members.

Mature Alb3 and Oxa1p are polytopic membrane proteins with five transmembrane domains. Alb3 is arranged in the thylakoid membrane with the N-terminus facing the thylakoid lumen and the C-terminus facing the stroma. Oxa1p is arranged in the mitochondrial inner membrane with the N-terminus facing the intermembrane space and the C-terminus facing the matrix. YidC is a polytopic membrane protein with six transmembrane segments and both the N and C termini facing in to the cytoplasm. Conserved regions are shown in black and non-conserved regions are shown in grey. Figure adapted from van Bloois *et al*., 2005.

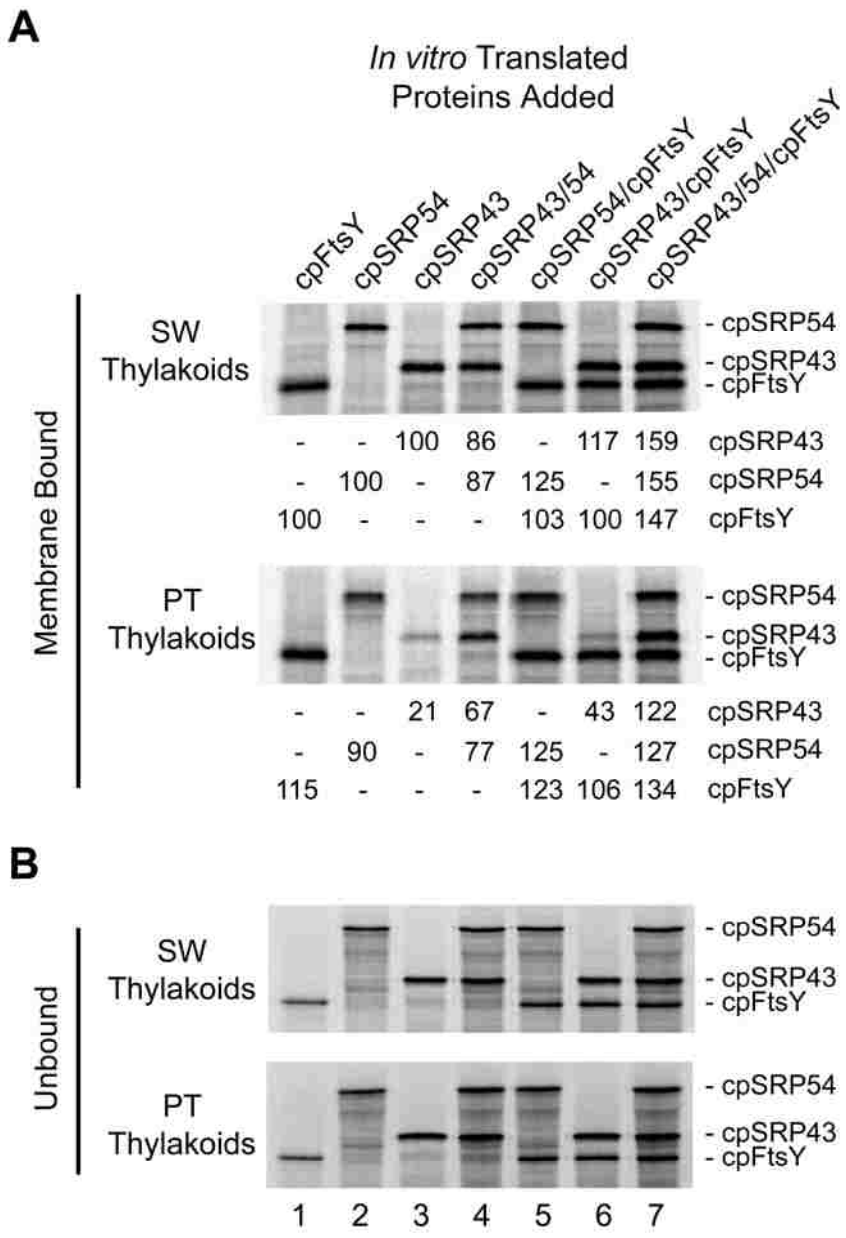

Figure 3.2. CpSRP43 binds a protease-sensitive thylakoid component.

A) Thylakoid membrane binding of radiolabeled cpSRP43, cpSRP54, or cpFtsY constructs (as indicated) was examined by incubation with SW or PT thylakoids. Thylakoids were re-isolated, washed and analyzed by SDS-PAGE and phosphorimaging. *In vitro* translation products were labeled differentially with S^{35}-methionine and unlabeled methionine such that equal signal represents equal molar quantities. **B**) Unbound samples were examined to verify that residual protease was not responsible for changes in amounts of thylakoid bound constructs.

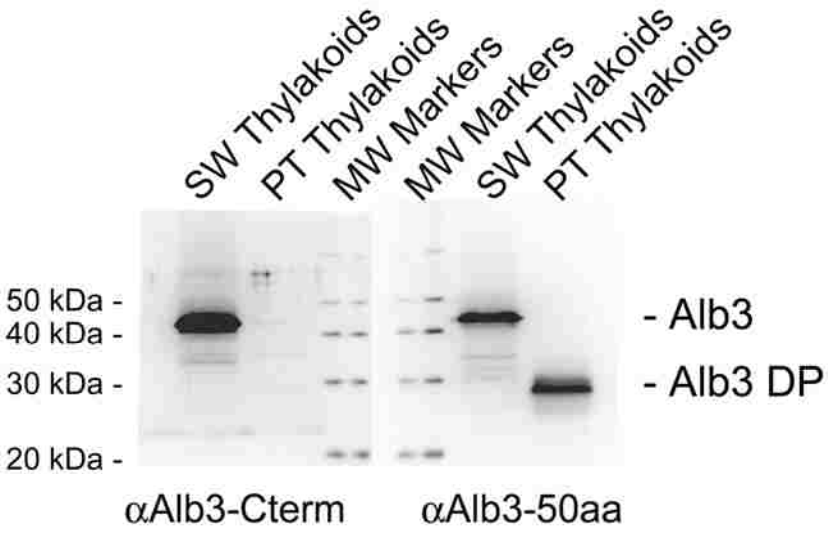

Figure 3.3. Protease-treatment removes the soluble Alb3 C-terminus.

Samples of both SW and PT thylakoids utilized in Figure 3.2 experiments were examined to verify complete protease-treatment of the membranes. Protease-treatment should result in conversion of Alb3 to Alb3-DP which indicates removal of an ~13 kDa soluble extension on the C-terminus of Alb3 (detected by αAlb3-Cterm).

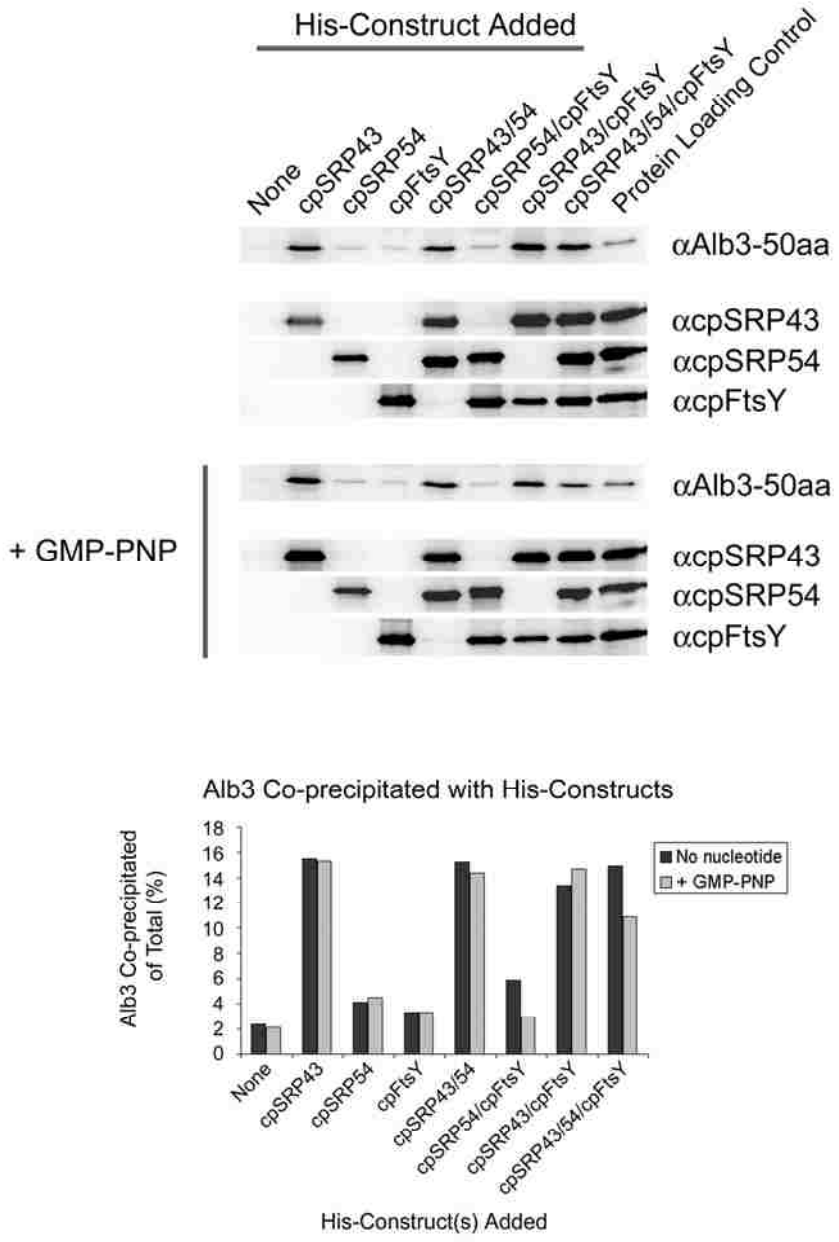

Figure 3.4. CpSRP43 coprecipitates Alb3 from thylakoid membranes.

Salt-washed thylakoids (75 µg Chl) were incubated with 10 µg of His-tagged constructs indicated (cpSRP43, cpSRP54, and cpFtsY). Assays were then solubilized in maltoside and used for precipitation with Talon metal affinity resin. Western blots of precipitated proteins are shown probed for the proteins indicated to the right. The last lanes (Protein Loading Control) contain thylakoid membranes for the αAlb3-50aa blots and 50 ng of His-tagged constructs. Protein loading control lanes were used for sizing and to compare relative amounts precipitated. The graph depicts the amount of Alb3 coprecipitated with the various His-tagged constructs. Total precipitated of available was calculated from the relative signal of total thylakoid lane and Talon eluate lanes.

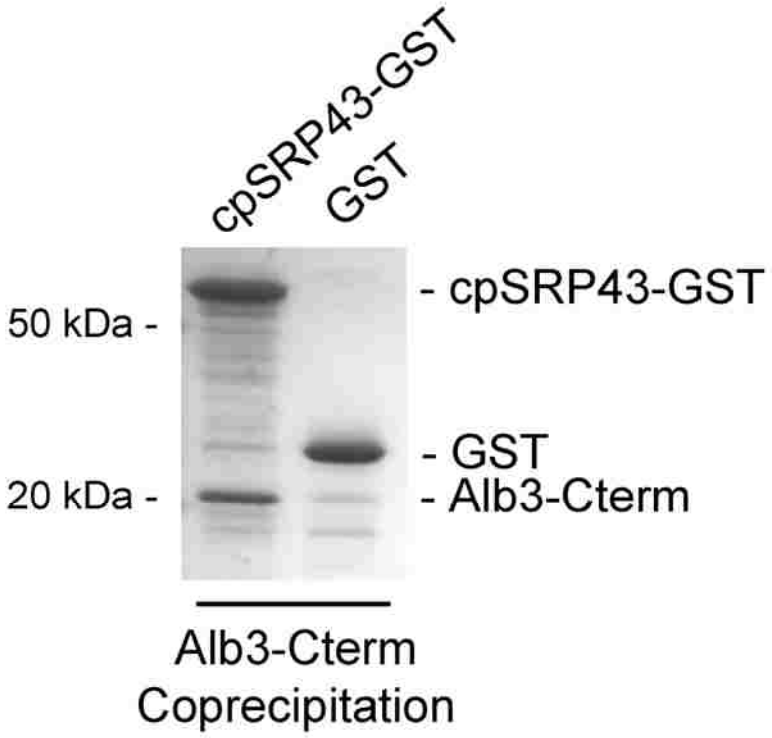

Figure 3.5. CpSRP43 specifically copurifies His-Alb3-Cterm.

Equimolar concentrations of cpSRP43-GST or GST alone were incubated with recombinant His-Alb3-Cterm and then recovered using Glutatione-Sepharose resin and eluted with 40 mM glutathione. The eluates from each assay were analyzed by SDS-PAGE and Coomassie blue staining. Data obtained by Nathan Lewis.

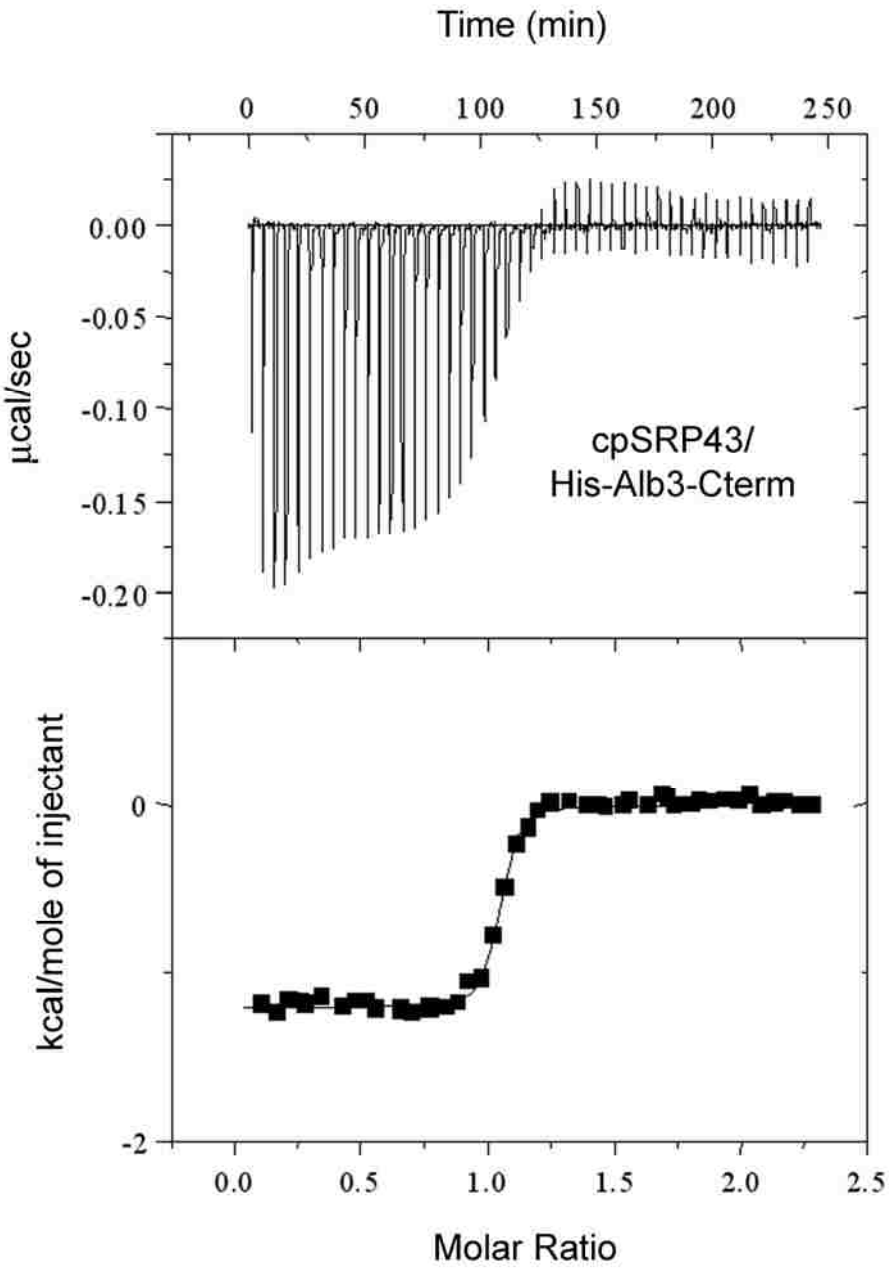

Figure 3.6. ITC curve indicates interaction between His-Alb3-Cterm and cpSRP43.

ITC curve showing binding of His-Alb3-Cterm to cpSRP43 at 25°C. The upper and lower panels show the raw and integrated data, respectively, of the titration of cpSRP43 with His-Alb3-Cterm as indicated. The solid line in the bottom panels represents the best-fit curve of the data (Microcal Origin). Background corrections were made in all spectra. These experiments were performed by Dakshinamurthy Rajalingam (Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR).

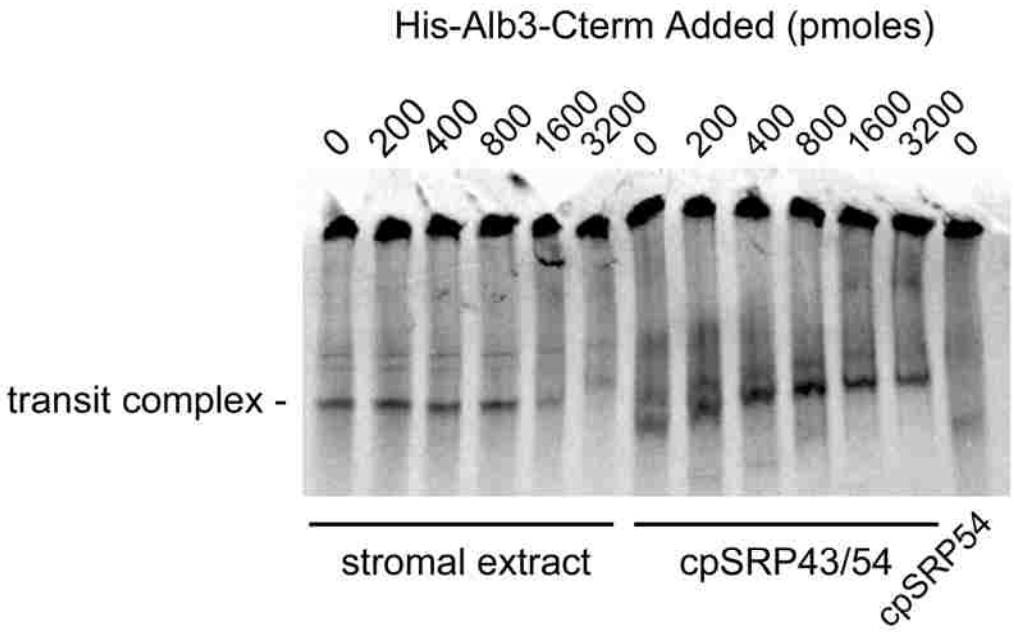

Figure 3.7. His-Alb3-Cterm affects transit complex stability.

In vitro translated pLHCP mixed with either stromal extract or recombinant purified cpSRP43 (100 pmol) and cpSRP54 (50 pmol) was incubated in the presence or absence of His-Alb3-Cterm (0-3200 pmol as indicated). Transit complex was tentatively identified by using native PAGE and phosphorimaging to compare assays conducted in the presence or absence of His-Alb3-Cterm.

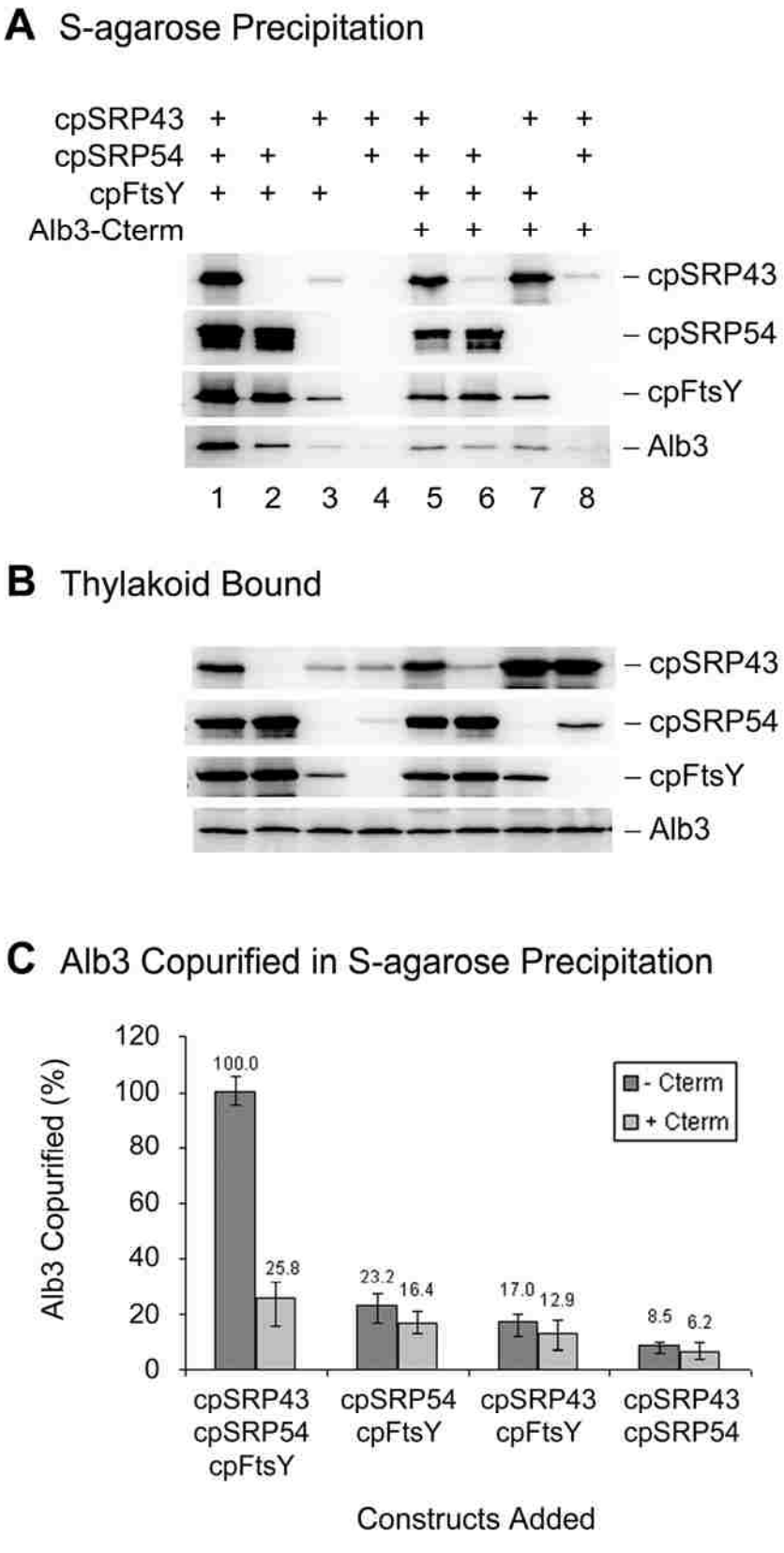
A S-agarose Precipitation
cpSRP43 + + + + + +
cpSRP54 + + + + + + +
cpFtsY + + + + + + +
Alb3-Cterm + + + +
– cpSRP43
– cpSRP54
– cpFtsY
– Alb3
1 2 3 4 5 6 7 8
B Thylakoid Bound
– cpSRP43
– cpSRP54
– cpFtsY
– Alb3
C Alb3 Copurified in S-agarose Precipitation
Alb3 Copurified (%)
120
100
80
60
40
20
0
100.0
25.8
23.2
16.4
17.0
12.9
8.5
6.2
- Cterm
+ Cterm
cpSRP43 cpSRP54 cpFtsY
cpSRP54 cpFtsY
cpSRP43 cpFtsY
cpSRP43 cpSRP54
Constructs Added

Figure 3.8. His-Alb3-Cterm competes for cpSRP membrane complex formation with Alb3.

A) CpSRP43 (10 µg) was pre-incubated in the presence or absence of 200 µg His-Alb3-Cterm. Salt-washed thylakoids (75 µg Chl) were incubated with 10 µg of constructs indicated (His-Alb3-Cterm-treated cpSRP43, cpSRP54, and S-cpFtsY) along with 0.5 mM GMP-PNP, such that assays were performed in the presence or absence of 200 µg His-Alb3-Cterm . Thylakoids were buffer washed and solubilized in maltoside. The soluble fraction was mixed with S-protein agarose to precipitated S-tagged cpFtsY and all coprecipitating proteins. Western blots of the samples were probed to identify the presence of proteins indicated to the right. **B**) Thylakoids with bound recombinant proteins were Western blotted to show relative amounts of soluble protein bound to the membranes. **C**) The level of Alb3 copurified with each assay was calculated from three separate experiments and is shown relative to Alb3 copurified with cpSRP43, cpSRP54, and cpFtsY.

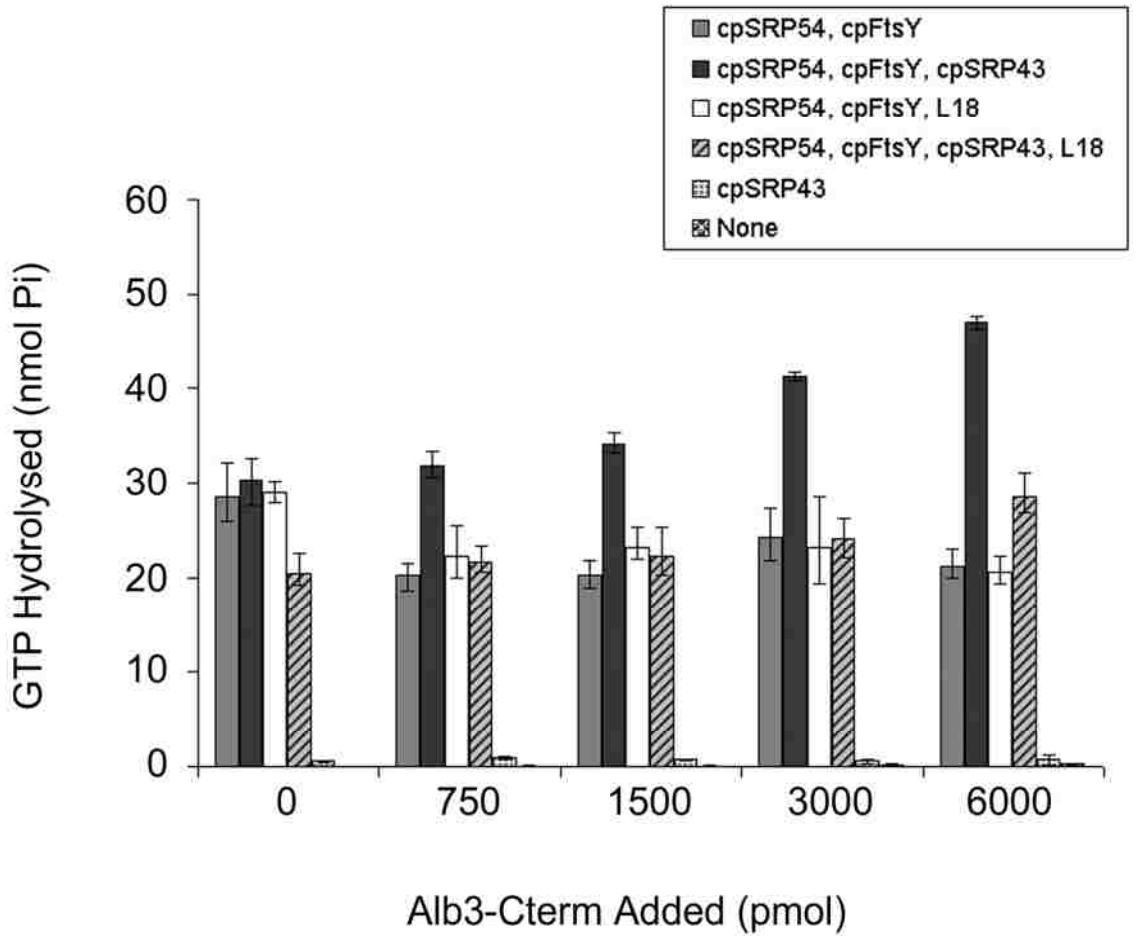

Figure 3.9 Alb3-Cterm stimulates GTP hydrolysis between the cpSRP GTPases in a cpSRP43-dependent manner.

The effect of His-Alb3-Cterm on the GTP hydrolysis activity of cpSRP54 and cpFtsY was examined in the presence or absence of cpSRP43 and/or L18 peptide. Assays contained 150 pmol of cpSRP43, cpSRP54, and cpFtsY, 750 pmol of L18, and 0-6000 pmol Alb3-Cterm as indicated with 2 mM GTP as described in "Materials and Methods." GTPase activity resulting in the release of inorganic phosphate (Pi) was determined according to Gonzalez and Romo, 1992 by using known phosphate standards. The average and standard deviation were calculated from three separate experiments.

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IV

CHLOROPHYLL BIOSYNTHESIS ENZYME GERANYLGERANYL REDUCTASE IS ASSOCIATED WITH ALB3

SUMMARY

Biogenesis of antennae light-harvesting complexes (LHCs) in thylakoid membranes requires proper routing and assembly of nuclear-encoded light-harvesting chlorophyll-binding proteins (LHCPs). In the presence of chlorophyll (Chl), LHCPs are routed to thylakoid membranes for integration and assembly via thylakoid translocase Alb3 by the chloroplast signal recognition particle (cpSRP). However, in the absence of Chl production, LHCPs do not accumulate in thylakoid membranes. Reciprocally, in the absence of cytosolic LHCP expression, Chl does not accumulate. Currently, no evidence has been published that directly links Alb3-dependent LHCP insertion/assembly with Chl biosynthesis. Previous examination of a crosslink-stabilized complex containing Alb3 by spectrometry indicated the presence of GGR, one of the last enzymes in Chl biosynthesis, as a component of the complex. We have utilized assays that reconstitute membrane-associated stages of cpSRP targeting to isolate Alb3 in complex with GGR. Our results demonstrate that two pools of Alb3 can be discerned. One pool binds cpSRP/cpFtsY and the other pool is enriched with GGR. This data provides the first evidence that Chl biosynthesis enzymes are in complex with Alb3, supporting the hypothesis that the final stages of Chl biosynthesis are coordinated with the assembly of proteins that require Alb3 for assembly.

INTRODUCTION

Light-harvesting complexes (LHCs) are composed of trimers of light-harvesting chlorophyll-binding proteins (LHCPs) bound to accessory pigments (i.e. xanthopylls, carotenoids, phycobilins) and of chlorophyll (Chl) *a* and *b* molecules that capture light energy in the form of excited electrons (Kuttkat *et al.*, 1995; Jensen *et al.*, 2007). This arrangement of Chls allows the energy of an excited electron to be passed from one Chl molecule to another, funneling the energy into a central photosystem protein complex.

LHCPs are encoded by nuclear DNA and include a chloroplast targeting peptide. Therefore, LHCPs are synthesized in the cytosol, imported into chloroplasts as full-length precursors, and targeted and integrated into thylakoids where they are assembled into trimers with Chl. The N-terminal chloroplast targeting peptide is removed by a stromal processing peptidase soon after chloroplast import. Imported LHCP is bound and transported across the stroma to thylakoid membranes by the chloroplast signal recognition particle (cpSRP). CpSRP is composed of a highly-conserved 54 kDa protein (cpSRP54) as well as a 43 kDa protein (cpSRP43) unique to chloroplast SRP. To accomplish the steps of protein transport, cpSRP works in combination with a membrane-associated SRP receptor protein (cpFtsY) and an integral membrane protein with translocase activity (Alb3). Briefly, cpSRP binds LHCP forming a soluble complex capable of transporting LHCP to the thylakoid membrane in an integration-competent state. Current research suggests that cpSRP/LHCP and cpFtsY together form a complex with Alb3, during which LHCP is released to Alb3 for insertion and assembly. Lastly, cpSRP54 and cpFtsY, both of which are GTPases, hydrolyze GTP in a concerted fashion, releasing cpSRP and cpFtsY for another round of targeting.

Peripheral light-harvesting centers of photosystems I and II in higher plants contain different specialized isoforms of LHCP proteins, which work together to facilitate the harvest of solar energy. *Lhca* and *Lhcb* designate genes corresponding to the LHCPs of photosystems I and II, respectively (Jansson *et al.*, 1992). Six *Lhca* and six *Lhcb* genes have been described to date (reviewed in (Jensen *et al.*, 2007; van Amerongen and Croce, 2008)). Though Lhcb1 has been the object of most *in vitro* studies, all of the Lhca and Lhcb members in *Arabidopsis thaliana* are nuclear-encoded (50-83% identity in mature protein) (Jensen *et al.*, 2007). Importantly, each contains a conserved 18 amino acid cpSRP43 binding region originally identified in *Pisum sativum* Lhcb1 (L18) (Cline, 2003). Due to the conservation of the cpSRP43-binding region, it is probable that like Lhcb1, LHCP homologues are also localized to thylakoids by the cpSRP-dependent targeting pathway. In agreement with this data, treatment of thylakoids using Alb3 antibody diminishes the integration of at least Lhcb1, Lhcb4.1, and Lhcb5 (Moore *et al.*, 2000; Woolhead *et al.*, 2001). Furthermore, cpSRP43 null mutants exhibit a specific reduction in chlorophyll and LHCPs ((Amin *et al.*, 1999; Klimyuk *et al.*, 1999).

Alb3 and its homologues in mitochondria and bacteria, Oxa1 and YidC, make up a conserved family of proteins that assist membrane insertion of a wide-range of integral membrane proteins. Members of the Alb3/Oxa1/YidC family have been described as 'membrane-localized chaperones' due to their apparent role in efficient folding and assembly of membrane proteins (Kuhn *et al.*, 2003; Ossenbuhl *et al.*, 2004). It has long been postulated that the chaperone functions of the Alb3/Oxa1/YidC family may be linked to the process of ligand attachment to newly inserted proteins (Hoober and Eggink, 2001; Cline, 2003; Kuhn *et al.*, 2003). In the case of LHCP insertion, Alb3 might hold

monomeric or trimeric proteins in a conformation that would allow Chl to bind appropriately. Chl association with LHCP is required for proper folding and stability in the bilayer. Within the Lhcb1 sequence, a highly conserved 'retention motif' has been identified that is thought to bind two Chl molecules during an early stage of insertion, thereby allowing the protein to remain in membranes long enough for further stabilization events, such as trimer assembly and additional Chl binding, to occur (Hoober and Eggink, 1999). Due to the low stability of 'free' Chl and the lack of evidence for a Chl 'storage' protein, the synthesis of Chl must be correlated with the synthesis/insertion/assembly of its binding proteins. In this context, enzymes involved in the last stages of Chl biosynthesis may be closely associated with LHCP insertion via Alb3 (for review see (Cline, 2003)). Enzymes that are involved in the conversion of Chl *a* to Chl *b* or synthesis and attachment of the tail moiety are probable suspects that may be associated with LHCP assembly.

In support of this idea, LHCP stability and chlorophyll synthesis appear to be correlated. In *Chlamydomonas reinhardtii*, LHCPs are sent to vacuoles for degradation in the absence of Chl synthesis (Park and Hoober, 1997). Chl *b*, in particular, appears to be important for the stability of light-harvesting complex of photosystem II. In *A. thaliana* and barley mutants lacking Chl *b*, LHCPs are expressed but not recovered in isolated chloroplasts (Murray and Kohorn, 1991; Preiss and Thornber, 1995; Reinbothe *et al.*, 2006). Chl *b* pigments are also required for inducing protease-resistant LHCP folding *in vitro* (Paulsen *et al.*, 1993). Furthermore, an analogue of Chl *b* promotes *in vitro* insertion of LHCPs into etioplast membranes (Kuttkat *et al.*, 1997).

LHCP assembly also appears to be critical for Chl stability. Chl *b* does not accumulate when synthesis of LHCPs in the cytosol is inhibited (Maloney *et al.*, 1989; Plumley and Schmidt, 1995). A *C. reinhardtii* mutant lacking Alb3 is almost devoid of LHCs and photosystem core polypeptides and suffers a nearly 70% reduction in Chl accumulation as well (Bellafiore *et al.*, 2002). Moreover, LHCP expression appears to influence the activity of certain Chl biosynthesis enzymes, namely those involved with biosynthesis steps at the membrane (Xu *et al.*, 2001). It is probable that LHCPs increase Chl accumulation by providing a protected binding site for Chl, which may subsequently activate late stage Chl biosynthesis enzymes as well as prevent Chl degradation.

The synthesis of Chl *a* and *b* can be divided into steps that take place in the stroma and those that take place at the membranes of the chloroplast inner envelope and thylakoid (see Figure 4.1A). As shown in Figure 4.1A, membrane-associated steps begin with Chl precursor protoporphyrinogen IX (for reviews see ((Beale, 1999; Cline, 2003)). Protoporphyrinogen oxidase, Mg cheletase, methyl transferase, and cyclase catalyze the conversion of protoporphyrinogen IX to protochlorophyllide. Protochlorophyllide is converted to chlorophyllide *a* (Chllide *a*) by protochlorophyllide oxidoreductase. Though the majority of Chl biosynthetic reactions appear to follow a linear progression, the order of the last stages depends on the availability of substrate (for reviews see (Beale, 1999; Beale, 2005)). Chl synthetase (aka Chl synthase) catalyzes the addition of an alcohol 'tail' to the tetrapyrrole ring, converting Chllide *a* to Chl *a*. Chl(ide) *a* oxidase (CaO) exchanges a methyl group on one ring for an aldehyde group, converting Chl *a* to Chl *b*. These reactions may happen in either order: I) Chllide *a* to Chllide *b* to Chl *b* or II) Chllide *a* to Chl *a* to Chl *b* (Figure 4.1A). For conjugation of the alcohol tail, Chl

synthase can use either the pyrophosphate ester of either phytol or a precursor of phytol, most commonly geranylgeranyl (GG) (Rudiger, 1997). If a phytol precursor such as GG is added, the final step in Chl biosynthesis is the conversion of the alcohol moiety added by Chl synthase to phytol by geranylgeranyl reductase (GGR). Reduction of the phytol tail by geranylgeranyl reductase is a stepwise progression as depicted in Figure 4.1B: GG to dihydroGG to teterahydroGG to phytol. Analysis of Chl biosynthesis *in vivo* reveals a reduction of Chl_{GG} to Chl_{phytol}, indicating that GGR is generally either the last or second to last enzyme (CaO activity may follow GGR reduction) required in synthesis of Chl (Soll *et al.*, 1983; Addlesee and Hunter, 1999; Chew *et al.*, 2008).

Currently, no evidence has been published that directly links Alb3-dependent LHCP insertion/assembly with Chl biosynthesis. Previous examination of a crosslink-stabilized complex containing Alb3 by spectrometry indicated the presence of GGR, one of the last enzymes in Chl biosynthesis, as a component of the complex. We have utilized assays that reconstitute membrane-associated stages of cpSRP targeting to isolate Alb3 in complex with GGR. Our results demonstrate that two pools of Alb3 can be discerned. One pool binds cpSRP/cpFtsY and the other pool is enriched with GGR.

MATERIALS AND METHODS

All reagents and enzymes used were purchased commercially. Plasmids used for *in vitro* transcription/translation of pLHCP (psAB80XD/4) (Cline *et al.*, 1989) have been described previously. Antibodies to the following proteins have also been described: Alb3-Cterm (Woolhead *et al.*, 2001), Alb3-50aa (Moore *et al.*, 2000), cpSecY (Mori *et al.*, 1999), Tha4 (Mori *et al.*, 1999), cpSRP43 (Moore *et al.*, 2003), and cpSRP54 (Moore *et al.*, 2003). Those against OE23, LHCP, Tha4, and cpSecY were generous gifts from Dr. Kenneth Cline (University of Florida, Gainesville). Antibodies to CaO were a generous gift from Judy Brusslan (California State University, Long Beach). Recombinant, purified cpSRP43-his, cpSRP54-his and Trx-His-S-cpFtsY were produced as described (Yuan *et al.*, 2002; Moore *et al.*, 2003).

Construction of a GGR Clones

A cDNA clone for pGGR was obtained by RT-PCR (reverse transcription-polymerase chain reaction) using total RNA from *A. thaliana*. Forward and reverse primers (Integrated DNA Technologies) were designed to match the precursor coding sequence of GGR starting with the predicted mature amino acid sequence MATTVTL and to include BamHI and HincII sites, respectively. The forward primer also added the bases cacg immediately preceding the start codon to facilitate efficient translation of in vitro transcribed mRNA. The resulting PCR fragment was restricted with BamHI and HincII and ligated into similarly-restricted pGEM-4Z to create the plasmid pGGR-pGEM-4Z.

A mature GGR expression clone (lacking the predicted stromal targeting domain) beginning AARAT and ending EKLSV* was amplified by PCR using forward and reverse primers designed to include BamHI and XhoI sites, respectively. The resulting PCR fragment was restricted with BamHI and XhoI and inserted in frame behind the coding sequence for Glutathione S-transferase (GST) in similarly-restricted pGEX-6P-2 (GE Healthcare) to produce GGR-pGEX-6P-2, which codes for the fusion protein GST-GGR. All cloned constructs were sequenced (Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR) to verify the fidelity of the PCR reaction.

GGR Expression, Purification, and Antisera Production

GST-GGR fusion protein was expressed in *E. coli* strain BL21 Star and purified by affinity to Glutathione Sepharose™ FastFlow (GE Healthcare). For increased purity, purified GST-GGR was separated on a 12.5% SDS-PAGE gel, excised, and electro-eluted into SDS-PAGE buffer using a Bio-Rad Model 422 Electro-Eluter with 12-15 kDa cutoff Clear Membrane Caps (Bio-Rad). Purified GST-GGR was then used as antigen to prepare polyclonal antibodies in rabbit (Cocalico Biologicals).

Antisera Specificity Verification

Isolated chloroplasts were lysed and separated by centrifugation (3200 x *g* for 8 min) into stroma and thylakoid fractions. Samples were solubilized in SDS solubilization buffer at a concentration equal to 0.25 mg Chl/ml and separated by SDS-PAGE before blotting to PVDF. Membranes were probed with GGR antisera or pre-immune sera and

visualized using HRP chemiluminescence as described in Analysis of Samples below. Competition for antibody binding to GGR on PVDF was done by incubating GGR antisera with the bacterial lysate containing either expressed GST (pGEX-6P-2) or GST-GGR (GGR in pGEX-6P-2) prior to probing blots for GGR detection.

CpFtsY Cloning and Antisera Production

The coding fragment for *A. thailiana* precursor cpFtsY (pcpFtsY) in pGEM-4Z (described in (Yuan *et al.*, 2002)) was excised from pcpFtsY4Z with KpnI and HindIII and ligated into appropriately-restricted pBAD (Invitrogen) forming pcpFtsYhis. This plasmid was then transformed into *E.coli* strain TOP10 (Invitrogen). The protein was expressed and purified on Talon™ Superflow™ affinity resin (Invitrogen) and used to generate rabbit polyclonal antibodies (Cocalico Biologicals) also used in (Moore *et al.*, 2003).

Construction of His-Alb3-Cterm Construct

A cDNA clone for PPF-1 (defined as Alb3 in *Pisum sativum*) was obtained by reverse transcription-polymerase chain reaction (PCR) using total RNA from *Pisum sativum*. Forward and reverse primers (Integrated DNA Technologies) matching the sequence for PPF1 (Accession #Y12618) were designed to include EcoRI and XbaI sites, respectively, for ligation into pGEM-4Z (Promega). The coding sequence for PPF1-Cterm, a 124-amino acid segment of PPF1 beginning at NNVLSTA and ending at SKRKPVA, was amplified by PCR from PPF-1-pGEM-4Z. The resulting PCR fragment was restricted with BamH1 and XbaI, then ligated into similarly-restricted pGEM-4Z to

produce the plasmid Alb3-Cterm-pGEM-4Z. Alb3-Cterm-pGEM-4Z was restricted with BamHI and SalI and the resulting Alb3-Cterm was inserted in-frame behind the coding sequence for glutathione S-transferase (GST) in pGEX-6P-2 (GE Healthcare) to produce the plasmid Alb3-Cterm-pGEX-6P2. Alb3-Cterm-pGEX-6P-2 was restricted with BamHI and XhoI and the resulting Alb3-Cterm was inserted in-frame behind the coding sequence for a 6-histidine tag in pET-32a (Novagen) to produce the plasmid Alb3-Cterm-pET-32a. Forward and reverse primers were designed to match the beginning and ending of the Alb3-Cterm (described above) and to include SphI and HindIII sites, respectively. The forward primer also included a 2 amino acid linker (SA), a Flag Tag, and a Thrombin cleavage site. The resulting PCR fragment was restricted with SphI and HindIII, then ligated into similarly-restricted pQE-80L (Qiagen) to create the plasmid Alb3-Cterm-pQE-80L. This plasmid was transformed into BL21* (Invitrogen) and used for expression of His-Alb3-Cterm. The protein was expressed and purified on Talon™ Superflow™ affinity resin (Invitrogen).

Preparation of Chloroplast Materials and Radiolabeled Precursors

In vitro transcribed and capped RNA was translated in the presence of [^{35}S] methionine using a wheat germ system to produce radiolabeled precursor proteins (Cline *et al.*, 1993). Translation products were diluted twofold and adjusted to import buffer (IB; 50 mM Hepes-KOH, pH 8.0, 0.33 M sorbitol) containing 30 mM unlabeled methionine. Intact chloroplasts were isolated from 9-10 day old pea seedlings (*Pisum sativum* cv. Laxton's Progress) and used to prepare thylakoids and stromal extract (SE) as

described (Cline *et al.*, 1993; Henry *et al.*, 1997; Moore *et al.*, 2003). Chlorophyll content was determined according to Arnon (Arnon, 1949).

Assays for Determination of Sub-Chloroplast Location

Isolated chloroplasts (equal to 50 µg chlorophyll) were pelleted and saved as the "C-" sample. Isolated chloroplasts (equal to 150 µg chlorophyll) were incubated in IB, 0.4 mg/ml thermolysin, and 2 mM $CaCl_2$ for 45 min. Protease-treated chloroplasts were re-isolated following the addition of EDTA to a final concentration of 25 mM EDTA. Protease-treated chloroplasts (equal to 100 µg chlorophyll) were lysed with 10mM Hepes/KOH (pH 8) for 5 min and split into three aliquots (each equal to 50 µg chlorophyll). One aliquot was saved as the "C+" sample. The second aliquot was separated into soluble and membrane fractions by centrifugation at 3200 x *g* for 8 min and saved as the "S" and "Th-" samples. The third aliquot was incubated in IB, 0.25 mg/ml thermolysin, and 1 mM $CaCl_2$ for 45 min and then mixed with EDTA to a final concentration of 25 mM EDTA and saved as the "Th+" sample. All samples were solubilized in SDS solubilization buffer at a concentration equal to 0.25 mg Chl/ml.

Complex Formation and Copurification Assays

Complexes between thylakoid membrane proteins and His-cpSRP54, Trx-His-S-cpFtsY, and cpSRP43 were formed by incubating 10 µg indicated purified proteins together with 0.5 mM GMP-PNP and salt-washed thylakoids equal to 75 µg chlorophyll at 25°C for 30 min in light. Complexes between thylakoid membrane proteins and His-cpSRP43 or His-Alb3-Cterm were formed by incubating the indicated pmol of the

indicated purified proteins with salt-washed thylakoids equal to 75 µg chlorophyll at 25°C for 30 min in light. Membranes were recovered by centrifugation, washed with IBM and resuspended in IBM. Thylakoids equal to 25 µg chlorophyll were removed and solubilized with SDS solubilization buffer (final volume 250 µl) for subsequent examination of bound recombinant proteins. The remaining membranes (equal to 50 µg chlorophyll) were solubilized in IB containing 1% n-Dodecyl β-D-Maltoside (Mal) and 1.5% BSA (final volume of 50 µl) 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) or TALON™ Superflow™ affinity resin as a 50% slurry in IB and incubated for 30 min at RT with gentle mixing. Afterward, the resin mixture was transferred to a 0.8 ml Centrifuge Column (Pierce) and washed four times with 0.5 ml 0.1% Mal in IB and one time with 0.5 ml IB. Copurified proteins were eluted in 100 µl SDS solubilization buffer.

For the comparison of thylakoid membrane proteins copurified with His-Alb3-Cterm from thylakoids solubilized before and after His-Alb3-Cterm binding, repurification was performed as described above except that assays were adjusted to 0.2% Mal in IB prior to His-Alb3-Cterm binding.

For purification of thylakoid membrane proteins following S-protein agarose purification, the unbound fraction from the S-protein agarose resin was recovered, added to 100 µl TALON™ Superflow™ affinity resin as a 50% slurry in IB and incubated for 20 min at RT with gentle mixing. The unbound fraction was recovered again, adjusted to 0.1% Mal in IB and mixed with the indicated pmol of His-Alb3-Cterm. Following 30 min incubation at 25°C, the membranes were added to 50 µl TALON™ Superflow™

affinity resin as a 50% slurry in IB and incubated for 30 min at RT with gentle mixing. Afterward, the resin mixture was transferred to a 0.8 ml Centrifuge Column and washed four times with 0.5 ml 0.1% Mal in IB and one time with 0.5 ml IB. Copurifying proteins were eluted in 100 µl SDS solubilization buffer.

Assays for Antibody Inhibition of Protein Transport into Thylakoids

Antibodies were used to inhibit protein transport into salt-washed thylakoids essentially as described in Mori et al. (Mori *et al.*, 1999; Moore *et al.*, 2000), except that thylakoids were resuspended in HKM (10 mM Hepes-KOH, pH 8.0, 10 mM $MgCl_2$) at 0.5 mg/ml chlorophyll before dilution to 0.4 mg/ml chlorophyll by the addition of 0.54 M phosphate (pH 7) containing sera. Briefly, thylakoids were incubated for 1 hr at 4°C with buffer alone or sera (13% of final volume) and then washed once with IBM (IB, 10 mM $MgCl_2$) to remove unbound antibody before resuspension in IBM to 1 mg/ml chlorophyll. Transport assays (150 µl final), conducted at 25°C for 30 min in light, were initiated by adding radiolabeled precursor protein (25 µl) to antibody-treated thylakoids (50 µg chlorophyll) mixed with SE (~0.5 mg protein), 5 mM MgATP, and 1 mM NaGTP. Afterward, recovered thylakoids were treated with thermolysin and finally dissociated in SDS-PAGE sample buffer at 1 mg/ml chlorophyll (Henry *et al.*, 1997).

Analysis of Samples

A portion of each sample (10 µl) from each assay was analyzed by SDS-PAGE followed by Western blotting or phosphorimaging. Molecular Dynamics image analysis software (Image Quant) was used for quantification of integration assays from

phosphorimages obtained using a Typhoon 8600 (GE Healthcare, formerly Amersham Biosciences). All Western blots were probed with Goat Anti-Rabbit IgG(H+L)-HRP Human/Mouse Adsorbed (Southern Biotech) and HRP chemiluminescence was produced using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Western blots were imaged using an Alpha Innotech Fluorchem™ IS-8900 using chemiluminescent detection. AlphaEase FC Stand Alone software (Alpha Innotech) was used for quantification of Fluorchem™ IS-8900 images. SDS-Page Standards (Invitrogen) were used to calculate molecular weights (MagicMark™ XP Western Standard for Western blots; Benchmark™ Protein Ladder for Coomassie-stained gels). Protein concentrations were estimated by Coomassie Blue staining of purified proteins using BSA as a standard.

RESULTS

Alb3 is copurified with His-Alb3-Cterm.

It has been documented that Alb3 is found is several oligomeric complexes in thylakoids, ranging in size from 145-700 kDa, of which the composition has not been determined (Moore, 2003). Similarly, studies of Alb3 homologues in *E. coli* and mitochondria, YidC and Oxa1p, respectively, have shown that these proteins form both homooligomeric complexes (Oxa1) (Nargang *et al.*, 2002) and complexes with other membrane proteins (YidC) (Houben *et al.*, 2004). Evidence from crystallography studies that the soluble periplasmic C-terminal domain of YidC forms a homodimer in solution, suggests that this region may be involved in the formation of higher order complexes (Ravaud *et al.*, 2008). For these reasons, it is sensible to anticipate the involvement of soluble interaction domains, such as the Alb3-Cterm, in forming higher order complexes. At the C-terminus of Alb3 is an ~13.8 kDa stromally-exposed region (Alb3-Cterm) that is likely to be involved in protein-protein interactions required for post-translational cpSRP-based protein targeting. We produced recombinant Alb3-Cterm with an N-terminal 6His affinity tag (His-Alb3-Cterm) for use in assays that reconstitute stages of cpSRP targeting. In utilizing this construct in cpSRP targeting assays with isolated *Pisum sativum* thylakoids, we noted that His-Alb3-Cterm associates with thylakoids (data not shown). To examine whether His-Alb3-Cterm forms complexes with Alb3 in thylakoid membranes and whether a potential interaction can be narrowed down to one of the stroma-facing domains (Alb3-50aa or Alb3-Cterm), salt-washed thylakoids were either pre-treated with antisera recognizing either a 50aa stroma-facing loop (αAlb3-50aa) or the soluble C-terminus of Alb3 (αAlb3-Cterm) or protease-treated (PT), washed to

remove unbound antisera, re-isolated, and incubated with His-Alb3-Cterm (+) as indicated. After incubating His-Alb3-Cterm with thylakoids, membranes were washed, solubilized with maltoside, and mixed with Talon resin to repurify His-Alb3-Cterm and all associated proteins. Interestingly, recombinant His-Alb3-Cterm is able to copurify Alb3 from pea thylakoids (Figure 4.2). Protease-treatment of thylakoid membranes, which results in removal of the C-terminus of Alb3 but leaves the remainder of Alb3 intact, greatly reduces the ability of His-Alb3-Cterm to copurify Alb3. As shown in Figure 4.2, pre-treatment of the thylakoids antisera raised against the Alb3-Cterm peptide also blocks copurification of Alb3 with His-Alb3-Cterm. Based on these results, it is reasonable to expect that the C-terminus of Alb3 may be involved in the formation of oligomeric complexes of Alb3. Based on these observations, we utilized the His-Alb3-Cterm construct as a tool to copurify Alb3 from thylakoids and characterize Alb3-containing membrane complexes.

Production of antisera for GGR, a previously-identified Alb3 crosslink adduct.

Alb3 forms large complexes within the thylakoid membrane as evidenced by BN-PAGE, gel filtration, and sucrose gradient centrifugation results. Crosslinking has also been utilized as another approach to characterize Alb3-associated proteins. Mass spectroscopy analysis of a crosslinked cpSRP/cpFtsY/Alb3-containing membrane complex isolated by repurification using an affinity tag indicated the presence of geranylgeranyl reductase (GGR), an enzyme that functions in chlorophyll biosynthesis (Moore, 2003).

Plant geranylgeranyl reductase, the *chl P* gene product, reduces (in a stepwise manner, as depicted in Figure 4.1) geranylated Chl to phytylated Chl and free geranylgeranyl diphosphate to phytyl diphosphate, which also serves as the side chain to chlorophylls, tocopherols, and plastoquinones (Keller *et al.*, 1998). In *A. thailiana*, the *chl P* gene (accession #AY059860.1) consists of 1404 base pairs and encodes a deduced product of 51.8 kDa with a chloroplast targeting peptide. Upon chloroplast entry, the chloroplast targeting peptide is cleaved forming mature geranylgeranyl reductase, predicted to be 410 amino acids in length (~45.5 kDa) in *A. thailiana*.

To examine conservation of GGR, the *A. thailiana chl P* gene was aligned with other known plant *chl P* genes using NCBI's blastp software (Tatusova and Madden, 1999). Accession numbers of aligned proteins are as follows: *A. thailiana* (AY059860); soybean, *Gliyne max* (AF068686); tobacco, *Nicotiana tabacum* (AJ007789); peach, *Prunus persica* (AY230212); common ice plant, *Mesembryanthemum crystallinum* (AF069318). *A. thailiana chl P* exhibits maximal identity with *Gliyne max* (87%), followed by *Nicotiana tabacum* (85%), *Prunus persica* and *Mesembryanthemum crystallinum* (both 84%).

For the purpose of confirming the presence of GGR in complex with Alb3, we produced recombinant *A. thailiana* GGR and used it to obtain antisera. GGR antiserum recognizes an ~47 kDa protein in salt-washed (SW) pea thylakoids (Figure 4.3). Other bands recognized by GGR antisera are also recognized by pre-immune antisera. Recognition of the ~47 kDa band is diminished in the presence of lysate from bacteria expressing GGR-GST, but not bacteria expressing GST alone. These results indicate that

the GGR antiserum recognizes *P. sativum* GGR on Western blots and is specific for GGR.

The addition of a phytol tail (final product of GGR) aids the stability of chlorophylls, tocopherols, and phylloquinones in the hydrophobic core of plastid membranes (Rosenberg, 1967). GGR-mediated steps in synthesis are compartmentalized in the thylakoid membranes for chlorophyll and the plastid envelope membranes for tocopherol and phylloquinone (Soll *et al.*, 1983). For this reason, it has been proposed that GGR partitions between the thylakoid and plastid envelope membranes, available to be recruited by either the chlorophyll or prenylquinone pathways. However, the sub-chloroplast localization of GGR has not been investigated to date. To determine the sub-chloroplast location of endogenous GGR, isolated pea chloroplasts were subfractionated using protease-treatment and centrifugation. Whole chloroplasts were protease-treated, lysed, and separated into stromal and thylakoid fractions via centrifugation. Isolated thylakoids were salt-washed to remove peripherally-associated proteins and then protease-treated for removal of unprotected protein domains. Equivalent amounts of total chloroplasts, protease-treated chloroplasts, stroma, thylakoids, and protease-treated thylakoids were probed using the GGR antisera (Figure 4.4). The GGR band is present in all fractions excluding stroma. GGR appears to be associated with membranes and partially-protease resistant, as the thylakoid fraction contains the ~47 kDa GGR band and protease-treated thylakoid fractions contain an ~30 kDa GGR degradation product.

GGR is co-purified with Alb3 using His-Alb3-Cterm.

With the idea that multiple functional pools of YidC exist in *E. coli (Samuelson et al*., 2000; Scotti et al., 2000; Facey et al., 2007), we anticipated that His-Alb3-Cterm and cpSRP could interact with distinct pools of Alb3. Based on the previous results, we expect His-Alb3-Cterm and His-cpSRP43 to copurify with Alb3 from thylakoids (Figure 4.2 and (Tzvetkova-Chevolleau *et al.*, 2007)). To compare the relative amount of Alb3 and potential membrane proteins (GGR, SecY) interacting with Alb3 that copurify with either cpSRP43 or His-Alb3-Cterm, salt-washed (SW) thylakoids were incubated with increasing amounts of either 6-His-tagged cpSRP43 or His-Alb3-Cterm. After incubating His-tagged proteins with SW thylakoids, membranes were washed, solubilized with maltoside, and mixed with Talon resin to isolate the His-tagged constructs and all associated proteins. As shown, recombinant His-cpSRP43 and His-Alb3-Cterm constructs are able to copurify Alb3 from SW thylakoids (Figure 4.5). The amount of Alb3 copurified with each component increases with the amount of His-tagged component added, indicating that Alb3 interacts specifically with cpSRP43 and His-Alb3-Cterm individually. Eluates of each assay were probed for copurified GGR as well as for another Chl biosynthesis enzyme, chlorophyll(ide) *a* oxidase (CaO). CaO does not appear to be copurified with cpSRP43 and small amounts of GGR (5-10% available) are copurified with cpSRP43. In contrast, GGR and CaO are copurified specifically with His-Alb3-Cterm; the amount of available GGR and CaO copurified with 1000 pmol His-Alb3-Cterm is 40% and 22%, respectively. Importantly, the presence of GGR is enriched between four- and eight-fold in the pool of Alb3 copurified with His-Alb3-Cterm as compared to the pool of Alb3 copurified with cpSRP43. Likewise, the amount of CaO is

highest in the His-Alb3-Cterm copurified pool of Alb3. These results suggest that at least one subset of Alb3 proteins are found in complex with chlorophyll biosynthesis enzymes.

GGR does not appear to be required for cpSRP-dependent targeting to Alb3.

To investigate whether GGR could be found in complex with cpSRP-associated Alb3, we isolated an Alb3-containing membrane complex containing soluble cpSRP components. Recombinant cpSRP and cpFtsY, each with unique affinity tags, are active in reconstituting LHCP integration and can be combined with the non-hydrolysable GTP analogue, GMP-PNP, and thylakoid membranes to form a stable thylakoid complex with Alb3 (Moore *et al.*, 2003). After incubating cpSRP, Trx-His-S-cpFtsY, salt-washed thylakoids, and GMP-PNP, membranes were washed, solubilized with maltoside, and mixed with S-protein agarose resin to isolate S-tagged cpFtsY and all associated proteins. This complex was probed for copurified GGR as well as for CaO. As expected, Alb3 is copurified with S-tagged cpFtsY in the presence of cpSRP components, cpSRP43 and cpSRP54. However, neither GGR nor CaO are copurified in detectable amounts with the cpSRP-containing membrane complex obtained in this manner (Figure 4.6A). Figure 4.6B shows that similar amounts of Alb3, GGR, and CaO were available for copurification in all assays. It should be noted that the amount of cpSRP43, cpSRP54, and cpFtsY bound to thylakoids is reduced when either GTPase (cpSRP54 or cpFtsY) is not present. These observations suggest that neither GGR nor CaO are tightly associated with the pool of Alb3 that is in complex with cpSRP targeting components, which is consistent with the low amounts of GGR copurified with cpSRP43 (Figure 4.5).

To assay whether GGR is required for LHCP targeting, we pre-treated thylakoid membranes with GGR antisera or GGR antisera and anti-rabbit antisera and reconstituted the targeting reaction *in vitro* as described (Moore *et al.*, 2000). Pre-immune sera for GGR as well as antisera against Alb3-Cterm, and OE23 were used as controls. As previously shown, Alb3-Cterm antisera inhibits LHCP integration (Moore *et al.*, 2000). Clearly, LHCP integration is not inhibited by the addition of GGR antisera alone or in combination with secondary antisera (Figure 4.7). These results suggest that GGR is not directly involved in LHCP targeting and integration. Then again, GGR antisera may not recognize GGR in a native folded state; α-GGR antisera was produced using purified GGR denatured prior to rabbit inoculation and does not immunoprecipitate GGR from salt-washed thylakoids (data not shown).

It is possible that GGR may associate with a pool of Alb3 not present in a complex with cpSRP/cpFtsY. Merely ~15% of the Alb3 in thylakoid forms a complex with cpSRP/cpFtsY when complex formation experiments are performed with saturating levels of cpSRP/cpFtsY. Consequently, we chose to investigate the possibility of there being a second pool of Alb3 that is not associated with cpSRP components, but is associated with GGR.

GGR and Alb3 are copurified with His-Alb3-Cterm using presolubilized thylakoids.

To determine if Alb3 and GGR are in a pre-existing pool or whether the addition of His-Alb3-Cterm induces an association between the two, we compared copurified fractions from thylakoids that were pre-solubilized with maltoside prior to His-Alb3-Cterm binding to those that were solubilized with maltoside after His-Alb3-Cterm

binding. As shown in Figure 4.8, His-Alb3-Cterm copurifies both GGR and Alb3 regardless of whether the thylakoids have been solubilized prior to His-Alb3-Cterm association. However, using pre-solubilized thylakoids, the amount of Alb3 copurified with 2000 pmol His-Alb3-Cterm is increased two-fold, while the amount of GGR copurified is reduced by ~50%. The observation of increased copurification of Alb3 from pre-solubilized membranes suggests Alb3 becomes more accessible to His-Alb3-Cterm following solubilization perhaps due to removal of an Alb3-bound protein or ribosome. The reduction in GGR copurification observed with the pre-solubilized thylakoids may mean that the Alb3/GGR complex is simply not as stable when membranes are solubilized; the duration between solubilization steps and affinity resin incubation were 0 min and 40 min for post-solubilized and pre-solubilized membranes, respectively. These results suggest both that a portion of the Alb3 that is copurified with His-Alb3-Cterm can be found in a pre-existing complex with GGR and that GGR is recruited to Alb3 in the presence of His-Alb3-Cterm. Alternatively, His-Alb3-Cterm may be able to interact with both Alb3 and GGR individually. However, we have not been able to identify an interaction between His-Alb3-Cterm and GST-GGR in solution using recombinant GGR.

GGR and cpSRP components associate with different pools of Alb3.

Since endogenous Alb3 can be copurified with His-Alb3-Cterm following thylakoid solubilization, we assayed for whether His-Alb3-Cterm associates with the same pool of Alb3 that is copurified with a thylakoid-bound, GMP-PNP-stabilized cpSRP43/54/cpFtsY complex. Saturating amounts of purified cpSRP43 and cpSRP54

His-tagged constructs and Trx-His-S-cpFtsY were combined with GMP-PNP and thylakoid membranes. After incubating cpSRP, cpFtsY, SW thylakoids, and GMP-PNP, membranes were washed, solubilized with maltoside, and mixed with S-protein agarose resin to isolate S-tagged cpFtsY and all associated proteins (Figure 4.9A). As expected, ~20% of the total added Alb3 was copurified with the GMP-PNP stabilized cpSRP targeting complex. S-protein agarose flow-through was then mixed with Talon resin for removal of remaining His-tagged cpSRP43, cpSRP54 or cpFtsY. To confirm saturation of Alb3 competent for cpSRP-dependent targeting and integration, a fraction of treated thylakoids were used for *in vitro* integration of LHCP. As shown in Figure 4.9A, LHCP integration is reduced by ~80% using treated thylakoids. The same thylakoids were then incubated with increasing amounts of His-Alb3-Cterm. After incubating His-Alb3-Cterm with solubilized thylakoids, membranes were mixed with Talon resin to isolate His-Alb3-Cterm and all associated proteins. As shown in Figure 4.9B, His-Alb3-Cterm is able to copurify Alb3 and GGR from thylakoids independent of whether a fraction of Alb3 had been previously removed. However, the amount of Alb3 copurified with His-Alb3-Cterm is reduced (33.3% reduction copurified with 2 nmol His-Alb3-Cterm) following the removal of Alb3 associated with cpSRP membrane complex. These results suggest that His-Alb3-Cterm is able to copurify the pool of Alb3 that interacts with cpSRP targeting components as well as a pool of Alb3 not available for cpSRP-dependent targeting. The amount of GGR copurified with His-Alb-Cterm is only slightly reduced (6.8% reduction in copurify with 2 nmol His-Alb3-Cterm) by the removal of cpSRP-associated Alb3. It should be noted that some cpSRP43, cpSRP54, and cpFtsY remained with the solubilized thylakoids, even after mixing with S-protein agarose and Talon resin,

as these constructs were copurified specifically with His-Alb3-Cterm (Figure 4.9B). This is not entirely surprising as the cpSRP components form a complex with Alb3 in the absence of substrate (Moore *et al.*, 2003).

To address whether the two pools of Alb3 of in a constant state of flux, we examined whether the pool of Alb3 that is associated with GMP-PNP-stabilized cpSRP complex decreases over time. Saturating amounts of purified cpSRP43, His-cpSRP54, and Trx-His-S-cpFtsY were combined with GMP-PNP and thylakoid membranes. After incubating cpSRP, cpFtsY, SW thylakoids, and GMP-PNP for the normal duration (30 min), membranes were washed twice and incubated for another 0-120 min. Following the indicated incubation, membranes were solubilized with maltoside and mixed with S-protein agarose resin to isolate S-tagged cpFtsY and all associated proteins (Figure 4.10). As expected, the amount of Alb3 was copurified with the GMP-PNP stabilized cpSRP targeting complex was stable for 120 min. Furthermore, GGR and CaO are not copurified with the S-tagged cpSRP complex, regardless of incubation time. These results suggest that the two pools of Alb3 are somewhat stable and that GGR and CaO are not recruited to the site of LHCP integration by the presence of cpSRP targeting components.

DISCUSSION

Though it has long been postulated that the insertion/assembly of Chl-binding proteins is coordinated with Chl biosynthesis, a link between the molecular machinery facilitating these processes had not been established (Paulsen *et al.*, 1993; Hoober and Eggink, 1999; Hoober and Eggink, 2001; Cline, 2003; Hoober *et al.*, 2007). Our investigation of a dynamic complex containing Alb3 and GGR, a late-stage Chl biosynthesis enzyme provides the first direct evidence of a link between Chl biosynthesis and the Alb3, supporting the hypothesis that the final stages of Chl biosynthesis are coordinated with the assembly of proteins that require Alb3 for assembly.

When we isolated a membrane complex containing Alb3 and GGR, we anticipated that this complex may also contain cpSRP targeting components. However, we show that GGR is not detectable in GMP-PNP-stabilized cpSRP membrane complex (Figure 4.6). Furthermore, the pool of Alb3 in complex with cpSRP targeting components can be removed from thylakoids, leaving a pool of Alb3 that can be isolated with GGR (Figure 4.9B). Consequently, the GGR/Alb3 association appears to be enriched within a pool of Alb3 not associated with cpSRP-targeting components. If GGR is not directly coupled with LHCP targeting and integration, we hypothesize that it may be necessary for ligand synthesis during LHCP assembly. Alternatively, Alb3 associated with GGR may be involved in the assembly of other Chl-containing thylakoid membrane complexes.

Several potential mechanisms exist for interaction of GGR and Alb3 as determined via copurification using His-Alb3-Cterm. Alb3-Cterm may be involved in the formation of Alb3 oligomers and, as such, capable of copurification of Alb3 and

tightly-associated thylakoid proteins (e.g. GGR). Alternatively, His-Alb3-Cterm may interact directly with GGR or another unidentified membrane protein that is tightly-associated with Alb3. Further examination of the behavior of Alb3 and its interaction partners is necessary to bring light to these mysteries.

It is likely that Alb3 complexes are dynamic and change with the stages of protein targeting, insertion, and assembly. Perhaps the pool of Alb3 available for cpSRP-dependent targeting is part of a larger complex with the pool of Alb3 coupled with GGR. This is consistent with the close relationship between Chl and LHCP stability in thylakoids and the observation that Alb3 can be separated into two pools, one that copurifies with GGR and one that does not. Though GGR does not appear to be involved in the cpSRP-dependent targeting reaction, per se, it will be interesting to determine whether the insertion/assembly of LHCP is dependent upon GGR activity. Presumably, a reduction in GGR activity would reduce the stability of inserted LHCP based on the fact that Chl attachment facilitates proper folding (Paulsen *et al.*, 1993).

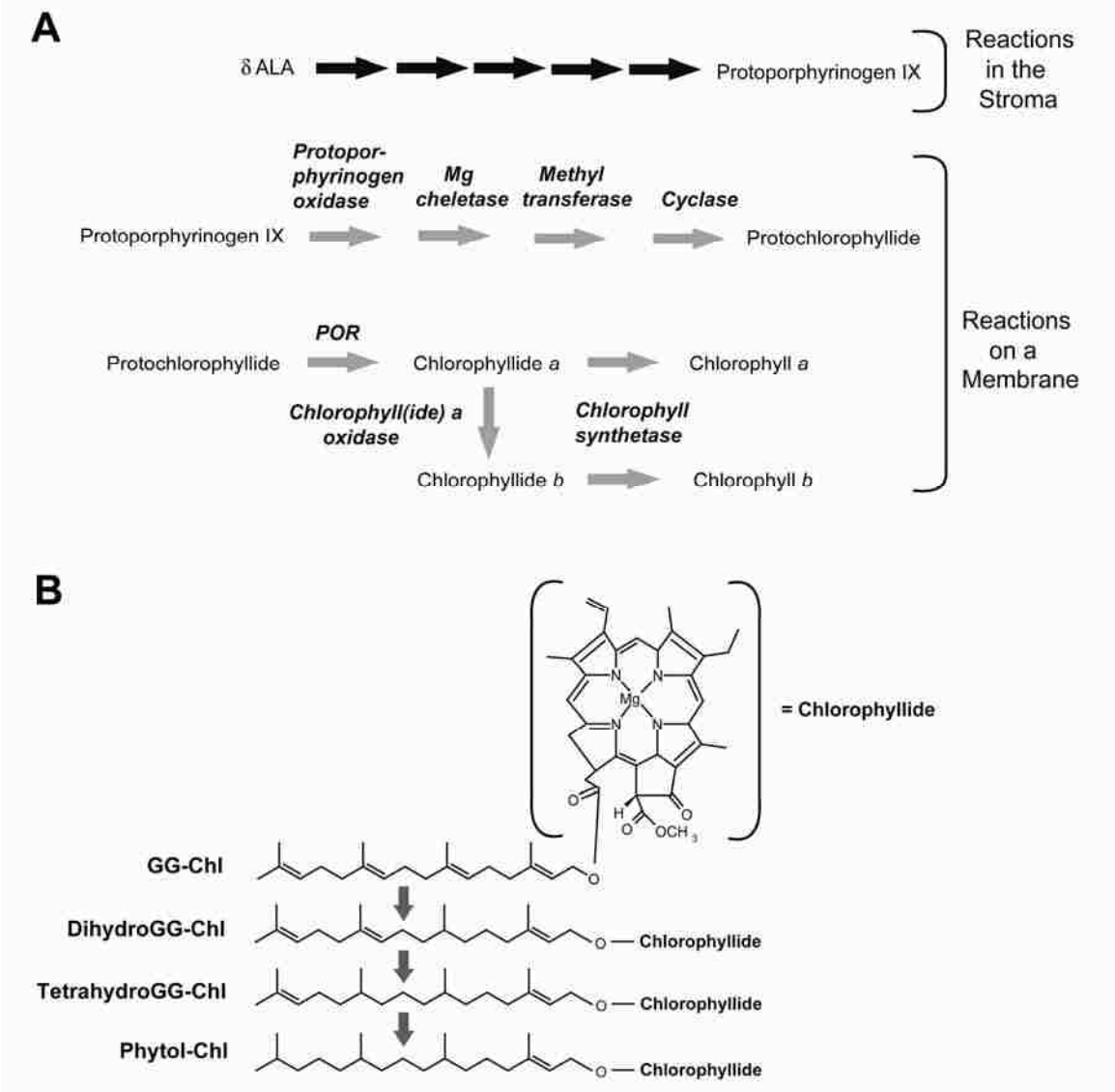

Figure 4.1 Topography of chlorophyll synthesis in higher plant chloroplasts.

A) Steps from ∂Aminolevulinic Acid (∂ALA) to protoporphyrinogen IX are catalyzed by enzymes located in the stroma and are designated by black arrows. Steps from protoporphyrinogen IX to chlorophylls *a* and *b* are carried out in association with a membrane, either the inner chloroplast envelope or the thylakoid membranes. Enzymes catalyzing these steps are bolded and gray arrows designate the steps. This figure is adapted from (Cline, 2003). **B**) The steps catalyzed by geranylgeranyl reductase are designated by grey arrows. Geranylgeranyl reductase can catalyze the stepwise reduction of geranylgeranyl-chlorophyll (GG-Chl) into phytol-chlorophyll (phytol-Chl) after the action of chlorophyll synthetase (shown above) or can catalyze the same reduction of geranylgeranyl to phytyl prior to attachment of chlorophyllide by chlorophyll synthetase.

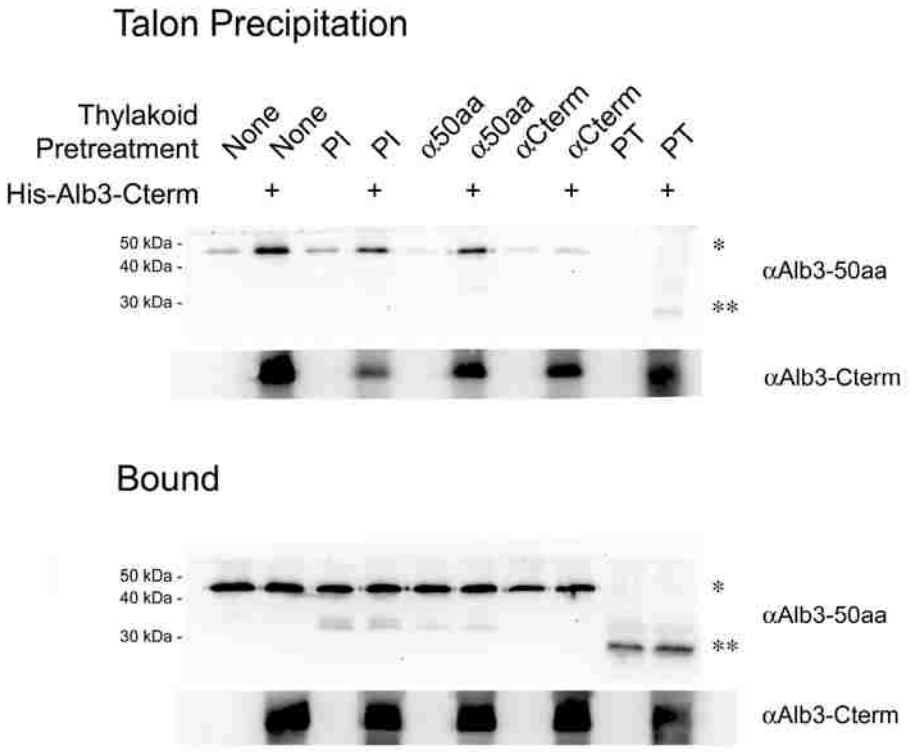

Figure 4.2 His-Alb3-Cterm copurifies Alb3 from thylakoid membranes.

Thylakoids were incubated with rabbit serum against proteins shown above (PI, preimmune serum) or protease-treated (PT), washed, and solubilized with maltoside. The soluble fraction was incubated in the presence (+) or absence (-) of His-Alb3-Cterm. Treated thylakoids were mixed with Talon metal affinity resin to isolate His-Alb3-Cterm and all copurifying proteins. Western blots of isolated proteins were probed for Alb3 using αAlb3-50aa (a soluble loop of Alb3). Likewise, blots of isolated proteins were probed for His-Alb3-Cterm using αAlb3-Cterm. Western blots of thylakoid samples were also probed using αAlb3-50aa to show similar starting amounts of Alb3 and the Alb3 degradation product resulting from protease-treatment. One asterisk (*) indicates intact Alb3 and two asterisks (**) indicate Alb3 reducing in size following proteolysis of its C-terminus.

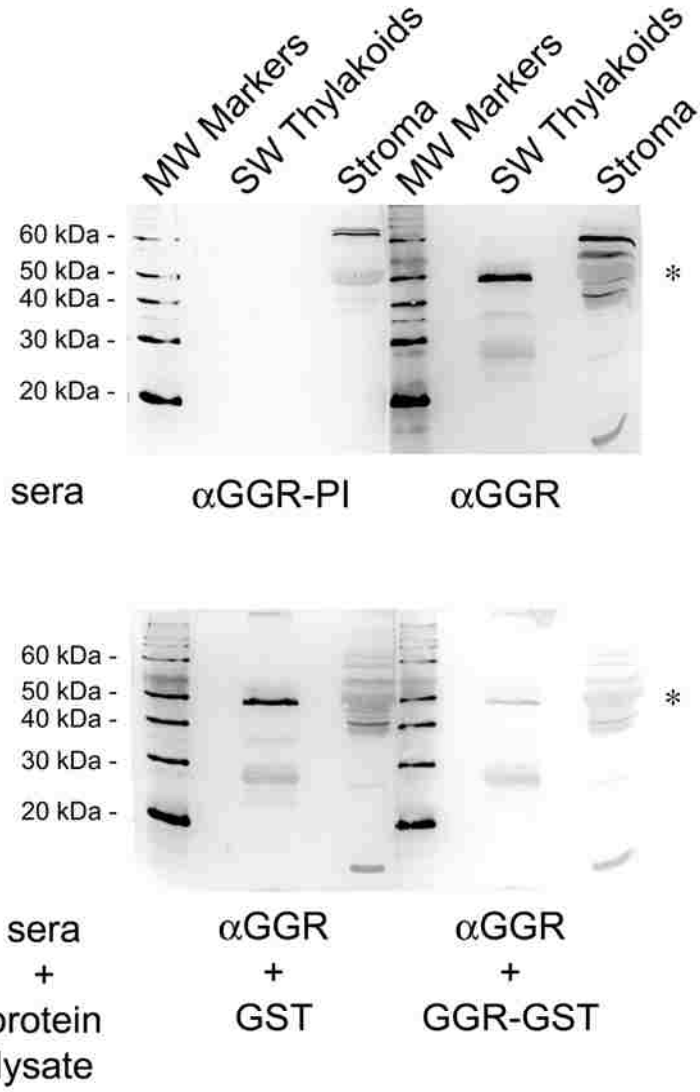

Figure 4.3 ***A. thailiana*** **GGR antisera recognizes a 47 kDa thylakoid protein.**

Top: Salt-washed thylakoids and stroma, separated by SDS-PAGE and electroblotted to membranes, were probed with preimmune antisera taken from rabbit prior to inoculation with GGR protein (αGGR-PI) or antisera obtained after inoculation (αGGR). A 47 kDa thylakoid protein (*) is recognized specifically by αGGR.
Bottom: SW thylakoids and stroma, separated by SDS-PAGE and electroblotted to membranes, were probed with αGGR in the presence of soluble lysate from bacteria expressing GST or GGR-GST. The 47 kDa band recognized by αGGR remains in the presence of GST alone, but not GGR-GST.

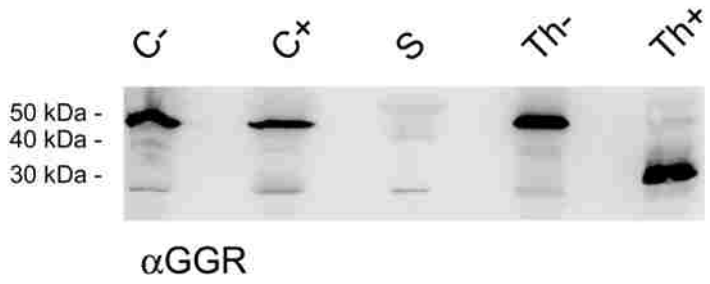

Figure 4.4 GGR subfractionates with thylakoids and contains a protease-sensitive domain.

Intact chloroplasts were subfractionated into chloroplasts (C-), protease-treated chloroplasts (C+), stroma (S), thylakoids (Th-), and protease-treated thylakoids (Th+). Samples, separated by SDS-PAGE and electroblotted to membranes, were probed with αGGR. The 47 kDa GGR protein is reduced to 30 kDa by protease-treatment.

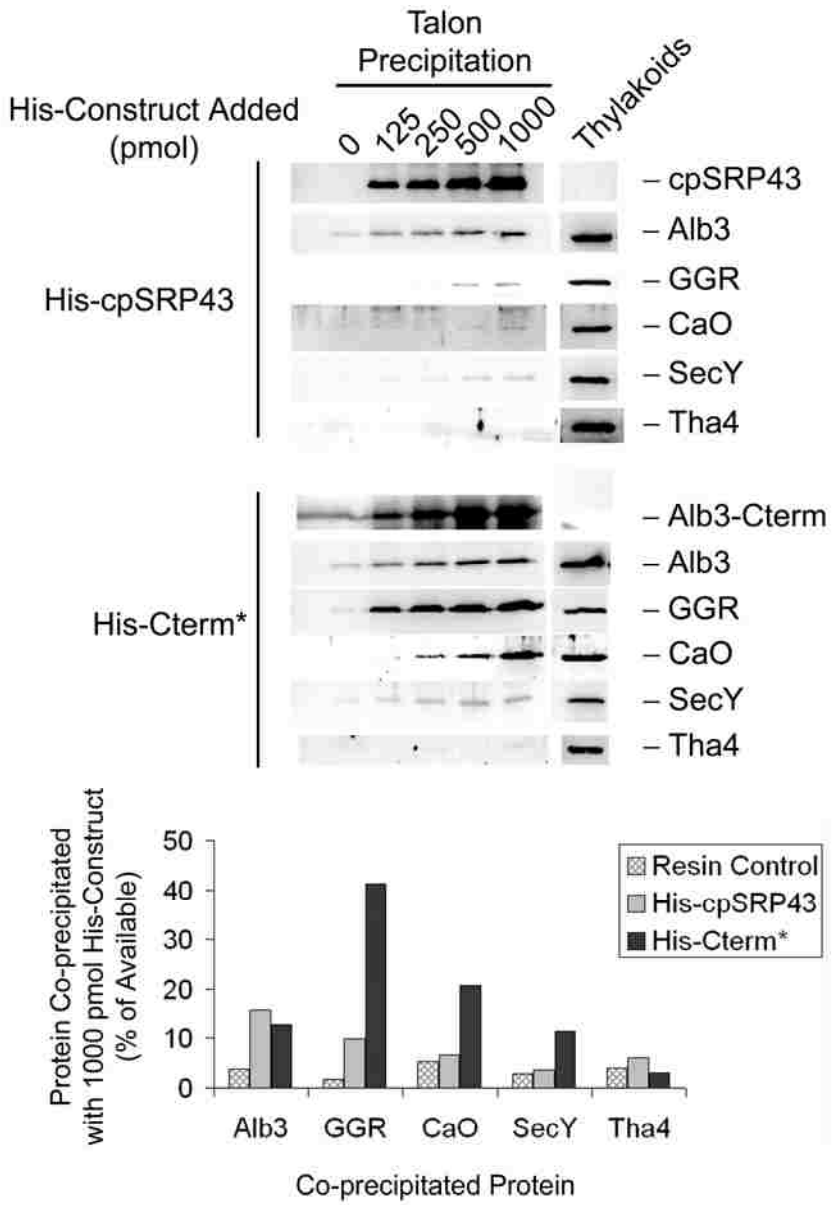

Figure 4.5 GGR is copurified with Alb3 using His-Alb3-Cterm but not His-cpSRP43.

Salt-washed thylakoids (75 µg Chl) were incubated with increasing amounts of either His-cpSRP43 (0-100 pmol) or His-Alb3-Cterm (*0-2000 pmol) as indicated. After washing, membranes were solubilized in maltoside and used for copurification assays with Talon affinity resin. Western blots of isolated proteins are shown probed for the proteins indicated to the right. The last lane (Thylakoids) contains thylakoid membranes with bound His-tagged constructs for sizing and to compare relative amounts isolated. The graph depicts the amount of thylakoid proteins copurified with the various His-tagged constructs. Approximate total isolated of available was calculated from the relative signal of total thylakoid lane and Talon eluate lanes.

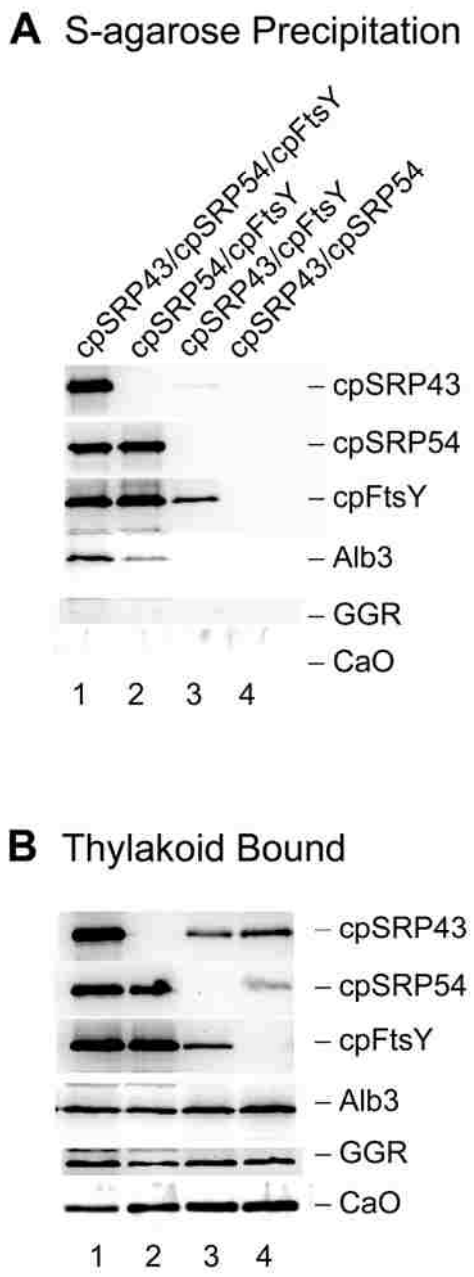

Figure 4.6 GMP-PNP-stabilized cpSRP/cpFtsY/Alb3 complex does not contain GGR.

A) Salt-washed thylakoids (75 µg Chl) were incubated with 10 µg each indicated protein (cpSRP43, His-cpSRP54, or Trx-His-S-cpFtsY) in the presence of 0.5 mM GMP-PNP. Thylakoids were buffer washed and solubilized in maltoside. The soluble fraction was mixed with S-protein agarose to isolate S-tagged cpFtsY and all copurifying proteins. Western blots of the samples were probed to identify the presence of proteins indicated to the right. **B**) Thylakoids with bound recombinant proteins were Western blotted to show relative amounts of soluble protein bound to the membranes.

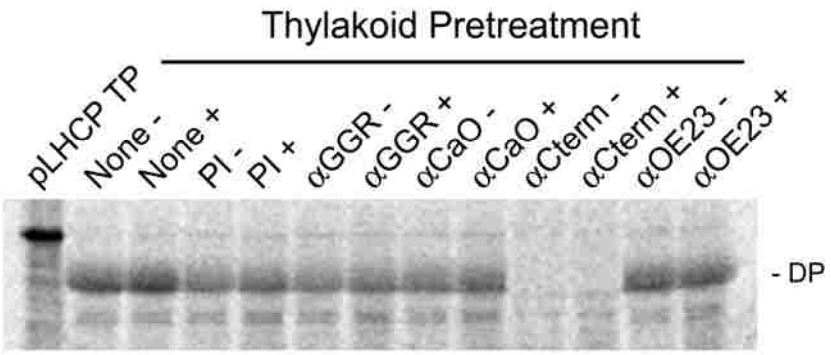

Figure 4.7 Anti-GGR serum does not inhibit LHCP integration.

Thylakoids were incubated with rabbit serum against proteins shown above (PI, Preimmune serum), washed, and incubated in the presence (+) or absence (-) of anti-rabbit IgG. After washing, the treated thylakoids were used in transport assays containing radiolabeled pLHCP. The correctly integrated protease-resistant degradation product of LHCP is indicated (DP) to the right of the phosphorimage.

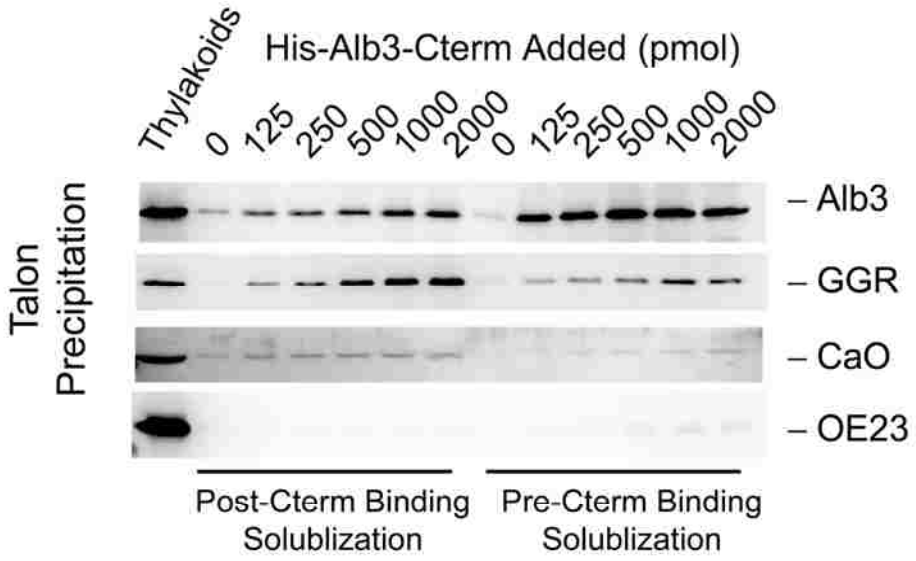

Figure 4.8 GGR and Alb3 are copurified using His-Alb3-Cterm from presolubilized thylakoids.

Salt-washed thylakoids (75 µg Chl) were solubilized before or after incubation as indicated with increasing amounts (0-2000 pmol as indicated) of His-Alb3-Cterm and used for copurification assays with Talon metal affinity resin. Western blots of isolated proteins are shown probed for the proteins indicated to the right. The first lane (Thylakoids) contains thylakoid membranes to compare relative amounts isolated.

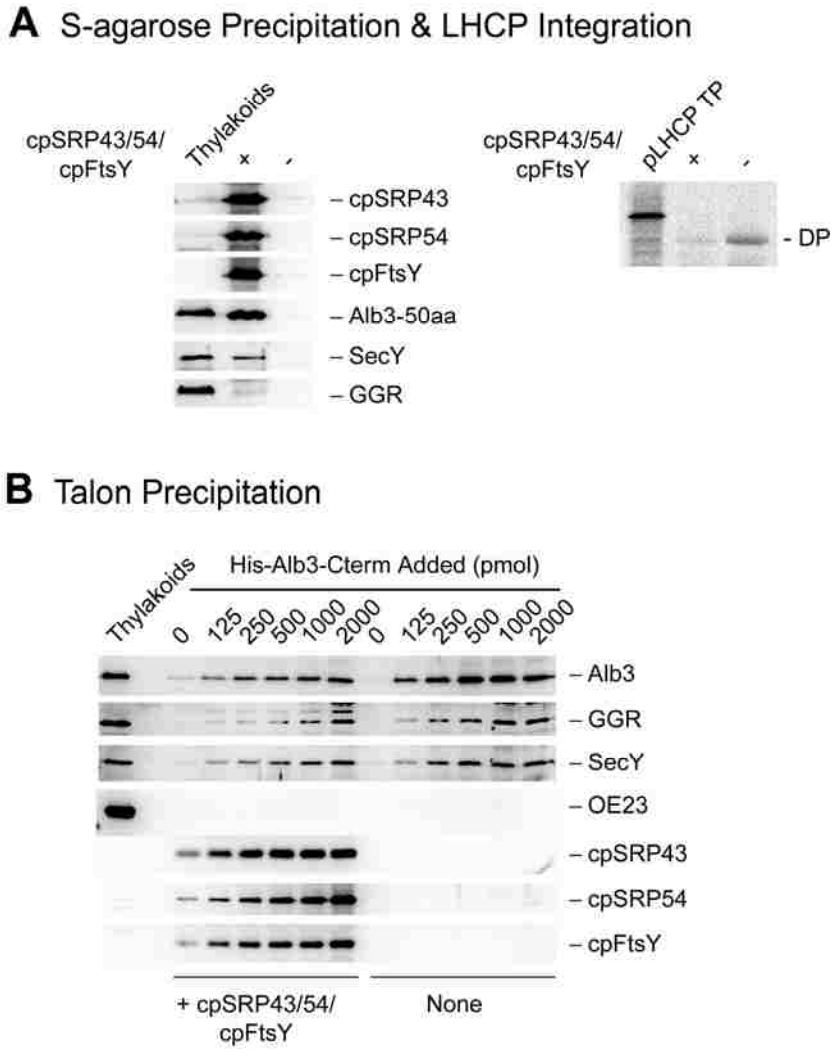

Figure 4.9 Two pools of Alb3 can be distinguished by copurification with cpSRP targeting components or GGR.

A) Salt-washed thylakoids (75 µg Chl) were mock-treated (-) or incubated with 10 µg each His-cpSRP43, His-cpSRP54, and Trx-His-S-cpFtsY (+) in the presence of 0.5 mM GMP-PNP. Thylakoids were buffer washed and solubilized in maltoside. The soluble fraction was mixed with S-protein agarose to isolate S-tagged cpFtsY and all copurifying proteins. Western blots of the samples were probed to identify the presence of proteins indicated to the right. The first lane (Thylakoids) contains thylakoid membranes to compare relative amounts isolated. A portion of treated thylakoids was also used in transport assays containing radiolabeled pLHCP. The correctly integrated protease-resistant degradation product of LHCP is indicated (DP) to the right of the phosphorimage. **B**) Treated thylakoids from the S-agarose unbound fraction were incubated with increasing amounts (0-2000 pmol as indicated) of His-Alb3-Cterm and used for copurification assays with Talon affinity resin. Western blots of isolated proteins are shown probed for the proteins indicated to the right. The first lane (Thylakoids) contains thylakoid membranes to compare relative amounts isolated.

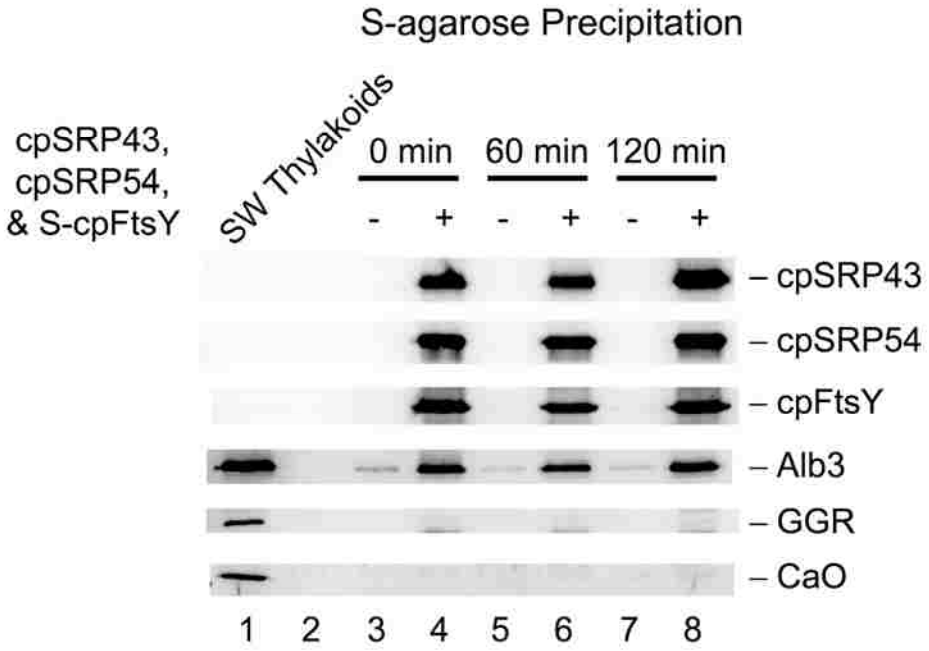

Figure 4.10 Composition of the cpSRP membrane complex is stable for the duration of copurification experiments.

Salt-washed thylakoids (75 µg Chl) were mock-treated or incubated with 10 µg each cpSRP43, His-cpSRP54, or Trx-His-S-cpFtsY in the presence of 0.5 mM GMP-PNP. Thylakoids were buffer washed and stored at room temperature for the time indicated. The maltoside-solubilized soluble fraction was then mixed with S-protein agarose to isolate S-tagged cpFtsY and all copurifying proteins. Western blots of the samples were probed to identify the presence of proteins indicated to the right. The first lane (Thylakoids) contains thylakoid membranes to compare relative amounts isolated.

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V

SUMMARY AND DISCUSSION

The signal recognition particle (SRP) pathway is a protein targeting system conserved in all kingdoms of life that is responsible for delivering both integral membrane proteins and secretory proteins to the appropriate translocons in target membranes. The most well studied examples of SRP-dependent targeting include proteins that are co-translationally targeted from the cytosol to the endoplasmic reticulum in eukaryotes and to the plasma membrane in prokaryotes (for reviews see (Keenan *et al.*, 2001; Doudna and Batey, 2004; Egea *et al.*, 2005; Pool, 2005; Bibi, 2007)). Unlike cytosolic SRPs that interact with the ribosome prior to interaction with the signal peptide, the chloroplast SRP is novel in the sense that it can function in the absence of a ribosome to bind and post-translationally target full-length proteins to the thylakoid membrane. While cpSRP is structurally and functionally specialized for post-translational protein targeting, the general targeting steps are similar to that of prokaryotic or mammalian SRPs.

The stages of SRP-dependent protein targeting can be divided into those that take place in the soluble phase (cytosol or stroma) and those that are membrane-associated events. For prokaryotic and mammalian SRPs, the targeting process is co-translational; therefore the cytosolic events begin with the recognition and association of SRP with a ribosome-nascent chain (RNC) complex. The SRP samples nascent chains for signal sequences by interacting with ribosomes at a discrete step in translation (Ogg and Walter, 1995). A highly-conserved 54 kDa subunit, SRP54, forms extensive contacts with ribosomal proteins L23 and L29 (near the nascent chain exit site) on non-translating ribosomes in *Escherichia coli* (Ullers *et al.*, 2003). In yeast, ribosomal proteins L25 and Rp125p have also now been implicated as a major interaction sites for SRP on the

ribosome and probably play a critical role in the recruitment of SRP to the ribosome (Grallath *et al.*, 2007; Dalley *et al.*, 2008). A RNC-bound SRP54 directly interacts with an exposed signal sequences via its C-terminal methionine-rich M-domain (Luetcke *et al.*, 1992). In eukaryotes, it has been shown that SRP binding pauses translation within the ribosome (Walter and Blobel, 1981; Wolin and Walter, 1989). Interaction with the RNC primes SRP for interaction with the SRP receptor (SR) protein at the target membrane (Gilmore *et al.*, 1982).

In the mammalian SRP system, the SRP consists of two GTPases, SRα and SRβ. The GTPase domain of SRα works in concert with the GTPase domain of SRP54 where the coordinated hydrolysis of GTP by both SRPα and SRP54 plays and integral part in the targeting cycle. The membrane-bound RNC-SRP-SR complex is stable until the signal sequence is transferred to an available Sec translocon (Song *et al.*, 2000). In the absence of a functional Sec translocon, SRP54 dissociation from signal sequences is blocked (Song *et al.*, 2000), suggesting that the Sec translocon regulates the GTP hydrolysis cycle of the SRP-SR complex at the stage of signal sequence dissociation from SRP54. It should be noted that SRβ also interacts with the ribosome and is involved in coordination of signal sequence release from SRP in the presence of an available translocon (Fulga *et al.*, 2001). Displacement of SRP from the ribosome and the signal sequence requires transfer of the substrate to the Sec translocon and GTP hydrolysis by SRP54 and SRα (Connolly and Gilmore, 1989). Transfer of the signal sequence and subsequent displacement of SRP allows translation to resume. The translating ribosome provides the driving force for pushing the substrate through the pore. To preserve the permeability barrier, an hsp70 homologue, BiP, is involved in sealing the luminal face of

the translocation channel in a reaction that is similar to the association/dissociation of Hsp70 with substrates (Hamman *et al.*, 1998; Haigh and Johnson, 2002; Alder *et al.*, 2005). SRP and SR are recycled through simultaneous GTP hydrolysis, releasing the SRP and SR for another round of targeting (Miller and Walter, 1993).

SRP-dependent targeting in archaea and eubacteria is somewhat simplified compared to mammalian targeting, in terms of SRP and SR components. In the mammalian system, the SRP consists of one RNA molecule (7SL RNA) and six proteins named according to their apparent molecular weight, SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72. The SRP54 component is considered the core of the SRP as it is conserved in all SRP systems examined to date and contains an essential GTPase activity. The corresponding SR consists of two GTPase proteins, SRα and SRβ. In archaea, the SRP contains an RNA molecule (7S RNA) and two proteins, SRP19 and SRP54. Archaea SR is simplified to an SRα homologue called FtsY. In comparison to mammalian SRP systems, systems found in eubacteria such as *E. coli* contain a shorter RNA molecule (4.5S RNA), a homologue of SRP54 known as Ffh (*f*ifty-*f*our *h*omologue), and FtsY, the SRα homologue.

Even with a dramatic reduction in complexity of targeting components, SRP-dependent protein targeting in *E. coli* appears to be very similar to that in mammalian cells with a few notable differences. *E. coli* SRP (Ffh + 4.5S RNA) interacts with RNC complexes in the cytosol; however translation is not paused in *E. coli* SRP targeting (Powers and Walter, 1997). Secondly, unlike the mammalian SR, FtsY is not anchored to a target membrane. Instead, FtsY has the ability to partition to membranes and is found in both the cytosol and on the plasma membrane. Despite these functional and structural

differences, research in *E. coli* SRP-dependent targeting has revealed much about the interactions between the core components, Ffh and FtsY, and regulation of the SRP GTP hydrolysis cycle.

The chloroplast SRP (cpSRP) is comprised of a conserved SRP54 (cpSRP54) homologue and an approximately 43 kDa protein (cpSRP43) unique to chloroplasts. . CpSRP in combination with and the SRα homologue, cpFtsY, facilitate the post-translational targeting of nuclear-encoded thylakoid proteins to the Alb3 translocase in thylakoid membranes. It should be noted that cpSRP54 and cpFtsY, independent of cpSRP43, are also utilized for the co-translational targeting of some chloroplast-synthesized proteins to an as of yet undefined translocon. The model substrate for post-translational cpSRP-dependent protein targeting is the gene product of *lhcb1*, a light-harvesting chlorophyll-binding protein (LHCP), herein referred to as LHCP.

The absence of a ribosome and presence of a unique SRP subunit in chloroplasts suggest both conserved and distinct mechanistic differences. From work conducted since the discovery of cpSRP, general steps of the targeting pathway have largely been uncovered. After the precursor form of LHCP is imported into the chloroplast, a stromal processing peptidase removes the chloroplast targeting peptide. CpSRP recognizes and binds to a cpSRP-binding motif in mature LHCP forming a transient intermediate termed transit complex (DeLille *et al.*, 2000; Tu *et al.*, 2000; Groves *et al.*, 2001). This transit complex subsequently interacts with the SRP receptor (cpFtsY) on the thylakoid membrane prior to interaction with the translocase, Alb3 (Moore *et al.*, 2003). Upon interaction of cpSRP, cpFtsY, and Alb3, the protein substrate is most likely transferred to Alb3, although an LHCP-Alb3 interaction has not been demonstrated. Simultaneous

GTP hydrolysis by cpSRP54 and cpFtsY releases the protein components for subsequent rounds of targeting.

Many questions remain concerning the orchestration and timing of membrane-associated cpSRP-dependent targeting events. How are the SRP components localized to the target membrane? Which components interact with the translocon? How is GTP hydrolysis regulated so that it only occurs at the right step in the targeting cycle? Which components and interactions trigger the hydrolysis of GTP by the SRP GTPases? Is LHCP insertion coordinated with Chl biosynthesis? Experiments described in chapters II-IV are aimed at understanding such events that take place at the membrane interface merging SRP-dependent protein targeting to Alb3-dependent insertion and assembly. What follows is a summary of the findings presented in chapters II-IV in light of currently published research findings. Additionally, a cpSRP-targeting model is presented with a discussion of the questions that remain to be addressed.

SRP Receptor Membrane Binding

In eukaryotes, SRα is anchored at the endoplasmic reticulum by its association with SRβ, an integral membrane component. For bacterial and chloroplast SRα homologues, FtsY and cpFtsY, no SRβ counterparts have been identified. Instead, both FtsY and cpFtsY are capable of partitioning on and off the target membrane. The membrane binding activity of FtsY was first thought to be housed within the large (~200 residues) N-terminal acidic domain (Zelazny *et al.*, 1997). Later, it was demonstrated that both *E. coli* FtsY and archaea *Haloferax volcanii* FtsY must contain two membrane binding domains, one of which resides in the NG domain (de Leeuw *et al.*, 2000; Lichi *et*

al., 2004). Together, both domains provide FtsY with the ability to preferentially interact with anionic head groups.

CpFtsY, in comparison to FtsY, contains a much shorter, less acidic N-terminal region (~20 residues), yet also exhibits the ability to partition on and off thylakoid membranes. It should be noted that the short N-terminal region found in cpFtsY is not novel; many other FtsY homologues (e.g. *Thermus aquaticus*) also contain a short N-terminal region (Ladefoged and Christiansen, 1997; Samuelsson and Zwieb, 1999). Sequence alignment reveals that residues of cpFtsY N-terminal to the NG domain are not conserved among SRP receptor proteins, with the exception of a double Phe motif (Phe195 and Phe196 in *E. coli* FtsY) commonly found in bacterial SRP receptors. Although the NG GTPase domain of *E. coli* FtsY ($FtsY_{NG}$) fails to support protein targeting, addition of the last A domain residue, Phe196 of a conserved double Phe motif ($FtsY_{NG+1}$), restores protein targeting *in vivo* (Eitan and Bibi, 2004). *In vitro* studies also show that $FtsY^{NG+1}$ retains the capacity to bind membranes and support integration of SRP-dependent substrates, though at significantly reduced levels compared to full-length FtsY (Angelini *et al.*, 2006). Due to the gain of function exhibited by $FtsY_{NG+1}$, we hypothesized that this conserved double Phe motif is necessary to support membrane binding and corresponding functions not only in *E. coli* FtsY, but also in FtsY homologues, including cpFtsY. For these reasons, we anticipated the identification and characterization of cpFtsY's membrane binding region would likely reveal a core structural requirement for membrane binding.

Our results indicate that the membrane association capacity of cpFtsY is housed in the N-terminal region of the mature protein (residues 41-56), which spans the

traditional A/NG domain delineation between residues 49 and 50 (chapter II). CpFtsY residues 41-56 are necessary for cpFtsY membrane association; upon removal of this region, *in vitro* translated cpFtsY does not associate with thylakoid membranes. Furthermore, when fused to the N-terminus of a soluble protein, cpFtsY residues 41-56 were demonstrated to be sufficient for anchoring routing and anchoring proteins to thylakoid membranes. Interestingly, a similar fusion corresponding to the same region in *E. coli* FtsY produced the same result. In both cpFtsY and *E. coli* FtsY, the conserved double Phe motif (Phe48 and Phe49 in cpFtsY; Phe195 and Phe196 in *E. coli* FtsY), was required for the membrane binding capacity of this region, suggesting a conserved binding mechanism.

Sequence alignments of the membrane binding motif in cpFtsY with FtsY homologues revealed at least four highly-conserved hydrophobic residues and three positively-charged residues arranged in a manner suggestive of an amphipathic helix (chapter II). The three-dimensional structure of peptides corresponding to cpFtsY's lipid binding motif confirmed an overall helical structure that is lost upon alanine-replacement of F48. Considering the predicted need to preserve the helical structure for lipid binding, we also demonstrated that each of the conserved hydrophobic and positively-charged residues identified by sequence alignments contribute to the function of the membrane binding region, as residue-replacement experiments of these residues resulted in appreciable reductions in membrane binding and even greater losses in integration efficiency.

Proteinaceous Membrane Binding Partners for SRP Receptors

It has been proposed that the membrane assembly of FtsY occurs in a two-step process involving initial association with phospholipid head groups and subsequent binding to a proteinaceous moiety (Millman *et al.*, 2001). In *E. coli*, the SecYEG translocon has been implicated as a 'membrane-bound receptor' for FtsY, as the formation of a stable FtsY/SecYEG translocon complex has been identified and shown to be necessary for substrate targeting/insertion reactions (Angelini et al., 2005, 2006; Weiche et al., 2008). In contrast, we demonstrate that cpFtsY binds thylakoid membranes in a non-saturable and protease-insensitive manner, suggesting that a membrane-bound receptor is not a requirement for cpFtsY membrane binding (chapter II). In addition, incubation of thylakoid membrane with cpSecY antisera does not inhibit cpSRP-dependent targeting of LHCP, which supports a model in which cpSecY is not required for cpFtsY function (Moore *et al.*, 2000; Moore *et al.*, 2003), although this does not rule out interaction with other membrane proteins such as Alb3.

Is SRP Receptor Membrane Partitioning Necessary for the Targeting Reaction?

Membrane association appears to be a critical function of FtsY, since removal of membrane binding capacity correlates with loss of SRP-dependent targeting (Zelazny *et al.*, 1997; Valent *et al.*, 1998; de Leeuw *et al.*, 2000). This may be explained, in part, by the fact that only membrane-associated FtsY supports the release of nascent chains from SRP to the translocon (Valent *et al.*, 1998). FtsY tethered to membranes is functional *in vivo* (Zelazny *et al.*, 1997), suggesting that the partitioning activity, in and of itself, is not strictly required. However, given the conserved nature of partitioning among bacterial

and chloroplast SRP receptor, the partitioning activity may play an as of yet unidentified role in protein targeting by the SRP.

Regarding the chloroplast system, our results consistently demonstrate that appreciable losses in cpFtsY membrane binding capacity are accompanied by corresponding losses in LHCP targeting (chapter II). This strongly suggests that association of cpFtsY with membranes is a necessary component of the cpSRP-dependent targeting cycle. The role of cpFtsY partitioning between the stroma and thylakoid fractions is yet to be determined. Our results demonstrating that tethering of cpFtsY to the thylakoid does not prevent cpFtsY from functioning in LHCP integration experiments suggests that the partitioning is not required (chapter II).

In confirmation of our findings concerning the cpFtsY membrane binding motif, recent reports have been published describing similar findings in FtsY homologues. The conserved lipid-binding motif, described as an amphipathic α-helix, was identified in *E. coli* FtsY and shown to be essential for FtsY function *in vivo* (Parlitz *et al.*, 2007). A crystal structure of cpFtsY at 1.75 Å resolution revealed an N-terminal amphipathic helix, as predicted, that is similar to that seen in *E. coli* FtsY (Stengel *et al.*, 2007). It has also been shown that a fusion of the N-terminal segments of *Streptomyces lividans* FtsY to the *E. coli* FtsY NG domain is functional *in vivo*, whereas the NG domain alone is not (Maeda *et al.*, 2008).

Membrane Binding Influences SRP Receptor GTPase Activity

It has been proposed that the lipid binding activity of FtsY is important for the regulation of SRP-dependent protein targeting (de Leeuw *et al.*, 2000). In previous

studies, lipid association was shown to induce a conformational change in FtsY (based on differential proteolysis) and greatly enhance its GTPase activity (de Leeuw *et al.*, 2000). Not surprisingly, cpFtsY's membrane-binding motif appears to contain a structural switch that modulates the ability of cpFtsY to partition to thylakoid membranes and function in the cpSRP targeting pathway. Similar to FtsY, the addition of liposomes increases the GTP hydrolysis activity of cpFtsY (chapter II). Interestingly, alanine-replacement of the conserved F48 residue results in high basal GTP hydrolysis activity and the loss of liposome-induced hydrolysis stimulation. These results agree with previously documented observations that the NG domain alone has a higher basal GTPase activity in solution than full-length FtsY, implicating the N-terminal A-domain as a repressor of GTP hydrolysis in the absence of a lipid bilayer (de Leeuw *et al.*, 2000). Furthermore, liposome-induced structural changes within the cpFtsY N-terminal peptide, as well as in a peptide corresponding to an aligned region within *E. coli* FtsY, suggest that the structural switch mechanism is conserved among SRP receptor homologues and plays a role in regulating FtsY functions unique to the membrane-bound state (chapter II).

Based on SR receptor roles in co-translational SRP targeting systems, it has been expected that cpFtsY facilitates the interaction of the cpSRP targeting complex with thylakoid membranes. While a membrane-associated cpSRP43/cpSRP54/cpFtsY complex can be repurified (Moore *et al.*, 2003), a stable interaction between cpSRP43/cpSRP54/LHCP (transit complex) and cpFtsY has not been observed in solution. Given current results, it seems likely that membrane binding facilitates cpFtsY's interaction with transit complex.

Translocation Channel Interactions

Once routed to the appropriate membrane, which components of the SRP targeting brigade facilitate interaction with the translocon? In the case of mammalian and *E. coli* SRP systems, a complex containing SRP54 and SRα (Ffh and FtsY in *E. coli*) form a complex with the Sec61 translocon (SecYEG in *E. coli*). Molecular modeling, based on the structure of the *T. aquaticus* Ffh-FtsY complex and the structure of an open *E. coli* translocon, has been utilized to predict the conformation and arrangement of the *E. coli* Ffh-FtsY complex with the SecYEG translocon (Chen *et al.*, 2008). The predicted model shows a shallow positively-charged cavity on the lateral surface of the SecYEG translocon which the authors propose may interact with FtsY's negatively-charged A domain. This is consistent with previously published observations that *E. coli* FtsY interacts in a functionally relevant manner with the SecYEG translocon (Angelini *et al.*, 2005; Angelini *et al.*, 2006).

The cpSRP system differs from bacterial and mammalian SRP systems in that it does not appear to utilize the available SecY homologue, cpSecY (Moore *et al.*, 2000; Moore *et al.*, 2003) and cpFtsY lacks the 'A' domain region of FtsY that is required for SecY binding in *E. coli*. Instead, cpSRP utilizes the translocase Alb3 (Moore *et al.*, 2000; Moore *et al.*, 2003) which is homologous to YidC and Oxa1 in bacterial and mitochondrial inner membranes, respectively. Though YidC/Oxa1/Alb3 homologues can vary in length quite dramatically (225-795 residues), all share a hydrophobic core region of about 200 residues. The conserved hydrophobic region has a conserved structure with five transmembrane segments broken up by hydrophilic loops (see Figure 3.1). Certain hydrophilic exposed regions are responsible for interacting with ribosomes or targeting

machinery, conceivably increasing the efficiency of the integration reaction. For example, the hydrophilic C-terminal extension of Oxa1 forms an α-helical domain essential for interacting with the ribosome during co-translational integration (Jia *et al.*, 2003; Szyrach *et al.*, 2003). A structure of the soluble, periplasmic, C-terminal extension of YidC reveals a hydrophobic cleft that appears to be a substrate binding cleft (Ravaud *et al.*, 2008).

Like Oxa1 and YidC, Alb3 contains a hydrophilic C-terminal extension. Steric hindrance of Alb3's C-terminus (Alb3-Cterm) using polyclonal antisera against Alb3-Cterm inhibits LHCP integration (Moore *et al.*, 2003), suggesting that interactions with Alb3-Cterm are directly involved in the cpSRP targeting reaction. Due to the fact that the cpSRP targeting machinery can be stabilized in complex with Alb3 in the absence of substrate, it is thought that Alb3 interacts with the cpSRP directly rather than through a substrate-mediated event (Moore *et al.*, 2003). This raises the question, which cpSRP components facilitate interaction with the Alb3 translocon?

In overall shape and charge distribution, cpSRP43 resembles helix 8 of the SRP RNA which is absent in chloroplasts (Stengel *et al.*, 2008). In agreement with this idea, cpSRP43, like the SRP RNA, interacts with cpSRP54 and plays a role in the regulation of GTP hydrolysis between cpSRP54 and cpFtsY (Peluso *et al.*, 2000; Goforth *et al.*, 2004; Siu *et al.*, 2007; Neher *et al.*, 2008). Additionally, cpSRP43 has been shown to interact with the substrate (LHCP) and cpSRP54 (Tu *et al.*, 2000; Jonas-Straube *et al.*, 2001; Goforth *et al.*, 2004), both important ribosomal roles in a co-translational SRP pathway. Thus, it has been proposed that cpSRP43 evolved both as a replacement for the SRP RNA and as a replacement for the ribosome in the post-translational cpSRP-dependent

targeting pathway (Goforth *et al.*, 2004; Stengel *et al.*, 2008). Because ribosomes have been shown to interact directly with Oxa1, it is plausible to hypothesize that cpSRP43 may interact with Alb3 (Jia *et al.*, 2003; Szyrach *et al.*, 2003). Indeed we have demonstrated that affinity-tagged cpSRP43 is able to specifically coprecipitate Alb3 from isolated thylakoid membranes (Tzvetkova-Chevolleau *et al.*, 2007).

Utilizing a recombinant construct corresponding to the soluble C-terminal region of Alb3 in assays that reconstitute cpSRP targeting activities, we have shown that cpSRP43 directly interacts with Alb3 via the hydrophilic C-terminal region of Alb3 (chapter III). It is attractive to propose that cpSRP43 facilitates the initial interaction of the targeting complex with Alb3. However, a stable complex containing cpSRP54, cpFtsY, and Alb3 can be formed without cpSRP43 or substrate, indicating that these proteins may also be capable of interaction with Alb3 (Moore *et al.*, 2003). However, cpSRP54 and cpFtsY individually do not appear to form a stable complex with Alb3; whereas his-tagged cpSRP43 copurifies ~15% total Alb3 from thylakoid membranes (chapter III). Interestingly, a soluble construct corresponding to the C-terminus of Alb3 is able to stimulate GTP hydrolysis between cpSRP54 and cpFtsY only in the presence of cpSRP43, indicating that cpSRP43 facilitates a response to Alb3 in cpSRP54 and cpFtsY (chapter III). These results suggest that cpSRP43 provides a translocon 'sensing' mechanism for the cpSRP and mediates key targeting events at the thylakoid membrane, such as release of the targeting complex from Alb3. Interestingly, biosensor analysis of the interactions between SRP, the SR, and the ribosome revealed that the SR has a 100-fold higher affinity for the ribosome than for the SRP (Mandon *et al.*, 2003). Based on these results, the authors proposed that the interactions with the ribosome and SR are

important for increasing the rate of the targeting reaction, while the SRP/SR and ribosome/translocon interactions are important for the reliability of proper targeting within the pathway. It is interesting to speculate that cpSRP43 may also interact with cpFtsY in a functionally relevant manner.

Given that the targeting processes are highly regulated, it seems likely that the interaction between cpSRP43 and the C-terminus of Alb3 is prohibited until the proper step in targeting is reached. We have determined that cpSRP43 is capable of interacting with a peptide corresponding to the C-terminus of Alb3 (Alb3-Cterm) in solution (chapter II); however, we presume that this interaction must take place at thylakoid membranes during a specific targeting step(s). It is possible that the cpSRP43/Alb3 C-terminus interaction takes place downstream of initial binding of the cpSRP targeting complex to another membrane receptor, such as a separate exposed Alb3 domain or another associated membrane protein. If this is the case, we would expect the Alb3-Cterm peptide neither to interact directly with transit complex nor inhibit the formation of a membrane complex containing cpSRP, cpFtsY, and Alb3. However, Alb3-Cterm peptide does appear to affect transit complex (chapter II), though whether it competes for transit complex formation or simply binds transit complex (resulting in lack of detection) remains to be determined. It should also be noted that, in the absence of LHCP substrate, Alb3-Cterm peptide also seems to influence the cpSRP targeting complex, as it appears to inhibit cpSRP43/cpSRP54/cpFtsY membrane complex formation with Alb3 (chapter II). Current studies to elucidate why Alb3-Cterm peptide does not inhibit LHCP integration *in vitro* are underway. The influence of Alb3-Cterm peptide on transit complex and on the cpSRP43-dependent stimulation of cpSRP54/cpFtsY GTPase activity are consistent

with a model in which cpSRP43 interaction with Alb3 C-terminus is required for LHCP release and GTP hydrolysis at the membrane. It will be interesting to determine whether this is true and, if so, whether Alb3 C-terminus binding is required for LHCP release from both cpSRP54 and cpSRP43.

Regulation of the SRP GTPase Cycle

Protein targeting in the eukaryotic SRP pathway is regulated by three GTPases, SRP54 and the α- and β-subunits of the SR. Distinct from SRP54 and SRα, the integral membrane protein SRβ is more closely related to the Arf GTPase subfamily (Miller and Walter, 1993). SRP54 and SRα make up a subfamily of G proteins that contain a classic GTPase G domain composed of conserved elements and an N-terminally adjacent N domain unique to the SRP subfamily of G proteins (Bourne *et al.*, 1991; Freymann *et al.*, 1997; Montoya *et al.*, 1997; Freymann and Walter, 2000; Chandrasekar *et al.*, 2008). Binding and hydrolysis of GTP involves the coordinated action of the GI-GIV elements, a conserved α-β-α structure known as the Insertion Box Domain, and GTP-binding loops. Unlike other GTPases, SRP54 and SRα (Ffh and FtsY, in *E. coli*) act reciprocally to stimulate the each other's GTPase activity. The Ffh/FtsY heterodimer displays a two-fold pseudo-symmetry with a joint GTP-binding cavity that allows for simultaneous GTP hydrolysis between the two proteins (Miller *et al.*, 1994; Powers and Walter, 1995; Egea *et al.*, 2004; Focia *et al.*, 2004). Extensive regulation is required to ensure spatial and temporal control of GTP hydrolysis during the SRP-dependent targeting cycle. At a minimum, this regulation is provided by the availability of GTP, substrate, ribosomes, SRP RNA, target membranes, and the translocon.

Unlike other GTPase subfamilies, SRP GTPases do not undergo large conformational changes between the GTP-bound and GDP-bound states (Bourne *et al.*, 1991; Montoya *et al.*, 1997; Freymann *et al.*, 1999; Gawronski-Salerno and Freymann, 2007; Reyes *et al.*, 2007). For SRP GTPases, the most dramatic conformational changes occur during heterodimer formation (Egea *et al.*, 2004; Focia *et al.*, 2004). It is the NG domains of Ffh and FtsY that are primarily responsible for heterodimer formation and the interaction face spans the surface of the N and G domains in both proteins (Freymann and Walter, 2000; Egea *et al.*, 2004; Focia *et al.*, 2004).

In the SRP GTPase cycle, the association of Ffh and FtsY appears to be the rate-limiting step rather than GTP hydrolysis (Peluso *et al.*, 2001). The SRP GTPases are distinct in that they exhibit both relatively low nucleotide affinity and hydrolysis rates as individual components, yet rapidly hydrolyze GTP as a heterodimeric complex (Peluso *et al.*, 2001; Shan and Walter, 2003). In fact, neither SRP nor SR stably binds GTP prior to formation of the SRP-SR complex assembly although SRP54 bound to the ribosome is thought to be in a GTP-bound state (Rapiejko and Gilmore, 1997). That said, GTP is stimulatory for complex formation; the rate of complex formation between Ffh and FtsY increases 10-fold when they are introduced in GTP-bound forms (Peluso *et al.*, 2001).

Recent evidence points to the ribosome nascent chain complex (RNC) and the SRP RNA as important regulators of the GTPase cycle in co-translational SRP-based protein targeting. While SRP54 alone has a low affinity for GTP, the presence of ribosome nascent chain (RNC) complexes significantly increases SRP54's affinity for GTP (Bacher *et al.*, 1996). Chemical crosslinking revealed conformational changes that occur as SRP interacts with the RNC (Pool *et al.*, 2002). During signal peptide binding,

SRP54 can be found close to the exit tunnel of the ribosome in close contact with ribosomal proteins L23a and L35. RNC-induced SRP conformational changes appear to promote not only GTP binding, but also SRP/SR heterodimer formation (Buskiewicz *et al.*, 2009). In the RNC- & GTP-bound state, SRP54 exhibits a much higher affinity for the SR (Bacher *et al.*, 1996). The highly conserved SRP RNA has now also been shown to facilitate complex formation between the signal sequence-bound SRP and SR, accelerating their association kinetics 400-fold (Peluso *et al.*, 2000; Peluso *et al.*, 2001; Bradshaw *et al.*, 2009). In fact, interaction of signal sequence-bound SRP with the SRP RNA induces a conformational switch in the SRP that mimics the conformational switch caused by the SRP/SR interaction, stabilizing an early intermediate SRP/SR interaction (Buskiewicz *et al.*, 2005; Buskiewicz *et al.*, 2005; Neher *et al.*, 2008; Zhang *et al.*, 2008; Bradshaw *et al.*, 2009).

Formation of the SRP GTPase heterodimer induces conformational changes in both proteins that prime the complex for GTP hydrolysis. In both GTPases, heterodimer formation also causes displacement of the N-termini, as evidenced by disorder in crystal structures and increases in protease accessibility in these regions, and a shift from an "open" to "closed" conformation, in terms of nucleotide specificity (Shepotinovskaya and Freymann, 2002; Shan and Walter, 2003; Egea *et al.*, 2004; Focia *et al.*, 2004). The overall rearrangement repositions catalytic residues in the active site pocket to form more extensive contacts with the bound nucleotide, activating the GTPases for hydrolysis (Powers and Walter, 1995; Egea *et al.*, 2004; Focia *et al.*, 2004; Shan *et al.*, 2004; Jaru-Ampornpan *et al.*, 2007; Reyes *et al.*, 2007; Chandrasekar *et al.*, 2008).

SRP GTPase activity is also regulated by association with membranes. With the exception of mammalian homologues, which are anchored at the membrane through an interaction with the SRβ subunit, SRα homologues partition between soluble and membrane phases. The association of FtsY with the bilayer has also been shown to induce conformational changes and stimulate GTPase activity (de Leeuw *et al.*, 2000). Our results indicate that interaction with the lipid bilayer also primes cpFtsY for GTP hydrolysis (chapter II). The mechanism for membrane-induced activation for GTP hydrolysis has been linked to conformational changes in a membrane-binding motif found near the N-terminus of cpFtsY (chapter II). This lipid-responsive membrane-binding motif appears to be conserved among bacterial and organellar FtsY homologues based on sequence alignments. This idea is supported experimentally by the observation that fusions of the corresponding region from *E. coli* FtsY and *Arabidopsis thailiana* cpFtsY behave similarly to tether unrelated proteins to thylakoid membranes and exhibit similar lipid-induced structural changes are exhibited by peptides corresponding to *E. coli* FtsY and *A. thailiana* cpFtsY membrane-binding motifs (chapter II). In the mammalian SRP system, nucleotide binding to SRβ is essential for both complex formation between SRα and SRβ and translocation of the targeted polypeptide, suggesting that SRβ plays a role in regulating transfer of the nascent chain from SRP to the translocon (Fulga *et al.*, 2001; Schwartz and Blobel, 2003). Since SRβ has only been identified in eukaryotic cells it is not surprising that many SRα homologues likely circumvent this problem by responding to lipid membranes with increased GTPase activity (de Leeuw *et al.*, 2000).

How is premature GTP hydrolysis between the SRP/SR GTPases prevented to ensure productive transfer of substrate to the translocation channel? Initially, interaction

with the SRP receptor allows the SRP-bound RNC complex to dock with an available translocon (Moller *et al.*, 1998). Briefly, the translocon binding site on the RNC is occupied by SRP54 (Beckmann *et al.*, 2001; Halic *et al.*, 2004) until formation of the SRP GTPase heterodimer, which induces conformational changes in SRP54 (Pool *et al.*, 2002) that move cpSRP54 out of the way to allow interaction between the ribosome and the translocon (Halic *et al.*, 2006). Membrane-bound SRP-RNC-SR complex remains in the GTP-bound conformation in the absence of an active translocation channel (Song *et al.*, 2000) suggesting that interaction with the translocon and release of the signal sequence is prerequisite for GTP hydrolysis. Similarly, the interaction of signal peptides with SRP-SR complex inhibits GTPase activity in the absence of an available Sec translocon (Miller and Walter, 1993; Rapiejko and Gilmore, 1997).

In chloroplast SRP, we also observe a reduction in GTP hydrolysis between cpSRP54 and cpFtsY in the presence of cpSRP43 and peptides corresponding to the cpSRP43-binding region in LHCP (chapter III), suggesting that the presence of LHCP substrate inhibits GTP hydrolysis via influence on cpSRP43. It is likely that the presence of an LHCP construct containing both cpSRP43- and cpSRP54-binding motifs would produce a more significant inhibition of GTP hydrolysis. Furthermore, our results show that the C-terminus of Alb3 is able to stimulate GTP hydrolysis between cpSRP54 and cpFtsY only in the presence of cpSRP43 (chapter III). Taken together, these findings support the idea that communication of cpSRP54/cpFtsY with the translocon and targeting substrate is mediated by cpSRP43 and its interaction with each of these components. It will be interesting to determine whether a translocation channel-bound ribosome facilitates similar GTP hydrolysis stimulation between the SRP and SR.

Current Model for SRP GTPase Regulation

In summary, much has been revealed about how the SRP GTPases work together to ensure efficient protein targeting in SRP-dependent pathways. A current model adapted from Shan and colleagues includes five steps with key regulatory points correlating with the conformational changes that occur during protein targeting (see Figure 5.1) (Shan *et al.*, 2004). Interaction of SR with the translocon induces large conformational changes at the NG domain interface, causing a shift from an open to closed conformation in terms of nucleotide specificity (step 1). A similar shift from an open to closed conformation occurs in SRP as it interacts with the ribosome (step 2). Formation of the SRP/SR heterodimer brings the ribosome-nascent chain to the membrane and induces conformational changes in SRP that expose the translocon binding site on the ribosome (step 3). Membrane binding and substrate release induce conformational changes that activate GTP hydrolysis (step 4). Reciprocal GTP hydrolysis drives dissociation of SRP and SR (step 5). Accordingly, each of the conformational changes and GTP binding/hydrolysis steps provide a means to ensure that every step, from binding of the signal peptide to release of substrate to the translocon, occurs properly and efficiently and unidirectionally.

The GTPase proteins of the chloroplast SRP, cpSRP54 and cpFtsY, have adapted to work efficiently with a fully-translated substrate (i.e. no ribosome) and without the SRP RNA moiety. CpFtsY contains a more tightly-packed N domain and a more extensive interface between the N and G domains, requiring a much smaller rotation (only 2 degrees instead of 10) upon heterodimer formation with cpSRP54 than bacterial

homologues (Chandrasekar *et al.*, 2008). This adaptation may explain why cpSRP54 and cpFtsY interact so efficiently without the RNA moiety required by their bacterial counterparts (Jaru-Ampornpan *et al.*, 2007). Our findings have identified a membrane binding motif in cpFtsY that is necessary for cpSRP-dependent targeting in a manner that is related to GTP hydrolysis regulation. Secondly, we have shown that cpSRP43 interacts with Alb3 and provides a mechanism for translocon 'sensing' required to facilitate Alb3-induced GTPase regulation of cpSRP54 and cpFtsY. As such, these data in combination with what is known from mammalian and bacterial SRP systems form the basis for the current model of cpSRP-dependent targeting to Alb3. Briefly, interaction of cpFtsY with the membrane induces conformational changes in cpFtsY that are stimulatory for interaction with cpSRP54 and subsequent GTP hydrolysis (step 1). A similar shift from open to closed conformation occurs in cpSRP54 as it forms transit complex with cpSRP43 and LHCP (step 2). Formation of the cpSRP54/cpFtsY heterodimer stabilizes transit complex at the membrane and allows cpSRP43 to interact with Alb3 (step 3). Membrane binding, cpSRP43 interaction with Alb3, and substrate release induce conformational changes in cpSRP54 and cpFtsY activating them for hydrolysis (step 4). Reciprocal GTP hydrolysis drives dissociation of cpSRP and cpFtsY (step 5).

Life After Targeting… Is LHCP Insertion/Assembly Coordinated with Chlorophyll Biosynthesis?

Our current understanding of the cpSRP-dependent delivery of LHCP far exceeds our understanding of the components and processes required for thylakoid membrane

insertion and assembly of LHCP. It has been demonstrated that in the absence of chlorophyll (Chl) synthesis, LHCPs do not accumulate in thylakoid membranes, but are routed to vacuoles for degradation (Park and Hoober, 1997). Chl b, in particular, has been correlated with proper membrane insertion and folding (Paulsen *et al.*, 1993; Kuttkat *et al.*, 1997). Reciprocally, in the absence of LHCP expression, Chl does not accumulate in thylakoid membranes (Maloney *et al.*, 1989; Plumley and Schmidt, 1995). Due to the low stability of 'free' Chl and the lack of evidence for a Chl 'storage' protein, the synthesis of Chl must be correlated with the synthesis, insertion, and assembly of its binding proteins (e.g. LHCPs). In agreement, LHCP expression has been shown to influence the activity of certain Chl biosynthesis enzymes (Xu *et al.*, 2001). Furthermore, a *Chlamydomonas reinhardtii* mutant lacking Alb3 is almost completely lacking LHCs and photosystem core polypeptides and exhibits a nearly 70% reduction in Chl accumulation (Bellafiore *et al.*, 2002).

It has long been thought that the chaperone functions of the Alb3/Oxa1/YidC family may be linked to the process of ligand attachment to newly inserted proteins (Hoober and Eggink, 2001; Cline, 2003; Kuhn *et al.*, 2003). In the case of LHCP insertion, Alb3 might hold LHCP or a trimer of LHCPs in a conformation that would allow Chl to bind appropriately. The fact that cpSRP43 null mutants exhibit specific reductions in Chl and LHCPs suggests that the regulation of Chl biosynthesis is coordinated with late-stages of LHCP targeting or the integration of LHCP into thylakoids via Alb3 (Amin *et al.*, 1999; Klimyuk *et al.*, 1999). However, no evidence has been published that directly links Alb3-dependent LHCP insertion/assembly with Chl biosynthesis.

Using mass spectrometry, we were able to identify geranylgeranyl reductase (GGR), one of the last enzymes to function in Chl biosynthesis, as a component of an Alb3-containing complex. Utilizing copurification experiments, we observe that GGR can be isolated with Alb3 from thylakoid membranes (chapter IV). We are able to isolate two pools of Alb3, one that is enriched in GGR and another that can be engaged by the cpSRP for LHCP protein targeting. These results are exciting, as they represent the first evidence of a direct interaction between Alb3 and enzymes required for Chl biosynthesis. However, this preliminary finding also precipitates many new questions. What is the purpose of the pool of Alb3 in complex with GGR? Do the two Alb3 pools function together, one to integrate LHCP and the other to facilitate Chl binding and trimer assembly? Does the GGR-enriched pool function in collaboration with Alb3-dependent co-translational integration of photosystem proteins? Are other late-stage Chl biosynthesis enzymes (e.g. Chl synthase) also in complex with Alb3? Most assuredly, the answers to these pressing questions will be useful in unraveling the mystery encompassing the integration and assembly of LHCP in thylakoid membranes.

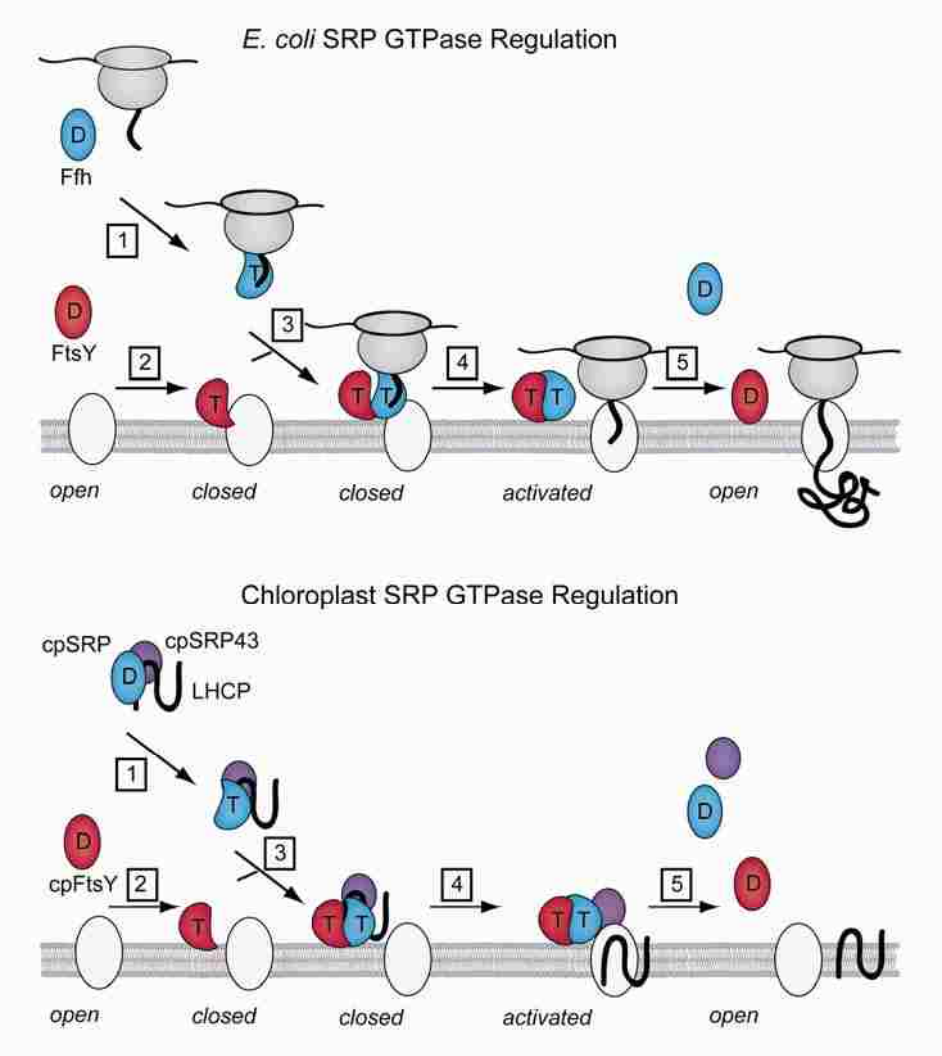

Figure 5.1. Current model for SRP GTPase regulation.

A) Step 1, SRP undergoes an open to closed conformational change, in terms of nucleotide specificity, upon association with the ribosome and nascent polypeptide. Step 2, the SRP receptor undergoes a similar open to closed conformational change upon association with the membrane translocon. Step 3, complex formation between SRP and its receptor delivers the ribosome-nascent chain to the membrane and induces conformational changes in SRP that expose the translocon binding site on the ribosome. Step 4, membrane binding and substrate release induce conformational changes that activate GTP hydrolysis. Step 5, reciprocal GTP hydrolysis drives dissociation of SRP and SR. This figure adapted from Shan et al., 2004. **B**) Based on similarities between cpSRP and co-translational SRP systems and GTPase proteins, the regulatory steps for cpSRP GTPase activity are anticipated to be similar. It should be noted that no structural evidence has been obtained to verify that the implied conformational changes take place in cpSRP54 and cpFtsY. However, the observations that both liposomes and cpSRP43 stimulate GTP hydrolysis between cpFtsY and cpSRP54 argue for the parallel conformational changes that are implied in steps 1 and 2.

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