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Thylakoid Protein Targeting/Insertion by a Signal Recognition Particle in Chloroplasts

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Thylakoid Protein Targeting/Insertion by a Signal Recognition Particle in Chloroplasts

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by

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ABSTRACT

Protein targeting is a fundamental cellular process that directs proteins from their site of synthesis to the site where they function. The signal recognition particle (SRP) dependent targeting pathway is conserved in both eukaryotes and prokaryotes where it co-translationally targets polypeptide chains emerging from ribosomes to the endoplasmic reticulum (eukaryotes) or cytoplasmic membrane (prokaryotes). A structurally unique form of SRP is found in chloroplasts where it functions to post-translationally bind and target a subset of integral thylakoid membrane proteins, the light harvesting chlorophyll binding proteins (LHCPs). Mature LHCPs bind chlorophyll a/b and function in photosynthetic light capture. Like many other chloroplast proteins, LHCPs are nuclear encoded and synthesized in the cytosol. Following their import into the chloroplast stroma, LHCPs associate with chloroplast SRP (cpSRP), which maintains LHCP solubility and initiates targeting of LHCP to the thylakoid membrane via an cpSRP receptor (cpFtsY) at the thylakoid membrane. Both cpSRP and cpFtsY are GTPases and associate at the thylakoid by a mechanism that requires GTP binding by both proteins. Subsequent insertion of LHCP into the lipid bilayer is mediated by a protein insertase Albino3 (Alb-3), which binds cpSRP to stimulate LHCP release from cpSRP and GTP hydrolysis by both cpSRP and its receptor. Work here has focused on studies to understand mechanistic details of the cpSRP targeting pathway and better understand the timing of targeting events at the membrane. The results provide support for a structure-based chronology of protein interactions between LHCP targeting substrates, cpSRP, cpFtsY, and Alb-3. They also demonstrate that GTP hydrolysis by cpSRP and its receptor at the membrane is not necessary for LHCP insertion by Alb-3, but serves to maintain an available pool of Alb-3 insertase at the membrane by

stimulating the exit of cpSRP targeting components following release of LHCP from cpSRP to

Alb-3.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

Alb-3-Cterm – Albino 3 protein C-terminus

Anks – ankyrin repeat

ATP – adenosine triphosphate

BSA – bovine serum albumin

cDNA – complementary DNA

CD – chromodomain

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

DP – degradation product representing trimeric form of LHCP

DP* – degradation product representing monomeric form of LHCP

ER – endoplasmic reticulum

EDTA – ethylene diamine tetra acetic acid

Ffh – fifty-four homologue

FRET – Forster resonance energy transfer

FtsY – SRα homologue in bacteria

GDP – guanosine diphosphate

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 $MgCl_2$

IPTG – isopropyl β–D-1-thiogalactopyranoside

ITC – isothermal titration calorimetry

kDa/kD – kiloDalton

L23 – *Arabidopsis thaliana* large ribosomal subunit protein L23

LB – Luria-Bertani

LHCP – light-harvesting chlorophyll-binding protein

M – mature form

Maltoside – n-Dodecyl β-D-Maltoside

min – minute

mM – millimolar

MWCO – molecular weight cut off

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 – precursor

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PD – pull down

PMF – proton motive force

PPL – preprolactin

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

RNA – ribonucleic acid

RNC – ribosome nascent chain complex

RT-PCR – reverse transcription PCR

SAXS – small angle X-ray scattering

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

SW – salt-washed

Tat – twin-arginine translocation

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

TC – transit complex

Tha4 – cpTat translocon subunit homologous to bacterial TatA

Tic – translocase at the inner membrane of the chloroplast

Toc – translocase at the outer membrane of the chloroplast

TM – transmembrane domain

TP – translation product

Trx-tag – thioredoxin tag

WT – wild-type

LIST OF PUBLISHED PAPERS

Henderson RC, Gao F, Jayanthi S, Kight A, **Sharma P**, Goforth RL, Heyes CD, Henry RL, Kumar TKS. Domain organization in the 54-kDa subunit of the chloroplast signal recognition particle. Biophys J 2016;111(6):1151-62.

Gao F, Kight AD, Henderson R, Jayanthi S, Patel P, Murchison M, **Sharma P**, Goforth RL, Kumar TK, Henry RL, et al. Regulation of structural dynamics within a signal recognition particle promotes binding of protein targeting substrates. J Biol Chem 2015 Jun 19;290(25):15462-74.

I. INTRODUCTION

Protein Targeting

Protein targeting or protein localization is the process by which proteins produced in the cytoplasm are transported to their destinations inside or outside the cell. Proper protein localization of nuclear encoded polypeptides from their site of synthesis in the cytosol to distinct membrane-bound compartments and organelles is crucial to maintain normal cellular function. The protein routing mechanisms that serve to localize newly made polypeptides rely on targeting sequences in the targeting substrate that are recognized by soluble and membrane-associated sorting components. In the case of chloroplasts (and mitochondria), which have a prokaryotic ancestry (64), sorting of proteins encoded by the organellar genome and synthesized by chloroplast ribosomes is accomplished by homologous sorting mechanisms found in modern day prokaryotes (9, 28, 31, 64). However, of the ~3000 proteins present in the chloroplast, only ~100 of these proteins are encoded by chloroplast DNA. Genes coding for the remainder of chloroplast proteins have since moved to the nucleus following the endosymbiotic event that gave rise to chloroplasts. Nearly all of the nuclear encoded chloroplast proteins are synthesized as full-length precursor proteins in the cytoplasm and contain an N-terminal targeting sequence or 'transit peptide', which directs the protein to chloroplast import machinery in the two envelope membranes. The import machinery forms a protein translocation channel composed of proteins that function as members of the TOC (Translocase of the outer membrane of chloroplast) and TIC (Translocase of the inner membrane of chloroplast) that recognize and engage transit peptides to facilitate precursor translocation across the envelope membranes (5, 38, figure 1.1). Transit peptides vary in length from 20 to > 100 residues and exhibit an abundance of hydroxylated residues as well as lacking acidic residues (25, 28). In the absence of additional targeting information, transit peptides direct proteins to the chloroplast stroma and are cleaved by

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a stromal processing protease. However, nuclear encoded thylakoid proteins must be routed from the stroma to the thylakoid where they are transported into or across the membrane.

Proteins that reside in the thylakoid lumen possess bipartite transit peptides with a stroma targeting and lumen targeting domain. Cleavage of the stroma targeting domain by a processing protease in the stroma produces a targeting pathway intermediate in the stroma that is also intermediate in size between the full-length precursor and the mature sized protein (25). The lumen targeting domain then directs proteins to the thylakoid where a Sec or TAT transporter, homologous to those in bacteria, transports proteins into the thylakoid lumen (69). The lumen targeting domain is structurally and functionally similar to bacterial signal peptides and are cleaved by a lumen processing protease to produce the mature sized protein in the thylakoid lumen. In contrast, nuclear encoded integral thylakoid proteins such as those that function in the photosystem 1 and 2 peripheral light harvesting protein complexes (LHCPs) contain information in the mature protein that is required for localization from the stroma to the thylakoid. Four distinct thylakoid localization pathways found in chloroplasts are-

Secretory Pathway (Sec pathway)

Precursor proteins which are translocated via chloroplast Sec pathway (cpSec) possess an N-terminal stroma targeting domain and a C-terminal lumen targeting domain (45, 46). Lumenal targeting domain consists of a charged N-terminal region, a core region of hydrophobic amino acid residues, and an A-X-A motif at the C-terminal region which is made up of polar residues. This motif serves as the cleavage site for the thylakoid processing peptidase (45). cpSec pathway is involved in the targeting of soluble proteins to the thylakoid lumen (e.g. Plastocyanin, OE33) as well as integration of thylakoid membrane proteins (e.g. Cytochrome f) (2, 34, 50). Key

components of chloroplast Sec pathway have been identified based on homology modelling (45). The components of Sec-protein transport machinery have been found in endoplasmic reticulum dependent targeting in eukaryotes (51), bacterial plasma membranes (70), in addition to the thylakoid membranes of plants and algal chloroplasts (6, 7). Protein translocation across the plasma membrane in bacteria consists of a membrane embedded Sec protein complex which is made up of SecY, SecE, SecG, SecF, SecD, YajC and a peripheral protein Sec A which is an ATPase (55, 59). SecYEG together form the core of membrane translocase in bacteria and SecA guides the unfolded protein to pass through the pore formed by SecYEG together. Bacterial Sec B serves as a chaperone and keeps the protein to be targeted in a targeting competent state. SecFDYajC forms another trimeric complex at the membrane which together with SecYEG aids in the smooth protein translocation across the membrane (45, 60). Chloroplast Sec targeting system contains only SecY (cpSecY), SecE (cpSecE) and SecA (cpSecA) homologues and they function very similar to the bacterial Sec targeting mechanism (37, 49, 57, 59, 73). cpSec E froms a complex at the thylakoid membrane with cpSecY and cpSecA serves as an ATPase and these together lead to the transport of thylakoid luminal proteins from stroma or to the integration of thylakoid membrane proteins (Figures 1.1 and 1.2). All substrate proteins of cpSec system have been known to be in their unfolded states. Studies have shown that SecA dependent translocation in chloroplasts could be inhibited by using antibodies against cpSecY which reiterates that these components work together in the system (46, 59). Translocation of thylakoid protein OE33 has also been shown to be inhibited when azide was used in the study (25, 30, 36). Azide is known as the inhibitor of bacterial SecA protein. cpSec pathway has also been shown to be involved in the co-translational targeting of the proteins like Cytochrome f, D1 which are synthesized by the chloroplast DNA using the stromal ribosomes (6).

Twin arginine translocation pathway (TAT pathway)

In chloroplast, an equal number of substrate proteins are targeted via cpSec pathway and cpTAT pathway whereas in bacteria, there are more substrate proteins which are targeted via Sec pathway and very few via TAT pathway (48). Substrate proteins like OE17, OE23, and Pftf are targeted by cpTAT pathway either in the lumen or integrated on the thylakoid membrane. The lumenal targeting domain of the substrate proteins for cpTAT pathway also contain charged N-terminal region, a core region of hydrophobic amino acid residues, and an A-X-A motif at the C-terminal region which is made up of polar residues (45). The only difference between the substrate protein for cpSec pathway and cpTAT pathway is that the substrate proteins for cpTAT pathway contain twin Arginine motif in their N terminal charged region. cpTAT pathway transports both folded and unfolded proteins across the membrane without the need of any soluble factors or nucleoside triphosphates, however, it requires the hydrogen ion gradient across the membrane to carry out its function (7, 48). This translocation system is present in chloroplasts of plants, algae and in bacteria. Fungi and animals lack this targeting system (6). Three integral membrane proteins- Tha4, Hcf106 and cpTatC are found on the thylakoid membrane of chloroplasts (48, figure 1.2). They are known as TatA, TatB and TatC in bacteria and are located on the cytoplasmic membrane. Maize Hcf106 (high-chlorophyll fluorescence 106) mutant plants showed deficiency in several thylakoid membrane complexes like photosystem I &II, cytochrome b6/f complex as shown in Settles et al.(61) and this plant was shown to be specifically defective in cpTat dependent targeting pathway (aka delta pH dependent pathway). The three subunits of cpTAT system exist in two sub-complexes on the thylakoid membrane. Subunits cpTatC and Hcf106 exist together as a receptor complex without Tha4 subunit. An active cpTat translocase machinery is formed as the substrate protein binds to the

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receptor complex and pH gradient is established leading to the binding of Tha4 with the receptor complex bound to the substrate. Inhibition of precursor protein binding as well as inhibition of protein translocation was observed in the assays where thylakoid membranes were pretreated with antibodies to Hcf106 or cpTatC whereas inhibition of protein translocation without any harm to protein binding was observed when thylakoids were pretreated with antibodies to Tha4 (45,). Studies have shown that oligomers of Tha4 (TatA in bacteria) are formed at the thylakoid membrane Tat translocase during protein transport making a flexible protein conducting channel (10, 21, 60). This explains how cpTat pathway (or bacterial TAT pathway) is able to transport folded proteins of different sizes into the thylakoid lumen or across bacterial plasma membrane. In-vitro import studies have shown that proton gradient force is essential for driving cpTat dependent targeting in chloroplasts or isolated thylakoid membranes (7, 41). However, *in vivo* studies in *Chlamydomonas reinhardtii* and tobacco protoplasts (14, 15) have shown that cpTat system works well even in the absence of proton gradient across the thylakoid membranes. Some more studies showed that neither electric potential nor proton gradient provide the driving force in cpTat transporting systems (14, 66). Further work needs to be done in order to answer this mystery.

Spontaneous pathway

This method of protein insertion is very different in its nature. It was first discovered in higher plant chloroplasts that CFo-II, the membrane component of ATP synthase complex found on the thylakoid membranes is post-translationally targeted via spontaneous insertion pathway (58, figure 1.1). Spontaneous insertion pathway leads to the insertion of single span membrane proteins in absence of stromal factors, nucleoside triphosphates (42) and is also independent of any proton gradient force across the membrane. Integration of such proteins into the protease

treated thylakoids proved that these proteins do not require any proteinaceous translocase component (56). Integration was also found to be unaffected in the presence of cpSec inhibitor-sodium azide which proved that CFo-II protein insertion is independent of cpSec machinery (58). CF0-II is a nuclear encoded chloroplast protein and is post translationally targeted to the chloroplasts from cell cytoplasm with the help of bipartite transit peptide which resembles those of the lumenal proteins (55). Absence of twin arginine motif in its signal peptide also rules out the possibility of cpTAT pathway aiding in the membrane insertion of this protein. The W and X protein subunits of photosystem II-PsbW, PsbX are also known to insert spontaneously into the thylakoid membrane (11). Presence of lumenal signal peptide in such proteins led to the speculation that signal peptide acts as a hydrophobic domain and helps in the insertion of proteins into the lipid bilayer by forming a loop intermediate (33). Once inserted, signal peptides are cleaved by the lumenal peptidases to give rise to the mature protein (67). Coat protein M13 which spans the inner membrane of virus infected bacterial cells is synthesized on polysomes as a precursor protein with an amino terminal signal peptide (11). This precursor inserts spontaneously into the plasma membrane where its signal peptide gets cleaved by the periplasmic signal peptidases leading to the mature form of protein (18)

Signal recognition particle pathway (SRP pathway)

Signal recognition particle machinery coordinates the targeting of secretory proteins or membrane proteins to their proper destinations in living systems and it functions co-translationally. The components of SRP pathway were first identified in mammalian cells in early 1980s (32, 71, 72,) and now this system is known to be present in all domains of life. SRP components initiate the targeting by binding to an N-terminal signal sequence on the emerging nascent polypeptide chain from the ribosomes. This binding step halts translation of the

polypeptide chain in eukaryotes. SRP-ribosome-nascent chain complex then interacts with the SRP receptor (SR) at the membrane in a GTP dependent manner (19, 32, 41). Translation of the nascent chain resumes while the polypeptide is directed into the Sec 61translocase on the endoplasmic reticulum in eukaryotes or into the SecYEG complex in prokaryotes leading to either translocation of the protein to enter into secretory pathway or integration of the protein on plasma membrane in prokaryotes (32). This is followed by the release of SRP and SR from ribosome-nascent chain complex. GTP hydrolysis by SRP and SRP receptor leads to the dissociation of the complex in order to recycle SRP components for next rounds of targeting (32).

Chloroplast SRP targeting (cpSRP) system functions post-translationally. The only known substrates which utilize chloroplast targeting system are the nuclear encoded integral thylakoid membrane proteins called light harvesting chlorophyll binding proteins (LHCPs). LHCPs consist of three transmembrane domains (TM) and they are found associated with photosystem II on the thylakoid membrane. LHCPs are synthesized in the cytoplasm and then imported into the chloroplast via translocation through the TOC and TIC envelope proteins. Once in the stroma, LHCPS are targeted post-translationally via chloroplast SRP machinery to the thylakoid membrane.

Mammalian SRP consists of a ribonucleoprotein which is made up of 7SL RNA and six polypeptides denoted as SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72(71,72, figure 1.3). This ribonucleoprotein complex is divided into two domains-S domain (consists of central portion of RNA together with SRP19, SRP54 and SRP68/72 heterodimer) and Alu domain (consists of heterodimer SRP19/14 and 5' and 3' terminal RNA regions) (23, 53). Bacterial SRP is much smaller in size than mammalian SRP. It consists of a single ribonucleoprotein Ffh

(homologue of mammalian SRP54) in complex with 4.5S RNA in Gram negative bacteria. Gram positive bacteria contain 6S RNA moiety in association with an additional protein HBsu and Ffh (4). In general, bacterial SRP lacks Alu domain found in mammalian SRP. Elongation arrest activity of mammalian SRP is led by its Alu domain (54, 62). SRP54/Ffh bind tightly to their respective RNA moieties and to the signal sequence of the nascent chain complexes.

Chloroplast SRP (cpSRP) is relatively simple in structure when compared to its mammalian or bacterial homologues. It consists of a heterodimer composed of a 54Kda subunit (cpSRP54) which is a homologue of mammalian SRP54 and a unique 43Kda subunit (cpSRP43). cpSRP lacks RNA moiety. Two pools of cpSRP54 have been found in the stroma of chloroplasts, one pool is associated with cpSRP43 and another pool is associated with chloroplast ribosomes (16) and appears to function in co-translation targeting of chloroplast DNA encoded proteins (1, 35). cpSRP43 is composed of three chromodomains (CD), one at the N terminus (CD1) and two at the C terminus (CD2 and CD3). The central region of the molecule is made up of four ankyrin (Ank) repeats (Ank1, Ank2, Ank3 and Ank4) (20, 22, 29). Studies have shown that it's CD2 domain of cpSRP43 which binds cpSRP54 resulting in a heterodimeric cpSRP formation (20, 29, 63, 26). Studies have shown that different domains of cpRP43 exhibit significant dynamics and that the flexibility of cpSRP43 is decreased when it binds to cpSRP54 (17).

SRP54/Ffh/cpSRP54 contain amino terminal four helix bundle N domain packed against a G domain which contains GTP binding site and a C-terminal methionine rich M-domain (74) which is known to interact with SRP RNA or cpSRP43 in case of chloroplasts. A flexible linker connects the two domains (24, 53). SRP54/Ffh is also known to bind the signal sequence of emerging polypeptide chain from ribosomes and in fact both types are shown to bind ribosomes while scanning for the emerging signal sequences via their N domains (53). Crosslinking data

has shown that M-domain of cpSRP54 interacts with the transmembrane domain- TM3 of the substrate LHCP during protein targeting (27).

Next component of SRP system is the SRP receptor (SR) which is found at the membrane of endoplasmic reticulum in mammalian cells (Figure 1.3). It is made up of two subunits SRα and SRβ both of which are GTPases (19, 41, 65). SRβ subunit is found embedded in the membrane. Bacterial SRP receptor is known as FtsY (homologue of SRα) and is found either in the cytoplasm or bound to the inner membrane in bacterial cells (12, 39). Some gram positive bacteria also contain a lipid binding domain in their FtsY (3). SRP receptor in chloroplasts is known as cpFtsY and is found in the stroma or at the thylakoid membrane. cpFtsY also contains a GTP binding domain like FtsY and SRα/SRβ and it binds to the thylakoid membrane via its N-domain (40). SRα is homologous to the *E. coli* and chloroplast FtsY.

In mammalian or bacterial co-translational SRP targeting, SRP directs nascent chain ribosome complex to the Sec translocase at the membrane as mentioned before. In chloroplast post translational SRP targeting, substrate is known to be directed to the Alb-3 translocase found on the thylakoid membrane. Alb-3 translocase belongs to the class of YidC/Oxa1 family of translocase (43)

In chloroplast post translational SRP targeting (Figure 1.4), precursor LHCP enters inside the chloroplast via TOC/TIC translocation pathway. Transit peptide of the precursor form of LHCP is cleaved by the stromal peptidases leading to a mature form of LHCP which then interacts with heterodimeric cpSRP to form a soluble transit complex in stroma. Transit complex maintains substrate LHCP in an integration competent state (52). LHCP binds to ankyrin region of cpSRP43 via an 18 amino acid stretch termed as L-18 and is found between the second and third

transmembrane domain of LHCP (13, 29, 68) and its speculated that it interacts with M-domain of cpSRP54 via its third transmembrane domain.

Transit complex is received at the thylakoid membrane by cpSRP receptor cpFtsY. cpFtsY interacts with N-G domain of cpSRP54 followed by GTP binding by both GTPases. This membrane bound targeting complex is next translocated to the chloroplast translocase Alb-3 which is thought to be responsible for LHCP integration into the thylakoid membrane as antibodies to Alb-3 blocked LHCP integration *in vitro* (44). GTP hydrolysis occurs to release cpSRP and cpFtsY into the stroma. There are still some unknown intricate details about LHCP targeting via cpSRP pathway. For example, what triggers the release of LHCP from the cpSRP/cpFtsy complex at the thylakoid membrane? What is the role of GTP hydrolysis in the LHCP targeting cycle, is it necessary for LHCP integration? What is the sequence of the protein-protein interactions which take place in the stroma or at the thylakoid membrane in order to result in a productive targeting cycle? Following chapters in this dissertation will help in answering these questions about the LHCP targeting pathway.
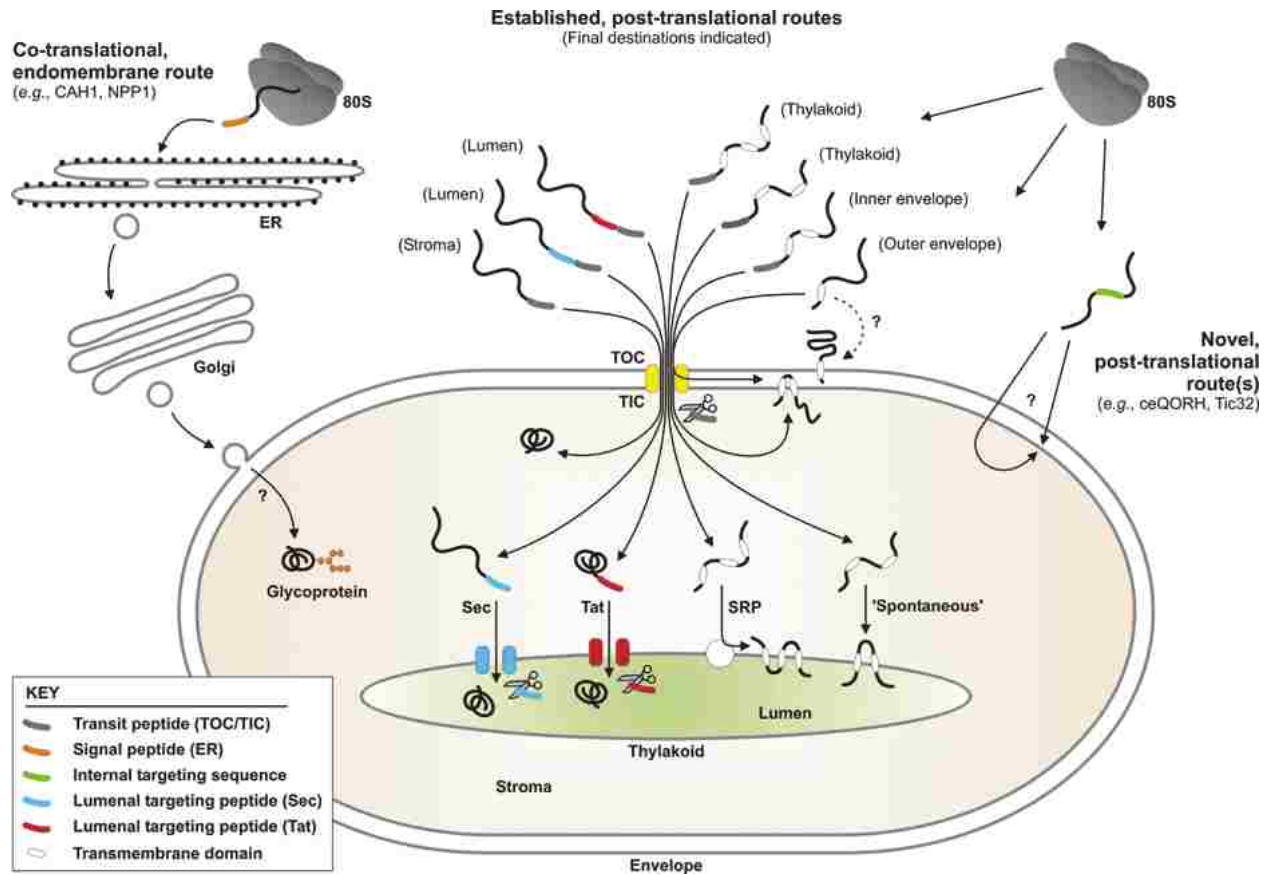
Figure 1.1: Overview of nucleus encoded chloroplast protein targeting pathways (28)

This figure represents various routes that the nuclear encoded chloroplast proteins can take based on their N-terminus targeting signals. Once inside the chloroplast, their transit peptides are cleaved off by the stromal peptidases and then protein can use one of four types of signaling pathways which will lead to the delivery of proteins either in the stroma, or on the thylakoid membrane or inside the lumen of thylakoid
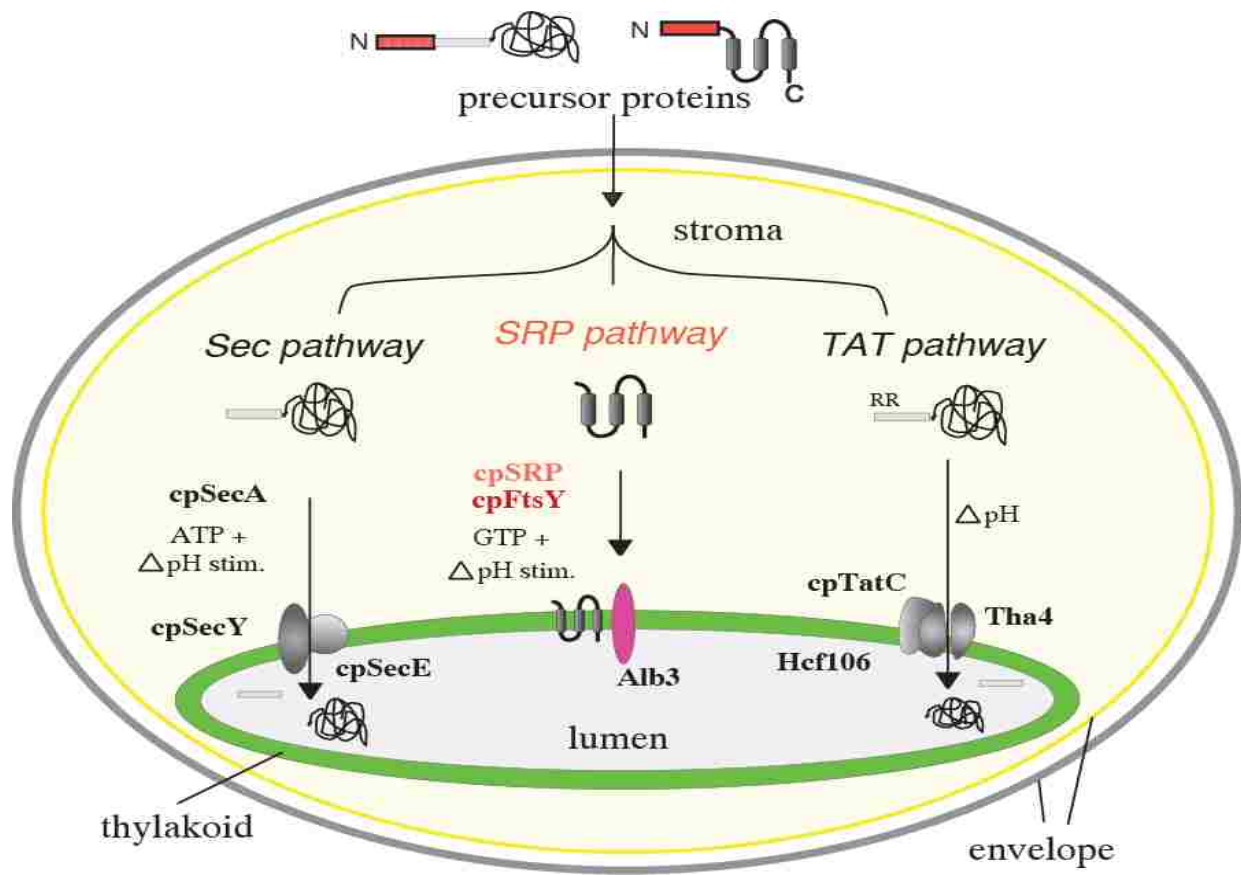
Figure 1.2: Detailed representation of the components of chloroplast protein targeting pathways

This figure represents various pathways that the nuclear encoded chloroplast proteins or chloroplast encoded proteins can utilize to undergo proper localization inside the chloroplasts.
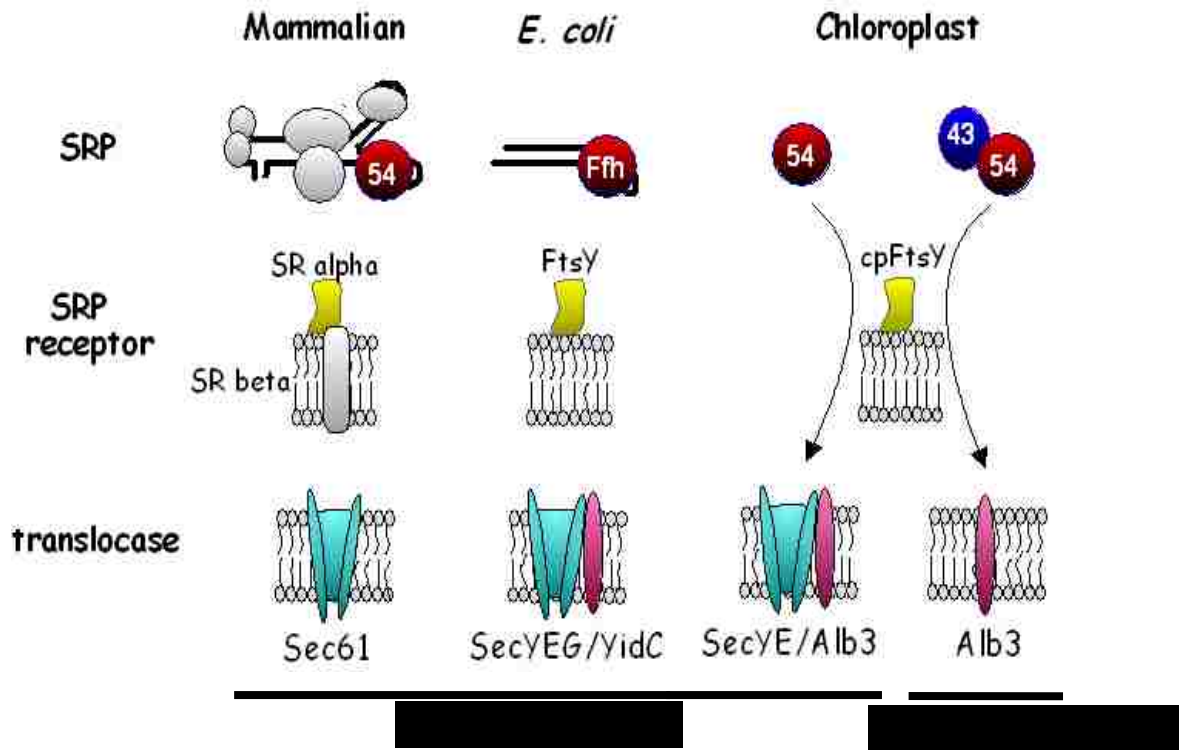
Figure 1.3: Components of signal recognition particle pathway

Components of the mammalian, *E. coli*, and chloroplast SRP systems are shown in the figure. Mammalian and bacterial SRPs contain an RNA moiety, while chloroplast SRP lacks RNA moiety and contains a unique 43-kDa protein subunit. A homologous SRP receptor protein is found in all organisms. Sec translocase is utilized by mammals and *E. coli*, while *E. coli* and chloroplast have homologous insertase proteins Yid C and Alb-3.
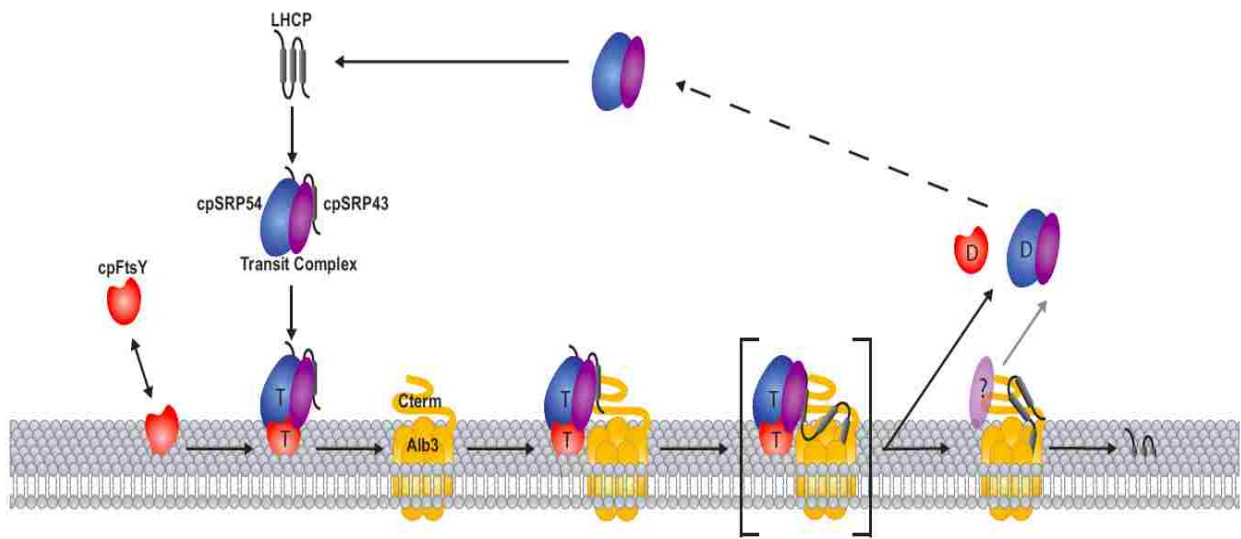
Figure 1.4: Current model of LHCP targeting via cpSRP pathway

LHCP forms a soluble transit complex with chloroplast signal recognition particle (cpSRP) inside the chloroplast stroma. This complex then interacts in a GTP bound state with cpFtsY GTPase at the membrane. This GTP bound complex targets the substrate LHCP to Alb-3 insertase at the membrane leading to the release and insertion of LHCP into the membrane.

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II. A STUDY TO DETERMINE THE BINDING SITE OF C-TERMINUS OF ALBINO 3 ON CHLOROPLAST SIGNAL RECOGNITION PARTICLE 43 (CPSRP43)

ABSTRACT

cpSRP43 is a signal recognition particle (SRP) protein found in post-translational targeting of light harvesting chlorophyll binding proteins (LHCP) in chloroplasts (23). It serves as an interaction bridge between the cpSRP targeting complex and the Alb-3 insertase to help coordinate insertion of LHCP into the thylakoid membrane. It is known that in co-translational targeting, ribosome interacts with both the targeting machinery and the insertase during peptide synthesis and insertion (21). Since the post-translational LHCP-SRP targeting system lacks a ribosomal component, it is speculated that cpSRP43 communicates with the Alb-3 insertase to carry out LHCP targeting events at the membrane, similar to the role of ribosome in co-translational targeting. It is known that cpSRP43 is comprised of three chromodomains and a four-ankyrin repeat region (15, 24). Further, it has been established that the C-terminal region of Alb3 binds to cpSRP43 (17). Based on these data, we hypothesize that negatively charged residues found in the ankyrin repeats of cpSRP43 are the physical sites which bind the C-terminus of the Alb-3 insertase. In order to test this hypothesis, we created six double point mutants of cpSRP43 where for each double point mutant, two negatively charged amino acids in the ankyrin region were converted to lysine or glycine. These double point mutants were examined for their ability to bind the substrate and to interact with Alb-3 insertase. Results showed that all of the mutants retained their ability to interact with the insertase but interestingly, three of the six mutants lost their ability to form soluble transit complex which is the first step in SRP dependent LHCP targeting pathway.

INTRODUCTION

Signal recognition particle (SRP) dependent protein targeting is present in all domains of life. It functions co-translationally in prokaryotes and eukaryotes leading to the delivery of nascent polypeptide chain to its target destination which could be an organellar membrane or the lumen of an organelle. Signal recognition particle (SRP) dependent targeting has also been found to target light harvesting chlorophyll binding proteins (LHCP) via an exclusive post-translational mechanism in chloroplasts. LHCPs are encoded by nuclear DNA yet function as integral thylakoid membrane protein complexes, i.e. the antenna complex for chloroplast photosystems I and II (8).

Chloroplast signal recognition particle (cpSRP) consists of a conserved 54KDa protein and a unique 43 KDa protein. CpSRP54 consists of a GTP binding domain at its amino terminal and a methionine rich M domain at its carboxyl terminal (10). CpSRP54 exists in two pools in the stroma of chloroplasts. One pool is associated with ribosomes and is involved in co-translational protein targeting. The second pool is associated with cpSRP43 and is involved in post-translational targeting. This second pool, comprised of cpSPR43 and cpSRP54, termed cpSRP is unique among signal recognition particles (SRPs) because it lacks an RNA moiety. CpSRP43 consists of an N-terminal chromodomain linked to four ankyrin repeat domains followed by chromodomain 2 and chromodomain 3 (Figure 2.1). CpSRP54 has been shown to interact with chromodomain 2 of cpSRP43 via its M-domain (12). CpSRP interacts with its receptor cpFtsY at the thylakoid membrane. Both cpSRP54 and cpFtsY are GTPases. Previous studies have shown that precursor form of LHCP (with an N-terminal signal peptide) enters the chloroplast where it undergoes processing by proteases to give rise to the mature form of LHCP, now lacking chloroplast signal peptide (16,22). Mature LHCP is then received by cpSRP in the stroma where

they form a soluble transit complex which then interacts with cpFtsY at the thylakoid membrane (via cpSRP54). This transit complex then interacts with Alb-3 insertase at the thylakoid membrane, leading to the release and insertion of LHCP into the membrane. Subsequent GTP hydrolysis by cpSRP54 and cpFtsY leads to the release of cpSRP and cpFtsY back into the stroma.

Alb-3 has been shown to be indispensable in LHCP targeting, antibodies to Alb-3 protein inhibit LHCP integration into the thylakoid membranes (19). The Alb-3 insertase belongs to YidC/Oxa1/Alb3 family of membrane protein translocases conserved in bacteria, mitochondria and chloroplasts respectively. Alb-3 is an integral membrane protein found in the thylakoid membrane of chloroplasts in green plant as well as in the thylakoid membranes of *Chlamydomonas reinhardtii*. In addition to Alb-3, a homologue, Alb-4, has been identified in *Arabidopsis thaliana* (29). Alb-4 shows 55% sequence similarity to Alb-3 and both are integral membrane proteins having five transmembrane domains (11). Analysis of Arabidopsis Alb-4 knock out mutants showed defective assembly of CF1CF0 ATPase complex, a thylakoid membrane protein complex, and reduction in ATP synthesis compared to the wild type plants (8). Alb-3 knock out Arabidopsis mutant plants produced yellow to white colored leaves when grown on carbon source containing media. These mutants lacked chlorophyll content, had fewer thylakoid membranes, were unable to grow in soil, and lacked proper thylakoid membrane biogenesis (8, 25). Crosslinking studies have shown that Alb-3 is associated with cpSecY, another thylakoid membrane protein, suggesting a role for Alb-3/cpSecY in co-translational protein targeting of subunits of chloroplast photosystem II protein (19, 27).

In recent years, numerous studies have been undertaken to understand the role of Alb-3 in post-translational LHCP protein via the chloroplast signal recognition particle (cpSRP) pathway (13,

14). It has been shown that Alb-3 interacts with cpSRP43 in order to release LHCP from the complex at the membrane (26) leading to LHCP integration into the membrane. This interaction is supported by the fact that cpSRP43 loses its ability to bind protease treated thylakoids (17) where the C-terminus portion of Alb-3 has been removed by protease. Also, we have data from previous studies which proves that C-term peptide has an affinity for cpSRP43-ankyrin region in nano-molar range whereas the same peptide does not show significant affinity for the chromodomain regions of cpSRP43 (17). It doesn't show any affinity for cpSRP54 or cpFtsY as well.

It has also been shown in protein binding assays that cpSRP43 is the key component involved in the interaction with translocase Alb-3 in thylakoids as it causes the release of LHCP from the cpSRP complex (17). Three dimensional structure of cpSRP43 shows negatively charged amino acid residues in its ankyrin repeats (Figure 2.1) and isothermal titration calorimetry (ITC) data supports the fact that ankyrin regions in cpSRP43 have affinity for Alb-3-C-terminus (Alb-3-Cterm) with nano-molar affinity (17). Also, multiple sequence alignments of Alb-3 shows the presence of four conserved regions augmented with positively charged residues on the C-terminus portion of Alb-3 that extends into the stroma (9).

Based on all the supporting data, it was hypothesized that it is the acidic patch found in the ankyrin regions on cpSRP43 which could be a potential site for the binding of C-terminus portion of Alb-3 insertase. If this step is understood, it will help in understanding the sequence of events which take place at the thylakoid membrane in order to release LHCP from the cpSRP/SR complex.

MATERIALS AND METHODS

All reagents and enzymes were purchased commercially. All DNA constructs were sequence verified by the Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock. Recombinant protein concentrations were determined by analyzing coomassie blue stained SDS PAGE gels on Alpha Innotech FluorChem IS-8900 using Alpha Ease FC Stand Alone software (Alpha Innotech).

<u>Construction of GST-cpSRP43 and mcpSRP43 Proteins</u>

Recombinant, purified GST-cpSRP43 and cpSRP43 were prepared as described previously (12, 28). Briefly, the coding sequence for mcpSRP43 from pGEM-pcpSRP43 was cloned into BamHI and EcoRI sites of pGEX-6P-2 (28). Expression plasmid was then transformed into *E.coli* BL21 star. Cells were cultured in Luria- Bertani (LB) medium, induced with 0.5mM isopropyl β–D-1-thiogalactopyranoside (IPTG) resulting in the expression of GST-mcpSP43 (28). Cell pellets were lysed, filtered, and then purified over a Glutathione-Sepharose column (GE Healthcare) and GST-mcpSRP43 was eluted in Glutathione elution buffer (20mM Glutathione, 100mM Tris, pH 8, 120mM NaCl). GST-mcpSRP43 in Glutathione elution buffer was desalted into HKM buffer (10mM HEPES-KOH, pH 8, 10mM $MgCl_2$) and stored at minus 80 degrees celsius.

For production of recombinant mcpSRP43, eluted GST-mcpSRP43 in Glutathione elution buffer as mentioned above, was desalted into 50mM Tris-HCl, 150mMNaCl, 1mM EDTA, 1mM dithiothreitol, pH 7 and incubated overnight with PreScission Protease^{TM} at 4 degrees celsius to cleave the GST tag (12). Cleaved cpSRP43 along with GST tag was then desalted into phosphate buffer saline and then purified over Glutathione-Sepharose column to elute cpSRP43 and to remove cleaved GST tag as well as the PreScission Protease. mcpSRP43, found in the flow-

through of the Glutathione-Sepharose column, was further desalted into HKM buffer and stored at minus 80 degrees celsius.

Construction of cpSRP43 Mutant Proteins

All six cpSRP43 mutant proteins as listed in table 2.1, Mutant 1 (AGA<u>GL</u>G<u>H</u>LD), Mutant 2 (LGA<u>GIG</u>VED), Mutant 3 (EDR<u>GV</u>G<u>A</u>VD), Mutant 4 (AGA<u>KL</u>K<u>H</u>LD), Mutant 5 (LGA<u>KIK</u>VED) and Mutant 6 (EDR<u>KV</u>K<u>A</u>VD) were full-length cpSRP43 proteins (GenBank accession number AAD01509) where in two acidic charges in the protein sequence were mutated into two basic charges (to lysine) or to glycine. For example Mutant 1 protein corresponds to mcpSRP43 D189G D191G which means that aspartic acid residues at positions 189 and 191 were mutated to glycine. See table 2.1 for other mutant descriptions. Briefly, primer-encoded point mutations were introduced into the coding sequence of mature cpSRP43 (starting with amino acid sequence AAVQRN) by PCR. The resulting product was cloned into pGEX-6P-2 (GE Healthcare) using BamHI and EcoRI sites. All constructs were sequence-verified by Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR. Each plasmid was then transformed into BL21 Star for isopropyl β–D-1-thiogalactopyranoside (IPTG) induced expression followed by affinity purification and tag cleavage as described above. Both GST tagged and cleaved versions of each mutant proteins were made. The concentration of purified protein was estimated by coomassie blue staining using bovine serum albumin (BSA) as a standard.

Construction of cpSRP54-His Protein

Recombinant cpSRP54-His was expressed and purified as described previously (28). Briefly, the coding sequence for mcpSRP54 was amplified from pNH2 (10) using a reverse primer that introduced six histidines at the C-terminus and the resulting PCR product was cloned into pPROLar.A122 using KpnI and HindIII sites to generate pPROLar-cpSRP54-His. Expression plasmid was then transformed into *E.coli* strain BL21 star and cells were cultured in LB medium, induced with 0.5mM isopropyl β–D-1-thiogalactopyranoside (IPTG) plus 0.2% arabinose resulting in the expression of cpSRP54-His (28). Cell pellets were lysed, filtered and then purified over Talon metal affinity resin to elute mcpSRP54-His in Talon elution buffer (50mM Na_2HPO_4, 0.3M NaCl, 150mM imidazole, pH 7). The eluted protein was desalted into 2XHKM + 200mM KCl. Eluted protein was diluted with an equal volume of glycerol prior to being aliquoted and stored at minus 80 degrees celsius.

Construction of cpFtsY Protein

Recombinant cpFtsY was expressed and purified as previously (28). Briefly, the coding sequence was amplified from pcpFtsY4Z and cloned into pET-32b (+) using NcoI and HindIII sites. The resulting clone expressed a thioredoxin (Trx) fusion protein Trx-His-Stag-cpFtsY. Expression plasmid was then transformed into *E.coli* BL21 star and cells were cultured in Luria-Bertani (LB) medium, induced with 0.5mM isopropyl β–D-1-thiogalactopyranoside (IPTG). Cell pellets were lysed, filtered and then purified over Talon metal affinity resin to elute Trx-His-Stag-cpFtsY in Talon elution buffer (50mM Na_2HPO_4, 0.3M NaCl, 150mM imidazole, pH 7). The eluted protein was desalted into 2XHKM + 200mM KCl. Eluted protein was diluted with an equal volume of glycerol prior to storage at minus 20 degrees celsius.

Construction of His-Stag-Alb-3-Cterm

The coding sequence for Alb-3-Cterm was amplified by PCR from His-FLAG-Alb3-Cterm-pQE-80L(17) with a reverse primer designed to match the C-terminus of Alb-3-Cterm and a forward primer designed to replace the FLAG tag (DYKDDDDK) with an S tag (KETAAAKFERQHMDS) resulting in a construct with a His6 tag, Ser-Ala linker, S tag, thrombin cleavage site, and the 124-amino acid segment of Alb-3 beginning at NNVLSTA and ending at SKRKPVA. This plasmid, referred to as His-Stag-Alb-3-Cterm-pQE-80L, was transformed into BL21 star. Bacterial cells were cultured in LB medium and induced with 1mM isopropyl β–D-1-thiogalactopyranoside (IPTG). Cell pellets were lysed, filtered and then purified over Talon metal affinity resin to elute His-Stag-Alb-3-Cterm in Talon elution buffer (50mM Na_2HPO_4, 0.3M NaCl, 150mM imidazole, pH 7). The eluted protein was desalted into HKM + 100mM KCl and stored at minus 80 degrees celsius in small aliquots (17).

Preparation of Chloroplasts

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 previously described (6). Chlorophyll (Chl) content was determined as described previously (2). Thylakoids were salt-washed (SW) two times with 1M potassium acetate in import buffer (IB: 50mM hepes-KOH, pH 8, 0.33M sorbitol) and then washed two times with import buffer containing 10mM $MgCl_2$ (IBM) as described previously (17). Thylakoids were resuspended at 1mg/ml chlorophyll in IBM buffer prior to use.

Preparation of Radiolabeled Precursors for in vitro Transcription and Translation

The plasmid used for *in vitro* transcription/translation of pLHCP (psAB80XD/4) has been described previously (5). L18 refers to the amino acid sequence of pLHCP starting form 189th amino acid up to 206th amino acid (VDPLYPGGSFDPLGLADD) and PPL is the endoplasmic reticulum-targeted protein preprolactin (7). The chimeric protein L18-PPL was produced as an in vitro transcribed/translated construct as described previously(7).

mcpFtsY Translated Product

Forward and reverse primers were designed to match the mature coding sequence of *A. thaliana* cpFtsY starting with the predicted mature sequence CSAGPSGF and including 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 as previously described (18).

M-domain of cpSRP54 Translated Product

The amino acid sequence for the M-domain of cpSRP54 begins with MGDVLS and ends with GSGN. The nucleotides coding for these amino acids were amplified from pNH2 (10) using XbaI and SmaI sites to create pGEM-3Z-Mdomain-cpSRP54 for *in vitro* transcription/translation (12).

In vitro transcribed and capped mRNA was translated in the presence of [^{35}S] methionine (4) using a wheat germ system to produce radiolabeled proteins (6). Precursor LHCP translation

product (TP), mcpFtsY translation product (TP), L18-PPL translated product(TP), M-domain of cpSRP54 TP were diluted with equal volume of 6mM unlabeled methionine in IB prior to use. Constructs were quantified by comparing the [^{35}S] signal from a given protein band as analyzed by SDS-PAGE and phosphor imaging (17).

Pull down Assay

GST tagged versions of cpSRP43 and mutants 1-6 (see table 2.1) were used in pull down assays. For pull-down assays involving recombinant proteins, 1000pmoles of GST-mcpSRP43 or mutant GST-cpSRP43 were incubated for 15 minutes at room temperature with 1500pmoles of Alb-3-Cterm or 1000pmoles of mcpSRP54-His in pull down buffer (10mM HEPES-KOH, pH 8.0, 50mM KOAc and 10mM MgCl$_2$) in a total reaction volume of 70µl. For pull-down assays involving *in vitro* transcribed and translated proteins, 200pmoles of GST-43s were incubated with 25µl of the translated products in a total reaction volume of 200µl. Next, 25µl or 70µl of 50% Glutathione sepharose slurry in HKM was added into each reaction, respectively and incubated by gently shaking for 30 minutes at room temperature. After incubation, beads were washed with wash buffer (20mM hepes-KOH, pH 8, 300mM KCl, 10mM MgCl$_2$ and 2% Tween20) two times, followed by a final wash with HKM. Co-precipitating proteins were eluted in 50µl of solubilization buffer (mostly comprised of Tris, Sodium dodecyl sulfate, β-mercaptoethanol, glycerol and water) and samples were analyzed by 12.5% SDS PAGE to separate the eluted proteins. Gels were visualized using coomassie blue staining and phosphor imaging as described below in Sample Analysis section.

Transit Complex Assay

Transit complex assays (TC) were performed as described in Payan and Cline, 1991 (20) with the following modifications. Transit complex was made by mixing 1μg (~25pmoles) of each cpSRP or 5μl of Stromal extract (4X), 10μl of 1:2 diluted *in vitro* translated radiolabeled pLHCP translation product (TP) and HKM in a total volume of 60μl. Proteins were incubated together at 26 degrees celsius for 30 minutes followed by centrifugation at 70,000 x g for 1 hour at 4 degrees celsius to remove aggregated pLHCP. The top 30μl of each centrifuged reaction was diluted with 10μl of 50% glycerol and loaded on 6% Native gel as described previously (17).

Integration Assay

Integration assays were carried out as described in Cline et al., 1993 (6) with minor modifications. Salt washed thylakoids equal to 25μg of chlorophyll, 1μg of cpFtsY, 1mM (final) GTP, 1μg of cpSRP54, 1μg cpSRP43, and 12.5μl of radiolabeled pLHCP translated product (TP) were added to IBM to make a total volume of 75μl. Samples were incubated at 26 degrees celsius for 30 minutes in the presence of light. Thylakoids were centrifuged at 4100 x g for 8 minutes at 6 degrees celsius, supernatant removed, and thylakoids resuspended in IB and treated with 12.5μl of thermolysin (2mg/ml thermolysin stock in 10mM $CaCl_2$) on ice for one hour. To stop the reaction, 100μl of 50mM EDTA in IB was added to thermolysin treated thylakoids followed by centrifugation at 4100 x g for 8 minutes at 6 degrees celsius. Pelleted thylakoids were solubilized using SDS buffer, heated, and analyzed by SDS-PAGE and phosphor imaging.

Sample Analysis

SDS PAGE and native gels were imaged using Typhoon FLA 9500 (GE Healthcare Life Sciences). OptiQuant software (GE Healthcare Life Sciences) was used to quantify signal from radiolabeled protein. All experiments were performed in triplicate. Bar graphs were generated using Microsoft Excel 2013. Error bars represent standard error of the mean (SEM). Protein concentrations were determined by analyzing coomassie blue stained SDS PAGE gels on Alpha Innotech FluorChem IS-8900 using Alpha Ease FC Stand Alone software (Alpha Innotech) as compared to bovine serum albumin (BSA) standards.

RESULTS

Mutants cpSRP43 D189G D191G, cpSRP43 D223G E225G and cpSRP43 D223K E225K Form Soluble Transit Complex

mpSRP43 D156G D158G (Mutant 3), mcpSRP43 D189K D191K (Mutant 4) and mcpSRP43 D156K D158K (Mutant 6) did not form transit complex. The remaining mutants- mcpSRP43D 189G D191G (Mutant 1), mcpSRP43 D223G E225G (Mutant 2) and mcpSRP43 D223K E225K (Mutant 5) did form transit complex (Figure 2.2). All six mutants were produced with the idea that the interaction between Alb-3 insertase and cpSRP43 could possibly be hampered by targeting the insertase binding/reacting site on cpSRP43. These mutations in cpSRP43 directly modified the predicted binding site. The designed cpSRP43 mutants were hypothesized to retain their ability to interact with cpSRP54 and LHCP. In other words, all of the mutants were expected to form heterodimeric cpSRP complex as well as transit complex. However, results showed that half of the mutants i.e. mutants 3, 4 & 6 lost the ability to form a soluble transit complex, thereby eliminating their ability to complete the LHCP insertion pathway.

It is known that transit complex is a trimeric complex where cpSRP43 and cpSRP54 bind LHCP. LHCP binds to the ankyrin region of cpSRP43 via an 18 amino acids long stretch that occurs before the third transmembrane domain (7, 17). CpSRP54 is known to bind chromodomain 2 (CD2) of cpSRP43 via its M domain (12). Mutations in the ankyrin region of cpSRP43, specifically at positions 156/158 and 189/191 somehow effected either interaction with cpSRP54 or with LHCP. In these mutants, aspartic acid residues were converted to either lysine or to glycine residues and these changes in the charged amino acids might have disturbed the electrostatic interactions between the binding partners in trimeric complex. In order to further

examine this, all six mutants were tested in pull down assays to assess their interaction with the individual components involved in transit complex formation.

All cpSRP43 Mutant Proteins Co-precipitate L18-PPL, M-domain of cpSRP54 and cpFtsY

The M-domain of cpSRP54 was used instead of full length cpSR54 as previously established (12). Similarly, the L18 region of LHCP, fused to the carrier protein PPL was used instead of full length LHCP as previously described (7). Data shows that all mutants were able to pull down the M-domain of cpSRP54 as well as L18 region of LHCP when tested in separate assays (Figure 2.4). Therefore, this showed that all interacting domains were able to bind each other individually but that the trimeric complex was not able to form. This finding suggests that there may be additional, as of yet unidentified, interactions involved in the transit complex formation.

All cpSRP43 Mutant Proteins Retain their Ability to Bind Alb-3-Cterm

Three dimensional modeling of cpSRP43 protein suggests that the all six mutants should be able to interact with cpFtsY in the quaternary complex comprised of cpSRP43-cpSRP54-LHCP-cpFtsY. Formation of this complex is viewed as a critical step prior to transfer of the LCHP cargo to the insertase at the thylakoid membrane. In order to check this, GST tagged versions of the cpSRP43 mutants were used to pull down cpFtsY (Figure 2.4). The data showed that all GST-cpSRP43 mutants pulled down cpFtsY at a level comparable to wild-type GST-cpSRP43. Importantly, even the mutants that were unable to form transit complex were still able to interact with cpFtsY (Figure 2.2 and 2.4).

Mutants cpSRP43D189GD191G, cpSRP43D223GE225G, cpSRP43D223KE225K Support

LHCP Integration

Next step was to check the ability of these mutants to integrate LHCP into the thylakoid

membrane. Results showed that mcpSRP43 D189G D191G (Mutant 1), mcpSRP43 D223G

E225G (Mutant 2) and mcpSRP43 D223K E225K (Mutant 5) were efficient in LHCP integration

whereas mpSRP43 D156G D158G (Mutant 3), mcpSRP43 D189K D191K (Mutant 4) and

mcpSRP43 D156K D158K (Mutant 6) showed reduced or no integration (Figure 2.3). LHCP

targeting is considered to be vectorial, a step by step method of post translational targeting where

the first step is to form transit complex and then transit complex interacts with cpFtsY at the

membrane and then this quaternary complex interacts with C-terminus of Alb-3 insertase leading

to release and insertion of LHCP. Hence, it makes sense that mutants which failed in the first

step of this targeting pathway (i.e. transit complex formation) could not lead to a successful

round of LHCP targeting. Therefore, mutants 3, 4 and 6 which did not form transit complex, lost

their ability or showed reduced ability to integrate LHCP into the thylakoid membranes. Mutants

1, 2 and 5 which formed transit complex, also proved efficient in LHCP integration into the

membranes (Figure 2.3). The hypothesis of this study was that these mutant versions of cpSRP43

would not interact with C-terminus of the Alb3 insertase at the membrane and hence there would

be no insertion of LHCP into the membrane. However, results showed that the mutants which

supported transit complex formation also supported LHCP integration. In order to further support

this result, we did a pull down assay using GST tagged cpSRP43 and mutants with the C-

terminus portion of the Alb-3 insertase. Pull down assay confirmed that all GST-cpSRP43

mutants were able to interact with C-terminus of insertase similar to wild-type GST-cpSRP43

(Figure 2.5). Hence, the mutations that were made in cpSRP43 were not sufficient to interrupt

cpSRP43 and Alb-3 insertase interaction.

DISCUSSION

In co-translational targeting of cytosolic proteins either to the bacterial plasma membrane or to the endoplasmic reticulum membrane in eukaryotes, signal recognition particle (SRP) system serves as a tool to carry the nascent ribosome chain complex to SecYEG/Sec61 translocase/insertase at the membrane which leads to the release/insertion of the protein into the lumen or into the membrane (1). Co-translational protein targeting machinery includes a translating ribosome, nascent polypeptide chain, SRP54 with RNA moiety, SRP receptor and a translocase. All these components together orchestrate successful protein delivery either inside the organelle or into the membrane.

SRP targeting system has also been observed in chloroplasts of higher plants where it comprises of a heterodimeric chloroplast SRP component (cpSRP54 and cpSRP43), cpSRP receptor (cpFtsY) and an insertase (Alb-3). Chloroplast SRP targeting machinery is unique in the sense that it lacks ribosomes, lacks RNA moiety, works post-translationally and has introduced a novel component-cpSRP43 into the targeting system. Only known substrate which utilizes this novel cpSRP targeting system is the light harvesting chlorophyll binding protein (LHCP). LHCP proteins are nuclear encoded and constitute about 50% of the protein bulk in thylakoids (26). Previous studies have shown that cpSRP43 and cpSRP54 bind to the imported LHCP in stroma and direct it to the thylakoid membrane in order to form cpSRP/LHCP/cpFtsY/Alb-3 complex. This targeting complex then undergoes a series of sequential arrangements leading to the release of cpSRP components from each other and the insertion of LHCP into the membrane. What triggers the release of LHCP at the membrane from the cpSRP component is unknown.

In co-translational targeting, ribosomes sense the presence of translocase and this leads to the release of polypeptide chain from the ribosomes into the translocase. Since cpSRP system lacks

ribosomes, so how does the targeting complex that has been loaded with the cargo (LHCP) sense the availability of Alb-3 translocase in order to release cargo at the membrane? Previous work proves that it is cpSRP43 in the targeting complex which communicates with Alb-3 in order to cause the subsequent release and insertion of LHCP into the thylakoid membrane. (17, 26). Ankyrin regions in cpSRP43 have been shown to be the binding site of C-terminus of Alb-3 insertase on cpSRP43. Based on structural information of cpSRP43, the ankyrin regions carry lot of negatively charged residues which we thought could be a potential site to interact with the positively charged residues found on C-terminus of Alb-3. Therefore, this study is designed to see if the interaction with C-terminus of Alb-3 can be lost when the negative charge on cpSR43 is replaced with positive charge or by neutralizing the negative charge on cpSRP43.

Results here have not supported the hypothesis because none of the cpSRP43 mutants which we designed lost their interaction with Alb-3. This can be explained based on the fact that may be more than two point mutations are needed to see the effect. However, it is interesting to note that some of the cpSRP43 mutants lost their ability to form transit complex when all three proteins (cpSRP43, cpSRP54 and LHCP) that are required to form transit complex were present in solution. It could mean that some other possible interactions are involved, may be between cpSRP54 and LHCP, which are necessary to hold LHCP in a soluble state in solution. The study also shows that cpSRP43 has an affinity for cpFtsY in solution even in the absence of cpSRP54.

Overall, this piece of information provides an insight into the new types of interactions which may take place in this overall unique system of protein targeting especially in the transit complex formation step in the pathway. In future, a different approach can be used to answer this question. Example includes inter-molecular FRET (Forster resonance energy transfer) technique where two dye molecules are placed on different proteins in order to understand relative

movements of proteins when present as a complex in solution and this can be narrowed down to

understand the specific sites involved in protein-protein interaction.

Mutants	Description
Mutant 1	cpSRP43 D189G D191G
Mutant 2	cpSRP43 D223G E225G
Mutant 3	cpSRP43 D156G D158G
Mutant 4	cpSRP43 D189K D191K
Mutant 5	cpSRP43 D223K E225K
Mutant 6	cpSRP43 D156K D158K

Table 2.1: Description of cpSRP43 mutants used in the study
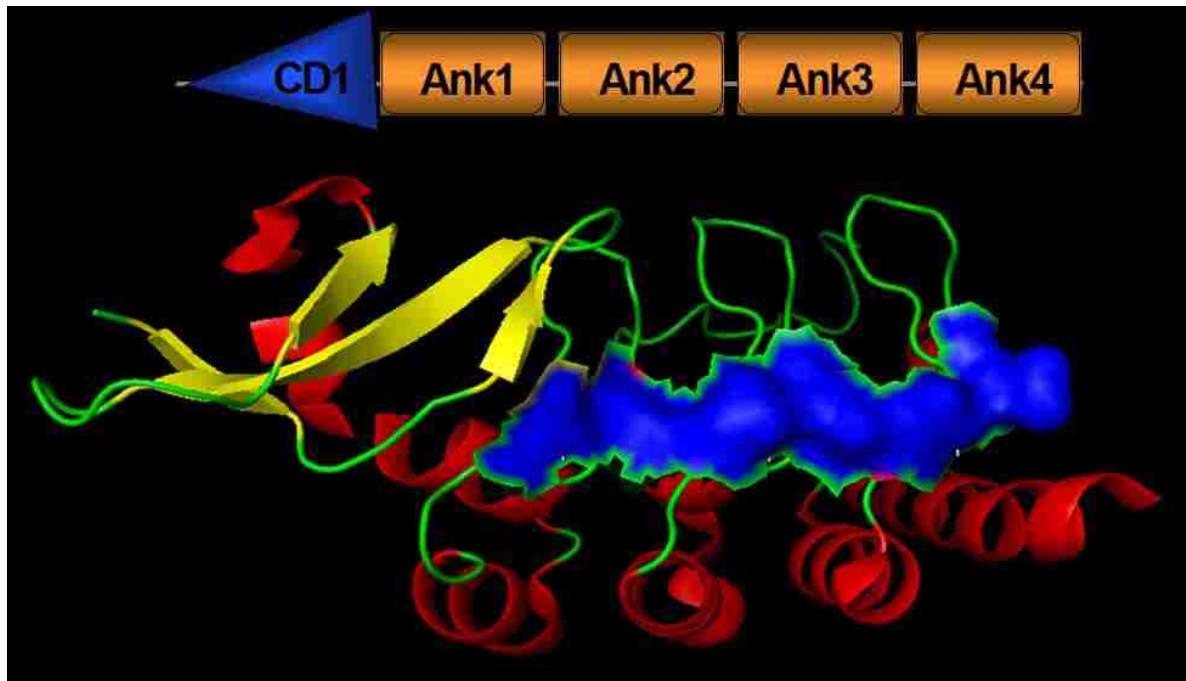
Figure 2.1: Three dimensional structure of cpSRP43

The figure here represents acidic patch (in blue) located on the ankyrin repeats (Ank 1-4) of cpSRP43. Ankyrin repeats are shown in red color and they are connected to each other via loops (in green), yellow color represents chromodomain1 (CD1) of cpSRP43.
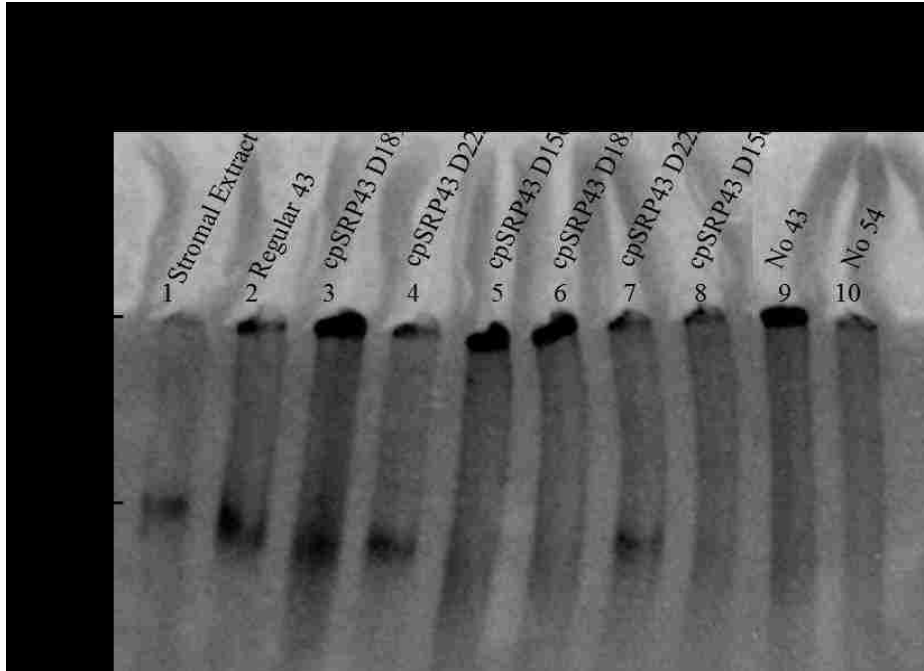
Figure. 2.2: Mutants cpSRP43 D189G D191G, cpSRP43 D223G E225G and cpSRP43D223K E225KK E225G/K form transit complex

Transit complex assay was performed using 1µg of recombinant cpSRP proteins and 10ul of 1:2 diluted *in vitro* translated radiolabeled LHCP. Lane 1, 2, 9 & 10 are the control lanes where lane 1 represents transit complex formed using stromal extract, lane 2 represents transit complex formed using wild type cpSRP43 and wild type cpSRP54, lanes 9 represents transit complex formed using wild type cpSRP54 and LHCP, lane 10 represents transit complex formed using wild type cpSRP43 and LHCP. Lanes 3-8 represent transit complex formed using mutant cpSRP43 proteins and wild type cpSRP54. Transit complex was examined using native page and phosphor imaging. Only one set of mutants (both lysine and glycine versions, lanes 4 & 7 using cpSRP43 D223G E225G and cpSRP43 D223K E225K) showed transit complex formation. cpSRP43 D189G D191G retained its transit complex forming ability when aspartic acid sites were mutated to glycines but lost its ability when same amino acids were switched with lysines cpSRP43 D189K D191K). CpSRP43 D156G D158G and cpSRP43 D156K D158K however, did not form transit complex at all. This experiment was repeated three times.

A.

B.

Integration Assay Quantification

(y-axis) % Integration relative to integration done using regular cpSRP43

(x-axis) Regular cpSRP43, cpSRP43 D189G D191G, cpSRP43 D223G E225G, cpSRP43 D156G D158G, cpSRP43 D189K D191K, cpSRP43 D223K E225K, cpSRP43 D156K D158K
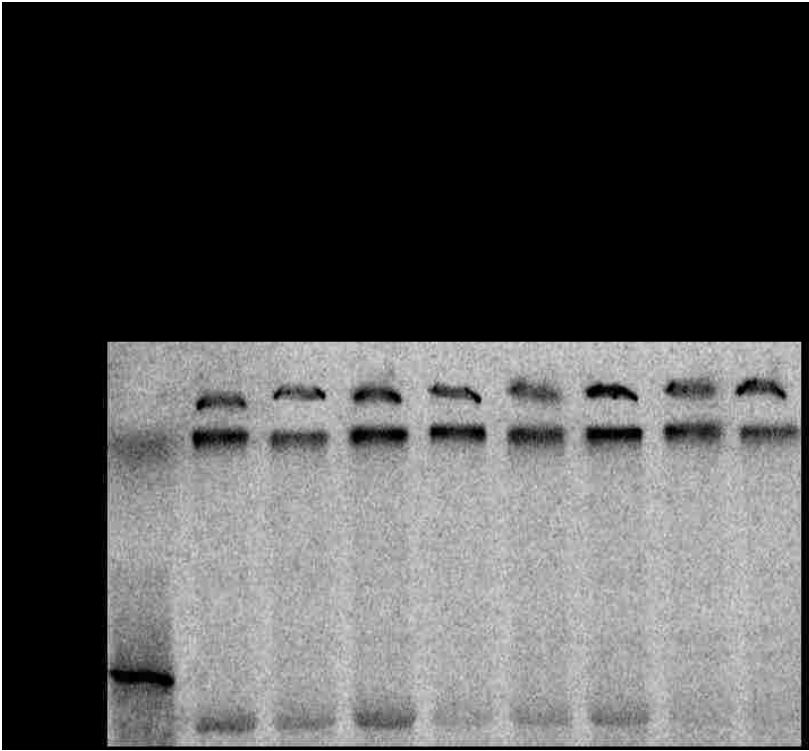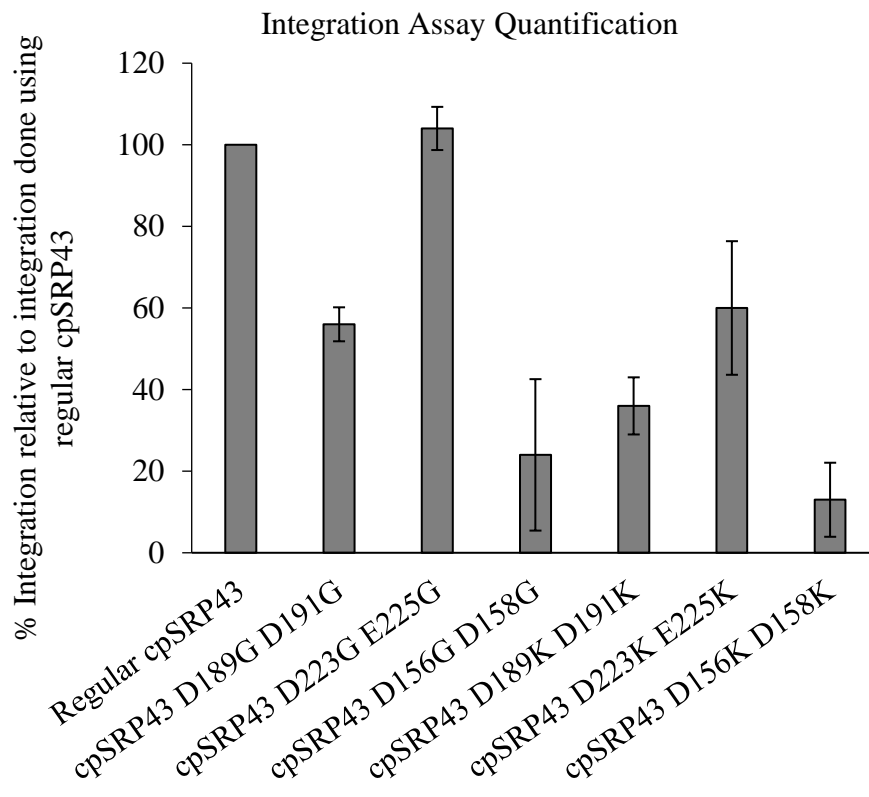
Figure 2.3: Mutants cpSRP43 D189G D191G, cpSRP43 D223G E225G, cpSRP43 D223K E225K support LHCP integration.

A. Seventy five microliters integration assays were performed using 1µg of cpSRP43 or cpSRP43 mutants as indicated, 25µg of salt washed thylakoids, 1µg of recombinant wild type cpSRP54, 1µg of recombinant wild type cpFtsY, 1mM GTP, and 12.5µl of 1:2 diluted *in vitro* translated radiolabeled pLHCP. Integrated LHCP represented as DP (degradation product) was analyzed using SDS PAGE and phosphor imaging. The experiment was repeated three times and quantified using Optiquant software.

B. Graph shows the percentage of LHCP integration relative to integration of LHCP using wild type cpSRP43 (Lane 2 on the gel). Control lane 9 from (A) represents integration lacking cpFtsY and serves as a background control for the quantifications. Data shows that cpSR43 D223G E225G and cpSRP43 D223K E225K are efficient in LHCP integration which is consistent with the transit complex data (Figure 2.2). CpSRP43 D156G D158G, cpSRP43 D189K D191K and cpSRP43 D156K D158K neither formed transit complex nor showed any significant LHCP integration. CpSRP43 D189G D191G showed reduced integration compared to cpSR43 D223G E225G and cpSRP43 D223K E225K.
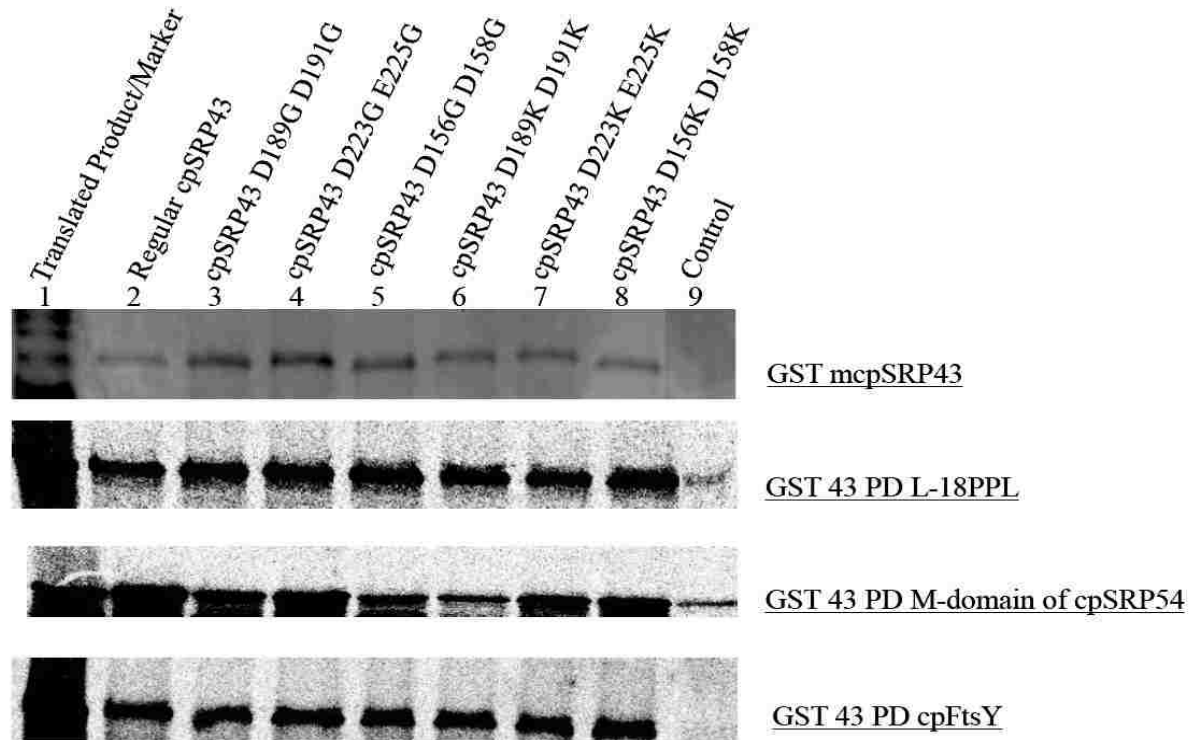
Figure 2.4: All cpSRP43 mutant proteins co-precipitate L18-PPL, M-domain of cpSRP54 and cpFtsY

Two hundred pmoles of recombinant GST tagged cpSRP43 or GST tagged cpSRP43 mutants were used to pull down 25µl of 1:2 diluted radiolabeled translation products (L-18PPL, M-domain of cpSRP54, or and cpFtsY). Gels were coomassie stained to quantify GST-cpSRP43 constructs. Phosphor imaging was used to quantify co-precipitated radiolabeled translation products. Lane 1 represents either the marker lane (on top-most gel) or the translation product lane (on remaining gels). Lane 9 on each gel represents the pull-down reaction where no cpSRP43 was added and represents the background binding of translation products to the Glutathione sepharose beads. Lane 2 represents pull down assays done using wild type GST-cpSRP43. Lanes 3-8 show the pull down results using mutant GST-cpSRP43. All cpSRP43 mutants were able to pull down L18-PPL, the M-domain of cpSRP54, and full length cpFtsY similar to wild type cpSRP43.
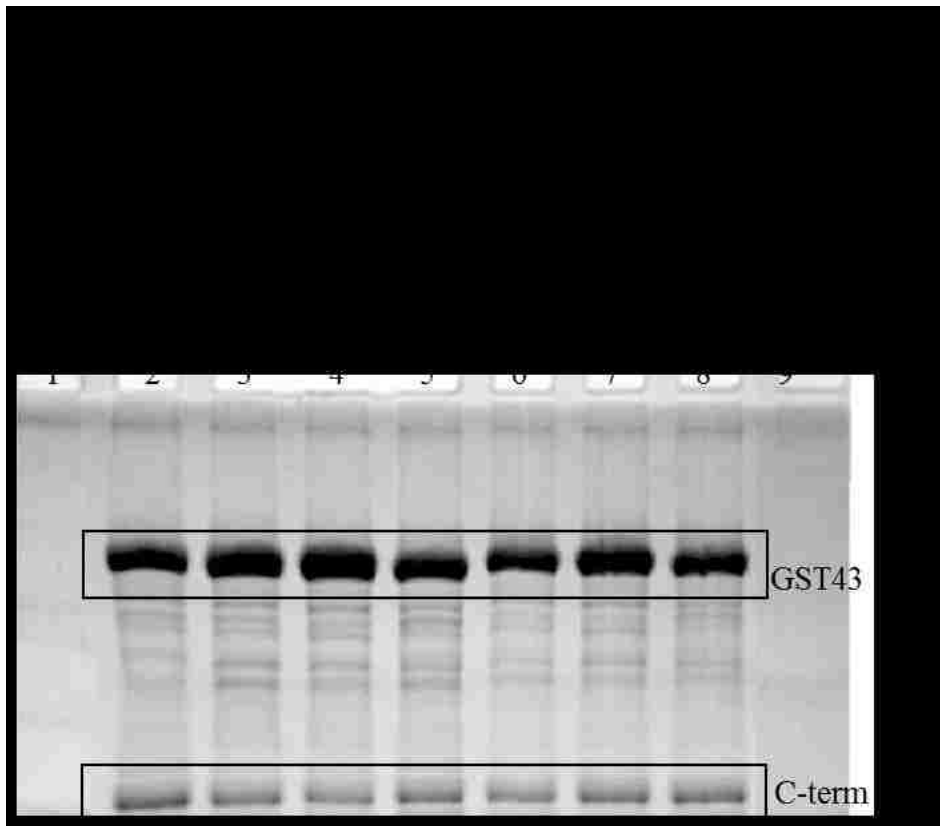
Figure 2.5: All cpSRP43 mutant proteins retain their ability to bind Alb-3-Cterm

One thousand pmoles of GST tagged cpSRP43 or GST tegged cpSRP43 mutants were co-incubated with 1500pmoles of Alb-3-Cterm protein. GST fusions and co-precipitating Alb3-Cterm were isolated using Glutathione sepharose. Samples were analyzed by SDS PAGE and coomassie stained to visualize protein bands. GST tagged cpSRP43 and all GST tagged cpSRP43 mutants were able to co-precipitate Alb-3-Cterm.

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III. CHLOROPLAST SIGNAL RECOGNITION PARTICLE 54 (CPSRP54) INTERACTS WITH THIRD TRANSMEMBRANE DOMAIN OF LIGHT HARVESTING CHLOROPHYLL BINDIDNG PROTEIN (LHCP)

ABSTRACT

Chloroplast SRP54 is a homologue of cytosolic SRP54/Ffh (fifty four homologue) and is known to be involved in post translational targeting of light harvesting chlorophyll binding (LHCP) proteins. Chloroplast SRP54 (cpSRP54) lacks key components (RNA moiety, ribosomes) which are used by its cytosolic homologues to carry out protein targeting of the secretory or membrane proteins. Study here aims to understand how cpSRP54 carries out its targeting role in chloroplasts. Homology modeling has predicted cpSRP54 as a dynamic component where its domains orient themselves in order to acquire all the functions of its missing partners in this system. In co-translational targeting, there is a direct contact between the targeting peptide and cytosolic SRP54/Ffh but here in our system of post translational targeting, this aspect of cpSR54 was unknown. Results in this study have demonstrated that cpSRP54 interacts with the substrate directly via its hydrophobic binding pocket and this is consistent with the co-translational targeting system. Any loss in the hydrophobicity of the binding pocket of cpSRP54 leads to its inability to interact with its substrate. The work done in this study has elucidated the significance of domain orientations in cpSRP54 which impact the overall role of cpSRP54 in the soluble phase of LHCP targeting cycle.

INTRODUCTION

Proteins synthesized in the cytoplasm are targeted either to the plasma membrane in bacteria or to the endoplasmic reticulum in eukaryotes via a conserved signal recognition particle (SRP) pathway (2, 13). Recognition of a hydrophobic signal sequence on ribosome nascent chain complex by the SRP marks the beginning of the targeting process (21). This is followed by binding of SRP to the ribosome nascent chain complex which subsequently leads to the association of SRP with its receptor (SR) at the membrane. Upon membrane binding, nascent chain ribosome complex is released from SRP-SR complex and transferred to the translocase at the membrane which subsequently leads to the insertion of the protein (16, 19). This entire targeting process consists of highly coordinated sequence of events in order to result in proper localization of the proteins. Co-ordination is achieved in terms of timely protein - protein interactions via proper conformational changes in the partner components involved in targeting.

In bacteria, SRP components consist of a 54-KDa protein called SRP54, referred as Ffh (fifty four homologue) bound to 4.5S SRP RNA, SRP receptor FtsY and a translocase Sec YEG at the plasma membrane (1). Ffh consists of amino terminal N domain that is found to be associated with Ras-like guanine triphosphatase (GTPase) G domain. N-G domains are connected to the third carboxyl terminal M domain via a linker (2, 14). N and G domains of Ffh are responsible for interacting with the SRP receptor FtsY in a GTP dependent manner. M domain binds to the 4.5S SRP RNA and is also known to bind the hydrophobic signal sequences of the nascent polypeptide chain (14).

A novel SRP mediated post translational targeting system has been observed in chloroplasts of green plants. The only known targeting substrate that utilizes this unique SRP pathway are light harvesting chlorophyll binding proteins (LHCPs). LHCPs are

nuclear encoded, integral thylakoid membrane proteins which are found associated with photosystems I and II and they are known to aid in photosynthetic light capture (5). Chloroplast SRP (cpSRP) machinery consists of a 54-KDa subunit of SRP called cpSRP54, SRP receptor cpFtsY, Alb-3 translocase and a unique 43-KDa SRP subunit called cpSRP43 (18). CpSRP43 and cpSRP54 together exist as a heterodimer in the stroma of chloroplasts. This system lacks ribosomes and RNA moiety.

Homology modeling has shown that cpSRP54 also contains four helix bundle N domain and five conserved GTPase domain called G domain. A flexible linker connects N-G domain to the carboxyl-terminal methionine rich M-domain. Despite of same domain organization as found in bacterial Ffh, cpSRP54 participates in a ribosome free environment to target LHCP. Also, there is no RNA binding site on cpSRP54. So how does cpSRP54 carry out its targeting role in this system?

Protein dynamics and protein structure studies have shown that cpSRP54 is a highly flexible protein with most of the flexibility present in the linker region which connects N-G domain to M-domain and a finger loop element which is present in the M-domain (in between first and second helices of M-domain, Figure 3.1). Data from modeling studies has shown that M-domain of cpSRP54 contains a hydrophobic grove which is formed by M-domain and this grove is predicted to read the hydrophobic residues found on the third transmembrane domain (TM3) of LHCP. In other words, M-domain is predicted to interact with TM3 of LHCP in the same way as M domain of cytosolic SRP54 (Ffh) interacts with the signal sequence of nascent polypeptide chain. This is supported by cross linking studies done in the past where cpSRP54 showed interaction with TM3 of LHCP in elongation arrested ribosomes (10).

Studies have shown that cpSRP43 interacts with L-18 motif of LHCP which is present between TM2 and TM3 domains of LHCP (20). CpSRP54 undergoes conformational changes when it binds to cpSRP43 and these conformational changes are predicted to have emerged from the interaction between the linker and the finger loop element of M domain which orients M domain in a state where it becomes accessible for the M domain to scan TM3 as it comes out of the cpSRP43 - LHCP binding grove. Also, there is supporting SAXS data which shows that M-domain of cpSRP54 lies relatively closer to the third transmembrane domain of LHCP in transit complex (Figure 3.2).

In this study, we are trying to narrow down TM3 binding site on cpSRP54 when cpSRP54 is in complex with LHCP and cpSRP43 in solution. We have created cpSRP54 mutants with either two point mutations in M domain grove or a 15 amino acid deletion mutation in the same grove with the hope that these mutants would either lose their ability to bind to the substrate or somehow will not be oriented in a state where substrate binding is accessible. Results are quite consistent with the structural data predictions.

MATERIALS AND METHODS

Cloning of Chloroplast SRP proteins

Primer-encoded Asn point mutations were introduced by polymerase chain reaction (PCR) into the mature cpSRP54 coding sequence (starting with amino acid sequence MAFGQL and including a C-terminal 6 His tag) at positions V339 (QTRA<u>V</u>AKMG) and L370 (AEKN<u>L</u>LVME) to make cpSRP54 V339N L370N. A loop deletion mutant, cpSRP54 Δ345-359, was made by splicing via overlap extension using internal overlapping primers to remove 15 amino acids (MTRVLGMIPGMGKVS). The resulting PCR products were cloned into pGEM-4Z (Promega, Madison, WI) using KpnI and HindIII sites for in vitro transcription/translation. CpSRP54 M-domain was produced as previously described in Goforth et al. (7). All constructs were sequence-verified by the Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock, AR.

Co-precipitation Assays

A protein binding assay was performed as described in Goforth et al. (7) using 100pmoles of GST-cpSRP43 and ^{35}S-labeled mcpSRP54 mutants produced by *in vitro* transcription/translation (Figure 3.3).

Transit Complex Formation

Mature-sized cpSRP54 or mutants of cpSRP54 described above were examined for their ability to support transit complex formation as described in Goforth et al. (7) with the following changes: 25pmoles of recombinant cpSRP43 was added to *in vitro* translated cpSRP54 or cpSRP54 mutant (indicated in Figure 3.4) along with ^{35}S-labeled *in vitro* translated pLHCP,

pLHCPΔTM3 (amino acids 1–206), or L18-TM3 (amino acids 189–269), which are described in

DeLille et al. (4). One-third of the assay was examined by native-PAGE, as described in Goforth

et al. (7). OptiQuant software from GE Healthcare and Life Sciences (Piscataway, NJ) was used

to quantify radiolabeled protein from Phosphor imaging.

RESULTS

GST tagged cpSRP43 Co-precipitates cpSRP54 V339N L370N

Previous work has shown that cpSRP54 interacts with chromodomain 2 of cpSRP43 via its M-domain (7) and it is shown that arginines at positions 461 and 462 in mature cpSRP54 are essential for this interaction (5). In co-translational targeting system, cytosolic SRP54 is known to bind hydrophobic signal sequences of the ribosome nascent chain complex via the hydrophobic residues which line up its signal sequence binding grove in the M-domain (15, 17, 22). Sequence analysis shows that M-domain of cpSRP54 also contains hydrophobic residues in its M-domain. In fact, modeling data shows that M-domain is arranged in the form of four helices with a finger loop between first and second helices and a large C-terminal extension (8, 9, 14). This together forms a hydrophobic signal sequence binding pocket. Also, LHCP contains three transmembrane domains-TM1, TM2 and TM3. TM3 has been known to be the most hydrophobic domain among all three transmembrane domains of LHCP (10). Crystal structure data also shows that TM3 exits cpSRP43 near its chromodomain 2 region and it is the same region on cpSRP43 which is known to interact with M-domain of cpSRP54 (11).

Based on this, it was hypothesized that hydrophobic grove found in M-domain of cpSRP54 interacts with TM3 domain of the substrate LHCP. If it is true, then any reduction in the hydrophobicity of M-domain binding grove will lead to loss of substrate loading ability of cpSRP54. In order to test this hypothesis, double deletion mutant of cpSRP54 was produced where valine and leucine at positions 339 and 370 in the M-domain were substituted by asparagine. Next, the mutant was tested for its interaction with cpSRP43 in a pull down assay (Figure 3.3). Lane 9 in figure 3.3 represents background binding of cpSRP54 V339N L370N to the GST tag and lanes 1, 2 represent pull down assay done using wild type cpSRP54. Results

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showed that the mutation did not alter the interaction between cpSRP43 and cpSRP54 V339N L370N mutant protein (Figure 3.3, lane 8).

Mutant cpSRP54 V339N L370N Forms Transit Complex with pLHCPΔTM3

Next, the mutant was tested for its ability to form transit complex using *in vitro* translated radiolabeled constructs of LHCP - full length LHCP, L18-TM3 and pLHCPΔTM3. All these LHCP constructs are known to form transit complex using full length cpSRP components (4). Results showed that mutant cpSRP54 V339N L370N lost its ability to form transit complex with full length LHCP and L18-TM3 (Figure 3.4, lanes 3 and 11). However, when pLHCPΔTM3 was used as a substrate, the mutant cpSRP54 V339N L370N regained its ability to interact and form transit complex (Figure 3.4, lane 7). This indicates that removal of TM3 from LHCP suppressed the mutation in cpSRP54 indicating that there could be a possible interaction between TM3 of LHCP and M-domain of cpSRP54. In fact, it suggests that M-domain of cpSRP54 acts as a driving force to orient cpSRP into a LHCP loading competent state where it must interact with TM3 of LHCP first to initiate transit complex formation. Since there was no loss of cpSRP43 binding using this mutant, it also gives an idea that cpSRP43 binds at a different site on cpSRP54 than the site where LHCP binds on cpSRP54. Lanes 4, 8 and 12 in figure 3.4 represent that substrate is not able to form a soluble complex if cpSRP components are not added to the assay, lanes 1, 5 and 9 show transit complex formation using wild type cpSRP54 and different LHCP constructs.

cpSRP54 Δ345-359 Interacts With cpSRP43 But Fails to Form Transit Complex

Modeling and FRET (Forster resonance energy transfer) data suggests that cpSRP54 exists in two possible conformations, one where M-domain is coupled to N-G domain and the other where

it is decoupled from N-G domain. This shuttling/positioning of the cpSRP54 domains take place

by the interaction between a flexible linker (which connects N-G domain to M-domain) and the

finger loop (between first and second helices) of the cpSRP54 M-domain. Since, our previous

mutant cpSRP54 V339N L370N showed that M-domain must interact or recognize TM3 of

LHCP first in order to initiate LHCP loading process. And for this interaction to take place, it is

essential that M-domain is positioned near the TM3 exit site on cpSRP43 (3). And for this to

happen, the flexible linker and finger loop must interact to orient M-domain properly.

In order to test this, a new cpSRP54 mutant- cpSRP54 Δ345-359 was designed where 15 amino

acid residues forming the finger loop (residues 345-359 in the mcpSRP54) were deleted from the

M-domain. It was hypothesized that the linker and finger loop are critical for positioning M-

domain of cpSRP54. If this is true, then this finger loop mutant would also lose its ability to

interact with LHCP. Therefore, to test the hypothesis, this mutant protein was tested for its

interaction with cpSRP43 and then for transit complex formation. Results showed that the finger

loop deletion mutant cpSRP54 Δ345-359 retained its ability to interact with cpSRP43 (Figure

3.3) like the previous mutant cpSRP54 V339N L370N but LHCP loading ability was completely

lost when transit complex assay was done using this cpSRP54 finger loop deletion mutant

cpSRP54 Δ345-359 with the three LHCP constructs as described before (Figure 3.4). This data

suggests that the interaction between finger loop of M-domain of cpSRP54 with the linker is

essential in orienting cpSRP54 in a state where M-domain can scan TM3 of LHCP as it comes

out of cpSRP43 in order to provide a hydrophobic binding grove to the TM3.

Taken together, these results confirm that cpSRP54 M-domain interacts with TM3 of LHCP by

providing a hydrophobic binding grove. Flexible elements present in cpSRP54 (linker and the

finger loop) interact with each other in order to orient cpSRP43-cpSRP54 in a LHCP loading

competent state in solution which is the first essential step in LHCP targeting. And this is

consistent with the data from co-translational targeting system where SRP54 binds to the signal

sequence of the nascent peptide chain emerging from the translating ribosome via the

hydrophobic M-domain pocket and gets ready to perform the co-translational targeting events.

DISCUSSION

In co-translational targeting system, SRP54/Ffh contains an RNA moiety and it also interacts with the hydrophobic signal sequence as it emerges out of the translating ribosomes. Elongation arrest occurs and SRP54/Ffh guides the ribosome nascent chain complex to the plasma membrane or endoplasmic reticulum where it interacts with the SRP receptor found at the membrane and transfers the ribosome nascent chain complex to the insertase at the membrane where elongation process resumes and the nascent chain is inserted into the membrane co-translationally (1). In our post-translational LHCP targeting system, not much is known about what interactions take place between cpSRP54 and the substrate LHCP. CpSRP54 lacks RNA moiety and there are no ribosomes involved in this type of targeting process. So how does the system work under these conditions for the successful targeting of LHCP?

In the past, much work has been done to understand the structure of cpSRP43 and its interaction with the substrate. In this study, we are trying to look at the possible interactions between cpSRP54 and LHCP. There is a crosslinking study which shows that cpSRP54 interacts with TM3 of LHCP in the ribosome bound complex (10).

This is the first study to directly look at the interaction between cpSRP54 and LHCP in solution. This study represents the domain organization of cpSRP54 and how orientations of the domain occur to promote the binding of cpSRP54 with its partners whether its cpSRP43 or LHCP. Results here show that cpSRP54 interacts with the third transmembrane domain of LHCP and this interaction is critical to keep cpSRP ready to form a complex with LHCP. Different conformational changes in between the cpSRP54 domains also play an important role in the targeting events. Previous work has shown that cpSRP43 is a dynamic protein and when it binds to cpSRP54, its flexibility decreases and its affinity to bind L-18 of LHCP increases (6).

In this study, we have tried to look at the role of conformational changes which occur in the cpSRP54 domains when it binds to cpSRP43 and to the substrate. This is the first study which confirms direct interaction between TM3 of LHCP and M-domain of cpSRP54 in transit complex. There are still many unanswered questions in this pathway. What happens to the N-G domain of cpSRP54 in transit complex, do those domains interact with cpSRP43 in the complex. How do the conformations of the proteins in the transit complex change when the targeting complex reaches the membrane? What is the factor that triggers this change? Much work is needed to understand the details of this pathway. However, present work provides a valuable insight in understanding the overall complicated process of LHCP targeting.
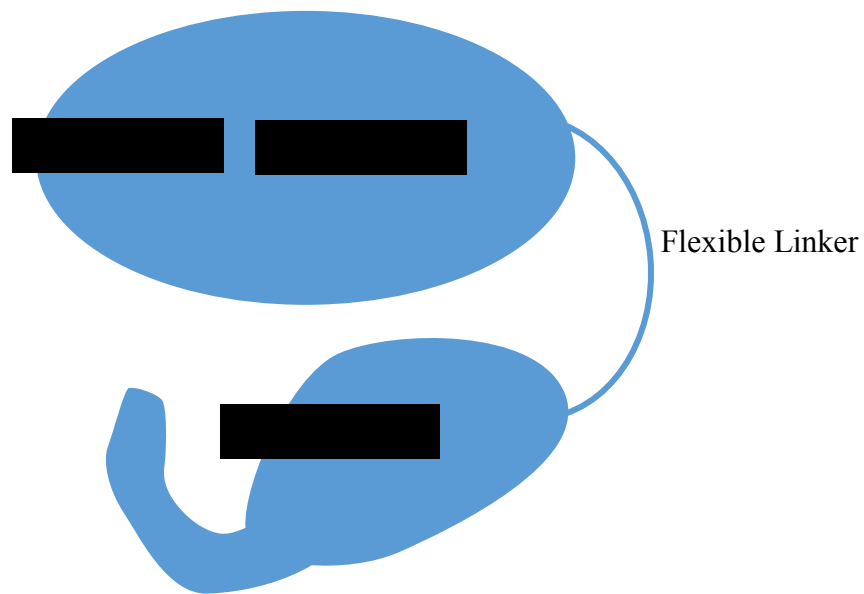
Figure 3.1: cpSRP54 domain organization

cpSRP54 is composed of three domains based upon homology. N-G domain is made up of four helix bundle N domain and a GTPase active G domain. N-G domain is connected to a C-terminal methionine rich domain called M-domain by a flexible linker.
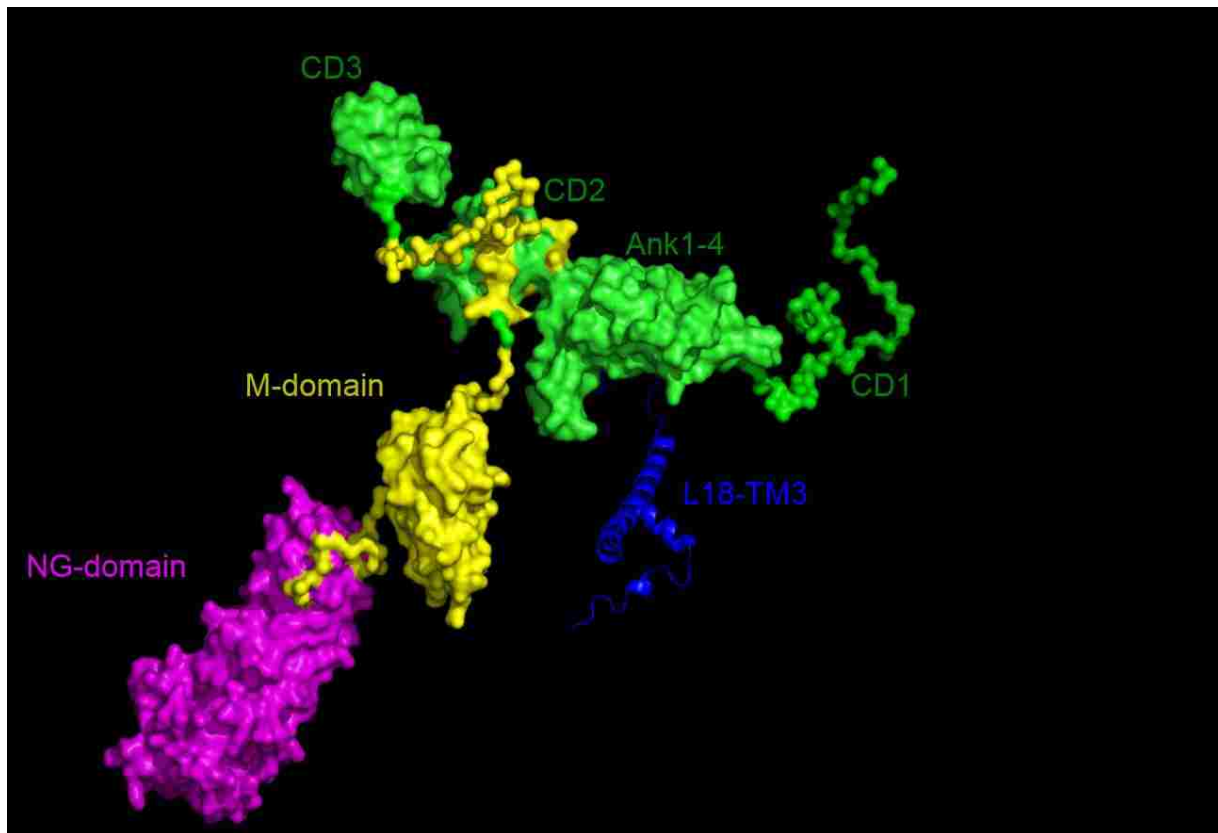
Figure 3.2: Structure of cpSRP using SAXS and existing domain structures

Figure here represents the positioning of cpSRP and L18-TM3 of LHCP in transit complex. Chromodomain 2 of cpSRP43 (in green) is bound to M-domain of cpSRP54 (in pink and yellow) and L18-TM3 (in blue) is bound to Ankyrin regions on cpSRP43. This data has been generated with the help from Dr. Suresh Kumar's lab.
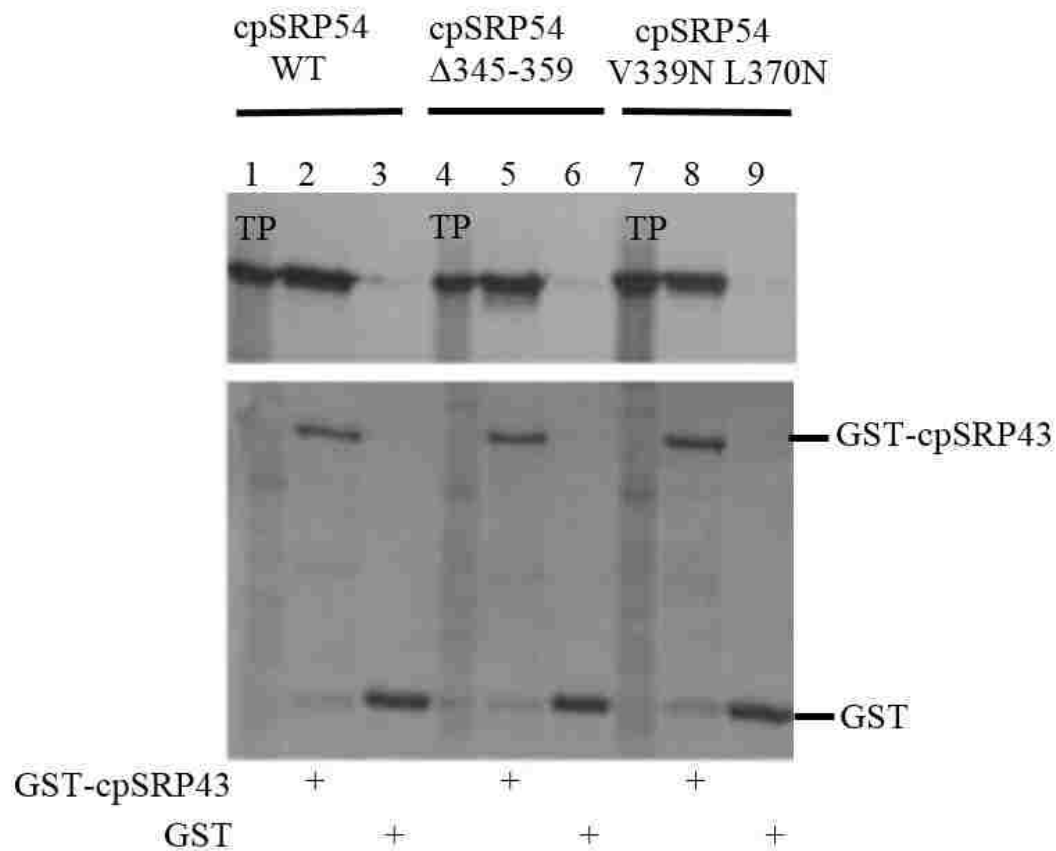
Figure 3.3: cpSRP54 M-domain mutants co-precipitate with GST-cpSRP43

In vitro translated radiolabeled cpSRP54WT (wild-type cpSRP54) or cpSRP54 mutants (cpSRP54 V339N L370N and cpSRP54 Δ345-359) were incubated with GST or GSTtagged cpSRP43 (GST- cpSRP43) as described in Materials and Methods. Proteins were recovered with Glutathione sepharose and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Radiolabeled translation product (*TP*) and co-precipitated cpSRP54 constructs were visualized by phosphor imaging (*top*) or by protein staining (*bottom*).

A.
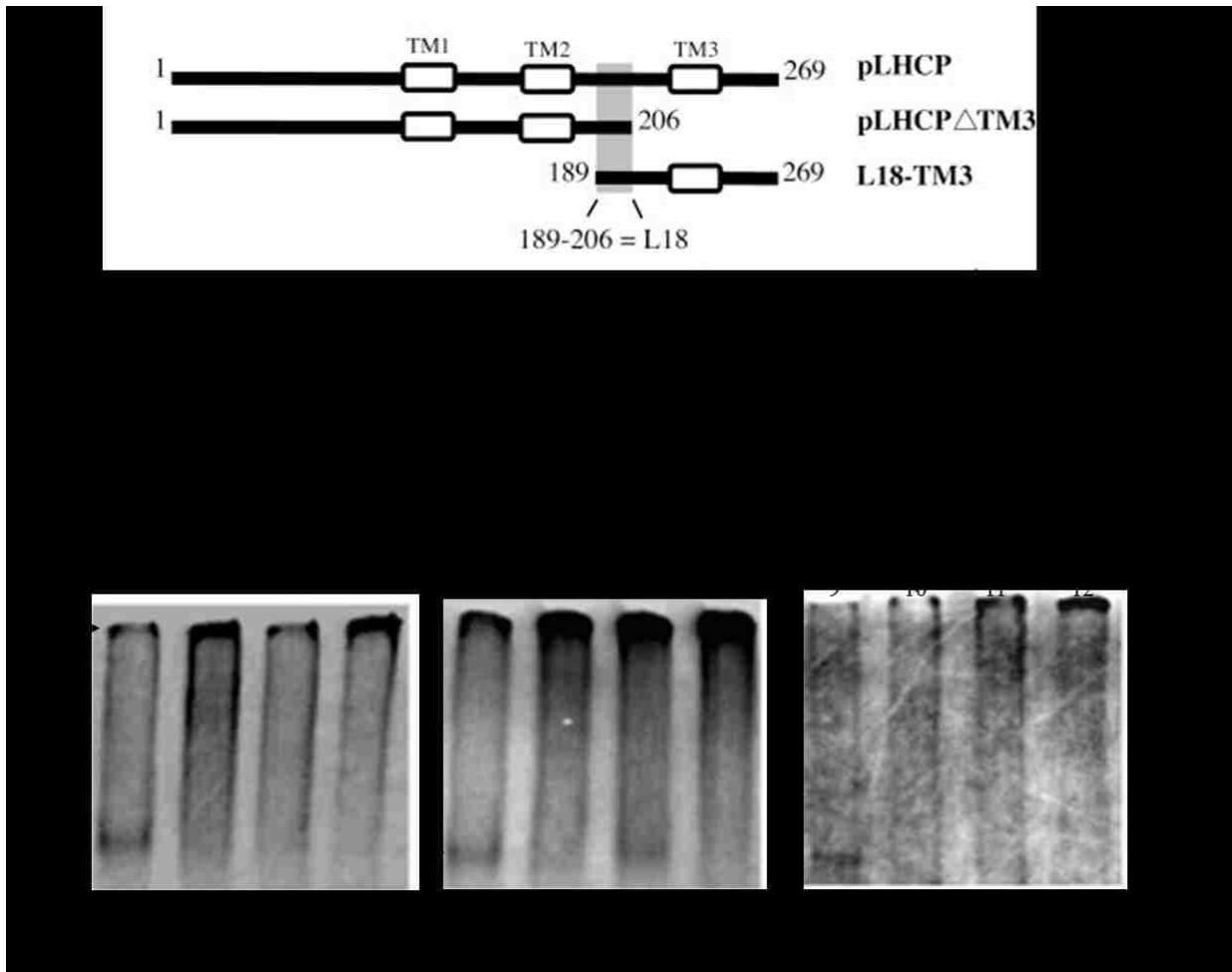
B.

Transit Complex Assay Quantification
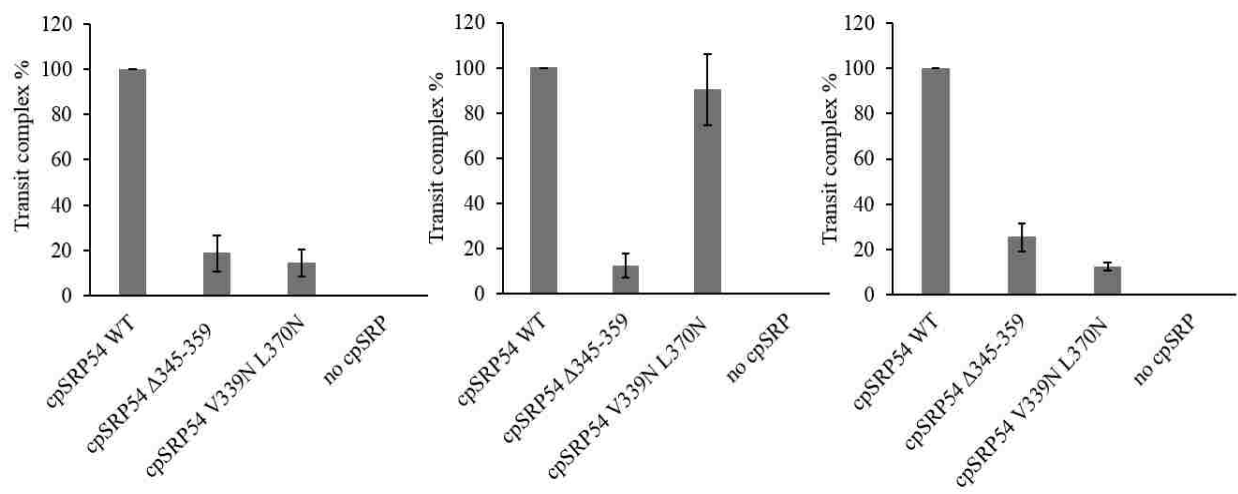
Figure 3.4: cpSR54 finger loop deletion mutant loses ability to form transit complex

A.The indicated cpSRP54 constructs were produced by *in vitro* translation and incubated with recombinant cpSRP43 and radiolabeled *in vitro* translated pLHCP or the indicated pLHCP deletion constructs, as described in the Materials and Methods. Transit complex containing the indicated radiolabeled LHCP construct was examined by native-PAGE/phosphor imaging as described in the Materials and Methods. The position of transit complex formed with each pLHCP construct is indicated (arrowhead) as is the position of aggregated LHCP constructs in the well.

B. Each transit complex assay was conducted in triplicate and quantified as described in the Materials and Methods. For each pLHCP construct, the relative amount of transit complex formed with each cpSRP54 mutant is shown. Error bars represent the standard deviation.

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IV.   GTP HYDROLYSIS IS NOT REQUIRED FOR LHCP INTEGRATION INTO THE THYLAKOLID MEMBRANES

ABSTRACT

Light harvesting chlorophyll binding proteins (LHCPs) are a family of nuclear encoded chloroplast thylakoid proteins that are synthesized in the cytosol, imported into the chloroplast stroma, and subsequently directed to the thylakoid membrane. LHCP targeting/insertion into thylakoid membranes requires GTP, a thylakoid protein insertase, and two prokaryote derived GTPases, chloroplast signal recognition particle (cpSRP) and its membrane receptor (cpFtsY). cpSRP binds LHCP in the stroma to form a soluble targeting complex that is directed to thylakoids owing to affinity of cpSRP for cpFtsY, an interaction requiring GTP and resulting in a membrane-bound targeting complex. LHCP insertion requires Albino-3 (Alb-3), a protein insertase that interacts with cpSRP and stimulates GTP hydrolysis by cpSRP/cpFtsY and to enable release of cpSRP from cpFtsY. The role and timing of GTP hydrolysis in LHCP insertion is less clear. Using assays that reconstitute LHCP integration into isolated thylakoids, we have demonstrated that GTP hydrolysis is not required for LHCP insertion from a membrane-bound targeting complex. GMPPNP, a non-hydrolysable analogue of GTP, is sufficient to support LHCP integration. Surprisingly, LHCP integration supported by GMPPNP, and not GTP, is sensitive to elevated concentrations of free cpSRP/cpFtsY, which interact at the membrane with Alb-3 and reduce Alb-3 availability for authentic interaction with cpSRP-bound LHCP targeting substrate. Our data supports a mechanism in which guanine nucleotide binding by cpSRP/cpFtsY is required for LHCP release from cpSRP upon interaction with Alb-3 whereas GTP hydrolysis serves at the membrane to release cpSRP/cpFtsY GTPases from each other and from Alb-3 for subsequent rounds of targeting. It is hypothesized that unproductive LHCP targeting is avoided by formation a GTP-stabilized cpSRP/cpFtsY/LHCP targeting complex at the membrane until Alb-3 becomes available.

INTRODUCTION

Protein localization is a vital process in all living cells that allows proteins to be directed from their site of synthesis in the cytosol to cellular sites where they must function. Many of the ancestral routing mechanisms that evolved in prokaryotes have adapted to serve in modern day eukaryotes. For example, a cytosolic signal recognition particle (SRP) targeting system that functions in prokaryotes to co-translationally localize integral membrane proteins to the cytosolic membrane, also directs soluble and membrane secretory proteins from the cytosol to the endoplasmic reticulum in eukaryotes by a conserved co-translational mechanism. Not surprisingly, critical structural and functional aspects of the co-translational SRP targeting system in prokaryotes and eukaryotes have been preserved. Specifically, cytosolic SRPs in prokaryotes and eukaryotes contain an RNA moiety associated with a 54KDa GTPase (SRP 54; Ffh in prokaryotes) that exhibits affinity for a conserved SRP receptor (FtsY in prokaryotes), which is also a GTPase.

In these co-translational targeting mechanisms, ribosome-associated SRP binds targeting substrates as they emerge from the ribosome owing to the ability of SRP54 to bind the hydrophobic region of signal sequences at the N-terminus of targeting substrates (14, 16). The SRP-ribosome-nascent chain complex docks at the membrane via the SRP receptor, an event that requires GTP binding by both cpSRP54 and its receptor. In eukaryotes, the ribosome interacts with both SRP and its receptor (17), but an available Sec translocase in the endoplasmic reticulum is required to stimulate dissociation of SRP54 from the targeting substrate's signal sequence and stimulate GTP hydrolysis by SRP54 and its receptor (22), an event that enables SRP to separate from its receptor at the membrane and engage in subsequent rounds of targeting. In prokaryotes, a homologous Sec translocase has been shown to act similarly in promoting the

concerted signal sequence release from SRP and handover of the translating ribosome to the Sec translocase from SRP targeting machinery (1).

A unique organellar SRP targeting system has evolved in chloroplasts to target light harvesting chlorophyll-binding proteins (LHCPs) to the chloroplast thylakoid membrane. LHCPs are a family of integral membrane proteins that bind chlorophyll a/b and are considered the most abundant membrane proteins on earth representing up to 50% of bulk protein in thylakoids. As nuclear encoded genes, LHCPs are synthesized as full-length precursor in the cytosol. Following their import and processing into the chloroplast stroma, mature-sized LHCPs remains soluble owing to the unparalleled ability of cpSRP in stroma to bind full-length targeting substrates. The cpSRP-LHCP complex in stroma, termed transit complex, represents the targeted form of LHCPs in chloroplasts. CpSRP is a heterodimer composed of a conserved 54kDa GTPase (cpSRP54) and a 43KDa protein (cpSRP43) unique to chloroplasts. The ability of transit complex to associate productively with thylakoid membranes relies on affinity of membrane-bound cpFtsY for cpSRP54 (18), a GTP requiring reaction stabilized when GTP is replaced by non-hydrolysable GTP (19). Unlike cytosolic SRPs, posttranslational substrate binding by cpSRP relies on cpSRP43, which has affinity for the L-18 motif present in each member of the LHCP protein family and located between the second and third transmembrane domains (TM) of LHCPs (8, 23). Recent data indicates that the while transit complex formation is initiated through cpSRP43-LHCP interaction, it is followed by association of cpSRP54 with LHCP-TM3 (12).

Once at the membrane, the transit complex docked with cpFtsY is believed to interact with the C-terminus of a protein insertase, Albino-3 (Alb-3), an interaction that takes place owing to affinity of the stroma-exposed Alb-3 C-terminus and cpSRP43 (9, 15). Although a homologous

Sec translocase functions in thylakoids to co- and post-translationally transport a specific subset of protein into or across the thylakoid membrane (2, 21), the Sec translocase is not thought to play a role in LHCP insertion (19).

Clues as to the role of Alb-3 interaction with cpSRP43 at the membrane come from the observation that peptide corresponding to the Alb-3 C-terminus induces GTP hydrolysis by cpSRP54/cpFtsY in a cpSRP43 dependent manner and also causes LHCP substrate release from cpSRP (15), a necessary step to promote LHCP insertion by Alb-3. What is not clear is the order of events triggered by Alb-3; is GTP hydrolysis needed for efficient LCHP release from cpSRP and subsequent insertion into thylakoids or does it simply serve to enable separation of cpSRP from its receptor, cpFtsY? It is also conceivable that LHCP insertion by Alb-3 relies on Alb-3 dissociation from cpSRP at the membrane, an event most likely tied to GTP hydrolysis by cpSRP54/cpFtsY and which allows dissociation of cpSRP54 from cpFtsY (19). Our results show that GTP hydrolysis is not required for LHCP integration; Alb-3 remains functional for LHCP insertion even when bound by cpSRP-cpFtsY. However GTP hydrolysis is needed to recycle the availability of Alb-3 for the next cpSRP loaded with LHCP cargo.

MATERIALS AND METHODS

All reagents and enzymes were purchased commercially. All DNA constructs were sequence verified by the Molecular Resource Laboratory, University of Arkansas for Medical Sciences, Little Rock. Recombinant protein concentrations were determined by analyzing coomassie blue stained SDS PAGE gels on Alpha Innotech FluorChem IS-8900 using Alpha Ease FC Stand Alone software (Alpha Innotech).

Construction of *in vitro* Transcribed and Translated pLHCP clone

Standard PCR techniques were used to add a Strep II tag (WSHPQFEK) to the N-terminus and three additional methionines to the C-terminus of the coding sequence for pLHCP (5). The resulting PCR product, Strep-pLHCP-3MC, was cloned into pGEM-4Z (Promega) using EcoRI and SalI for *in vitro* transcription and translation as for pLHCP as described previously (5, 15) Translation products were diluted with equal volume of 60 mM unlabeled methionine in import buffer (IB: 50mM hepes-KOH, pH 8.0, 0.33M sorbitol) prior to use.

Cloning, Expression and Purification of Recombinant Proteins

Recombinant, purified proteins Trx-His-S-tagged cpFtsY and cpSRP43 were produced and isolated as described previously (11, 24). A C-terminal Strep II tag (WSHPQFEK) was added to the coding sequence of mcpSRP54-His (10, 11) by standard PCR techniques. The resulting PCR product was cloned into pPROLar.A122 plasmid (Clontech Laboratories, Inc.) using KpnI and HindIII sites to generate a plasmid coding for mcpSRP54-His-Strep as described previously for mcpSRP54-His (24). Soluble mcpSRP54-His-Strep was purified via Talon metal affinity resin (Clontech Laboratories, Inc.), concentrated using 30kDa molecular weight cut off (MWCO) vivaspin centrifugal concentrator (GE Healthcare Life Sciences) followed by secondary

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purification over Strep-tactin (EMD) gravity flow resin per manufacturer's protocols. The

resulting mcpSRP54-His-Strep was desalted into HKM buffer (10mM hepes-KOH, pH 8.0,

10mM MgCl$_2$) with 100mM KCl and aliquots were stored at minus 80 degrees celsius till use.

Chloroplast SRP Formation

cpSRP complex was prepared by combining equimolar amounts of cpSRP43 and cpSRP54-His-

Strep and incubating overnight at 4 degrees celsius. After incubation, the complex was purified

by gel filtration column HiLoad36/60 Superdex75 (Amersham Biosciences) to yield cpSRP in

HKM buffer (10mM hepes-KOH, pH 8.0, 10mM MgCl$_2$) plus 100mM KCl as per Moore et al

2003 (19).

Preparation of Salt washed Thylakoids

Intact chloroplasts were isolated from 10 to 12 days old pea seedlings (*Pisum sativum* cv.

Laxton's Progress) and used to prepare thylakoids as described previously (6). Chlorophyll

content was determined as described previously (3). Thylakoids were salt-washed (SW) two

times with 1M potassium acetate in import buffer (IB: 50mM hepes-KOH, pH 8.0, 0.33M

sorbitol) and then washed two times with import buffer containing 10mM MgCl$_2$ (IBM buffer) as

described before (15). Thylakoids were resuspended at 1mg/ml chlorophyll in IBM buffer prior

to use.

Transit Complex Assay

Transit complex (TC) assays were performed as described in Payan et al 1991 (20) with

following modifications. Transit complex was made by mixing 0.5μg of preformed cpSRP, 20μl

of 1:2 diluted *in vitro* translated radiolabeled Strep-pLHCP-3MC translation product, and HKM

(10mM hepes-KOH, pH 8.0, 10mM MgCl$_2$) buffer in a total volume of 45μl. The reaction size

was scaled dependent on integration assays needs. Proteins were incubated together at 26 degrees celsius for 30 minutes followed by apyrase (Sigma-Aldrich) treatment or control (no apyrase) treatment. For apyrase treatment, one microliter of apyrase (in 10mM hepes-KOH, pH 7.0, 50mM NaCl, 0.1mM $MgCl_2$, 0.1mM Dithiothreitol and 50% glycerol) was added per 12.5µl of 1:2 diluted translation product (TP). The samples were incubated on ice for 15 minutes. After incubation, 2.5µl of 50% glycerol was added to a 15µl aliquot of transit complex for native gel analysis. Increasing concentrations of preformed cpSRP (0.0125µg, 0.025µg, 0.05µg, 0.1µg, 0.2µg, 0.5µg, 1.0µg and 2µg) were used to perform transit complex assays as shown in figure 4.3A and figure 4.4A.

Integration Assay

Integration assays were carried out as described in Cline et al 1993 (6) with few modifications. Seventy five microliters total volume assays containing IBM, salt washed thylakoids equal to 25µg of chlorophyll, 5µg of cpFtsY, and 1mM (final) nucleotides (GMPPNP or GTP) were incubated with or without 1 Unit of apyrase on ice for 30 minutes. After incubation, 30µl aliquots of apyrase treated or control (no apyrase) transit complex assays (as described above) were added as indicated to the integration reaction mixture. Samples were incubated at 26 degrees celsius for 30 minutes in the presence of light. Thylakoids were centrifuged at 4100 x g for 8 minutes at 6 degrees celsius and treated with 12.5µl of thermolysin (2mg/ml thermolysin stock in 10mM $CaCl_2$) on ice for an hour. Hundred microliters of 50mM EDTA (ethylenediamine tetra acetic acid in import buffer) was added to thermolysin treated thylakoids followed by centrifugation at 4100 x g for 8 minutes at 6 degrees celsius. Pelleted thylakoids were solubilized using SDS buffer and heated for SDS-PAGE analysis. Amounts equivalent to 13.8µg of chlorophyll per sample were analyzed by SDS PAGE and phosphor imaging. A second

approach was used to perform integration assays (Figure 4.5 and figure 4.6) where salt washed thylakoids were treated with excess preformed cpSRP, nucleotides (GMPPNP or GTP), cpFtsY and apyrase prior to transit complex addition. All integration assays in this approach received transit complex formed as described above by using only one concentration of preformed cpSRP (0.5μg).

Sample Analysis

SDS PAGE and native gels were imaged using Typhoon FLA 9500 (GE Healthcare Life Sciences). OptiQuant software (GE Healthcare Life Sciences) was used to quantify signal from radiolabeled protein. All experiments were performed in triplicate. Bar graphs were generated using Microsoft Excel 2013. Error bars represent standard error of the mean (SEM).

RESULTS

GMPPNP, a Non-hydrolysable Analogue of GTP is Sufficient for LHCP Integration

It has been demonstrated in SRP mediated co-translational protein targeting that GTP hydrolysis is required to recycle SRP/SR complex in order to carry out subsequent cycles of targeting (7). To understand the role of guanine nucleotide in SRP mediated post-translational targeting of LHCP, we reconstituted LHCP integration assays using salt washed thylakoids (from *Pisum sativum)*, pre-formed transit complex, cpFtsY, in the presence of GTP or GMPPNP (a non hydrolysable analogue of GTP) or no added nucleotides (Figure 4.1, lanes 1-3). These assays were also conducted in the presence of apyrase (Figure 4.1, lanes 4-6). Apyrase hydrolyses nucleoside triphosphates into nucleoside monophosphates (13) but it cannot hydrolyze GMPPNP. GTP present in lanes 4, 5 & 6 (Figure 4.1) has been hydrolyzed by apyrase. Quantification was done by repeating the experiment three times. Integration assay where no additional nucleotides were added was set to 100% (Fig. 1, lane 3). Data shows that additional GTP stimulates LHCP integration by about ~70% (Figure 4.1, lane 1). GMPPNP also supports LHCP integration (Figure 4.1, lane 5), although to a reduced level. This suggested that GTP hydrolysis may not be required for LHCP integration as integration is supported by a non-hydrolysable GTP analogue.

GMPPNP is Not a Limiting Factor in LHCP Integration

By comparing lanes 3 and 5 in figure 4.1 it is clear that GMPPNP supported integration is reduced by ~55%. To account for loss in integration activity in the presence of GMPPNP, we conducted integration by using different concentrations of GMPPNP (Figure 4.2) in the presence of apyrase. Results showed that GMPPNP concentration as low as $30\mu M$ is sufficient to saturate

integration activity, and, we used 1mM GMPPNP previously, (Figure 4.1). This suggested that GMPPNP concentration is not a limiting factor to account for loss in GMPNP supported LHCP integration.

Excess free cpSRP limit LHCP integration in presence of GMPPNP

We also conducted integration assays using different concentrations of cpSRP to further understand the loss in GMPPNP supported integration. We have used *in vitro* translated LHCP and recombinant cpSRP in our transit complex assays, this may serve as a source of excess free cpSRP that is not bound to the substrate. Previous studies have shown that cpSRP and cpFtsY lock together in the presence of GMPPNP and occupy Alb-3 translocation binding sites (19). This may be the reason behind loss of GMPPNP supported integration in our experiments where excess free cpSRP is blocking the Alb-3 translocase binding sites in the presence of GMPPNP. To test this, we formed transit complex using different concentrations of cpSRP (Figure 4.3A). More cpSRP resulted in more transit complex formation. Integration assays were also done in presence of GTP and transit complex formed as mentioned above. As expected, more transit complex resulted in more GTP supported integration (Figure 4.3B). Similarly, we also performed transit complex assays and integration assays using different concentrations of cpSRP in presence of apyrase and GMPPNP, respectively. Apyrase treated transit complex assays showed increasing trend (Figure 4.4A) as shown by non-apyrase treated transit complex assays (Figure 4.3A). Integration done in the presence of GMPPNP and apyrase using apyrase treated transit complex with different concentrations of cpSRP first showed an increasing trend but then at a higher cpSRP concentration, integration dropped down (Figure 4.4B). This confirmed that excess cpSRP reduces integration activity by occupying Alb-3 translocase sites in presence of GMPPNP.

Thylakoids pre-incubated with excess cpSRP also limit GMPPNP supported integration

In order to reconfirm previous result, we repeated above assays by pre-incubating salt washed thylakoids with excess cpSRP and then added transit complex formed by using $0.5\mu g$ of cpSRP either in the presence of GTP or a combination of GMPPNP and apyrase, respectively. Data showed that excess free cpSRP did not decrease GTP supported integration (Figure 4.5A) but did limit integration in the presence of GMPPNP and apyrase (Figure 4.5B). These results elucidated the fact that excess free cpSRP in the presence of GMPPNP reduces translocase availability to the targeting complex. GTP hydrolysis is not required for LHCP integration but at the same time hydrolysis shows a stimulatory effect on integration. This suggests a downstream role of hydrolysis, similar to what happens in co-translational targeting where hydrolysis leads to release of cpSRP and cpSRP receptor components from each other to carry out subsequent rounds of protein targeting.

DISCUSSION

In this report, we have examined the role of guanine nucleotide (GTP) in post-translational LHCP targeting pathway, which relies on a unique post-translational SRP system. Despite the fact that components are lacking in the cpSRP targeting system whose interactions control the timing of GTP hydrolysis in cytosolic SRP targeting to the endoplasmic reticulum (ribosome, SRP RNA, an insertase), our data indicates that the targeting substrates are transferred from the SRP to the translocase prior to the need for GTP hydrolysis despite the absence of conserved proteins. Presumably this is true of the cytosolic co-translational SRP in prokaryotes, although the details surrounding the timing of substrate release from SRP in *E. coli* relative to GTP hydrolysis is less clear.

It has been established in co-translational protein targeting in bacteria, that ribosome nascent chain complex interacts with bacterial SRP54 (Ffh in bacteria) via the primary interaction site between ribosomal L-23 protein and N-domain of SRP54. This complex interacts with bacterial SR FtsY, which then directs the ribosome nascent chain complex to protein conducting channel SecYEG, located on the bacterial cytosolic membrane (cell membrane). SecYEG triggers release of nascent chain from SRP/SR complex by associating with the ribosomal protein L-23 leading to displacement of SRP54 from the ribosome. Conformational changes in the SRP54 (Ffh in bacteria) and SR FtsY promoted by SecYEG leads to the release of nascent chain from the ribosome into the SecYEG channel as well as GTP hydrolysis to liberate SRP components. Studies have also shown that in the eukaryotic co-translational protein targeting system, ribosome nascent chain complex in association with eukaryotic SRP54 and SR deliver ribosome nascent chain complex to translocase Sec61 in the rough endoplasmic reticulum membrane,

thereby leading to hydrolysis of GTP which leads to the recycling of eukaryotic SRP and SR into the cytoplasm. All the events are well synchronized to avoid any futile targeting.

Our results here in post-translational protein targeting system are also consistent with the findings of co-translational protein targeting system that role of GTP hydrolysis is to recycle SRP and its receptor into the chloroplast stroma for further rounds of protein targeting. Since our system lacks ribosome, so what is it that triggers release of LHCP from transit complex? It has been published before that C-terminus of Alb-3 translocase interacts with cpSRP43 and that in the transit complex it's the cpSRP43 that is interacting with LHCP via binding of ankyrin region with the L-18 region of LHCP. cpSRP54 (M-domain) is bound primarily via chromodomain 2 to cpSRP43 and cpFtsY is bound to cpSRP54. Therefore, when transit complex-FtsY complex at the membrane comes in contact with C-terminus of Alb-3, C-terminus interaction with cpSRP43 may serve as a trigger to release LHCP from the complex to the translocase. Interaction of cpFtsY with the thylakoid membranes may lead to conformational changes in cpFtsY/cpSRP54/Albino-3 association which further aid in the LHCP release process. All the events lead to the release of LHCP to the translocase Alb-3 followed by LHCP integration into the membrane. GTP hydrolysis occurs to recycle cpSRP and cpFtsY into the stroma. Therefore, cpSRP43 may behave like a ribosome in our system.

What happens to cpSRP/cpFtsY complex at the membrane when LHCP integration process is being going on, does LHCP integration and SRP recycling occur simultaneously or is it more like a sequential process where first LHCP is released and integrated into the membrane and then GTP hydrolysis occurs to recycle cpSRP? Our data shows that LHCP integration occurs in the absence of hydrolysis which may favor the fact that cpSRP/cpFtsY may still remain associated with the membrane/Alb-3 until LHCP fully integrates into the membrane to ensure proper

localization and then GTP hydrolysis occurs to recycle SRP/SR components into the stroma. Our results also show that when LHCP integration was done in the presence of GTP or GMPPNP (a non-hydrolysable analogue of GTP), more LHCP integrated into the thylakoid membrane in presence of GTP than in the presence of GMPPNP. This reduction in GMPPNP supported integration in the absence of hydrolysis could be attributed to the presence of cpSRP/cpFtsY complex at the membrane which may have occupied the available translocase sites on Alb-3, hence no recycling occurs thereby leading to the loss of subsequent rounds of LHCP integration, hence the low level of integration. This leads to a possibility that cpSRP/cpFtsY complex interaction sites on Alb-3 translocase could be different from what is needed for LHCP release and integration. But these sites are crucial for the attachment of targeting complex to the translocase in order to initiate targeting events at the membrane. Further work is needed to understand the protein-protein interactions that occur at the membrane when targeting complex is associated with the Alb-3 translocase just prior to the release of LHCP.

Our data points to another feature of the Alb-3 insertase, namely that the cpSRP does not have to depart the insertase for the insertase to function properly. Yet, newly targeted substrates fail to insert when cpSRP/cpFtsY complexes remain associated with Alb-3. Therefore, a major function of GTP hydrolysis is not only the recycling of cpSRP/cpFtsY targeting components for new rounds of targeting, but GTP hydrolysis appears critical to continue the availability of Alb-3 for new targeting substrates.

Also based on the high level of insertion achieved without the ability for cpSRP recycling (insertion with GMPPNP, figure 4.1, lane 5), our data suggests that under the conditions insertion was examined, recycling of cpSRP during the 30 minutes assay does not play a major role in reaching the levels of observed insertion. Rather, the major limitation was the level of

available insertase owing to arrest of cpSRP/cpFtsY complexes titrating Alb-3. This points to the

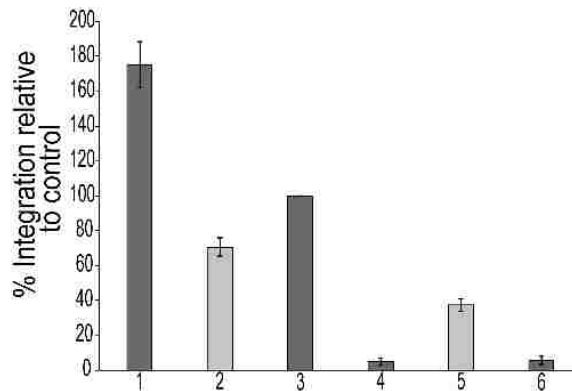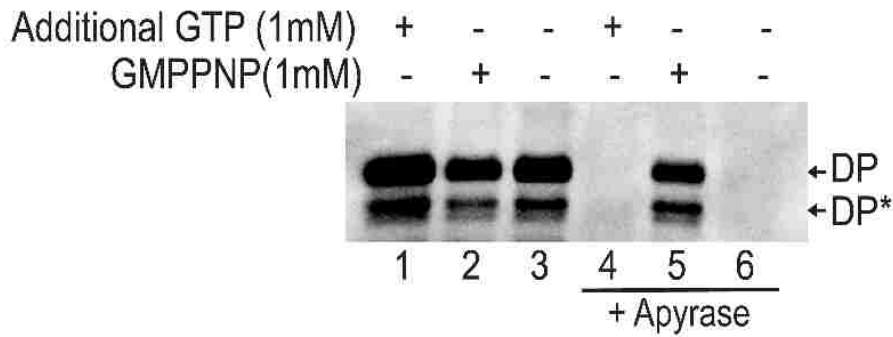physiological need to rapidly remove cpSRP/cpFtsY from Alb-3 to maintain its availability.

Figure 4.1: GTP hydrolysis is not required for LHCP integration

Salt washed thylakoids (25μg of chlorophyll) were incubated with additional GTP (1mM, lanes 1 & 4), GMPPNP (1mM, lanes 2 & 5) or no additional GTP (lanes 3 & 6) and 5μg of recombinant cpFtsY. All samples contain 0.15mM residual GTP from the translation mixture. Samples 4, 5 & 6 were apyrase treated prior to transit complex (TC) addition. TC was made using 0.5μg of recombinant cpSRP & *in vitro* translated radiolabeled LHCP. Apyrase treated TC was added to samples 4, 5 & 6. Integrated LHCP was examined using SDS PAGE & Phosphor imaging as a degradation product (DP). Full-length translation product (TP) runs higher than DP on SDS PAGE gel. DP* refers to monomeric form of LHCP in the membrane. Graph depicts percentage of integration relative to the level of integration in lane 3 where no additional GTP was added and is determined from three separate experiments.
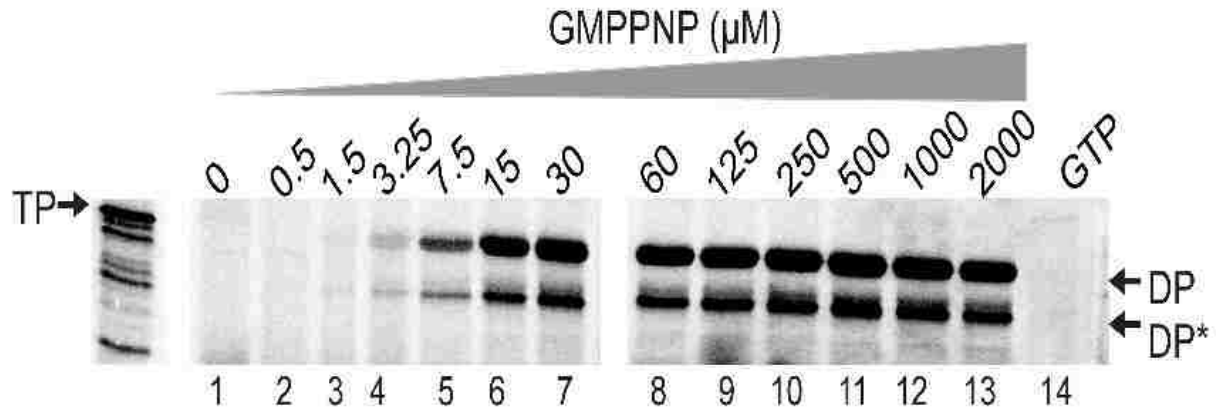
Figure 4.2: GMPPNP is not a limiting factor in LHCP integration

To account for low level of LHCP integration in lane 5 compared to lane 1 in figure 4.1, integration assay was done using increasing amounts of GMPPNP (in µM), as shown in the figure. Salt washed thylakoids (25µg of chlorophyll) were incubated with increasing amounts of GMPPNP (lanes 2- 13), lane 14 contains 1mM additional GTP, 5µg of cpFtsY and apyrase before adding transit complex (TC). Transit complex was formed as described in the legend to figure 4.1 followed by incubation with apyrase and then added to the thylakoids mix. Phosphor imaging of integrated LHCP in the form of degradation product (DP) are shown in the figure. Full-length translation product (TP) runs higher than DP on SDS PAGE gel. DP* refers to monomeric form of LHCP.
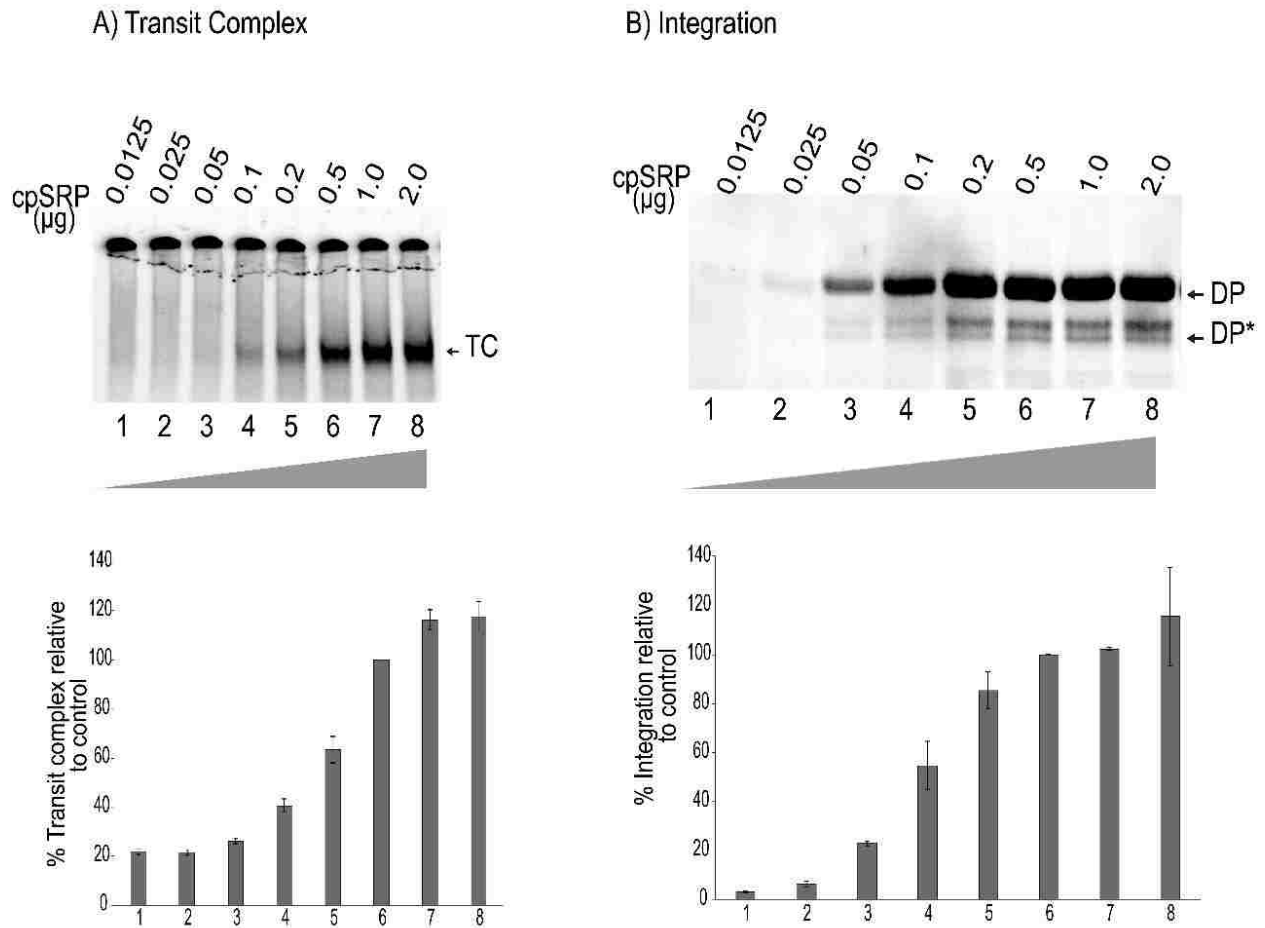
Figure 4.3: Transit complex formed with increasing amounts of cpSRP results in increasing levels of GTP supported LHCP integration.

A. Transit complex (TC) was formed using increasing amounts of recombinant cpSRP and *in vitro* translated radiolabeled LHCP as shown in the figure. Transit complex formation was examined using native PAGE and Phosphor imaging. The levels of transit complex formed using different concentrations of cpSRP were calculated form three separate experiments and is shown relative to transit complex formed using 0.5μg of cpSRP (lane 6).

B. Integration assay was done by incubating salt washed thylakoids (25μg of chlorophyll) with additional GTP (1mM), 5μg of cpFtsY before adding transit complex (TC). Transit complex formed as mentioned in figure 4.3A was added to the thylakoids mix. Integrated LHCP was examined using SDS PAGE & Phosphor imaging as a degradation product (DP). Full-length translation product (TP) runs higher than DP on SDS PAGE gel. Graph shows percentage of LHCP integration relative to integration of LHCP using 0.5μg of cpSRP based on calculations from three separate experiments. DP* refers to degradation product representing monomeric form of LHCP.
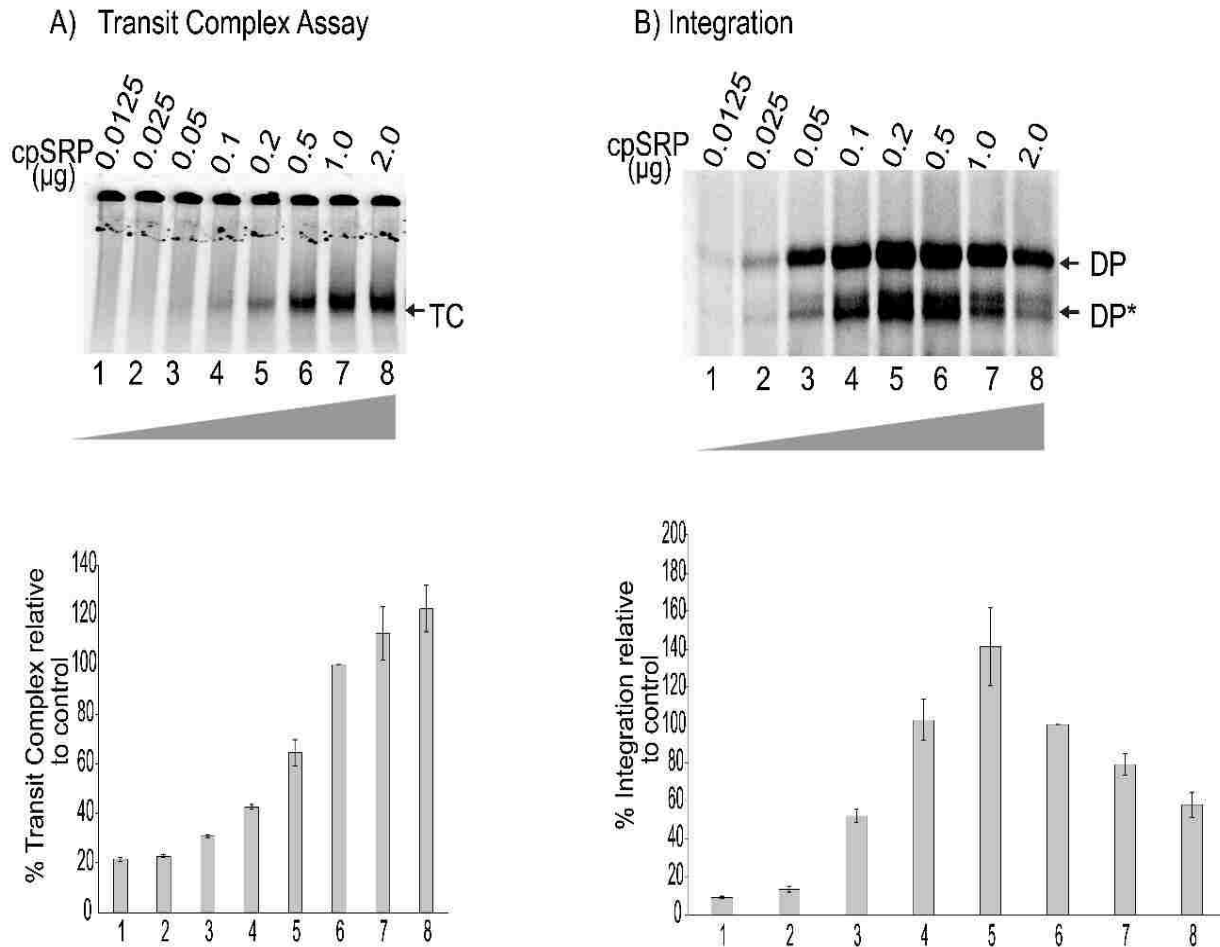
Figure 4.4: Transit complex formed with increasing amounts of cpSRP limits LHCP integration in the presence of GMPPNP and apyrase.

A. Transit complex was formed as mentioned in the legend to figure 4.3A followed by apyrase treatment, examined using native PAGE and Phosphor imaging. Graph depicts percentage of transit complex (TC) relative to transit complex formed using 0.5μg of cpSRP (lane 6) based on results from 3 separate experiments.

B. Integration assay was conducted as mentioned in the legend to figure 4.3B in the presence of GMPPNP and apyrase and examined via Phosphor imaging. Efficiency of integrated LHCP was calculated from 3 separate experiments and is presented relative to integration efficiency using 0.5μg of cpSRP. Full-length translation product (TP) runs higher than degradation product (DP) on SDS PAGE gel. DP* refers to degradation product representing monomeric form of LHCP.
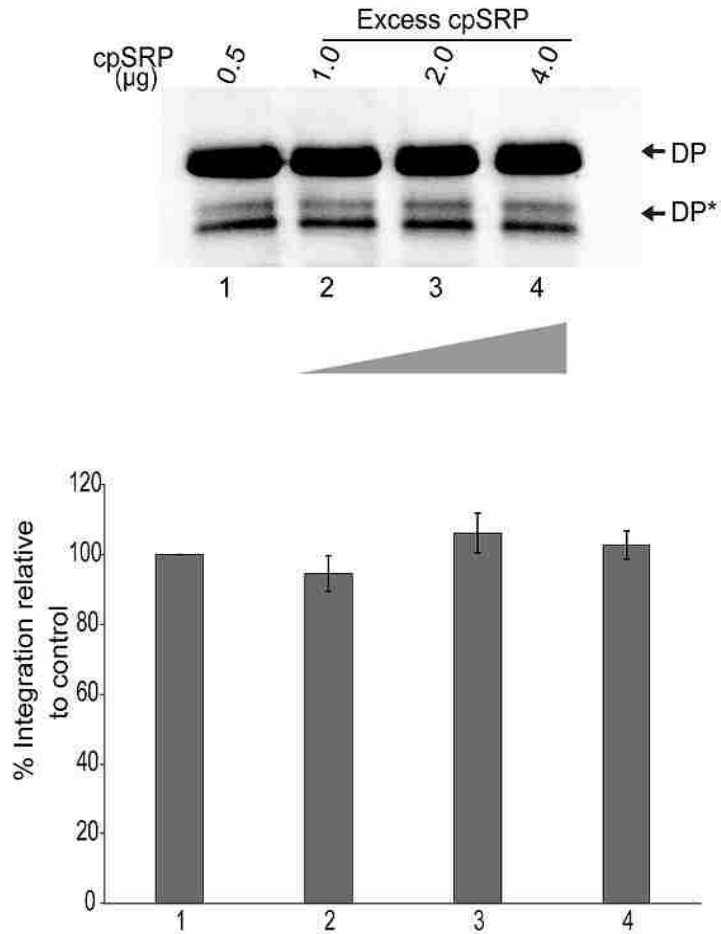
Figure 4.5: Thylakoids pre-incubated with excess cpSRP, do not show decrease in LHCP integration in the presence of GTP

Salt washed thylakoids (25μg of chlorophyll) were pre-incubated with increasing amounts of cpSRP (lanes 2-4), additional GTP (1mM), 5μg of cpFtsY before adding transit complex. Transit complex made using 0.5μg of recombinant cpSRP and *in vitro* translated radiolabeled LHCP was added to the pre-treated thylakoids. Integrated LHCP was examined using SDS PAGE & Phosphor imaging as a degradation product (DP). Full-length translation product (TP) runs higher than degradation product (DP) on SDS PAGE gel. Graph shows integration percentage relative to percentage of integration obtained using 0.5μg of cpSRP (lane 1 which was not incubated with excess cpSRP) as calculated from three separate experiments. DP* refers to degradation product representing monomeric form of LHCP
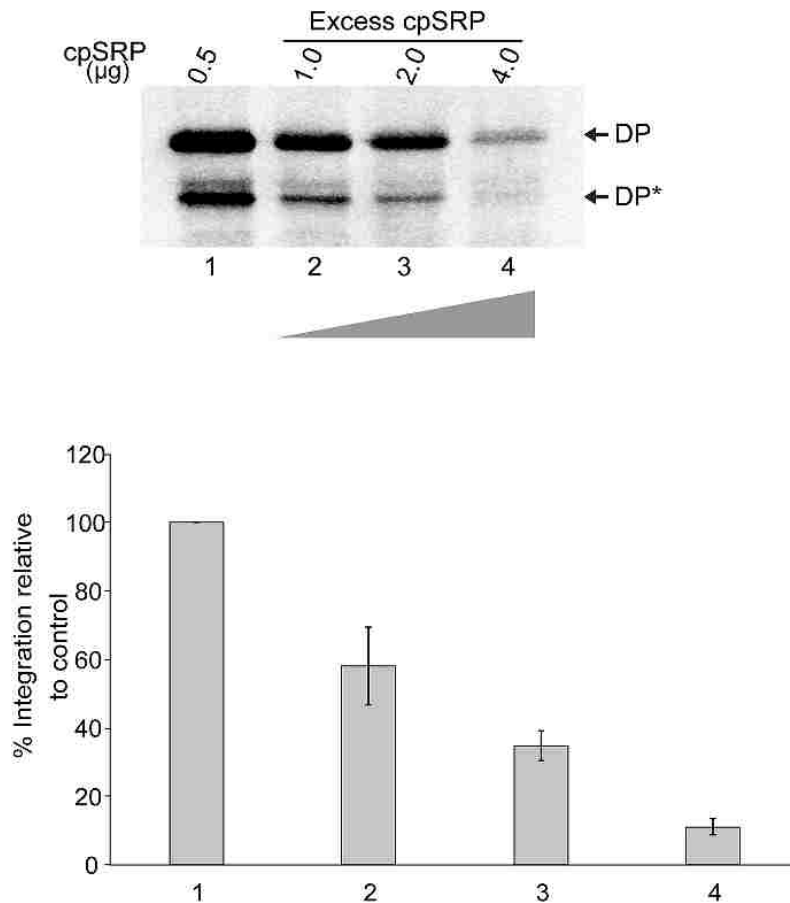
Figure 4.6: Thylakoids pre-incubated with excess cpSRP limit LHCP integration in the presence of GMPPNP & apyrase

Salt washed thylakoids (25μg of chlorophyll) were incubated with increasing amounts of cpSRP (lanes 2-4), GMPPNP, 5μg of cpFtsY and apyrase before adding transit complex. Apyrase treated transit complex was formed as mentioned in the legend to figure 4.5 and then added to the pre-treated thylakoids. Integrated LHCP was examined using SDS PAGE and Phosphor imaging as a degradation product (DP). Full-length translation product (TP) runs higher than DP on SDS PAGE gel. Three separate experiments were conducted to determine percentage of integration relative to level of integration obtained using 0.5μg cpSRP (lane 1 which was not incubated with excess cpSRP). DP* refers to degradation product representing monomeric form of LHCP.

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V. CONCLUSION AND FUTURE DIRECTIONS

Research work done here has tried to answer the questions that we asked in Chapter 1 regarding the role of different components in chloroplast signal recognition particle (cpSRP) pathway. This system lacks ribosome, RNA moiety of SRP, yet it has divided the functions of missing components very wisely among the available protein machinery of the pathway.

In chapter 2, we have tried to look at the binding site between cpSRP43 and C-terminus of Alb-3 translocase, although we did not succeed in finding the exact region of interaction on cpSRP43 but this study hinted at the presence of other types of previously unknown interactions between binding partners in soluble phase of the SRP cycle. Results suggested that these interactions could be responsible for proper orientation of the binding partners at various steps of the targeting cycle in order to result in a productive LHCP targeting. For example, some of cpSRP43 mutants lost their interaction with either full length cpSRP54 or full length LHCP or both in soluble phase of the pathway. However, there was no loss of the binding ability of these mutants when they were tested in pull down assays utilizing only the known interacting domains on the partner proteins (e.g. M-domain of cpSRP54, L-18 domain of LHCP) in solution. More work still needs to be done in understanding the role of Alb-3 translocase in chloroplast targeting cycle and this could possibly be achieved by combining biophysical techniques like intermolecular FRET (Forster resonance energy transfer) with molecular biology tools to look at the conformational changes that Alb-3 can cause in the targeting complex.

In chapter 3, we have utilized a wonderful combination of bio-physical and bio-functional techniques to confirm that chloroplast signal recognition particle 54 (cpSRP54) binds to the third transmembrane domain (TM3) of LHCP when it is associated with cpSRP43 and LHCP in transit complex in solution. Studies have also shown that both subunits of cpSRP are dynamic in nature and when combined together, both help in orienting each other in a conformation which is more

favorable for the binding events to take place in solution or at the membrane, thereby avoiding any futile targeting step in the cycle.

In chapter 4, we have looked at the role of nucleotide in GTP hydrolysis in the targeting cycle and results suggested that GTP hydrolysis is not required for LHCP integration. This result is consistent with the role of nucleotide hydrolysis in cytosolic SRP targeting in mammalian system and bacteria. GTP hydrolysis is essential to keep LHCP targeting cycle going as it replenishes stroma with the recycled cpSRP as well as available Alb-3 translocase at the membrane to continue targeting events.

There is still some work needed in this area to understand the protein-protein interactions at the membrane, to confirm if translocase Alb-3 is involved in co-translational targeting of chloroplast encoded proteins and if it does, then how do the two types of targeting events compete in finding the available translocase.

V.   APPENDIX

UNIVERSITY OF ARKANSAS.

January 30, 2014

MEMORANDUM

TO: Dr. Ralph Henry

FROM: W. Roy Penney
              Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #:   10044

Protocol Title: "Protein Targeting to the Chloroplast Thylakoid Membrane"

Approved Project Period: Start Date: January 26, 2014
                          Expiration Date: January 25, 2017

The Institutional Biosafety Committee (IBC) has approved the Renewal of Protocol 10044, "Protein Targeting to the Chloroplast Thylakoid Membrane". You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.