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The Mechanism of LHCP Insertion Into Thylakoid Membranes

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by

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ABSTRACT

The light harvesting chlorophyll a/b-binding proteins (LHCPs) are the most abundant membrane proteins. LHCP is a nuclear encoded protein which is targeted to the thylakoid membranes by chloroplast signal recognition particles (cpSRP). Insertion into thylakoid membranes is facilitated by the cpSRP receptor cpFtsY and the Alb3 translocase. Work here focused on understanding the molecular events of LHCP insertion into the thylakoid membranes. Specifically, we sought to develop a tool to detect the insertion of the lumen-localized loop of LHCP into thylakoid membranes, which relies on cleavage of the loop by a thylakoid lumen processing protease. We also sought to understand effects of lumenal loop insertion mutations in LHCP on trimerization and association with photosystem II.

Our data shows that insertion of the D1 processing site, a cleavage site in the D1 protein of photosystem II, in the lumenal loop of LHCP functions as a tool to detect the integration of LHCP into the thylakoid membranes. Cleavage of the D1 processing site is performed by lumen protease, C-terminal processing protease. Cleavage of the processing site is also independent of thermolysin treatment of the thylakoid membranes.

Our data also shows that insertion of the OE33 signal peptide site or the D1 processing site in the lumenal loop of LHCP affects LHCP assembly into trimer. Insertion of the OE33 or the D1 cleavage site after amino acid 134 of LHCP results in formation of trimeric and monomeric LHCP upon integration into thylakoid membranes. Interestingly, this mutation also prevents LHCP assembly into photosystem II. The slow assembly of trimer and lack of photosystem II association appears to be unique to the mutation at position 134.

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ABBREVIATIONS

ΔpH- pH gradient across a membrane

Alb3- Albino 3 protein

ATP- adenosine triphosphate

Chl- chlorophyll

cpSec- chloroplast secretory

cpSRP- chloroplast signal recognition particle

cpSRP43- 43 kD subunit of the cpSRP

cpSRP54- 54 kD subunit of the cpSRP

cpFtsY- chloroplast FtsY homologue (cpSRP receptor)

cpSecA, E, Y- chloroplast SecA, E, Y

cpTatC- cpTat subunit C

CSI- cleavage site insertion

DLU- digital light units

DNA- deoxyribonucleic acid

DP- degradation product

ER- endoplasmic reticulum

Ffh- fifty-four homologue

FtsY- SRα homologue in bacteria

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, 10 mM $MgCl_2$

kD- kiloDalton

LHC- light- harvesting complex

LHCP- light- harvesting chlorophyll a/b- binding protein

m- mature form

Min- minute

OE33- 33 kD component of the oxygen evolving complex

p- precursor

PCR- polymerase chain reaction

PS-I, PS- II- photosystem I, II

RNA- ribonucleic acid

RNC- ribosome nascent chain complex

Sec- secretory

SecA- cytosolic chaperone in Sec pathway

SecB- cytosolic chaperone in Sec pathway

SecEGY- E, G, Y subunits of the bacterial Sec translocon

SecGDF- G, D, F, subunits of the bacterial Sec translocon

SDS- sodium dodecylsulfate

SDS- PAGE- SDS- polyacrylamide gel electrophoresis

SOE- splicing by overlap extension

SP- signal peptide

SR- SRP receptor

SRα, SRβ- α and β subunits of the SR

SRP- signal recognition particle

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

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

Thy- thylakoid membrane

TM- transmembrane domain

TP- translation product

UV- ultraviolet

INTRODUCTION

In eukaryotic cells many nuclear encoded proteins are synthesized in the cytosol and routed to membrane bound organelles. This molecular event is an important process which directs proteins to their functional destination. Entrance into the organelle is the final stop for some proteins, however additional routing is required to target proteins destined for the inner membranes of organelles such as chloroplasts and mitochondria. Proteins targeted to the inner membranes have targeting and membrane translocation mechanisms that reflect physical characteristics of the targeting substrate. Proteins inserted into the inner membranes often associate with other membrane proteins as part of an assembly process that yields a fully functional protein complex. Work conducted here focused on the insertion pathway of light harvesting chlorophyll-a/b binding proteins into the thylakoid membranes, which assemble into light harvesting complexes that subsequently associate with photosystem I or II.

Protein Sorting to the Chloroplast

The chloroplast is a specialized organelle found in photosynthetic eukaryotes (e.g. higher plants), which contains chloroplast DNA that codes for proteins transcribed and translated in the stroma. However, the vast majority of chloroplast proteins are nuclear encoded, synthesized as full-length precursors in the cytosol, and enter the chloroplast using a general protein translocase in the outer and inner chloroplast envelope that recognizes a cleavable amino terminal transit peptide (Heazlewood *et al.*, 2005). Once the protein reaches the chloroplast stroma, the transit peptide is cleaved by a stromal processing protease. Although some proteins remain in the stroma, four different targeting pathways route thylakoid proteins from the stroma for insertion into the thylakoid lipid bilayer or translocation into the thylakoid lumen (depicted in Figure 1).

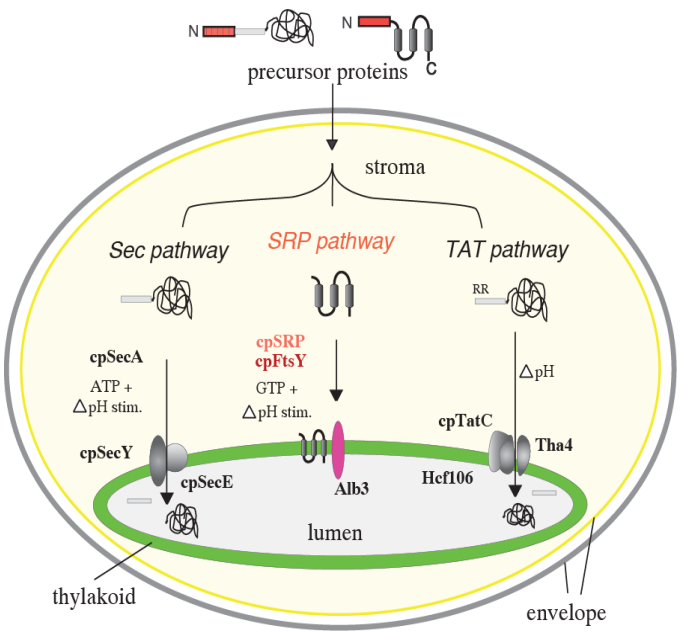

Figure 1. Nuclear encoded proteins are directed to the thylakoid membranes by precursor-specific pathways.
Synthesis of precursor proteins with an N-terminal chloroplast targeting domain (depicted by red box) occurs in the cytosol. Translocase located in the chloroplast envelope allows proteins to enter the stroma where the targeting domain is removed by a stromal protease. A thylakoid targeting domain, depicted by gray box directs proteins across the thylakoid membrane by the cpSec, or cpTAT pathway. Integral thylakoid proteins rely on the spontaneous insertion pathway (not shown) or a chloroplast SRP pathway in the case of LHCP localization. Adapted from (Henry et. al, 2007).

Spontaneous Insertion

The spontaneous thylakoid insertion pathway allows proteins to insert into the lipid bilayer without the need for soluble or membrane protein components. The CFoII subunit of ATP synthase is spontaneously inserted into the thylakoid membranes of chloroplasts (Michl et al., 1994). CFoII is synthesized in the cytosol with a bipartite transit peptide that contains an envelope targeting domain followed by a hydrophobic signal sequence, which directs the stromal pathway intermediate to the thylakoid membrane (Herrmann et al., 1993). Using isolated thylakoids, insertion is not inhibited by the ionophore nigericin or by protease pretreatment of thylakoids known to prevent protein insertion by the other three thylakoid transport pathways. Proteins that utilize this pathway possess a single transmembrane spanning domain. Insertion is thought to rely on formation of a helical hairpin structure held together by interaction of the hydrophobic signal sequence and the transmembrane domain (Engelman and Steitz, 1981). The protein is then spontaneously inserted into the membrane exposing the

signal sequence cleavage domain to the lumen where signal cleavage by the thylakoid processing peptidase takes place.

Secretory Pathway

The chloroplast secretory (cpSec) pathway is homologous to the bacterial Sec pathway and is used to translocate a subset of thylakoid lumen proteins across the thylakoid membrane. The best-studied cpSec targeting substrates are nuclear encoded and utilize a bipartite transit peptide. The cpSec pathway requires cpSecA, an ATPase homologous to SecA in bacteria. CpSecA is found in the stroma and contains a signal peptide binding site and acts as a chaperone leading the protein to the translocon complex, cpSecE and cpSecY (Laidler et al., 1995). Once the cpSecA-substrate complex reaches the thylakoid membrane, cpSecA binds to a translocon composed of cpSecE/cpSecY. The substrate can pass through the translocon in the presence of ATP and a proton gradient, although dependence on the proton gradient is substrate specific. A cycle of hydrolysis and release of ATP is necessary to cause conformational changes to occur in cpSecA that drives the substrate through the pore of the translocon. Exposure of the protein's thylakoid targeting peptide in the lumen allows the lumenal processing peptidase to cleave the signal sequence from the mature protein. Plastocyanin is an example of a nuclear encoded photosynthetic protein that enters the thylakoid lumen by the sec dependent pathway. The lumen targeting signal peptide used to route cpSec substrates generally contain a positively charged amino acid at the N-terminus, a hydrophobic region, and a polar region at the C-terminus followed by a cleavage site. The mature domain must be in an unfolded state to pass through the cpSecY/E translocation pore. No additional stromal proteins appear necessary to keep the substrate in an unfolded state.

The sec dependent pathway in bacteria is very similar to the events which take place in the chloroplast. However, additional components of the pathway have been identified in bacteria which have not been found in the chloroplast. In bacteria the sec translocon has an additional component, SecG, and a SecDF complex associated with the translocon. In addition to the transport protein SecA, bacterial systems also use SecB which associates with substrates and may help the substrate remain unfolded during translocation/insertion. The translocation of some substrates in bacterial systems requires yet

another component. Cytochrome o oxidase is a substrate of the sec pathway in *E. coli* that requires the help of YidC, a membrane protein that participates in translocation. YidC associates with the sec translocon SecEGY to help insert transmembrane domains of cytochrome o oxidase into the membrane (du Plessis *et al.*, 2006).

Twin Arginine Transport Pathway

The chloroplast twin arginine transport (cpTAT) pathway, first identified in mutant maize, translocates folded proteins across the thylakoid membrane. Identification of the cpTAT pathway led to identification of a homologous transport system in bacteria. Substrates of this pathway contain a twin arginine motif found in the signal peptide region of bipartite transit peptides, similar to the twin arginine motif required for TAT pathway transport in E. coli targeting substrates (Ser/Thr-Arg-Arg-X-Phe-Leu-Lys, where X is any polar amino acid) giving the pathway its name (Berks, 1996). The cpTAT pathway is also referred to as the delta pH (ΔpH) pathway since translocation is dependent upon a proton gradient across the membrane. In chloroplasts, the cpTAT translocase is composed of the membrane proteins Tha4, Hcf106, and cpTatC (TatA, TatB, and TatC, respectively in prokaryotes) (Lee et al., 2006). TatC is a transmembrane protein that associates with Hcf106 in the thylakoid membrane. The substrate docks to the TatC through an interaction with the signal peptide twin arginine motif. Once the TatC-Hcf106-substrate complex is formed the substrate is passed to Tha4 in a ΔpH dependent step. Tha4 is a channel that translocates a TAT substrate in a folded state. Upon entering the lumen, the TAT substrate transit peptide is cleaved by a processing protease.

In *E. coli* eight proteins have been found lacking the TAT signal sequence, but are translocated via the TAT pathway. Such proteins follow the hitchhiker mechanism, forming a complex with another substrate containing the TAT signal sequence (Lee et al., 2006). All eight *E. coli* proteins are redox proteins that are often transported with its partner. For example, HybC is a hydrogenase that forms a complex with its redox partner HybO. Only HybO contains the TAT sequence responsible for leading the HybO/HybC complex to the plasma membrane for translocation (Lee et al., 2006) by TatABC.

Signal Recognition Particle Pathway

Signal recognition particle (SRP) was first discovered in mammalian cells along with the signal recognition receptor (SR) and shown to target nascent membrane and secretory proteins from the cytosol to the endoplasmic reticulum by a co-translational targeting mechanism (Walter and Blobel, 1980; Gilmore et al., 1982; Walter and Blobel 1982). Since then, the SRP/SR targeting mechanism has been found in numerous organisms in all three domains of life. In eukaryotes and prokaryotes, cytosolic SRPs all contain an RNA moiety, but differ in the number of protein subunits and the structure of SR. For instance, six SRP subunits (SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72) are found in mammals and only one SRP subunit (Ffh; fifty four homologue) is found in bacteria (Walter and Blobel, 1980). Despite differences in complexity, the key components of the SRP pathway are universally conserved.

In eukaryotes, SRP54 (named by molecular weight) associates with the 7S RNA moiety and functions to bind N-terminal signal sequences of newly made polypeptides as they emerge from a translating ribosome. SRP54 bound to a ribosome-nascent chain complex (RNC) interacts with SR at the surface of the ER membrane bringing with it the entire mRNA-RNC complex. In eukaryotes, the SR is composed of two subunits, SRα and SRβ. SRP binding to SR takes place through interaction between SRP54 and SRα, both of which are GTPases (Krieg et al, 1986; Keenan et al, 2001). GTP is hydrolyzed during SRP-SR interaction and is believed to release the ribosome-nascent chain (RNC) complex from SRP allowing the RNC to associate with a nearby translocon in the ER. The RNC and translocon association re-initiates translation to allow co-translational protein transport into or across the ER membrane.

There are differences in the prokaryotic homologs of SRP54 and SRα/SRβ that result in slight changes in the molecular events of the SRP pathway. In prokaryotes, the homologs of SRP54 and SRα/SRβ are Ffh and FtsY, respectively. Ffh binds to 4.5S RNA and GTP prior to recognizing the signal sequence on the nascent chain. Unlike in eukaryotes, translation of the nascent chain does not halt upon binding of Ffh. Eukaryotic 7SL RNA (bound to SRP54) contains an Alu domain that is bound by SRP9 and SRP14 which arrest translation during transport of the RNC complex to the membrane (Strub et al., 1991). Prokaryotic 4.5S RNA lacks an Alu domain and no homologs of SRP9/SRP14 have been identified

in prokaryotes. These additional components found in eukaryotes are thought to increase efficiency during translocation. The membrane receptor FtsY is a single polypeptide that can be found in the plasma membrane or in the cytosol. It has GTPase activity that serves the same purpose as in eukaryotes. FtsY has a less complex structure than the two subunit SRα/SRβ receptor. Although there differences in structure and pathway components between the eukaryotic and prokaryotic SRP pathways, Ffh and FtsY can efficiently substitute for SRP54 and SRα *in vitro* (Powers and Walter, 1997). This demonstrates that the SRP and SR interactions are evolutionarily conserved and have great impact on the pathway (Keenan et al., 2001).

LHCP Targeting To Thylakoids Uses a Novel Organellar SRP

Light harvesting chlorophyll a/b binding proteins (LHCPs) are a family of nuclear encoded chloroplast proteins that are inserted into the thylakoid membranes post-translationally by an SRP in chloroplasts (cpSRP). LHCPs are the only known substrates for the post-translation SRP pathway. LHCPs have three transmembrane domains and a mature size of 20-28 kD. The pea cab80 gene product, hereafter referred to as LHCP, codes for the most abundant of the Photosystem II (PS-II)-associated LHCPs. Precursor LHCP (30 kD) is synthesized in the cytosol with a cleavable transit peptide allowing it to transverse the chloroplast envelope. During or soon after its entry into the stroma, LHCP is bound by cpSRP, which is composed of two subunits, cpSRP54 and a novel cpSRP43 unique to chloroplasts. The cpSRP54-cpSRP43-LHCP complex, termed 'transit complex', serves as the soluble targeted form of LHCP in the chloroplast. Transit complex is directed to the thylakoid membrane where it docks with the SRP receptor, cpFtsY, a reaction that relies on GTP binding by both cpSRP54 and cpFtsY (Moore et al., 2003). Association of the transit complex with cpFtsY at the thylakoid recruits the translocon Albino 3 (Alb3). Binding of the Alb3 C-terminus to cpSRP43 stimulates LHCP release from cpSRP and stimulates GTP hydrolysis by cpSRP54/cpFtsY causing release of cpSRP from its receptor to recycle cpSRP for subsequent rounds of targeting (Falk et al., 2010; Moore et al., 2000). Stable insertion of LHCP relies on the presence of chlorophyll (Kuttkat et al., 1997). In steps not fully understood, LHCP is inserted into the thylakoid membranes, bound by chlorophyll, and assembled into trimeric LHCP (Cline,

1988; Kuttkat et al., 1995). Trimeric LHCP then associates with the PS-II complex to participate in energy transfer during photosynthesis.

Past research has been done to understand the assembly of monomeric LHCP into trimer in the thylakoid membranes. The monomeric and trimeric forms of LHCP can be distinguished upon protease treatment of the thylakoids (Kuttkat et al., 1995). Trimeric LHCP is protease resistant, except for 6 kD on the N-terminus of pLHCP, resulting in a 24 kD protein referred to as degradation product (DP). The stroma-exposed N-terminus of integrated monomeric LHCP exhibits increased sensitivity to protease resulting in a 20 kD protein degradation product, DP*. LHCP mutagenesis studies have identified regions necessary for trimerization. Hobe et al. determined that mutations in the stroma exposed N terminus of *Pisum sativum* mature LHCP at amino acids positions W16 and/or Y17 and R21 inhibit the formation of trimer (Hobe et al., 1995). The lumen-exposed C-terminus of LHCP also appears critical to LHCP trimer assembly; replacement of W222 with a histidine abolishes trimerization, although the hydrophobic residue phenylalanine is tolerated (Kuttkat et al., 1996).

Despite having a general understanding of LHCP targeting to thylakoid by cpSRP, the mechanism by which LHCP is inserted by Alb3 and subsequently assembled into a trimeric light harvesting complex remains largely unexplored. For instance, do all three LHCP transmembrane domains (TMs) insert simultaneously, or does insertion require stepwise insertion of each TM? Is Alb3 responsible for insertion of all three TMs or does insertion of one or more TMs take place in an Alb3-independent manner, possibly at a step in the targeting mechanism that precedes Alb3 association with the membrane-associated the cpSRP-LHCP-cpFtsY complex? In this study, we constructed a tool to answer questions concerning the molecular event of LHCP insertion into the thylakoid membranes. This tool was developed to detect insertion of the TMs by inserting a signal peptide cleavage site from PS-II proteins OE33 or D1 into the lumenal loop of LHCP between TM1 and TM2. Cleavage of the processing site by a thylakoid processing peptidase indicates TM1 and TM2 have crossed the thylakoid membrane. Construction of these mutant LHCPs also have an effect on LHCP trimer assembly and trimer association with PS-II. Disruption of the helix ^{133}VWFKAGSQIFS at amino acid position 134 by the OE33 cleavage

site insert forms a low molecular weight degradation product referred to as DP^a in addition to DP and DP*. Furthermore, the trimers formed by mutations at position 134 are unable to associate with PS-II.

MATERIALS AND METHODS

Construction Of Precursor LHCP Mutants

Coding sequences for precursor LHCP (pLHCP) containing signal peptidase cleavage site insertions (CSI-SP) were constructed by polymerase chain reaction using splicing by overlap extension (PCR-SOE) (Horton et al., 1989). Construction of all CSI pLHCP insertion mutants utilized overlapping internal primers to introduce the Pisum sativum OE33 signal peptide cleavage site SGASAEG following amino acid 122, 134, or 145 of pLHCP to produce CSI-SP_{122}, CSI-SP_{134}, and CSI-SP_{145}. External forward and reverse primers for pLHCP introduced EcoRI and SalI restriction sites, respectively allowing restricted SOE-PCR products to be ligated into similarly restricted pGEM-4Z using T4 DNA ligase. Ligation products were subsequently transformed into E. coli strain TB1.

Overlapping internal primers were also used in PCR-SOE reactions to construct pLHCP clones that introduce the coding sequence for Pisum sativum D1 processing site (EVMHERNAHNFPLDLAAVEAPSING). CSI-$D1_{122}$ and CSI-$D1_{134}$ in pGEM-4Z code for D1 cleavage site insertions following amino acid 122 and 134 of pLHCP.

pLHCP mutant name	Protein inserted	Insertion after pLHCP residue
CSI-SP_{145}	OE33	145
CSI-SP_{122}	OE33	122
CSI-SP_{134}	OE33	134
CSI-$D1_{122}$	D1	122
CSI-$D1_{134}$	D1	134

Table 1. pLHCP mutant constructs.
All five pLHCP mutants are listed by name. The source of the inserted protein and location in pLHCP is also listed.

All pLHCP CSI mutants were sequenced (DNA Sequencing Laboratory, University of Arkansas for Medical Sciences, Little Rock, Arkansas) to verify the fidelity of primer synthesis (Integrated DNA Technologies) and DNA polymerase (Genesee Scientific) used for PCR-SOE.

Chloroplast and Thylakoid Isolation

Pea seedlings 9-12 days old were used to isolate intact chloroplasts, prepare thylakoids, and stroma extract as previously described (Cline et al., 1993). Chloroplasts were lysed by resuspending chloroplasts to 1 mg/ml chlorophyll in HKM (10 mM HEPES-KOH pH 8, 10 mM MgCl2) and incubating for 5 min on ice. Lysate was then adjusted by adding an equal volume of Import Buffer (IB: 50 mM HEPES-KOH, pH 8.0 and 330 mM sorbitol). Thylakoids were isolated by centrifugation at 3200 x g for 8 min and buffer washed twice with IB containing 10 mM $MgCl_2$ (IBM). The chlorophyll (Chl) concentration was determined as described by (Arnon, 1949) using a UV- visible spectrophotometer (BioSpec- 1601).

Production of Radiolabeled Proteins by In Vitro Transcription/Translation

In vitro transcribed capped mRNA was translated and radiolabeled proteins were produced by in vitro translation using a wheat germ system in the presence of radiolabeled S^{35}- methionine (Chu et al., 2004; Cline et al., 1993). Translation products for wildtype pLHCP and pLHCP mutants were diluted two fold with 60 mM unlabeled methionine in IB.

Transit Complex Assay

Transit complex assays included 1 µg of cpSRP43 and cpSRP54, 1.5 mM MgATP (final), and 5 µl radiolabeled pLHCP translation product. HKM was used to bring the final volume to 20 µl. HKM was used for negative controls without SRP. The reaction was incubated at 25°C for 15 min followed by addition of 5 µl 50% glycerol with bromophenol blue. Samples were analyzed by 6% native gel and phosphor imaging.

Integration Assays

Integration assays were conducted by mixing buffer washed thylakoids (equal to 25 µg Chl) in IBM, 5 mM (final) MgATP in IB, 1 mM (final) NaGTP in IB, 12.5 µl of 1:2 diluted radiolabeled pLHCP translation product prepared as described above, and 1 µg each of recombinant cpSRP43, cpSRP54, and cpFtsY. IBM was used to bring the final volume to 75 µl. The reaction was incubated at 25°C for 30 min in the presence of light. Thylakoid membranes were pelleted at 3200 x g for 8 min followed by protease treated with 12.5 µl of 2 mg/ml thermolysin and 10 mM $CaCl_2$ for 45 min at 4°C. Thermolysin was

inactivated by adding 50 mM EDTA in IB. Protease treated thylakoid membranes were recovered by centrifugation at 3200 x g for 8 min. Thylakoid membranes were resuspended in 20mM EDTA in H_2O then solubilized in 1 + 1+ 1 with Lithium dodecyl sulfate at 4°C for 30 min. 10 µg Chl samples were analyzed by SDS-PAGE and phosphor imaging.

Import Assays

Import assays were conducted using intact chloroplasts (equal to 100 µg Chl) in IB, 10 mM MgATP (final), and 50 µl of 1:2 diluted radiolabeled translation product. IB was added to bring the final volume to 300 µl. The reaction was incubated at 25°C for 10 min in the presence of light. Chloroplasts were centrifuged at 3200 x g for 8 min through a 35% Percoll gradient. Intact chloroplasts were washed in IB and recovered by centrifugation at 1000 x g for 5 min. HKM was used to lyse chloroplasts at 4°C for 5 min. After the chloroplasts were lysed, IB was added and thylakoids membranes were centrifuged at 3200 x g for 8 min. Thylakoid membranes were suspended in 0.5 ml IB and protease treated with12.5 µl of 2 mg/ml thermolysin in 10 mM $CaCl_2$ at 4°C for 45 min. Following protease treatment 50mM EDTA was added to inactivate the protease, and thylakoid membranes were centrifuged at 3200 x g for 8 min. Thylakoid membranes were suspended in 20mM EDTA in H_2O and solubilized in 1 + 1 + 1 with Lithium dodecyl sulfate at 4°C for 30 min. 10 µg Chl samples were analyzed by SDS-PAGE and phosphor imaging.

LHC Assembly Assays

Following import of LHCP constructs into isolated chloroplasts and recovery using a 35% Percoll cushion as described above, intact chloroplasts were washed in 1 ml IB, recovered by centrifugation at 1000 x g, and lysed by resuspension in HKM (200 µL). Following 5 min incubation at 4°C, thylakoid membranes were collected by pelleting at 3200 x g for 8 min. The 50 µg chlorophyll pellet was solubilized in 5 µl of glycerol, 5 µl of 10% Maltoside in IB, and 40 µl of HKM at 4°C for 30 min. 7 µg Chl samples were analyzed by non-denaturing polyacrylamide gel electrophoresis (native gel) with 0.05% maltoside as described by (Bass and Bricker, 1988) to separate Photosystem I and II. The native gel was then analyzed by UV- transillumination and phosphor imaging.

Imaging Tools and Quantification of Radiolabeled Wildtype and Mutant LHCP

SDS-PAGE and native gels were imaged using a Cyclone Plus (PerkinElmer) and Optiquant Software (PerkinElmer). Native gels with 0.05% maltoside were imaged using UV- Transilluminator (UVP) and Cyclone Plus. The relative amounts of radiolabeled proteins were quantified from phosphor images using wild type LHCP as a control or by comparison to a known amount of translation product.

RESULTS

LHCP Mutant Constructs

In order to create a tool to detect the insertion of the lumenal loop of LHCP, a processing site (derived from the OE33 signal peptide or D1 processing site) was inserted in the lumenal loop (after amino acid 122, 134, or 145). Figure 2A and 2B depict the location of the insertion sites in the LHCP model.

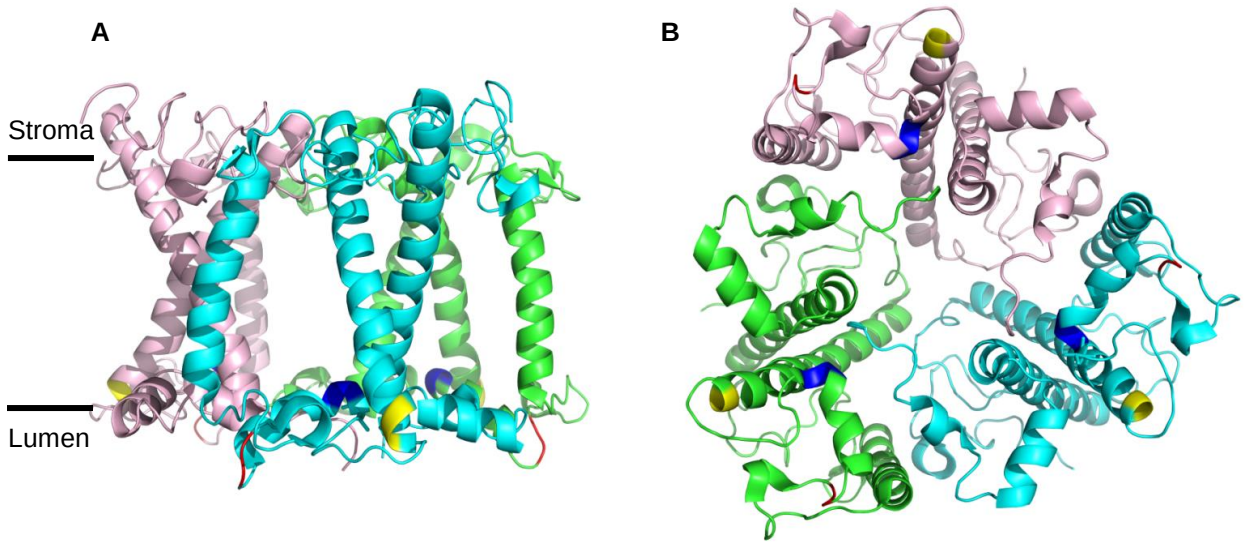

Figure 2. LHCP trimeric complex.
The trimeric LHCP membrane complex (adapted from Standfuss et al. 2005) is shown with cleavage insertion sites highlighted. The three LHCPs are colored pink, cyan, and green. The cleavage sites are colored yellow, blue, and red. The complex is shown **(A)** inside the membrane and **(B)** the lumenal side.

Cleavage Site	Amino Acids	Color
122	L, S	Yellow
134	W, F	Blue
145	G, G	Red

Table 2. Color scheme of cleavage sites in LHCP trimeric complex.
The OE33 or D1 processing site in LHCP constructs was placed at the amino acid position indicated and in-between the two listed amino acids. Both amino acids listed are colored in the LHCP trimeric complex in Figure 2.

Insertion of OE33 Signal Peptide/ D1 Processing Site Does Not Inhibit Transit Complex Formation

All five constructed mutants were translated in a wheat germ system much like wildtype LHCP as shown in Figure 3. The mutation in the lumenal loop of LHCP does not disrupt transit complex formation with cpSRP 43 and cpSRP 54 heterodimer (Figure 4).

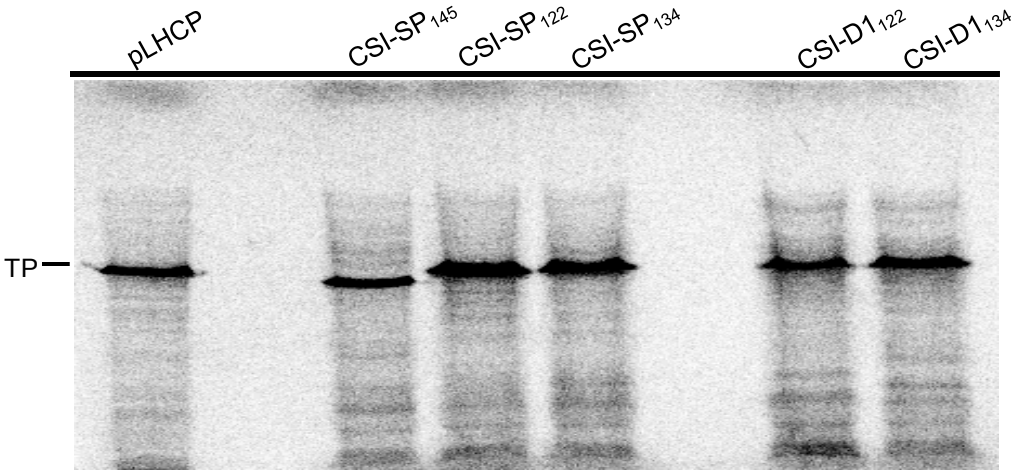

Figure 3. Translation of radiolabeled pLHCP mutants is comparable to wildtype pLHCP.
In vitro transcribed mRNA of pLHCP and pLHCP mutants were in vitro translated as described in Materials and Methods. Translation products (TP) were diluted with SB to 1:80 (final) and 10 µl were loaded on a SDS- polyacrylamide gel.

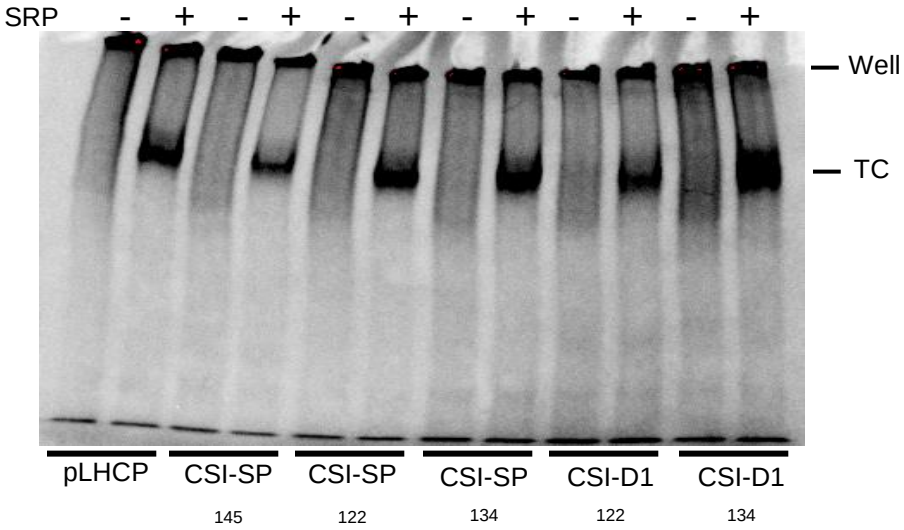

Figure 4. LHCP mutants form transit complex.
Wildtype pLHCP forms transit complex with cpSRP43 and cpSRP54, visible on a native gel. pLHCP mutant constructs were incubated in the presence or absence of SRP. The phosphor image of the native gel is shown with transit complex indicated as (TC).

Integration of LHCP Mutants Into Isolated Thylakoid Membranes

Buffer washed thylakoids were used to analyze LHCP mutants for cleavage of the processing site and integration deficits. As depicted in Figure 5A, LHCP mutants integrate into isolated thylakoids. Radiolabeled bands of integration, degradation product (DP), are present and indicative of trimeric LHCP. Also present are DP* and DP^{a} bands for wildtype LHCP and some of the other mutants. DP* is approximately 20kD and indicative of monomeric LHCP. DP^{a} is slightly smaller in size and is indicative of another conformation of LHCP. The percent of integration for the OE33 signal peptide mutants (CSI-$SP_{122,}$ CSI-SP_{134}, and CSI-SP_{145}) was approximately 2 times less than wildtype LHCP. The percent of integration for the D1 processing site mutants (CSI-$D1_{122,}$ CSI-$D1_{134}$) was greatly reduced, approximately 32 times less and 7 times less respectively, compared to wildtype LHCP integration (Figure 5B). No apparent cleavage of the OE33 signal peptide or the D1 processing site was present consistently.

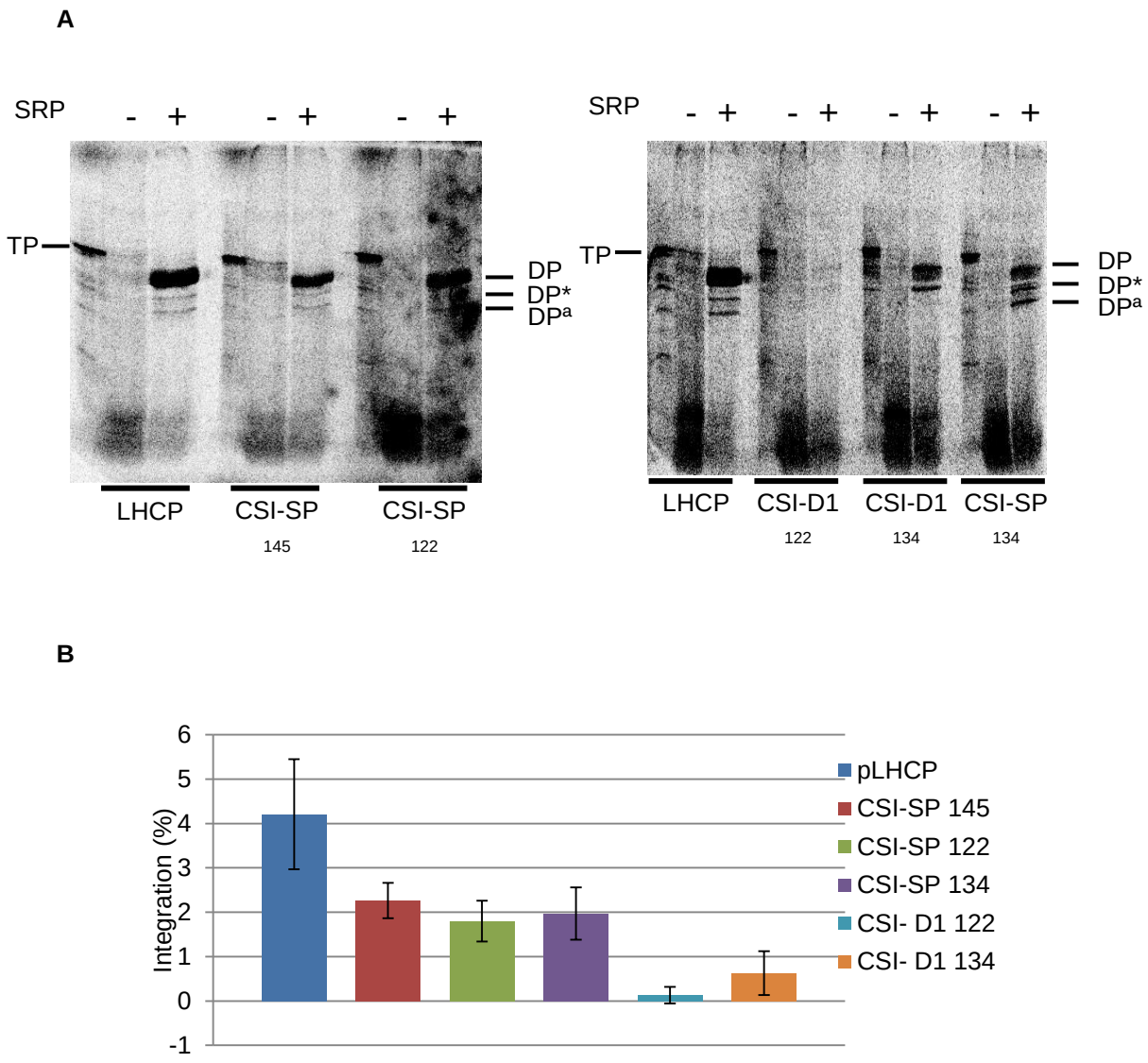

Figure 5. Mutation of LHCP lumenal loop between TM1 and TM2 affect LHCP integration. Stable integration of LHCP into thylakoids occurs in the presence of cpSRP43 and cpSRP54 forming degradation product (DP) indicative of protease resistant trimeric LHCP. **(A)** Radiolabeled pLHCP and mutant constructs were integrated into thylakoids in the presence or absence of cpSRP 43 and cpSRP 54. Monomeric LHCP is protease sensitive and forms smaller product, denoted DP*. LHCP in and unknown confirmation forms product DP^{a}. Translation product is marked as TP. **(B)** The amount of integration produced by wildtype LHCP and LHCP mutants were based on three separate experiments. The intensity of TP measured in Digital Light Units (DLUs) on the gel and the amount of 1:2 TP present for each protein were used to determine the amount of DLUs in the loaded 10 µl sample on the gel. The amount of DLUs in all degradation products (DP, DP*, and DP^{a}) for each protein were divided by the amount of DLUs in TP (adjusted for missing methionines removed after protease treatment), and multiplied by 100, and summed. This represents the amount of TP which integrated into the thylakoid membranes. The average percent integration for three assays is depicted.

Behavior Of LHCP Mutants Imported Into Intact Chloroplasts

Isolated chloroplasts were also used to analyze LHCP mutants for cleavage of the processing site and integration deficits, under in vivo like conditions. Like LHCP, all mutants contain DP, approximately 24kD, indicative of integration and trimeric LHCP (Figure 6A). In addition to DP, CSI-SP_{134} and CSI-$D1_{134}$ both consistently contain DP* bands which represents approximately 17% and 20%, respectively, of the total amount of integration (Figure 6C). CSI-D1 122 consistently displays low levels of integration, and DP* represents approximately 28% of the total integration. CSI-SP_{134} also contains a DP^a band, which represents approximately 14% of the total integration (Figure 6D). The total percent integration (sum of DP, DP*, and DP^a) for OE33 signal peptide mutants was approximately 2.5 times less than wildtype LHCP. The total percent integration for CSI-$D1_{134}$ was about 3 times less and CSI-$D1_{122}$ was about 7 times less than wildtype LHCP. Although the D1 mutants showed less DP, cleavage of the inserted processing site was indicated by the presence of lower molecular weight products. These mutants may be used as tools to indicate the integration of the lumenal loop of LHCP into thylakoid membranes in future studies.

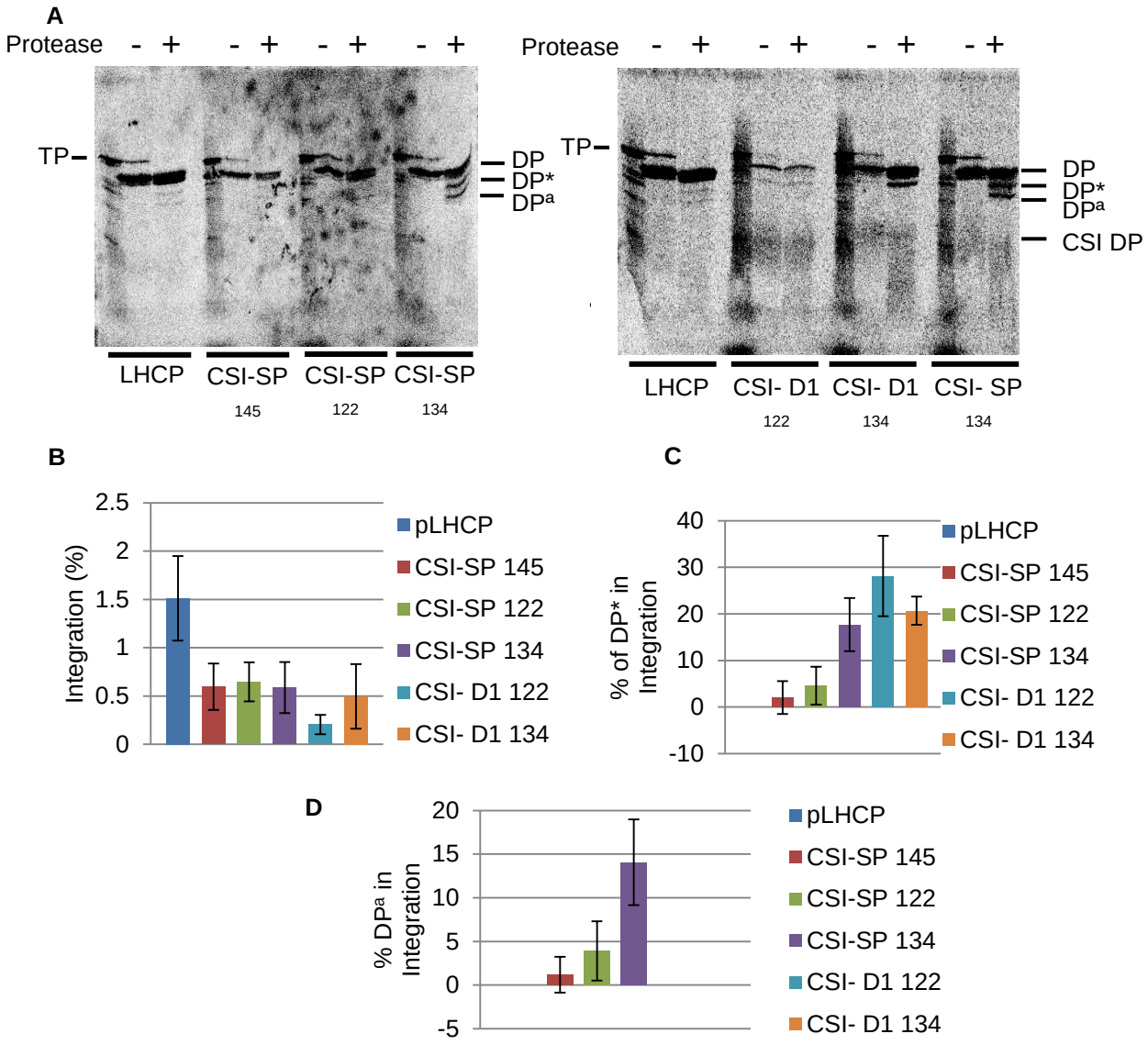

Figure 6. Mutation of pLHCP lumenal loop affects LHCP integration in intact chloroplasts. Radiolabeled pLHCP and mutant constructs were imported into intact chloroplasts with 10mM (final) MgATP. **(A)** Properly inserted LHCP forms a largely protease resistant trimer referred to as degradation product (DP). Monomeric LHCP is sensitive to protease and is labeled as DP*. LHCP in an unknown conformation is sensitive to protease, this degradation product is denoted DP^a. Each LHCP mutant contains a cleavage site insertion. Processing of this site results in low molecular weight bands labeled CSI DP. **(B)** The amount of integration was calculated from three separate assays. The intensity of TP measured in Digital Light Units (DLUs) on the gel and the amount of 1:2 TP present for each protein was used to determine the amount of DLUs in the loaded 10 µl sample on the gel. The amount of DLUs in all degradation products (DP, DP*, and DP^a) for each protein were divided by the amount of DLUs in TP (adjusted for missing methionines), multiplied by 100, and summed to calculate the total integration in an assay. This represents how much translation product integrated into the thylakoid membranes. **(C)** The amount of DP* was divided by the total integration and multiplied by 100. **(D)** The amount of DP^a was divided by the total integration and multiplied by 100. CSI-SP$_{145}$ did not consistently display DP* and DP^a, which accounts for the margin of error.

Association Of LHCP Mutants With Photosystem II

Radiolabeled LHCP and mutants were imported into intact chloroplasts. The chloroplasts were lysed and recovered, and then run on a native gel (0.05% maltoside final). Gel electrophoresis separated PS-I and PS-II into two distinct bands as shown in Figure 7A (Bass and Bricker, 1988). The native gel was place on a UV-transilluminator, allowing chlorophyll containing proteins in PS-II to fluoresce (Figure 7B). A gel treated under similar conditions was then analyzed by phosphor imaging to determine whether the radiolabeled LHCP was associated with PS-II, as shown in Figure 7C. Wildtype LHCP, CSI-SP_{145} and CSI-SP_{122} show a distinct radiolabeled band at PS-II. CSI-SP_{134} displays a distinct band not associated with PS-II. CSI-$D1_{134}$ appears to be in multiple states and not completely associated with PS-II due to broad stretch of radiolabeled signal. No clear band present for CSI-$D1_{122}$, however this mutant consistently displayed very low levels of integration compared to wildtype LHCP.

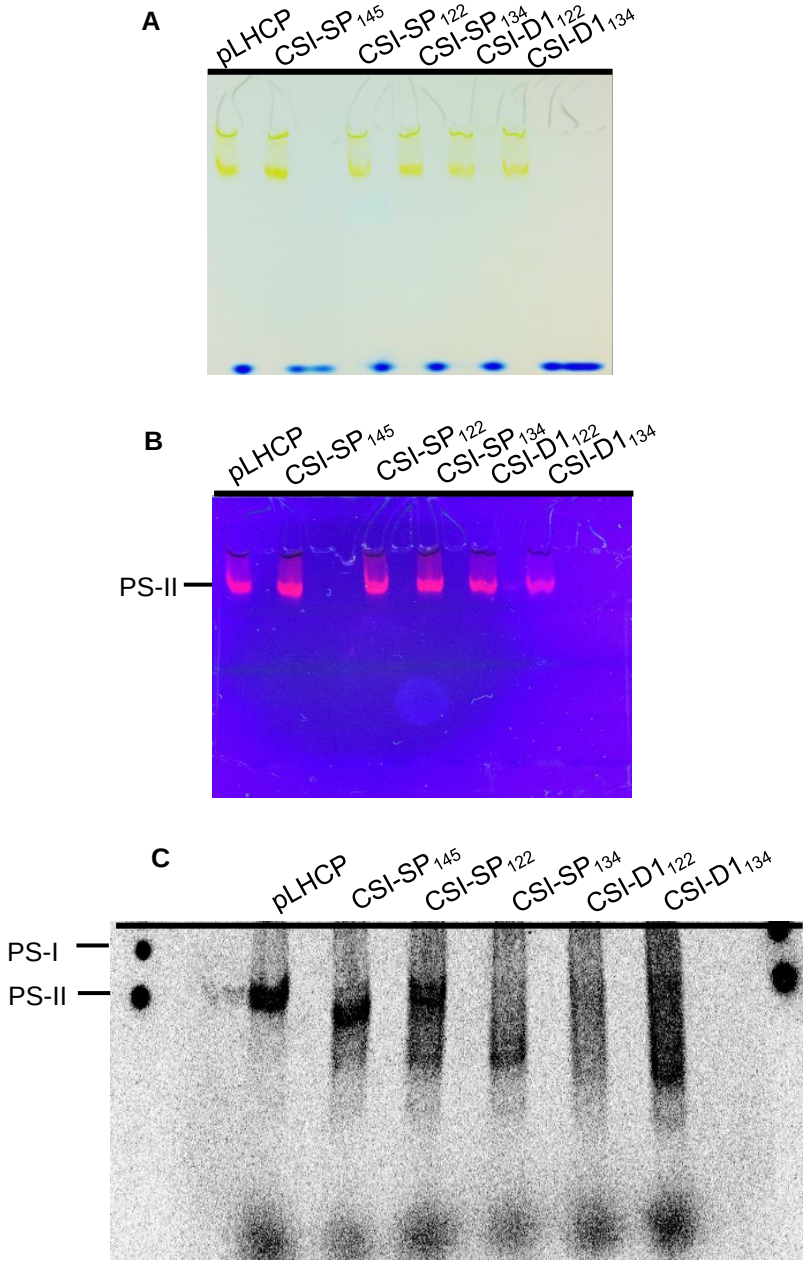

Figure 7. LHCP mutants lose association with Photosystem II.
Radiolabeled pLHCP constructs were imported into chloroplasts. Chloroplasts were lysed; thylakoids were recovered and solubilized in maltoside. Thylakoid protein complexes were examined using native gel electrophoresis in the presence of 0.05% maltoside. **(A)** Gel electrophoresis separated Photosystem complexes I and II. **(B)** PS-II complexes were identified by chlorophyll fluorescence using excitation with UV illumination (360 nM). Photosystem II fluoresces when exposed to UV light (Bass and Bricker, 1988) and is labeled PS-II. **(C)** A native gel treated under the same conditions as the native gel pictured in Figure 7a was analyzed by phosphor imaging to determine the PS-II association of radiolabeled LHCP mutants. The position of green bands corresponding to photosystems I and II (denoted PS-I and PS-II, respectively) is shown.

Cleavage Site Processing Requires Integration Into The Thylakoid Membrane

In order to promote processing of the cleavage site of CSI-SP $_{134}$, the mutant was allowed to sit at 4°C for 45 min after import of radiolabeled LHCP into intact chloroplasts. During this 45 min, half of the mixture was protease treated. The control in this experiment was not allowed to sit for 45 min and did not receive protease treatment of the chloroplasts. Results in Figure 8 show processing of the OE33 cleavage site increased during the additional 45 min incubation. The cleavage products are insensitive to protease treatment of the chloroplasts and thylakoid membranes, indicating this material is stably integrated into the thylakoid membranes.

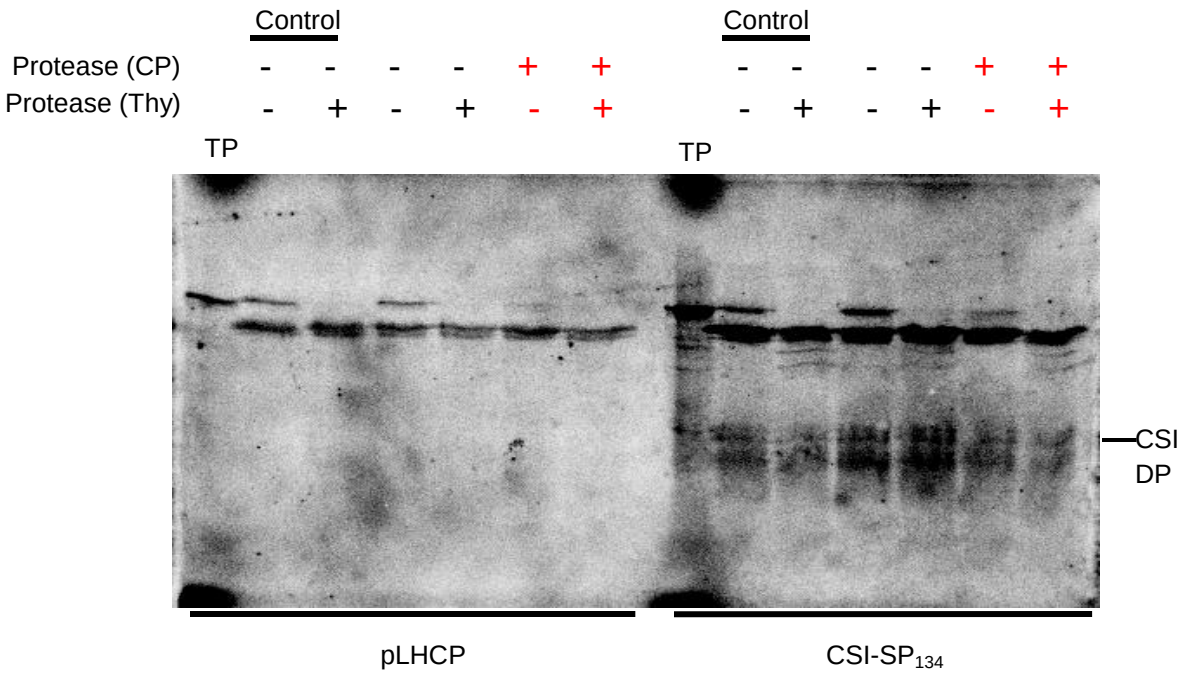

Figure 8. Cleavage site processing requires integration into the thylakoid membrane.
Following import of radiolabeled pLHCP (wild type) and CSI-SP$_{134}$ into intact chloroplasts, a portion of the chloroplasts (CP) were treated with protease to remove radiolabeled protein from the chloroplast surface. Thylakoids (Thy) recovered were examined before and after protease treatment. The translation product lanes are labeled TP. Red +/- indicates lanes which contain isolated thylakoids. Black +/- indicates lanes which contain chloroplasts.

DISCUSSION

The molecular events of LHCP insertion into the thylakoid membrane are not fully understood. Several key membrane steps have been identified such as, formation of a stable LHCP-SRP54/43/FtsY complex alone and in association with the Alb3 translocase, and chlorophyll binding which are all essential for LHCP insertion. Whether or not Alb3 is required to insert all three transmembrane domains is not known. It is possible that a portion of LHCP inserts upon arrival at the thylakoid, perhaps spontaneously, while still associated with cpSRP and that Alb3 is required for insertion of the remaining uninserted protein in a subsequent step.

In this study, we constructed LHCP mutants to develop a tool to detect LHCP insertion into the thylakoid membrane, which could then be used to examine insertion when Alb3 availability is restricted. Introduction of a processing site should yield two cleavage products upon exposure to the lumen-localized thylakoid processing protease (Nilsson and von Heijne, 1991). All constructs, CSI-SP_{122}, CSI-SP_{134}, CSI-SP_{145}, CSI-$D1_{122}$, and CSI-$D1_{134}$ were properly inserted into thylakoid membranes. The data demonstrates that insertion of *Pisum sativum* D1 processing site at positions 122 and 134 of pLHCP does yield cleavage of the intended processing site as seen in Figure 6a. Mutants CSI-$D1_{122}$ and CSI-$D1_{134}$ can therefore be used in future projects to determine the timing of insertion of LHCP TMs. Consistent cleavage of the CSI-SP constructs was not observed.

Surprisingly, introduction of the OE33 or D1 processing site at position 134 in pLHCP affected trimer assembly. Integration of CSI-SP_{134} and CSI-$D1_{134}$ led to multiple DPs upon post treatment with protease. The presence of bands designated as DP and DP* indicate that both trimeric and monomeric forms of LHCP are formed (Kuttkat et al., 1995). Position 134 of pLHCP is a part of a small helix, 133VWFKAGSQIFS. The OE33/D1 insert was placed between 134W and 135F, two large hydrophobic amino acids. The simultaneous occurrence of DP and DP* suggests that trimer assembly is slowed by the processing site insertion and suggests that position 134 is necessary for efficient trimer assembly. Additional mutations in this helical region could help determine whether position 134 alone is critical to trimer assembly or if this residue is part of a trimer assembly motif. In past studies, Kuttkat et al. determined that sites at the N-terminus and C-terminus of LHCP were important for trimerization, however no mutations were made in the lumenal loop between TM1 and TM2 (Kuttkat et al., 1995; Kuttkat et al.,

1996). The presence of a prominent DP* band, indicative of integrated monomeric LHCP, was not observed with the other LHCP CSI constructs.

An additional DP, referred to as DP^a, is observed only in CSI-SP$_{134}$ upon insertion and protease treatment of the thylakoids. DP^a has a lower molecular weight migrating just below DP*. A DP of this size has not been previously reported. DP^a likely represents a form of LHCP inserted in an unknown conformation that allows more of the N terminus of LHCP to be removed by protease treatment resulting in a truncated form of DP* (Kuttkat et al., 1995). Observing this new DP as a result of the insertion mutation of pLHCP confirms that position 134 is important for proper LHCP assembly in the membrane.

The trimeric LHCP formed by CSI-SP$_{134}$ and CSI-D1$_{134}$ loses association with PS-II. Wildtype LHCP forms a trimer after insertion into the thylakoids, and then associates with PS-II to participate in energy transfer during photosynthesis. Following solubilization of thylakoids with maltoside, intact PS-II is observable by electrophoresis as a green band that fluoresces under UV irradiation (Bass and Bricker, 1988). PS-I migrates as a separate green band and lacks fluorescence due to inherent fluorescence quenching. Association of radiolabeled LHCP with PS-II was analyzed on a native gel as shown in Figures 7a and 7b. PS-II is visible upon exposure to UV light. Trimeric wildtype LHCP that was integrated into the thylakoids is present in the PS-II band (Figure 7b). However, trimer formed by mutant CSI-SP$_{134}$ is observed migrating separate from the PS-II band further down the gel. The amount of trimer formed by CSI-SP$_{134}$ is comparable to CSI-SP$_{145}$ and CSI-SP$_{122}$ which are both found in the PS-II band. We conclude that CSI-SP$_{134}$ trimer has lost association with PS-II. CSI-D1$_{134}$ appears to be in multiple states, and not within the PSII band. pLHCP position 134 appears to be important for trimer association with PS-II. The helical region around site 134 may be important for a stable interaction with PS-II.

In this project we successfully constructed a tool for detection of LHCP insertion. This tool can be used in future assays to detect the insertion of TM1 and TM2 and requirements for this insertion event, e.g. is Alb3 availability required for insertions of TM1/TM2. Position 134 in pLHCP was also identified to be important for efficient trimer assembly and subsequent association with PS-II. This finding was unexpected, yet exciting. In this context, the CSI-SP134 mutant represents a tool to better understand the molecular events of LHCP trimer formation. Is site 134 of pLHCP involved in a direct association with PS-II? Does any disruption of the helical region near site 134 result in loss of trimer formation and PS-II

association? What conformation is DP^a in? Only further investigation can yield answers to the mechanism of LHCP insertion, assembly, and PS-II association.

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