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I am submitting herewith a thesis written by Sarah Jean Wright entitled "Probing Precursor Interactions with the Chloroplast Import Apparatus." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

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**Probing Precursor Interactions with the Chloroplast Import
Apparatus**

A Thesis Presented for
the Master of Science
Degree

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Sarah Jean Wright
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Abstract

The majority of plastid proteins are nuclear-encoded and imported post-translationally. A cleavable N-terminal extension, the transit peptide, targets these preproteins to the plastid. Transit peptides show very little primary sequence homology, yet are able to direct the precursor protein to interact with the protein components of the translocation complexes located within the inner and outer membranes of the chloroplast. In this study, a semi-conserved motif of the transit peptide, (F/W)(P/G)h(R/K) has been targeted for deletion in order to probe its importance. Two corresponding regions were deleted in the transit peptide of the precursor to the small subunit of Rubisco (prSSU) from tobacco: FTGLK and FPVSR. Both full and partial deletions were made in each region, resulting in the following 6 deletions: FTGLK, FTG, GLK, FPVSR, FPV, and VSR. The mutant preproteins as well as WT precursor (prSSU) and mature protein (mSSU) were overexpressed and purified in *E. coli* and the import competence evaluated by *in vitro* chloroplast import competition assays. Both of the full deletions caused a marked decrease in ability to compete for import, whereas the partial deletions had less of an effect. One aspect of import that could be compromised is transit peptide interactions with the Toc GTPase components of the translocon, Toc34 & Toc159. Recent work has shown a complex interaction between these GTPases and their substrates: preproteins/transit peptides and GTP/GDP. Although the Toc GTPases have been proposed to function as “gate-keepers”, neither the psToc34 crystal structure nor the low-resolution Toc complex structure reveal the dynamics of Toc-Toc or Toc-preprotein interactions during preprotein binding and translocation. In order to investigate the interactions between the soluble cytosolic domain of the Toc GTPase psToc34 and the transit peptide, analytical ultracentrifugation (AUC) was used. Using sedimentation velocity centrifugation, the presence of a homodimer of psToc34 was observed. The population of the homodimer increased in the presence of the transit peptide substrate, and decreased in the presence of nucleotide substrate. AUC of the mutant transit peptides with psToc34 showed that they were unable to influence the oligomeric state of psToc34 to the extent of WT transit peptide.

Table of Contents

Chapter 1 Introduction and General Information.....	1
Protein import into chloroplasts.....	1
General import pathway.....	3
Toc complex.....	6
Toc75	7
Toc64	8
Toc 159	8
Toc34	10
Crystal structure and implications	12
GTPase activity.....	14
Core complex	16
Tic complex	18
Tic 110	18
Tic 40	19
Tic 22 and 20	19
Soluble factors	20
Lipid components of the chloroplast outer envelope.....	21
Signal processing peptidase and presequence proteases.....	22
Transit peptide	22
Characteristics of transit peptides	23
FGLK motif	24
Rubisco	26
Analytical ultracentrifugation	26
Summary	29
Chapter 2 Materials and Methods	30
Plant growth and chloroplast isolation.....	30
Chlorophyll measurement.....	31
Generation of Δ FTGLK and Δ FPVSR prSSU mutants	31

Subcloning of the transit peptide region into the pTYB2 vector	34
Colony screen of ligation product	34
Purification of prSSU from inclusion bodies	36
<i>In vivo</i> radiolabeling of WT prSSU	37
Expression and purification of SStpNt and mutants	37
MALDI-TOF of peptides	38
Over expression and purification of the cytosolic domain of psToc34	39
<i>In vitro</i> chloroplast protein import competition	41
Data analysis	43
AUC analysis of psToc34	43
Data analysis	44
Protein measurement	45
Gel electrophoresis	45
Chapter 3 Analysis of the Role of the FGLK Semi-Conserved Sequence of the Transit	
Peptide in Chloroplast Import	48
Abstract	48
Introduction	49
Results	51
Generation and purification of prSSU mutants	51
Import competition and IC ₅₀ determination for ³⁵ S-prSSU, prSSU, mSSU, prSSUΔFPVSR, prSSUΔFPV, prSSUΔVSR, prSSUΔFTGLK, and prSSUΔFTG ..	55
Discussion	58
Deletion of the FGLK motif	58
Effect of deletion of the FGLK motif on chloroplast import	60
Chapter 4 AUC Analysis of PsToc34 with Nucleotide and Transit Peptide Ligands	
Abstract	62
Introduction	63
AUC analysis of dimerization	65
Results	66
Subcloning of the transit peptide region into the pTYB2 vector	66

Expression and purification of SStp mutants.....	68
MALDI-TOF of peptides.....	70
Expression and purification of cytosolic domain of Toc34.....	72
PsToc34 exhibits concentration dependent homodimerization	73
Nucleotide addition increases psToc34 homodimerization	73
Characterization of the effect of nucleotide analogs on psToc34 homodimerization	75
Transit peptide addition decreases psToc34 homodimerization.....	77
Characterization of the effect of mutant transit peptides on psToc34 homodimerization	77
Discussion.....	80
psToc34 exhibits concentration dependent homodimerization.....	80
GTP increases the concentration of dimeric Toc34.....	81
Transit peptide addition increases the population of monomeric Toc34.....	83
Transit peptides lacking the FGLK motif show a decreased influence on the population equilibrium of psToc34 Δ TM	84
Chapter 5 Conclusions and Future Directions	86
Conclusions.....	86
Future directions	89
References.....	92
Vita.....	103

List of Tables

Table 1 Primer sequences	32
Table 2 Long distance PCR set up.....	33
Table 3 Direct colony PCR screen.....	35
Table 4 Chloroplast import competition assay	42
Table 5 Tris/tricine gel for electrophoresis of low molecular weight peptides	47
Table 6 IC ₅₀ of WT prSSU and deletion mutants	57

List of Figures

Figure 1-1 General import pathway for chloroplast precursor protein import.	4
Figure 1-2 Structure of psToc34 Δ TM.....	13
Figure 1-3 Reconstructed electron microscopy image of the Toc core complex	17
Figure 1-4 Alignment of transit peptides from the small subunit of Rubisco	25
Figure 1-5 Sedimentation coefficient distribution of an SV simulation of a monomer-dimer fast association.....	28
Figure 3-1 Sequence and mutagenesis of the transit peptide of prSSU.....	52
Figure 3-2 Purification profile and final soluble prSSU, mSSU, and mutant proteins.....	54
Figure 3-3 <i>In vitro</i> chloroplast import competition assay of full-length motif deletions.	56
Figure 3-4 <i>In vitro</i> chloroplast import competition assay of partial motif deletions.....	59
Figure 4-1 Direct colony PCR screen of insertion candidates.....	67
Figure 4-2 Purification profile and MALDI-TOF of SStpNt.....	69
Figure 4-3 Tris/Tricine gel and MALDI-TOF of SStpNt deletions.....	71
Figure 4-4 PsToc34 exhibits concentration dependent homodimerization.....	74
Figure 4-5 SV analysis of psToc34 with nucleotide substrate and analogs.....	76
Figure 4-6 Addition of transit peptide shifts the population equilibrium of psToc34	78
Figure 4-7 Deletion mutant transit peptides do not influence the population equilibrium of psToc34 to the same extent as WT.	79
Figure 5-1 Model of Toc34 interactions mediated by the transit peptide and nucleotide substrates.....	88

Chapter 1 Introduction and General Information

Photosynthesis uses the sun's energy to fix carbon, storing light energy in the form of chemical bonds. This process, necessary for life on earth, occurs in photosynthetic prokaryotes as well as in chloroplasts, organelles unique to eukaryotes that perform photosynthesis. Early endosymbiotic events in which a proteobacterium and later a photosynthetic cyanobacterium were engulfed created the modern mitochondria and chloroplasts of eukaryotic cells (Weeden 1981). These once-free-living bacteria survived phagocytosis and through evolution eventually transferred much of their genetic information to the host cell, resulting in a much more permanent relationship. Today, more than 90% of the proteins of chloroplasts and mitochondria are synthesized in the cytosol as preproteins with an N-terminal targeting sequence or transit peptide and are targeted to their respective organelles post-translationally (Bhushan, Kuhn et al. 2006). Both organelles also possess a double membrane which is a remnant of the original endosymbiotic event and must be traversed by proteins targeted to either the chloroplast or mitochondria. The chloroplast accomplishes translocation of preproteins across the two membranes using translocons assembled at each membrane, Toc (Translocon of the outer envelope membrane of chloroplasts) and Tic (Translocon of the inner envelope membrane of chloroplasts), mirroring the mitochondrial Tom and Tim complexes. The mechanism(s) by which specificity of targeting to each of the organelles is achieved remains largely mysterious, however is presumably mediated by interactions of the corresponding N-terminal presequences.

Protein import into chloroplasts

It has been well established that the majority of chloroplast proteins are nuclear encoded and synthesized on free ribosomes in the cytosol of the cell, making post-translational import into the chloroplast necessary. Chloroplasts do have their own genome, but it contains only 120-135 genes, ~80 of which encode proteins, the remainder encoding chloroplast tRNA and rRNA (Lopez-Juez and Pyke 2005). In *Arabidopsis*, a recent study

predicted that about 2,100 of the nuclear-encoded proteins contain a transit peptide and are targeted to the chloroplast (Richly and Leister 2004). These nuclear-encoded proteins are synthesized as precursor proteins or preproteins, higher molecular weight proteins with an N-terminal transit peptide about 30-100 amino acids in length which serves as a cleavable targeting sequence (Dobberstein, Blobel et al. 1977). The transit peptide is both necessary and sufficient for precursor targeting and import into the chloroplast and is cleaved off upon import.

Protein import across the double membrane of chloroplasts is mediated by multimeric protein complexes present in the outer and inner envelope membranes denoted Toc and Tic (Translocon of the outer/inner chloroplast membrane, respectively), the assembly of which is probably dynamic. The proteinaceous components are denoted by a combination of their respective translocon (Toc or Tic) and their apparent molecular masses. Early studies of the translocation apparatus in isolated pea chloroplasts used crosslinking during the different stages of import to determine the necessary components of the translocon. From these studies along with antibody inhibition of import, the Toc import complex was determined to consist of two GTPases, Toc34 and Toc159, and the channel protein Toc75 (Perry and Keegstra 1994; Kouranov and Schnell 1997). Further characterization of the core complex using electron microscopy (EM) determined the complex to be roughly spherical with four pores surrounding a finger-like central domain and the stoichiometry of Toc75:Toc34:Toc159 4:4:1 respectively (Schleiff, Soll et al. 2003). Another protein has been added more recently, Toc64, which associates with the core complex transiently and is proposed to interact with chaperones delivering preproteins (Sohrt and Soll 2000; Qbadou, Becker et al. 2006). The Tic components are not as well characterized as the Toc components, but include Tic22, Tic20 (Kouranov, Chen et al. 1998), Tic40, and Tic110 (Stahl, Glockmann et al. 1999; Kovacheva, Bedard et al. 2005). While biochemical methods have historically been used to characterize and evaluate the Toc and Tic components and their roles in chloroplast protein import, *in vivo* approaches using *Arabidopsis thaliana* are now taking advantage of genomic and/or genetic approaches to further probe the functions of these proteins (Bauer, Chen et al. 2000; Hiltbrunner, Grunig et al. 2004).

General import pathway

The majority of plastid-targeted preproteins that possess an N-terminal transit peptide follow the general import pathway shown in Fig. 1-1, which is mediated by the Toc and Tic complexes (Bruce 2001; Reumann, Inoue et al. 2005). Recent data does suggest that other pathways may exist, with a proteomics study identifying nuclear-encoded chloroplast proteins lacking canonical transit peptides and another study identifying an ER to Golgi to chloroplast protein transport pathway (Kleffmann, Russenberger et al. 2004; Radhamony and Theg 2006). However, these alternative pathways are still a matter of dispute and are not the subject of this study.

There are several possibilities for preprotein targeting from the cytosol to the outer membrane of the chloroplast and interaction with the Toc complex. The first depicted in Fig. 1-1(1a) is the recognition and binding of a phosphorylated transit peptide by a 14-3-3 chaperone, forming a guidance complex along with Hsp70 that delivers the transit peptide to the Toc receptors (May and Soll 2000). Another possibility is denoted (1b) in Fig. 1-1, showing the interaction of the preprotein with a soluble form of the receptor Toc159, which then presumably binds Toc34 and assembles into the Toc complex (Wallas, Smith et al. 2003). A third possibility shown as (1c), most likely for specific classes of preproteins, is recognition and binding by the Hsp90 molecular chaperone, which then binds to Toc64 (2f) and is guided to Toc34 (5), assembling the Toc complex (Qbadou, Becker et al. 2006). A fourth possibility (2a), is specific interaction of the transit peptide with chloroplast-specific lipids, which have been shown to interact and cause a conformational change in the transit peptide, increasing the α -helical content (Pinnaduwaage and Bruce 1996; Wienk, Wechselberger et al. 2000), and possibly spurring interaction with the Toc receptors. Finally, the preprotein may require none of these aids in order to form a productive interaction with the Toc receptors, but may bind directly to the Toc receptors. It has been suggested that different precursors may utilize different targeting mechanisms, which is likely in light of recent data demonstrating that Toc159 from *Arabidopsis* specifically interacts with highly expressed photosynthetic proteins and not with other representative chloroplast proteins (Smith, Rounds et al. 2004).

Figure 1-1 General import pathway for chloroplast precursor protein import.

Preproteins are synthesized in the cytosol with an N-terminal transit peptide, shown here are two different classes of preproteins, each with three domains in red, green and yellow and purple, green, and blue respectively. There are multiple mechanistic possibilities for import depicted. (1a) Interaction of the phosphorylated (black dot) transit peptide with a soluble guidance complex containing a 14-3-3 protein and Hsp70. (1b) Interaction of the preprotein and soluble Toc159. (1c) Interaction of preprotein and Hsp90. (2a) Partitioning of the precursor out of the cytoplasm onto the chloroplast surface via a nucleotide-independent interaction of the transit peptide with chloroplast-specific lipids, such as MGDG, SL and PG. (2b, 2c, 2d, 2e, 2f) Direct interaction of the precursor with the Toc components, possibly facilitated by recognition of the Hsp70 guidance complex or Hsp90. Initial interaction may be with the full-length Toc159 (2b), Toc64 (2c) or (2f), or the heterooligomeric Toc translocon (2d), or guidance by soluble Toc159 to the translocon (2e). (3) Peptide/lipid interactions resulting in reciprocal changes in both the transit peptide helical structure (green) and the lipid phase preference of the envelope (shown as an inverted micelle). (4) Recognition and interaction of membrane-associated transit peptide with Toc86/159 receptor. (5, 6) Lateral movement and/or transfer of the transit peptide from its initial association with Toc86/159 and/or Toc64 to interact with Toc34 and Toc75, resulting in the assembly of a Toc translocon, localized at a contact site of both the inner and outer envelopes. This also illustrates (6) the sequential or concurrent GTP-driven insertion of transit peptide into Toc75. (7) Preprotein translocation across the outer membrane in a nucleotide-dependent manner using molecular motors and a push-pull mechanism. (8) Complete translocation of preprotein across the inner membrane requiring higher levels of nucleotide and using stromal molecular motors. (9,10) Upon import, the transit peptide is cleaved by SPP, followed by complete degradation of the transit peptide by PreP1 and 2 and proper folding of the mature domain. This figure is adapted from (Bruce 2001).

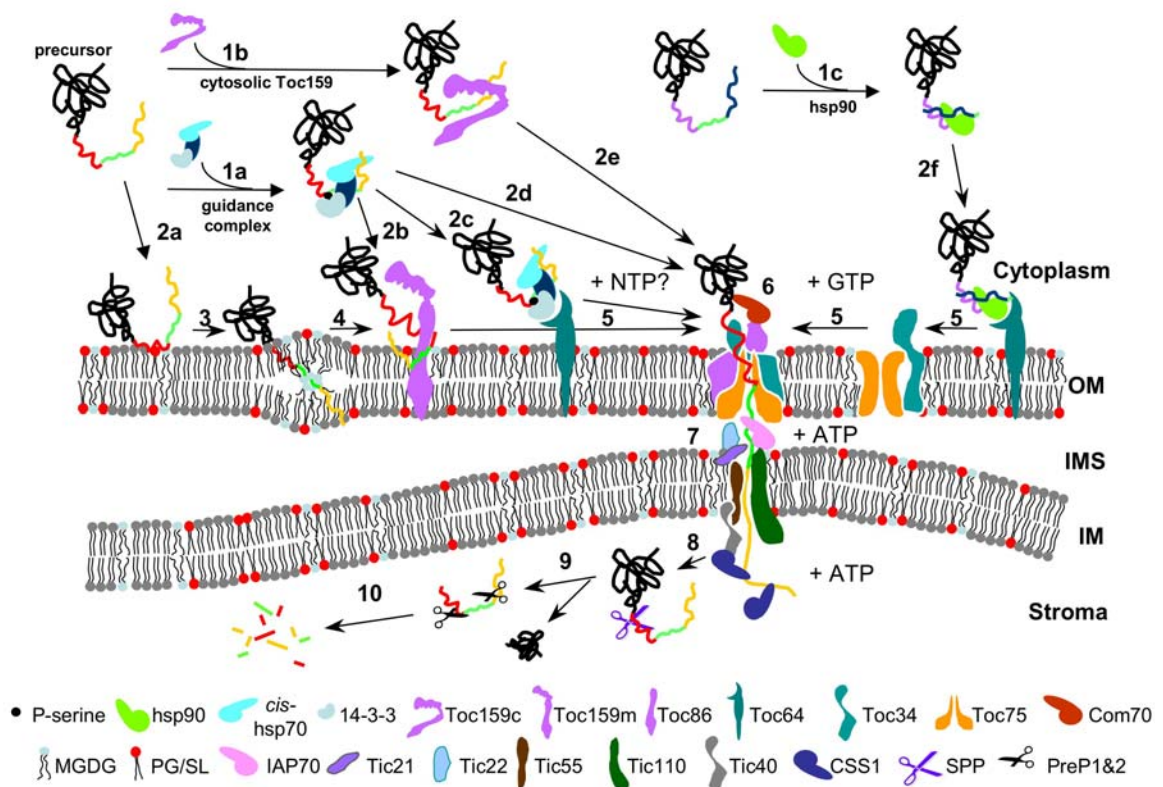


Figure 1-1 General import pathway for chloroplast precursor protein import.

For the translocation of preproteins across the chloroplast membranes the general Toc/Tic import pathway requires the input of energy, and can be divided into three stages according to the amount of energy needed at each stage. Studies utilizing isolated chloroplasts and *in vitro* import of preproteins have contributed to the characterization of the energy requirements of import using crosslinking as well as antibody inhibition to arrest import at different points followed by isolation of the complex to determine the components present at each step. The first stage occurs at the outer membrane, steps (5) and (6) in Fig. 1-1, where preproteins weakly interact with the Toc receptors and is energy independent and reversible (Perry and Keegstra 1994). Upon addition of low levels of ATP ($<100\ \mu\text{M}$), the second stage of import occurs, shown as step (7) in Fig. 1-1 wherein early translocation intermediates are formed in which the preprotein has begun to make contacts with the Toc75 channel protein as well as the assembled proteins of the Tic complex (Ma, Kouranov et al. 1996; Nielsen, Akita et al. 1997). GTP is also required for the formation of early import intermediates at this stage, probably driving translocon assembly and/or preprotein insertion into the translocon channel, but is not required for the translocation of preproteins (Olsen and Keegstra 1992; Young, Keegstra et al. 1999). In order to accomplish complete translocation of the preprotein, ATP is required at levels $> 100\ \mu\text{M}$, step (8) in Fig. 1-1, a requirement possibly due to the involvement of hsp70 or hsp100 stromal chaperones in the import process (Theg, Bauerle et al. 1989; Jackson-Constan, Akita et al. 2001). Finally, upon import into the stroma of the chloroplast (steps 9 and 10 in Fig. 1-1), the transit peptide is cleaved from the mature domain by the stromal processing peptidase (SPP) and completely degraded by the presequence proteases PreP1 and 2, and the folding and assembly process is initiated for the mature domain (Richter and Lamppa 1999; Bhushan, Stahl et al. 2005).

Toc complex

Preprotein recognition and translocation across the outer envelope of the chloroplast double membrane is mediated by the Toc complex (Translocon of the outer envelope membrane of chloroplasts). Initial identification of the Toc components was accomplished by crosslinking of early import intermediates, resulting in the isolation of

the receptors Toc34 and Toc86/159 and the channel protein Toc75 crosslinked to preprotein (Perry and Keegstra 1994). When these three proteins were reconstituted into liposomes they were able to form an import-competent pore, suggesting that they are the core components of the translocon (Schleiff, Jelic et al. 2003). A third receptor, Toc64, was identified, exhibiting transient association with the complex in the presence of GTP, but its role has been hotly debated because genetic knockout mutants in the moss *Physcomitrella patens* showed very little effect (Sohrt and Soll 2000; Rosenbaum Hofmann and Theg 2005). However, recent data has shown that Toc64 does act as a general import receptor, serving as a docking site for Hsp90-bound preproteins (Qbadou, Becker et al. 2006). Therefore, these four proteins are major components of the Toc complex and will be discussed in detail below.

Toc75

The pore-forming channel protein of the translocon, Toc75, is the most abundant protein of the outer membrane of chloroplasts and was originally identified by isolation of early preprotein import intermediates with only low levels of ATP supplied (Schnell, Kessler et al. 1994). Structural studies using proteases and computational predictions suggest that it forms a β -barrel lined with either 16 or 18 transmembrane β sheets (Sveshnikova, Grimm et al. 2000; Schleiff, Soll et al. 2003). Initial patch-clamp analysis of Toc75 indicated that the pore size was only about 8-10 Å, suggesting that preproteins had to be completely unfolded in order to pass through (Hinnah, Hill et al. 1997). Earlier data contradicted this, showing that DHFR (dihydrofolate reductase) fused to a transit peptide was import competent even when methotrexate was bound, indicating that it was at least partially folded during translocation (America, Hageman et al. 1994). Further electrophysiological characterization with better reconstitution of Toc75 in planar lipid bilayers confirmed that partially-folded proteins would fit through the constriction site of the channel, which was found to be about 15 Å (Hinnah, Wagner et al. 2002). Interestingly, the same study also found that the channel itself distinguished between a chloroplast transit peptide and a mitochondrial signal sequence, presumably based on both charge and structure of the peptide due to the observation that a synthetic peptide with the same charge as the

mitochondrial sequence but with a structure characteristic of a transit peptide was able to interact with the channel at a level intermediate to the mitochondrial and chloroplast peptides. However, this is the only study that supports recognition specificity of Toc75 for chloroplast preproteins, and further data is needed to confirm such a role for Toc75.

Toc64

Toc64 was identified as a protein component of the Toc complex later than the other receptors, and is not considered to be part of the core complex, being only dynamically associated in the presence of GTP (Sohrt and Soll 2000; Schleiff, Soll et al. 2003). It was originally proposed to mediate docking of cytosolic chaperone-bound preproteins due to the presence of three tetratricopeptide repeats (TPR) which are similar to motifs found in other proteins that interact with Hsp90 and Hsp70 chaperones (Sohrt and Soll 2000). This role has recently been confirmed by work demonstrating recognition of Hsp90 by the TPR domain of Toc64 (Qbadou, Becker et al. 2006). The same study demonstrates a GTP-dependent interaction of the TPR domain of Toc64 with Toc34, suggesting that the mechanism of preprotein transfer from Toc64 to either Toc34 or the assembled translocon is mediated by a transient GTP-dependent association, followed by preprotein translocation by the core complex.

Toc 159

Toc159 was originally identified as Toc86 due to its proteolytic sensitivity in isolated chloroplast suspensions, on which most of the original studies were performed (Bolter, May et al. 1998). Toc159 is a large protein that can be divided into three domains: a central GTPase domain or G-domain, which is highly homologous to the G-domain of Toc34, an N-terminal acidic or A-domain whose function is unknown, and a C-terminal membrane or M-domain (Chen, Chen et al. 2000). In pea, the system in which the current study was performed, only one isoform of Toc159 has been identified, however, in *Arabidopsis* several family members have been identified and are identified by a preceding at for *Arabidopsis thaliana*: atToc90, atToc120, atToc132, and atToc159

(Hiltbrunner, Bauer et al. 2001). In addition, it appears that these isoforms in *Arabidopsis* serve as substrate-specific receptors, which will be discussed in detail below.

Although it is accepted that Toc159 is a preprotein receptor, it is a matter of debate whether it or Toc34 serves as primary receptor. Experimental data supports both sides of the debate. The classical cross-linking data identified Toc159 (identified as Toc86) as the major protein cross-linked with preproteins arrested in early stages of translocation, prompting the notion that Toc159 was the site of the initial interaction (Ma, Kouranov et al. 1996). The role of Toc159 as the primary receptor is also supported by the more recent observation of a cytosolic form of Toc159 which docks with Toc34 and inserts into the membrane in a GTP-dependent manner (Smith, Hiltbrunner et al. 2002). The cytosolic form of atToc159 in *Arabidopsis* has also been shown to bind chloroplast preproteins in a specific manner, adding further support to the idea that Toc159 is the primary receptor, handing the preprotein off to Toc34 (Smith, Rounds et al. 2004). These studies indicate that Toc159 serves as the primary receptor, even serving as a cytosolic mediator of preprotein targeting to the chloroplast membrane followed by translocon assembly and translocation of the preprotein. However, the existence of the cytosolic Toc159 is not accepted by everyone in the field, and data has been presented indicating that the soluble form is a result of improper fractionation methods and Toc159 is in reality an integral membrane protein at all times (Becker, Jelic et al. 2004). In addition, cross-linking data has also shown that preproteins interact with Toc34 transiently and in an energy-independent manner, while Toc159 can be crosslinked to preproteins throughout the early stages of translocation, suggesting that Toc34 is the site of the initial interaction (Kouranov and Schnell 1997). Also, more recent data using reconstituted proteoliposomes showed that Toc34 and the channel protein Toc75 could not import preproteins without Toc159 but that Toc159 and Toc75 in the absence of Toc34 were import competent, suggesting that Toc34 may function in initial binding while Toc159 is the motor driving translocation of preproteins (Schleiff, Jelic et al. 2003).

One possible explanation for the confusing results concerning the primary receptor status of Toc159 is suggested by recent data indicating that the different atToc159 family members serve as substrate specific preprotein receptors. AtToc159 and its family

members atToc90, atToc120, and atToc132 show high similarity to one another, and are almost completely conserved throughout the G and M domains (Ivanova, Smith et al. 2004). The most variability between the isoforms is found in the A domain, which could be responsible for either binding specific preprotein subclasses or interacting with different Toc34 isoforms (Jarvis and Robinson 2004). Expression of atToc159 is much higher than the other isoforms in all tissues of *Arabidopsis*, indicating that it is probably the major receptor while the others play minor roles in preprotein recognition or import (Hiltbrunner, Bauer et al. 2001). Recent *in vitro* binding experiments have shown that atToc159 binds photosynthetic proteins specifically while atToc132 was shown to bind a nonphotosynthetic protein preferentially over a photosynthetic protein, and these observations were supported by a proteomics comparison of plants lacking one or the other receptor (Ivanova, Smith et al. 2004; Kubis, Patel et al. 2004; Smith, Rounds et al. 2004). The functional specialization of the atToc159 homologues suggests that different Toc components probably assemble for import of different preproteins, which could explain some of the different observations concerning Toc assembly and the order of interactions with preproteins.

Toc34

Toc34 was originally identified in crosslinking studies, associated with preproteins during energy-independent binding or early import intermediates in the presence of low levels of ATP indicating that it is involved in the early stages of binding and translocation of the preprotein (Schnell, Kessler et al. 1994; Kouranov and Schnell 1997). Toc34 is composed almost entirely of a GTP-binding (G) domain, which is homologous to the central G-domain of Toc159 (Reddick, Vaughn et al. manuscript in preparation). However, unlike Toc159 which is a much larger GTPase and has two additional domains (discussed above), Toc34 only has a short stretch of hydrophobic amino acids C-terminal to the G-domain which anchors it to the outer membrane, leaving the majority of the protein exposed to the cytosol (Kessler, Blobel et al. 1994). The cytosolic domain has been shown to interact specifically with transit peptides in addition to binding and hydrolyzing GTP (discussed in further detail below), supporting the role of Toc34 as a

nucleotide-regulated receptor of the Toc complex (Sveshnikova, Soll et al. 2000; Becker, Jelic et al. 2004).

Like Toc159, more than one homologue of Toc34 has been found in *Arabidopsis* as well as in many other plants including maize (*Z. mays*), rape seed (*B. napus*), potato (*S. tuberosum*), tomato (*L. esculentum*), poplar (*P. tremula*), and the moss *Physcomitrella patens* (Voigt, Jakob et al. 2005). In *Arabidopsis*, two genes have been identified, encoding atToc33 and atToc34, with atToc33 considered the ortholog of pea Toc34 (Jackson-Constan and Keegstra 2001). AtToc33 is expressed at levels about 5-fold higher than atToc34 in leaves, but the two proteins are differentially expressed in roots, stems, and flowers, suggesting that atToc33 function is more important in young, actively growing green tissues (Jarvis, Chen et al. 1998; Gutensohn, Schulz et al. 2000). *In vitro* binding assays demonstrated that the two homologues do show specificity towards different transit peptides, however the binding affinities varied greatly, with some transit peptides interacting strongly with just one receptor and others interacting equally with both receptors, suggesting that the receptors are not totally exclusive for specific preproteins but can compensate for one another to an extent (Jelic, Soll et al. 2003). However, due to the differential expression in photosynthetic versus non-photosynthetic tissues, the protein composition of mutant *Arabidopsis* lacking atToc33 (*ppi1*) and WT plants was compared using mass spectrometry and photosynthetic proteins were found to be decreased whereas non-photosynthetic proteins were increased (Kubis, Baldwin et al. 2003). This data along with similar observations concerning the Toc159 homologues suggests that different Toc receptors may assemble for translocation of different types of preproteins, at least in *Arabidopsis* (Jelic, Soll et al. 2003). Interestingly, the organism that is used in the current study, garden pea, is one of the few experimental systems in which multiple isoforms of Toc34 have not been observed (Hirohashi and Nakai 2000; Fulgosi, Lepedus et al. 2005; Voigt, Jakob et al. 2005).

Crystal structure and implications

The crystal structure of the cytosolic domain of Toc34 from *Pisum sativum* (psToc34 Δ TM) has been determined, providing new insight into the mechanisms of chloroplast receptor-mediated import of preproteins. In addition, due to the homologous nature of the G-domains of both Toc34 and Toc159, the crystal structure can be used as a guide to the structure of each of the Toc GTPases (Bedard and Jarvis 2005; Reddick, Vaughn et al. manuscript in preparation). The crystal structure determined by Sun et al., shown in Fig. 1-2, exhibited a dimeric arrangement with bound GDP molecules in the active sites although the protein was purified without any exogenously supplied nucleotide. Even more intriguing, at least four residues of each monomer, Pro169, Asp170, Tyr132, and Arg133 are involved in the nucleotide binding pocket of the other monomer, shown in Fig. 1-2(B), suggesting the possible involvement of nucleotide regulated dimerization in the import mechanism (Kessler and Schnell 2002; Sun, Forouhar et al. 2002). In addition, Arg133 is in a position that suggested a possible role as a catalytic arginine finger, common in activating proteins that associate with GTPases, stimulating their activity, which will be discussed in more detail below. Due to this arrangement, dimerization was proposed to cause reciprocal activation of GTP hydrolysis (Sun, Forouhar et al. 2002). Furthermore, the bound GDP molecules in the crystal structure appear unable to exchange out of the dimeric arrangement due to an obstructing loop from the other Toc34 molecule, suggesting that the dimer must be interrupted for nucleotide exchange to occur (Kessler and Schnell 2002). PsToc34 has also been shown to exist as a homodimer using size exclusion chromatography and native gel electrophoresis, indicating that the homodimeric structure is not artificially induced due to crystal packing (Sun, Forouhar et al. 2002; Weibel, Hiltbrunner et al. 2003). Furthermore, since the G-domains of both Toc34 and Toc159 receptors are similar, it was thought that the interaction may be heterodimeric *in vivo*, and this idea was supported by several *in vitro* binding experiments that showed the G-domains of the two receptors do interact in a GTP-dependent manner (Smith, Hiltbrunner et al. 2002; Weibel, Hiltbrunner et al. 2003). The crystal structure therefore raises the possibility that nucleotide-regulated

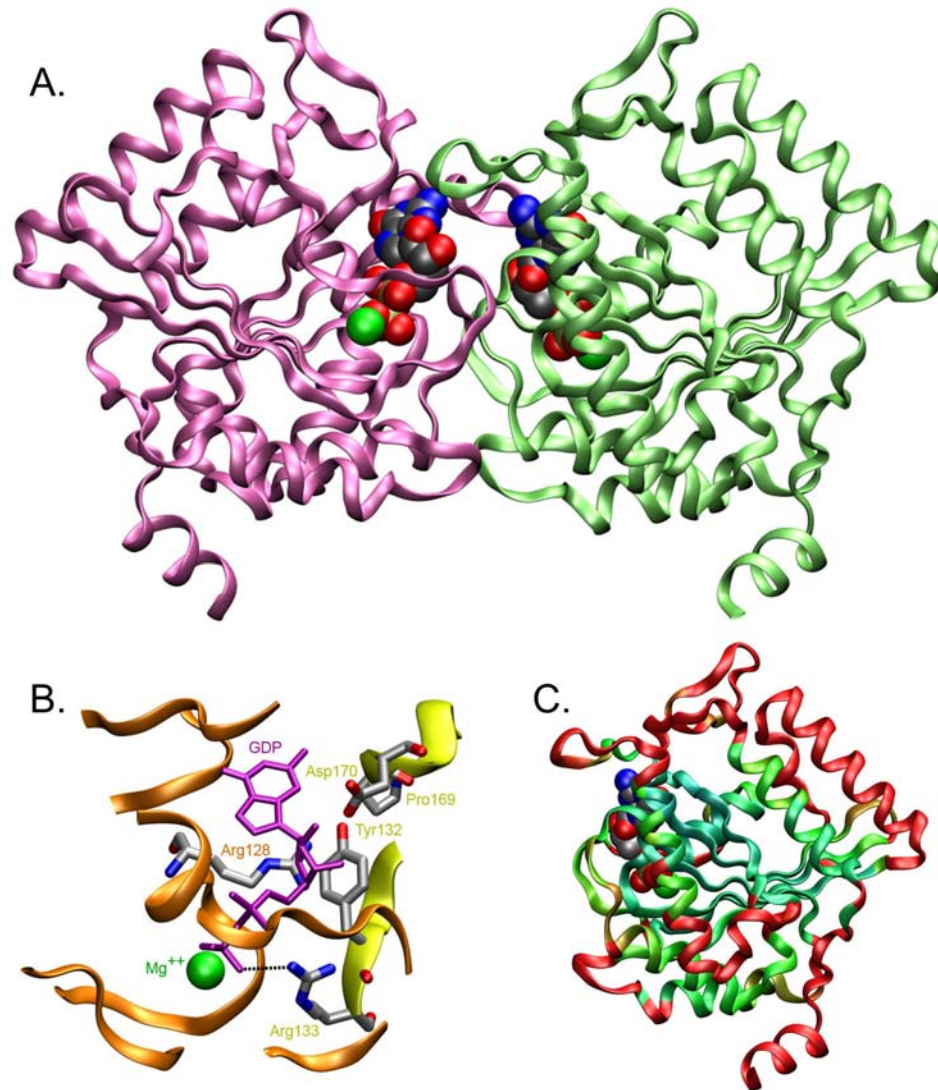


Figure 1-2 Structure of psToc34ΔTM

(A) Two Toc34 molecules are shown in a two-fold plane of symmetry with their respective bound GDP molecules shown and Mg²⁺ metal cations shown in green. To emphasize the crossover of each monomer into the active site of the other monomer, one Toc34 molecule is colored entirely in green and the other is shown in purple. (B) The nucleotide binding site of one monomer (orange) is shown with participating residues from each monomer (2nd monomer shown in yellow) labeled in their respective colors. (C) Novel regions of the Toc34 GTPase compared to other small GTPases (i.e. Ras, Rho, Rab) are shown in red. Modeling by Michael Vaughn.

homo and/or heterodimerization of the Toc GTPases may be an important step in preprotein import into chloroplasts. In addition, the structure of psToc34 when compared to other small GTPases such as Ras has novel regions shown in red in Fig. 1-2(C), including a large region at the N-terminus at the top of the monomer, which could correspond to a transit peptide binding region (Reddick, Vaughn et al. manuscript submitted). The crystal structure of Toc34 in general has raised many new questions while providing a few clues that may contribute greatly to the investigation of the mechanism of the Toc GTPases and the regulatory role of GTP in preprotein import, which will be discussed in the next section.

GTPase activity

Early studies of preprotein import indicated that GTP plays a necessary role in the early steps of translocation, promoting the formation of early import intermediates (Kessler, Blobel et al. 1994; Young, Keegstra et al. 1999). Since then, many experiments have been undertaken with the goal of determining the role of GTP in the Toc GTPases' interactions with preproteins and translocon assembly. The resolution of the crystal structure, discussed in detail above, also contributed a great deal of detailed information, revealing a dimeric arrangement of the Toc34 GTPase as well as specific residues of the nucleotide binding pocket.

Taken together, recent studies have shown a complex interaction between the two GTPases and their apparent substrates, the transit peptide of chloroplast targeted preproteins and the nucleotides GTP and GDP. Heterodimerization of the G domains of Toc34 and Toc159 has not only been shown to occur, but also has been shown to be stimulated by the addition of GDP, supporting the idea of nucleotide regulation of the dimer (Smith, Hiltbrunner et al. 2002; Weibel, Hiltbrunner et al. 2003). In contrast, the interaction of preproteins with the GTPases is promoted by GTP, suggesting that nucleotide binding and hydrolysis may serve as a switch, regulating the binding affinity for transit peptides and alternately the homo/heterodimeric binding partner (Sveshnikova, Soll et al. 2000). GTP has also been shown to decrease binding of a transit peptide that could not be translocated due to an N-terminal tag, suggesting that GTP binding may

represent a commitment step in the import of preproteins (Subramanian, Ivey et al. 2001). Furthermore, both transit peptides and preproteins have been shown to stimulate GTP hydrolysis of the Toc GTPases, suggesting that they also play a role in regulation of activity (Jelic, Sveshnikova et al. 2002; Becker, Jelic et al. 2004).

Many small GTPases are regulated by the association of a GTPase activating protein (GAP) and/or a GTPase exchange factor (GEF), and it has been proposed that either the monomers composing the Toc34 homo/heterodimer act as GAPS for each other, or the transit peptide may alternatively act as a GAP or GEF (Sun, Forouhar et al. 2002; Becker, Jelic et al. 2004). While several experiments have attempted to resolve this question, results have been largely unclear. Mutagenesis of a residue necessary for dimerization, Arg128, in psToc34 Δ TM resulted in lack of dimerization and decreased GTP hydrolysis, indicating that Toc34 might serve as its own GAP (Sun, Forouhar et al. 2002). However, mutagenesis of Arg130 in the *Arabidopsis* homologue atToc33, equivalent to the putative GAP-like arginine finger in psToc34, eliminates dimerization but does not have any affect on GTPase activity, directly contradicting any function as a GAP (Weibel, Hiltbrunner et al. 2003). Furthermore, the observation that preproteins increase GTPase activity suggests that they may act as GAPs rather than the dimeric partner (Jelic, Sveshnikova et al. 2002). It is difficult to distinguish between GAPs and GEFs if the final hydrolysis product is the only data collected because either activation or nucleotide exchange can result in more hydrolyzed nucleotide. However, data from our lab using an assay that follows rate of nucleotide exchange indicates that there is no difference when transit peptide is added, supporting the role of transit peptide as a GAP (Reddick, Vaughn et al. manuscript in preparation). Unfortunately, there is no structural information concerning the interaction of transit peptides with the Toc GTPases, so the mechanism of activation is yet to be determined. However, the structure of psToc34 when compared to other small GTPases such as Ras, Rho, or Rab does have a novel region at the N-terminus that would presumably be exposed to the cytosol, shown in red in Fig. 1-2(C) at the top of the monomer, which could correspond to a transit peptide binding region.

Core complex

Four major protein components of the Toc complex have been described: Toc159, Toc75, Toc64, and Toc34. However, the core complex of the outer chloroplast membrane is generally accepted to be composed of just three proteins: the two GTPase receptors Toc159 and Toc34, and the channel protein, Toc75 (Kouranov and Schnell 1997). There is some debate concerning the stoichiometry of the assembled components, and several methods have been used to isolate the core complex in order to determine protein ratios. Schleiff et al. purified the core complex from pea chloroplast outer membranes using sucrose gradient centrifugation. Using this method, they isolated a complex with an apparent mass of ~500 kD, which they analyzed using cryo electron microscopy and two-dimensional structural analysis, representative results of which are shown in Fig. 1-3. The EM analysis revealed at least 8 different classes of particles, however rotational averaging to obtain the image shown in Fig. 1-3 was performed on the most prevalent class, representing ~50% of the particles. From this analysis, they concluded that the translocon was roughly spherical, containing four channels surrounding a finger-like central core (Schleiff, Soll et al. 2003). Additional biochemical analysis of the core complex including Coomassie-staining of SDS-PAGE gels, immunolabeling, and radiolabeling using cysteine-specific radioactive N-ethylmaleimide (NEM) indicated that the stoichiometry of Toc159:Toc75:Toc34 was 1:4:4-5 respectively, leading them to suggest that the observed channels were the four Toc75 proteins each associated with a Toc34 protein and arranged around one Toc159, proposed to be the “motor” of the translocon (Schleiff, Soll et al. 2003). This arrangement is further supported by their observation of up to four gold-labeled transit peptides associated with core complexes in the presence of GTP, indicating that four preproteins may be inserted into the translocon at one time. However, the resolution is not high enough to conclusively determine the location or identity of each of the protein components using this method.

Another recent study by Kikuchi et al. used blue native gel electrophoresis to analyze core complexes by running detergent solubilized isolated pea chloroplasts directly on BN-PAGE. This study determined the apparent molecular mass to be ~800-1,000 kD, double the mass determined by the previous study. In addition, the stoichiometry was

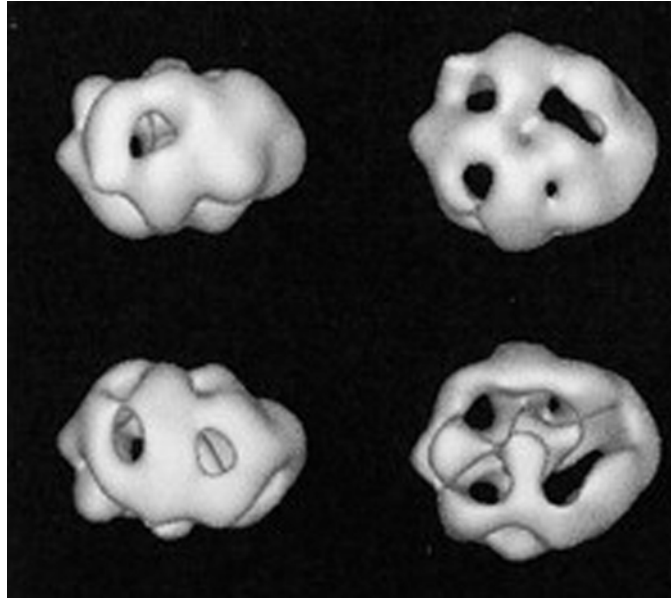


Figure 1-3 Reconstructed electron microscopy image of the Toc core complex

The Toc core complex was isolated from pea chloroplast outer membranes and exhibited GTP-dependent import competence. The stoichiometry of protein components was determined to be 1:4:4-5 between Toc159:Toc75:Toc34, with a total apparent molecular mass of about 500 kD. The three dimensional map was obtained from transmission electron micrographs by random conical tilt reconstruction, showing four channels around a central finger-like core region. Adapted from Schleiff, Soll et al. 2003.

found to be 1:3:3 for Toc159:Toc75:Toc34, slightly different from the previously determined numbers (Kikuchi, Hirohashi et al. 2006). The authors conclude that there are two possibilities: either they have identified a super-complex composed of two Toc159 proteins and six each of Toc75 and Toc34, or there are additional unidentified protein components associated with their core complex (Kikuchi, Hirohashi et al. 2006). Despite the need for further investigation to resolve the differences in absolute numbers, it seems clear that the core complex is composed of one Toc159 GTPase receptor in association with multiple Toc75 and Toc34 proteins present in equal ratios.

Tic complex

Translocation of preproteins through the inner membrane is accomplished by the Tic complex (Translocon of the innner envelope membrane of chloroplasts). The Tic complex is not as well characterized as its Toc counterpart, in part due to dynamic behavior of the protein components. It has been shown that preproteins can be translocated across each membrane independently under conditions of osmotically induced organelle shrinkage (Scott and Theg 1996), but it is commonly accepted that the typical mechanism involves preprotein promoted formation of contact sites between Toc and Tic complexes and simultaneous translocation of preprotein across both membranes into the stroma of the chloroplast (Schnell and Blobel 1993; Perry and Keegstra 1994). The most studied Tic proteins include Tic110, Tic40, Tic22, and Tic20, which will be discussed in detail below.

Tic 110

Tic110 is one of the most studied components of the Tic complex and was isolated in early studies cross-linked with preprotein import intermediates (Akita, Nielsen et al. 1997). The role of Tic110 is still a matter of debate although the stromal domain has been shown to form pores when incorporated into liposomes, suggesting that it is the pore-forming component of the Tic complex (Heins, Mehrle et al. 2002). However, the same structure is not found *in vivo*, and Tic20 (discussed below) has also been proposed to form the channel. Tic110 has also been implicated in chaperone recruitment mediated by

its large stromal domain and may serve as a binding and assembly site for preproteins as they are translocated into the stroma and folded by molecular chaperones (Inaba, Li et al. 2003). Despite the controversy, Tic110 is unquestionably an integral part of the Tic complex, as loss is embryo lethal, with embryo arrest occurring at the globular stage (Kovacheva, Bedard et al. 2005). Furthermore, *in vivo* studies of *Arabidopsis* dominant negative mutants also indicated that Tic110 is necessary for formation of Tic complexes and translocation of preproteins across the inner membrane of chloroplasts (Inaba, Alvarez-Huerta et al. 2005).

Tic 40

Tic40 was first identified as a component of the Toc complex, crosslinked to preproteins arrested during import (Wu, Seibert et al. 1994) and later in Tic complexes tightly associated with Tic110 and definitively localized to the inner membrane (Stahl, Glockmann et al. 1999). A later study found Hsp93 tightly associated as well as Tic110, and this along with the presence of a TPR (tetratricopeptide) domain for protein-protein interactions and the observation that Tic40 is analogous to HSP70-interacting proteins suggested a role recruiting chaperones (Chou, Fitzpatrick et al. 2003). In addition, *Arabidopsis* knock-out mutants showed a chlorotic phenotype and imported preproteins at a slower rate, exhibiting the same phenotype as another knock-out mutant for Hsp93, and supporting the role of Tic40 as a chaperone recruiter (Kovacheva, Bedard et al. 2005).

Tic 22 and 20

Tic22 and Tic20 are thought to be important components of the Toc/Tic contact sites and were originally isolated crosslinked to arrested late import intermediates (Kouranov and Schnell 1997). Tic22 is a weakly associated inner membrane protein that localizes to the inter-membrane space, while Tic20 is an integral membrane protein of the inner membrane, and both have been shown to interact with Toc components during preprotein translocation (Kouranov, Chen et al. 1998). In addition, antisense knockouts of Tic20 in *Arabidopsis* resulted in a chlorotic phenotype and reduction of preprotein import at levels

that corresponded to the degree of reduction of expression of Tic20, indicating that it is an important component of the Tic complex (Chen, Smith et al. 2002). Tic20 has also been proposed to form part of the channel of the Tic complex because it has four putative transmembrane α -helices that insert deeply into the membrane, but this has not been confirmed to date.

Soluble factors

The interaction between transit peptides and soluble targeting factors in the cytosol has been a matter of ongoing debate. Over 75% of chloroplast transit peptides have been found to possess binding domains for either 14-3-3 proteins or Hsp70 protein chaperones, and some have been found to possess both (Ivey, Subramanian et al. 2000; May and Soll 2000; Zhang and Glaser 2002). The 14-3-3 proteins require phosphorylation of substrate to bind, and several preproteins have been shown to possess a putative phosphorylation motif $(P/G)X_n(K/R)X_n(S/T)X_n(S^*/T^*)$, where the asterisk denotes the site of phosphorylation and n can be 0-3 residues (Waagemann and Soll 1996). In addition, chloroplast preproteins translated in a wheat germ lysate have been shown to be phosphorylated and associated with 14-3-3 and Hsp70 proteins in a guidance complex (Waagemann and Soll 1996). The same study found that preprotein associated with a guidance complex was imported at a higher rate than the same preprotein without the guidance complex, however dephosphorylation of the transit peptide was required for complete import. Due to the observation that plant mitochondrial presequences are not phosphorylated, it has been suggested that tp phosphorylation could confer organelle targeting specificity. However, phosphorylation of the preprotein has been shown by other studies to be unnecessary, as mutagenesis of the predicted phosphorylation site resulted in no loss of chloroplast targeting and import (Nakrieko, Mould et al. 2004). Recently, the molecular chaperone Hsp90 was also implicated as a soluble factor that participates in delivery of preprotein to the Toc64 receptor of the chloroplast import apparatus, suggesting that different preproteins may interact with different subsets of chaperones, subsequently interacting with different Toc receptors (Qbadou, Becker et al. 2006). Also important to note is the fact that not all transit peptides have been shown to

possess chaperone binding domains, indicating that chaperone binding may not be required for targeting and import, but may have a role in targeting of highly expressed preproteins, preventing their build-up in the cytosol.

Interestingly, the motif(s) being investigated in the current study of prSSU are located on either side of the proposed transit peptide phosphorylation motif and even includes several amino acids of the motif. However, it is important to consider that the current study uses *in vitro* experiments with isolated chloroplasts and purified preproteins made in *E. coli*, eliminating the possibility of contributions from soluble cytosolic factors or phosphorylation of the preprotein. In addition, it has been shown that *in vitro* import into isolated chloroplasts lacking any cytosolic factors proceeds at a rate that is agreeable with that required for physiological chloroplast development (Pilon, Weisbeek et al. 1992; Dabney-Smith, van Den Wijngaard et al. 1999).

Lipid components of the chloroplast outer envelope

The chloroplast is surrounded by two membranes, presumably due to the original endosymbiotic event when the bacteria was phagocytosed by the host cell. The outer membrane has a very high lipid/protein ratio (about 80% lipids), having fewer proteins than other membranes of the cell (Block, Dorne et al. 1983) so that the lipids of the membrane may be the defining characteristic of the chloroplast membrane. Interestingly, the chloroplast is also unique in that the membranes contain lipids not typically found in eukaryotic cell membranes: MGDG, digalactosyldiacylglycerol (DGDG), both of which are galactolipids, sulfolipid (SL), and phosphatidylglycerol (PG), which is negatively charged. The chloroplast is the only organelle that exhibits the galactolipids MGDG and DGDG exposed to the cytosol of the cell (Bruce 2001). It has been proposed that these unique characteristics may provide a mechanism for the cell to distinguish between precursors targeted to the organelle, with chloroplast specific lipids inducing a conformational change in the transit peptide allowing recognition and import of the preprotein into the chloroplast. Several studies have supported this idea, showing that the transit peptide interacts with chloroplast outer membrane lipids, specifically MGDG, and has also been shown to adopt a more α -helical conformation in the presence of these

lipids (Pinnaduwaige and Bruce 1996; Wienk, Wechselberger et al. 2000). In addition, an *Arabidopsis* mutant deficient in the plastid lipid digalactosyl diacylglycerol (DGDG) was reduced in ability to import preproteins targeted to the chloroplast interior (Chen and Li 1998), indicating that lipids probably play a role in preprotein import.

Signal processing peptidase and presequence proteases

The transit peptide is cleaved off of the preprotein during or shortly after translocation into the chloroplast stroma by a zinc metallopeptidase, the stromal processing peptidase (SPP) (Richter and Lamppa 1999). The function of SPP is indispensable, with *Arabidopsis* antisense mutants showing a high percentage of seedling lethal phenotypes and survivors showing severely reduced ability to import preproteins, indicating that SPP function may influence even early stages of import (Zhong, Wan et al. 2003). SPP is soluble and has been shown to interact specifically with the C-terminus of transit peptides, resulting in specific cleavage followed by release of the mature domain (Richter and Lamppa 2002). The same study determined that the interaction of the transit peptide and SPP is terminated by a second cleavage that removes the C-terminus of the tp followed by release of the subfragments. Isolation of the subfragments has been difficult, indicating that degradation occurs rapidly after release. Interestingly, two separate metallopeptidases were recently isolated, presequence protease (PreP) 1 and 2, which are targeted to both mitochondria and chloroplasts and were found to further degrade targeting sequences in both organelles (Moberg, Stahl et al. 2003; Bhushan, Stahl et al. 2005). Following the removal of the transit peptide, the mature domain of the preprotein folds and assembles if it is a subunit of a larger complex or is further targeted to the thylakoid membrane or lumen with the aid of secondary targeting signals (Richter and Lamppa 1998).

Transit peptide

Targeting of the great majority of nuclear encoded precursor proteins to the chloroplast after synthesis in the cytosol is accomplished by the addition of an N-terminal transit peptide which has been shown to be both necessary and sufficient for targeting and

import. The transit peptide is considered necessary for targeting and import because chloroplast targeted preproteins will no longer be imported into the chloroplast if the transit peptide region is removed (Archer and Keegstra 1990; Dabney-Smith, van Den Wijngaard et al. 1999). In addition, transit peptides are considered sufficient for targeting and import because in most cases they can be attached to a non-chloroplast targeted protein, GFP for example, resulting in import of the protein into chloroplasts, indicating that the transit peptide contains all the information needed for chloroplast targeting and import (Smeekens, van Steeg et al. 1987; Lee, Kim et al. 2002). The transit peptide is cleaved after import, resulting in a mature protein that undergoes folding and assembly in the chloroplast. Recent studies have identified chloroplast proteins that lack a cleavable transit peptide, but these probably do not utilize the general import pathway which is the subject of the current study, and will not be discussed further (Radhamony and Theg 2006).

Characteristics of transit peptides

The most prominent characteristic of transit peptides is that they are highly heterogeneous in length, composition, and organization, making it very difficult to decipher the information that they must contain in order to accomplish targeting and import of their respective preproteins into chloroplasts. Chloroplast transit peptides range from 25 to 150 amino acids in length and very little primary sequence homology is apparent between them (Zhang and Glaser 2002). However, a few features are shared: most have an uncharged N-terminus and a basic central and/or C-terminal region (May and Soll 2000; Bhushan, Kuhn et al. 2006). They also tend to be enriched in the hydroxylated amino acids serine and threonine and small nonpolar residues such as alanine, and lack the positively charged residues aspartate and glutamate (von Heijne, Steppuhn et al. 1989). Due to the lack of primary sequence homology and the long length of transit peptides, it has been suggested that they may be composed of domains that interact with different factors during targeting and import and that these domains may overlap, causing a lack of homology between different transit peptides (Pilon, Wienk et al. 1995; Rensink, Pilon et al. 1998). The variability in primary sequences also led to

another idea that the defining information contained within the targeting peptides may be structural rather than sequence specific. Unfortunately, chloroplast transit peptides have proven difficult to study by NMR or crystallization due to their unstructured nature, resulting in very few determined structures (Bruce 2000). For the few transit peptides that have been characterized, the structural information indicates that chloroplast transit peptides are unstructured only until exposed to membranes or membrane-mimetic lipids, at which time they form helices (Wienk, Czisch et al. 1999; Bruce 2001).

FGLK motif

Despite the general lack of primary sequence homology between transit peptides, conserved regions have been observed in many transit peptides. Initial examination of a small number of transit peptides resulted in the proposal of a loosely defined motif: h(P/G)h(R/K), or hydrophobic residue, proline or glycine, hydrophobic residue, and a positively charged residue, which was identified in both the precursor to the small subunit of Rubisco and light harvesting chlorophyll a/b protein (LHCP)II (Karlin-Neumann and Tobin 1986). Shortly afterwards, a loosely conserved motif, FGLK, was identified in the C-terminal domain of the transit peptide of Ferredoxin from *Silene pratensis* and was found to be present in the transit peptide of Ferredoxin from other plants as well as transit peptides from other precursor proteins (Pilon, Wienk et al. 1995). Fig. 1-4 shows an alignment of the transit peptides of the small subunit of Rubisco from a wide range of organisms. The two regions showing the highest similarity between the different transit peptides are a region containing the FGLK motif and a region that surrounds the site of specific recognition and cleavage of the transit peptide by stromal processing peptidase (SPP).

Studies using both *in vitro* and *in vivo* methods have supported the idea that the FGLK motif may be important for preprotein import into chloroplasts. Early studies found that removal of portions of the transit peptide containing this motif resulted in loss of import efficiency, suggesting that the motif serves a role in chloroplast protein import (Pilon, Wienk et al. 1995). More recent studies have also implicated the FGLK motif; recent *in vivo* work targeting GFP reporter proteins fused to a transit peptide in *Arabidopsis* leaf

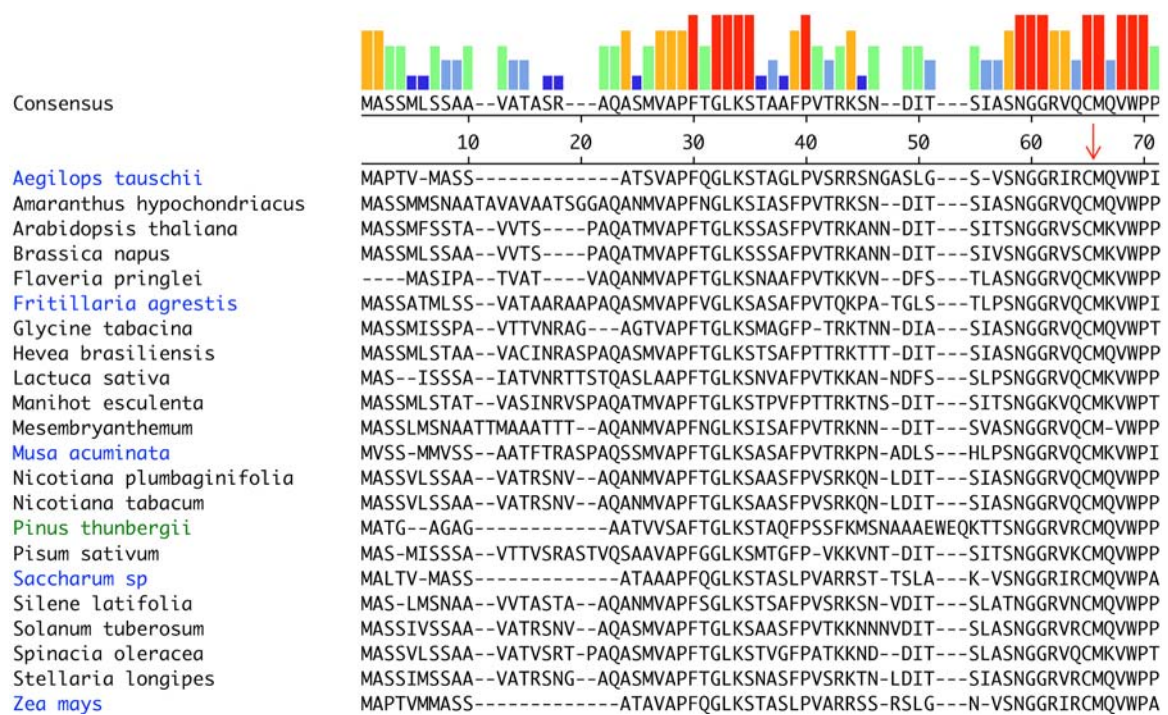


Figure 1-4 Alignment of transit peptides from the small subunit of Rubisco

Transit peptides of the small subunit of Rubisco from different organisms are shown aligned using MegAlign software from DNASTar. The level of conservation between transit peptides is indicated by the color and height of the bars above each residue, with the tall red bars indicating the most conserved residues and blue or no bar indicating the least conserved residues. Transit peptides from several different organisms are shown, with the genus and species of monocots in blue text, angiosperms in green, and dicots in black. The SPP cleavage site at the C-terminus is indicated by the red arrow.

protoplasts focused on 10-12 amino acid blocks, both replacing them with alanine and deleting them completely (Lee, Lee et al. 2006). Two sequences in one of the blocks were found to be absolutely critical for targeting and import, FP and RK, which are part of the FGLK motifs in this transit peptide. These studies clearly suggest that the FGLK motif plays an important role in chloroplast protein import, yet no studies to date have specifically studied these motifs, a goal undertaken in the current study.

Rubisco

Rubisco, ribulose 1,5 biphosphate carboxylase/oxygenase is the most abundant protein in the world and catalyzes the first step of photosynthetic CO₂ assimilation and photorespiratory carbon oxidation. It is the rate-limiting step in carbon-fixation of photosynthesis and is notoriously inefficient at its job, a characteristic that is compensated for by making large amounts of the enzyme; about 50% of the total protein in chloroplasts is Rubisco. The holoenzyme is composed of 8 large subunits which are present in the chloroplast genome and 8 small subunits that are present on the nuclear genome and must be imported into the chloroplast (Spreitzer 2003). The small subunits are synthesized in the cytosol as a larger molecular weight precursor, prSSU (precursor to the small subunit of Rubisco), which has a transit peptide and is targeted and imported into the chloroplast for assembly with the large subunits into the holoenzyme. PrSSU was the first chloroplast preprotein demonstrated to undergo post-translational import and remains one of the most well-studied chloroplast precursor proteins (Dobberstein, Blobel et al. 1977).

Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) is a classical technique for the study of protein interactions as well as self-association, and has the advantage of being able to observe these interactions in solution without any added modifications. The analytical ultracentrifuge is similar to a conventional preparative centrifuge, but is equipped with an optical system allowing the observation of the protein distribution during the centrifugation. This permits the observation of the spatial concentration gradients that

result from the applied gravitational field (Lebowitz, Lewis et al. 2002). There are two types of AUC, sedimentation equilibrium (SE) which has traditionally been used to determine molar mass and study self-association and heterogeneous interactions, and sedimentation velocity (SV), which is typically used for the identification of oligomeric state as well as hydrodynamic shape of proteins (Lebowitz, Lewis et al. 2002). SE uses lower centrifugal forces than SV, allowing sedimentation to achieve equilibrium with diffusional transport, causing net transport of the macromolecule to cease.

However, for fast or weak protein-protein associations, SE analysis may be problematic, making SV analysis preferable (Schuck 2003). SV is carried out at higher centrifugal force, causing the macromolecules to migrate downward, depleting the proteins at the meniscus and forming a concentration boundary that moves toward the bottom of the centrifuge cell as a function of time. The rate of migration and concentration boundary shape will be determined by both the size and shape of the macromolecule (Brown and Schuck 2006). Furthermore, since the migration due to the centrifugal force is opposed by molecular friction, sedimentation depends on hydrodynamic properties of the molecules, reflecting the low-resolution structure of protein complexes, and enabling the detection of conformational changes (Schuck, Perugini et al. 2002). In addition, a very important feature of sedimentation analysis of protein interactions is the fact that faster sedimenting components migrate through a solution of the components that sediment at a slower rate, enabling reversible reactions to occur throughout the time scale of sedimentation and allowing weak or transient interactions to be characterized (Schuck 2003; Brown and Schuck 2006).

In fact, in the case of interacting molecules, SV direct boundary modeling, or $c(s)$ analysis (discussed in detail in Materials and Methods), can also be used to determine the time scale of the reaction. A rapidly occurring reaction ($k_{\text{off}} \sim 10^{-4}$ - 10^{-3}) will result in apparent sedimentation coefficients that are concentration dependent and distributed between the upper and lower sedimentation values. An illustration of such an interaction is shown in Fig. 1-5. In contrast, a slow reversible reaction ($k_{\text{off}} \sim 10^{-5}$) will result in sharp peaks at constant sedimentation coefficients corresponding to the sedimentation values of the monomer and higher order oligomer (Schuck 2003). Examples in which SV

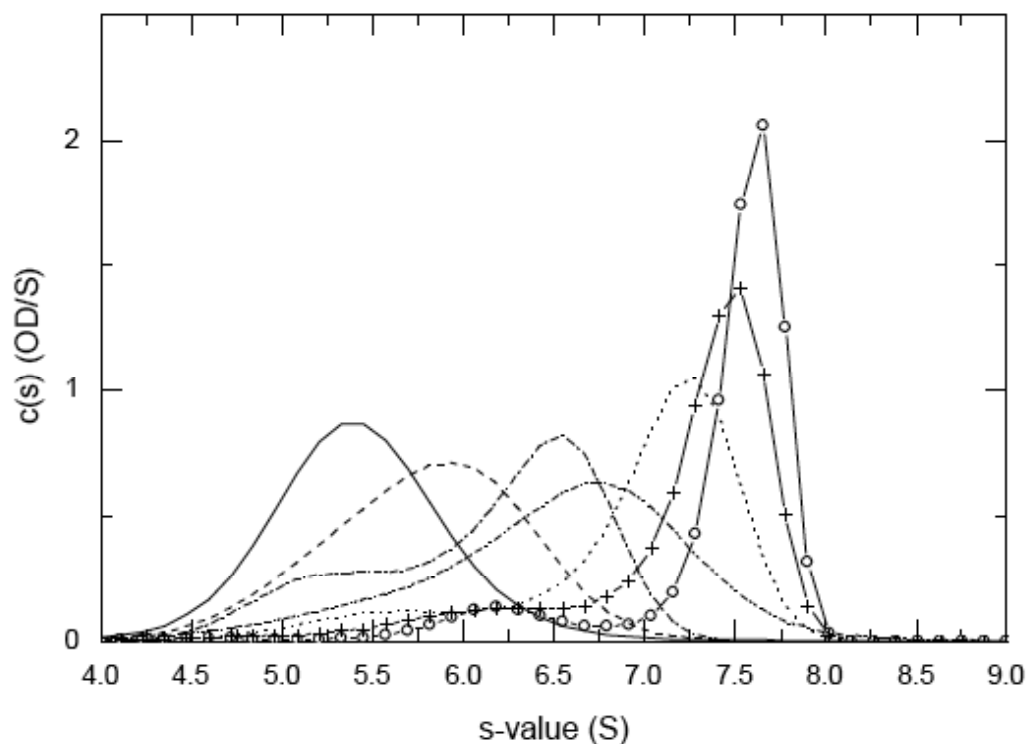


Figure 1-5 Sedimentation coefficient distribution of an SV simulation of a monomer-dimer fast association.

Sedimentation profiles of a simulated monomer-dimer system demonstrating fast association ($k_{\text{off}} \sim 10^{-3}$) were subjected to direct boundary modeling $c(s)$ analysis. Concentrations shown are 0.2 μM (solid line), 0.5 μM (dashed line), 1 μM (dash-dotted line), 2 μM (dash-dot-dot line), 5 μM (dotted line), 10 μM (+), and 20 μM (circles). The $c(s)$ distributions were normalized to make comparison easier. Figure adapted from Schuck, 2003.

ultracentrifugation has been used in conjunction with direct boundary modeling c(s) analysis to characterize complex protein interactions include ligand-induced association of regulatory proteins, formation of amyloid fibers, structural requirements for ligand-binding of membrane receptor proteins and many others (Nourse, Trabi et al. 2004; Khursigara, De Crescenzo et al. 2005; Howlett, Minton et al. 2006; Mok and Howlett 2006).

Summary

Photosynthesis performs the necessary job of converting energy from the sun into usable chemical energy, fixing carbon dioxide and generating oxygen, supporting all life on earth (Kessler and Schnell 2006). All of this occurs in the chloroplast of eukaryotes, an organelle that originated from an ancient endosymbiotic event and has since transferred much of its genetic information to the cell. Proteins necessary for chloroplast biogenesis are therefore synthesized in the cytosol and must be targeted and imported into the chloroplast in order for proper chloroplast development. The current study focuses on the early stages of import: the interaction of the transit peptide of the precursor protein with the chloroplast import apparatus. In order to probe the importance of a putative FGLK motif, corresponding regions of the transit peptide were deleted in prSSU from tobacco. The ability of the mutant precursor proteins to compete for import into isolated chloroplasts was then characterized. Furthermore, the mutant transit peptides were then purified in soluble form without the mature domain to biochemically characterize their interaction with a GTPase receptor of the Toc complex, psToc34. PsToc34 was found to exhibit complex interactions with both of its substrates, nucleotide and transit peptide, and this characteristic was exploited to test the ability of the transit peptides to influence the receptor. This study provides new insight into the importance of the FGLK motif for both general import and specific transit peptide/receptor interactions.

Chapter 2 Materials and Methods

Plant growth and chloroplast isolation

Chloroplasts were isolated from dwarf pea (*Pisum sativum*) seedlings 10-12 days old (Thomas Laxton var. 2968 from Shumway Quality Seed, Graniteville, SC). Dry pea seeds (approximately 300 ml/flat) were imbibed over night with aeration and planted in horticultural vermiculite in 12 X 24 X 5 inch flats. Plants were grown in EGC growth chambers with 160 $\mu\text{E}/\text{m}^2/\text{sec}$ cool white fluorescent light. In order to reduce starch accumulation, plants were grown on a 12 hr light/12 hr dark cycle. For chloroplast isolation, leaves were harvested at the end of a dark cycle and chopped in a Cuisinart food processor with 2-3 sec bursts until uniformly reduced to 3-5 mm pieces. The leaf tissue was then thoroughly homogenized in the presence of Grinding Buffer (GB: 330 mM Sorbitol, 2mM MgCl_2 , 2 mM MnCl_2 , 4 mM EDTA, 0.2% w/v BSA, 50 mM HEPES-KOH, pH 7.3), the volume to be used being 3X the weight in grams of leaves harvested. A Polytron was used to homogenize the tissue in 2-3 sec bursts 3-4 times. The homogenate was filtered through 4 layers of cheesecloth and 1 layer of MiraCloth. The filtrate was centrifuged at 2,000 x g for 6 min in a Sorvall GS-3 rotor using a Sorvall RC26 Plus centrifuge at 4°C to collect the chloroplasts. The pellet was resuspended in 5-10 ml of Import Buffer (IB: 330 mM Sorbitol, 50 mM HEPES-KOH, pH 8), and gently layered onto 50% Percoll (Pharmacia) continuous gradients that had been prepared by mixing 2 X IB and Percoll at equal volumes and centrifuging for 30 min at 20,000 x g in a Sorvall SS 34 rotor with the brake off. Loaded gradients were centrifuged at 6,000 x g for 14 min in a Sorvall HB-4 swinging bucket rotor with the brake off, collecting intact chloroplasts into a dark-green band near the bottom of the gradient. Intact chloroplasts were collected using a large-bore syringe needle, diluted with 3 X their volume of 1 X IB, and centrifuged at 3,500 x g for 6 min in the Sorvall HB-4 rotor to remove Percoll. The pellet of intact chloroplasts was resuspended with approximately 4 ml 1 X IB and the chlorophyll content assessed as described below.

Chlorophyll measurement

Chlorophyll concentration was determined in order to bring the chloroplasts to the 1 mg chlorophyll/ml concentration used for chloroplast import assays. Chlorophyll a and b content was determined by chlorophyll extraction with 80% acetone: 50 µl of chloroplast suspension was added to 5 ml 80% acetone and centrifuged at 14,000 x g for 3 min in a microcentrifuge to remove starch. Absorbance measurements at 663 for chlorophyll a and 645 nm for chlorophyll b were obtained and used to calculate total chlorophyll content with the following equation (Bruce, Perry et al. 1994).

$$\text{Chlorophyll}(mg/ml) = \frac{((8.02 \times A_{663}) + (20.2 \times A_{645}))}{0.05ml \times 1000 \mu g/mg} \times 5ml$$

After chlorophyll concentration was determined, chloroplasts were diluted with 1 X IB to obtain a 1 mg chlorophyll/ml final concentration.

Generation of Δ FTGLK and Δ FPVSR prSSU mutants

Site-directed mutagenesis of prSSU in the pET11d vector was performed using the Stratagene QuikChange kit (Stratagene, La Jolla, CA). Primers were designed that had 9-15 bases on either side of the portion to be deleted and lacked the bases to be deleted. Both forward and reverse primers were designed and were synthesized by IDT (Coralville, IA). Table 1 shows the sequences of the primers used to introduce each deletion. Long distance PCR using ExTaq DNA polymerase (Takara Bio Inc., Shiga, Japan) was performed in an Eppendorf thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) using the following cycling conditions:

1. 95° 1 min.
2. 95° 30 sec.
3. 54° 1 min.
4. 68° 12 min.
5. repeat steps 2-4 35 times
6. 4° hold

The long distance PCR reaction set up is shown in Table 2. The PCR product was digested with DpnI (NEB, Beverly, MA), a restriction enzyme that digests methylated

Table 1 Primer sequences

Primer name	Strand	Length	Sequence 5' → 3'
ΔFTGLK	+	21	GGTTGCACCTTCAGCTGCCTC
ΔFTGLK	-	20	GAGGCAGCTGAAGGTGCAAC
ΔFTG	+	27	GGTTGCACCTCTTAAGTCAGCTGCCTC
ΔFTG	-	26	GAGGCAGCTGACTTAAGAGGTGCAAC
ΔGLK	+	33	GGTTGCACCTTTCACCTCAGCTGCCTCATTCCC
ΔGLK	-	25	GGCAGCTGAAGTGAAAGGTGCAACC
ΔFPVSR	+	24	CAGCTGCCTCAAAGCAAAACCTTG
ΔFPVSR	-	21	GGTTTTGCTTTGAGGCAGCTG
ΔFPV	+	19	GCTGCCTCATCAAGGAAGC
ΔFPV	-	30	GCTTCCTTGATGAGGCAGCTGACTTAAGGC
ΔVSR	+	30	CAGCTGCCTCATTCCCTAAGCAAAACCTTG
ΔVSR	-	27	GGTTTTGCTTAGGGAATGAGGCAGCTG
N-NdeIns	+	27	GGTAGATACATATGGCTTCCTCAGTTC
C-SmaIns	-	26	GTAAATTGGTGGCCCGGGCTGCATGC
T7 Universal	+	17	TAATACGACTCACTATA
Intein reverse	-	24	GAGGTTGGTAATAAGGTCATGGGT

All primers were synthesized by IDT (Coralville, IA), received as lyophilized powder, and resuspended to 500 ng/μl in distilled, autoclaved H₂O. These were the 5 X stocks and were kept frozen at -20°C. Working stocks of each primer were made by diluting a small aliquot to 100 ng/μl to prevent contamination of primer stocks.

Table 2 Long distance PCR set up

50 µl PCR reaction	
10 X ExTaq Buffer	5 µl
prSSU template	1 µl
Primer forward (100 ng/µl)	1.25 µl
Primer reverse (100 ng/µl)	1.25 µl
10 mM dNTPs	1 µl
ddH ₂ O	40.25 µl
ExTaq	0.25 µl

and hemimethylated DNA, at 37°C for one hour to remove the parental plasmid. The digested product was then used to transform Nova Blue competent cells. Candidates were selected, plasmid prepped, and confirmed by automated DNA sequencing in one direction using the T7 universal primer listed in Table 1. DNA sequencing results were analyzed using SeqMan software (DNASTAR, Madison, WI).

Subcloning of the transit peptide region into the pTYB2 vector

The transit peptide of the small subunit of Rubisco was subcloned into the pTYB2 vector to enable purification of soluble transit peptide without any additional tags that could interfere with biochemical analysis of peptide structure and function. The transit peptide of the small subunit of Rubisco from *N. tabacum* (SstpNt) was obtained by PCR amplification of the transit peptide region of the full-length precursor (pET11-prSSU). The primers N-NdeIns and C-SmaIns in Table 1 were used to insert an N-terminal *NdeI* site and a C-terminal *SmaI* site. The PCR product was restriction digested with *NdeI* and *XmaI* (an isoschizomer of *SmaI*) using these sites and subcloned into the pTYB2 vector (New England BioLabs, Beverly, MA, USA), introducing a C-terminal cleavable Intein tag and a chitin-binding domain. This construct permitted one-step purification of the protein using a chitin column, described below.

Colony screen of ligation product

Initial screening of the ligation products to confirm insertion of the gene into the pTYB2 vector was accomplished by colony screening. PCR amplification of the ligation product using the T7 universal primer and the intein reverse primer shown in Table 1 resulted in a 222 bp product if the transit peptide wasn't present and a 349 bp product if the transit peptide was inserted. The PCR reaction set up is shown in Table 3. The PCR screen was performed in an Eppendorf thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) using the following cycling conditions:

1. 94° 4 min.
2. 94° 30 sec.
3. 55° 20 sec.

Table 3 Direct colony PCR screen

25 µl colony screen	
5 X GoTaq Buffer	5 µl
5% Triton X-100	1 µl
10 mM dNTP's	0.25 µl
T7 universal primer (100 ng/µl)	0.5 µl
INTEIN reverse primer (100 ng/µl)	0.5 µl
Taq polymerase	0.1 µl
ddH ₂ O*	17.65 µl

* *E. coli* colony is added directly to ddH₂O in the PCR tube before adding remaining ingredients

4. 72° 30 sec.
5. repeat steps 2-4 35 times
6. 72° 7 min.
7. 4° hold

The PCR products were visualized by running on a 6% Acrylamide, 1 X TAE (Tris, acetic acid, EDTA) gel and staining with Ethidium Bromide. Colonies that produced the larger product were selected for plasmid prep and automated sequencing in both directions using the T7 universal primer and Intein reverse sequencing primer.

Purification of prSSU from inclusion bodies

The pET11-prSSU, mSSU, and the prSSU deletion constructs were used to transform *E. coli* [BL21(DE3)RIL] competent cells. An induction screen was performed on at least 4 transformed colonies in 1 ml of Luria Broth (LB) with 150 µg/ml Ampicillin to choose the colonies producing the most protein. After choosing the best producing colonies, 1 L cultures of cells were grown in LB at 37° with shaking at 225 rpm until a cell density giving an OD₆₀₀ of 0.6 was reached and were induced with 1 mM IPTG (Iso-Propyl-β-Thio Galactopyranoside) for 3-4 hours. The cells were pelleted by centrifugation and resuspended for lysis in ice cold Buffer A (50 mM Tris-HCl, 5 mM MgCl₂, pH 7.6). PrSSU as well as the other constructs are found predominantly in inclusion bodies when over expressed in *E. coli*, making purification relatively simple and resulting in high yields of pure protein. Cells were lysed by passing through a French Press (Thermo Electron Corporation, Waltham, MA) two to three times at 20,000 psi followed by centrifugation at 40,000 x g for 20 min. in a Sorvall SS 34 rotor. Inclusion bodies were isolated to homogeneity by 3-4 rounds of washing in Buffer A with 0.1% Triton X-100 followed by 4-5 rinses in Buffer A without detergent, resuspending thoroughly using a Dounce homogenizer and centrifuging at 40,000 x g for 10 min. in a Sorvall SS 34 rotor each time. The final purified inclusion body pellets were then solubilized in 8 M Urea containing 50 mM DTT and 20 mM Tris-HCl, pH 8. Remaining impurities were removed by centrifugation at 40,000 x g for 30 min. The concentrations of purified and solubilized prSSU, mSSU, and mutants were determined by the Bradford method as

described in the protein measurement section below, and were brought to 2 mg/ml, aliquotted and stored at -80°C .

***In vivo* radiolabeling of WT prSSU**

WT prSSU was radiolabeled with ^{35}S -methionine by performing cell growth in methionine and cysteine-deficient DMEM (Dulbecco's Modified EAGLES Medium) (ICN, Ohio). Cells were first grown in LB until a cell density corresponding to an OD_{600} of 0.5 was achieved. They were then pelleted, washed once in methionine-deficient DMEM, and finally resuspended in the DMEM. After allowing cells to deplete their methionine sources by growing in methionine-deficient DMEM for 2-3 hours, they were induced using 1 mM IPTG. After 15 min of induction, 200 $\mu\text{Ci}/5$ ml culture of methionine/cysteine Trans- ^{35}S label was added (ICN). The Trans label is added after the initial 15-min induction to ensure that most of the label is incorporated into the protein of interest rather than the T7 RNA polymerase, which is also induced at high levels upon addition of IPTG. The induction was allowed to continue for 3 hours with the ^{35}S -trans label being the only source of methionine and cysteine, forcing incorporation of these radioactive amino acid residues into translated proteins. Radioactively labeled prSSU in inclusion bodies was then purified in the same manner mentioned above, with the following changes. To lyse cells, a probe sonicator was used (Misonix Sonic 550 probe ultrasonicator), sonicating cells on ice for 2 min total sonication time using a cycle of 5 sec on and 10 sec off. After thoroughly washing the inclusion body bound preprotein as described for the inclusion body purification in the previous section, the final pellet was resuspended in 1-2 ml of 8 M Urea with 50 mM DTT and 20 mM Tris-HCl, pH 8. The specific activity was calculated and corrected for the purity of the protein as determined by scanning densitometric analysis of an 18% SDS-PAGE gel.

Expression and purification of SStpNt and mutants

The construct was transformed into competent *E. coli* strain ER2566 and grown at 37°C in 1-2 L of Luria Broth media with 150 $\mu\text{g}/\text{ml}$ Ampicillin to an O.D._{600} of 0.6. IPTG was added to 1 mM to initiate induction of the fusion protein, and induction was continued at

a lowered temperature of 25°C for 14-16 hours. Cells were harvested by centrifugation, resuspended in 25-30 ml lysis buffer per liter of cell culture being prepped (20 mM Na-phosphate (pH 8), 0.5 M NaCl, 1mM EDTA, and 0.1% Triton X-100) with protease inhibitors (100 µg/ml PMSF, 1 µM leupeptin, and 1 µM pepstatin) and lysed by probe sonication (Misonix Sonic 550 probe ultrasonicator) set at medium power (5 out of 10) on ice for 3 min total sonication time, in 10-sec bursts. The lysate was centrifuged for 30 min at 20,000 g in a Sorvall SS 34 rotor, and the supernatant was loaded onto a chitin matrix column (5 ml volume/ liter prepped, NEB, Beverly, MA). After thorough washing of the column with ice-cold column buffer (20 mM Na-phosphate (pH 8), 0.5 M NaCl, 1mM EDTA, 100 µg/ml PMSF, 1 µM leupeptin, and 1 µM pepstatin), the buffer was replaced with ice-cold phosphate buffer (1 mM Na₂HPO₄, 0.17 mM KH₂PO₄, pH 7.5) with 50 mM β-ME to induce cleavage. To prevent loss of transit peptide due to intein self-cleavage, the buffer replacement step was not allowed to go longer than 30 min before capping off the column and incubating at 4°C. SStpNt was eluted in three 6 ml elutions after 24 h of on-column cleavage with 50 mM β-ME at 4°C and lyophilized to concentrate before being analyzed on a 19.2% Tris/Tricine SDS-PAGE gel stained with Coomassie Brilliant Blue as described in the gel electrophoresis section. One liter of cell culture routinely yielded 15-30 mg of transit peptide. Storage of peptide was at -20°C as a lyophilized powder.

MALDI-TOF of peptides

The peptides were analyzed by MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry to confirm purity and correct cleavage. MALDI-TOF MS was performed on a Bruker Daltonics MicroflexTM mass spectrometer, with Apomyoglobin used as an internal standard for calibration. Lyophilized standard protein and transit peptide were resuspended to a final concentration of 50 pmol/µl in 10 µl of 50% ACN v/v with 0.1% TFA, v/v. The samples were mixed with 10 µl of CHCA (α-cyano-4-hydroxycinnamic acid, Sigma-Aldrich, St. Louis, MO) matrix suspended at 10mg/ml in 50% ACN, 0.1% TFA v/v, 1 µl was spotted on the plate, and they were dried thoroughly in a vacuum dessicator for one hour at room temperature. Mass spectra were

acquired in positive ion mode accumulating 100 nitrogen laser pulses/ spectrum. The resulting mass value data was analyzed using the FindPept program which is freely accessible on the web at: <http://ca.expasy.org/tools/findpept.html>. This program allows the user to input the peptide amino acid sequence and possible modifications as well as the mass values from MS analysis and identifies the peptide fragments (or intact peptide) that correspond to the experimentally obtained mass values.

Over expression and purification of the cytosolic domain of psToc34

A construct containing the cytosolic domain of psToc34 lacking the C-terminal transmembrane domain (psToc34 Δ TM) in the pET21d vector obtained from Prof. Jurgen Soll (Munich, Germany) was used to transform the BL21(DE3)RIL strain of *E. coli* competent cells. The transmembrane domain has been deleted in this construct by inserting a C-terminal His tag immediately following residue D243, providing a straightforward method of protein isolation using a Co²⁺ metal affinity column, described below. After performing an induction screen on at least four colonies in small 1 ml LB cultures with 150 μ g/ml Ampicillin to choose the colonies that produced the most protein, the cells were grown at 37°C in 2-3 L of Luria Broth media with 150 μ g/ml Ampicillin added to an OD₆₀₀ of 0.5 or less. This step was found to be extremely important, as overgrowth at any stage of cell culture lead to production of insoluble protein. IPTG was added to 1 mM, and induction was continued at the lowered temperature of 25°C for 3 hours. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Sodium Phosphate pH 7, 300 mM NaCl, 0.5% Triton X-100, 5 mM β -ME), and lysed by passage through a French Press (Thermo Electron Corporation, Waltham, MA) at least 2-3 times at 20,000 psi. Benzonase (Promega, Madison, WI) was added to digest DNA, using 1 μ l of Benzonase for every 10 ml of lysed cells and incubating for 15 min on ice. Insoluble cellular debris was separated out by centrifugation at 20,000 x g for 30 min. The clarified cell extract was loaded onto a Cobalt-Sepharose column that had been prepared as follows: 2 ml of Sepharose fast flow resin (Amersham Biosciences, Piscataway, NJ) was used for every L of cell culture being processed, placed

in a low pressure column, rinsed with 5 column volumes (CV) of water, and charged with cobalt metal by adding 1 CV of 0.3 M CoCl_2 and incubating for 5-10 minutes with rocking, followed by extensive rinsing with at least 15 CV of water to remove unbound metal, and equilibrated with 5 CV of column buffer (50 mM Sodium Phosphate pH 7, 300 mM NaCl, 1 mM β -ME). The clarified cell extract was loaded onto the Co^{++} metal affinity column using a slow flow rate of 1 ml/min at room temperature to encourage binding. The column was washed thoroughly with column buffer, using at least 20 column volumes and a flow rate of 2-3 ml/min. Because long-term storage and stability of the eluted protein was problematic, a method for maintaining the protein on the column resin in 20% glycerol in single-use amounts was devised. The buffer was first replaced with 1 X GBS (20 mM Tricine/KOH pH 7.6, 1 mM MgCl_2 , 50 mM NaCl, 1mM β -ME) with 20% glycerol by running at least 5 column volumes through the column. The column was then closed off and mixed by inversion to distribute the resin-bound protein equally throughout the column. The column resin was then divided equally between 2 ml spin columns (BioRad Laboratories, Hercules, CA) and frozen at -20°C until the protein was needed. To elute the protein, a spin column was thawed on ice and washed with 1 X GBS without glycerol by placing the spin column into a 2 ml Eppendorf tube and spinning at 7,000 rpm in a microcentrifuge. After repeating this wash step 3 times, the glycerol was removed and the protein could be eluted. Elution was performed by placing the spin column into a fresh 2 ml Eppendorf tube, adding 400 μl of 1 X GBS with 300 mM Imidazole, incubating 5 min, and spinning at 7,000 rpm in a microcentrifuge for 30 sec. Four elutions were collected, with most of the protein in the first and second elutions. Protein concentration was determined by Bradford assay as described in the protein measurement section and was immediately diluted to 2 mg/ml using 1 X GBS. Dialysis to remove imidazole was performed using SnakeSkin Dialysis Tubing (Pierce, Rockford, IL) in 1 X GBS at 4°C , changing the buffer 3 times and letting it go overnight to guarantee that it had reached equilibrium.

***In vitro* chloroplast protein import competition assay**

The chloroplast protein import competition assay was performed with freshly isolated intact chloroplasts at 25 µg/ml chlorophyll final concentration. In order to determine the import competency of the mutant precursor proteins, the competition assay kept WT ³⁵S radiolabeled prSSU at 100 nM while increasing the concentration of unlabeled preprotein competitors. Five concentrations were tested for each competitor protein: 0 nM, 30 nM, 100 nM, 300 nM, and 600 nM. Nucleotide in the form of Mg-ATP (3 mM) was added from a freshly prepared 100 mM stock solution at levels sufficient to support import of the preprotein. Urea concentrations were kept below 600 mM to prevent inhibition of import (Cline, Henry et al. 1993). Table 4 shows conditions for all import competition assays. The assay was started by adding chloroplasts to the assembled import mixture and was incubated at room temperature for 20 minutes with occasional mixing to keep chloroplasts suspended. To terminate the import competition assay, 700 µl of ice-cold 1 X IB was added to dilute out the precursor and ATP and to rapidly lower the temperature, halting import, and the tubes were placed on ice. Intact chloroplasts were immediately reisolated away from unimported and unbound precursor proteins by layering the samples over 900 ml of 40% Percoll in 1 X IB and centrifuging in a Sorvall swinging bucket tabletop centrifuge at 3,500 x g for 6 min. The supernatant containing free precursor proteins and lysed chloroplasts was discarded and the pellet containing intact chloroplasts was resuspended in 1 ml 1 X IB. Samples (50 µl) were removed for protein concentration determination by BCA (described in the Protein Measurement section below) and the remainder (950 µl) was centrifuged again as in the previous step. The pellet was resuspended in 25 µl ddH₂O, 25 µl of 4 X SSB (sample solubilizing buffer) was added, and finally the samples were boiled for 3 min. Samples were brought to equal protein concentration by adding ddH₂O, the volume to add determined by the BCA results. Finally, samples were run on 18% polyacrylamide gels (described in the Gel Electrophoresis section below) overnight or until the green chlorophyll band was 2/3 down the gel and the dye front had run off. The gels were dried on a gel-dryer and placed on storage phosphor screens for 16-24 hours. Visualization was accomplished by scanning the screens with a storage phosphor system, the Storm PhosphorImager

Table 4 Chloroplast import competition assay

<i>300 μl rxn</i> <i>μl to add</i>	<i>competitor concentration</i>				
	<i>0 nM</i>	<i>30 nM</i>	<i>100 nM</i>	<i>300 nM</i>	<i>600 nM</i>
1 X IB	185.8	185.8	185.8	185.8	185.8
2 X IB	19.6	19.6	19.6	19.6	19.6
1M DTT	3.0	3.0	3.0	3.0	3.0
100 mM Mg⁺⁺-ATP	9.0	9.0	9.0	9.0	9.0
8M Urea	6.1	4.3	none	4.3	2.4
chloroplasts	75	75	75	75	75
20 μM ³⁵S-prSSU	1.5	1.5	1.5	1.5	1.5
1 mg/ml competitors	-	1.8*	6.1*	1.8	3.7

* add from 0.1 mg/ml competitor stock solution, not 1 mg/ml stock solution

(Molecular Dynamics, CA), and quantification of band intensity was performed using ImageQuant analysis software (Molecular Dynamics).

Data analysis

Analysis of the chloroplast import competition assays was accomplished by exposing the dried gels to phosphor screens, scanning on the PhosphorImager, and quantifying with ImageQuant. Import was expressed as percent of WT ³⁵S-labeled prSSU imported without any competitor present. At least three repeats of each import assay were performed, which consisted of 3 separate competition assays (300 µl reaction volume competition as shown in Table 4) using the same chloroplast prep. At least two WT control competitions (prSSU competitor with ³⁵S-prSSU) were performed for each chloroplast prep and all deletion competitions were graphed along with their respective WT competitions. This was done to minimize the effects of uncontrollable variability in different chloroplast preps. Data was assembled and nonlinear regression analysis performed using GraphPad Prism (GraphPad Software, Inc.).

AUC analysis of psToc34

Analytical Ultracentrifugation (AUC) analysis was used to follow the formation of homodimers of psToc34, one of the GTPase receptors of the translocon on the outer chloroplast membrane. Sedimentation velocity analyses used a Beckman Optima XL-1 analytical ultracentrifuge with both absorbance and interference optical detection systems. After dialyzing samples extensively into 1 X GBS as described above, 400 µl was loaded into epon double sector centerpieces. For experiments at different concentrations, all dilutions were made using the dialysate. Experiments analyzing the effect of nucleotides had the same concentrations of nucleotide added to both sample and dialysis buffer for the last hour of dialysis. When included, transit peptides were dialyzed alongside psToc34 using minidialysis cups with a molecular weight cut-off (MWCO) of 3,500 daltons. For all experiments, dialysate was used as the reference buffer loaded in the reference sector of the centerpiece. Sample cells were loaded into the rotor, placed in the ultra centrifuge, vacuum turned on, and were allowed to equilibrate to 25°C for at

least 1 hour. After fully equilibrated, samples were centrifuged at 50,000 rpm and data was collected using interference optics and/or absorbance optics at 280 nm.

Data analysis

Analysis of the sedimentation velocity data was performed using the program SEDFIT, which is freely available for download on the World Wide Web (www.AnalyticalUltracentrifugation.com). SEDFIT uses Lamm equation modeling to carry out size distribution analyses (Dam and Schuck 2004). The velocity profiles are analyzed by direct boundary modeling using distributions of Lamm equation solutions $c(s)$ as follows:

$$a(r,t) \cong \int_{s_{\min}}^{s_{\max}} c(s) x_1(s, F, r, t) ds + \beta(t)$$

where $a(r,t)$ denotes the measured absorbance or interference scan profiles at position r at time t , $b(r)$ and $\beta(t)$ are the systematic offsets (Schuck 2000), and $x_1(s, F, r, t)$ are the solutions of the Lamm equation, which is shown below:

$$\frac{\partial x}{\partial t} = \frac{1}{r} \frac{\partial}{\partial r} \left[r D \frac{\partial x}{\partial r} - s \omega^2 r^2 x \right]$$

where ω denotes the rotor velocity, D is the diffusion coefficient, and s is the sedimentation coefficient. The diffusion and sedimentation coefficients are related by the frictional ratio $F = (f / f_0)$ and the hydrodynamic scale relationship,

$$D(s) = \frac{\sqrt{2}}{18\pi} k T s^{-1/2} (\eta F)^{-3/2} ((1 - \bar{v}\rho) / \bar{v})^{1/2}$$

where η is the solvent viscosity and ρ is the density (determined to be 1.0003 at 25°C using an Anton Parr densitometer by Prof. Jochen Weiss, UMass Amherst), and $\bar{v}$ is the partial specific volume of the macromolecules, calculated by inputting the protein sequences into the program SEDNTERP, a public domain software which is freely available on the World Wide Web at www.jphilo.mailway.com. The nonlinear regression using distributions of Lamm equation solutions $c(s)$, shown in the first equation above, was combined with the determinations of the best fit weight average frictional ratio, the best fit meniscus position, and the algebraic calculation of systematic time-invariant and

radial-invariant noise components and maximum entropy regularization using a confidence level of > 0.8 (Dam and Schuck 2004). The resulting sedimentation coefficient distributions, expressed in Svedberg units [S], were imported into Microsoft Excel and the highest peak was normalized to 1. Data was then graphed using GraphPad Prism (GraphPad Software, Inc.).

Protein measurement

Protein concentrations of all Toc GTPases were determined using the Bradford method with the Coomassie Plus Assay Reagent (Pierce, Rockford, IL). Protein concentrations of transit peptides and chloroplast import assays for normalization were determined using BCA (bicinchoninic acid) reagents (Pierce, Rockford, IL). Bovine serum albumin (BSA, Pierce, Rockford, IL) was used as the protein standard for all protein concentration assays. For protein solutions that had the reducing agents DTT or β -ME in excess of the amounts tolerated by the BCA assay, the Compat-Able kit (Pierce, Rockford, IL) was used to precipitate the protein, followed by resuspension in a buffer tolerated by the BCA assay (water, unless otherwise noted), and the standard BCA assay was performed.

Gel electrophoresis

The PCR products of the colony screens were visualized using a 6% acrylamide gel (1 ml 30% acrylamide, 6 ml 1 X TAE, 50 μ l APS, 25 μ l TEMED) without a stack, poured in 0.75 mm-spaced Bio-Rad MiniProtean gel plates. Gels were run in the MiniProtean apparatus (BioRad Laboratories, Hercules, CA) with 1 X TAE (40 mM Tris, 20 mM Acetic Acid, 1mM EDTA) used as the gel running buffer at 80 V for approximately 45 min. The gels were then incubated in 0.1% Ethidium Bromide in ddH₂O for 5 min., followed by a quick rinse in ddH₂O for 1-2 min. DNA was then visualized and photographs taken using the UVP BioImaging system (Upland, CA).

General SDS-PAGE utilized 15% acrylamide separating gel and 5% acrylamide stacking gel and 1 X Tris/glycine running buffer, (25 mM Tris/glycine pH 8.3, 0.1% SDS) for protein separation and gels were run at 20-30 mA/small gel. After the Bromophenol blue of the sample buffer ran off the bottom of the gel, the gel was stopped and proteins were

stained by incubation in warmed (15 sec at maximum power in a microwave oven) Coomassie stain (10% acetic acid, 50% methanol, 0.25% Coomassie Brilliant Blue R 250) for 15 min, followed by warmed (same procedure) destain buffer (10% acetic acid, 50% methanol) for 15 min or until background was sufficiently reduced. Pictures were taken to document the gels using the UVP BioImaging System (Upland, CA).

For chloroplast import competition assays, large gels were run at low speeds to improve resolution. The separating gel used was 18% acrylamide with a 5% acrylamide stacking gel along with the same 1 X Tris/glycine running buffer described above. Gels were run at 4-12 mA/gel overnight until the blue ran off the bottom and the visible green bands of chlorophyll had run 2/3 of the way down the gel. Gels were stopped at this point and proteins fixed by incubating gels in gel fixative solution (10% acetic acid, 50% ethanol) for at least 15 min. Gels were then dried on a gel-dryer (BioRad Laboratories, Hercules, CA) and subsequent steps are described in the *in vitro* chloroplast import competition assays section above.

The transit peptide constructs (SStpNt and mutants) are approximately 6,100 daltons or less, so a 19.2% Tris/tricine gel system was used for better resolution of the low molecular weight peptides. The recipes for the separating and spacer gel solutions are listed in Table 5. The 48% acrylamide solution with 32:1 acrylamide:bisacrylamide ratio is not commercially available, so it was made in the lab by combining 48.02 g of acrylamide and 1.48 g bisacrylamide and bringing to a final volume of 100 ml in ddH₂O. The gel buffer is 3 M Tris-HCl pH 8.45. Both the separating gel (with glycerol) and spacer gel (without) is prepared for pouring, APS and TEMED are added, and mixing well and moving quickly, the separating gel is poured to fill approximately 4 cm, then the spacer gel is layered on top to fill about 2 cm. Isopropanol is layered on top and the gel polymerizes 15-20 min. The 5% stacking gel is poured as for a normal Tris/glycine gel. The tris/tricine gel system uses an anode buffer (0.2 M Tris-HCl pH 8.9), which is placed in the lower chamber, and a cathode buffer (0.1 M Tris-HCl, 0.1 M Tricine, 0.1% SDS, pH 8.25), which is placed in the upper chamber of the gel apparatus. The gels are run at 30 mA/ small gel for about 2-3 hours or until the Bromophenol blue reaches the bottom before staining as described above for standard gels.

Table 5 Tris/tricine gel for electrophoresis of low molecular weight peptides

Ingredients	19.2% separating gel	10% spacer gel
48% acrylamide 32:1 *	1.5 ml	0.3125 ml
Gel buffer*	1.25 ml	0.5 ml
Water	0.375 ml	0.6875 ml
80% glycerol	0.625 ml	0
10% APS	18.75 μ l	7.5 μ l
TEMED	1.875 μ l	0.75 μ l
20% SDS	18.75 μ l	7.5 μ l

*acrylamide preparation and buffer components are described in the text

Chapter 3 Analysis of the Role of the FGLK Semi-Conserved Sequence of the Transit Peptide in Chloroplast Import

Abstract

Despite the fact that transit peptides show very little primary sequence homology, they are able to direct chloroplast precursor proteins to interact with one or more protein components of the translocation complex located within the inner and outer membranes of the chloroplast, resulting in targeting and import of these nuclear-encoded preproteins into the plastid. Inspection of chloroplast targeted precursor proteins resulted in the suggestion that there may be conserved domains of the transit peptide that are important for targeting to chloroplasts (Karlin-Neumann and Tobin 1986). Analysis of transit peptide sequences from multiple preproteins and different organisms led to the refinement of the suggested framework block to an amino acid sequence consisting of FGLK, or more loosely defined, an aromatic residue, a turn-promoting residue, a hydrophobic residue, and a positively charged residue (Pilon, Wienk et al. 1995). In this study, possible semi-conserved regions of the transit peptide resembling this motif have been identified and targeted for deletion in order to probe the importance of these regions in interactions with the chloroplast import apparatus. Two corresponding domains were targeted and deleted in the transit peptide of the precursor to the small subunit of Rubisco (prSSU) from tobacco: FTGLK and FPVSR. Both full and partial deletions were made for each region, resulting in the following 6 mutant preproteins: Δ FTGLK, Δ FTG, Δ GLK, Δ FPVSR, Δ FPV, and Δ VSR. The mutant preproteins, WT precursor (prSSU), and mature protein (mSSU) were over expressed and purified from *E. coli*. The import competence of the mutant preproteins was evaluated by chloroplast import competition assays testing the ability of the preprotein to inhibit import of ^{35}S -labeled prSSU into isolated chloroplasts. Both the Δ FTGLK and the Δ FPVSR deletions showed a marked decrease in ability to compete for import. For each motif, the importance of the two halves is also demonstrated by the reduced ability of each of the partial motif deletion mutants to

compete for import. However, deletion of the second half of the motif seemed to reduce the ability to compete for import more than the deletion of the first half, indicating that these residues may play a more important role in determining the import competence of the preprotein. This study provides clear evidence of the importance of each of these motifs and their components for targeting and import of preproteins into chloroplasts.

Introduction

The great majority of chloroplast proteins are nuclear encoded and synthesized as preproteins in the cytosol of the cell. Targeting to the chloroplast is mediated by an N-terminal transit peptide, which is cleaved upon import resulting in the mature protein. Although it is well known that transit peptides are both necessary and sufficient for the chloroplast targeting of these preproteins, the defining characteristics of the transit peptide remains a mystery. Following numerous studies, some general trends have emerged out of the highly divergent amino acid sequence, length, and organization exhibited by transit peptides: they tend to have an extremely high content of hydroxylated amino acid residues such as serine and threonine, and very few acidic amino acids such as aspartate and glutamate (Bruce 2000).

Chloroplast preproteins have also been compared to mitochondrial presequences due to the similar endosymbiotic origin of both organelles and subsequent gene transfer to the host cell. Many similarities exist between the chloroplast transit peptides and the mitochondrial presequences, yet the cell must distinguish between the two in order to correctly target preproteins to their respective organelles. It has been observed that chloroplast transit peptides are generally longer than mitochondrial presequences (average 58 and 42 residues, respectively) and that the uncharged N-terminus is unique to the chloroplast transit peptide (Bhushan, Kuhn et al. 2006). This led the authors to investigate whether removal of the uncharged N-terminus might result in miss-targeting of chloroplast preproteins to the mitochondria, however removal resulted in complete loss of *in vivo* targeting to either organelle, indicating that this region is important for both correct targeting and miss-sorting. Structural differences have been observed, with the mitochondrial presequences exhibiting amphipathic α -helices that have been shown to be

necessary for import (Roise 1988; Endo, Shimada et al. 1989), whereas chloroplast transit peptides are unstructured until exposed to membranes, at which time they also form helices (Wienk, Czisch et al. 1999; Bruce 2001). The variability in primary sequences led to the idea that the defining information contained within the targeting peptides may be structural rather than sequence specific. Unfortunately, little data is available describing the structural characteristics of chloroplast transit peptides and they have proven difficult to study by NMR or crystallization due to their unstructured nature, resulting in only a small handful of determined structures (Bruce 2000).

Variability in primary sequences has also led to the proposal that transit peptides may contain domains with different and possibly overlapping functions required for targeting and import such as lipid interaction, chaperone binding, and interaction with the GTPase receptors of the translocon (Wienk, Wechselberger et al. 2000; Zhang and Glaser 2002; Becker, Jelic et al. 2004). Despite the fact that these domains remain ill defined, it has been generally accepted that transit peptides contain three distinct regions: an uncharged N-terminus depleted in R and L, a central domain that lacks acidic residues and has high S/T content, and a C-terminal domain enriched in positively charged residues (Bruce 2000; Bhushan, Kuhn et al. 2006). Furthermore, a loosely conserved motif, FGLK, was identified in the C-terminal domain of the transit peptide of Ferredoxin from *Silene pratensis* and from other plants as well as transit peptides from other precursor proteins (Pilon, Wienk et al. 1995). This motif corresponded well with a more loosely defined motif suggested by earlier research: h(P/G)h(R/K), or hydrophobic residue, proline or glycine, hydrophobic residue, and a positively charged residue, which was found in a smaller sample size of transit peptides (Karlin-Neumann and Tobin 1986).

Recent work attempting to identify critical motifs for preprotein targeting and import into chloroplasts has utilized *in vivo* targeting of GFP reporter proteins fused to a transit peptide and transiently expressed in *Arabidopsis* leaf protoplasts (Lee, Lee et al. 2006). This study targeted 10-12 amino acid blocks, both replacing them with alanine and deleting them completely. The authors conclude that multiple sequence motifs exist that share complex interrelationships because they see a lack of effect of almost all single block substitutions but a large loss of targeting efficiency (50% or greater) when more

than one block is substituted with alanine or deleted. However, one block did seem to be required for targeting, and was found to have two critical sequences, FP and RK, which are part of the motif discussed above, h(P/G)h(R/K).

In the current study, we specifically target this motif in the transit peptide of the small subunit of Rubisco (prSSU), which has two regions that satisfy the criteria separated by four residues. We delete each motif entirely and in two halves, generating six prSSU mutants. We then evaluate the import efficiency of these preproteins by using them as competitors of radiolabeled prSSU in *in vitro* chloroplast import competitions, demonstrating that the mutants are severely compromised in their ability to compete for import. This observation indicates that the deleted regions are required for efficient preprotein import into chloroplasts.

Results

Generation and purification of prSSU mutants

Deletions targeting the FGLK semiconserved sequence motif were introduced into the transit peptide region of the precursor protein of the small subunit of Rubisco (prSSU) from *Nicotiana tabacum* as shown in Fig. 3-1. The transit peptide is 60 amino acids in length and like other chloroplast targeting transit peptides can be divided roughly into three domains: an N-terminal uncharged domain, a central domain enriched in prolines and possibly containing FGLK motif(s), and a C-terminal domain enriched in positively charged amino acids that may form an amphiphilic β -strand (Bruce 2001). The uncharged amino terminal domain of prSSU from tobacco is easily identified, and is indicated in Fig. 3-1 by overlining. In contrast, this transit peptide does not have an easily identifiable concentration of positively charged residues at the C-terminus, having positively charged residues evenly distributed throughout the internal region instead. Nonetheless, this transit peptide does have two regions close to the center of the transit peptide that correspond to the FGLK motif, therefore both were removed using long distance PCR. The more N-terminal motif, FTGLK, was removed completely, generating prSSU Δ FTGLK, and also in two halves, generating prSSU Δ FTG and prSSU Δ GLK.

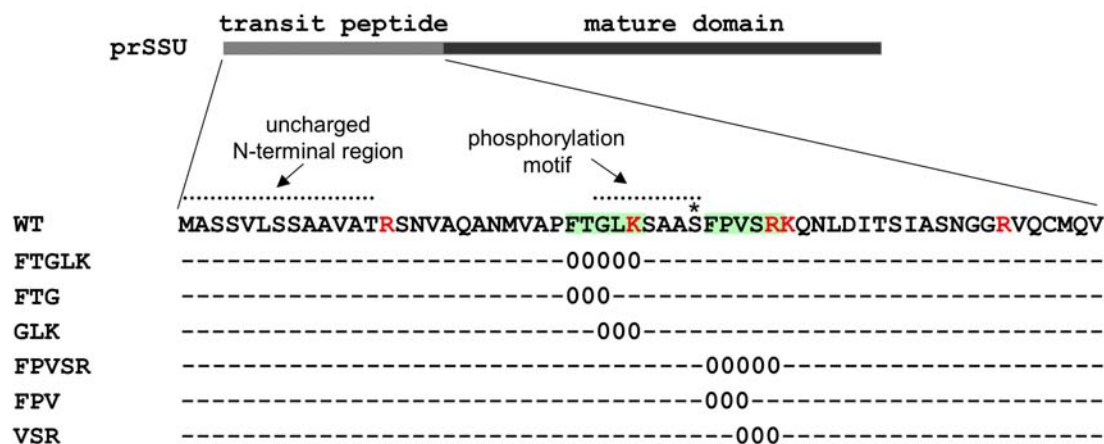


Figure 3-1 Sequence and mutagenesis of the transit peptide of prSSU.

The transit peptide region of the small subunit of Rubisco is represented by the grey rectangle. The mature domain is shown in black. The amino acid sequence of the transit peptide region is shown with the FGLK-like domains highlighted in green. The deletions made to eliminate these domains are shown as 0's, with the remaining unaltered sequence indicated by dash marks. Additional features of the transit peptide region are also indicated, with the uncharged N-terminal region overlined, the positively charged residues in red, and the putative phosphorylation motif overlined, with the serine residue that is the site of phosphorylation indicated by an asterisk.

Immediately following the FTGLK motif is the putative phosphorylation site of the transit peptide, indicated by an asterisk in Fig. 2-1. The FTGLK motif actually overlaps with the proposed phosphorylation motif, overlined in Fig. 2-1 (Nakrieko, Mould et al. 2004). The second motif is closer to the C-terminus, starting at residue 35, following the serine that is potentially modified. It, too was deleted completely, generating prSSU Δ FPVSR, and in two halves, generating prSSU Δ FPV and prSSU Δ VSR. These constructs as well as WT (wild-type) prSSU and mSSU (the mature domain of prSSU, without the transit peptide) in pET11d were used to transform competent *E.coli* cells and protein expression was induced with IPTG. These proteins over expressed in *E.coli* form inclusion bodies, allowing for straightforward purification to near homogeneity. After induction, the total cell lysate contained a prominent band that runs at about 23 kD (Figure 3-2A, lane T). This is very close to the predicted molecular weight of prSSU Δ FPV, 19,967.8 daltons. When the cells were lysed using the French press and centrifuged at 20,000 x g, the soluble portion had no band at 23 kD, indicating that prSSU is almost totally insoluble and exists as inclusion bodies. The inclusion bodies were purified to near homogeneity by washing first with detergent-containing buffer, followed by buffer without detergent. The inclusion bodies were finally solubilized in 8 M Urea and centrifuged to remove any remaining impurities, resulting in >98% purity in most cases, determined by scanning densitometric analysis of an SDS-PAGE gel. This method of purification routinely yielded 35-60 mg of pure protein per liter of culture. Figure 3-2 shows a representative purification profile of the prSSU Δ FPV construct (A) and the final purified protein for all of the constructs (B). The increased mobility of the deletion mutants due to lower molecular weight can be seen, particularly in the case of Δ FTGLK and Δ FPVSR. Note that mSSU (the mature domain of the small subunit of Rubisco), which lacks the transit peptide, runs at about 15,000 kD, which agrees with its predicted molecular weight of 14,559.7 daltons.

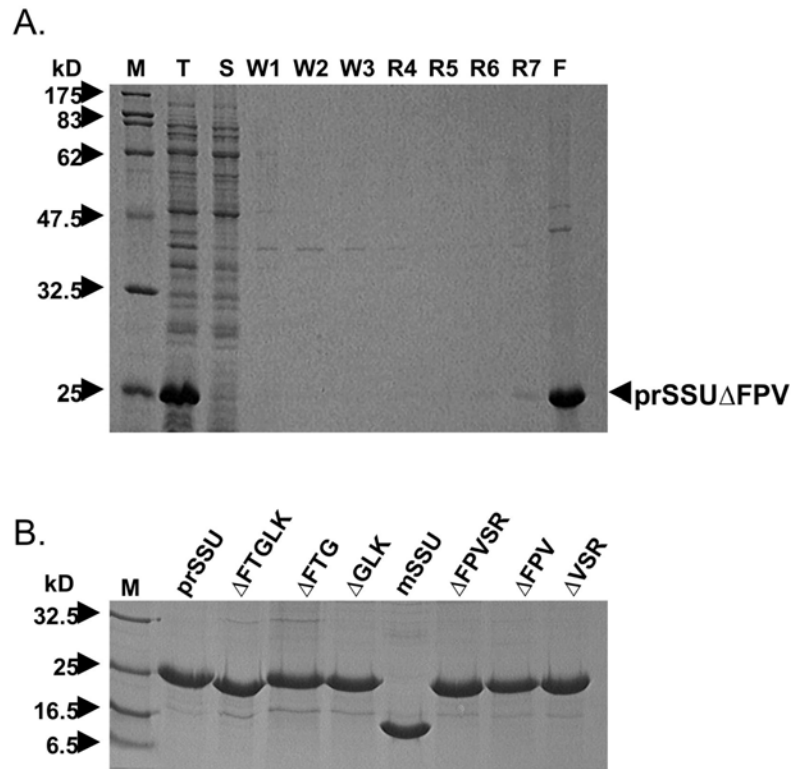


Figure 3-2 Purification profile and final soluble prSSU, mSSU, and mutant proteins.

(A.) A Coomassie stained SDS-PAGE gel showing the purification of prSSU Δ FPV. M denotes the broad range marker (NEB) and the molecular weights are specified in kD. T denotes total starting material after cells were induced for 3 hours with IPTG. S is the soluble portion after cell lysis and centrifugation. W1 through W3 represents the supernatant after washing the inclusion bodies with Buffer A with Triton X-100. R4 through R7 is the supernatant after the inclusion bodies were “rinsed” with Buffer A without detergent and centrifuged at 20,000 x g. F is the final protein after solubilization in 8 M Urea, purified to near homogeneity. (B.) A Coomassie stained SDS-PAGE gel showing prSSU, mSSU, and all of the deletion mutants after purification and solubilization in 8 M Urea. All proteins were brought to 1 mg/ml and 5 μ g loaded on the gel.

Import competition and IC₅₀ determination for ³⁵S-prSSU, prSSU, mSSU, prSSUΔFPVSR, prSSUΔFPV, prSSUΔVSR, prSSUΔFTGLK, and prSSUΔFTG

In order to determine the effect of the motif deletion on import competence, the purified mutant proteins as well as prSSU and mSSU were used as competitors of ³⁵S-labeled prSSU for *in vitro* import assays using isolated intact chloroplasts. The concentration of the ³⁵S-labeled prSSU was kept constant at 100 nM while the concentration of the competitor protein was increased from 0 to 600 nM. Import of the labeled prSSU can be traced as accumulation of radioactive mSSU because processing and cleavage of the transit peptide occurs rapidly after the preprotein is imported into the chloroplast. Ability of the competitor to compete for import results in a decrease of processed radioactive mSSU as more competitor is added to the import reaction. Fig. 3-3 shows the results of at least three independent replicates of *in vitro* import competition assays for each of the full deletions, prSSUΔFTGLK and ΔFPVSR. Both deletions severely reduce ability to compete for import, and this is reflected in their extremely high IC₅₀ values, shown in Table 6. IC₅₀ is defined as the concentration of inhibitor required for inhibition of 50% of activity. If the inhibitor is competing for the same binding site and is identical to its competitor, for example cold prSSU competing against radiolabeled prSSU, the IC₅₀ of the inhibitor should be equal to the amount of competitor present. In our experiments, radiolabeled prSSU is present at 100 nM, so the IC₅₀ of unlabeled prSSU competing for the same import apparatus should be 100 nM. The IC₅₀ of WT prSSU is 147 nM, which is slightly higher than the theoretical IC₅₀ of 100 nM indicating that the concentration determination of either the cold prSSU competitor or, more likely, the labeled prSSU, is not exactly correct. A high IC₅₀ (> 100 nM) indicates that more unlabeled precursor protein is required to compete for import with the radiolabeled WT prSSU, meaning the competitor has decreased import competence. Both of the preprotein mutants with the full deletions have very similar IC₅₀ values that are more than tenfold higher than WT prSSU, indicating that they are severely compromised in their ability to compete for import. In these assays the mature domain of the small subunit of Rubisco, mSSU, is used as a

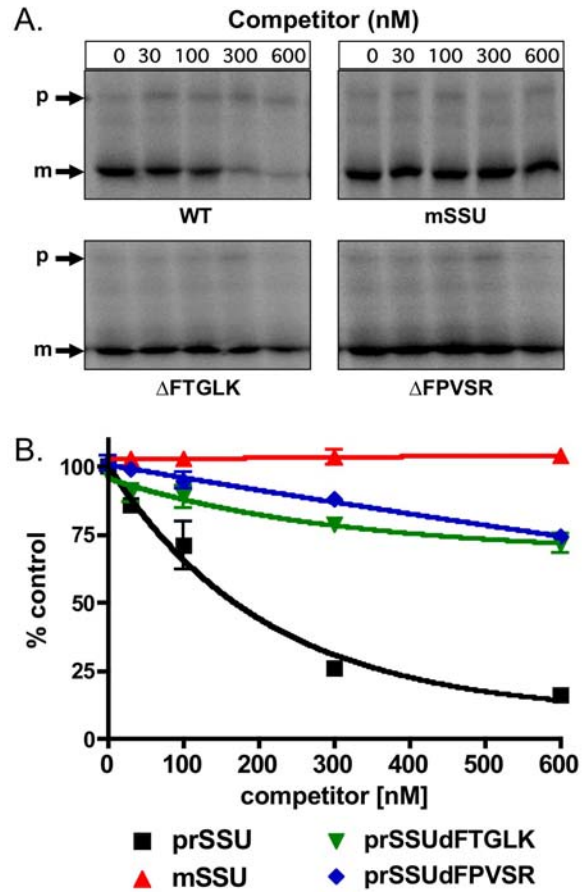


Figure 3-3 *In vitro* chloroplast import competition assay of full-length motif deletions.

(A.) Phosphor Images of SDS-PAGE gels of representative chloroplast import competition assays between ^{35}S -prSSU and precursors with the full motif deleted. Gels were dried and exposed to phosphor screens. Competitor concentration is indicated in nM, labeled prSSU was held constant at 100 nM throughout. P indicates bound precursor and m denotes mature protein. (B.) Graphical analysis of import competitions between ^{35}S -prSSU and precursors with deletions of the full motif. Quantification of the bands of mature protein was performed and import was expressed as percent of WT ^{35}S -labeled prSSU imported without any competitor present (control).

Table 6 IC₅₀ of WT prSSU and deletion mutants

Competitor	IC ₅₀ (nM)
prSSU	147±26
ΔFTGLK	1745±335
ΔFPVSR	1728±213
ΔFTG	341±88
ΔGLK	948±186
ΔFPV	747±73
ΔVSR	1081±272
mSSU	No inhibition

IC₅₀ is the concentration of inhibitor required to inhibit 50% of activity and was calculated by GraphPad PrismTM using import competition data from Fig. 3-3 & 3-4.

negative control because it lacks the transit peptide region necessary for targeting and import into chloroplasts, making it unable to compete for import into chloroplasts (Dabney-Smith, van Den Wijngaard et al. 1999). Figure 3-4 shows representative Phosphorscreen images of *in vitro* chloroplast import competition assays as well as graphical analysis of at least 3 replicates of the competition assays for the partial motif deletions, prSSU Δ FTG, Δ GLK, Δ FPV, and Δ VSR. The most N-terminal deletion, Δ FTG has the least effect on ability to compete for import as can be seen from the graphical analysis as well as the apparent IC₅₀ for this deletion. The IC₅₀ for this mutant is 341 nM, which is only two-fold the IC₅₀ value of WT prSSU, 147 nM. The graphical analysis of the import competition assays indicates that the other partial deletions behave similarly to one another, severely affecting the ability of the preproteins to compete for import. This is also reflected by their higher IC₅₀ values between 747 and 1081 nM. Interestingly, the two partial deletions that remove the second half of each motif, Δ GLK and Δ VSR, result in a competition curve that is more linear in appearance and also have the highest IC₅₀ values of the partial deletions. Also interesting is the behavior of Δ FPV, which seems to be intermediate between Δ FTG and the others, still exhibiting a nonlinear inhibition curve like Δ FTG but unable to compete very well for import as indicated by the IC₅₀ value closer to those of Δ GLK and Δ VSR. These results suggest that the positively charged residues in the second half of the motif may serve a more important role in import, but the first half of the motif also contributes to the function of the transit peptide region in preprotein import.

Discussion

Deletion of the FGLK motif

Analysis of transit peptide sequences from multiple preproteins and different organisms led to the observation of a conserved motif consisting of FGLK, or more loosely defined, an aromatic residue, a turn-promoting residue, a hydrophobic residue, and a positively charged residue (Pilon, Wienk et al. 1995). Previous studies have not targeted this motif specifically for investigation, so the current study was aimed at investigating the

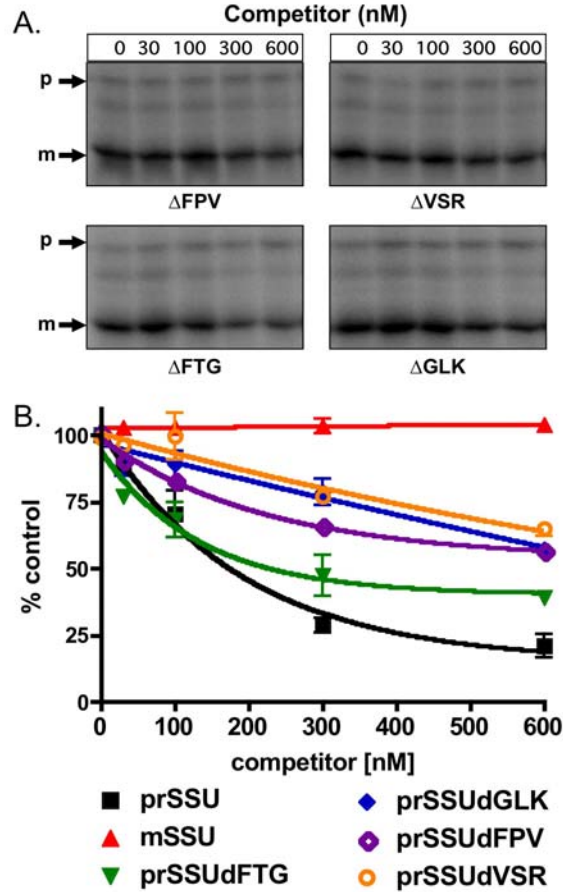


Figure 3-4 *In vitro* chloroplast import competition assay of partial motif deletions.

(A.) Phosphor Images of SDS-PAGE gels of representative chloroplast import competition assays between ^{35}S -prSSU and partial motif deletion preproteins. Gels were dried and exposed to phosphor screens. Competitor concentration is indicated in nM, labeled prSSU was held constant at 100 nM throughout. P indicates bound precursor and m denotes mature protein. (B.) Graphical analysis of the import competitions between ^{35}S -prSSU and precursors with partial motif deletions. Quantification of the bands of mature protein was performed and import was expressed as percent of WT ^{35}S -labeled prSSU imported without any competitor present (control).

importance of this motif for chloroplast import of the precursor protein of the small subunit of Rubisco (prSSU). This precursor has two regions that satisfy the motif and both full and partial deletions were made for each, resulting in the following 6 mutant preproteins: Δ FTGLK, Δ FTG, Δ GLK, Δ FPVSR, Δ FPV, and Δ VSR. The mutant preproteins, WT precursor (prSSU), and mature protein (mSSU) were over expressed and purified from *E. coli*.

Effect of deletion of the FGLK motif on chloroplast import

The import competence of the mutant preproteins was evaluated by chloroplast import competition assays testing the ability of the preprotein to inhibit import of ^{35}S -labeled prSSU into isolated chloroplasts. Both the Δ FTGLK and the Δ FPVSR deletions showed a marked decrease in ability to compete for import, reduced almost to the level of the negative control, mSSU. For each motif, deletion of the second half of the motif seemed to reduce the ability to compete for import more than the deletion of the first half. In both cases, the second half of the motif contains a positively charged residue, which is recognized as a defining characteristic of chloroplast transit peptides (Bruce 2000). Unfortunately the role of the positively charged residues in transit peptides is as yet unknown and chloroplast import assays, while providing general information on import competence, do not provide much information on what aspect of import is compromised. However, these results are supported by recent *in vivo* work targeting GFP reporter proteins fused to a transit peptide in *Arabidopsis* leaf protoplasts (Lee, Lee et al. 2006). This study targeted 10-12 amino acid blocks, both replacing them with alanine and deleting them completely. Two critical sequences in one of the blocks were found to be critical for targeting and import, FP and RK, which are part of the motif discussed above, h(P/G)h(R/K), and are targeted for deletion in the current study. In their study, a region roughly corresponding to the FPVSR motif of the current study was indispensable for *in vivo* targeting of the GFP construct (Lee, Lee et al. 2006). However, they also found that a region roughly corresponding to the more N-terminal FTGLK motif in the current study was not necessary, and was probably redundant as deletion of this region in combination with other regions resulted in loss of targeting and import. This apparent disagreement

between *in vivo* and *in vitro* experimental results has been observed many times, and it has been suggested that this may be due to the emphasis placed on different aspects of preprotein targeting and import by each type of analysis (Bruce 2000).

The deletion of the FTGLK motif also interrupts a proposed phosphorylation motif (P/G) X_n (K/R) X_n (S/T) X_n (S*/T*), where the asterisk denotes the site of phosphorylation and n can be 0-3 residues (Waegemann and Soll 1996). Phosphorylation of this site in transit peptides has been shown to enable binding of 14-3-3 chaperones, forming a complex along with Hsp70 that is involved in the chloroplast import pathway in *in vitro* studies utilizing wheat germ translation systems (May and Soll 2000). However, other studies have shown that phosphorylation is not important for targeting to chloroplasts, demonstrated by *in vivo* targeting of GFP using a transit peptide mutated to remove the phosphorylation motif (Nakrieko, Mould et al. 2004). Studies have also altered transit peptides to decrease affinity for both 14-3-3 proteins and Hsp70 chaperones, resulting in no reduction in *in vitro* import into isolated chloroplasts (Rial, Ottado et al. 2003). However, since this study as well as our own used isolated chloroplasts and purified proteins, no cytosolic chaperones are present. Therefore, although these studies indicate that chaperone binding is unnecessary for import competence, it cannot be definitively concluded that chaperones are not important for targeting, at least in some cases. Finally, although the specific factors of preprotein targeting and import that are compromised by the deletion of these motifs cannot be determined by the present study, we have provided clear evidence of the importance of each of these motifs and their components for import of preproteins into chloroplasts.

Chapter 4 AUC Analysis of PsToc34 with Nucleotide and Transit Peptide Ligands

Abstract

Transit peptides show very little primary sequence homology, yet they are able to direct the precursor protein to interact with one or more protein components of the translocation complex of the chloroplast. This study has targeted a possible semi-conserved FGLK motif of the transit peptide for investigation and two corresponding domains were deleted in the transit peptide of the precursor to the small subunit of Rubisco (prSSU) from tobacco: FTGLK and FPVSR. Both the FPVSR and FTGLK deletions showed a marked decrease in ability to compete for import, indicating that these motifs are required for efficient preprotein import into chloroplasts. One aspect of import that could be compromised is the interaction between the transit peptide and the Toc GTPase components of the translocon, Toc34 & Toc159. Recent work has shown a complex interaction between these GTPases and their substrates: the transit peptide region of chloroplast targeted preproteins and the nucleotides GTP and GDP. Although the Toc GTPases have been proposed to function as “gate-keepers”, neither the psToc34 crystal structure nor the low-resolution Toc complex structure reveal the dynamics of Toc-Toc interactions during preprotein binding and translocation. In order to investigate the interactions between the soluble cytosolic domains of the Toc GTPase psToc34 and the transit peptide, analytical ultracentrifugation (AUC) was used. A construct allowing purification of soluble transit peptide lacking the mature domain was used to allow biochemical studies without the contributions of the mature domain and most importantly without the urea necessary to solubilize full-length prSSU. Using velocity sedimentation, the presence of a homodimer of psToc34 was observed and was populated by the addition of the transit peptide (SS_{tp}) substrate, and depopulated by the addition of nucleotide substrate. AUC of the mutant transit peptides with psToc34 showed that they were unable to influence the oligomeric state of psToc34 to the extent of the WT transit peptide.

Introduction

The majority of chloroplast proteins are synthesized in the cytosol of the cell as a higher molecular weight precursor protein with an N-terminal transit peptide which mediates targeting and import of the preprotein into the chloroplast. The number and complexity of the interactions that the transit peptide region of chloroplast preproteins is suspected to participate in makes characterization a daunting task. A soluble form of the transit peptide that could be easily purified in large amounts would enable biochemical characterization of these interactions, simplifying the job and isolating the contributions of the transit peptide from those of the mature domain. Precursor targeting and import is a complex process involving several stages or steps, and the transit peptide is believed to be vital throughout, making its characterization very important for elucidation of the mechanism by which organelle specific targeting and import occurs.

The first steps may occur in the cytosol, with the transit peptide interacting with cytosolic chaperones such as 14-3-3 proteins or Hsp70 chaperones. Over 75% of chloroplast transit peptides have been found to possess binding domains for one or the other class of protein chaperones, and some have both (Ivey, Subramanian et al. 2000; May and Soll 2000; Zhang and Glaser 2002). Recently, the molecular chaperone Hsp90 was also implicated in delivery of preprotein to a receptor of the chloroplast import apparatus (Qbadou, Becker et al. 2006). However, not all transit peptides have been shown to possess chaperone binding domains, suggesting that chaperone binding may not be necessary for targeting and import of all preproteins.

It has also been observed that the transit peptide interacts with chloroplast outer membrane lipids, adopting a more α -helical conformation (Pinnaduwa and Bruce 1996; Wienk, Wechselberger et al. 2000). The lipid content of chloroplasts and mitochondria are different, and this may provide a mechanism for the cell to distinguish between precursors targeted to each organelle, with chloroplast lipids inducing a conformational change in the transit peptide allowing recognition and import of the preprotein. In addition, an *Arabidopsis* mutant deficient in the plastid lipid digalactosyl diacylglycerol (DGD) was unable to import preproteins targeted to the chloroplast

interior (Chen and Li 1998), indicating that lipids may play an important role in preprotein import.

The transit peptide has also been shown to interact with the GTPase gatekeepers of the translocon: Toc34 and Toc159 (Schleiff, Soll et al. 2002; Becker, Jelic et al. 2004); this will be more thoroughly discussed below. Following translocation across the double membrane and into the stroma of the chloroplast, the transit peptide interacts with the stromal processing protease (SPP) and is cleaved, allowing further targeting to the thylakoids or folding of the mature domain into active protein (Richter and Lamppa 2002). Due to the number of interactions that transit peptides seem to be capable of, a tool allowing biochemical characterization of the transit peptide in a soluble form and in the absence of the mature domain was needed. The current study utilized such a construct, producing the WT transit peptide of the small subunit of Rubisco from *Nicotiana tabacum* (SStpNt) as well as a C57Y mutant that does not undergo disulfide-mediated dimerization and the two full-length motif deletions, SStpNt Δ FTGLK and Δ FPVSR.

The most-studied interactions of the transit peptide are those with the proteinaceous components of the translocon. Early studies used crosslinking or antibody inhibition during the different stages of import to determine the necessary components of both the transit peptide and the translocon. From these initial studies, the core import complex was determined to consist of two GTPases, Toc34 and Toc159, and the channel protein Toc75 (Perry and Keegstra 1994; Kouranov and Schnell 1997). Toc34 and Toc159 have much in common: they both have a conserved GTP-binding domain (G-domain) and have both been shown to directly interact with preproteins (Becker, Jelic et al. 2004).

Recent work has shown a complex interaction between these two proteins and their substrates: the transit peptide region of chloroplast targeted preproteins and the nucleotides GTP and GDP. The crystal structure of the cytosolic domain of Toc34 exhibited a dimeric arrangement with bound GDP molecules in the active sites suggesting the possible involvement of nucleotide regulated dimerization in the import mechanism (Kessler and Schnell 2002; Sun, Forouhar et al. 2002). Furthermore, *in vitro* binding studies support the possibility of heterodimerization between the G domains of Toc34

and Toc159 and provide evidence that this dimerization is more favorable in the presence of GDP, lending support to the idea of nucleotide regulation of the dimer (Smith, Hiltbrunner et al. 2002; Weibel, Hiltbrunner et al. 2003). However, despite the psToc34 crystal structure and the binding studies, the dynamics of the Toc-Toc interactions during preprotein binding and translocation remain largely unsolved. The current study investigates the interactions of one of the Toc GTPases from pea, psToc34 Δ TM (lacking the transmembrane domain) with its substrates using sedimentation velocity analytical ultracentrifugation (AUC). PsToc34 was first analyzed to confirm homodimerization and then was further characterized in the presence of different nucleotides as well as WT and mutant transit peptides. The transit peptide deletion constructs SStpNt Δ FTGLK and Δ FPVSR were evaluated for their ability to interact with and induce changes in the homodimer of psToc34 Δ TM and clearly demonstrated the importance of these motifs in the interaction with one of the gatekeepers of the translocon.

AUC analysis of dimerization

Sedimentation velocity (SV) analytical ultracentrifugation (AUC) analysis of protein interactions can give information about the association scheme (monomer-dimer, monomer-trimer, etc.), sedimentation coefficients, as well as the hydrodynamic shape of the monomeric and oligomeric components (Lebowitz, Lewis et al. 2002). Migration of the macromolecules towards the bottom of the cell due to the centrifugal force is opposed by molecular friction, therefore sedimentation depends on hydrodynamic properties of the molecules, reflecting the low-resolution structure of protein complexes, and enabling the detection of conformational changes (Schuck, Perugini et al. 2002). Furthermore, the fact that faster sedimenting components are constantly bathed in a solution of the components that sediment at a slower rate also enables reversible reactions to occur throughout the time scale of sedimentation making it possible to characterize weak or transient interactions (Schuck 2003; Brown and Schuck 2006). All of these characteristics made SV analysis of our system attractive. PsToc34 has been found to exhibit complex interactions with two substrates: nucleotides and the transit peptide region of precursor proteins, and has also been shown to form homo/heterodimers (Schleiff, Jelic et al. 2003;

Weibel, Hiltbrunner et al. 2003; Becker, Jelic et al. 2004). Therefore, the current study undertook the characterization of the cytosolic domain of the GTPase receptor, psToc34 Δ TM, analyzing concentration dependence of the homodimer as well as the effect of nucleotide and transit peptide substrates using SV AUC analysis. Both substrates had a large effect on the oligomeric status of psToc34, clearly supporting a role for both nucleotides and transit peptides in the assembly status of the GTPase components of the translocon of the outer chloroplast membrane. The mutant transit peptides lacking the FGLK motifs were also analyzed and were found to exhibit less of an effect on the monomer-dimer equilibrium of psToc34 Δ TM, indicating that these motifs are necessary for transit peptide function.

Results

Subcloning of the transit peptide region into the pTYB2 vector

The transit peptide of the small subunit of Rubisco was subcloned into the pTYB2 vector to enable purification of soluble transit peptide without any additional tags that could interfere with biochemical analysis of peptide structure and function. The transit peptide of the small subunit of Rubisco from *N. tabacum* (SstpNt) was obtained by PCR amplification of the transit peptide region of the full-length precursor (pET11-prSSU). The PCR product was restriction digested with *NdeI* and *XmaI* (an isoschizomer of *SmaI*) using these sites and subcloned into the pTYB2 vector, which introduced a cleavable Intein tag and a chitin-binding domain (CBD) onto the C-terminus of the transit peptide, permitting one-step affinity purification using a chitin column. Another advantage of this construct is that no exogenous proteases are required to cleave the transit peptide from the affinity tag, with the intein undergoing specific self-cleavage in the presence of reducing agents such as DTT or β -Mercaptoethanol (β -ME) and releasing the transit peptide from the column bound intein-CBD tag. An initial direct colony PCR screen was used to evaluate success of subcloning and to select colonies for plasmid prep and sequencing. Figure 4-1 shows a 6% acrylamide gel of the results of the direct colony PCR screen for the SSstpNt Δ FPVSR construct. Colonies that produced a larger PCR product

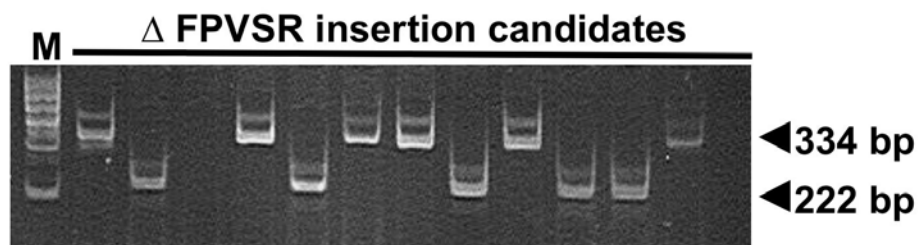


Figure 4-1 Direct colony PCR screen of insertion candidates

Direct colony screening was performed using T7 forward and INTEIN reverse primers, resulting in a larger PCR product if the transit peptide insert was present in the vector. PCR products were run on a 6% Acrylamide, 1 X TAE gel and visualized using Ethidium Bromide. M denotes the marker, a 100 bp ladder from Gibco. Both 222 bp (unsuccessful insertion) and 334 bp (successful insertion) PCR product sizes are indicated.

were selected and sequenced using an automated sequencer and were found to be inserted correctly in almost every case. The initial direct colony PCR greatly reduced the numbers of candidates that had to be plasmid prepped and sequenced. This method was used successfully to subclone WT transit peptide and the transit peptides with FTGLK and FPVSR deleted, resulting in the following constructs: SStpNt (small subunit transit peptide from *Nicotiana tabacum*), SStpNtC57Y, SStpNtΔFTGLK/C57Y, and SStpNtΔFPVSR. It should be noted that SStpNtΔFTGLK has an additional mutation at its C-terminus, C57Y, which mutates the cysteine at residue 57 to a tyrosine. The same mutation of the WT transit peptide has been made and serves two purposes: first, it reduces disulfide-mediated dimerization of transit peptides observed during protein isolation, and secondly, it provides a tyrosine to aid in determination of transit peptide concentration using A₂₈₀.

Expression and purification of SStp mutants

The constructs discussed above were transformed into competent *E. coli* strain ER2566 and protein expression was induced with IPTG at low temperature for an extended period of time to increase solubility of the large fusion protein and to reduce degradation. After overnight induction, a major band appeared in the total cell lysate (lane 2, Fig. 4-2), running at about 60 kD and corresponding well with the predicted molecular mass of the transit peptide fusion protein with the intein and chitin binding domain of 62.9 kD. After cell disruption by sonication, the band of interest was present in both the pellet and the soluble fractions (lanes 3 and 4, respectively, Fig.4-2), indicating either that the cells were incompletely lysed or that some of the fusion protein was present as inclusion bodies. However, most of the fusion protein was present in the soluble fraction, and was bound by the chitin affinity column, as is apparent on the gel indicating the lack of the band in the flow-through (lane 5, Fig.4-2). No 60 kD protein can be seen in the wash (lane 6, Fig.4-2), indicating that the CBD strongly binds the chitin affinity matrix of the column. Elution of the transit peptide is triggered by incubation of the column in the presence of 50 mM β-ME overnight at 4°C, which induces the intein self-cleavage

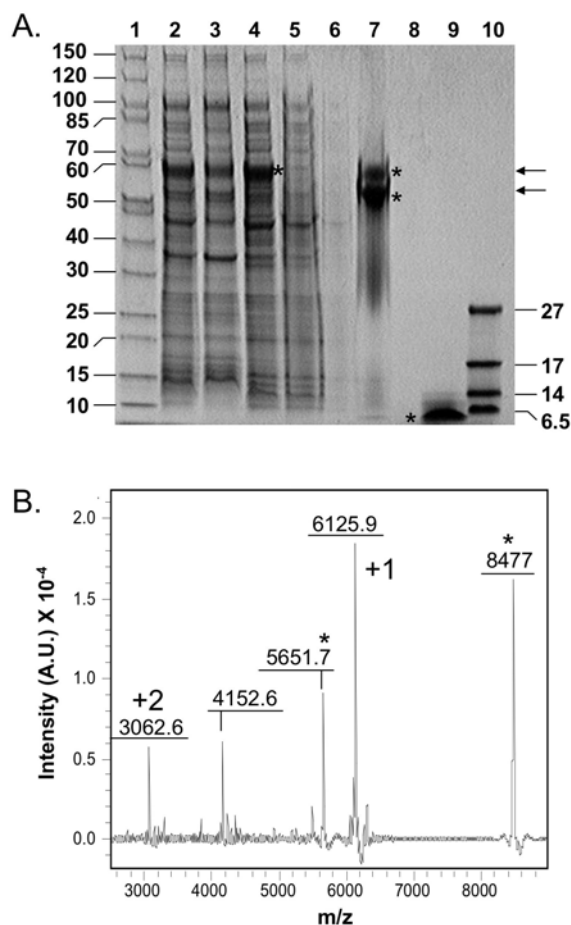


Figure 4-2 Purification profile and MALDI-TOF of SStpNt

(A.) Transit peptide was purified using affinity chromatography. Marker (lane 1), total protein, pellet, soluble fraction, flow through, wash (lanes 2-6), column matrix (lane 7), elution before and after lyophilization to concentrate (lanes 8-9), and peptide marker (lane 10) were separated by SDS-PAGE and visualized by Coomassie Blue staining. (B.) Maldi-Tof of SStpNt was performed on a Bruker Daltonics MicroFlexTM mass spectrometer, with Apomyoglobin used as an internal standard for calibration. The +2 and +3 charged Apomyoglobin species can be seen at 8477 m/z (avg. MW of Apomyoglobin is 16952) and 5651.7 m/z, respectively. The +1 and +2 charged SStpNt species are found at 6125.9 and 3062.6 m/z. The species at 4152.6 is a fragment resulting from proteolysis or transcription from an alternative start site at a second methionine.

reaction and releases the transit peptide from the column bound intein-CBD. In order to evaluate the success of elution, an aliquot of the chitin matrix is removed and run on the gel (lane 7, Fig.4-2). Two dominant bands can be seen bound to the matrix: the top band running at about 60 kD and corresponding to the intein-CBD tag with transit peptide still attached and the lower band running at about 55 kD corresponding to the intein-CBD tag (predicted MW of 56.9 kD), indicating successful cleavage of the transit peptide occurred. The transit peptide is eluted in a large volume and is at very low concentrations until lyophilization is performed to concentrate the peptide and to remove β -ME. Lane 8 of the gel shows the elution before lyophilization and lane9 shows the elution after it has been lyophilized to powder and resuspended at 1/6th of its original volume. The major band is apparent at about 6 kD, which corresponds to the predicted MW of the transit peptide of 6125 daltons. This purification method was used for both of the deletion mutants SStpNt Δ FTGLK and SStpNt Δ FPVSR and the SDS-PAGE of the purified peptides are shown in Fig. 4-3. For better resolution of the low molecular weight peptides, a Tris/Tricine gel system for peptides and Coomassie staining was used to visualize the final products. The purity and integrity of the transit peptide preps were evaluated using both the Tris/Tricine gels and MALDI-TOF MS, which is discussed below, and were >95% pure.

MALDI-TOF of peptides

MALDI-TOF MS of transit peptides was performed with apomyoglobin used as an internal standard on a Bruker-Daltonics MicroflexTM mass spectrometer. The spectrum of the WT SStpNt is shown in Fig. 4-2(B). The +2 and +3 charged species of the apomyoglobin internal standard can be seen at 8477 m/z (avg. MW of Apomyoglobin is 16952) and 5651.7 m/z, respectively. The +1 and +2 charged SStpNt species are found at 6125.9 and 3062.6 m/z. The FindPept tool (<http://ca.expasy.org/tools/findpept.html>) was used to identify the peptides that correspond to the main peaks. The largest peak at 6125.9 m/z corresponds to the full-length transit peptide, while the minor peak at 4152.6 m/z represents either an alternative translation initiation at Met 22 or possibly a degradation product where short N- and C-terminal regions were proteolytically removed

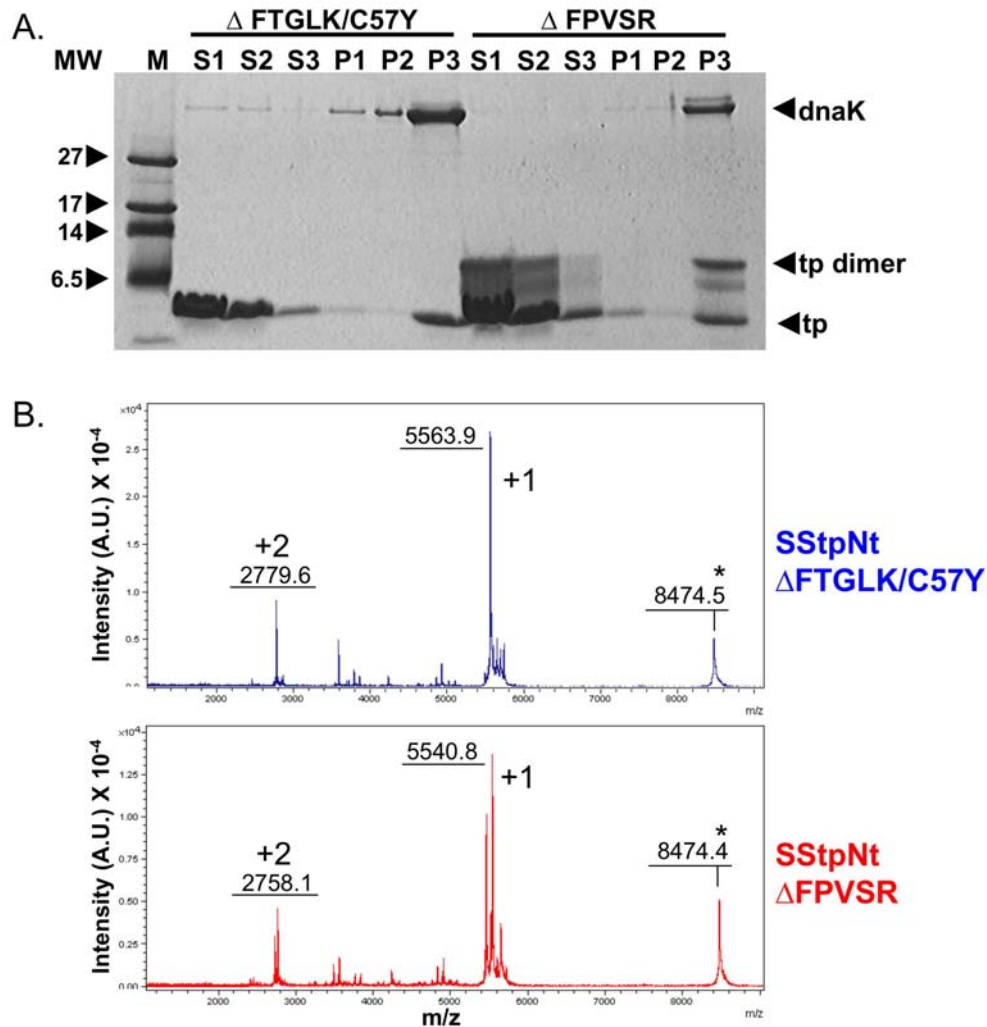


Figure 4-3 Tris/Tricine gel and MALDI-TOF of SStpNt deletions

(A.) Coomassie stained 19.2% Tris/Tricine gel of deletion mutants. M denotes the peptide marker, S1 through S3 denote soluble fraction of lyophilized and resuspended elutions 1-3, and P1 through P3 indicate the pellet fraction of the elutions. Respective deletion peptides are indicated above the gel image with a bar above all corresponding elutions. (B.) MALDI-TOF analysis of the peptides was performed on a Bruker Daltonics MicroFlexTM mass spectrometer, with Apomyoglobin used as an internal standard for calibration. The +2 charged Apomyoglobin species can be seen at 8474 m/z. The +1 and +2 charged SStpNt Δ FTGLK/C57Y species are found at 5563.9 and 2779.6 m/z. The +1 and +2 charged SStpNt Δ FPVSR species are found at 5540.8 and 2758.1 m/z.

during expression and purification (Fig. 4-2 B). The spectra of the two transit peptide deletion mutants are shown in Fig. 4-3 B. The +2 charged Apomyoglobin species can be seen at 8474 m/z in both spectra. The +1 and +2 charged SStpNt Δ FTGLK/C57Y species are found at 5563.9 and 2779.6 m/z, respectively. The +1 and +2 charged SStpNt Δ FPVSR species are found at 5540.8 and 2758.1 m/z. Interestingly, the SStpNt Δ FPVSR peptide exhibits two major peaks at 5540.8 and 5465 m/z. The difference between these peaks corresponds to the mass of a molecule of β -ME covalently attached to the C-terminal cysteine at residue 57. The lack of this extra peak in the MS spectrum of the SStpNt Δ FTGLK/C57Y mutant that lacks this C-terminal cysteine further supports this explanation of the extra species. The MALDI-TOF spectra confirm that the transit peptides were purified to near homogeneity and are full-length.

Expression and purification of cytosolic domain of Toc34

Expression of the cytosolic domain of Toc34 was induced by addition of IPTG to large cultures of *E.coli* strain BL21(DE3)RIL transformed with psToc34 Δ TM in pET21d. The temperature was lowered to increase the solubility of the overexpressed protein and cells were harvested after 3 hours of induction. PsToc34 Δ TM has a C-terminal 6-His tag, which allows easy affinity purification using a Cobalt-Sepharose column. Because protein solubility at high concentrations over an extended period of time was an issue, column bound protein was aliquotted into smaller spin columns and stored at -20° C in the presence of glycerol until the day before an experiment. Glycerol, which was problematic for analytical ultracentrifugation experiments, could then be easily removed by washing the column before eluting the protein using 300 mM imidazole. Immediately after eluting the protein, it was diluted to 2 mg/ml and dialyzed to remove the imidazole. The dialysis buffer was saved to serve as the reference buffer for the sedimentation velocity experiments discussed below. Concentration after dialysis was measured using the Bradford assay and the purity was assessed by SDS-PAGE and Coomassie staining.

PsToc34 exhibits concentration dependent homodimerization

PsToc34 Δ TM was analyzed using sedimentation velocity analytical centrifugation at a range of different concentrations, shown in Fig. 4-4. The typical protocol for analysis of possible concentration dependent self-association of proteins is to run at least three concentrations: 0.05 mg/ml, 0.5 mg/ml, and 1 mg/ml. This was done as well as a full concentration range to more fully characterize the concentration dependence of the homodimerization behavior of psToc34 Δ TM, and the results are shown in Fig. 4-4B in the left hand and center graphs. Toc34 seems to exhibit a fast association ($k_{\text{off}} \sim 10^{-3}$), as the peak distribution does not sit at two distinct sedimentation coefficients, but is distributed between the two bracketing sedimentation coefficients that are seen at the highest (dimer S value) and lowest (monomer S value) protein concentrations (Dam, Velikovskiy et al. 2005). To further characterize this concentration dependent homodimerization, additional concentrations were analyzed and the point at which psToc34 seems to be equally distributed between monomeric and dimeric forms was observed to be around 0.4 mg/ml as can be seen in Fig. 4-4B in the left-hand graph. This is further illustrated by the graph on the right that shows the relationship between monomer and dimer peak heights and protein concentration. Notice that the line showing the increase in dimer peak height as concentration increases crosses the line showing the simultaneous decrease in monomer peak height at slightly less than 0.5 mg/ml, suggesting that this is the equilibrium midpoint of psToc34 Δ TM. Therefore, all future experiments looking at the effect of ligands on Toc34 oligomeric status used the 0.4 mg/ml concentration (corresponding to 13.5 μ M) in order to maximize the observable shifts caused by addition of ligand.

Nucleotide addition increases psToc34 homodimerization

In order to analyze the effect of nucleotide substrate on the population equilibrium of psToc34, an excess of GTP (200 μ M) was added to the dialysis buffer and the sample and allowed to dialyze for at least 1 hour. The dialysis buffer with 200 μ M GTP was then used as the reference buffer to eliminate contributions of the nucleotide itself to the

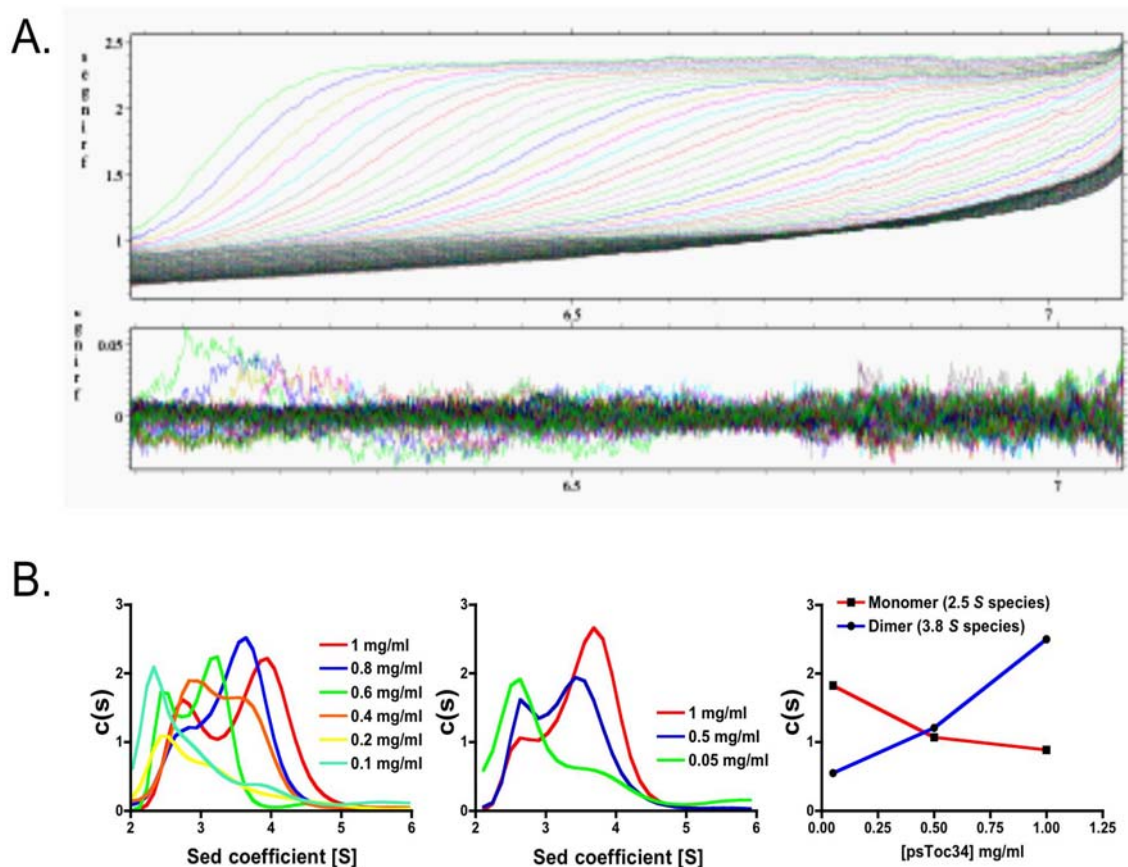


Figure 4-4 PsToc34 exhibits concentration dependent homodimerization.

(A.) Representative sedimentation velocity data with global fits is shown at the top. The resulting residuals for the analysis is shown below. Scans shown were collected approximately 10 min. apart. (B.) Sedimentation coefficient distributions for a large range of concentrations of psToc34ΔTM, indicative of a fast association are shown in the left hand panel. The center panel shows the apparent sedimentation coefficient for the lowest concentration (0.05 mg/ml) is about 2.2-2.5 S, the highest concentration (1 mg/ml) is 3.7-4 S, and a concentration in between (0.5 mg/ml) shows S values intermediate to the monomeric and dimeric S values. The right panel shows the relationship between psToc34ΔTM concentration and the population of monomeric or dimeric apparent sedimentation coefficient.

difference between reference and sample. Samples were subjected to sedimentation velocity ultracentrifugation and data was collected using the interference optics because nucleotide absorbs at low wavelengths, raising the background and making A_{280} data uninterpretable. Data was analyzed as described in Materials and Methods, and the resulting sedimentation data is shown in Fig. 4-5. PsToc34 was kept at 0.4 mg/ml for all samples and the sample without any nucleotide added is shown in red. GTP addition results in a shift of the sedimentation coefficient to the right, indicating that more homodimer is formed (blue trace, Fig. 4-5).

Characterization of the effect of nucleotide analogs on psToc34 homodimerization

Since it is known that psToc34 is a GTPase, further analysis of the effect of other non-substrate nucleotides as well as nucleotide analogs was of interest. Dialysis of other nucleotides and analogs was performed as described for GTP and the respective dialysis buffer was used as reference for each sample. The interference optics detection system was used due to background nucleotide absorbance at 280 nm which was impossible to differentiate from the protein absorbance, rendering absorbance optics detection unusable for these experiments. Data was analyzed as described in Materials and Methods. Addition of GDP (green trace, Fig. 4-5) resulted in a small shift of the sedimentation coefficient to the right, which is unexpected since it is generally accepted that isolated psToc34 has a bound GDP. This may indicate that a small population lacks bound GDP and forms a dimer upon binding GDP, contributing the shift in oligomeric status. However, this study cannot distinguish whether that is what is really occurring, and it is also possible that this small shift is due to the increase in Mg^{2+} ions from the Mg-GDP since psToc34 also coordinates a Mg^{2+} ion. Interestingly, the result of addition of GMP-PNP, which is a nonhydrolyzable analog of GTP, is very similar to the addition of XTP, which is supposed to be a hydrolyzable analog (yellow and orange traces, respectively, Fig. 4-5). This GTPase seems unable to hydrolyze XTP or GMP-PNP and the sedimentation coefficient it shifted to the left, indicating that the monomeric population is increased. Finally, addition of the nonsubstrate nucleotides ATP and ADP (light blue and

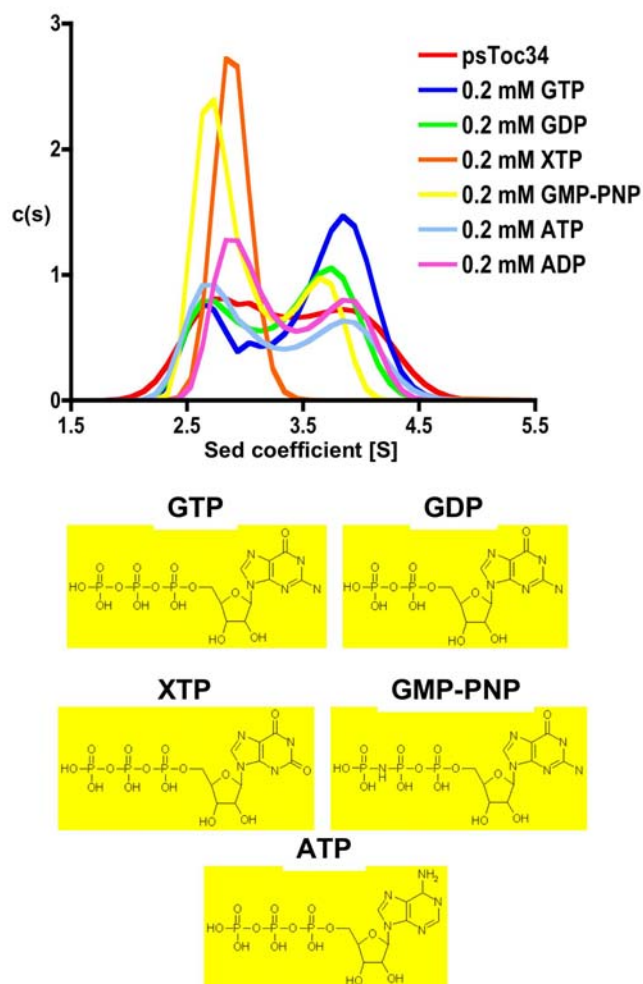


Figure 4-5 SV analysis of psToc34 with nucleotide substrate and analogs.

PsToc34 Δ TM (0.4 mg/ml) was dialyzed in the absence of nucleotide (red trace) or in the presence of an excess (200 μ M) of the indicated nucleotide, followed by SV ultracentrifugation at 50,000 rpm at 25°C. Data collected using interference optics was analyzed using the continuous distribution $c(s)$ model in SEDFIT, resulting in the sedimentation coefficients shown above. Species with S values < 3.5 are considered monomeric and species exhibiting S values > 3.5 are dimeric. The structures of the nucleotides tested are shown at the bottom.

mauve traces, respectively, Fig.4-5) have very little effect on the distribution of the sedimentation coefficient, although ADP does seem to shift the population equilibrium slightly to the left, indicating that some binding may be occurring, although this is not supported by any other data.

Transit peptide addition decreases psToc34 homodimerization

PsToc34 has also been shown to interact with transit peptides (Schleiff, Soll et al. 2002), therefore we wanted to test the effect of transit peptides on the observed population equilibrium. Full-length prSSU is insoluble and must be solubilized in urea, making analytical ultracentrifugation data interpretation much more difficult. For this reason, we used the transit peptide alone (SStpNt) in these experiments. After resuspending the lyophilized peptide in the dialysis buffer (described in Materials and Methods), SStpNt was dialyzed exhaustively alongside psToc34. After dialysis, SStpNt concentration was determined by BCA (described in Materials and Methods), and 10 X the psToc34 molar concentration was added to psToc34 at a constant concentration of 0.4 mg/ml (corresponding to 13.5 μ M). Fig. 4-6 shows psToc34 alone (red trace), transit peptide alone (blue trace), and psToc34 with transit peptide added (green trace), which shifts the sedimentation coefficient dramatically to the left, indicating that the psToc34 population is shifted towards the monomer in the presence of the transit peptide.

Characterization of the effect of mutant transit peptides on psToc34 homodimerization

Since it was established that the transit peptide triggered a change in the population equilibrium of psToc34, we wanted to test the motif deletion mutants, SStpNt Δ FTGLK and Δ FPVSR. Purification and dialysis of the deletion mutants was performed exactly the same as for WT SStpNt, described in Materials and Methods and above. After dialysis, transit peptide concentrations were determined by BCA (described in Materials and Methods), and 10 X the psToc34 molar concentration was added to psToc34 at a constant concentration of 0.4 mg/ml or 13.5 μ M. Fig. 4-7 shows psToc34 alone (black trace), with the SStpNtC57Y transit peptide (red trace), and with the deletion transit peptides Δ FTGLK and Δ FPVSR added (blue and green traces respectively). The SStpNtC57Y

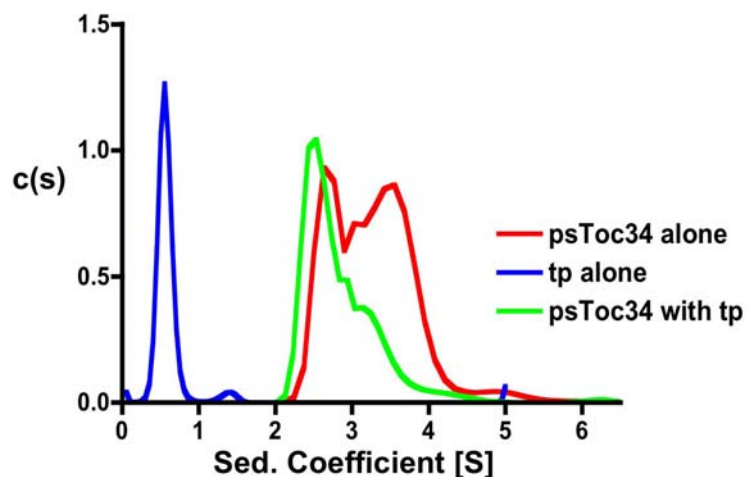


Figure 4-6 Addition of transit peptide shifts the population equilibrium of psToc34

PsToc34 (0.4 mg/ml, equal to 13.5 μM) and SStpNt transit peptide (tp, 135 μM) at a 10-fold higher molar concentration were analyzed individually (red and blue traces, respectively), and after mixing in the same cell (green trace) by centrifugation at 50,000 rpm at 25°C. Interference optics were used to collect SV data, followed by direct boundary modeling $c(s)$ analysis with the program SEDFIT. Upon addition of transit peptide, there is a large shift in the apparent sedimentation coefficient towards the monomeric population.

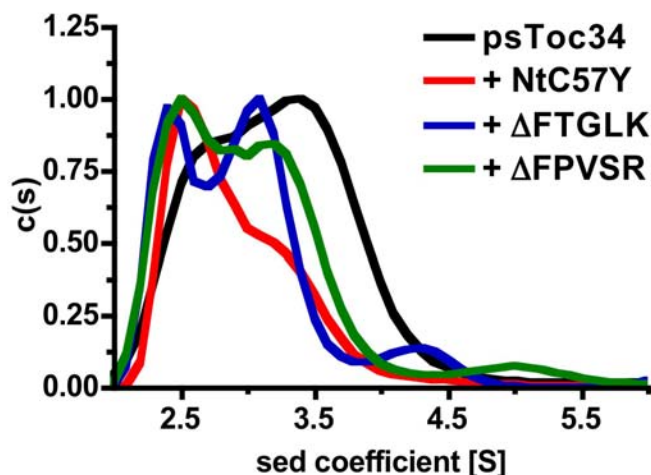


Figure 4-7 Deletion mutant transit peptides do not influence the population equilibrium of psToc34 to the same extent as WT.

SV analysis of psToc34 alone (black trace) and in the presence of SStpNtC57Y (behavior equivalent to WT transit peptide), red trace, and deletion mutants SStpNtΔFTGLK (blue trace) and ΔFPVSR (green trace). Transit peptides were added at 10-fold the molar concentration of psToc34 and allowed to equilibrate at 25°C for at least 1 hour before analyzing by SV at 50,000 rpm using interference optics. Data was analyzed using direct boundary modeling $c(s)$ analysis by the program SEDFIT and the highest peak normalized to 1 to make comparison easier.

transit peptide has the C-terminal cysteine removed to eliminate disulfide-mediated dimerization and a tyrosine introduced to enable concentration determination using tyrosine fluorescence at A_{280} . It behaves identically to WT SStpNt, shifting the sedimentation coefficient to the left indicating that the psToc34 population is shifted towards the monomer. In contrast, neither of the deletion mutants were able to shift the population equilibrium of psToc34 to the degree that the WT transit peptide did. However, they do exhibit interesting behavior, with a sedimentation coefficient distribution that appears to be intermediate between the behavior of psToc34 in the absence of transit peptide and in the presence of WT transit peptide. Although they do not shift the equilibrium very much, demonstrated by the presence of two peaks indicating both monomer and dimer are maintained at near equilibrium, a tightening of the peaks does occur along with a general decrease in the sedimentation coefficients, indicating that the transit peptides are probably binding psToc34 but are unable to influence oligomeric status. Further analysis using alternative methods is needed to thoroughly evaluate this behavior.

Discussion

psToc34 exhibits concentration dependent homodimerization

The crystal structure of psToc34, discussed in detail in Chapter 1, showed a dimeric arrangement, indicating that the GTPase may undergo dimerization (Sun, Forouhar et al. 2002). It has also been established by several other methods including SEC and BN-PAGE that psToc34 can exist in an equilibrium between a monomer and a dimer (Sun, Forouhar et al. 2002; Weibel, Hiltbrunner et al. 2003). In addition, due to the similarity of the G-domains of Toc34 and Toc159, the other GTPase receptor, other studies have tested the ability of the two to heterodimerize, determining that they interact in a GTP-dependent manner (Smith, Hiltbrunner et al. 2002; Weibel, Hiltbrunner et al. 2003). However, previous studies have not characterized this equilibrium quantitatively, failing to determine whether it exhibits concentration dependence and what the critical concentration is. Therefore, the behavior of the cytosolic domain of psToc34 was evaluated using sedimentation velocity AUC, which allows the study of reversible protein

interactions in solution and without the addition of probes that could inhibit or modify the interactions. The sedimentation velocity analysis is interesting because it shows that psToc34 exists in a population equilibrium that is concentration dependent. The distribution of the concentration-dependent peaks at S values intermediate between the monomer and dimer S values also indicates that the association is rapidly reversible. The broadening and shifting of the peaks is due to the behavior of the rapidly interacting species: a dimer will form, begin to sediment more quickly, then dissociate, with the monomers sedimenting at a slower rate, causing diffusional broadening of the peaks (Schuck 2003). In contrast, if the interaction were slow, longer than the time scale of sedimentation (6-8 hours using our conditions), but still concentration dependent, the peaks would be at constant positions, varying only in peak height at the different concentrations (Schuck 2003). Our analysis confirms the previously observed dimerization of psToc34 and further characterizes it as a fast association which is concentration dependent.

GTP increases the concentration of dimeric Toc34

It has long been known that GTP plays a necessary role in the early steps of translocation, promoting the formation of early import intermediates (Kessler, Blobel et al. 1994; Young, Keegstra et al. 1999). The resolution of the crystal structure of psToc34 Δ TM revealed details that were both expected and surprising: that the Toc GTPase receptors Toc34 and Toc159 are a novel class of GTPase proteins had long been proposed, however the dimeric arrangement of the Toc34 molecules was surprising. Furthermore, the bound GDP molecules in the crystal structure appear unable to exchange out of the dimeric arrangement without large conformational changes or complete dissociation (Kessler and Schnell 2002). For these reasons, it was of interest to determine the effect of substrate nucleotide, GTP on the observed concentration equilibrium of psToc34 Δ TM.

The structural details of the crystal structure not only prompted the proposal of homo/heterodimerization of the GTPases but also suggested that reciprocal activation of nucleotide hydrolysis was possible; in effect the function of each monomer as a GTPase activating protein (GAP) for the other monomer, discussed in detail in Chapter 1 (Sun,

Forouhar et al. 2002; Becker, Jelic et al. 2004). However, data from our lab has determined that the GTPase activity of psToc34 Δ TM is not concentration dependent, indicating that it probably does not serve as its own GAP (Reddick, Vaughn et al. manuscript in preparation). Nonetheless, dimerization of the Toc GTPases at the outer chloroplast membrane is undoubtedly physiologically important, as heterodimerization of the G domains of Toc34 and Toc159 is known to occur, and has also been shown to be stimulated by the addition of GDP, supporting the idea of nucleotide regulation of the dimer (Smith, Hiltbrunner et al. 2002; Weibel, Hiltbrunner et al. 2003). The results of the current study indicated that addition of GTP resulted in the increased population of the dimeric species, which is consistent with nucleotide regulation of dimer formation, but seems to contradict the previously observed effect of GDP. However, the hydrolysis of GTP to GDP probably occurs much faster than the time course of the sedimentation velocity experiment, indicating that the results may actually be due to population of GDP bound protein.

In order to resolve whether the increase in the dimeric population is due to GTP or GDP in the active site of psToc34 Δ TM, non-hydrolyzable analogues of GTP were used in the sedimentation velocity AUC assay. The non-hydrolyzable analog GMP-PNP showed a large shift towards the monomeric population, indicating that GTP inhibits the formation of the dimer, which agrees with the work of Sveshnikova et al. Surprisingly, XTP, which is considered a hydrolyzable analog, shows the strongest effect, causing the entire population of psToc34 to become monomeric. Other nucleotide competition assays in our lab determined that Toc34 has an affinity for XTP that is equal to its affinity for GTP and 3-fold higher than the affinity for GDP, indicating that the excess of XTP added replaces any bound GDP (Reddick, Vaughn et al. manuscript submitted). The same assay determined the affinity of Toc34 for the non-hydrolyzable analog GMP-PNP to be almost 6 times lower than affinity for either GTP or XTP and about half of the affinity for GDP, indicating that the amounts of GMP-PNP added may not have completely replaced any GDP already present in the active site of psToc34. This could explain the presence of some remaining dimeric Toc34 species when GMP-PNP is present in the sedimentation velocity experiment. In addition, although it has not been determined whether XTP is

hydrolyzed by Toc34, the similarity of the shift to that of the GMP-PNP sample indicates that it is probably not hydrolyzed. Furthermore, the complete shift to monomeric *s* values compared to the partial shift caused by GMP-PNP is probably due to the difference in affinities of Toc34 for the two nucleotides. Taken together, these results suggest that nonhydrolyzed GTP increases the likelihood that psToc34 will exist as a monomer, while nucleotide hydrolysis seems to increase the likelihood that psToc34 will participate in a homo/heterodimer. The other nucleotides tested showed very little effect on the equilibrium distribution of Toc34. This is supported by earlier studies that determined psToc34 to be a GTPase, as well as recent data from our lab indicating that Toc34 has very low affinities for UTPs and other nucleotides (Sveshnikova, Soll et al. 2000; Reddick, Vaughn et al. manuscript submitted).

Transit peptide addition increases the population of monomeric Toc34

The interaction of preproteins with the GTPases is promoted by GTP, suggesting that nucleotide binding and hydrolysis may serve as a switch, regulating the binding affinity for transit peptides and alternately the homo/heterodimeric binding partner (Sveshnikova, Soll et al. 2000). Furthermore, transit peptides alone as well as preproteins have been shown to stimulate GTP hydrolysis of the Toc GTPases, suggesting that they also play a role in regulation of activity (Jelic, Sveshnikova et al. 2002; Becker, Jelic et al. 2004). For these reasons, investigation of the effect of the transit peptide on the equilibrium population of psToc34 Δ TM using SV analysis was undertaken. Upon addition of the transit peptide, a dramatic shift in the population towards the monomer is observed. It should be noted that it has been proposed that the transit peptide region of precursor proteins in fact serve as a GAP rather than the other Toc34 molecule. Unfortunately, it is a complicated task to distinguish between GAPs and GEFs due to the difficulty in determining whether activation of hydrolysis activity or nucleotide exchange is occurring if the final hydrolysis product is the only data collected, as both can result in an increase in hydrolyzed nucleotide. Data from our lab using an assay that follows rate of nucleotide exchange indicates that while there is an increase in hydrolysis rate, there is no difference in exchange when transit peptide is added, supporting the role of the transit peptide as a

GAP (Reddick, Vaughn et al. manuscript submitted). Unfortunately, there is no structural information concerning the interaction of transit peptides with the Toc GTPases, so the mechanism of activation is yet to be determined. However, this data along with the SV analysis indicates that the transit peptide region plays an important role in the regulation of the GTPase activity and oligomeric status of the Toc receptor psToc34.

Transit peptides lacking the FGLK motif show a decreased influence on the population equilibrium of psToc34 Δ TM

Due to the observed effect of the transit peptide on the population equilibrium of psToc34 Δ TM, investigation of the effect of a mutant transit peptide was of interest. The deletion of either of the two FGLK motifs in the transit peptide region of the precursor protein drastically decreased their ability to compete for import in *in vitro* chloroplast import competition assays (described in Chapter 3). For both of these reasons, the soluble, truncated transit peptides SStpNt Δ FTGLK and Δ FPVSR were tested for the ability to influence the population equilibrium of psToc34 Δ TM using SV analysis. The results of the AUC analysis were very interesting due to a dual effect that the mutant transit peptides seemed to exert on psToc34 Δ TM's apparent sedimentation coefficient. First, neither of the deletion mutant tps shifted the population equilibrium of the GTPase to increase the population of the monomer like the WT tp did. However, a change in peak shape can be seen, with a general tightening of the two monomer and dimer peaks accompanied by a general shift of both towards smaller sedimentation coefficients. This tightening of the peaks could be indicative of either a conformational change or a slowing of the monomer-dimer association rate, or a combination of both. The current study cannot differentiate between the two, however, these results do indicate that the mutant tps interact with psToc34 Δ TM, causing a change in the c(s) distribution, reflected most clearly in the peak on the left (monomeric psToc34 Δ TM) which matches almost exactly both the S value and the shape of the peak caused by addition of WT tp. Also clear from these results is that the mutant tps are unable to increase the population of psToc34 Δ TM monomers. From these results it seems that the effect of the transit peptide on psToc34

must be twofold: first a binding event followed by a conformational change that decreases the likelihood that psToc34 will participate in homo/heterodimerization.

Chapter 5 Conclusions and Future Directions

Conclusions

This study has clearly shown that the FGLK motif, consisting of an aromatic residue, a turn-promoting residue, a hydrophobic residue, and a positively charged residue, is important for the transit peptide region of the precursor of the small subunit of Rubisco (prSSU) to maintain proper function, demonstrated both in *in vitro* chloroplast import competition assays and in biochemical characterization of its interaction with the cytosolic domain of one of the Toc GTPase receptors psToc34 Δ TM. The decreased ability of either of the precursors lacking the motif, prSSU Δ FTGLK and Δ FPVSR to compete for import into isolated chloroplasts demonstrates that these motifs perform an essential function.

The import behavior of the precursors with partial deletions of the motif may also give some indication as to the importance of the components of the motif. In each case, deletion of the second half of the motif that contained a positively charged residue seemed to reduce the ability to compete for import more than the deletion of the first half of the motif, containing the aromatic residue. The prevalence of positively charged residues in the C-terminus is recognized as a defining characteristic of chloroplast transit peptides (Bruce 2000). Unfortunately, the exact role of these positively charged residues is unknown, although it has been postulated that they could mediate initial interactions of the transit peptide with chloroplast anionic lipids (Pilon, Wienk et al. 1995; Pinnaduwege and Bruce 1996). Interestingly, the aromatic residue in the first half of the motif has also been shown to be involved in interactions with the lipid components of the chloroplast outer membrane (Zhang 2003). However, given the complexity of the interactions transit peptides participate in during preprotein targeting and import, it is likely that these residues have multiple functions.

The results of the analytical ultracentrifugation sedimentation velocity analysis supports the conclusion that the FGLK motif is also important for interaction with the GTPase receptors of the translocon of the outer chloroplast membrane. While the precise

mechanism of regulation of the GTPase receptors remains undetermined, the results indicate that a nucleotide and transit peptide dependent switch is most likely involved. While the Toc receptors have previously been found to undergo hetero and homodimerization (Sun, Forouhar et al. 2002; Weibel, Hiltbrunner et al. 2003), the current study has more quantitatively characterized the homodimerization of psToc34 Δ TM, determining that it is a fast, reversible event that is concentration dependent. The effect of GTP substrate on the dimerization is complex, but from the effect of nonhydrolyzable GTP analogs it can be tentatively concluded that the nonhydrolyzed triphosphate form of the nucleotide increases the likelihood that psToc34 will be monomeric, while the hydrolyzed diphosphate increases the population of psToc34 dimer. These results indicate that the nucleotide hydrolysis state acts as a switch that influences the equilibrium population distribution of psToc34, probably due to a conformational change. In addition, the transit peptide region of prSSU has also been shown to have an effect on the likelihood that psToc34 is a monomer or a dimer, shifting the population drastically towards the monomer. This information indicates that the transit peptide also acts as a regulator, and probably arbitrates a conformational change upon binding to the receptor that causes the population to shift towards the monomer. This suggests that the transit peptide can mediate the disassembly of the Toc GTPase receptors, possibly in order to activate preprotein exchange and nucleotide hydrolysis, triggering the insertion of the preprotein into the translocon for complete import into the chloroplast. The effect of successful preprotein/Toc34 interactions in this scenario can be compared to a key: if the correct contacts are made, the door is unlocked, and translocation into the chloroplast can occur. Fig. 5-1 presents a model of the mechanism of Toc34 interactions with the core complex, taking into account the conclusions drawn from the AUC characterization of the effect of its substrates, both nucleotides and preproteins, on the tendency of psToc34 to participate in dimerization.

Interestingly, the effect of transit peptides lacking the FGLK motif in the SV ultracentrifugation analysis is not so easily quantifiable. They seem to interact with the Toc receptor, yet are unable to influence the population equilibrium. It is possible that the deletions remove a portion of a domain or a specific class of residue necessary for the full

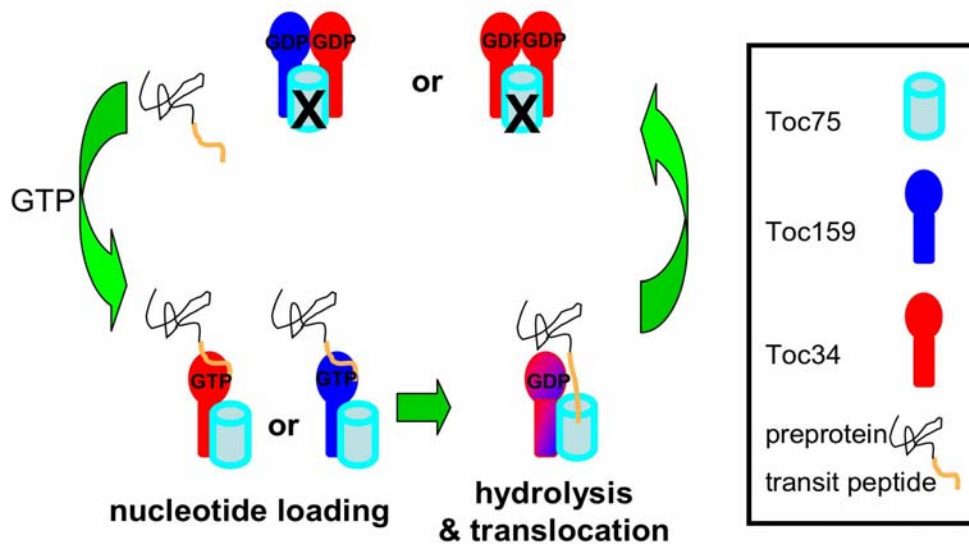


Figure 5-1 Model of Toc34 interactions mediated by the transit peptide and nucleotide substrates.

The GDP-bound form of psToc34 forms a dimer, which may be either a hetero or homodimer with the other Toc GTPase, Toc159. The transit peptide binds to a GTPase, forming the contacts necessary to change its conformation and disrupt the dimer. The opening of the dimer both provides access to the OM translocation channel, Toc75, and allows GDP to diffuse out and GTP to diffuse in, providing the energy for initial insertion of the preprotein into the translocon. Complete translocation could be accomplished in several ways: first, successive rounds of GTP hydrolysis could provide the energy to push the preprotein through the translocon. However, it has been observed that nonhydrolyzable GTP analogs do not inhibit *in vitro* translocation, suggesting other mechanisms are used (Young, Keegstra et al. 1999). Another possibility is that chaperones in the IMS and then in the stroma pull the preprotein through, supported by the observation that ATP is required in increasing amounts for formation of import intermediates and complete translocation. Whatever mechanism(s) is/are used, after translocation occurs the GDP-bound Toc34 re-associates into a dimer, closing the translocon until another preprotein binds successfully.

transit peptide/receptor interaction, allowing the altered peptides to bind but leaving them unable to cause the necessary conformational change that disrupts the dimer-promoting arrangement of Toc34. In the key scenario described above this could be compared to putting your key into the wrong keyhole: the key fits, but cannot negotiate the required interactions in order to open the door. This could explain the large decrease in the ability of the preproteins lacking these motifs to compete for import into chloroplasts.

Future directions

The debate concerning the existence of transit peptide domains has been ongoing for the past twenty years and will probably continue into the future. While the current study has clearly demonstrated that removal of the FGLK regions has a deleterious effect on preprotein import as well as transit peptide interaction with Toc34, additional data is needed to settle this issue. Despite the clear decrease in import competence shown in the current study, it does not resolve exactly what aspect(s) of import is negatively impacted. First, further characterization of the *in vitro* chloroplast import behavior could be helpful. For example, radioactive labeling of the mutant precursor proteins would enable direct characterization of their import behavior rather than their ability to compete for import. Labeled mutant precursors could then be evaluated for their ability to engage the translocon by using only low levels of ATP ($< 100 \mu\text{M}$) to support binding but not translocation. This could help to determine whether the FGLK motif is most important in the binding stage, or whether it is involved in multiple stages of preprotein import.

It is also interesting to note that the Lee et al. study identified a region roughly corresponding to the FPVSR motif of the current study as being indispensable for *in vivo* targeting of a GFP construct in *Arabidopsis* protoplasts (Lee, Lee et al. 2006). However, they found that a region roughly corresponding to the FTGLK motif in the current study was not necessary, and was probably redundant as deletion of this region in combination with other regions resulted in loss of targeting and import. This apparent disagreement between *in vivo* and *in vitro* experimental results has been observed many times, and it has been suggested that this may be due to the emphasis placed on different aspects of preprotein targeting and import by each type of analysis (Bruce 2000). The major

differences between the two include the lack of any possible cytosolic factors as well as no other organelles to which the altered preproteins could be mis-targeted in the *in vitro* system. The *in vitro* system tests the ability of the altered transit peptides to engage a translocon in the absence of factors that have in fact been proposed to play a role in preprotein targeting (Ivey, Subramanian et al. 2000; Jackson-Constan, Akita et al. 2001; Qbadou, Becker et al. 2006). This means that it reflects a simplified system, which can be advantageous for initial evaluation, but should be followed by more rigorous characterization. The current study has addressed this issue by constructing a soluble truncated transit peptide lacking the mature domain for further biochemical characterization. Although this has proven to be a powerful tool, enabling the characterization of the effect of the transit peptide region on one of the receptor GTPases, further characterization of the transit peptide would be helpful.

For example, the motifs being investigated in the current study of prSSU are located on either side of a proposed phosphorylation motif and even includes several amino acids of the phosphorylation motif. This could partially explain the differences between the current study and the *in vivo* study of Lee et al. if chaperones could bind and assist targeting of one of these altered preproteins but not the other. For this reason, these altered transit peptides should be evaluated for their ability to interact with putative cytosolic chaperones. Although this could not have played a role in their decreased ability to compete for import in the current study, investigating the chaperone binding activity would still contribute to the elucidation of the mechanisms of preprotein targeting and import into chloroplasts.

The lipid interactions of the transit peptides lacking the FGLK motif should also be investigated. The *in vivo* work of Lee et. al. also indicated that removal of the region including the more C-terminal motif resulted in drastically increased lipid interaction as well as reduced targeting to the chloroplast (Lee, Lee et al. 2006). Several other experiments have also determined that the C-terminus of transit peptides mediates interactions with chloroplast lipids (Pilon, Wienk et al. 1995; Pinnaduwege and Bruce 1996). Further mapping of the residues or motifs necessary for interaction with lipids representative of the chloroplast outer membrane as well as the effect of such interactions

on the transit peptide could clear up many questions about the importance of such interactions.

Finally, since it has been suggested that transit peptide secondary structure rather than primary sequence may be the defining characteristic that allows it to mediate preprotein targeting and import (Bruce 2001), the structural characteristics of transit peptides should be evaluated. It has been determined that transit peptides are largely unstructured in aqueous environments and attain a more α -helical structure upon exposure to membrane-mimetic environments (Wienk, Czisch et al. 1999; Bruce 2000), however further refinement of structural details has been elusive. The interaction of mitochondrial presequences with the receptor Tom20 of the mitochondrial outer membrane has been mapped out using NMR (Muto, Obita et al. 2001), suggesting that a similar method could contribute to the study of preprotein/receptor interactions in chloroplast import as well.

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Vita

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