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I am submitting herewith a dissertation written by Amanda Carole Dabney Smith entitled "Modulators of transit peptide activity in targeting and translocation of precursors into plastids: the mature domain, lipids, and Toc/Tic components." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree Doctor of Philosophy, with a major in Biochemistry, Cellular, and Molecular Biology.

Barry D. Bruce, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost and
Dean of Graduate Studies

MODULATORS OF TRANSIT PEPTIDE ACTIVITY IN TARGETING AND TRANSLOCATION OF PRECURSORS INTO PLASTIDS: THE MATURE DOMAIN, LIPIDS AND TOC/TIC COMPONENTS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee

Amanda Carole Dabney Smith
August 2001

DEDICATION

This dissertation is dedicated to my husband

Martin K. Smith

and

to my mother

Eloise Harlan Cheatham

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ABSTRACT

The import of nuclear-encoded precursor proteins into chloroplasts occurs via a cleavable, N-terminal targeting sequence known as the transit peptide. To test the influence of the mature domain of the small subunit of Rubisco during import *in vitro*, the precursor (prSSU), the mature domain (mSSU), the transit peptide (SStp) and three C-terminal deletion mutants (Δ52, Δ67, and Δ74) of prSSU were expressed and purified from *Escherichia coli*. Activity was then evaluated by inhibition of import of ^{35}S-prSSU to show that removal of C-terminal prSSU sequences inhibits its interaction with the translocation apparatus. Import studies demonstrated that prSSU and Δ52 were processed and accumulated within the chloroplast, whereas Δ67 and Δ74 were rapidly degraded. Import-competent proteins were also able to induce anion channel closure for PIRAC (Protein Import Related Anionic Channel). Although the C-terminal deletion mutants were less effective at inducing channel closure upon import, they did not affect the mean duration of channel closure. In addition the same proteins, as well as the precursors to the 33 and 23 kD subunits of the oxygen evolving complex of photosystem II, prOE33 and prOE23, respectively, were used in liposome dye release assays to investigate the interaction between precursors and chloroplast outer membrane lipids. Chloroplast precursor proteins do interact with liposomes mimicking the chloroplast outer envelope lipids through a process mediated through the transit peptide and requiring the presence of non-bilayer forming lipids and anionic lipids. The interaction of the transit peptide with liposomes involves electrostatic interactions between the peptide and the anionic lipids in the liposome. From this study, two additional precursors were shown to be

membrane active, prOE33 and prOE23 Finally to investigate the *in vivo* activity of prSSU transit peptide, we designed green fluorescent protein (GFP) chimeras with the precursor to the small subunit of Rubisco (prSSU-GFP). GFP alone or prSSU-GFP could be expressed in a transient assay in onion epidermal cells. These experiments lay the groundwork for expression of mutant precursors fused to GFP in either onion epidermal to explore the effect mutations in the transit peptide have on targeting and import relative to the wildtype precursor.

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Chapter 1-General Introduction

A typical plant cell may contain millions of individual protein molecules. If such a cell is to function properly, it must maintain a process to ensure the correct targeting of those proteins to specific cellular locations. Locations within the plant cell can be divided into three broad groups: the cytoplasm, membrane systems, and metabolic compartments. Soluble proteins are located in all subcellular metabolic compartments including the cytosol, vacuole, mitochondrial matrix, peroxisomes, nucleus, endoplasmic reticulum (ER) lumen, cisternae of the Golgi apparatus, chloroplast stroma and thylakoid lumen. Membrane proteins are located in all lipid bilayers in the cell including the plasma membrane, the nuclear membrane, the endoplasmic reticulum, the Golgi, the tonoplast, the membranes of the mitochondria, and the membranes of the chloroplast. Targeting to proper subcellular locations often means that the protein has to cross at least one membrane to get where it needs to go. Transporting soluble proteins across membranes presents special challenges to a cell because of the hydrophobic nature of the membrane and the mostly hydrophilic nature of the soluble protein. Cells therefore require machinery to sort each protein and direct it to its proper location.

SORTING SIGNALS

How do cells direct all of these proteins to their correct subcellular location? All nuclear-encoded proteins, except those that remain in the compartment where they were translated, i.e. the cytoplasm, have one or more targeting domains that act as an address label (Raikhel and Chrispeels 2000). Targeting domains are usually short peptides or

amino acid motifs, which are often located at the N terminus of a protein, but can also be located at the C terminus or elsewhere in the protein. Specific subcellular machinery (proteins or protein complexes) interacts with the targeting information to translocate the protein into, or retain it in, the proper compartment. For example, proteins targeted to the ER have an N-terminal extension, called a signal peptide, which directs the protein cotranslationally to the ER membrane through interaction with a signal recognition particle (SRP; Johnson and van Waes 1999). Proteins targeted to mitochondria have an N-terminal extension, called a presequence, which directs proteins post-translationally to translocation machinery of the outer membrane of the mitochondria (Voos et al. 1999). Proteins targeted to the chloroplast also have N-terminal extensions, called transit peptides, which direct proteins post-translationally to the translocation machinery of the outer membrane of the chloroplast envelope (Chen and Schnell 1999; Keegstra and Cline 1999). Transport of proteins into the ER, mitochondria, and chloroplasts will be discussed in more detail later. Other examples of targeting domains are shown in Table 1-1.

The first step in sorting for all proteins made in the cytosol is to separate them into one of two groups. The first group of proteins is released from ribosomes into the cytosol and may remain in the cytosol or is targeted to a subcellular location such as mitochondria, chloroplasts, peroxisomes or nuclei. In addition, they face the problem to fold or not to fold. Premature folding may prevent the protein from being targeted or folding may occur incorrectly. Cytosolic factors or molecular chaperones often bind to newly synthesized cytosolic proteins to prevent misfolding and/or aggregation or to direct

Table 1-1. **Types of targeting domains**. Hydrophobic, **basic,** acidic, and hydroxylated amino acids are indicated.

Function of Signal Peptide	Example of Signal Peptide	Cleavable sequence
Export into ER	H_3N-MLTAMLLSCVLLLALPPTLG-	yes
Retention in ER lumen	-**K**DEL-COOH	no
Import into nucleus	-PP**KKKRK**V-	no
Import into mitochondria	H_3N-MLSLRQSI**R**FF**K**PATRTLCSS**R**YLL-	yes
Import into peroxisomes	-SKL-	no
Import into chloroplasts	H_3N-MASSVLSSAAVATRSNVAQANMVAPFTG LKSAASFPVS**RK**QNLDITSIASNGGRVQC-	yes
Export into thylakoid lumen	H_3N-(stromal targeting domain)- GAAAVGS**K**VSPADA	yes

the newly synthesized protein to its final destination (Fink 1999; Lund 1995). Typically one targeting domain directs the protein to its subcellular location, however, for some proteins destined for mitochondria or chloroplasts, one targeting domain directs the protein to the organelle while a second targeting domain directs the protein to its suborganellor location. Examples of suborganellar-localized proteins in mitochondria include proteins localized to the intermembrane space, which have bipartite targeting sequences (Beasley et al. 1993; Braun et al. 1992). Examples of suborganellar-localized proteins in the chloroplast include proteins localized to the thylakoid or thylakoid lumen (Clausmeyer et al. 1993; Ko and Cashmore 1989; Michl et al. 1994). The second group of proteins contains signal peptides and is targeted to the ER and the secretory pathway, which in plant cells includes retention, secretion or localization to the plasma membrane

or to the vacuole (Di Sansebastiano et al. 1998; Holwerda et al. 1992; Johnson and van Waes 1999).

Successful targeting to a subcellular location often involves crossing at least one membrane (Fig. 1-1). Getting soluble proteins across a membrane presents unique challenges to a cell because of the inherent differences between the membrane environment and the soluble protein. To get around this, translocation across a membrane is mediated by a proteinaceous pore or channel through which the targeted protein passes, usually in an unfolded form (Fig. 1-1; Chen and Schnell 1999; Raikhel and Chrispeels 2000; Schatz and Dobberstein 1996; Voos et al. 1999). Schatz and Dobberstein (Schatz and Dobberstein 1996) identified five universal steps of protein transport across a membrane common to all general protein translocation systems. [(1)]the transported protein typically carries an N-terminal signal and is recognized by cytosolic factors that deliver it to specific receptors on the target membrane; [(2)]the protein usually remains partly unfolded during transport; [(3)]the signal sequence opens a hetero-oligomeric transmembrane channel that is gated both across and within the plane of the membrane; [(4)]the channel is coupled to a peripherally attached protein translocation motor that is driven by the hydrolysis of nucleoside triphosphate and transports the protein across the membrane; and [(5)]the protein folds on the *trans* side of the membrane with the help of chaperones or folding enzymes.

Schatz and Dobberstein (1996) also divided protein transport into two categories, which differ in their evolutionary origin: export processes and import processes (Table 1-2). Export processes occur when a protein is transported from the cytosol out to the

Fig. 1-1. General model of translocation of proteins across a membrane. *A*, Transport of proteins can occur either post-translationally or cotranslationally involving cytosolic factors or molecular chaperones. *B*, Interaction of the preprotein with a hetero-oligomeric translocation pore induces translocation (cyan). *C*, A peripheral translocation motor provides the energy (yellow). *D*, Chaperones on *trans* side mediate folding of the newly transported protein (tan and yellow).

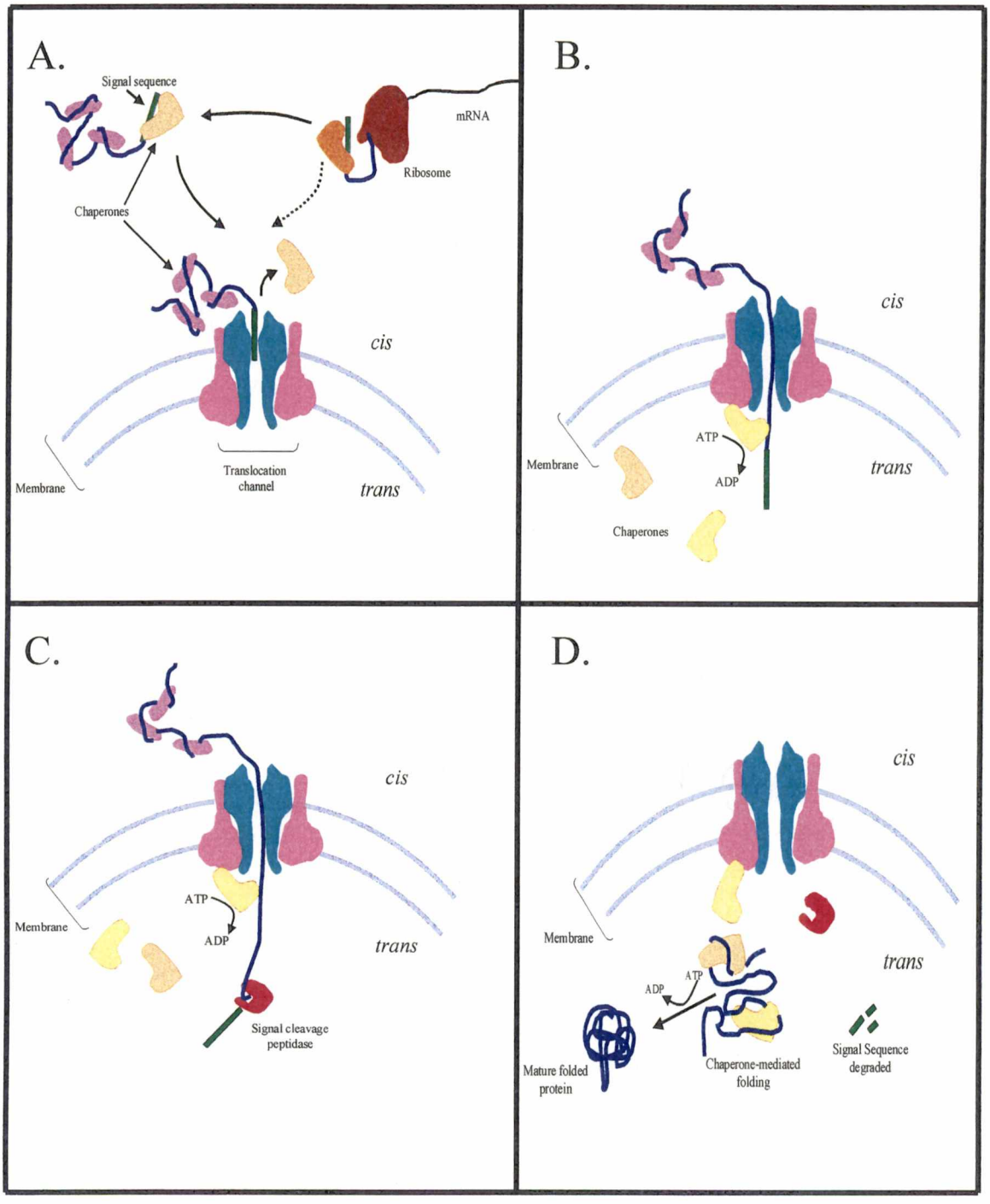

A.
Signal sequence
mRNA
Ribosome
Chaperones
cis
Translocation channel
Membrane
trans
B.
cis
ATP
ADP
Membrane
trans
Chaperones
C.
cis
ATP
ADP
Membrane
trans
Signal cleavage peptidase
D.
cis
Membrane
trans
ADP
ATP
Mature folded protein
Chaperone-mediated folding
Signal Sequence degraded

Table 1-2. **Major cellular translocation systems.** Translocation is divided into three categories: **import systems,** export systems, and *gated processes.*

nslocation-competent membranes	*cis*	Direction of translocation →→→	*trans*
Outer chloroplast membrane	**Cytoplasm**	→→→	**IMS**
Inner chloroplast membrane	**IMS**	→→→	**Stroma**
Outer mitochondrial membrane	**Cytoplasm**	→→→	**IMS**
Inner mitochondrial membrane	**IMS**	→→→	**Matrix**
eroxisomal membrane	**Cytoplasm**	→→→	**Peroxisome**
Endoplasmic reticulum	Cytoplasm	→→→	ER lumen
Prokaryotic plasma membrane	Cytoplasm	→→→	Periplasm
Inner mitochondrial membrane	Matrix	→→→	Inner membrane
Thylakoid membrane	Stroma	→→→	Thylakoid lumen
Nuclear envelope	*Cytoplasm*	→→→ ←←←	*Nucleoplasm*

periplasm or a topologically equivalent area, such as the lumen of the ER. Import processes occur when a protein is transported from the cytoplasm into some other cellular compartment, such as the peroxisome, mitochondria, or plastid. Transport of proteins into the lumen of the ER, *Escherichia coli* secretion into the periplasm, and transport of proteins across the thylakoid into the thylakoid lumen are examples of export processes. Transport of proteins across the chloroplast envelope, across membranes of the mitochondria, and transport to the peroxisome/glyoxisome are examples of import processes. A third category of protein transport, gated transport such as that found in the nucleus, will not be discussed here because it is not transport into a compartment topologically different from the cytosol, but has been reviewed recently (Gorlich 1997; Kohler et al. 1999; Yoneda 1997). In addition, vesicle-mediated protein transport, such as secretion of proteins or targeting to the vacuole, utilizes the secretory system after proteins are exported to the lumen of the ER. The major compartments of the secretory system consist of the ER, the Golgi complex, and the vacuole. All of these compartments and the plasma membrane are connected by membrane traffic, which can occur by direct contact or budding and fusion of vesicles (Vitale and Denecke 1999; Vitale and Raikhel 1999). Vesicle-mediated protein transport is very different from the export/import processes discussed above and as such is outside the scope of this discussion. The reader is referred to recent reviews on the process (Vitale and Denecke 1999; Vitale and Raikhel 1999).

As stated above, the general features of protein sorting (Fig. 1-1) seem to apply to any organelle and will be discussed in greater detail for transport of proteins into the chloroplast. In order to fully appreciate the nuances of transport of proteins into the

chloroplast, however, a brief discussion of more well defined protein transport systems is in order.

Features of targeting proteins to the ER, mitochondria or chloroplast have many similarities. [1]All three mechanisms require a sorting signal as part of a precursor form of the protein to be targeted. [2]Cytosolic factors also play a role, although to varying degrees. [3]All three utilize a receptor system on the cytosolic face of the target membrane. [4]Translocation occurs through heteroligomeric channels in the membrane in an energy-dependent manner. [5]All three have a protein translocation motor, which drives transport with NTP hydrolysis.

In contrast, however, each system has key unique features. [a]The signal sequence for each process is unique. [b]Targeting of proteins to the ER occurs co-translationally, whereas targeting to mitochondria or chloroplasts occurs post-translationally. [c]Translocation into the ER involves crossing one membrane, whereas, translocation into mitochondria or chloroplasts involves two membranes. [d]Translocation of proteins into chloroplasts occurs at contact sites between the outer and inner envelope membranes, whereas transport across the outer and inner membranes of mitochondria are distinct. [e]Transport of proteins into the mitochondria is dependent upon a membrane potential, whereas transport into the ER or transport into the chloroplast is not.

The most striking difference between export into the ER and import into chloroplasts and mitochondria is that chloroplasts and mitochondria transport systems arose in association with endosymbiotic gene transfer from the genome of the endosymbiont to the nucleus. Exactly what has driven this transfer of genes from the organelle to the nuclear genome is unclear. The various mutational pressures on the

endosymbiont genome due to oxidative DNA damage, lack of recombination, and genetic bottle necking, because chloroplasts and mitochondria are both clonal and asexual, have probably all contributed to a situation where, if the genes remain in the organelle, deleterious mutations would have accumulated, ultimately killing the endosymbiont (Allen and Raven 1996; Lynch 1996; Lynch 1997; Muller 1964). Whether the import systems of chloroplasts or mitochondria arose as unique protein transport systems or were modified from existing translocation systems or are a combination of both has yet to be determined. The fact still remained however, that those organelles needed, for full functionality, proteins that were no longer encoded by genes located in the organelle and mechanisms to return those necessary proteins had to evolve.

TARGETING PROTEINS TO THE ER

Signal Peptides

As peptides are synthesized on the ribosome, proteins, which are destined to enter the secretory pathway, contain an N-terminal targeting domain called a signal peptide, which directs the ribosome to bind to the ER (Walter and Johnson 1994). Signal peptides can vary in length from about 15 to >50 amino acids, but they do have common features (von Heijne 1986c; von Heijne 1987). They typically have an N-terminal region that is hydrophilic (basic) followed by a hydrophobic stretch of 6-15 amino acids and a C-terminal flanking region (Martoglio and Dobberstein 1998; Schatz and Dobberstein 1996; von Heijne 1985). The hydrophobic stretch, the h-region, has been shown to be the most critical part of the signal peptide (von Heijne 1985). The flanking C-terminal region typically has polar residues as well as helix-breaking proline and glycine residues. The

signal peptide cleavage site is also found in the C-terminal region and is usually identified by the presence of small uncharged residues at positions –3 and –1 (von Heijne 1983; von Heijne 1984; von Heijne 1986b). On the immediate N-terminal side of the h-region, a polar region is found often having a net positive charge (von Heijne 1986a; von Heijne 1986b). Most variation in length occurs in the N-terminal region, which is different from chloroplast transit peptides where variability occurs in the central domain. Fig. 1-2 is a schematic representation of different sorting sequences.

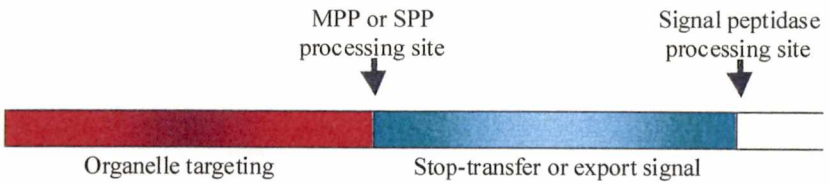
In addition to region similarities between signal peptides, they also have secondary structure similarities. The general theme is that signal peptides form a mostly hydrophobic α-helix through the h-region, especially in a hydrophobic environment such as trifluorethanol (TFE), sodium dodecylsulfate (SDS) micelles or lipid monolayers (Briggs et al. 1986; Briggs and Gierasch 1984; Bruch et al. 1989; Emr and Silhavy 1983; Jones et al. 1990). By following tryptophan fluorescence, McKnight, et al., (1991) were able to demonstrate that h-region of the signal peptide of LamB of *Escherichia coli* buried itself into lipid vesicles while the N- and C-terminal regions were more interfacial. Membrane activity of signal peptides is important for insertion of proteins into membranes in either spontaneous or translocon-mediated fashion (Walter and Johnson 1994). Membrane activity is common to ER signal peptides, mitochondrial presequences, and chloroplast transit peptides.

Fig. 1-2. Types of sorting signals. Export sequences are shown in blue, import sequences in red. Vertical lines represent hydrophobic residues; zig-zag lines are β-strands; plus sign indicates basic amino acids; OH represents hydroxylated amino acids. (Adapted from Schatz and Dobberstein, 1996).

Export signal sequence

Mitochondrial presequence

Chloroplast transit peptide



Bipartite signal sequence

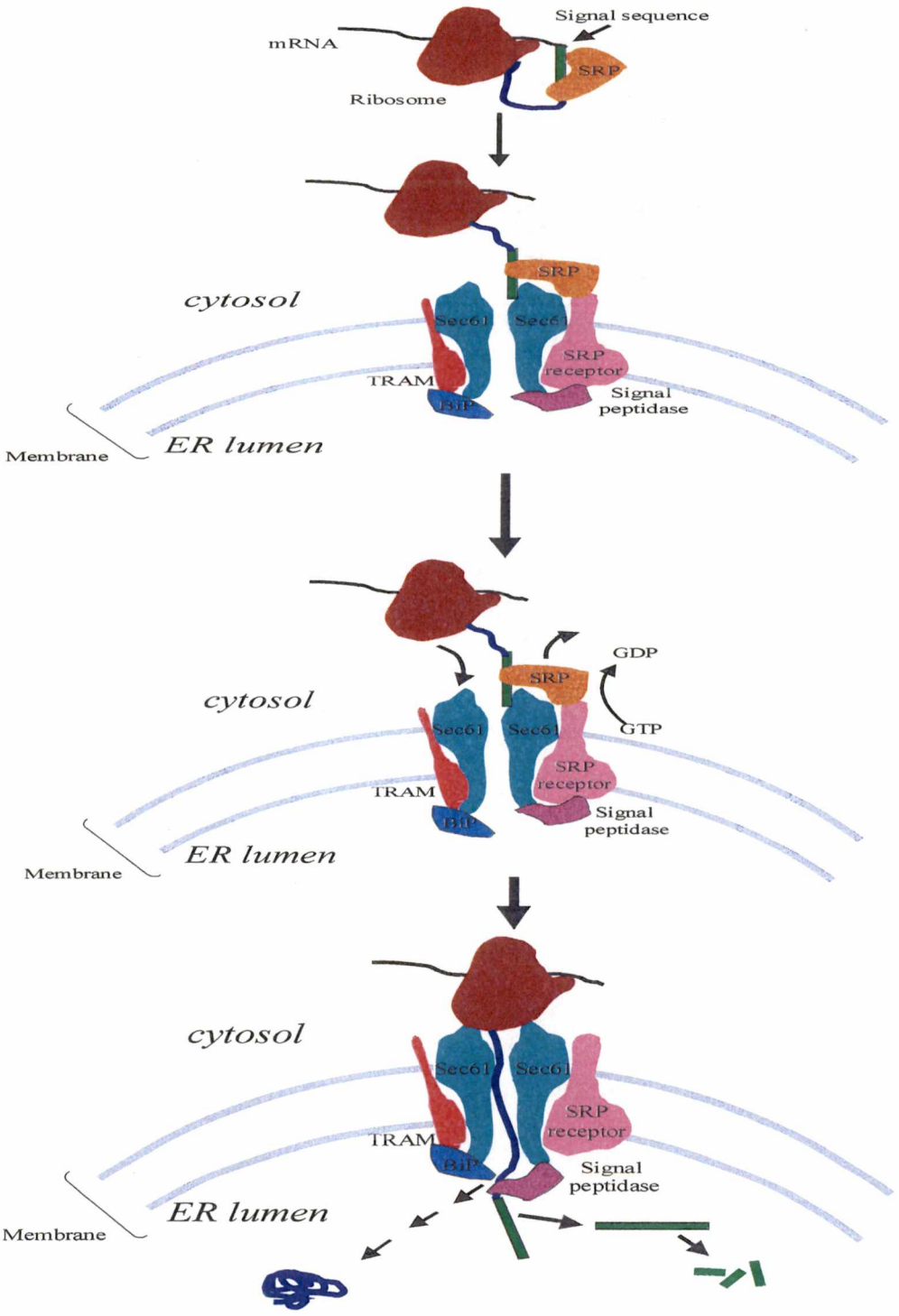
Cytosolic Factors

Targeting of proteins by signal peptides to the ER membrane (of yeasts) can occur post translationally or cotranslationally and can be dependent on SRP/SRP receptor or be SRP independent (Johnson and van Waes 1999; Walter and Johnson 1994). SRP-independent targeting to the ER utilizes the chaperone SecB in prokaryotes and Sec62/63 in yeast and is post-translational (Brodsky et al. 1995; Brodsky and Schekman 1993; Deshaies et al. 1991; Johnson and van Waes 1999; Musch et al. 1992; Sanders et al. 1992; Walter and Johnson 1994).

SRP-dependent targeting involves recognition of the signal peptide on the nascent polypeptide by SRP. SRP recognizes the signal peptide largely through the hydrophobicity of the h-region. Sequences that engage SRP tend to be more hydrophobic than those that do not utilize SRP (Hatsuzawa et al. 1997; Ng et al. 1996; Ulbrandt et al. 1997; Valent et al. 1998; Zheng and Gierasch 1996). Once SRP binds to the signal peptide on the nascent polypeptide, it causes a pause in translation (Fig. 1-3; Lipp et al. 1987; Lutcke 1995). Continuing synthesis at this point might allow the emerging protein to begin folding incorrectly, which would not be productive. SRP then recruits the polysome complex to the ER membrane by interacting with its membrane SRP receptor. This allows the ribosome to contact Sec61p, the translocation pore (Luirink and Dobberstein 1994; Lutcke 1995; Ogg et al. 1992).

SRP is a ribonucleic acid:protein complex, which has 6 protein subunits and a 5.6S RNA. One subunit, which is particularly conserved from bacteria to buffalo, is the 54 kD subunit (Ffh). This Ffh subunit has GTPase activity and recognizes the h-region of

Fig. 1-3. Model of cotranslational translocation into the ER. *A*, As the nascent peptide is synthesized, the signal peptide is bound by SRP, halting translation, and directing the polysome to the membrane of the ER. *B*, GTP hydrolysis by SRP and its receptor cause transfer of the signal peptide to the translocation pore as well as SRP release and ribosome binding to the translocation pore. *C*, Translation continues and the newly synthesized protein emerges into the lumen of the ER, to be folded with the aid of chaperones (BiP).


Signal sequence
mRNA
SRP
Ribosome
cytosol
Sec61
Sec61
SRP receptor
TRAM
BiP
Signal peptidase
Membrane
ER lumen
GDP
GTP

signal peptides (Bacher et al. 1996; Freymann et al. 1997; Lutcke et al. 1992). GTP hydrolysis has been shown to play a role in transfer of the nascent polypeptide into the translocation pore. This is mediated by the interaction of SRP with its receptor, SRPR or docking protein (Bacher et al. 1996; Freymann et al. 1997).

Membrane and Lumenal Components

The SRP binds to the target membrane via a hetero-oligomeric receptor complex, which has α and β subunits. Interestingly, both SRPRα and SRPRβ also have GTPase activities (Bacher et al. 1996; Freymann et al. 1997). How GTP functions to "switch" the signal peptide from a nascent peptide to a translocation intermediate is not known. However the coordinate hydrolysis of multiple GTP molecules could provide significant energy for molecular reorganization. The role of GTP in ER transport is particularly important to plastid protein transport since the translocation apparatus of plastids also contains multiple GTPases, as described below. Nonetheless, GTP hydrolysis provides the energy needed to transfer the signal peptide of the nascent polypeptide to the translocation pore. Once GTP hydrolysis occurs, SRP and the β subunit of SRP receptor release the polypeptide into the translocation pore and SRP dissociates (Bacher et al. 1996; Freymann et al. 1997).

Concomitantly, interaction of SRP with its receptor allows the ribosome to interact with the translocation pore, mostly comprised of Sec61. Sec61 is a heterotrimer of three subunits, α, β, γ (Gorlich et al. 1992a; Gorlich et al. 1992b; Gorlich and Rapoport 1993; Sanders et al. 1992). Another protein that was found to be required for translocation of most, but not all, polypeptides is TRAM (translocation-associated-

membrane protein; Gorlich et al. 1992a). Protein translocation or integration could be achieved by reconstitution of these four proteins into proteoliposomes, thus indicating that they comprise the core of the translocation pore (Gorlich et al. 1992a; Gorlich et al. 1992b; Gorlich and Rapoport 1993; Nicchitta and Blobel 1990).

Release of SRP promotes binding of the ribosome to the translocation pore (Fig. 1-3) so that cotranslational translocation can occur (Johnson and van Waes 1999; Lyman and Schekman 1996). At this point, translation resumes and the elongating polypeptide is pushed through the translocation pore. As the signal peptide emerges on the lumenal side of the pore, it is recognized by the signal peptidase, located adjacent to the pore, which cleaves the signal peptide from the rest of the emerging polypeptide (Johnson and van Waes 1999; Lyman and Schekman 1996; Nothwehr et al. 1989; Rapoport 1986; von Heijne 1984). Additionally, if the emerging protein has sites for glycosylation, oligosaccharyltransferases (OST), also adjacent to the pore, can glycosylate the nascent chain as it is translated (Johnson and van Waes 1999; Nilsson and von Heijne 1993).

As translocational translation continues, chaperones bind to the emerging chain to assist in folding the protein. Soluble lumen chaperones include BiP and calnexin both of which assist in folding the newly synthesized protein (Brodsky and Schekman 1993; Flynn et al. 1991; Hebert et al. 1998; Jannatipour and Rokeach 1995; Nguyen et al. 1991; Scidmore et al. 1993; Tatu and Helenius 1997; Vogel et al. 1990). BiP not only aids in folding of newly synthesized polypeptide, but was shown to seal the translocation pore when translocation was not active thereby gating the translocon perpendicular to the membrane channel and preventing any passage of molecules, ions, or protons between the cytoplasm and ER lumen (Hamman et al. 1998). In addition, other proteins can also

interact with the emerging nascent chain for different purposes. These include protein disulfide isomerase (PDI), which aids in proper folding by catalyzing formation of disulfide bonds (Joly and Swartz 1994; Kamitani et al. 1992; Ostermeier et al. 1996); calreticulin, which works with calnexin to fold glycosylated proteins (Jannatipour and Rokeach 1995; Molinari and Helenius 2000); and ERp57, which is a member of the PDI superfamily and has similar function in the folding of glycoproteins (Elliott et al. 1997; High et al. 2000; Molinari and Helenius 1999; Oliver et al. 1999).

Thus transport of proteins into the lumen of the ER involves the export of proteins from the cytoplasm into an area that is topologically distinct from the cytoplasm. This is very similar to the Sec system of bacteria. Many components are conserved between the two. ER signal peptides and bacterial signal peptides are interchangeable (Wickner and Leonard 1996). Both systems utilize cytosolic factors (SRP or Ffh) or molecular chaperones (Hsp70 or SecB) to target the protein and prevent misfolding, respectively (Economou 1998; Economou 1999; Wickner and Leonard 1996). In addition both transport systems utilize a heterotrimeric core protein complex for translocation of which the components are strikingly similar (Wickner and Leonard 1996). In contrast protein secretion using bacterial Sec system almost exclusively involves post-translational transport of proteins, whereas export into the ER mainly involves co-translational protein transport; and Sec export requires the use of a translocation motor (SecA) which drives transport through ATP hydrolysis, whereas for co-translational ER export, protein synthesis by the ribosome is the driving force (Economou 1998; Economou 1999; Vitale and Denecke 1999; Wickner and Leonard 1996).

TARGETING PROTEINS TO THE MITOCHONDRIA

Another protein transport mechanism as highly studied as export of proteins into the ER, is import of proteins into the mitochondria. Almost all eukaryotes have mitochondria, which, as the site of cellular respiration and ATP synthesis, is the energy center of the cell. Like chloroplasts, mitochondria are semiautonomous organelles possessing their own genome, yet they, too, have lost most of the genes coding for proteins required for mitochondrial function to the nucleus (Unseld et al. 1997). Proteins encoded by those genes that have relocated to the nucleus are, therefore, synthesized in the cytoplasm and must be post-translationally targeted to the mitochondria. In plants, targeting proteins to the mitochondria may present new levels of complexity, which do not exist in animals or yeasts, because the plant cell has to be able to distinguish between mitochondrial and chloroplastic signal sequences.

Superficially, mitochondrial and chloroplastic targeting and protein translocation appear very similar, and yet they have only gross similarities. For example, proteins destined for the matrix of the mitochondrion must traverse two membranes, just like proteins destined for the stroma of chloroplasts; and transport occurs through protein complexes located in both the outer and inner membranes in both systems. Closer inspection of the nuances of protein transport into either organelle reveals many key differences. Translocation components in the outer and inner membrane of mitochondria show no sequence similarity with translocation components of the outer and inner membrane of chloroplasts. Only the Hsp70's (the translocation motor and/or for folding of newly imported proteins) are conserved between the two organelles (Karlin and Brocchieri 1998). For proteins destined for the inner membrane of mitochondria, the

presequence can act as a signal-anchor sequence during transport across the inner membrane and the protein is released into the inner membrane from the translocation complex (Koehler et al. 1998; Koehler et al. 2000; Sirrenberg et al. 1996). Proteins destined for the inner membrane of chloroplasts, however are typically targeted to the stroma and directed back to the membrane in an as yet undiscovered manner. Finally, import of precursors into the matrix of the mitochondria requires a membrane potential (Martin et al. 1991; Ungermann et al. 1996), which is not present for import of precursors into the stroma.

Presequences

Proteins destined for the mitochondria are synthesized in the cytoplasm as higher molecular weight precursors. They contain an N-terminal extension, the presequence, which serves to target the protein to the mitochondria (Haucke and Lithgow 1997; Koehler 2000; Lithgow et al. 1995; Schatz 1996). Similar to proteins targeted to the ER or chloroplast, the presequence is both necessary and sufficient and is cleaved during or after transport of most precursors into the organelle (Pfanner et al. 1997; Schatz 1996; Voos et al. 1999). The presequence of mitochondria precursor proteins also shows a high degree of variability within its sequence between precursors similar to the signal peptide and the transit peptide.

Even though there is little sequence similarity among presequences of mitochondrial precursor proteins, several common features have been identified. Presequences are about 20-60 amino acids in length for non-plant precursors and about 7-10 amino acids (on average) longer for plant precursors (Glaser et al. 1998; Neupert

1997; Sjoling and Glaser 1998). Presequences are also noted by the presence of basic and hydroxylated amino acids and a lack of acidic and aromatic residues. Plant presequences are unique in that they have a significantly higher content of serine residues (17%) relative to yeast (7%), mammals (3%), or *Neurospora crassa* (10%) (ref. Sjoling and Glaser 1998). Presequences tend to have basic residues located at the N-terminal half of the sequence, followed by a short linker and a mostly hydrophobic C-terminal half, whereas hydroxylated residues are fairly evenly distributed along the length (Glaser et al. 1998; Schatz and Dobberstein 1996; Sjoling and Glaser 1998). Presequences form an amphiphilic α-helix at the N terminus, and a hydrophobic helix at the C terminus, although that is only prediction based on a large hydrophobic moment of the C-terminal region (Fig. 1-2; Neupert 1997). Plant presequences seem to follow these tendencies except that they are longer and have more variation in the C terminus around predicted cleavage sites as well as more variation in the linker region between the N and C termini (Glaser et al. 1998; Sjoling and Glaser 1998).

The presequence does have membrane activity (Leenhouts et al. 1995; Rietveld et al. 1986; Roise et al. 1988; Tamm and Bartoldus 1990) especially for membranes or bilayers containing cardiolipin and anionic lipids. Membrane activity is the ability of a molecule, in this case peptide, to interact and/or associate with a membrane through electrostatic and hydophobic interactions between the peptide and the membrane. There is disagreement, however, as to which end of the presequence predominantly interacts with the lipids (Golding and O'Shea 1995; Hammen et al. 1996; Leenhouts et al. 1995; Leenhouts et al. 1996). Recent NMR evidence suggests that interaction of presequence with Tom20 (translocator of the outer membrane of mitochondria subunit of 20 kDa) is

through mostly hydrophobic residues, either the hydrophobic face of the amphipathic N-terminal helix or the C terminal hydrophobic helix of the presequence (Abe et al. 2000; Muto et al. 2001). In contrast, however, a prevailing model of presequence progression into the translocation channel from the membrane receptors is the 'acid chain' model. This model suggests that acidic patches on the cytosolic face of outer membrane receptors and translocation components serve to interact with the basic, helical N terminus of presequence in a manner of increasing affinity as the presequence "surfs" toward the translocation channel from receptors to translocation components (Komiya et al. 1998; Schatz 1997). In either scenario, the presequence may still interact with lipid components of the membrane establishing some secondary structure that then enhances interaction with the receptor components (Hammen et al. 1996).

Cytosolic Factors

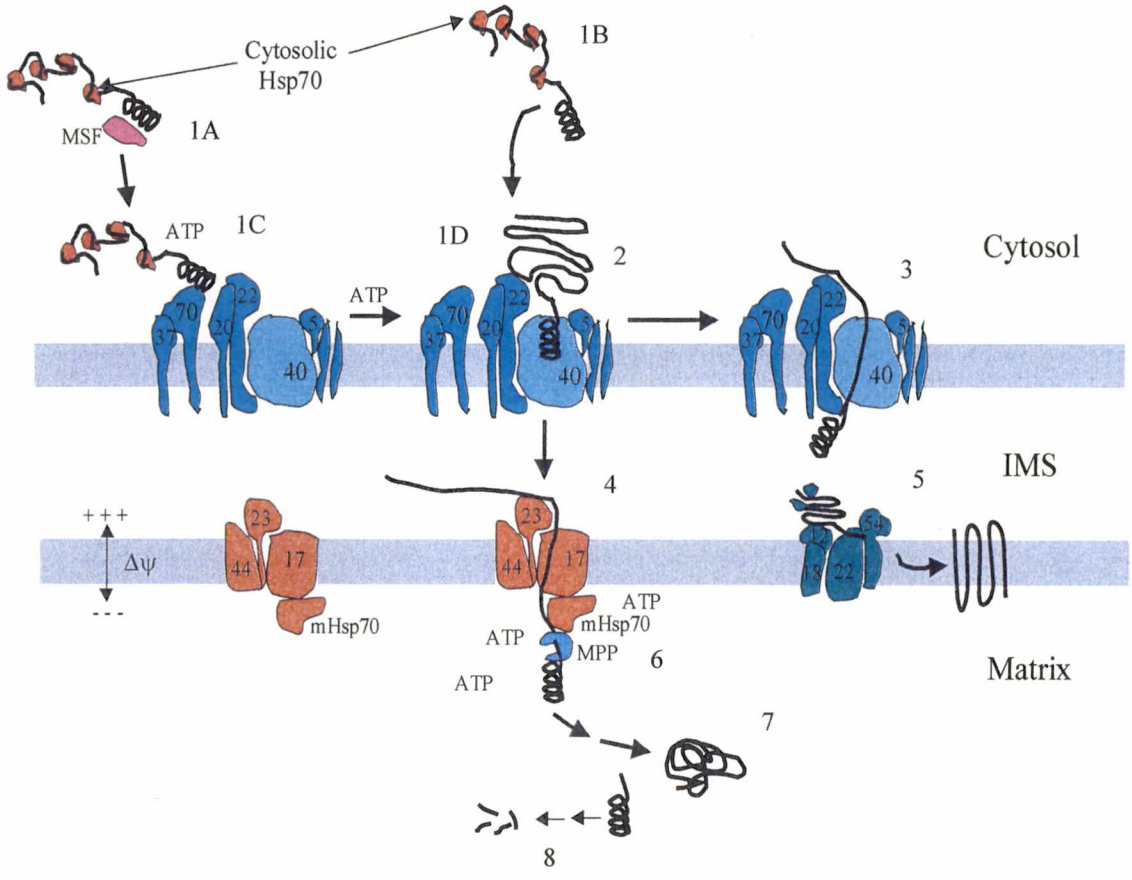
After translation of the mitochondrial precursor, chaperones play a role in targeting the protein to the outer membrane of the mitochondria. Transport of proteins into the mitochondria requires that the precursor be at least partly unfolded (Neupert 1997; Schatz 1996; Schatz and Dobberstein 1996; Whelan and Glaser 1997), and chaperones are known to bind to unfolded proteins to prevent them from folding prematurely (Fink 1999; Hartl 1995; Hartl-FU 1997; Mihara and Omura 1996a; Mihara and Omura 1996b). Two chaperones, cytosolic Hsp70 and mitochondrial import stimulating factor (MSF) play important roles in targeting certain subsets of precursors. Cytosolic Hsp70 plays a role in delivering mitochondrial precursors to the Tom20/22 preprotein receptor of the outer mitochondrial membrane in an ATP-independent manner

(Fig. 1-4; Komiya et al. 1997). Interaction of precursor and cytosolic Hsp70 are thought to occur mostly through the non-native protein, presumably to prevent misfolding or aggregation, not necessarily through the presequence (Glaser et al. 1998; Neupert 1997; Schatz 1996; Whelan and Glaser 1997). MSF, on the other hand, is a 14-3-3 protein, which preferentially binds phospho-serine in the presequence of mitochondrial precursors, delivering the precursors to the Tom70 preprotein receptor also in the outer mitochondrial membrane in an ATP-dependent manner (Fig. 1-4, Steps 1A and 1B; Komiya et al. 1997; Omura 1998).

Even though cytosolic Hsp70 and MSF have distinct roles during targeting of precursors to mitochondria, they are not, however, obligate (Hajek and Bedwell 1994; Murakami et al. 1988). Mitochondrial precursors are imported in the absence of cytosolic factors *in vitro*, but the presence of the cytosolic factors does prolong import, presumably by prolonging import competence of the precursors (Hajek and Bedwell 1994; Murakami et al. 1988). The chaperones probably maintain the precursor in an unfolded or loosely folded state, because chemically denatured proteins are imported in the absence of the chaperones whereas reversibly folded precursors are impeded in import until unfolded again (Rassow et al. 1989; Verner and Lemire 1989).

MSF is not the only protein involved in targeting proteins to the mitochondria. In mammals another protein shown to interact with presequences was identified and named the precursor binding factor (PBF; Murakami and Mori 1990). PBF was identified as being distinct from MSF because its interaction with presequence was not ATP-dependent, nor was it sensitive to *N*-ethylmaleimide (NEM; Murakami and Mori 1990).

Fig. 1-4. Model of mitochondrial protein import. Import into mitochondria occurs in several steps. *1A*, Presequence may be recognized by MSF and Hsp70 or *1B*, by cytosolic Hsp70 alone. *1C*, MSF-bound precursors interact with membrane receptors Tom70/37 in an ATP-dependent manner. *1D*, Precursors bound by Hsp70 are directed to the Tom20/22 receptor complex in an ATP-independent manner. *2*, The presequence is transferred to the translocation pore composed of Tom40 and is translocated across the outer membrane. *3*, The presequence can then interact with one of two inner membrane complexes. *4*, Precursors destined for the matrix are transported across the Tim23/17 complex. *5*, Whereas precursors destined for the inner membrane are transported across the Tim22/18 complex. *6*, a matrix-localized peripheral translocation motor (mHsp70) drives import into the matrix. *7*, Matrix proteins are folded with the aid of chaperones and the presequence is degraded, *8*.


Cytosolic
Hsp70
MSF
1A
1B
ATP
1C
1D
2
3
Cytosol
ATP
70
37
20
22
40
5
4
IMS
5
+++
Δψ

23
44
17
mHsp70
ATP
MPP
6
Matrix
7
8

Membrane Components and Translocation Motor

When the precursor encounters the mitochondrial membrane (either through direction from a chaperone or otherwise), two receptor complexes are waiting to interact with it (Kiebler et al. 1993; Kiebler et al. 1990; Mayer et al. 1995a; Neupert 1997; Schatz 1996; Schneider et al. 1991; Steger et al. 1990). Four distinct proteins have been identified in yeast and *N. crassa,* and they are Tom70 (translocator of the outer membrane of mitochondria followed by the apparent molecular weight), Tom37, Tom22, and Tom20 (Kiebler et al. 1993; Kiebler et al. 1990; Mayer et al. 1995a; Neupert 1997; Schatz 1996; Schneider et al. 1991; Steger et al. 1990). If the mitochondrial membrane is solubilized with non-denaturing detergents, two receptor complexes are isolated, a Tom70/Tom37 heterodimer and a Tom22/Tom20 complex (Gratzer et al. 1995; Haucke et al. 1995a; Haucke et al. 1995b; Kiebler et al. 1993; Kiebler et al. 1990; Mayer et al. 1995a; Schlossmann and Neupert 1995; Schneider et al. 1991; Steger et al. 1990). Both complexes associate with each other through tetratricopeptide repeat motifs found on Tom70, Tom37, and Tom20 (Haucke et al. 1995a). Tetratricopeptide repeats are 34 amino acid long degenerate sequences, which mediate protein-protein interactions through their tendency to form α helices (Lamb et al. 1995).

While deleting Tom70 causes a reduction in growth of yeast (Schlossmann et al. 1994), Tom37 was shown by genetic deletion in yeast to be non-essential because yeast lacking Tom37 do not show delayed growth, but it does crosslink to Tom70 and enhances import of ADP/ATP carrier protein (AAC; Gratzer et al. 1995). Tom37 is not found as part of the Tom components in *N. crassa* (Kunkele et al. 1998). Only a few proteins such

as AAC, cytochrome c_1, F_1-ATPase β subunit, and the phosphate translocator have been shown to utilize the Tom70/Tom37 receptor system (Hines et al. 1990; Sollner et al. 1990; Steger et al. 1990). MSF brings proteins to the Tom70/Tom37 complex in an ATP-dependent manner and releases the precursor to the receptor with ATP hydrolysis (Komiya T 1996).

The majority of mitochondrial precursors interact with the Tom20/Tom22 receptor (Neupert 1997; Schatz 1996). Recent NMR data have identified a hydrophobic binding cleft in Tom20, which binds hydrophobic regions of the presequence, either the hydrophobic face of the amphipathic, N-terminal α-helix or the hydrophobic C-terminal helix (Abe et al. 2000; Muto et al. 2001). These receptor components were first discovered, however, by inhibition by antibodies against outer membrane proteins of the mitochondria and assaying for protein import in *N. crassa* (Schneider et al. 1991; Sollner et al. 1989). Genetic knockouts of Tom20 lead to severe growth defects implicating its role in protein import into mitochondria (Harkness et al. 1994a; Harkness et al. 1994b). Deletion of Tom22 (Lithgow et al. 1994; Nargang et al. 1995) was lethal indicating that almost all proteins imported into mitochondria require Tom22. Tom22 can be crosslinked to Tom40, the translocation pore, to comprise most of the general import pore (Dekker et al. 1996; Dekker et al. 1998; Kiebler et al. 1990; Sollner et al. 1992), further demonstrating its importance in mitochondrial protein import.

Components of the mitochondrial import apparatus (both Tom and Tim) were initially identified in yeast and the fungus *N. crassa*. In plants, homologs for most of these components were found in potato mitochondria, by blue native gel electrophoresis and immunoaffinity chromatography, except no homologs of Tom37 or Tom22 were

found, and an additional 9 kD protein was seen that is not seen in complexes from other organisms (Jansch et al. 1998). The absence of Tom37 may not be surprising since *N. crassa* also is without Tom37 (Kunkele et al. 1998) and it was shown to be non-essential in yeasts (Gratzer et al. 1995). The absence of a Tom22 homolog, however was very surprising since Tom22 is thought to play such a critical role by acting as both receptor and part of the translocation channel and is highly conserved among organisms without plastids. Subsequently, homologs of Tom22 have been identified using iterative BLAST analysis (Macasev et al. 2000). Most interesting, however, was that the plant homologs identified had more variation than Tom22 from non-plant organisms. In addition the plant Toms22 are smaller ranging in size from 11.1 kD (in cotton) to 9 kD (in potato) (Macasev et al. 2000). Therefore the additional 9 kD protein that Jansch, et al., identified is actually the Tom22 homolog (Jansch et al. 1998). The size difference is attributed to plant Tom22 lacking the large acidic cytosolic face, which is so critical for binding presequence in other organisms (Macasev et al. 2000). The authors suggest that loss of the very acidic domain in plant Tom22 may allow for discrimination between highly basic presequences and somewhat less basic chloroplast transit peptides (Macasev et al. 2000). This discrimination could be assayed by ability of plant Tom22 to bind chloroplast transit peptide relative to the ability of a non-plant Tom22 to interact with transit peptide.

Specific recognition of mitochondrial precursors by membrane receptors is only the first step of the import process (Fig. 1-4, Steps 1C and 1D). Successful recognition leads directly to the second step, the insertion of the presequence into the outer membrane (Fig. 1-4, Step 2) . Tom40 is an essential integral membrane protein and is the central component of the outer membrane translocase (Baker et al. 1990; Kiebler et al.

1990; Vestwebber et al. 1989), which has been shown to form a stable hydrophilic channel that translocates precursors in proteoliposomes (Hill et al. 1998). Tom40 and four other proteins, Tom22, Tom7, Tom6, and Tom5, form a ~400 kD complex in yeast called the general import pore (Fig. 1-4; Dekker et al. 1996; Dekker et al. 1998; Kiebler et al. 1990; Sollner et al. 1992). Transfer of proteins across the outer membrane may be driven mostly by successive interaction with several import components according to the 'acid chain hypothesis' (Dietmeier et al. 1997; Komiya et al. 1998; Pfanner et al. 1997; Schatz 1997). Tom22, Tom20, and Tom5 have all been shown to have acidic patches on their cytosolic faces which may serve to 'surf' or direct the basic presequence to the general import pore (Dietmeier et al. 1997; Komiya et al. 1998; Pfanner et al. 1997; Schatz 1997). In addition, the intermembrane space faces of Tom22 and Tom40 can bind presequences *in vitro*, through acidic patches, suggesting a mechanism of pulling the presequence through the pore by electrostatic interaction (Bolliger et al. 1995; Mayer et al. 1995b).

While movement across the outer mitochondrial membrane may or may not require energy, movement across the inner membrane is accomplished by a completely different principle. Once translocation of the presequence across the outer membrane has occurred (Fig. 1-4, Step 3), the precursor can be imported across the inner membrane by at least two different mechanisms based upon the presequence and the ultimate location of the protein (Fig. 1-4, Steps 4 and 5). Proteins destined for the matrix utilize the Tim23/Tim17 complex, while some inner membrane localized proteins use the Tim22 complex (Koehler 2000; Kubrich et al. 1994; Sirrenberg et al. 1996).

Import across the inner membrane into the matrix (via Tim23/Tim17) has strict energy requirements in the form of a membrane potential ($\Delta\psi$) and matrix localized peripheral translocation motor consisting of mitochondrial Hsp70 (mHsp70; Berthold et al. 1995). Two mechanisms have been offered to explain Hsp70-driven protein import into the matrix. One is the 'ratchet' model, which states that mHsp70 binds to the protein as it comes across the inner membrane. mHsp70 binding to the protein is sufficient for making the movement of the protein vectorial because backward movement out of the pore by the precursor is prevented by the bound mHsp70 on the emerging peptide (Schneider et al. 1994; Simon et al. 1992; Ungermann et al. 1996). The other proposed mechanism involves 'pulling' the protein across the inner membrane by mHsp70 binding to the targeted protein in an extended conformation and binding to components of the inner membrane pore for leverage. ATP hydrolysis provides the conformational shift needed to generate vectorial movement of the targeted protein. Several reports demonstrate binding of mHsp70 to the precursor as it comes through the pore (Gambill et al. 1993; Kang et al. 1990; Voos et al. 1994; Zhang et al. 1999). In addition, mHsp70 binding to Tim components has also been seen (Kronidou et al. 1994; Rassow et al. 1994; Schneider et al. 1994; Voos et al. 1996). Finally, von Ashen, et al., (1995) demonstrated that ATP hydrolysis causes a conformational shift in mHsp70 which could provide the leverage needed to vectorially transfer the precursor into the matrix.

TARGETING PROTEINS TO THE CHLOROPLAST

Like mitochondria, plastids are also semiautonomous organelles containing a genome which codes for about 60-200 proteins (Martin et al. 1998) depending upon the host.

Martin and Herrmann (1998) predict that there are probably 2000-5000 individual kinds of proteins in a fully functional plastid. For comparison, the evolutionary relative *Synechocystis* PCC6803 contains more than 3000 genes (Kaneko et al. 1996; Kaneko and Tabata 1997). Additionally, analysis of the sequence of the *Arabidopsis* genome predicts that ~2100 (>95% specificity) genes code for chloroplast precursors (Initiative 2000). For contrast, the *Arabidopsis* genome sequence predicts ~4200 (>95% specificity) genes that contain signal peptides to target to the ER, and ~2900 (~400 >95% specificity) genes that code for mitochondrial precursors (Initiative 2000). If the plastid genome of higher plants contains genes for ~80 (Martin and Herrmann 1998; Martin et al. 1998) proteins, the rest of the proteins necessary for function are coded for by genes that have relocated to the nucleus. Plastids, therefore, must import the vast majority of their proteins to gain functionality. The process of protein import into plastids is presumably very similar for the different types of plastids since each plastid subtype arises from the proplastid (Fig. 1-5), yet most protein import studies have utilized chloroplasts because of their abundance and importance. The following discussion of chloroplast protein import details different aspects of protein targeting to the chloroplast by looking at the transit peptide, cytosolic factors involved, membrane components, and the role of membrane lipids.

Import of the vast majority of precursor proteins into the chloroplast from the cytosol is thought to occur through one general import mechanism (Fig. 1-6; Archer and Keegstra 1990; Bruce 2000; Chen and Schnell 1999; Keegstra and Cline 1999; Keegstra and Froehlich 1999). Briefly, after translation, the precursor is targeted to the outer membrane of the chloroplast either through peptide/lipid interactions of the transit peptide with membrane lipids or by delivery from cytosolic factors, which recognize and

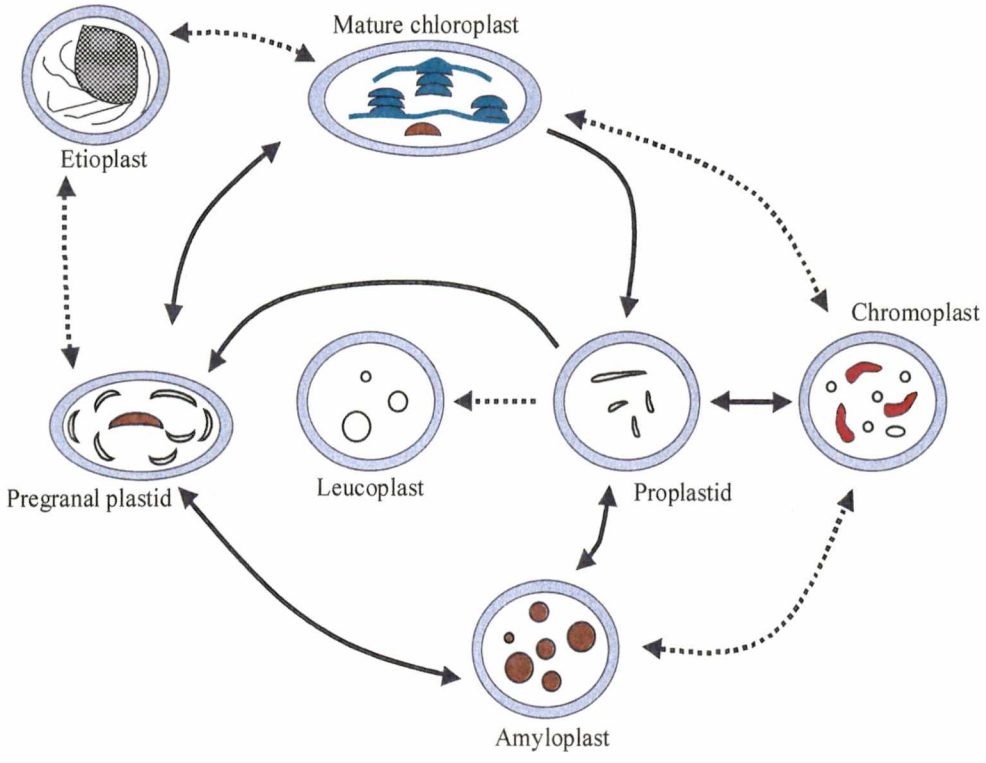
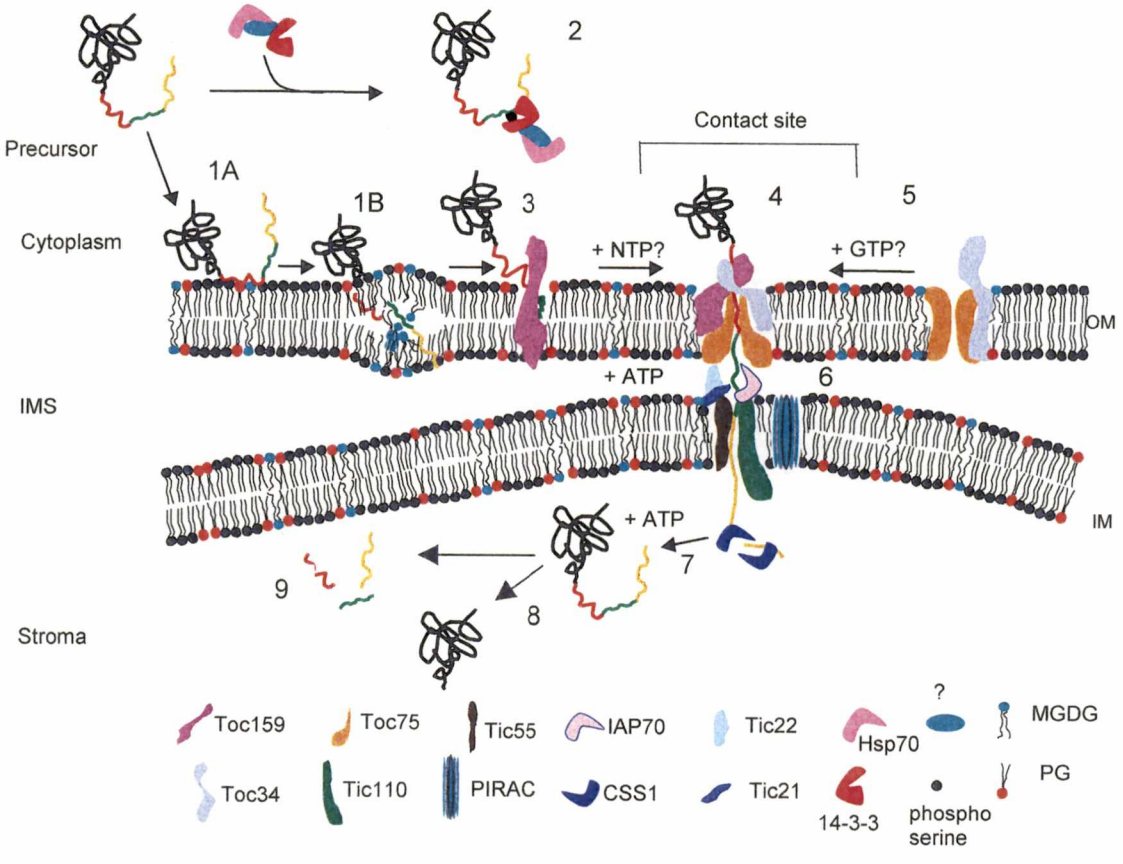


Fig. 1-5. **Subtypes of plastids.**

Fig. 1-6. Model of chloroplast protein import. Several steps have been identified for protein translocation into chloroplasts. *1A-B*, Precursors interact with the membrane through the transit peptide, particularly anionic and non-bilayer forming lipids. *2*, A guidance complex recognizes the transit peptide. *3*, Precursors from Steps 1 or 2 interact with receptor proteins in the membrane. *4-5*, The receptors complex with the translocation pore to form an active translocation complex. *6*, Translocation occurs at contact sites. *7*, Stromal translocation motors, ctHsp70, drive import. *8*-9, The transit peptide is cleaved and degraded, while the mature protein is folded.


Precursor
1A
2
Contact site
1B
3
4
5
Cytoplasm
+ NTP?
+ GTP?
OM
+ ATP
6
IMS
+ ATP
IM
9
7
8
Stroma
Toc159
Toc75
Tic55
IAP70
Tic22
?
Hsp70
MGDG
Toc34
Tic110
PIRAC
CSS1
Tic21
14-3-3
phospho serine
PG

bind the transit peptide and deliver the precursor to the translocation apparatus in the membrane (Steps 1 and 2). Interaction of precursors with membrane lipids may place the precursor in the appropriate conformation or location to then interact with protein components (i.e. precursor receptor Toc159) of the membrane (Step 3). Translocation component nomenclature arises from its location (Toc = Translocator of the outer envelope membrane of chloroplasts, Tic = Translocator of the inner envelope membrane of chloroplasts) and its apparent molecular weight (Schnell et al. 1997). Interaction with cytosolic factors may direct the precursor to protein receptors (Steps 2-3), which then complex with additional translocation components in an energy-dependent manner to generate an active translocation complex (Steps 4 and 5). Translocation occurs through the outer and inner membranes at contact sites between the two (Step 6) in an energy-dependent manner. Once the precursor has traversed both membranes, the transit peptide is cleaved by a stromal processing protease and degraded (Steps 8 and 9). Concomitantly or subsequently, the mature protein can fold to become active or the intermediate can be further targeted to suborganellar locations (Step 8). Different aspects of protein translocation into chloroplasts will be explored in greater detail.

Transit Peptides

Almost all nuclear-encoded proteins destined for the chloroplast are synthesized as higher molecular weight precursors with an N-terminal extension called a transit peptide. The transit peptide is both necessary and sufficient to direct import of proteins into the chloroplast (Anderson and Smith 1986; Mishkind et al. 1985; Van den Broeck et al. 1985), although the mature domain does contribute to import efficiency (Comai et al.

1988; Dabney-Smith et al. 1999; Ko and Ko 1992; Nawrath et al. 1994). As stated earlier, the nuclear *Arabidopsis* genome sequence predicts ~2100 chloroplast proteins (Initiative 2000) by the presence of putative transit peptides. Chloroplast proteins were predicted by the presence of transit peptides using the neural network TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2000). TargetP has an accuracy of prediction of ~85% for chloroplast transit peptides based on using known precursors and their transit peptides (Emanuelsson et al. 2000).

Even though a neural network has been trained to predict transit peptides with some accuracy, sequence similarity among transit peptides is not the basis for prediction. Very little sequence similarity exists between known, sequenced transit peptides; but transit peptides can be divided into three functional domains. Transit peptides have an uncharged N-terminal domain of about 10 amino acids which may be an Hsp70 binding site (Ivey and Bruce 2000; Ivey et al. 2000; Rial et al. 2000), a central domain of variable length lacking acidic residues but containing basic and hydroxylated amino acids, and a C-terminal domain of about 10 amino acid residues predicted to form an amphiphilic β strand which may be a site for membrane interaction (Pinnaduwage and Bruce 1996).

In addition to lack of sequence similarity, transit peptide structure has been somewhat elusive until recently. Circular dichroism (CD) and nuclear magnetic resonance (NMR) studies of transit peptides in an aqueous environment, suggest that they have no secondary structure but rather exist in a random coil conformation (Horniak et al. 1993; Pilon et al. 1992b; Wienk et al. 1999). In addition, earlier attempts to predict secondary structure of transit peptides led von Heijne and Nishikawa (1991) to suggest that transit peptides had evolved specifically to maximize random coil formation to

promote interaction with the translocation machinery. More recent evidence, however, (Horniak et al. 1993; Krimm et al. 1999; Lancelin et al. 1994; Pilon et al. 1992b; Wienk et al. 1999) suggests that transit peptides may have little or no secondary structure in an aqueous environment, but adopt secondary structure (such as alpha helix) when placed in hydrophobic environments such as aqueous trifluoroethanol (TFE; Krimm et al. 1999; Lancelin et al. 1994) or detergent/lipid micelles (Endo et al. 1992; Horniak et al. 1993; Wienk et al. 1999).

The gain of secondary structure provides an attractive hypothesis to explain why transit peptides (or mitochondrial presequences) can be so diverse in sequence yet still be able to successfully engage the chloroplast outer envelope to be translocated. In the absence of secondary structure the transit peptide may be a substrate for a cytosolic factor or molecular chaperone, but when the chaperone recruits the transit peptide to the membrane, the transit peptide can adopt secondary structure in the hydrophobic environment making it a more suitable substrate for the membrane receptors. A similar mechanism is utilized by peptides involved in host defense against bacterial pathogens, which often have no apparent structure in an aqueous environment, yet adopt secondary structure (either amphipathic α-helical or β-strand) structure when introduced to the hydrophobic membrane environment (Oren and Shai 1998; Shai 1999). These 'antimicrobial peptides' vary in size and type but are usually small peptides of less than 60 amino acids, and exert their action by permeabilizing the target membrane (Sitaram and Nagaraj 1999).

Several types of antimicrobial peptides exist based on their secondary structure in hydrophobic environment, from linear amphipathic α-helices to β-sheet to forming

cysteine knots (Epand and Vogel 1999). In addition antimicrobial peptides can be antibacterial or lytic to eukaryotic cells (hemolytic) or both (Epand and Vogel 1999). Mellitin, from bee venom, is a 26 amino acid peptide which exhibits mostly (amphipathic) helical structure with a bend predicted from residues 11-15 (Dufourcq et al. 1986; Sitaram and Nagaraj 1999). This helix-bend-helix conformation is reminiscent of the NMR secondary structures of transit peptides of the precursor to ferredoxin (prFd) from *Silene pratensis* (Wienk et al. 1999) and the predicted secondary structure (helicalwheel prediction by DNAStar) of the transit peptide of the precursor to the small subunit of Rubisco (prSSU) from *Pisum sativum* (Bruce 2000). In addition, the transit peptides of the precursors of Rubisco activase and Fd from *Chlamydomonas reinhardtii* have also been analyzed by NMR to show formation of an α-helix at the C terminus and N terminus, respectively, in a hydrophobic environment (Lancelin et al. 1994; Lancelin et al. 1996). The algal α-helices are amphiphilic, and entire transit peptide from algal sources tends to be much shorter than transit peptides from higher plants (Franzen et al. 1990; Kindle 1998). Krimm, et al., (1999) predict that the presence of these coil-helix or helix-coil structures could be repeated in order to provide a level of distinction for higher plant transit peptides thus resulting in longer length.

The similarities between antimicrobial peptides and transit peptides are not limited to secondary structure formation in hydrophobic environments. Antimicrobial peptides tend to be positively charged (from the presence of arginine and lysine residues) with an absence of acidic residues such as aspartate and glutamate (Dathe and Wieprecht 1999; Epand and Vogel 1999; Sitaram and Nagaraj 1999). Transit peptides are also distinguished by possession of a net positive charge with a lack of acidic residues,

especially in the central domain (von Heijne and Nishikawa 1991; von Heijne et al. 1989). Some antimicrobial peptides also have an elevated hydroxylated amino acid residue content (Dathe and Wieprecht 1999; Epand and Vogel 1999; Sitaram and Nagaraj 1999), whereas transit peptides also have a higher than typical hydroxylated amino acid content (von Heijne et al. 1991; von Heijne and Nishikawa 1991; von Heijne et al. 1989). In addition, plants also possess their own form of lytic peptides, defensins or thionins (Broekaert et al. 1995; Epand and Vogel 1999). Plant defensins are some of the largest antimicrobial peptides at 50 amino acids, and they are also cationic. They are distinguished by the presence of cysteines with a propensity to form disulfide bonds between molecules. Structural analysis of plant defensins indicates that they possess mostly β-sheet structure with an α-helical structural motif at one end (Epand and Vogel 1999). For comparison, early prediction of transit peptide structure proposed an amphiphilic β-strand at the C terminus (Archer and Keegstra 1990; von Heijne et al. 1989). In addition, Reumann, et al., postulate that transit peptides arose evolutionarily from virulence factors (antimicrobial and/or hemolytic peptides) secreted by the ancestral cyanobacterial cell based on the correlation of current activity of the translocation pore protein Toc75 with the activity of its ancestral homologs (Reumann et al. 1999).

Based on these pieces of evidence, it is enticing to hypothesize that, after endosymbiosis, when a gene was duplicated to the nucleus, it acquired targeting information that is derived from defense peptides whose function is to permeabilize membranes. One possibility is that transit peptides evolved to be more membrane active than lytic to preserve the new symbiosis that was evolving. This would account for the ability of transit peptides to interact with membranes for targeting and ultimately

translocation, which will be discussed in detail later, while not destroying the membrane in the process. One way this could be achieved is by increasing the distance between membrane active helices by varying the central domain. The central domain of transit peptides appears to be the most variable and diverse part when comparing sequences from different organisms (Archer and Keegstra 1990; von Heijne et al. 1991; von Heijne and Nishikawa 1991; von Heijne et al. 1989). Membrane activity of transit peptides may serve as an initial mechanism of targeting promoting subsequent interaction of the precursor with the translocation apparatus of the membrane. Another way of tempering the membrane activity of the new transit peptide is to shorten the amphipathic and/or helical regions. While this may not remove the lytic properties of the transit peptide, it may change the MIC (minimal inhibitory concentration) to a concentration so high as to be above physiological. The transit peptide would still be membrane active, but it would probably never accumulate in cells to be lytic as well. The possible role of membrane activity of transit peptides will be explored in more detail in Chapter 3.

Cytosolic Factors

As discussed earlier, for translocation of proteins into the lumen of the ER or the matrix of the mitochondrion to occur, the precursor must first encounter the organelle. For targeting proteins to the ER, the encounter with the organelle is mediated by SRP which binds to the signal sequence and helps direct the nascent peptide and ribosome to the membrane (Zheng and Nicchitta 1999). For precursors destined for the mitochondria two chaperones, hsp70 and the 14-3-3 protein mitochondrial import stimulating factor (MSF), play an important role in directing the encounter with the mitochondrial outer membrane

translocation apparatus (Hachiya et al. 1993; Komiya et al. 1994; Komiya et al. 1996; Mihara and Omura 1996b).

Until recently (May and Soll 2000), chloroplast import was thought to be unique from other protein translocation systems because it lacked, or did not require, participation by cytosolic factors (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1990; Pilon et al. 1992a; Subramanian et al. 2001). In studies where overexpressed precursors and isolated chloroplasts are incubated together, one can detect import of the overexpressed precursor and processing to the mature form (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1990; Pilon et al. 1992a; Subramanian et al. 2001). Import of these overexpressed precursors was measured kinetically (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1990) and the translocation rates measured are thought to be similar to rates that would be required in a cell of a plant for normal maintenance of the chloroplast (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1992c). These *in vitro* import rates lend credibility to the use of isolated organelles for the study of import processes into the chloroplast as well as demonstrating there is no obligate need for cytosolic factors during targeting.

While cytosolic factors or chaperones may not be obligate participants in precursor targeting to the chloroplast, there is mounting evidence that they are present (Fig. 1-6; May and Soll 2000; Waegemann et al. 1990). One group (May and Soll 2000; Waegemann et al. 1990) has reported involvement of cytosolic components that they have identified as a guidance complex containing at least an Hsp70 and a 14-3-3 protein (May and Soll 2000). Hsp70's are known to bind to newly synthesized protein to help them to fold or to keep them unfolded to help maintain them in an import or targeting

competent form (Endo et al. 1996; Lain et al. 1994; Terada et al. 1995). 14-3-3 proteins, on the other hand, bind to a recognition site containing a phosphorylated serine residue and serve to act as linker proteins in cellular processes such as signal transduction (Aitken et al. 1992; May and Soll 2000; Muslin et al. 1996). As discussed earlier (Fig. 1-4), an Hsp70 plays a role in delivering mitochondrial precursors to the Tom20/22 preprotein receptor of the outer mitochondrial membrane (Komiya et al. 1997). Additionally, as discussed previously, MSF is a 14-3-3 protein, which preferentially binds phospho-serine in the presequence of mitochondrial precursors delivering the precursors to the Tom70 preprotein receptor also in the outer mitochondrial membrane (Komiya et al. 1997; Omura 1998).

Even though cytosolic Hsp70 and MSF have distinct roles during targeting of precursors to mitochondria, they are not obligate as was discussed earlier (Hajek and Bedwell 1994; Murakami et al. 1988). Mitochondrial precursors are imported in the absence of cytosolic factors, but the presence of the cytosolic factors does prolong import, presumably by prolonging import competence of the precursors (Hajek and Bedwell 1994; Murakami et al. 1988). The chaperones probably maintain the precursor in an unfolded or loosely folded state, because chemically denatured proteins are imported in the absence of the chaperones whereas reversibly folded precursors are impeded in import until unfolded again (Rassow et al. 1989; Verner and Lemire 1989).

Translocation apparatus

Considerable progress has been made in identifying components of the translocation apparatus in chloroplast envelope membranes mostly by chemical crosslinking and mild-

detergent membrane solubilization. Using these techniques, researchers have been able to identify components of the outer membrane, inner membrane, and molecular chaperones involved. In addition, homologs for all components that have been identified so far are found in *Arabidopsis* (Jackson-Constan and Keegstra 2001).

For translocation of precursors to occur, the earliest events involve encountering the outer membrane of the chloroplast (Fig. 1-6, Steps 1-3). Translocation occurs by a three-step process: binding, early import intermediate, and import, which can be distinguished by NTP requirements. Initial interaction of precursors to the chloroplast translocation apparatus can occur in the presence of low levels (Olsen and Keegstra 1992) or absence (Ma et al. 1996; Perry and Keegstra 1994) of NTP. Interaction in the absence of NTP, considered binding, displays weak affinity of precursor for components in the membrane and can only be detected when trapped through the use of chemical crosslinking (Ma et al. 1996) and may reflect precursor interaction with the membrane lipids (Fig. 1-6, Steps 1A and 1B). Presence of low levels of ATP (<100 μM) in the intermembrane space drive formation of a more stable interaction of precursor with membrane components (Fig. 1-6, Step 3), called an early translocation intermediate (Olsen and Keegstra 1992). Through the use of chemical crosslinking, the precursor interacts with outer membrane components, which were named Toc159, Toc75, and Toc34 (Akita et al. 1997; Kouranov and Schnell 1997; Ma et al. 1996; Nielsen et al. 1997). Toc159 was originally identified as an 86 kD protein anchored in the outer membrane (Kessler et al. 1994; Ma et al. 1996), but has since been demonstrated to be a larger 159 kD transmembrane protein (Bolter et al. 1998; Chen and Schnell 1999).

Toc159 and Toc34 form the primary contacts with the precursor in early stages of import (Akita et al. 1997; Kouranov and Schnell 1997; Perry and Keegstra 1994; Sveshnikova et al. 2000). Both Toc159 and Toc34 contain structurally related cytosolic GTP-binding domains defining a distinct family of GTPases (Hirsch et al. 1994; Kessler et al. 1994; Seedorf et al. 1995). Toc159 was originally thought to be the precursor receptor and Toc34 was postulated to play a role in regulating the transition from energy-independent binding to later energy-dependent import steps through a cycle of GTP binding and hydrolysis, but was not originally described as a part of the preprotein receptor complex (Hirsch et al. 1994; Kouranov and Schnell 1997; Ma et al. 1996).

Recently, however, new data suggest that Toc34 may also play a role as a precursor receptor *in vitro* in a GTP-dependent manner (Sveshnikova et al. 2000). Sveshnikova, et al. (2000) were able to demonstrate using immunoprecipitation assays that soluble, cytosolic domain of Toc34 preferentially interacts with phosphorylated transit peptide in a specific manner. They suggest that transit peptide binding only occurs when Toc34 has GTP bound and GTP release or GTP/GDP exchange occurs to release the preprotein towards the translocation channel (Sveshnikova et al. 2000). Toc34 binds prSSU but loses affinity for prSSU as portions of the mature domain are removed (E. Schleiff, personal communication). One interesting hypothesis to explain the presence of two precursor receptors in the membrane is that for maximal import efficiency to occur, Toc159 and Toc34 come together at the translocation channel, Toc75, to promote import. Another is that Toc159 and Toc34 may each serve to direct different subsets of precursors to the translocation channel. More evidence supports the former hypothesis,

particularly the ability of a single precursor to crosslink to both proteins (Akita et al. 1997; Kouranov and Schnell 1997; Perry and Keegstra 1994).

Once the precursor interacts with Toc159 or Toc34 (or both), it is transferred to Toc75 (Fig. 1-6, Steps 5 and 6) which serves as the translocation channel of the outer membrane (Hinnah et al. 1997; Kouranov and Schnell 1997; Ma et al. 1996). Strong evidence that Toc75 was the protein conducting channel came from crosslinking to Toc75 of early import intermediates, which are inserted across the membrane, (Kouranov and Schnell 1997; Ma et al. 1996). Additional even more compelling evidence arose when Hinnah, et al., (Hinnah et al. 1997) reconstituted Toc75 into proteoliposomes and demonstrated voltage-sensitive ion conductance. This conductance was selectively regulated by the presence of a chloroplast precursor providing strong evidence for transit peptide-mediated channel activity (Hinnah et al. 1997). A caveat to Toc75 being the only component of the translocation channel is that the conductance measured is indicative of a fairly small pore diameter of 8-9 Å. Protein conducting channels in other systems such as the ER (40-60 Å) and mitochondria (~22 Å) are much larger (Hamman et al. 1997; Hill et al. 1998; Johnson and van Waes 1999). A pore as small as Toc75 is proposed to form would require almost complete unfolding of precursors to traverse the membrane. Furthermore, Toc159 also crosslinks (in regions other than the cytosolic face) with early import intermediates, suggesting that it may also play a role in forming the pore (Kouranov and Schnell 1997). Wu et al, (1994) identified another outer membrane component, Toc36, which also crosslinks to an early import intermediate arrested during import (Ko et al. 1995; Wu et al. 1994). Other evidence challenging the pore size of 8-9

Å include import of folded precursors (America et al. 1994; Clark and Theg 1997; Ottado and Ceccarelli 1998). Clearly, more work needs to be done to resolve this issue.

Recently, another outer membrane component was identified as part of the translocation apparatus. Toc64 is an integral membrane protein characterized by the presence of tetratricopeptide repeats (TPR) on its cytosolic surface (Sohrt and Soll 2000). Remember that TPR's are stretches of amino acids which form helices and are involved in protein:protein interactions (Haucke et al. 1995a). Several mitochondrial import receptors have TPR segments, which act as docking sites for cytosolic factors directing precursors to the mitochondrial import apparatus (Haucke et al. 1995a; Lamb et al. 1995). Toc64, therefore, may function as a docking protein for the guidance complex directing precursors to the import apparatus.

Precursor translocation is thought to occur through both the outer and inner membranes at once (Fig. 1-6, Step 7) (Pain et al. 1988; Pain et al. 1990; Schnell and Blobel 1993). Three inner membrane components have been identified, Tic110, Tic20, and Tic22, which associate with the outer membrane complex to form a functional supercomplex. Presence of a separate Tic complex has not been detected suggesting that they only complex when the outer membrane components are present further supporting the 'contact site' theory of import into chloroplasts (Kouranov et al. 1998). Tic110 was the first identified inner membrane component. While it is an integral membrane protein, the bulk of it protrudes into the stroma and has been postulated to be an anchor site for stromal chaperones involved in translocation and folding of importing precursors (Jackson et al. 1998; Kessler and Blobel 1996). A precise function for Tic110 is unclear, but Kessler and Blobel (Kessler and Blobel 1996) demonstrated that its hydrophilic

stromal domain interacts with molecular chaperones, particularly cpn60. Other studies, however, do not indicate an interaction with cpn60, but rather, suggest an interaction with ClpC, an hsp100 molecular chaperone (Akita et al. 1997; Nielsen et al. 1997). Clearly more still needs to be done to learn the complete function of Tic110.

The two other inner membrane components, which associate with outer membrane components are Tic22 and Tic20. Tic22 is a peripheral membrane protein associated with the outer surface of the inner envelope membrane. It has no known homologs, but it does crosslink to precursors as they are imported into the chloroplast (Kouranov et al. 1998; Kouranov and Schnell 1997). Kouranov, et al., (1998) postulate that Tic22 may play a role in recognizing the precursor as it enters the intermembrane space and directing it to the inner membrane import machinery, as well as providing the link between the two envelope membranes to promote formation of the contact sites.

Tic20 is an integral membrane protein, which is predicted to span the inner membrane with three helical transmembrane domains. Kouranov, et al., (Kouranov et al. 1998) postulate that, based on its predicted topology, it could be part of the translocation pore of the inner membrane. Indirect support for this hypothesis is based on the observation that crosslinking of precursors to Tic20 increases at later stages of protein import when the translocating peptide chain has inserted across the inner envelope membrane (Kouranov et al. 1998).

Additional membrane components, which have been identified include Tic55 and Tic40. Tic55 is unique in that it contains a Reiske-type Fe-S center typically found in proteins involved in redox reactions (Caliebe et al. 1997). Tic55 was found associated with translocation components by blue native gel electrophoresis and

immunoprecipitation, as well as cofractionation by affinity chromatography. A role for redox potential in import is unclear at this point, however.

Tic40 from pea is a homolog of a protein described earlier from *Brassica napus* as either Cim/Com44 or Toc36 (Ko et al. 1995; Pang et al. 1997; Wu et al. 1994). Tic40 can be covalently crosslinked to Tic110, indicating its close physical proximity to an established translocon component (Stahl et al. 1999). Previously, Tic40 had been localized to both the outer and inner membranes, but the work of Stahl, et al. (1999) provides strong evidence that it is only localized to the inner membrane. According to Stahl, et al., (1999) Tic40 shows some sequence similarities to Hsp70 interacting proteins (Hip's), which serve to link Hsp70 and Hsp40 and regulate Hsp70 ATPase activity (Hohfeld et al. 1995). Tic40 might, therefore, have a role in recruiting molecular chaperones to the inner envelope, which would promote the movement of precursor proteins across the membrane or assist them in folding.

Involvement of Lipids

Signal sequences for targeting to the ER, mitochondria and chloroplast all exhibit membrane activity. The h-region of signal peptides can bury themselves into liposomes and monolayers consisting of zwitterionic and anionic lipids. The presequence of mitochondria can interact with anionic lipids, especially cardiolipin, which is also non-bilayer forming. Chloroplast transit peptides also have an affinity for anionic or non-bilayer lipids, especially the polar lipid monogalactosyl diacylglycerol (MGDG). These findings do not seem surprising when one considers the endosymbiotic theory of mitochondria and chloroplasts. Prokaryotic membranes contain both anionic and non-

bilayer forming lipids such as phosphatidylglycerol, phosphatidylethanolamine, cardiolipin and MGDG (Dame and Shapiro 1979; Khomutov et al. 1990; Mozharov et al. 1985; Shibuya et al. 1985).

Lipids comprise approximately 80% of the chloroplast outer envelope membrane the remaining components being proteins (Block et al. 1983; Poincelot 1973). Not only is the outer envelope membrane protein depleted, but it also contains unique lipids such as galactolipids and sulfolipid unlike any other organelle (Allen et al. 1964; Bishop et al. 1971; Poincelot 1973). Galactolipids tend to be highly unsaturated and are only found in the chloroplast (Joyard et al. 1991). Historically, the lipids in this membrane have been thought to be barriers to the inside. Evidence is mounting, however, that targeting sequences can interact with and disrupt synthetic bilayers and liposomes, which mimic the chloroplast outer envelope membrane, *in vitro* (Chupin et al. 1994; Horniak et al. 1993; Pinnaduwage and Bruce 1996; van't Hof and de Kruijff 1995; van't Hof et al. 1993).

The work of de Kruijff, using fragments of the transit peptide of Rubisco (van't Hof et al. 1991) or the transit peptide or precursor to ferredoxin (Chupin et al. 1994; Pilon et al. 1995; van't Hof and de Kruijff 1995; van't Hof et al. 1993), has shown that precursors can perturb lipid monolayers or disrupt liposomes when they contain anionic lipids, such as phosphatidylglycerol (PG) or sulfolipid (SL), or galactolipid, particularly monogalactosyl diacylglycerol (MGDG). Pinnaduwage and Bruce (1996) demonstrated that the precursor and transit peptide to the small subunit of Rubisco was also membrane active and able to induce dye release from liposomes with dye entrapped. On the other hand, however, synthetic peptides of the transit peptide (stromal targeting domain only)

of plastocyanin did not show high affinity for liposomes (Endo et al. 1992). A possible explanation for this result, however, is that the thylakoid targeting domain, which was not present in the peptides used, works in tandem with the stromal targeting domain to provide membrane activity.

What purpose might the presence of anionic lipids or MGDG serve? Recalling that membrane active peptides tend to be helical and amphipathic and tend to have a net positive charge or are hydrophobic, one can envision that electrostatic interactions or hydrophobic interactions with anionic lipids may help stabilize the targeting domain in a conformation that can be recognized by translocation component receptors.

In addition, some lipids where found to form non-bilayer or inverted micelle structures when purified from their native environment. Examples include MGDG, phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA) (Dowhan 1997; Lee 1977; Verkleij et al. 1984). Because the ratio of the volume of the head group to the volume of the acyl chain groups, v_h/v_{ac} , is so small, these lipids do not pack in a lamellar array, but rather the headgroups pack together with the tails protruding producing an inverted micelle (Bruce 1998; de Kruijff 1997). Non-bilayer lipids in membranes are thought to add curvature to the membranes and may also provide localized regions of membrane instability (Gruner 1985). In addition to being non-bilayer forming, MGDG is also polar, which could provide a point of noncovalent interaction with polar regions of proteins (Fig. 1-6, Step 1). Currently, no evidence exists demonstrating the physical interaction between transit peptides and non-bilayer lipids, but one might envision a scenario as depicted (Fig. 1-6, Step 1). Whatever the reason, mitochondrial and chloroplastic signal sequences have limited ability to interact with

vesicles containing only neutral, nonpolar lipids such as phosphatidylcholine (Golding and O'Shea 1995; Hoch 1992; Leenhouts et al. 1996; Rietveld et al. 1986; Roise et al. 1988; Van Venetie and Verkleij 1982; van't Hof and de Kruijff 1995; van't Hof et al. 1991; van't Hof et al. 1993). Transit peptides do have net positive charges as well as containing hydroxylated amino acids, which would explain why they are more interactive with anionic and polar lipids.

SUMMARY

Transport of proteins across cellular membranes presents unique challenges to cells. Work in this lab is focusing on the early events in import into chloroplasts, which lead to actual translocation of a precursor across the membrane. The subsequent chapters of this work lay some groundwork for how precursors may find themselves in the proper situation to engage the translocation apparatus of chloroplasts by looking at mature domain involvement and transit peptide interaction with membrane lipids.

Chapter 2-Materials and methods for subsequent chapters

MATERIALS AND METHODS

Plant Growth and Chloroplast Isolation

Dwarf pea seedlings (*P. sativum,* Laxton Progress #9) were grown and chloroplasts isolated at 14 days after germination as described in Bruce *et al.* (Bruce et al. 1994). Briefly, leaves and stems were harvested and pulse-chopped in a Cuisinart processor for very short bursts (1-2 s each) until a chopped parsley consistency was achieved. The chopped tissue was then moved to an electric homogenizer and homogenized in the presence of ~300-500 ml of Grinding Buffer (GB; 330 mM sorbitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 4 mM EDTA, 0.2% BSA, 50 mM HEPES-KOH, pH 7.3) in 2-3 s bursts 3-4 times. The resulting brie was filtered through 4 layers cheesecloth and two layers MiraCloth. The filtrate was centrifuged at 2000 xg and the supernatant discarded. The pellet was resuspended in 15-20 ml in 1X Import Buffer (IB; 330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0) and gently placed over Percoll (Pharmacia) continuous gradients. Gradients were prepared by mixing equal parts 2X IB and Percoll and centrifuging for 30 min at 20,000 xg. The loaded gradients were centrifuged at 5800 xg for 14 min. The lowest band was harvested as intact plastids using a large-bore needle. The fractions were pooled (if totaling less than 15 ml) or washed with IB 3X fraction volume separately to remove the Percoll. The plastids were then pelleted by centrifugation at 2000 xg for 6 min. The supernatant was discarded, and the pellet was gently resuspended in ~3 ml 1X IB.

Chlorophyll Measurement

Chlorophyll measurements were made by dissolving 5 µl purified plastid in 5 ml 80% acetone by vortexing for 20s. The acetone extract was centrifuged at ~18,000 xg on a bench-top mircocentrifuge for 2 min to pellet starch granules. Photometric readings were made using the supernatant at 645 nm and 663 nm of light and the chlorophyll content calculated using the equation

$$Chlorophyll\ (mg/ml) = \frac{[8.65 \times A_{663} + 20.2 \times A_{645}]}{0.05 \times 1000} \times 5\ ml$$

Suspensions were further diluted in 1X IB to give 1 mg/ml chlorophyll.

Generation of C-terminal Deletions

The cDNA coding for the full-length precursor of the small subunit of Rubisco was isolated from pET11-prSSU (Klein and Salvucci 1992) using *Bam*HI and *Nco*I, and subcloned into the same sites of pET23d (Novagen). To linearize pET23-prSSU in preparation for Exonuclease III digestion, XhoI was used. α-phosphorothioate NTP's were used with Klenow fragment of DNA polymerase to fill in the overhangs generated by XhoI digestion. To generate single 5' overhang, HindIII was used to digest the 3' of the linearized plasmid. DNA was ethanol precipitated after each digestion. Exonuclease III digestion was performed using the Erase-a-Base Kit (Promega). Briefly, Exonuclease III was added to linear DNA with a single 5' overhang and allowed to digest for timed periods (30s). The reaction was stopped by removing aliquots to buffer containing S1 nuclease. Samples were then ethanol precipitated and the DNA was resuspended in TE

buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was treated with Klenow again to prepare for ligation. Ligation went for 1 hr at room temperature. DNA samples were then transformed into competent *E. coli.* To determine the extent of the deletions, direct colony PCR was performed using forward, T_7 promoter, and reverse, T_7 terminator, primers (T_7 universal primers, Novagen). Plasmid DNA from select colonies was isolated and sequenced using an ABI automated sequencer. Three deletion mutants, prSSΔ52 (Δ52), prSSΔ67 (Δ67), and prSSΔ74 (Δ74) were generated that lacked 52, 67, and 74 amino acids, respectively, at the C-terminus. The Δ74 deletion contained an additional 6 amino acids (ATVVRM) at the C-terminus due to read through into vector sequences. All other regions of the precursor were unchanged.

Preparation of Precursor Proteins, Mature Domain and the Transit Peptide

Plasmids containing prSSU, mSSU, and prSSU deletions were expressed and the proteins isolated from the BL21[DE3] strain of *E. coli* as described in Pinnaduwage and Bruce (Pinnaduwage and Bruce 1996). Briefly, the bacteria were grown at 37°C with shaking (250 rpm) under selection at 200 μg/ml ampicillin until OD_{600} of 0.6 was achieved. The cells were then induced with 1 mM IPTG (isopropyl-β–D-thiogalactopyranoside) for 2.5 hrs. The cells were then pelleted at 5000Xg and the supernatant was poured off. The cells were resuspended in ~30 ml of Buffer A (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.6) and frozen at –80°C for at least 2 hr and thawed. Cells were subjected to at least 2 freeze/thaw cycles. Cells were then sonicated with a probe sonicator for 5 min in 30s – 1 min pulses with at least 30s between. The samples were centrifuged at 37,000 xg for 15 min. The supernatants were poured off and saved. The pellets were resuspended in Buffer

A + 1% Triton X-100 and processed with sonication and centrifugation as before. This was repeated at least 2 additional times, saving the supernatants. The pellets were then washed at least 3 times with Buffer A to remove detergent. The final inclusion body pellet was then solubilized in 8 M urea + 50 mM DTT. The transit peptide of prSSU (SS-tp) was expressed and purified from the BL21 strain of *E. coli* as described in Pinnaduwage and Bruce (Pinnaduwage and Bruce 1996) or as described in Subramanian and Bruce (2001).

In vivo Radiolabeling of prSSU and Deletions

The BL21[DE3] strain of *E. coli* was used for generating radiolabeled prSSU and prSSU deletion proteins. The cells were grown for 8 h in Dulbeccio's Modified EAGLES Medium (DMEM, BioWhittaker) without cysteine and methionine, followed by incubation with Tran^{35}S-Label (5-14 mCu) metabolic labeling reagent (ICN) for at least 4 h. The inclusion bodies were isolated using BugBuster™ Protein Extraction Reagent (Novagen) and solubilized in 8 M urea + 50 mM DTT similar to the process above. Specific activity was 1-5 x 10^6 dpm/μg protein.

In Vitro Protein Import Assays

Kinetic import assays were performed by incubating increasing concentrations of ^{35}S-labeled prSSU with freshly prepared chloroplasts (50 μg chlorophyll) in the presence of 1X IB containing 5 mM Mg-ATP, 10 mM DTT. Urea concentration was kept constant at 300 mM and was shown not to affect import (data not shown). Addition of ^{35}S-prSSU started the assay. After 10 min at RT, the assay was terminated by rapid dilution in ice-

cold 1X IB (5-fold) and immediately placing the samples on ice in the dark. Intact plastids were reisolated according to Bruce *et al.* (Bruce et al. 1994) and were prepared for SDS-PAGE. Briefly, the entire assay was layered on 40% Percoll cushions and centrifuged at 1000 xg for 6 min to pellet intact plastids. The supernatant was aspirated and the pellets were resuspended in 1 ml of 1X IB and recentrifuged at 1000 xg for 6 min. The supernatant was aspirated and the pellets were resuspended in 2X SSB.

In Vitro Protein Import Competition Assays

Competition import assays were performed by incubating 100 nM ^{35}S-prSSU and increasing concentrations of cold competitor proteins with freshly prepared chloroplasts as described above. Competitions proceeded for 15 min at RT. To start the assay, ^{35}S-prSSU and the competitor protein were added in rapid succession (<3 s between additions). The assay was terminated by rapid dilution in ice-cold 1X IB (5-fold) and placed on ice in the dark. Intact plastids were reisolated according to Bruce *et al.* (Bruce et al. 1994).

Time Course of Import and Degradation

Time course import assays of ^{35}S precursors were performed using 300 nM of each precursor as described above. Assays were performed at RT in the dark. Addition of the ^{35}S-precursor started the assay and 100 µl samples were removed at timed intervals, diluted 10-fold with ice-cold 1X IB, and placed on ice in the dark. Intact chloroplasts were reisolated from each sample as described by Bruce *et al.* (Bruce et al. 1994). These samples represent the re-purified chloroplast sample. Also at each time interval, 50 µl of

the import reaction was removed and immediately mixed with 50 µl 2X SB and boiled for 3 min. These samples represent the total import reaction.

Polyacrylamide Gel Electrophoresis for Import Assays

Equal protein amounts of the import assay samples were electrophoresed through 10-20% gradient polyacrylamide gels until the dye front reached the extreme bottom edge. Gels were then fixed in 10% acetic acid, 30% methanol for at least 30 min. Gels were then dried onto Whatman filter paper at 60°C for at least 2 h. Dried gels were subjected to radiolabel detection by filmless autradiography (Instant*Imager*, Packard Instruments).

Data Analysis

Data analysis was performed by electronic, filmless autoradiography (Instant*Imager*, Packard Instruments), and quantification of signal by Instant*Imager* analysis software (Packard Instruments). Signals were reported as cpm's and converted to molecules mSSU/chloroplast/min using the counting efficiency of the instrument, the specific activity of the precursor, the cysteines and methionines per molecule, the number of chloroplasts per ml import reaction, and the length of import time. The counting efficiency of the Instant*Imager* was calculated relative to measurements made using a liquid scintillation counter using the formula

$$Efficiency = \frac{net\ CPM\ \ prSSU\ \ I.I.}{net\ DPM\ \ prSSU\ \ L.S.C.}$$

using a known amount of protein. The DPM of the liquid scintillation counter (L.S.C.) was calculated according to methods preprogrammed into the machine. The counting

efficiency of the Instant*Imager* was determined to be 0.1%. Once the counting efficiency of the Instant*Imager* had been established, that could be used to determine DPM of a sample which was then converted to DPM/μg precursor/min. The data were analyzed using GraphPad Prism™ (GraphPad Software, Inc.) computer software for enzyme kinetics and the K_m and V_{max} determined using non-linear regression. These calculations were performed unless otherwise noted.

Converting CPMs (from Instant Imager) to DPMs

$$DPM = \frac{CPM\ prSSU}{Efficiency}$$

Calculated Specific Activity each time a new prSSU prep was made (from Scintillation Counter data)

$$SA\ (DPM/\mu g) = \left[\frac{DPM/\mu l}{\mu g/\mu l\ protein}\right]$$

$$DPM/\mu l = \left(\frac{total\ DPM}{\mu L}\right) * \%\ prSSU/lane$$

To calculate molecules mSSU/DPM

$$\left[\left(\frac{DPM}{\mu g}\right)\left(\frac{20300\ g}{mole}\right)\left(\frac{1\times10^{6}\ \mu g}{1\ g}\right)\left(\frac{1\ mole}{6.022\times10^{23}\ molecules}\right)\right]^{-1} = \left(\frac{DPM}{molecule}\right)^{-1}$$

$$\left(\frac{molecules\ mSSU}{DPM}\right) = \left(\frac{molecules}{DPM}\right)\frac{9sites/prSSU}{5sites/mSSU}$$

To calculate DPM/μg protein loaded on gel/min of assay time

$$\frac{DPM/\mu g}{\min} = \left(\frac{DPM\ in\ lane}{\mu g\ loaded}\right)\left(\frac{1}{time\ (\min)}\right)$$

To calculate Chloroplasts(cts)/ml

Volume of grid square on hemocytometer = $0.2\ mm \times 0.2\ mm \times 0.1\ mm$

$$= (0.004\ mm^3) * \left(\frac{1\ ml}{1000\ mm^3}\right)$$

$$= 0.000004\ ml$$

$$\frac{cts}{ml} = \left(\frac{Avg\ count/grid}{0.000004\ ml}\right) * d.f.$$ d.f.=dilution factor

To calculate mg cts protein/ml (to convert cts/ml to cts/μg protein and ultimately to calculate cts/lane on gel) calculate μg chloroplast protein/ml by using a BCA (Pierce) of chloroplasts diluted 1:10 and 1:5 and average.

To calculate number of chloroplasts per μg protein:

$$\frac{chloroplasts}{\mu g\ protein} = \left(\frac{cts}{ml}\right)\left(\frac{1}{mg\ cts\ prot/ml}\right)\left(\frac{1mg}{1000\mu g}\right)$$

To calculate the number of chloroplasts loaded per lane on gel:

$$\frac{cts}{lane} = \left(\frac{cts}{\mu g\ prot}\right) * \mu g\ loaded$$

To calculate number of molecules mSSU per assay per min:

$$\frac{molecules\ mSSU/assay}{\min} = \left[DPM * \left(\frac{molecules\ mSSU}{DPM}\right)\right] \div time$$

To calculate molecules mSSU per chloroplast per minute:

$$\frac{molecules\ mSSU/cts}{\min} = \left(\frac{molecules\ mSSU/assay(lane)}{\min}\right) \div \left(\frac{cts}{lane}\right)$$

Dixon Analysis

For Dixon analysis, competition import reactions were performed as described above with either 60 nM or 180 nM ^{35}S-precursor plus increasing concentrations of cold competitor. Rates were calculated in molecules mSSU/chloroplast/min as described above and the inverse plotted as a function of inhibitor concentration. Determination of K_i values was performed by linear regression analysis of the plot of 1/V vs. [I] at the two fixed prSSU concentrations where the intersection of the lines is given as $-K_i$.

Electrophysiological Measurements

Standard patch clamp technique was used to measure current across the chloroplast envelope as previously described by our collaborators (Dabney-Smith et al. 1999; van den Wijngaard et al. 1999). Electrodes were made from borosilicate glass by a two-step pull and extensively fire-polished. Electrodes were filled with buffer containing 2.5 mM TES/KOH, pH 7.2, 250 mM KCl, and 2 mM $CaSO_4$, leading to a 10-fold KCl gradient. Electrode resistances were typically around 30 MΩ. To clarify the inactivation of PIRAC during protein import, proteins were added to the pipette filling solution (i.e. cytoplasmic equivalent) to 0.5 mM for >5 min before electrophysiological experiments were started.

Currents were measured with an Axopatch 200B patch clamp amplifier (Axon Instruments). The data were filtered at a cut-off frequency of 1 kHz, using an 8-pole Bessel filter (internal filter of the Axopatch 200B). The filtered data were digitized at 10 kHz using a CED 1401 + and analyzed with the Patch and Voltage Clamp software (Cambridge Electronic Design). Potentials were given with regard to the pipette interior, the bath was kept at ground, using a 250 mM KCl agar bridge. To determine the

distributions of open and closed time durations of PIRAC, a module was developed in the matrix-calculating software Matlab (Mathworks, Inc.). This module uses the 50% threshold method to identify transitions of the channel between the open and the closed state. The distributions were fitted with multiexponential probability density functions using the maximum likelihood method as previously described (van den Wijngaard et al. 1999).

Protein measurements

Purified proteins were quantified by commercial Bradford reagent (Bio-Rad). Quantification of total chloroplast protein was performed using the BCA Protein Quantification Assay (Pierce Chemicals).

Liposome preparation

Lipid vesicles were prepared by mixing appropriate amounts (Table1) of lipids (5 mM total) in chloroform. The solvent was evaporated at ~45°C under a stream of nitrogen gas for 30 min and the samples were vacuum dessicated overnight. The dried lipid film was rehydrated in 0.1X phosphate-buffered saline (PBS, 0.0017 M K_2HPO_4, 0.05M KH_2PO_4, 0.015M NaCl, pH 8.0) containing 1 mM EGTA, 0.02% azide and 200 mM calcein. Small unilamellar vesicles were prepared by vortexing the lipid mixture and then sonicating using a bath sonicator for 5-10 min. At least two additional sonication events were performed with a 2-4 h interval. Free unincorporated calcein was removed by chromotagraphy on a Bio-Gel A0.05m column equilibrated with the PBS/EGTA/azide

buffer. Liposomes eluted in the void volume fractions showed a calcein fluorescence quenching of >70-80%.

Fluorescence Quenching Measurements

Fluorescence of liposomes was measured in a solid glass 96-well plate (Konte Glass) using a Wallac Victor^2 workstation (PerkinElmer Life Sciences, Gaithersberg, MD) outfitted with narrow bandpass filters for excitation at 490 ± 10 nm and emission at 514.5

$$\%\ Quench = \left(1 - \frac{F_o}{F_t}\right) \times 100\%$$

± 10 nm. Fluorescence quenching was calculated from the formula

where F_o and F_t are the fluorescence of the liposome sample before and after addition of 0.1% Triton X-100, respectively.

Protein-induced Release of Calcein from Liposomes

Liposomes containing entrapped calcein were incubated at room temperature for 1 h at room temperature with various concentrations of different proteins as indicated in the respective figure legends. The fluorescence measurements were performed as described above. The percentage of calcein release was calculated using the formula

$$\%\ \mathrm{Re}lease = \frac{F - F_o}{F_t - F_o} \times 100\%$$

where F_o and F are the fluorescence before and after addition of the protein, respectively, and F_t is the total fluorescence after addition of 0.1% Triton X-100. All measurements were done in triplicate and data was analyzed using GraphPad Prism software.

Coomassie Brilliant Blue Staining and Western Blot Analysis of Proteins

To detect proteins by Coomassie Brilliant Blue staining, proteins were separated by SDS-PAGE (10-20% acrylamide) and stained in a CBBstaining solution (10% acetic acid, 50% methanol, 0.25% Coomassie Brilliant Blue R250) for 20 min and destained in a solution of 50% methanol, 10% acetic acid for 45 min. For western blot analysis, proteins (2 μg) were separated by SDS-PAGE and transferred electrophoretically to PVDF membrane. The membrane was blocked with TBST + 3% non-fat dry milk (25 mM Tris-HCl pH 7.6, 3 mM KCl, 137 mM NaCl, 0.1% Tween 20) for at least 1 h. The primary antibody was added at a titer of 1:1000 and incubated for 1 h. After washing again with TBST + 3% non-fat dry milk, the blot was incubated with the secondary antibody, donkey anti-rabbit IgG-AP (1:10,000) for 30 min. The membrane was then washed with TBS (25 mM Tris-HCl pH 7.6, 3 mM KCl, 137 mM NaCl) for 30 min, changing buffer twice. The membrane was then rinsed with alkaline phosphatase (AP) buffer for 10 min. New AP buffer was added and AP substrates BCIP and NT were added to a final dilution of 1:200. Incubation occurred for ~20 min or until bands developed. The membrane was finally rinsed with deionized, distilled H_2O and dried.

Preparation of prSSU-GFP

Standard molecular cloning procedures (Sambrook et al., 1989) were employed. Bluescript-derived vector, pAVA393, containing the cDNA for green fluorescent protein (GFP) was a gift from A. von Arnim (von Arnim et al. 1998). This vector also contains a dual 35S promoter from cauliflower mosaic virus (CaMV), the translational leader sequence from tobacco etch virus (TEV), and the 35S polyadenylation signal from CaMV. To prepare the vector for subcloning, it was linearized by digestion with NcoI and dephosporylated using calf intestinal alkaline phosphatase. The vector was then purified by agarose gel electrophoresis using the GeneClean III kit (Qbiogene, Carlsbad, CA).

prSSU insert was prepared by PCR amplification from pET11-prSSU using forward primer 5'-GGAGATATCCATGGCTTCCTCAGTTC-3' and reverse primer 5'-GGAACTATATCCATGGGTAGCCTTCTG-3', which served to insert NcoI sites at the prSSU start codon and replace the stop codon with an NcoI site. The PCR fragment was digested with NcoI and purified by agarose gel electrophoresis using the GeneClean III kit. Quantification of linear vector and purified fragment was by visualization against standards of known mass (BstEII digest of known mass of λ DNA).

Ligations were performed using 3:1 insert:vector molar ratio (the vector:insert mass ratio is 9:1) at 16°C for 4 h. Ligation samples were transformed into competent NovaBlue [DE3] *E. coli* cells. Colonies were screened by mini-prep and restriction digestion with NsiI and XbaI, which allowed detection of insert orientation. NsiI was specific for prSSU and XbaI was specific for GFP. Positives were selected for sequencing and sequenced using an ABI automated sequencer. Two sets of sequencing primers were

used. One set allowed sequencing the junction of the N-terminal region of prSSU and the translational leader in the vector. The forward primer used was 5'-CAAAACAAACGAATCTCAAGCAATCAAGC-3' and the reverse primer was 5'-GCCACACCTGCATTGCACTCTTCC-3'. The other primer set allowed sequencing of the prSSU-GFP junction. The forward primer used was 5'-CCCAAGTGTTGGCTGAGGTGGAAGAGGC-3' and the reverse primer was 5'-CCCTTAAGCTCGATCCTGTTGACGAGGG-3'. DNA samples, which were positive for both primer sets, were used to prepare more DNA in anticipation of biolistics and concentrated to a final concentration of 1mg/ml.

Transient Expression in Onion Cells

Transient expression assays in onion epidermal cells using particle bombardment (Bio-Rad, Richmond, CA) were carried out as described by von Arnim and Deng (von Arnim and Deng 1994). Briefly, tungsten particles (Aldrich) were prepared by mixing 50 mg tungsten powder/ ml 95% ethanol, EtOH, (20 ml total) and sonicating with a probe sonicator for 5 min in 1 min pulses with 30 s rest in between. The sonication cycle was repeated twice and the tungsten was washed with 95% EtOH, resuspended, dispensed into 1 ml aliquots, and stored at –20°C.

To prepare two biolistic shots of GFP vectors bound to the tungsten beads, 1 mg of tungsten is washed extensively (3X) in deionized, distilled H_2O (ddi H_2O) and resuspended in the original volume with H_2O. 4.8 μg DNA was added to the beads along with $CaCl_2$ and spermidine to give 1 M $CaCl_2$ and 15 mM spermidine (free base). DNA was incubated with the beads for 20 min in ice with occasional vortexing and tapping to

resuspend the tungsten particles. After incubation, 95% EtOH was added in excess (200 µl) and the tungsten was pelleted in a tabletop microfuge. Then the pellet was washed three times in 95% EtOH and finally resuspended in 15 µl 95% EtOH. Biolistics was performed on either onion epidermal cells or *Arabidopsis* leaves on MS salts plates using either the empty GFP vector or the prSSU-GFP vector. Biolistics was performed using a PDS1000/He system (BioRad, Richmond, CA) with 1100 p.s.i. rupture disk. After bombardment, the plates were incubated in the dark, overnight, at room temperature.

Fluorescence and Confocal Laser-Scanning Microscopy

Samples for fluorescence microscopy were wet-mounted on microscope slides and examined on a Zeiss Axiovert 135 inverted microscope with a 100-W mercury arc lamp with a filter cube (Chroma FITC-cube CZ917) with an additional block of infra-red emission (Chroma; 485±10-nm excitation; 505-nm beam splitter; 535±20-nm band-pass emission). Images were acquired through a 10× objective with a 12-bit MicroMax cooled CCD camera (Princeton Instruments, Trenton, NJ) operated by IPLab software (Signal Analytics, Vienna, VA) on a Macintosh 7100 PowerPC. Images were processed for printing via Microsoft PhotoEditor software and printed on a Lexmark 3200 inkjet printer. For laser-scanning confocal microscopy (LSCM), samples were prepared as for fluorescence microscopy and examined on a Leica confocal microscope.

Chapter 3 The C-terminus of a chloroplast precursor modulates its interaction with the translocation apparatus and PIRAC.

ABSTRACT

The import of proteins into chloroplasts involves a cleavable, N-terminal targeting sequence known as the transit peptide. Although the transit peptide is both necessary and sufficient to direct precursor import into chloroplasts, the mature domain of some precursors has been shown to modulate targeting and translocation efficiency. To test the influence of the mature domain of the small subunit of Rubisco during import *in vitro*, the precursor (prSSU), the mature domain (mSSU), the transit peptide (SS-tp) and three C-terminal deletion mutants (Δ52, Δ67, and Δ74) of prSSU were expressed and purified from *E. coli*. Activity was then evaluated by competitive import of ^{35}S-prSSU. Both IC_{50} and K_i values consistently suggest that removal of C-terminal prSSU sequences inhibits its interaction with the translocation apparatus. Non-competitive import studies demonstrated that prSSU and Δ52 were properly processed and accumulated within the chloroplast, whereas Δ67 and Δ74 were rapidly degraded via a plastid-localized protease. The ability of prSSU-derived proteins to induce inactivation of the Protein-Import-Related Anion Channel (PIRAC) was also evaluated. Although the C-terminal deletion mutants were less effective at inducing channel closure upon import, they did not effect

INTRODUCTION

Many chloroplast proteins are nuclear-encoded and produced in the cytosol of the plant cell. Nuclear-encoded chloroplast proteins are predominantly synthesized as larger molecular weight precursors containing an N-terminal transit peptide. The transit peptide (tp) is a cleavable N-terminal signal sequence that mediates precursor binding and import into the chloroplast via an ATP-dependent mechanism. Following transport of the precursor through the chloroplast envelope, the tp is cleaved by the stroma processing peptidase to generate the mature protein. Transport of precursors across the chloroplast envelope has been shown to involve distinct protein complexes within the outer membrane (Toc: translocation apparatus of the outer membrane of chloroplasts) and inner membrane (Tic: translocation apparatus of the inner membrane of chloroplasts) (Hirsch et al. 1994; Perry and Keegstra 1994; Schnell et al. 1997; Schnell et al. 1994). These two complexes are believed to interact at inter-membrane contact sites, which enables precursor translocation in a single coordinated step (Bruce and Keegstra 1994; Chen and Schnell 1999).

Recently, an inner membrane localised anion channel, PIRAC, has been shown to be involved in chloroplast protein import. PIRAC (Protein Import Related Anion Channel) is a 50 pS anion channel that becomes inactivated during chloroplast protein transport via a mechanism that requires both a functional transit peptide and a stromal ATP source (van den Wijngaard and Vredenberg 1997). The exact role of PIRAC in

protein import into the chloroplast is not defined as yet, however, at least three different mechanisms may explain channel inactivation: (i) PIRAC may function as the protein import channel in the inner membrane of chloroplasts and inactivation represents a shift from ion conductance to protein conductance; (ii) PIRAC may be intimately associated with translocation components of the inner membrane such that a precursor-induced conformational change results in inactivation of the ion channel; or (iii) the formation of contact sites between Toc and Tic during translocation causes transient inactivation of the ion channel. Further investigation is needed to elucidate the precise role of PIRAC in protein import into the chloroplast.

Current models suggest that transit peptides are both necessary and sufficient to direct the targeting and import of precursor proteins into the chloroplast. However, direct experimental evidence is not entirely consistent with these models. For example, the attachment of transit peptides to unrelated precursors confers proper targeting to some, but not all proteins (Kavanagh et al. 1988; Ko and Ko 1992; Lubben et al. 1989; Wasmann et al. 1986). In addition, no common motif has been identified among the several hundred transit peptides that have been sequenced (von Heijne et al. 1991). To explain this apparent enigma, it has been postulated that a common secondary or tertiary structure involving the mature domain might provide both targeting specificity and translocation efficiency (Bruce and Keegstra 1994). Consistent with this notion, chimeras constructed between the transit peptide and various portions of the mature domain have been shown to improve import efficiency (Kavanagh et al. 1988; Wasmann et al. 1986). Also, deletion of C-terminal sequences from various precursors has been shown to interfere with one or more steps of the translocation pathway, depending on the precursor

studied and the extent of the C-terminal deletion (Ko and Ko 1992). These studies collectively suggest that contributions from the mature domain play an important role in facilitating the interaction of transit peptides with the translocation apparatus. However, a more systematic and quantitative study is needed to identify mature domain sequences that directly influence chloroplast import.

In the current work, we have evaluated the import activity of the precursor to the small subunit of Rubisco (prSSU), the mature domain (mSSU), the full-length transit peptide, (SS-tp), and three prSSU mutants that lack 52, 67, or 74 C-terminal amino acids, respectively. Using these proteins as inhibitors of the import of radiolabeled precursor, we demonstrated that the transit peptide is absolutely required for interaction with the translocation apparatus. However, a specific region within the mature domain strongly influences this interaction. In addition, we demonstrated that sequences within the C-terminus of prSSU also influence the interaction between the transit peptide and PIRAC. The findings indicate that the C-terminus of prSSU plays an important role in protein translocation.

RESULTS

Generation and Purification of C-terminal deletions

To determine the effect of prSSU C-terminal sequences in chloroplast protein import, serial C-terminal deletions were generated and characterized. Out of the several hundred clones originally generated, only a small subset were characterized further, based on deletion length and ease of purification from inclusion bodies when expressed in *E. coli* (data not shown). The location of these deletions and the size of the resulting protien are

shown in Figure 3-1. Variants of prSSU that lack 52, 67, and 74 amino acids at the C-terminus were denoted Δ52, Δ67, and Δ74. We were unable to detect a deletion that yielded an insoluble protein shorter in length than the Δ74 deletion, suggesting that sequences within this region are directly involved in inclusion body formation. The purification of prSSU, mSSU, GST-tp and SS-tp was reported previously (Pinnaduwage and Bruce 1996). The Δ52, Δ67, and Δ74 deletions were isolated by a similar method and analyzed for purity by SDS-PAGE (Figure 3-2, left panel). The apparent molecular weights of Δ52, Δ67, and Δ74 are 18.9 kDa, 16.7 kDa, and 9.8 kDa, respectively, which differ from the calculated molecular weights of 15.3 kD, 12.5 kD, and 11.6 kD for the deletions respectively. We have no plausible explanation for these differences.

To confirm that the deletions were prSSU derived, immunoblot analysis was performed using antibodies to the native Rubisco small subunit purified from pea, α-SSU (Figure 3-2, right panel). Two deletions, Δ52 and Δ67, were recognized by α-SSU, similar to prSSU and mSSU. Interestingly, Δ74 was not recognized by the antibody, but had been confirmed by DNA sequencing to be correct. The deletions were derivatives of prSSU and were used for subsequent experiments without further purification.

In vitro import kinetics

To define the kinetic parameters of prSSU import *in vitro,* the rate of import (processing to mSSU) as a function of prSSU concentration was determined. The amount of ^{35}S-prSSU substrate is varied from 10 nM to 1.6 μM in a 10 min *in vitro* import assay (Figure

Fig. 3-1. Comparison of mSSU, prSSU, GST-tp, SS-tp, & C-terminal deletions. The position and length of the transit peptide and mature domain have been drawn to scale. The region corresponding to the full-length transit peptide is shown with a gray fill and the region corresponding to the mature domain is shown in black. The small white block between GST and SS-tp represents two amino acids (GS) introduced during sub-cloning. Glutathione-S-transferase protein is shown with the diagonal fill pattern and has been condensed for space constraints. The amino acid sequences for prSSU, mSSU, and the deletions (prSSΔ52, prSSΔ67 and prSSΔ74) were obtained from DNA sequence analysis of the cloned genes. The actual molecular weight, calculated using the Protean function in DNAStar (Lasergene, Madision) is shown in Daltons on the right.

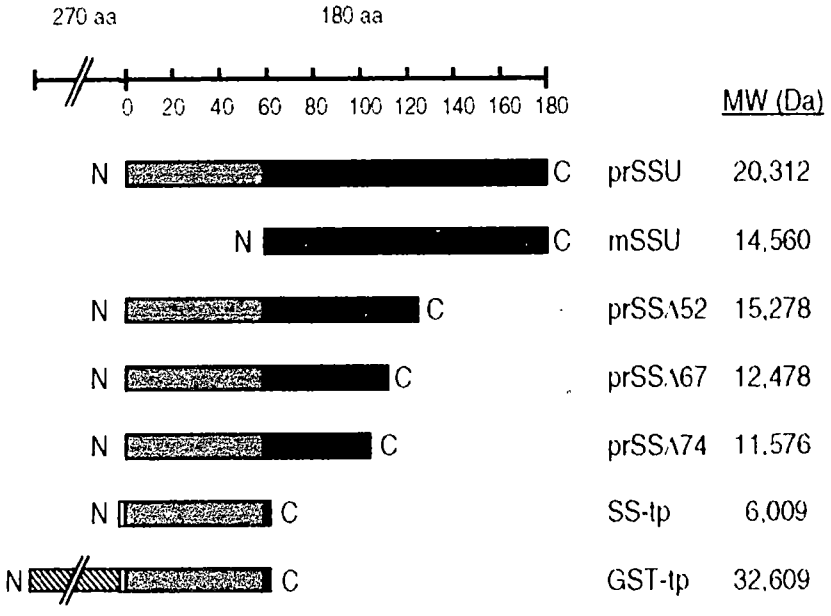
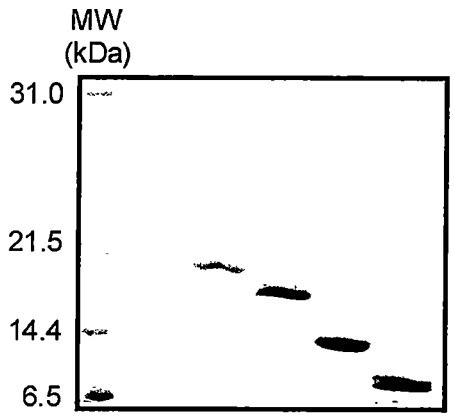
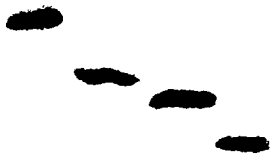

270 aa
180 aa
0 20 40 60 80 100 120 140 160 180
MW (Da)
N C prSSU 20,312
N C mSSU 14,560
N C prSSΔ52 15,278
N C prSSΔ67 12,478
N C prSSΔ74 11,576
N C SS-tp 6,009
N C GST-tp 32,609

Fig. 3-2. Purification of E. coli-expressed prSSU, mSSU, Δ52, Δ67, Δ74, GST-tp, & SS-tp. *A*, Overexpressed prSSU, mSSU, Δ52, Δ67, Δ74, were purified to near homogeneity. SDS-PAGE and CBB stain of purified precursors (5 μg total protein loaded). Purified protein loaded in each lane is shown at the top of the figure. *B*, Western blot of (A) using antibodies against the native small subunit of Rubisco from *Pisum sativum*. Arrow to the right of western blot indicates location of prSSΔ74 on Coomassie Brilliant Blue stained gel.



CBB



3-3A). This time point was within the linear range of the time course of import (data not shown). Three independent import assays were performed and the amount of mSSU product was directly quantified (Figure 3-3B). The average product formed at each substrate concentration was analyzed by non-linear regression (Figure 3-3B), which permitted the determination of K_m (282±55 nM) and V_{max} (~9887±735 molecules mSSU/cts/min) values. Although the K_m did not deviate significantly between separate chloroplast preparations, the V_{max} was found to vary between chloroplast preparations by ~2-fold.

Import competitions and IC_{50} determination for prSSU, mSSU, GST-tp, and SS-tp

To determine which sequences of prSSU are both necessary and sufficient for chloroplast import, purified prSSU, mSSU, GST-tp, and SS-tp were used as inhibitors of ^{35}S-prSSU import *in vitro*. The concentration of ^{35}S-prSSU used in the competition assays was 100 nM, which is well below the measured K_m. Figure 3-4A illustrates representative import assays for each unlabeled peptide, and direct quantification of three independent assays for each peptide is shown in Figure 3-4B. Both mSSU and GST-tp failed to inhibit import of ^{35}S-prSSU, even at the highest concentrations tested. The inability of GST-tp to compete for the import of prSSU suggests that the transit peptide requires a free N-terminus to productively engage the translocation apparatus. Since both prSSU and its full-length transit peptide effectively inhibit import (Figure 3-4A), these data clearly indicate that transit peptide is both necessary and sufficient to direct import into chloroplasts *in vitro*. However, the IC_{50} for SS-tp (570±111 nM) was ~7-fold higher than the IC_{50} for unlabeled prSSU (80±15 nM), indicating that the transit peptide

Fig. 3-3. *In vitro* import kinetics. *A,* Electronic autoradiogram of a representative import reaction as a function of increasing concentrations of ^{35}S-prSSU. The concentration of prSSU used in each import reaction is shown at the top of the gel. Import reactions were conducted as described in Materials and Methods. Chloroplast loading was normalized by BCA protein measurements. *B,* Quantitative analyses of import data using increasing concentrations of ^{35}S-prSSU. Inset shows the linear regression of the data represented as an Eadie-Scatchard Analysis. Data are presented as mean ± the standard deviation.

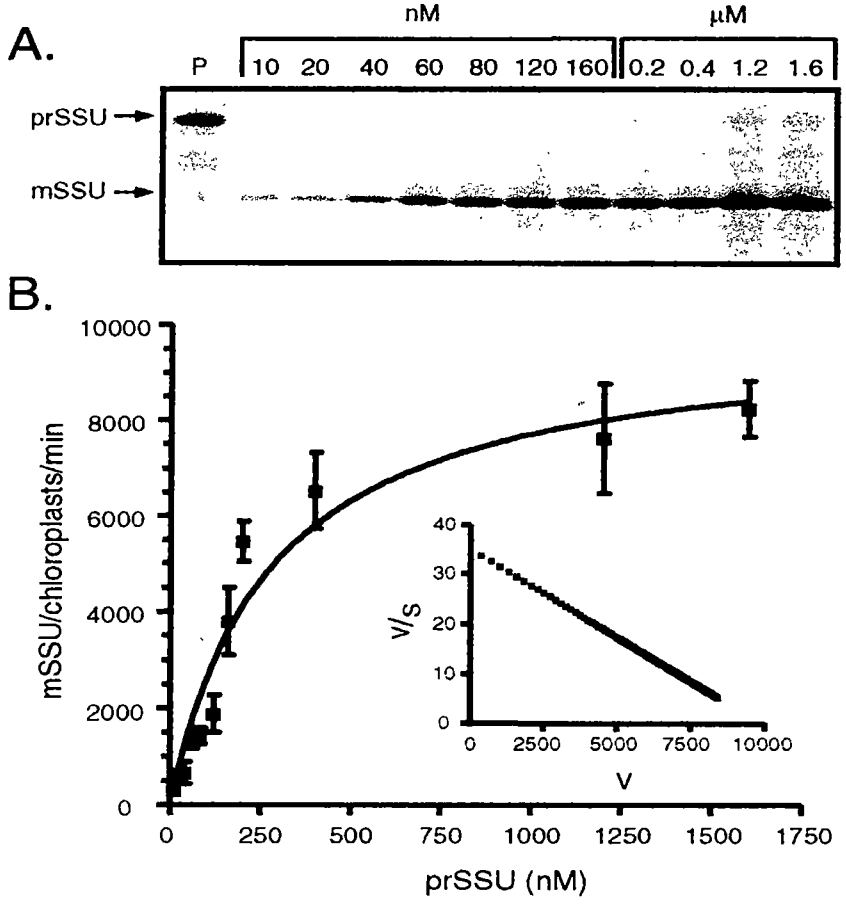
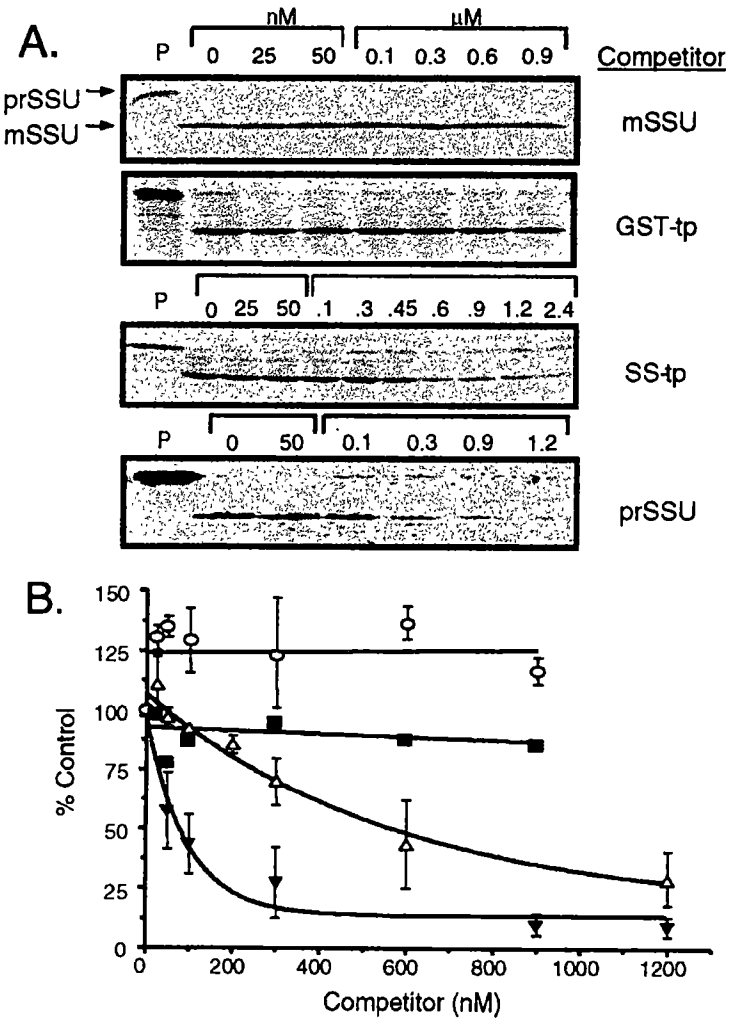

A.
nM
μM
P
10 20 40 60 80 120 160
0.2 0.4 1.2 1.6
prSSU
mSSU
B.
mSSU/chloroplasts/min
10000
8000
6000
4000
2000
0
0 250 500 750 1000 1250 1500 1750
prSSU (nM)
V/S
40
30
20
10
0
0 2500 5000 7500 10000
V

Fig. 3-4. Import competitions and IC_{50} determination for prSSU, mSSU, GST-tp, and SS-tp. *A,* SDS-PAGE analysis of import competitions between ^{35}S-prSSU and cold competitors mSSU, GST-tp, SS-tp, and prSSU. The concentration of competitor in each reaction is shown at top of the figure. The ^{35}S-precursor was 100 nM for all reactions. Reactions proceeded for 10 minutes and were halted by rapid dilution. *B,* Graphical analysis of import competitions between radiolabeled prSSU and competitors mSSU (O), GST-tp (■), SS-tp (Δ), and prSSU (▼). Samples were normalized to a no unlabeled petide. All except GST-tp represent the average of three independent experiments. The data were analyzed as described in the Materials and Methods.


A.
nM
μM
P
0 25 50
0.1 0.3 0.6 0.9
Competitor
prSSU
mSSU
mSSU
GST-tp
P
0 25 50 .1 .3 .45 .6 .9 1.2 2.4
SS-tp
P
0 50 0.1 0.3 0.9 1.2
prSSU
B.
% Control
150
125
100
75
50
25
0
0 200 400 600 800 1000 1200
Competitor (nM)

alone engages the import machinery less effectively than the full length precursor. Taken together, these data suggest that sequences in the mature domain of prSSU act *in cis* to modulate or enhance the import activity of the transit peptide.

Import competitions and IC_{50} determination of C-terminal deletions

To identify the regions within the mature domain of prSSU that facilitate import, prSSU C-terminal deletions (Δ52, Δ67, and Δ74) were used as competitive inhibitors in ^{35}S-prSSU import assays. All three deletions were tested with the same preparation of chloroplasts to minimize experimental variability. Decreasing levels of mSSU accumulation with increasing peptide concentration indicated that all three precursor mutants were able to inhibit ^{35}S-prSSU import into the chloroplast (Figure 3-5A). However, the longer precursor mutants were more effective than the shorter mutants. Quantification of three independent experiments (Figure 3-5B) resulted in IC_{50} calculations of 33±15.3, 145±26.9, and 305±40.8 nM for the Δ52, Δ67, and Δ74 mutants, respectively. Plotting these values as a function of mature domain length illustrates the relationship between precursor length and activity as a competitive inhibitor (Figure 3-6). Although removal of the last 52 amino acids from the C-terminus of prSSU did not significantly reduce the affinity of the precursor for the translocation apparatus, removal of an additional 15 amino acids increases the IC_{50} >4.5-fold and removal of an additional seven amino acids increases the IC_{50} by ~9- fold. These findings suggest that sequences between the C-termini of the Δ52 and Δ74 mutants contribute significantly to the process of chloroplast protein targeting/translocation.

Fig. 3-5. Import competitions and IC_{50} determination of C-terminal deletions. *A,* SDS-PAGE analysis of import competitions between ^{35}S-prSSU and truncated precursors. The concentration of competitor in each reaction is shown at top of the figure. The ^{35}S-precursor was 100 nM for all reactions. Reactions proceeded for 10 minutes and were halted by rapid dilution. *B,* Graphical analysis of import competitions between ^{35}S-prSSU and truncated precursors Δ52 (●), Δ67 (□), Δ74 (▼). Samples were normalized to a no competitor control. The data were analyzed as described in the Materials and Methods.

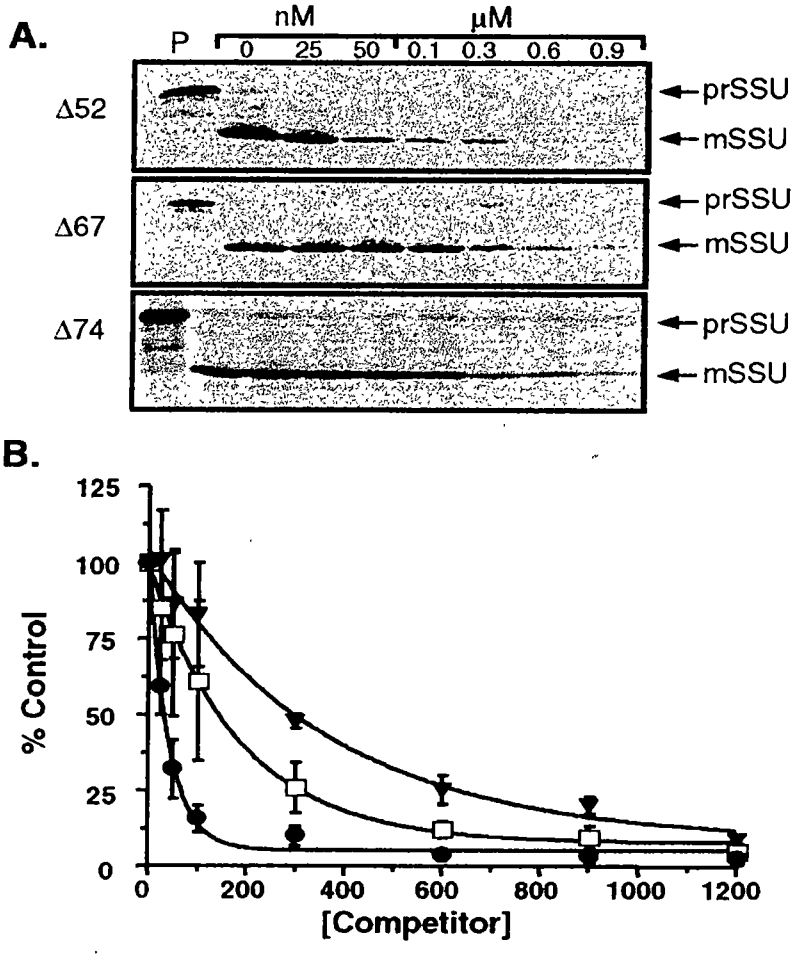
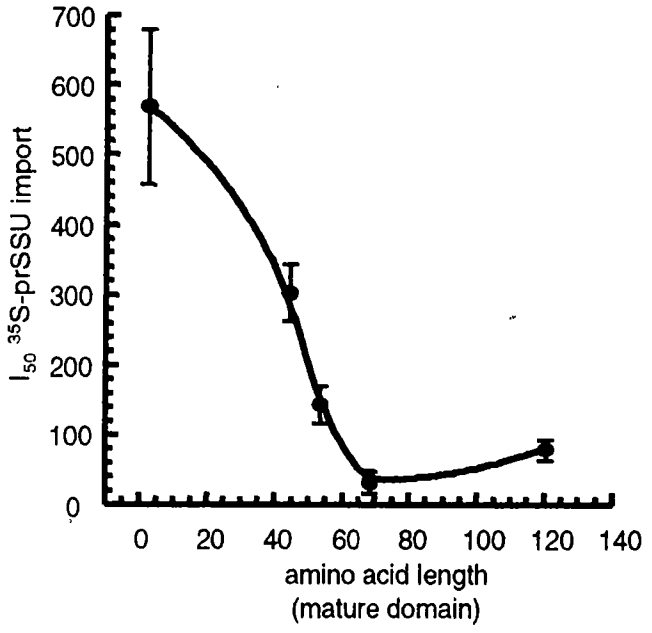

A.
nM
μM
P
0
25
50
0.1
0.3
0.6
0.9
Δ52
Δ67
Δ74
prSSU
mSSU
prSSU
mSSU
prSSU
mSSU
B.
% Control
125
100
75
50
25
0
0
200
400
600
800
1000
1200
[Competitor]

Fig. 3-6. Relationship of mature domain length and IC_{50}. Plot of IC_{50} of competitor as a function of length of the mature domain of SS-TP (2 aa in mature domain), Δ74 (46 aa in mature domain), Δ67 (53 aa in mature domain), Δ52 (68 aa in mature domain), and prSSU (120 aa in mature domain), and their respective IC_{50} values from Table 1. Graph was generated using Kaleidagraph (Abelbeck Software). Data are presented as mean ± the standard deviation.


700
600
500
400
300
200
100
0
I_{50} ^{35}S-prSSU import
0
20
40
60
80
100
120
140
amino acid length
(mature domain)

Dixon analysis of K_i values for prSSU and C-terminal deletions

To determine the inhibition constant, K_i, for prSSU and the three C-terminal deletion mutants, Dixon analysis was performed using ^{35}S-prSSU as substrate and unlabeled prSSU, Δ52, Δ67 or Δ74 as inhibitors of import. ^{35}S-prSSU was fixed at 60 and 180 nM and the inhibitor concentration was varied from 50 nM to 1.2 μM (Figure 3-7A). Data were fit by linear regression (Figure 3-7B) and the intersection point of the lines is defined as $-K_i$. The K_i values for prSSU (105 nM), Δ52 (66 nM), Δ67 (110nM) and Δ74 (437 nM) are consistent with the IC_{50} values for each competitor (Table 3-1). Successive deletion of sequences within the prSSU mature domain results in less effective import inhibition, suggesting that an element within the mature domain modulates targeting and/or translocation efficiency.

Attenuated import competition by the prSSU deletion mutants could result from disrupted binding at the chloroplast surface, impaired translocation through Toc and Tic, or some combination of these processes. To explore a binding-specific effect, binding studies were performed with prSSU and the precursor mutants at low levels of ATP (data not shown). The binding results demonstrated that removal of 52, 67, and 74 amino acids from the C-terminus did not significantly affect binding of these precursors to the chloroplast presumably via Toc components.

Import time course of prSSU and C-terminal deletions

Except for mSSU and GST-tp, all of the proteins tested have been shown to compete for the import of ^{35}S-prSSU. To directly determine whether the C-terminal deletion mutants

Fig. 3-7. Dixon analysis of K_i values for prSSU and C-terminal deletions. *A,* Representative SDS-PAGE analysis of import competitions between ^{35}S-prSSU and prSSU. The concentration of ^{35}S-precursor was fixed at either 180 nM or 60 nM while varying the concentrations of the competitor as shown at the top of the figure. Truncated precursors were also used as competitors (data not shown; see Table 1 for K_i values). Reactions proceeded for 10 minutes and were halted by rapid dilution. *B,* Graphical analysis of import competitions between radiolabeled prSSU (60 nM, O; 180 nM, ▲) and prSSU. The data were analyzed using GraphPad Prism™ (GraphPad Software, Inc.) computer software for enzyme kinetics by linear regression.

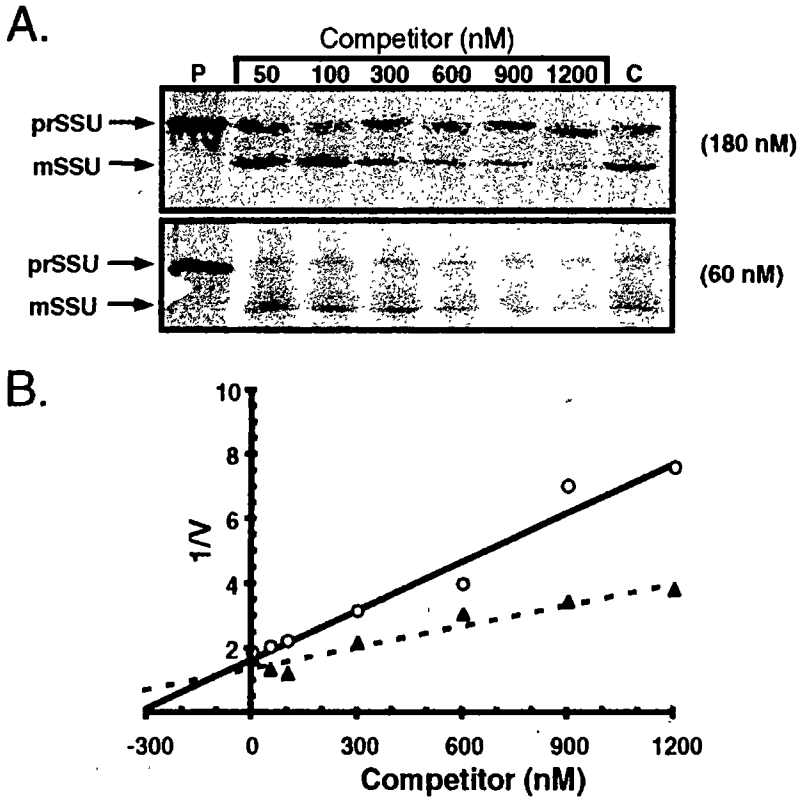

A.
Competitor (nM)
P
50
100
300
600
900
1200
C
prSSU
mSSU
(180 nM)
prSSU
mSSU
(60 nM)
B.
1/V
10
8
6
4
2
-300
0
300
600
900
1200
Competitor (nM)

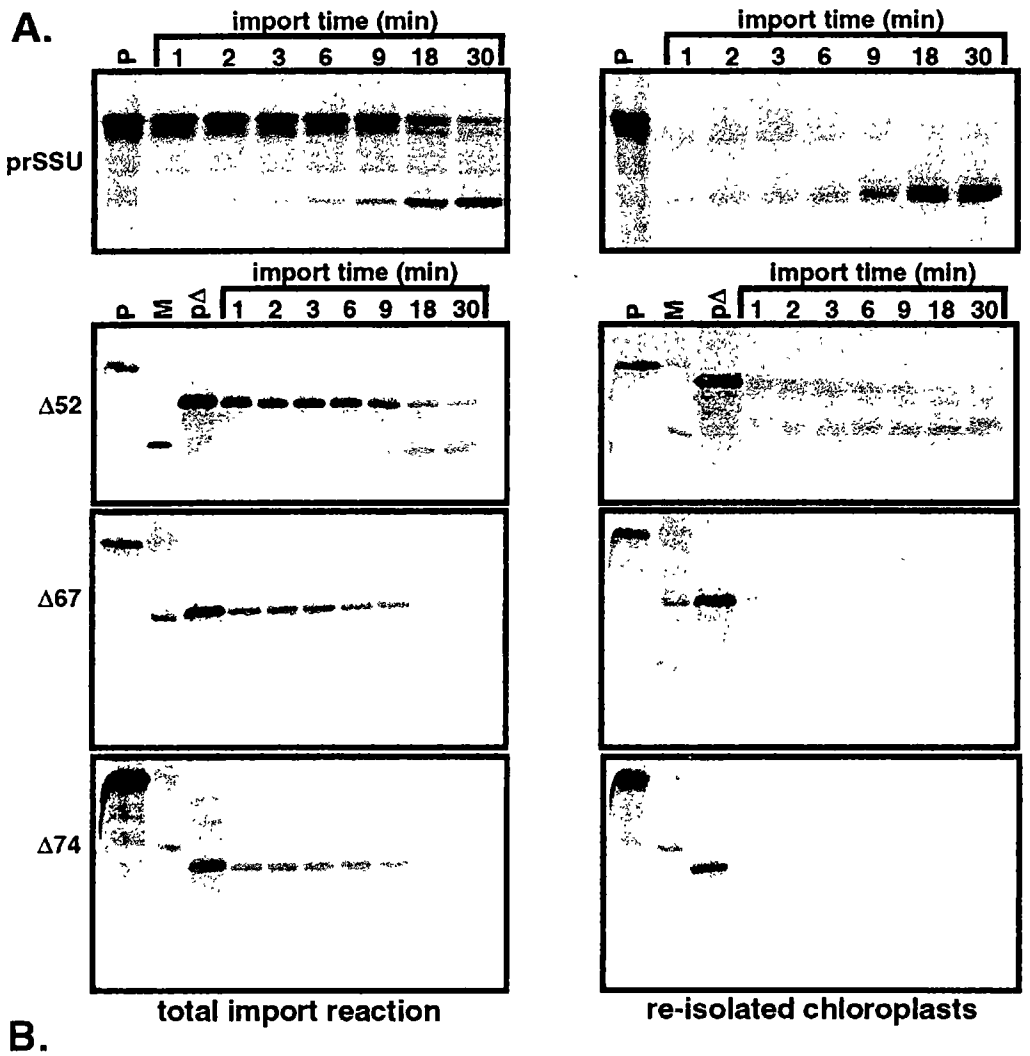
Table 3-1. **Inhibition of import is affected by regions in the mature domain.** Comparison of precursor length and import competence. IC_{50} and K_i were calculated by GraphPad Prism™ of previously described data.. IC_{50} is defined as the concentration of inhibitor required to inhibit 50% of activity. K_i is defined as the concentration of competitor required to bind half of the total binding sites on an enzyme at equilibrium in the absence of substrate.

Competitor	IC_{50} nM	K_i nM
Control	NA	NA
mSSU	no inhibition	no inhibition
prSSU	80 ± 15.4	105.1 ± 20.3
prSS Δ52	33 ± 15.3	66.2 ± 30.7
prSS Δ67	145 ± 26.9	110.0 ± 20.4
prSS Δ74	305 ± 40.8	436.5 ± 58.3
SS-tp	570 ± 111.4	ND
GST-tp	no inhibition	no inhibition

of prSSU are competent for chloroplast import *in vitro*, the truncated precursors were metabolically labeled with ^{35}S and used as substrates in import time course experiments. Monitoring the level of ^{35}S-labeled protein remaining in both the reaction mixture and within re-isolated chloroplasts permits direct quantification of the substrate remaining at each time point, as well as the amount of imported/ processed mature form. In this way, both the import rate and the extent of mSSU accumulation for each precursor may be determined and directly compared.

The four autoradiogram panels on the left of Figure 3-8A indicate the precursor pool size remaining in each import reaction as a function of import time. Quantification of these data is shown in Figure 3-8B, where the amount of precursor was normalized to the 1 min time point allowing comparison between the different precursors. All four proteins, prSSU, Δ52, Δ67, and Δ74, were imported at a similar rate, as seen in 3-8A and

Fig. 3-8. Import time course of prSSU and C-terminal deletions. *A,* SDS-PAGE analysis of time course import reactions of ^{35}S-prSSU and ^{35}S-truncated precursors (Δ52, Δ67, and Δ74). The concentration of ^{35}S-precursor was 300 nM for all reactions. Left panels: 5 μl of total import reaction from time points indicated at the top of the figure. Import reactions proceeded for the indicated time and were halted by dilution (1:2) with 1X SSB and boiling immediately. Right panels: 35 μg protein from reisolated chloroplasts from time points indicated at the top of the figure. Reactions proceeded for the indicated time and were halted by rapid dilution. *B,* (Left) Graphical analysis of import time course showing decrease of precursors: prSSU (■), prSSΔ52 (▽), prSSΔ67 (●), and prSSΔ74 (✳). (Right) Graphical analysis of import time course showing increase of accumulation of mature protein: mSSU (□), mSSΔ52 (●), mSSΔ67 (✳), and mSSΔ74 (Δ). All data were analyzed using GraphPad Prism™ (GraphPad Software, Inc.) computer software for enzyme kinetics by linear regression.

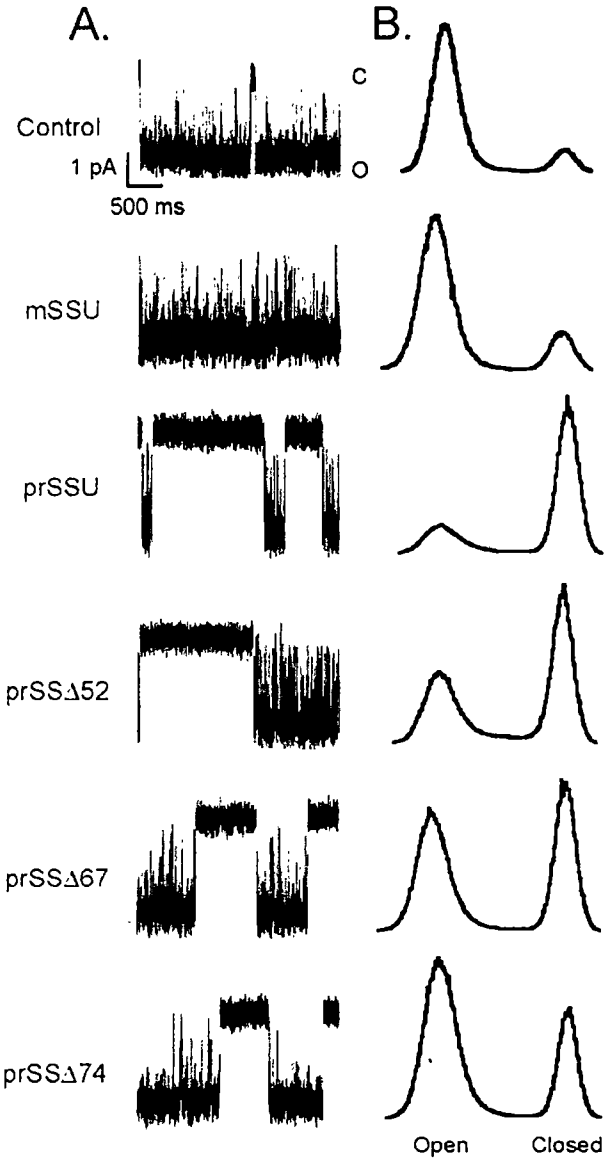

A.
import time (min)
p
1 2 3 6 9 18 30
prSSU
M
pΔ
Δ52
Δ67
Δ74
total import reaction
re-isolated chloroplasts
B.
prSSU (%control)
time (min)
mSSU (%maximum)

quantified in 3-8B. Re-isolation of the chloroplasts, however, demonstrated that only full-length prSSU and the Δ52 precursor imported and accumulated as mature, processed proteins. Although the level of Δ67 and Δ74 precursors clearly decreased as a function of import time, these mutants failed to accumulate to detectable levels as a processed form in the stroma. Taken together, these results indicate that all four precursor proteins import successfully into the chloroplast but that the Δ67 and Δ74 truncated precursors are subject to rapid degradation following import. Neither the identity nor the sub-organellar location of the protease responsible for this degradation is known.

Interaction of prSSU and C-terminal deletions with PIRAC

Thus far, we have demonstrated that sequences within the mature domain of prSSU enhance the efficiency of transit peptide-mediated import into chloroplasts. These findings implicate an interaction of the precursor with transport complexes, Toc and Tic. However, the anion channel, PIRAC, is also involved in chloroplast protein import since a functional transit peptide and full length precursor have been shown to inactivate the channel (van den Wijngaard et al. 1999). To evaluate whether precursor interaction with PIRAC is also modulated by sequences in the mature domain, single channel recordings of PIRAC conductance were measured in the presence of prSSU, mSSU, and the prSSU C-terminal deletion mutants. Figure 3-9A shows the single channel recordings of PIRAC conductance for a control excised patch and for an excised patch after protein addition. Figure 3-9B represents the all points amplitude histogram of these recordings. Addition of prSSU to the envelope patch decreased the open probability (P_O) of the channel from 0.81±.04 in the control situation to 0.17±.08, indicating that prSSU induces channel

Fig. 3-9. Interaction of prSSU and C-terminal deletions with PIRAC. *A*, Single channel recordings of the absence (control) or presence of 50 nM protein (mSSU, prSSU, prSSΔ52, prSSΔ67, or prSSΔ74) in the pipette filling solution. Recordings were made at a holding potential of –20 mV. *B*, All point amplitude histograms of single channel recordings of PIRAC in the absence and presence of the different proteins.


A.
B.
C
O
Control
1 pA
500 ms
mSSU
prSSU
prSSΔ52
prSSΔ67
prSSΔ74
Open
Closed

inactivation. In contrast, addition of mSSU induced no significant change in PIRAC activity, as indicated directly in Figure 3-9A, and in the failure of this protein to decrease the P_O measured in the all points amplitude histogram in Figure 3-9B. The deletion mutants of prSSU, however, induced PIRAC inactivation in a manner that was proportional to the length of the mature domain of the precursor. The P_O of PIRAC in the presence of Δ52, Δ67, and Δ74 was 0.45±.07, 0.53±.07, and 0.71±.07, respectively (Table 3-2). These data indicate that the transit peptide is necessary for PIRAC inactivation, as was shown previously (van den Wijngaard et al. 1999; van den Wijngaard and Vredenberg 1997), and that the removal of the C-terminus of the precursor attenuates this interaction.

Under control conditions (i.e. in the absence of any precursor) and in the presence of mSSU, three closed channel states with characteristic mean durations of 0.35±.03, 2.41±.98, and 29.1±6.8 ms and two open channel states with mean durations of 0.88±.34 and 47.2±9.6 ms have been detected for PIRAC (Table 3-2) (van den Wijngaard et al. 1999). However, in the presence of prSSU, an additional closed state (τ_{4C}) with a much longer duration was detected (van den Wijngaard et al. 1999). The duration of this new, long-lived closed state of PIRAC is 989±214 ms. All of the truncated precursors induced a similar long-lived closed state with a mean duration of ~1300±400 ms (Table 3-2). The observation that full-length prSSU and the deletion mutants decreased the P_O of PIRAC, yet did not significantly change the duration of τ_{4C}, suggests that these precursors interact with PIRAC via a common mechanism but that their affinity for the channel is proportional to the length of the mature domain. Whether the difference in affinities results from an attenuation of direct precursor interaction with PIRAC or, alternatively, is

Table 3-2. Effect of precursor addition on the P_o and duration times for PIRAC- Comparison of the probability of channel opening in the absence (control) or presence of 50 nM protein (mSSU, prSSU, prSSΔ52, prSSΔ67, and prSSΔ74). Durations of the closed (τ_C) and open (τ_O) states are also given as the mean ± the standard deviation.

	P_O	τ_{1C}	τ_{2C}	τ_{3C}	τ_{4C}	τ_{1O}	τ_{2O}
Control	0.81 ± 0.04	0.35 ± 0.03	2.41 ± 0.98	29.1 ± 6.8	Not present	0.88 ± 0.34	47.2 ± 9.6
MSSU	0.83 ± 0.03	ND	ND	ND	ND	ND	ND
PrSSU	0.17 ± 0.08	0.33 ± 0.03	0.76 ± 0.16	5.12 ± 1.03	989 ± 214	0.92 ± 0.11	16.2 ± 0.3
prSS Δ52	0.45 ± 0.07	0.34 ± 0.04	1.53 ± 0.62	6.97 ± 2.62	1298 ± 366	0.79 ± 0.51	22.5 ± 1.7
prSS Δ67	0.53 ± 0.07	0.34 ± 0.04	1.31 ± 0.49	9.13 ± 2.51	1559 ± 676	1.74 ± 1.52	27.3 ± 1.5
prSS Δ74	0.71 ± 0.07	0.29 ± 0.01	1.02 ± 0.31	7.95 ± 1.34	1362 ± 351	0.73 ± 0.22	38.2 ± 5.7

a secondary result of a diminished interaction with components of either Toc and/or Tic will require additional study.

DISCUSSION

Although a great number of different chloroplast precursors have been identified (von Heijne 1992), only a few have been characterized in detail by *in vitro* studies. The current study is the first to directly compare and quantify the ability of a full-length precursor (prSSU), mature protein (mSSU), and isolated transit peptide(SS-tp) to interact with the chloroplast translocation apparatus.

Kinetic analysis of prSSU

The precursor for the small subunit of Rubisco is one of the best studied precursors, yet kinetic analysis of its *in vitro* import has not been reported previously. Compared with the few other precursors whose import kinetic parameters have been determined experimentally (prLHCP, prFd, prOEE23, and prOEE33), the K_m of prSSU import is intermediate between the value reported for prFd (120 nM) and prOEE23 (400 nM). The V_{max} of prSSU import is also comparable to that reported for prLHCP. In contrast, the V_{max} of prSSU import is significantly less than the V_{max} reported for either prFd (~22,000 molecules/chloroplast/min) (Pilon et al. 1992) or prOEE23 (>40,000 molecules/chloroplast/min) (Cline et al. 1993). These differences may reflect genuine disparities in the rate or efficiency of import for the various precursors, or alternatively, they may represent differential responses to chemical denaturation agents used for

solubilization of the precursor. As discussed previously by Pilon *et al.* (Pilon et al. 1992), however, the rates of translocation measured *in vitro* are quite close to the rates required *in vivo* for normal maintenance of chloroplast biogenesis, which lends credibility to the use of *in vitro* import studies as a means of exploring the mechanism of chloroplast protein transport.

Effect of GST Fusion on SS-tp Activity

An interesting and significant finding from our *in vitro* import studies is that the transit peptide of prSSU failed to function as a competitive inhibitor of import when GST was fused to the N-terminus of the transit peptide. This finding suggests that addition of a 26.6 kDa folded fusion protein inhibits interaction of the N-terminus of the transit peptide with one or more components of the targeting/translocation apparatus. Although the precise mechanism of this apparent interaction is unknown, one possibility is that the committed step in binding/translocation occurs when the N-terminus of the precursor traverses both envelope membranes and gains accessibility to the stroma. Once the N-terminus of the transit peptide stretches into the stroma, binding by one or more molecular chaperones may prevent escape and provide a mechanism for unidirectional ATP-dependent translocation. Evidence from our laboratory indicates that chloroplast transit peptides function *in vitro* as substrates for hsp70 (Ivey and Bruce 2000; Ivey et al. 2000), and that > 80% of stromal-targeted precursors in the CHLPEP database contain high affinity hsp70 binding sites at their N-termini (Ivey et al. 2000; von Heijne 1992). If this interaction occurs *in vivo,* then binding by hsp70 in either the stroma (Marshall et al.

1990) or the IMS (Schnell et al. 1994) would require a free transit peptide N-terminus, and fusion to GST would predictably block import.

An alternative explanation for the lack of import activity by GST-tp is that the GST domain may interfere with interactions between the transit peptide and lipid components of the chloroplast envelope. Two independent laboratories have shown that the N-terminus of transit peptides are capable of interacting directly with chloroplast-specific lipids (Pilon et al. 1995; Pinnaduwage and Bruce 1996). The GST-tp fusion protein used in this study was shown previously to inhibit the interaction of SS-tp with liposomes whose composition mimics the outer envelope (Pinnaduwage and Bruce 1996). Regardless of the molecular mechanism our findings clearly suggest that accessibility of the N-terminus of SS-tp is required for interaction with one or more components of the chloroplast translocation apparatus. Several investigations into the role of different domains of chloroplast transit peptides have also shown that the N-terminus is required for precursor import *in vitro* (Lawrence and Kindle 1997; Pilon et al. 1995), *in vivo* (Kindle 1998; Rensink et al. 1998), and for directing the import of certain fusion proteins *in vitro* (Wasmann et al. 1986). An intriguing aspect of these reports is that N-terminus of some transit peptides is often the least conserved region of the entire presequence (Rensink et al. 1998).

Diminished activity of SS-tp vs. prSSU

Although popular models contend that the transit peptide is both necessary and sufficient to support the import of chloroplast-destined precursors (Bruce and Keegstra 1994), several reports indicate that the addition of mature domain sequences increases the

targeting/translocation activity of certain transit peptides (Kavanagh et al. 1988; Lubben et al. 1989; Wasmann et al. 1986). We also found that sequences within the mature domain of prSSU enhance import efficiency, only when fused *in cis* with the transit peptide. The minimal, requisite structural elements for engaging the translocation apparatus are contained within the transit peptide, however, not the mature domain. In direct support of this conclusion, short synthetic peptides derived from the SS-tp sequence competitively inhibit prSSU import (Perry et al. 1991), and similarly, the full-length ferredoxin transit peptide has been shown to import into chloroplasts with no associated passenger protein (van't Hof and de Kruijff 1995). The full length precursor was a 7-fold more effective inhibitor of *in vitro* chloroplast import, however, and deletion of specific mature domain sequences within the context of prSSU significantly impaired this activity, strongly implicating this mSSU sequence in a beneficial *cis* interaction with the transit peptide during chloroplast targeting/translocation. The physical/chemical basis for this interaction remains unknown, however.

Effect of C-terminal deletions

Both the IC_{50} and the K_i values calculated from our experiments indicate that removal of sequences at the C-terminus of prSSU decrease the affinity of the protein for one or more components of the translocation apparatus. This effect is not strictly a linear function of precursor length, however, as shown in Figure 3-6. Removal of the last 52 amino acids of prSSU may have enhanced import potential, yet removal of a few additional sequences progressively and significantly weakened the ability of the precursors to compete for import. In addition, the Δ52, Δ67, and Δ74 mutations did not affect precursor binding to

the chloroplast. Unlike full length prSSU and the Δ52 mutant however, the Δ67 and Δ74 precursors did not accumulate in the stroma, suggesting that the decrease in the pool amount may be due to rapid degradation. These observations contrast with the findings of a previous study of prSSU import, which indicate that removal of 26 and 47 amino acids from the C-terminus completely abolish import (Ko and Ko 1992). Even at the earliest point in import time course experiments, the authors did not detect binding or import of the mutant precursors and attributed no import to abolished binding as opposed to rapid degradation. One major difference between that report (Ko and Ko 1992) and our study is that we utilized purified proteins rather than *in vitro* translated protein. A potential explanation for the discrepancies in the findings from these two studies, therefore, is that components in the wheat germ extract may interfere with the import of the mutant precursors.

Stromal assembly vs. degradation of the truncated small subunit

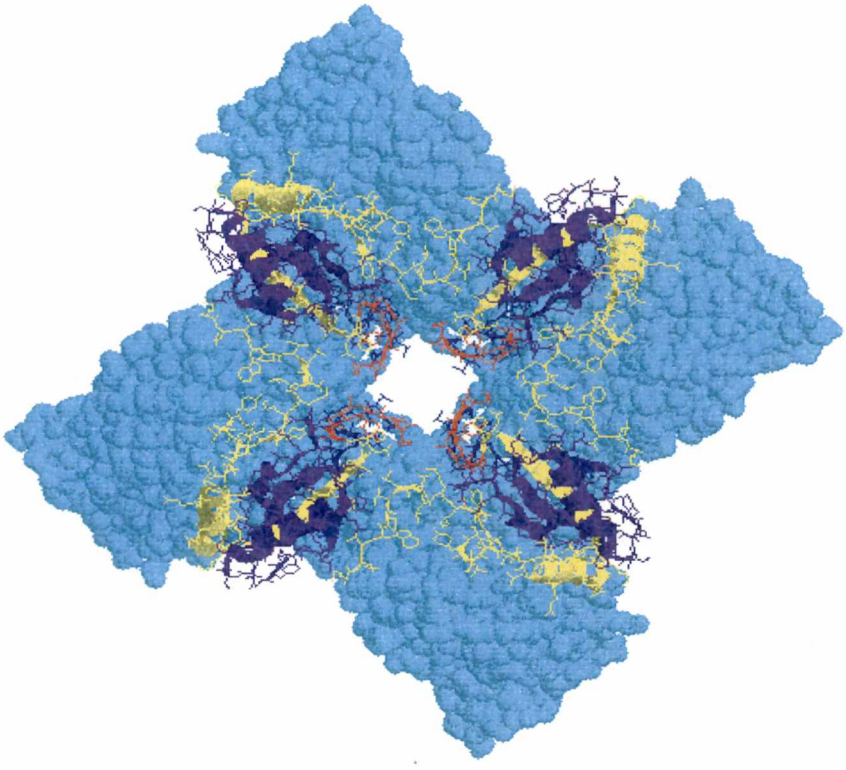
Direct import of our radiolabeled prSSU mutants into isolated chloroplasts indicated that wild-type prSSU and the Δ52 mutant clearly engaged the translocation apparatus, traversed the chloroplast envelope, and were properly processed into stable, appropriately-sized mature forms. In contrast, neither the precursor, mature form, nor discrete degradation products of the radiolabeled Δ67 and Δ74 mutants were detected. The rate at which all four precursors decreased in the total import reaction was comparable, however, suggesting that each precursor was successfully imported into the chloroplast. Degradation of the Δ67 and Δ74 precursors outside the chloroplast is highly unlikely since the organelle preparations were Percoll-purified and extensively washed.

A probable explanation is that the Δ67 and Δ74 mutants were degraded in the stroma following import. Mistargeted proteins, incorrectly translated proteins, non-functional proteins, or overproduced subunits are proteolytically degraded in the chloroplast to prevent accumulation of non-functional or potentially toxic subunits. Analysis of the Rubisco holoenzyme 3D structure (Taylor and Andersson 1997) indicates that the deletions in Δ67 and Δ74 remove significant structural elements that intimately contact adjacent large subunits (Figure 3-10). The Δ52 mutation, however, removes a portion modeled to be uninvolved in interaction with large subunit. The failure of mΔ67 and mΔ74 to assemble properly into the holoenzyme may result, therefore, in rapid degradation via a post-translocational mechanism. Supporting degradation in the stroma, Spreitzer, et al., (Spreitzer et al. 2001) demonstrated that changing a critical arginine (arg71) resulted in drastically lowered expression of Rubisco in *Chlamydamonas*. Arg71 in *Chlamydamonas* small subunit corresponds to arg65 in spinach or tobacco. The deletions used in this study had 71 (Δ52), 56 (Δ67, and 49 (Δ74) amino acids remaining as the mature domain. Therefore Δ67 and Δ74 may have removed sufficient portions of the mature domain to be no longer able to intimately contact large subunit, and they are degraded.

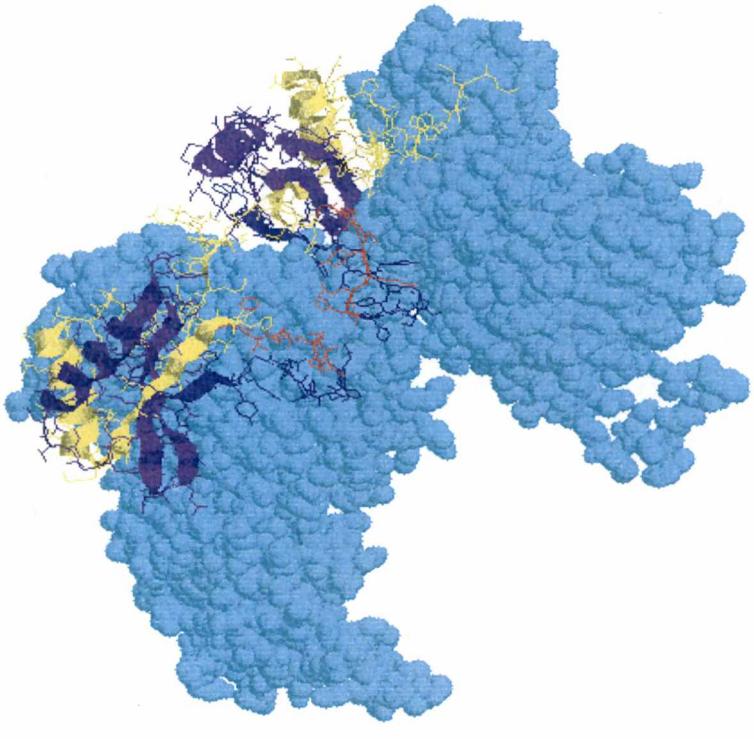
The protease responsible for mΔ67 and mΔ74 degradation is not known, however a likely candidate is the plastid homologue (ClpC/P) to the bacterial Clp family of serine proteases. ClpC is the regulatory subunit and ClpP the proteolytic subunit. Although both subunits are stromally localized (Shanklin et al. 1995), they have also been shown to co-purify with the chloroplast translocation apparatus (Akita et al. 1997; Nielsen et al. 1997) and were implicated in degradation of mistargeted aberrant prOE33

Fig. 3-10. Structural model of Rubisco small and large subunit interactions. *A,* Four each large and small subunits of Rubisco from spinach are modeled using RasMol showing the proposed orientation of the entire small subunit (ribbon) with large subunit (spacefill). In addition, the amino acids corresponding to C-terminal deletions are shown: Δ52 in purple, Δ67 in red, Δ74 in blue. The remaining small subunit is in yellow. View is looking down on the top of the complex. *B*, Same coloration as in *A* with two large and two small subunits removed to accentuate the interaction of small subunit with large subunit.

A.



B.



(Halperin and Adam 1996). Further experiments are needed to determine what protease is responsible for proteolytic degradation of mΔ67 and mΔ74.

Effect on PIRAC

Previous studies have demonstrated that PIRAC is inactivated during ATP-dependent protein translocation into the chloroplast (van den Wijngaard et al. 1999; van den Wijngaard and Vredenberg 1997). This inactivation is reflected by the presence of a precursor-induced long-lived closed state that is absent in the control patches. The fact that PIRAC is localized in the inner envelope and is inactivated by cytosolic-localized precursors or transit peptides in the presence of stromal-localized ATP suggests that PIRAC is either a component of Tic or is in some way intimately associated with the Tic complex. All of the precursors and transit peptides tested to date (van den Wijngaard et al. 1999; van den Wijngaard and Vredenberg 1997) induced a similar long-lived closed state, suggesting that the duration of closure is independent of transit peptide/precursor length. A dynamic aspect of the translocon, other than the time needed for completion of polypeptide translocation may determine the duration of PIRAC closure. The effect of the C-terminal deletions of prSSU on the P_O of PIRAC may result from removing a critical structural element that is required both for precursor interaction with Toc/Tic and PIRAC, or alternatively, it may be a secondary effect of reduced affinity for a component in the outer envelope, which precedes interaction with PIRAC.

Concluding remarks

Conflicting evidence in the literature has caused debate over the role of the C-terminus of chloroplast precursor proteins in targeting/translocation across the chloroplast envelope. In this first study to directly compare the targeting/translocation activity of a purified precursor, its mature domain, transit peptide, and multiple C-terminal deletion mutants, we have verified a basic tenet of chloroplast import models that the transit peptide is absolutely necessary for import. Our data also demonstrate quantitatively that the interaction of prSSU's transit peptide with the translocation apparatus is positively modulated by sequences within the mature domain, which function *in cis*. Moreover, we have partially mapped the regions within the mature domain that are particularly important for this interaction. In addition to facilitating a transit peptide-mediated interaction with components of the translocation apparatus, Toc and Tic, sequences within the mature domain of prSSU also contribute to the precursor's affinity for PIRAC. The structural basis for interactions between disparate regions of the precursor and these distinct chloroplast membrane complexes remains to be determined.

Chapter 4-Chloroplast precursor interaction with artificial membranes requires polar, H_{II} and anionic lipids

ABSTRACT

Import of proteins into the chloroplast is an indispensable process for chloroplast viability. Interaction of artificial membranes (liposomes) with purified chloroplast precursors and transit peptides was studied to investigate precursor-lipid interactions prior to import into chloroplasts. *Escherichia coli*-expressed forms of the precursor of the small subunit of Rubisco (prSSU), three deletion mutants of prSSU:Δ52, Δ67, Δ74; mature small subunit, and transit peptide of prSSU (SStp), as well as the precursors to the 33 and 23 kD subunits of the oxygen evolving complex of photosystem II, were used in dye release studies to further examine the specificity of interaction with chloroplast outer membrane lipids prior to translocation. Artificial bilayers, with a lipid composition mimicking the outer membrane of chloroplasts, were used to demonstrate that chloroplast precursor proteins do interact with chloroplast outer envelope lipids through a process which is mediated through the transit peptide and requires the presence of non-bilayer lipids and anionic lipids. Ability of these proteins to interact with liposomes of different lipid compositions including vesicles devoid of non-bilayer lipids, vesicles containing different non-bilayer lipids, and vesicles devoid of galactolipids was also investigated. From this study, two additional precursors were shown to be membrane active, the precursors to the 33 and 23 kD subunits of the oxygen evolving complex; adding to the very short list of precursors previously shown to be membrane active.

INTRODUCTION

Most proteins in plastids are nuclear encoded and as such have to be post-translationally translocated into the plastid. Targeting of chloroplast proteins occurs through the presence of an N terminal extension of the mature peptide, the transit peptide, which is cleaved after translocation of the precursor protein. Translocation is energy-dependent and occurs post-translationally through proteinaceous components of the chloroplast outer and inner membranes at contact sites between those membranes (for reviews see Chen and Schnell 1999; Keegstra and Cline 1999; Keegstra and Froehlich 1999). Transit peptides are both necessary and sufficient to direct a protein to the chloroplast membrane for translocation. Much emphasis has been placed on discovering the membrane-bound components involved in translocation such as Toc159, Toc75, Toc34 (Toc = translocator of the outer membrane of chloroplasts) and Tic110, Tic55, Tic22, and Tic20 (Tic = translocator of the inner membrane of chloroplasts; Chen and Schnell 1999; Keegstra and Cline 1999; Keegstra and Froehlich 1999; May and Soll 1999; Schnell et al. 1997). The role and function of each of these components in the mechanism of translocation once the precursor has reached the translocation apparatus are just beginning to be elucidated (Chen and Schnell 1999; Keegstra and Froehlich 1999; May and Soll 1999; Schnell et al. 1997).

The steps involved prior to engaging the actual translocation apparatus, however, are less clear. Until recently (May and Soll 2000) chloroplast import was thought to be unique from other protein translocation systems because it either lacked or did not require participation by cytosolic factors (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1990; Pilon et al. 1992a; Subramanian et al. 2001). In studies where purified precursors

and isolated chloroplasts are incubated together, one can detect import of the purified precursor and processing to the mature form (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1990; Pilon et al. 1992a; Subramanian et al. 2001). Import of these purified precursors was measured kinetically (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1990) and the translocation rates measured are thought to be similar to rates that would be required in a cell of a plant for normal maintenance of the chloroplast (Cline et al. 1993; Dabney-Smith et al. 1999; Pilon et al. 1992c). These *in vitro* import rates lend credibility to the use of isolated organelles for the study of import processes into the chloroplast as well as demonstrating there is no obligate need for cytosolic factors during targeting.

In addition to *in vitro* import assays using purified precursors, many cleavable, N-terminal targeting domains are membrane active. Signal peptides, which target proteins to the endoplasmic reticulum (ER), form mostly hydrophobic helices and can interact with monolayers or liposomes containing zwitterionic or anionic phospholipids (Demel et al. 1990; Killian et al. 1990a; Killian et al. 1990b; Yun and Kim 1989). Furthermore mitochondrial presequences and chloroplast transit peptides have also been shown to disrupt monolayers or liposomes. Presequences, because of their high positive charge and amphiphilic α-helical nature, interact with monolayers or liposomes containing anionic phospholipids and non-bilayer lipids as well as with cardiolipin, which is both non-bilayer and anionic (Skerjanc et al. 1987; Tamm 1991; Wang and Weiner 1994; Yu et al. 1994). Chloroplast transit peptides, on the other hand, have elements of both types of targeting domains. They typically have an N terminus that is uncharged and may form an α-helix, a central domain containing basic and hydroxylated amino acids, and a C

terminus that has little charge and is predicted to form a β-strand (von Heijne et al. 1991; von Heijne and Nishikawa 1991). Previous work on two transit peptides, the transit peptide of the precursor to ferredoxin (tpFd) and the transit peptide of the precursor to the small subunit of Rubisco (SStp), demonstrated an ability of those precursors to interact with monolayers and liposomes containing anionic phospholipid and the plastid-specific lipid monogalactosyl diacylglycerol (MGDG) (Bruce 1998; Chupin et al. 1994; Horniak et al. 1993; Pinnaduwage and Bruce 1996; van't Hof et al. 1991; van't Hof et al. 1993).

In recent years, the transit peptide has come under intense scrutiny to try to shed light on targeting and plastid recognition by the precursor. Several hundred transit peptides have been identified and sequenced (von Heijne et al. 1991), and they all have the same function of targeting proteins to the chloroplast. Unfortunately, no sequence similarity exists among these transit peptides. The lack of primary sequence similarity suggests that functional homology may stem from secondary or tertiary structure of the precursors. One hypothesis states that transit peptides have evolved to contain a minimum of secondary structure so that they remain quite flexible as random coils to engage the translocation apparatus (von Heijne and Nishikawa 1991).

More recent evidence, however, suggests that transit peptides are random coil structures until they encounter the membrane at which time they change conformationally to form secondary structures such as helices (Horniak et al. 1993; Pilon et al. 1992b; van't Hof et al. 1993). This hypothesis is attractive because it would provide a way for many different transit peptides to converge upon a similar structure in the presence of lipid in the membrane ultimately allowing them to engage the same translocation apparatus.

Historically, emphasis was placed on the protein components of the membrane as providing the recognition and translocation of precursors with the lipid bilayer itself merely providing the boundary of the organelle. This emphasis overlooks possible contributions to the targeting process by the specific interaction between one or more regions of the transit peptide and the lipid components of the membrane. Chloroplasts are bounded by a double bilayer membrane system, which contains lipids unique to the organelle. While chloroplast membranes do contain such common lipids as phosphatidylcholine, they are devoid of phosphatidylethanolamine (PE) and cardiolipin (CL). They are comprised of galactolipids, such as the polar lipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) and the negatively charged lipids phophatidylglycerol (PG) and sulfolipid (sulfoquinovosyl diacylglycerol; SL) (Siebertz et al. 1979). MGDG, DGDG and SL are unique in that they are not phospholipids, and they are only found in plastid membranes. MGDG and DGDG have galactose (one and two moieties, respectively) esterified to the R_3 position of the glycerol backbone, and SL is a galactolipid derivative containing sulfate at the 6' position of the galactose moiety (Somerville et al. 2000).

Recent evidence suggests a role for MGDG and for the anionic lipid PG in precursor translocation (Horniak et al. 1993; Pinnaduwage and Bruce 1996; van't Hof et al. 1993). Both groups have demonstrated the membrane activity of transit peptides and the requirement for MGDG while Horniak, et al. (1993) also demonstrated a need for anionic lipid. Horniak, et al. (1993) and van't Hof, et al. (1993) looked at transit peptide activity with a monolayer system as well as a liposome dye release system. They used lipid extracts from pea thylakoid as well as commercially prepared lipids to assay

membrane activity of transit peptide. Yet they did not test transit peptide in the presence of other non-bilayer forming lipids such as PE, which is not found in chloroplast membranes, or CL, which is found in mitochondria membranes (Siebertz et al. 1979). In addition, previous studies have utilized only two precursors, the precursor to ferredoxin, prFd, and the precursor to the small subunit of Rubisco, prSSU.

The present study looks at the affect of non-bilayer forming lipids upon membrane activity of prSSU as well as demonstrating the membrane activity of two additional precursors: the precursors to the 33 and 23 kD subunits of the oxygen-evolving complex, prOE33 and prOE23, respectively. In addition, this study demonstrates that liposome disruption by import-competent proteins is mediated through non-bilayer and anionic lipids. Interactions between import-competent proteins and liposomes utilize electrostatic forces. Possible interactions between precursors and membrane lipids will be discussed.

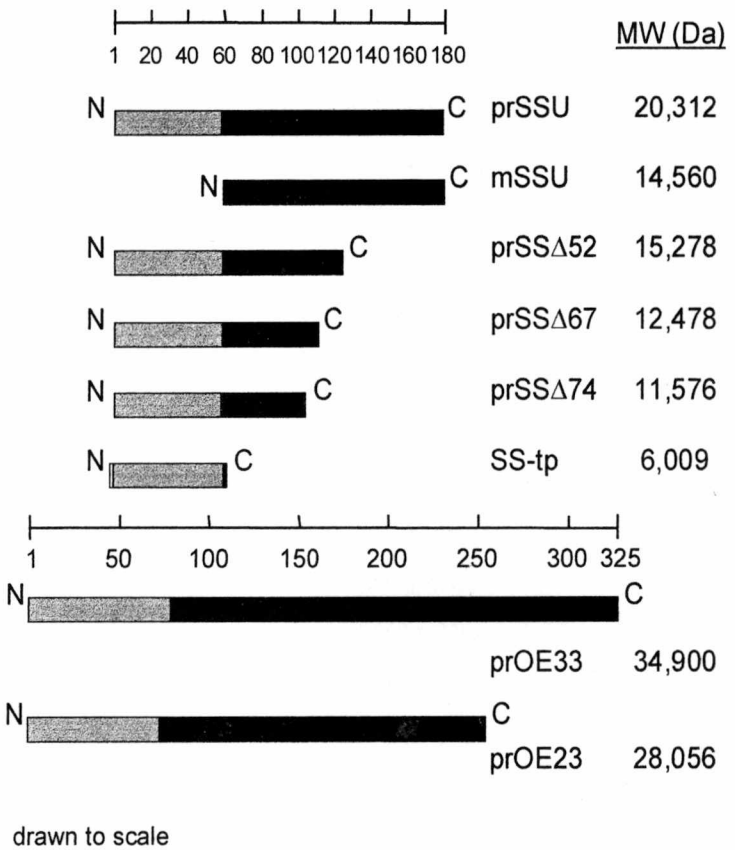
RESULTS

Overexpression and purification of precursors, transit peptide and mature domain

Fig. 4-1A gives a schematic diagram of proteins used in this study. Proteins were expressed in *E. coli* and purified to near homogeneity from inclusion bodies according to methods discussed in Chapter 2 (Fig 4-1B). The apparent molecular massses of prSSΔ52, prSSΔ67, and prSSΔ74 are 17.0, 15.8, and 10.3 kDa, respectively, which differ from the calculated molecular masses of 15.3, 12.5, and 11.6 kDa, respectively. Additionally, prSSU, prOE33, and prOE23 also have apparent molecular masses larger than their

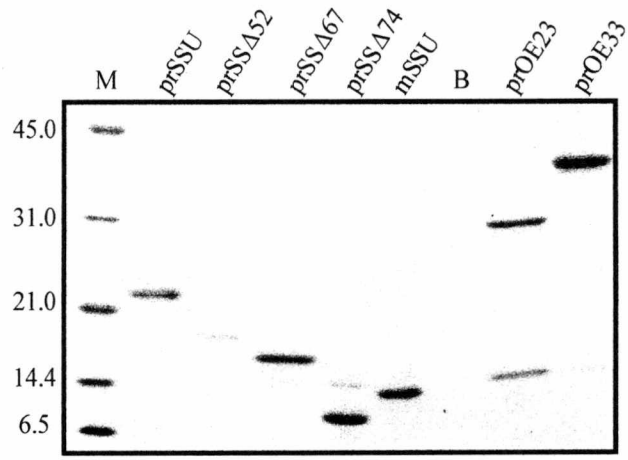
Fig. 4-1. **Comparison of prSSU, mSSU, prSSΔ52, prSSΔ67, prSSΔ74, prOE33, prOE23 and SStp**. A, The position and length of the transit peptide has been drawn to scale. The region corresponding to the full-length transit peptide is shown in *gray*, and the region corresponding to the mature domain is shown in *black*. The *small white block* at the beginning of SStp represents two amino acids introduced during subcloning. The amino acid sequences for prSSU, mSSU, prSSD52, prSSD67, and prSSD74 were obtained from DNA sequence analysis of cloned genes. The amino acid sequences for prOE33 and prOE23 were obtained from NCBI Proteins database (Accession numbers: P27665 and P16059, respectively). The lower bands in pOE33 and pOE23 lanes may be degradation products or carryover from previous lanes. The actual molecular weight is shown in Daltons on the right. B, Overexpressed prSSU, prSSΔ52, prSSΔ67, prSSΔ74, mSSU, prOE23, and prOE33 were purified to near homogeneity. SDS-PAGE and Coomassie Brilliant Blue stain of purified proteins (2 μg loaded for all; except prSSΔ52, 0.5 μg loaded). Purified protein loaded in each lane is shown at the *top*.

A.



drawn to scale

B.



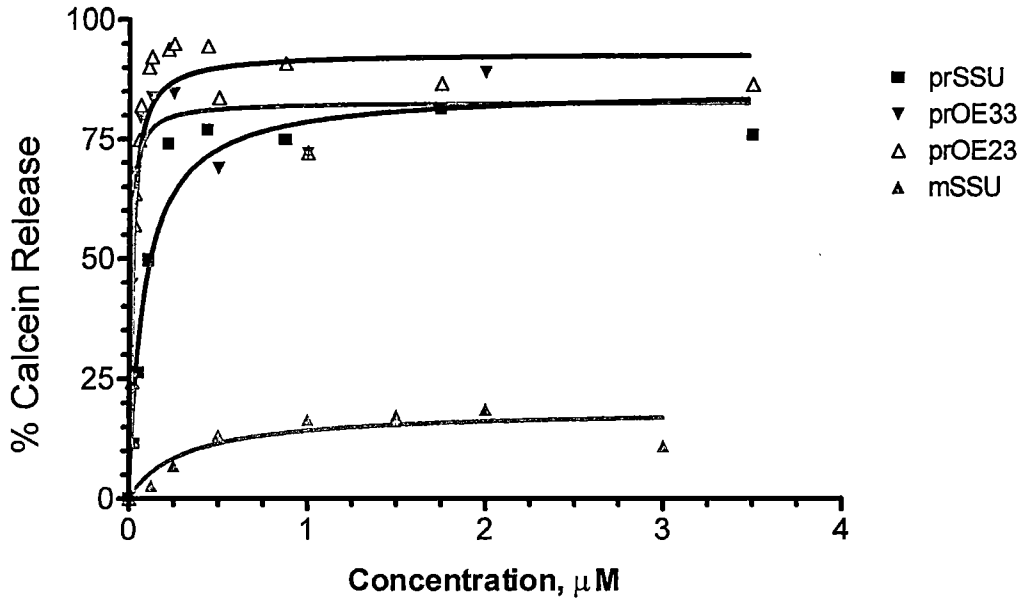
respective calculated molecular masses. We have no explanation for these abnormal electrophoretic mobilities.

Liposome disruption by precursors of the oxygen-evolving complex

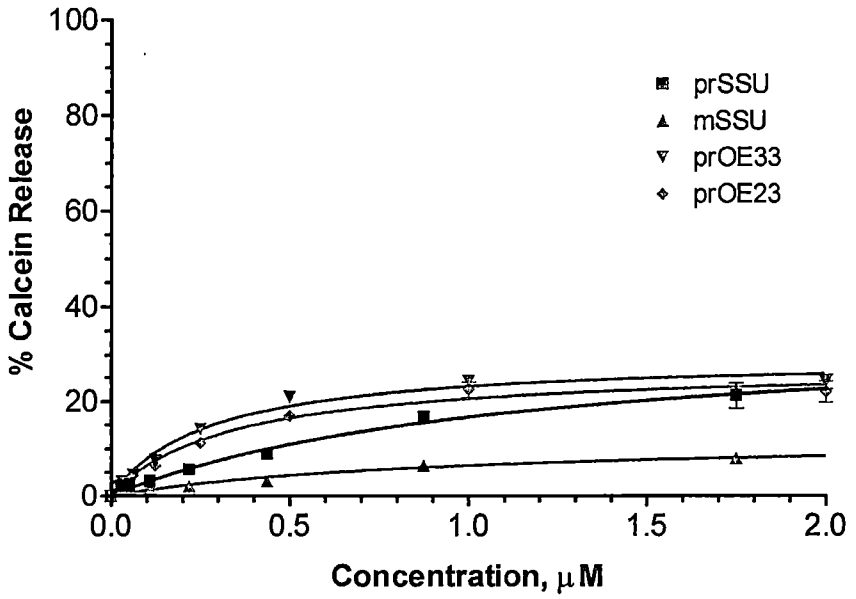
Almost all of the studies of membrane activity by chloroplast precursors have utilized prFd and prSSU. In order to gain a more complete understanding of the membrane interaction process additional precursors need to be included. To this end we overexpressed and purified from *E. coli* precursors to the 33 kD and 23 kD subunits of the oxygen evolving complex of Photosystem II in thylakoid membranes (prOE33 and prOE23, respectively) to assay for membrane activity using fluorescent dye-entrapped small unilamellar vesicles (SUV's or liposomes). If the protein has the ability to interact with liposomes mimicking the outer membrane of the chloroplast envelope (COM, Table 4-1), disruption of the membrane occurs causing dye to be released. Calcein dye is self-quenching at concentrations above ~50 mM, such as would be found inside the prepared liposome, yet fluoresces at ~517 nm upon dilution. Disruption of the membrane either through protein interaction or Triton X-100 causes a rapid dilution of the dye that can be followed by measuring fluorescence. An increase in fluorescence due to dye released as a result of protein interaction with the liposome membrane has been normalized to a Triton X-100 control which represents 100% dye release due to total disruption of the liposomes. Figure 4-2A shows that all of the precursors are indeed able to induce dye release from liposomes mimicking the outer membrane of the chloroplast envelope. Additionally, neither is able to effectively induce dye release from PC liposomes (Fig 4-

Figure 4-2. **Precursors to subunits of the oxygen evolving complex are able to induce dye release.** A, Precursors to subunits of the oxygen evolving complex (prOE33 and prOE23) were incubated for 1 hr at room temperature with liposomes mimicking the outer membrane of the chloroplast envelope (Table 1). Percent dye release (see Materials and Methods) plotted against protein concentration for prSSU (■), mSSU (▲), prOE33(▼) and prOE23(Δ). B, prOE33 and prOE23 were incubated for 1 hr at room temperature with PC liposomes (Table 1). Percent dye release (see Materials and Methods) plotted against protein concentration for prSSU (■), mSSU (▲), prOE33 (▼) and prOE23(◆)

A.


OM
100
75
50
25
0
% Calcein Release
0
1
2
3
4
Concentration, μM
prSSU
prOE33
prOE23
mSSU

B.


PC liposomes
100
80
60
40
20
0
% Calcein Release
0.0
0.5
1.0
1.5
2.0
Concentration, μM
prSSU
mSSU
prOE33
prOE23

2B) suggesting that prOE33 and prOE23 utilize the same mechanism of dye release as prSSU. All assays were done in 0.1X PBS, pH 8.0, unless otherwise noted.

Attenuation of precursor activity is not due to loss of lipid interaction

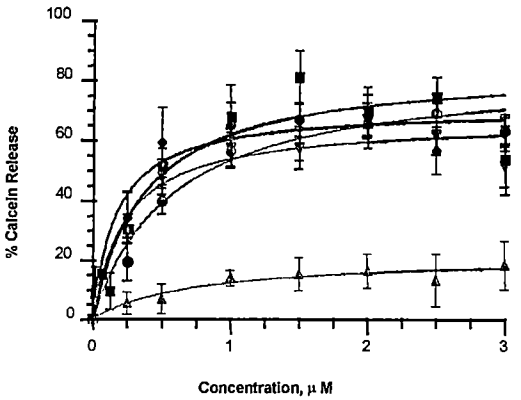
We also investigated the ability of the C-terminal deletions to interact with liposomes comprised of lipids mimicking the outer membrane of chloroplasts. Fig. 4-3A shows that as protein concentration increases, dye release from COM liposomes also increases resulting in ~80% dye release for prSSU relative to the Triton X-100 control. The mature domain alone is not able to induce much dye release (~20%) whereas the different deletions are able to induce dye release to levels comparable to that of the full-length precursor. This suggests that removal of portions of the mature domain, while affecting import competence, does not affect the initial interaction of the precursor with the membrane. Fig. 4-3B demonstrates that the transit peptide alone is sufficient to induce dye release. While the mature domain alone is able to mediate low levels of dye release through liposome disruption, the majority of dye release from a precursor is mediated by its transit peptide alone. By contrast, precursor proteins were not able to induce dye release from PC liposomes (Fig. 4-3C and 4-3D).

Liposome disruption by precursors requires MGDG

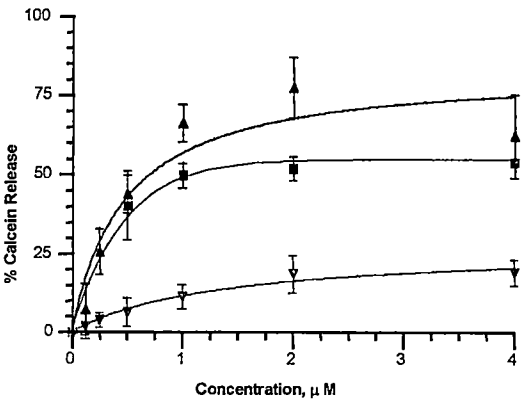
One of the unique features of chloroplast membranes is the presence of the polar, non-bilayer H_{II} lipid monogalactosyl diacylglycerol (MGDG). To test if H_{II} lipids promote lipid disruption, liposomes were prepared in 0.1X PBS, pH 8.0, with (COM) or without (OM-MG) MGDG (Table 1). Fig. 4-4A also shows that import-competent proteins are

Figure 4-3. **Liposome disruption is mediated through the transit peptide.** Precursors and mSSU were incubated for 1 hr at room temperature with liposomes mimicking the outer membrane of the chloroplast envelope (COM; Table 1). A, Percent dye release (see Materials and Methods) plotted against protein concentration for prSSU (■), mSSU (▲), prSSΔ52 (•), prSSΔ67 (▼), and prSSΔ74 (◆). Protein concentrations were from 125 nM to 3 μM. B, Percent dye release (see Materials and Methods) plotted against protein concentration for prSSU (▲), mSSU (▼), and SStp (■). Protein concentrations were from 125 nM to 4 μM. C, Precursors and mSSU were incubated for 1 hr at room temperature with PC liposomes (Table 1). Percent dye release (see Materials and Methods) plotted against protein concentration for prSSU (■), mSSU (▲), prSSΔ52 (▼), prSSΔ67 (◆), and prSSΔ74 (•). Protein concentrations were the same as in A. D, Percent dye release (see Materials and Methods) plotted against protein concentration for prSSU (■), mSSU (▲), and SStp (•) Protein concentrations were from 55 nM to 1.75 μM.

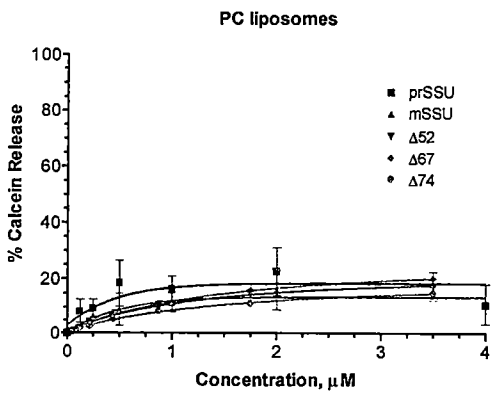
A.


% Calcein Release
Concentration, μ M

B.


% Calcein Release
Concentration, μ M

C.


PC liposomes
prSSU
mSSU
Δ52
Δ67
Δ74
% Calcein Release
Concentration, μM

D.

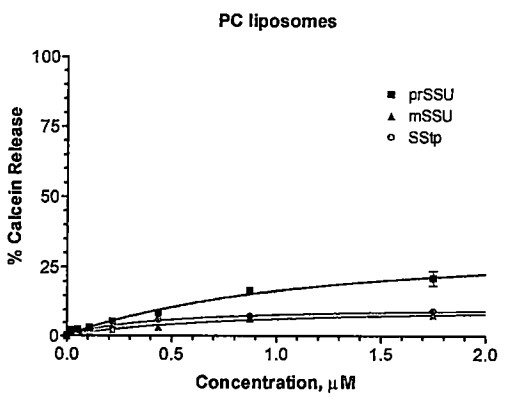
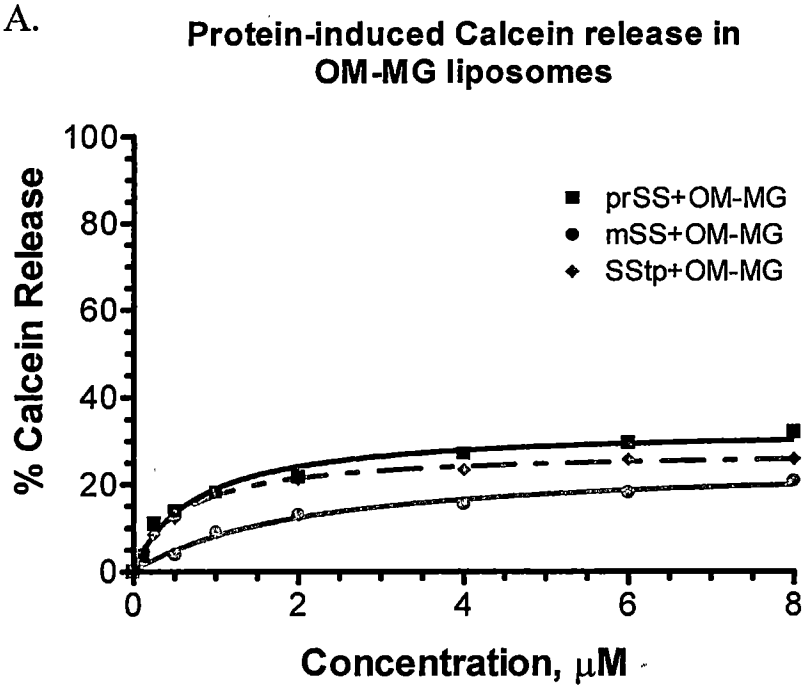

PC liposomes
prSSU
mSSU
SStp
% Calcein Release
Concentration, μM

Table 4-1. **Lipid composition of liposomes.**

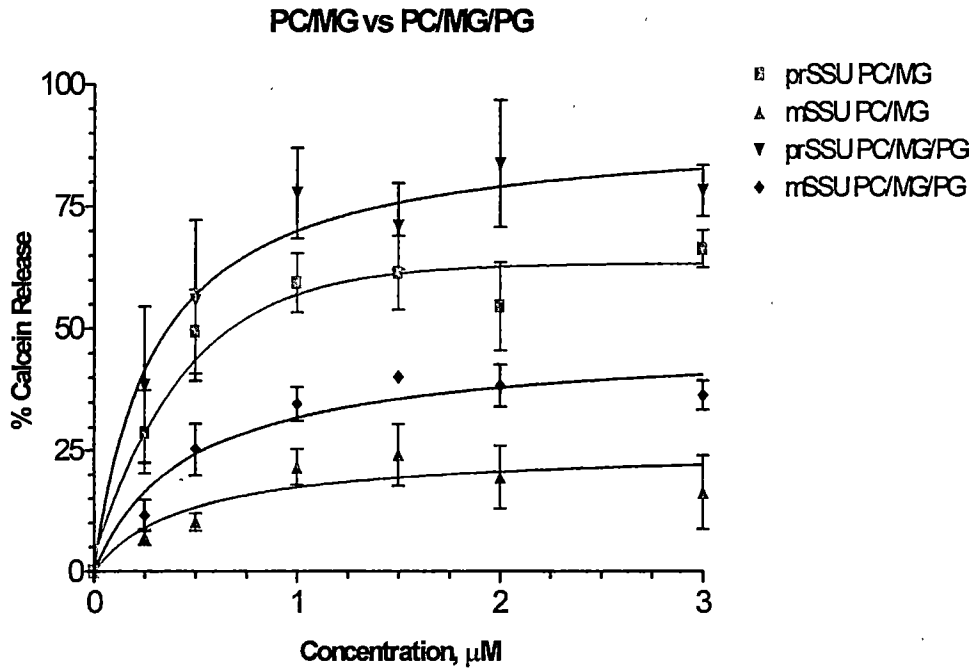
Lipid	COM Mol%	PC Mol%	OM-MG Mol%	PC/MG Mol%	PC/MG/PG Mol%	PC/PE Mol%	PC/PE/PG Mol%	PC/CL Mol%	PC/CL/PG Mol%
PC	35	100	60	80	70	80	70	80	70
PG	10	-	10	-	10	-	10	-	10
DGDG	30	-	30	-	-	-	-	-	-
MGDG	20	-	-	20	20	-	-	-	-
PE	-	-	-	-	-	20	20	-	-
CL	-	-	-	-	-	-	-	20	20
SL	5	-	-	-	-	-	-	-	-

Figure 4-4. **Liposome disruption requires MGDG.** prSSU and mSSU were incubated for 1 hr at room temperature with OM-MG, PC/MG or PC/MG/PG liposomes (Table 1). A, Percent dye release (see Materials and Methods) plotted against protein concentration for prSSU (■), mSSU (●), and SStp (◆) with liposomes lacking MGDG (OM-MG). B, Percent dye release plotted against protein concentration for prSSU and mSSU with PC/MG liposomes with (PC/MG/PG) or without (PC/MG) PG. prSSU PC/MG (■), mSSU PC/MG (▲), prSSU PC/MG/PG (▼), and mSSU PC/MG/PG (◆).

A.


Protein-induced Calcein release in OM-MG liposomes
% Calcein Release
100
80
60
40
20
0
0
2
4
6
8
Concentration, μM
prSS+OM-MG
mSS+OM-MG
SStp+OM-MG

B.


PC/MG vs PC/MG/PG
% Calcein Release
100
75
50
25
0
0
1
2
3
Concentration, μM
prSSU PC/MG
mSSU PC/MG
prSSU PC/MG/PG
mSSU PC/MG/PG

unable to induce dye release from vesicles lacking MGDG. In addition, Fig. 4-4A also demonstrates that the presence of the polar lipid (DGDG) in the absence of non-bilayer lipid (MGDG) also does not promote dye release. These data suggest that the MGDG lipids in the liposomes mediate the ability of import-competent proteins to induce dye release.

Disruption of liposomes is enhanced by anionic lipids

Since we saw a dramatic difference between liposomes containing or lacking MGDG, we wanted to see if other H_{II} lipids could also promote dye release from liposomes interacting with import competent proteins. Liposomes were prepared in 0.1X PBS, pH 8.0, with other non-bilayer lipids (8:2 PC/H_{II} molar ratio) such as phosphatidylethanolamine (PE), which is not found in plastid membranes, or cardiolipin (CL), which is present in mitochondria membranes (Table 4-1). These liposomes were made with (7:2:1 PC/H_{II}/PG molar ratio) or without the anionic phospholipid, phosphatidylglycerol (PG) to see if the presence of charge affected dye release. Fig. 4-4B shows dye release induced by prSSU and mSSU from liposomes containing PC/MG lipids and with or without PG. In PC/MG liposomes, prSSU was able to induce dye release yet not as high as that of COM (compare Fig 4-2B and Fig 4-4B). prSSU was able to induce dye release from PC/MG vesicles about two-fold higher than it could in OM-MG liposomes (Fig. 4-4A and 4-4B). About 20 mol% of MGDG could be incorporated into liposomes. This amount is sufficient to induce significant dye release (compare 80% dye release in COM to 60% dye release in PC/MG). However, addition of the anionic lipid PG restored dye release levels to that of COM (Fig. 4-4B). Dye release induced by

mSSU, which does not contain targeting information, was significantly lower than prSSU in PC/MG liposomes with or without PG. The presence of PG in liposomes did increase the ability of mSSU to induce dye release (compare Fig. 4-2B and Fig. 4-4B). This suggests that the mature domain does contain some ability to interact with the membrane albeit less than SStp.

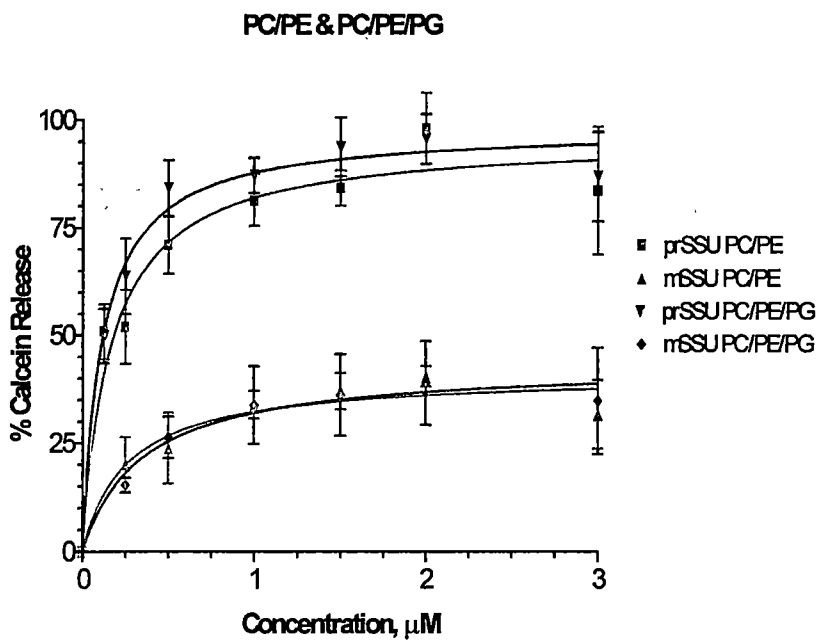
By the same token, when PE was used in liposomes instead of MGDG, the import competent precursor was able to induce dye release to about 90% of Triton X-100 control (Fig. 4-5A), whereas the mature domain alone could only cause low levels of dye release. These data suggest the requirement for the transit peptide as providing the majority of the interaction with the non-bilayer lipid. Interestingly, the presence or absence of PG did not affect dye release. By contrast, liposomes prepared using CL instead of MGDG either with or without PG were highly interactive with both proteins demonstrating ~100% dye release (Fig. 4-5B). Again, the presence or absence of PG did not change the affect. Interestingly, interaction with CL liposomes is no longer mediated through the transit peptide alone. mSSU was able to induce dye release just as effectively as prSSU when assaying CL liposomes.

Liposome disruption by import-competent proteins involves electrostatic interactions

To investigate further the role of the anionic lipid, PG, in liposome disruption, COM liposomes were pre-equilibrated in PBS buffer, pH 8.0, containing additional NaCl (15-165 mM) and incubated with 500 nM of prSSU, SStp, or mSSU. The starting NaCl concentration was 15 mM, the typical composition of 0.1X PBS buffer. Fig. 4-6 demonstrates that as NaCl concentration in the buffer increased, dye release induced by

Figure 4-5. **Liposome disruption requires H_{II} lipids.** A, Increasing concentrations of prSSU and mSSU were incubated for 1 hr at room temperature with PC/PE or PC/PE/PG liposomes (Table 1). Percent dye release plotted against protein concentration for prSSU PC/PE (■), mSSU PC/PE (▲), prSSU PC/PE/PG (▼), and mSSU PC/PE/PG (◆). B, Increasing concentrations of prSSU and mSSU were incubated for 1 hr at room temperature with PC/CL or PC/CL/PG liposomes (Table 1). Percent dye release plotted against protein concentration for prSSU PC/CL (■), mSSU PC/CL (▲), prSSU PC/CL/PG (▼), and mSSU PC/CL/PG (◆)

A.


PC/PE & PC/PE/PG
% Calcein Release
100
75
50
25
0
0
1
2
3
Concentration, μM
prSSU PC/PE
mSSU PC/PE
prSSU PC/PE/PG
mSSU PC/PE/PG

B.

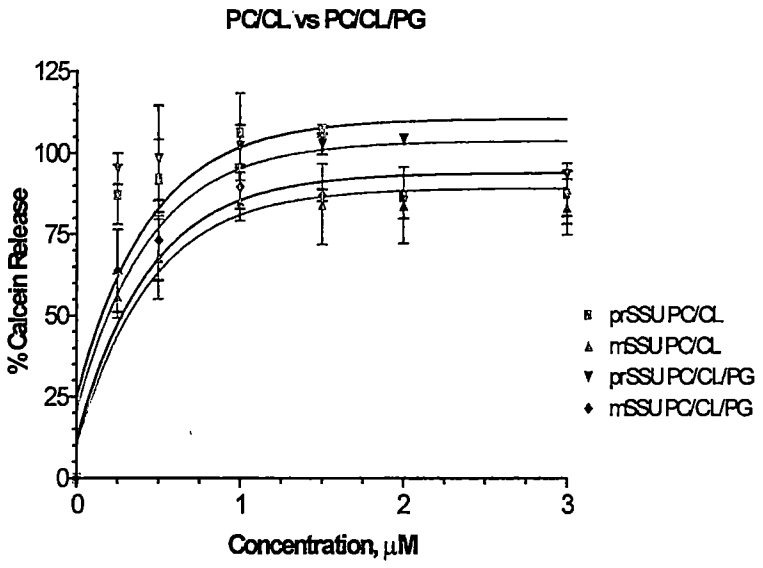
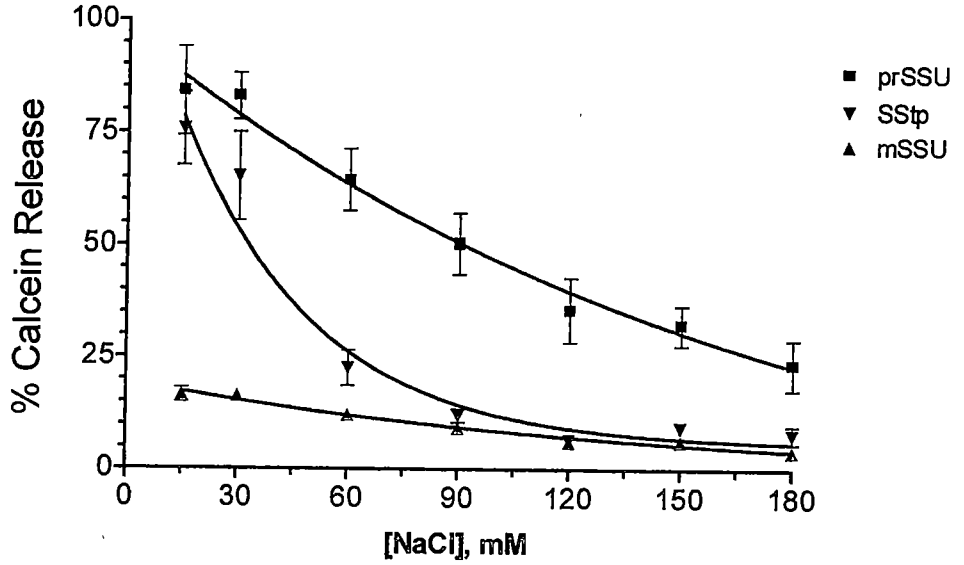
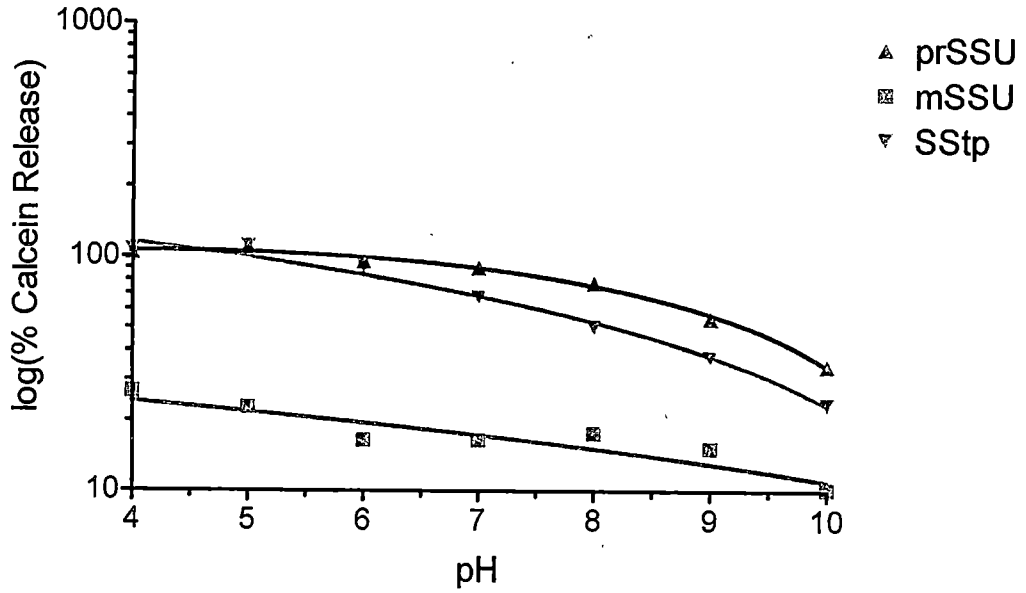

PC/CL vs PC/CL/PG
% Calcein Release
125
100
75
50
25
0
0
1
2
3
Concentration, μM
prSSU PC/CL
mSSU PC/CL
prSSU PC/CL/PG
mSSU PC/CL/PG

Figure 4-6. **Liposome disruption involves electrostatic interactions.** *A*, COM liposomes were preequilibrated 0.1X PBS with increasing concentrations of NaCl (15-180 mM) for 15 min before incubating with 500 nM prSSU (■), mSSU (▼) or SStp (▲) for 1 hr at room temperature. *B*, COM liposomes were preequilibrated in 0.1X PBS with increasing pH (4-10) for 15 min before incubating with 500 nM prSSU (▲), mSSU (■) or SStp (▼) for 1 hr at room temperature.

A.


100
75
50
25
0
% Calcein Release
0
30
60
90
120
150
180
[NaCl], mM
prSSU
SStp
mSSU

B.


1000
100
10
log(% Calcein Release)
4
5
6
7
8
9
10
pH
prSSU
mSSU
SStp

import competent proteins decreased from ~85% to ~25% for prSSU and from ~80% to ~10% for SStp. The dye release induced by the mature domain alone also decreased although not as significantly. While the decrease in dye release as NaCl concentration increases was linear for prSSU and mSSU, it was not linear for SStp suggesting the transit peptide was more sensitive to the ionic strength of its environment than prSSU or mSSU.

In addition, pH also affected dye released induced by import-competent proteins. COM liposomes were pre-equilibrated with 0.1X PBS buffer, pH 4-10, for 15 min and incubated with 500 nM of prSSU, SStp, and mSSU. At acidic pH, liposomes were completely disrupted by import-competent proteins to the levels of the Triton X-100 control. Dye release decreased as pH increased suggesting disruption of electrostatic interactions.

DISCUSSION

Precursors interact with artificial membranes

In the present study we demonstrate that two precursors for components of the oxygen-evolving complex (prOE33 and prOE23) were able to interact with liposomes mimicking the outer membrane of the chloroplast envelope adding to the list of precursors which contain membrane active components. Further analysis of these precursors should be done to confirm that the transit peptide is responsible for the interaction, as is the case for prSSU and prFd. This work provides evidence that chloroplast precursors may indeed have secondary or tertiary structure homology even though there does not seem to be any

primary sequence similarity. Having similar secondary structure in the presence of the membrane would provide a way for the many diverse forms of transit peptide to be able to converge upon a single general import pathway to gain entry into the organelle.

Dye disruption from liposomes is mediated mostly through the transit peptide

Previously (Dabney-Smith et al. 1999) we demonstrated that removal of C-terminal portions of the mature domain of the precursor to the small subunit of Rubisco decreased the ability of the protein to compete with wildtype precursor. Several possibilities exist to explain why removal of the C terminus altered ability of the precursor to compete with the wildtype precursor for import into chloroplasts. (1) The level of competition may be proportional to the resident time of the precursor in the translocon such that full length precursors are better competitors than truncated ones or (2) the mature domain may contain structural information which modulates the proper transit peptide conformation thus enabling transport or (3) the mature domain may contain structural information which directly facilitates import of the precursor through direct interaction of the precursor with membrane lipids or Toc/Tic components. The first possibility is unlikely based on the ability of truncated precursors to induce long-state anion channel closure of similar duration times as the wildtype (Dabney-Smith et al. 1999). These data suggest that once the precursor has engaged the translocation apparatus, import is similar to that of wildtype. These data also suggest that removal of the C terminus has altered affinity of the precursor for some aspect of translocation such as membrane lipids or Toc/Tic components.

One aim of this study was to investigate if removal of portions of the C terminus of

prSSU resulted in decreased ability to compete for import because of decreased ability to interact with the membrane. Here we showed that interaction with the membrane by C-terminal deletions of prSSU was comparable to full-length prSSU demonstrating that the decrease in ability to compete for import was not due to altered ability to interact with the membrane. Additionally we have evidence that the decrease in competition is not due to changing the resident time in the translocation apparatus (Dabney-Smith et al. 1999), but rather due to a decrease in affinity for proteinaceous components of the translocon such as Toc34 (Schleiff et al, manuscript in preparation). Here we demonstrated that the lipid interaction was mediated mostly through the transit peptide. The mature domain alone may be able to mediate low levels of dye release through liposome disruption, but the largest amount of dye release was mediated by the transit peptide alone. This supports previous studies using ferredoxin and its precursor and transit peptide (van't Hof et al. 1993), which also demonstrated that the interaction was mediated through the transit peptide and not the mature domain (either apo- or holoferredoxin.) This study also directly supports work by Pinnaduwage and Bruce (1996) who showed the transit peptide of prSSU mediated interaction with liposomes containing MGDG.

The antimicrobial peptide, mellitin, from bee venom, is a 26 amino acid mostly (amphipathic) helical structure with a bend predicted from residues 11-15 (Dufourcq et al. 1986; Sitaram and Nagaraj 1999). This helix-bend-helix conformation is reminiscent of the NMR secondary structures of transit peptides of the precursor to ferredoxin (prFd) from *Silene pratensis* (Wienk et al. 1999) and the predicted secondary structure of the transit peptide of the precursor to the small subunit of Rubisco (prSSU) from *Pisum sativum* (Bruce 2000). In addition, the transit peptides to the precursors of

Rubisco activase and Fd from *Chlamydomonas reinhardtii* have also been analyzed by NMR to show formation of an α-helix at the C terminus and N terminus, respectively, in a hydrophobic environment (Lancelin et al. 1994; Lancelin et al. 1996). The algal α-helices are amphiphilic, and even though the entire transit peptide from algal sources tend to be much shorter than transit peptides from higher plants (Franzen et al. 1990; Kindle 1998). Krimm, et al., (1999) predict that the presence of these coil-helix or helix-coil structures could be repeated to provide a level of distinction for higher plant transit peptides thus providing them with their longer length.

The similarities between antimicrobial peptides and transit peptides are not limited to secondary structure formation in hydrophobic environment. Antimicrobial peptides tend to be positively charged (from the presence of arginine and lysine residues) with an absence of acidic residues such as aspartate and glutamate (Dathe and Wieprecht 1999; Epand and Vogel 1999; Sitaram and Nagaraj 1999). Transit peptides are also distinguished by possession of net positive charge with a lack of acidic residues, especially in the central domain (von Heijne and Nishikawa 1991; von Heijne et al. 1989). Some antimicrobial peptides also have an elevated hydroxylated amino acid residue content (Dathe and Wieprecht 1999; Epand and Vogel 1999; Sitaram and Nagaraj 1999), whereas transit peptides also have a higher than typical hydroxylated amino acid content (von Heijne et al. 1991; von Heijne and Nishikawa 1991; von Heijne et al. 1989). In addition, plants also possess their own form of lytic peptides, defensins or thionines (Broekaert et al. 1995; Epand and Vogel 1999). Plant defensins are some of the largest antimicrobial peptides at 50 amino acids, but they are also cationic. They are distinguished by the presence of cysteines with a propensity to form disulfide bonds

between molecules. Structural analysis of plant defensins indicates that they possess β-sheet structure with an α-helical structural motif (Epand and Vogel 1999). For comparison, transit peptides typically have at least one cysteine residue and early prediction of transit peptide structure proposed an amphiphilic β-strand at the C terminus (Archer and Keegstra 1990; von Heijne et al. 1989).

Based on these pieces of evidence, it is enticing to hypothesize that, after endosymbiosis, when a gene was duplicated to the nucleus, it acquired targeting information that is derived from defense peptides whose function is to permeabilize membranes. One possibility is that transit peptides evolved to be more membrane active than lytic to preserve the evolving symbiosis. This would account for the ability of transit peptides to interact with membranes for targeting and ultimately translocation, while not destroying the membrane in the process. One way this could be achieved is by increasing the distance between membrane active helices by varying the central domain. The central domain of transit peptides appears to be the most variable and diverse part when comparing sequences from different organisms (Archer and Keegstra 1990; von Heijne et al. 1991; von Heijne and Nishikawa 1991; von Heijne et al. 1989). Membrane activity of transit peptides may serve as an initial mechanism of targeting, promoting subsequent interaction of the precursor with the translocation apparatus of the membrane. Another way of tempering the membrane activity of the new transit peptide is to shorten the amphipathic and/or helical regions. While this may not remove the lytic properties of the transit peptide, it may change the MIC (minimal inhibitory concentration) to a concentration so high as to be above physiological. The transit peptide would still be membrane active, but it would probably never accumulate in cells to be lytic as well.

Transit peptide interaction with liposomes occurs through electrostatic interactions

When interacting with PC/MG liposomes, prSSU, mSSU, and SStp all displayed increased ability to induce dye release in the presence of anionic PG suggesting electrostatic interactions are involved. Interactions of prSSU, mSSU, and SStp with liposomes at increasing NaCl or at increasing pH all showed a decrease in dye release confirming the disruption of electrostatic interactions. SStp appeared to be more sensitive to the ionic strength of its environment than did prSSU or mSSU. This is probably due to the overall net charge of SStp of +4 at pH7.0 (P_i 11.0), while prSSU and mSSU have overall net charges of +1 and 0, respectively (P_i 7.7 and P_i 7.0, respectively). Because of its high net charge, SStp would be more sensitive to the ionic strength of its environment. The shift seen between prSSU and SStp at increasing pH cannot be explained.

How might transit peptides interact with membrane lipids? Transit peptides are thought to be mostly random coil in aqueous environment (for review see Bruce 2000). However, increasing evidence shows that in the presence of a membrane environment, the transit peptide adopts secondary structure especially in the form of amphipathic α-helices (Bruce 1998; Endo et al. 1992; Krimm et al. 1999; Wienk et al. 1999). The presence of charged lipids (i.e. PG) and polar lipids (i.e. MGDG) in the membrane would provide areas of interaction with the polar, charged face of the helix. In addition, the presence of a H_{II} lipid (such as MGDG) would provide a local 'looseness' of lipid packing which would make the interaction of the hydrophobic face of an amphipathic helix with acyl chain of lipids more feasible. Dependence upon the presence of anionic and/or non-bilayer lipids in a membrane for a peptide to be membrane active is seen for

presequences of mitochondrial precursors (Hammen et al. 1996; Leenhouts et al. 1994; Swanson and Roise 1992; Yu et al. 1994), signal peptides of endoplasmic reticulum (ER) precursors (Demel et al. 1990; Jones et al. 1990; Keller et al. 1992; Killian et al. 1990b), and antimicrobial peptides (Bechinger 1999; Dathe and Wieprecht 1999; Shai 1999).

Possible roles of nonbilayer-forming lipids in membranes

Previously only three chloroplast precursors, precursor to ferredoxin (prFd) (Horniak et al. 1993; van't Hof and de Kruijff 1995; van't Hof et al. 1993), precursor to the small subunit of rubisco (prSSU) (Pinnaduwage and Bruce 1996; van't Hof et al. 1991), and precursor to plastocyanin (prPC) (Endo et al. 1992) have been studied for their ability to interact with membranes. The stromal targeting domain of the transit peptide of prPC was shown to develop secondary structure in the presence of membrane environment, yet it was not able to disrupt the membrane (Endo et al. 1992). The precursor to ferredoxin (prFd) has been extensively studied and shown to required both anionic and polar, non-bilayer lipids to be membrane active (Horniak et al. 1993; van't Hof and de Kruijff 1995; van't Hof et al. 1993). Pinnaduwage and Bruce (1996) used the precursor to the small subunit of Rubisco to also demonstrate a need for polar, nonbilayer lipids. This study investigates the effect of other non-bilayer-forming lipids such as unsaturated PE and CL. PE is zwitterionic and has a tendency to be non-bilayer-forming when it is unsaturated (Lugtenberg and Peters 1976). CL is both non-bilayer-forming and anionic (Lehninger et al. 1993). prSSU was able to induce dye release from both types of liposomes as well as from liposomes containing MGDG. This suggests that H_{II} phase lipids are important in mediating interaction between the precursor and the membrane.

PE is not found in chloroplast membranes but is present in membranes of the ER, Golgi apparatus, and mitochondria (Douce et al. 1984). Additionally, CL is found in mitochondrial membranes (Smaal et al. 1985). Import-competent protein based dye release from liposomes containing PE showed a loss of enhancement upon the addition of PG. In addition, CL liposomes were reactive with the non-import competent protein, mSSU. Those liposomes have lost requirement for targeting information, which is in contrast to the MG and PE containing vesicles. Because prSSU can interact with liposomes composed of these other nonbilayer-forming lipids, and evidence that import-competent proteins do not interact with liposomes containing lamellar, polar lipids (DGDG) suggests that the hydrogen bonding that occurs only occurs when the transit peptide can embed into the bilayer, i.e. in the presence of non-bilayer lipid. This suggests that a hierarchy exists such that non-bilayer lipid composition > anionic lipid composition > polar lipid composition. The presence of non-bilayer lipids in the membrane may serve as a mechanism of nonspecific membrane recognition, which allows the precursor to gain access to the membrane. The greater specificity then lies in whether or not appropriate charge and polarity are present, such as PG and MGDG/DGDG, to promote productive interaction with the membrane to lead to interaction with protein components for translocation.

Concluding remarks

Two transit peptides of chloroplast precursors, tpFD and SStp, are membrane active. In this study we increased the list of chloroplast precursors which are membrane active as demonstrated by interaction with calcein-entrapped liposomes mimicking the outer

membrane of the chloroplast envelope. The thylakoid-localized precursors, prOE33 and prOE23, are able to induce dye release comparable to prSSU suggesting they utilized the same mechanism for interaction. We also demonstrated that the interaction is modulated through the transit peptide. Changes in the mature domain (through deletion of the C terminus) did not affect dye release. In addition, we demonstrate involvement of non-bilayer lipids in the interaction. Interaction of import-competent proteins with PE and CL liposomes did cause dye release, however any effect of adding anionic PG was lost. Targeting information was required for interaction with PE liposomes, yet interaction with CL liposomes seemed more non-specific having lost requirement for targeting information. Import-competent protein interaction with liposomes mimicking the outer membrane of chloroplast envelopes was dependent upon the presence of non-bilayer lipids, anionic lipids and polar lipids, specifically non-bilayer lipids > anionic lipids > polar lipids. The structural mechanism of interaction of import-competent proteins with membranes needs to be determined.

Chapter 5- Preliminary Results

ABSTRACT

Our current understanding or how precursors are targeted to the chloroplast has relied mostly on using either biochemical or *in vitro* import assays. While these approaches have provided considerable information on kinetics, energetics, and mechanistic details of protein targeting and translocation into chloroplasts, only now are *in vivo* studies being employed to confirm these observations on protein targeting and translocation into chloroplasts *in vivo*. *in vivo* approaches have utilized transgenics to test the activity of protein targeting. To further our understanding of *in vivo* activity of prSSU transit peptide, we designed green fluorescent protein (GFP) chimeras with the precursor to the small subunit of Rubisco (prSSU-GFP). GFP alone or prSSU-GFP could be expressed in a transient assay in onion epidermal cells. Additionally GFP was expressed in *Arabidopsis* leaf tissue which may provide a second trasient assay for prSSU-GFP. These experiments lay the groundwork for expression of mutant precursors fused to GFP in either onion epidermal cells or *Arabidopsis* leaf tissue to explore the effect mutations in the transit peptide have on targeting and import relative to the wildtype precursor.

INTRODUCTION

Targeting proteins to chloroplasts is a crucial process for the plant cell because so many of the proteins needed for function are encoded by nuclear genes (Chen & Schnell 1999, Keegstra & Cline 1999, Keegstra & Froehlich 1999, May & Soll 1999). Proteins destined

for the chloroplast are synthesized in the cytoplasm as higher molecular weight precursors with an N-terminal extension, the transit peptide, which is both necessary and sufficient to direct import of proteins into the chloroplast. Targeting is only the first step for import of a protein into the chloroplast, however. Post-translational translocation of the precursor across the outer and inner membranes of the chloroplast envelope must occur for the protein to reach its final destination (Chen & Schnell 1999, Keegstra & Cline 1999, Keegstra & Froehlich 1999, May & Soll 1999). Translocation occurs at contact sites between the outer and inner membrane (Archer & Keegstra 1990, Pain et al 1988, Schnell et al 1990) through protein components located in the membrane (Kouranov et al 1998, Lubeck et al 1996, Perry & Keegstra 1994, Schnell et al 1997, Sohrt & Soll 2000, Stahl et al 1999, Sveshnikova et al 2000). These membrane components are called Toc, for translocator of the outer membrane of chloroplasts, and Tic, for translocator of the inner membrane of chloroplasts (Schnell et al 1997). Transport of proteins across the envelope can be divided into three steps based on different energy requirements. The first step is an energy independent binding of the precursor to the membrane or Toc components in the membrane (Ma et al 1996 and Chapter 4, this work). The second step is a GTP or ATP-dependent (<100 μM) early import intermediate, in which the precursor is in a stable association with the import machinery, but is still outside the chloroplast because it is exogenous-protease sensitive (Olsen & Keegstra 1992). The third step is complete translocation or import of the protein into the chloroplast. This step requires higher amounts of stromally localized ATP (>100 μM) and results in accumulation of exogenous-protease-insensitive, processed forms of the precursor in the stroma (Olsen & Keegstra 1992, Theg et al 1989).

Almost all of the information acquired about protein import into the chloroplast has occurred using *in vitro* assays with isolated organelles and either purified precursors or *in vitro* translated precursors. While these assays have been shown to be physiologically relevant with import rates comparable to what would be expected *in vivo* (Cline et al 1993, Dabney-Smith et al 1999, Pilon et al 1992), not much focus has been placed on *in vivo* protein import into chloroplasts. A few studies have employed genetic approaches to identify mutants which have altered chloroplast protein import. Jarvis, et al., (Jarvis et al 1998) identified an *Arabidopsis* mutant affected in plastid protein import (ppi1), which corresponded to mutations in Toc34. Bauer, et al., (Bauer et al 2000) identified a second ppi mutant (ppi2) in *Arabidopsis* which had a defective Toc159. Another group, Jarvis, et al., (Jarvis et al 2000) identified an MGDG synthase mutant in *Arabidopsis* which had altered MGDG content in plastid membranes and was defective in protein import.

There is evidence that import of precursors into chloroplasts *in vitro* and *in vivo* is not faithful. Rensink et al., (1998) investigated the role of transit peptide domains from prFd by generating deletion mutants within the transit peptide and transforming *Arabidopsis*. While their wildtype prFd construct was imported and processed both in transgenic plants and in isolated organelles, the deletion mutants behaved differently (Rensink et al 1998). None of the deletion mutants were able to import into isolated organelles, but some could import to varying degrees in transgenic plants. In addition, studies in *Chlamydomonas* have revealed similar results. Lawrence and Kindle (1997) performed similar experiments as Rensink, et al., (1998) by creating deletion mutants in the transit peptide of the precursor to plastocyanin (prPC). The deletions severely

inhibited import of wildtype prPC *in vitro* but were still able to accumulate in the chloroplast of *Chlamydomonas* (Lawrence & Kindle 1997). These studies indicate that while *in vitro* studies have been important in understanding some aspects of import, they may be somewhat out of context in others, such as structure/function relationships of transit peptides.

To begin to understand how alterations in the transit peptide can affect targeting *in vivo*, we have designed chimeric proteins with the precursor to the small subunit of Rubisco, prSSU, and green fluorescent protein, GFP. Utilizing GFP provides a way to follow accumulation of prSSU either in stroma or in cytoplasm, initially using wildtype, but ultimately transit peptide mutants as well. These transit peptide mutants would alter the structural flexibility of the transit peptide by either adding helix breakers or removing them. Eventually, we would like to follow accumulation of precursors containing these transit peptide mutations *in vivo* and compare those patterns of accumulation with wildtype, which may be possible in the same cell using spectrally distinguishable fluorescent proteins, ultimately providing insight into the structure/function relationship of the transit peptide. The current study suggests that prSSU-GFP fusion protein can be directed to punctate forms presumed to be plastids in onion epidermal cells. Use of onion epidermal cells has several benefits including easier viewing of GFP due to lack of chlorophyll autofluorescence, ease of tissue manipulation (no plant culture required), large cells for ease of visualization, requires only an overnight incubation, and cost effectiveness. In addition, localization of GFP to biolistically transformed *Arabidopsis* leaf tissue suggests another avenue of pursuit as a transient assay for chloroplast protein

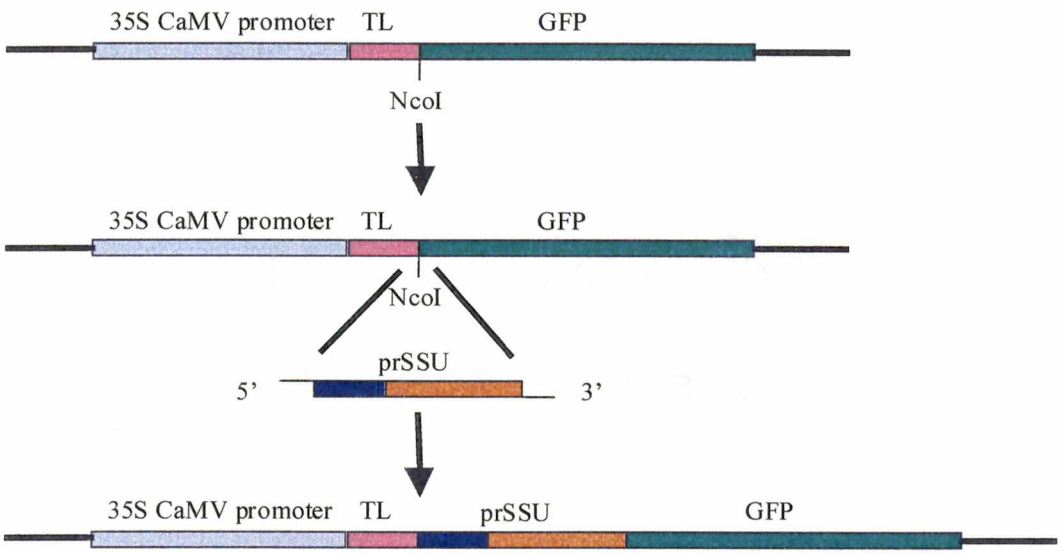
import *in vivo*. Possible corrective measures to improve the transient assay will be discussed.

RESULTS

Generation of prSSU-GFP constructs

To generate fusions between the precursor of the small subunit of Rubisco (prSSU) and GFP, we used base vectors pAVA393, pAVA554, and pAVA574 (von Arnim et al 1998). pAVA393 contains green fluorescent protein, while pAVA554 contains yellow fluorescent protein (YFP) and pAVA574 contains cyan fluorescent protein (CFP). CFP and YFP are mutational changes in GFP which alter the fluorescent properties resulting in different excitation wavelengths (~430 nm for CFP and ~525 nm for YFP) and emission wavelengths (~460 nm for CFP and ~535 nm for YFP) from GFP (excitation 490 nm and emission ~515 nm) (Clontech, Palo Alto, CA). All three vectors confer ampicillin resistance, a dual 35S CaMV promoter, a translational enhancer sequence from tobacco etch virus (TEV), the fluorescent protein coding sequence, and the 35S CaMV transcriptional terminator. The subcloning strategy used to fuse prSSU to the N terminus of GFP is outlined in Fig. 5-1. The same cloning strategy was used for fusing prSSU to the N terminus of CFP or YFP as well as for fusing mutant precursors for future investigations. Positive clones for proper insert orientation were sequenced to confirm that the junction between prSSU and GFP were in the same frame.

Fig 5-1. Construction of prSSU-GFP chimeras. prSSU was PCR amplified from pET11-prSSU (see Chapter 2) with primers which inserted an NcoI site at both ends preserving the start codon, but removing the stop codon. GFP vectors pAVA393 (GFP), pAVA554 (YFP) or pAVA574 (CFP) were linearized at unique NcoI sites and prSSU was inserted. Orientation of the insert was confirmed by restriction digestion at NsiI and XbaI. Shown is the scheme for prSSU and GFP. The schemes for CFP and YFP were identical. TL (pink) is the translational enhancer sequence from TEV. The prSSU transit peptide is shown in blue and the prSSU mature domain is orange. The dual 35S CaMV promoter is colored lavender and the GFP is green.


35S CaMV promoter
TL
GFP
NcoI
35S CaMV promoter
TL
GFP
NcoI
prSSU
5'
3'
35S CaMV promoter
TL
prSSU
GFP

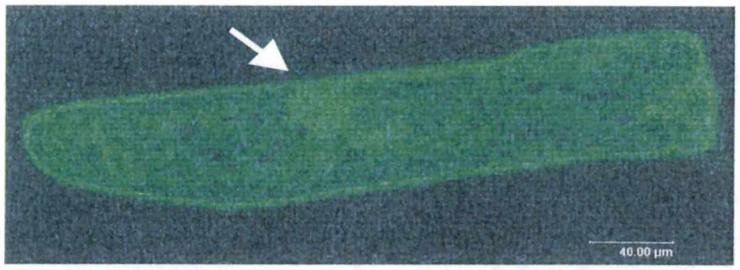
Transient Expression of GFP-Fusion Proteins in Onion Epidermal Cells

Since prSSU contains a functional transit peptide, making an N-terminal fusion of prSSU to GFP should result in localization of GFP to plastids. Fusion genes encoding the full-length prSSU-GFP were transiently expressed in onion epidermal cells. Fluorescence analysis indicates that prSSU-GFP localizes to areas presumed to be leucoplasts in onion epidermal cells (Fig. 5-2B, yellow arrows). By contrast transient expression of GFP alone resulted in localization to the cytoplasm and the nucleus but not the vacuole (Fig. 5-2A). Transient expression of prSSU-GFP consistently resulted in a regular punctate pattern. Optical sectioning using CLSM demonstrated that the punctate regions of fluorescence were localized to around the edge of the cell as would be expected for leucoplasts with the presence of a large vacuole (Fig. 5-2B and 5-2D). The majority of prSSU-GFP fusion targeted to areas presumed to be leucoplasts, although in some cells a large fluorescent aggregate was observed (Fig. 5-2B, arrowhead). GFP illuminating the nucleus may be as a result of localization of GFP to the nucleus but could be due to a large protein aggregate immediately adjacent. prSSU is likely to form aggregates because it is being expressed behind a dual strength constitutive promoter, 35S CaMV. In addition, prSSU may aggregate if it cannot be imported due to saturation of the available plastid Toc components or because necessary cytosolic factors are not present. Furthermore, prSSU itself exhibits low solubility when overexpressed in *E. coli*, and may do the same when overexpressed in *Arabidopsis*. Localization to the nucleus is demonstrated in Fig. 5-2A, where GFP appears inside the nuclear envelope. One solution may be to quantitate relative fluorecent densities of same dimensional areas within prSSU-GFP cells and control GFP cells to determine difference of expression.

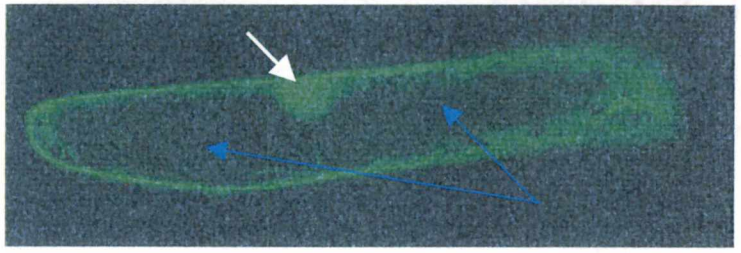
Fig. 5-2. Transient expression of prSSU-GFP in onion epidermal cells. *A*, Onion cell expressing GFP alone, white arrow indicates nucleus. *B*, Same cell as in (A), showing the vacuole, cyan arrows. *C*, Onion cell expressing prSSU-GFP. Leucoplasts are indicated by yellow arrows. The nucleus is indicated by the white arrow and possible protein aggregation is indicated by the arrowhead. *D*, Same cell as in (C) showing the vacuole, cyan arrows.

A.

GFP

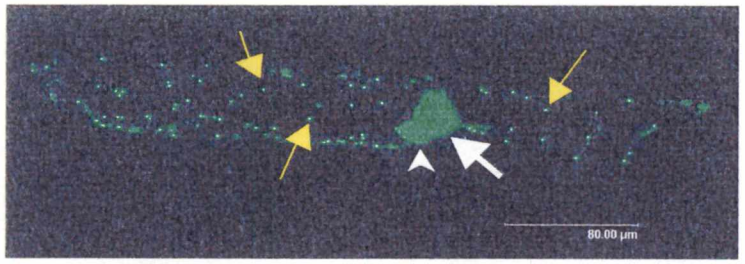


B.

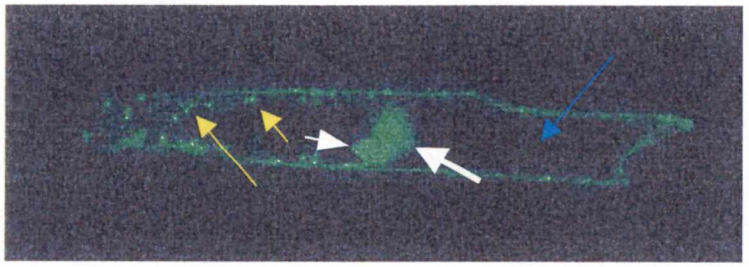


C.

prSSU-GFP



D.



Transient prSSU-GFP Expression in Arabidopsis *leaf tissue*

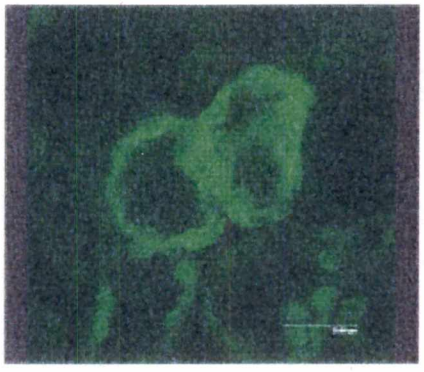
Since leucoplasts do not contain chlorophyll, we need a way to confirm localization of prSSU-GFP to the plastid. To simplify to where prSSU-GFP localizes, particle bombardment of *Arabidopsis thaliana* leaf tissue was performed essentially the same as for onion epidermal cells. Figure 5-3 shows transient expression of GFP in *Arabidopsis* leaf tissue. Fig. 5-3A shows GFP fluorescence, Fig. 5-3C shows chlorophyll autofluorescence, and Fig. 5-3B is an overlay of the two. In the overlay, the two colors do not superimpose indicating that GFP (white arrows) and chlorophyll (yellow arrows) are not localized to the same place. GFP is localized outside the chloroplasts in this control image. Unfortunately, transient expression of prSSU-GFP was not observed in leaf tissue bombarded with that construct, but this may provide a way to follow prSSU-GFP into chloroplasts *in vivo*.

DISCUSSION

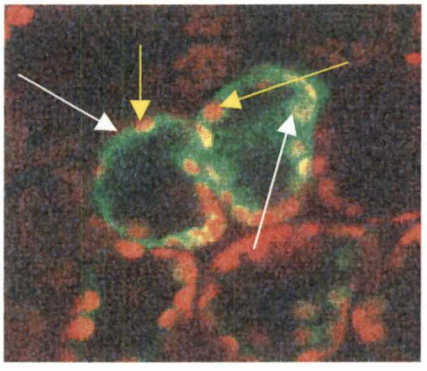
Targeting and translocation of chloroplast precursors has been studied in great detail using *in vitro* import assays and isolated organelles. While *in vitro* assays have been extremely useful in gaining insight into protein translocation, they are non-physiological nonetheless. In addition, inconsistencies between *in vitro* and *in vivo* targeting have been observed (Kindle & Lawrence 1998, Rensink et al 1998). Until advances in molecular techniques, *in vivo* investigations were difficult to perform. Now it is possible to investigate protein targeting and translocation to chloroplasts *in vivo* using techniques of genetic transformation either transiently, as was done here, or by stable integration of DNA into the genome through *Agrobacterium*-mediated methods.

Fig. 5-3. Transient expression of GFP in *Arabidopsis* leaf tissue. *A*, GFP expression in leaf tissue. *B*, Overlay of (A) and (C). *C*, Chlorophyll autofluorescence. Images were made on a Leica CLSM at 40X. White arrows indicate GFP and the yellow arrows indicate chlorophyll autofluorescence.

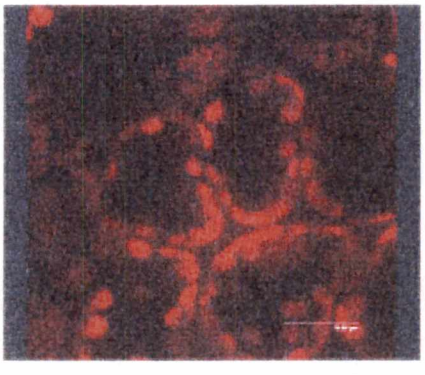
A.



B.



C.



As a component of Rubisco, small subunit and its precursor have been extensively studied (for example, Broglie et al 1984, Dabney-Smith et al 1999, Highfield & Ellis 1978, Smrcka et al 1991, Spreitzer et al 2001), while studies on the precursor's targeting and translocation activities *in vivo* are lacking. This demonstrates the utility of using *in vivo* assays for the future for studying the effects of mutation within the transit peptide on targeting and/or translocation within the cell.

Transient Expression in Onion Epidermal Cells

Onion epidermal cells are easily visualized by low objective (10X) light microscopy because they are fairly large cells, about 300-500 μm in length. While onion epidermal cells contain leucoplasts, they lack chlorophyll-containing chloroplasts. Recently, however, two different laboratories demonstrated targeting of GFP fusion chloroplast-localized proteins to punctate regions presumed to be leucoplasts, supporting our results that the process of targeting and translocation to leucoplasts share common Toc components and recognize the prSSU transit peptide (Kohchi et al 2001, Moller et al 2001). These experiments set precedence for presumed localization of prSSU-GFP to leucoplasts. Similar to what was seen here, both groups saw a regular punctate pattern of transient expression of the GFP fusion protein, which contrasts with the overall diffuse pattern for GFP alone. In contrast to what was observed here, however, both groups only saw the regular punctate pattern and no fluorescence in the nucleus or large intensely fluorescing regions presumably fusion protein aggregates (Kohchi et al 2001, Moller et al 2001). Possible explanations for the fluorescence seen in or on the surface of the nucleus

include [1]no localization to the nucleus, but rather large aggregation of fusion protein in the cytoplasm immediately adjacent, [2]residual localization due to supersaturation by very efficient prSSU-GFP expression because the gene is behind the dual 35S CaMV promoter, or [3]mistargeting of the prSSU-GFP fusion protein to the nucleus possibly due to saturation of plastid translocation components.

In order to try to correct for possibility (1) or (2), we can vary the amount of DNA used for the bombardment of tissue to minimize the amount of DNA coated onto a bead that enters a cell. Minimizing the DNA coated on the beads decreases the amount of DNA that would get into a cell by biolistic methods, potentially decreasing the expression level of the fusion protein. Decreasing the fusion protein level may prevent aggregates from forming, while still allowing targeting. Aggregates may be due to expression levels that are so high as to generate excess fusion protein saturating the translocation apparatuses in the leucoplasts in the cell. If too much fusion protein is present such that some of it remains in the cytoplasm, aggregation of the excess may occur. In addition, we could bombard with less tungsten particles or shorten the time between bombarding the tissue and analysis.

A similar scenario could be described for localization of prSSU-GFP to the nucleus. If expression levels are so high such that copious amounts of fusion protein are present, some of it may end up in the nucleus. GFP does localize to the nucleus when it is by itself (von Arnim et al 1998). One explanation may be because the nuclear pore may not be a barrier to GFP. If the plastid translocation machinery was 'bottlenecked' with too much fusion protein, excess fusion protein may be targeted to the nucleus by the same signals that target non-fusion GFP to the nucleus.

Another way to prevent aggregation or mistargeting of the fusion protein would be to slow down protein expression by incubating bombarded tissue at 4°C. Lowering the temperature of the cells will decrease the rate of transcription and translation as well as protein translocation. If optimal temperature conditions are determined, we could optimize translocation with synthesis of the fusion protein to minimize protein aggregation or mistargeting due to excessive amounts.

Yet a third way to try to curtail protein synthesis would be to alter the transcriptional or translational efficiency by which the prSSU-GFP chimera is expressed. Reducing the dual 35S CaMV promoter region to a single 35S CaMV promoter region may decrease the transcriptional efficiency thus lowering fusion protein synthesis. Additionally, the 35S CaMV promoter could be replaced with an inducible promoter such as the estrogen receptor-based chemical-inducible promoter generated by Zou et al (2000). Manipulating or removing the translational enhancer sequence could serve to reduce translation efficiency also reducing fusion protein synthesis.

The possibility does exist that none of the suggestions above will prevent localization to the nucleus. This may be because leucoplasts do not have active photosynthetic components and therefore may not target or translocate prSSU efficiently. If that is a possibility, the transit peptide alone could be used to target GFP (i.e., without the mature domain of small subunit). Alternatively, a different transit peptide could be used, preferably from a precursor not involved in photosynthesis.

Transient Expression in Arabidopsis *leaf tissue*

One caveat to localization of prSSU-GFP to leucoplasts in onion epidermal cells is the absence of a straightforward way to confirm that localization was indeed to the plastid. This caveat is due to the absence of chromophores such as chlorophyll, which have autofluorescence. To circumvent this problem, transient expression in *Arabidopsis* leaf tissue was pursued. Particle bombardment was the same as for onion epidermal cells for GFP vector with or without prSSU. While GFP expression was detected in control tissue, transformation efficiency was low. Only two cells were seen, from four small (1 cm X 0.5 cm) leaves bombarded (four each for the GFP control and prSSU-GFP). Interestingly, those transformed cells were adjacent to each supporting work by Wymer et al. (2001), and Crawford and Zambryski , (2001) among others, that GFP is able to move from one cell to another through plasmodesmata. Unfortunately, no transformed cells were seen for the prSSU-GFP chimera. In this case where transformation efficiency was low or not at all, manipulating the amount of DNA bound to the tungsten beads would be the first thing to troubleshoot. If not enough DNA was present on the beads, expression may be low or not at all. In addition if too much DNA is present that may serve to 'silence' expression of prSSU-GFP (Niwa et al 1999). Manipulating the amount of DNA bound to the tungsten particles or the amount of particles may help circumvent this problem.

Alternatively, *Arabidopsis* may be a difficult tissue to bombard. Bombardment may cause too much tissue damage to the leaves such that cells are too damaged to express the transgene. If that proves to be the case, protoplasts from tobacco or *Arabidopsisi* could be generated and transformed by electroporation or using PEG (polyethylene glycol) (Bates 1995, Bottin et al 1999, Mathur & Koncz 1998).

Conclusions and Future Directions

These experiments lay the foundation for studying targeting and translocation processes *in vivo*. Preliminary transient expression of prSSU-GFP in onion epidermal cells demonstrates that we could use this technology to follow import of mutant precursors, which have altered import characteristics, relative to wildtype, *in vitro*. The *in vivo* assay would indicate whether changes in the transit peptide, which do affect *in vitro* import, also affects *in vivo* import. This would lend insight into structure/function relationships of transit peptides. Experiments to be performed include transiently expressing the deletion mutants generated in Chapter 3 as GFP fusions. Since those deletions compete to varying levels *in vitro*, and they seemed to have decreasing affinities for Toc34 (Schleiff, et al., manuscript in preparation), they may have altered accumulation patterns *in vivo*. Another set of experiments to perform is transiently expressing two precursors, for example wildtype and one mutant, fused to two different fluorescent proteins with non-overlapping emmision wavelengths. This could allow direct comparison between accumulation patterns of the wildtype precursor and the mutant precursor by quantitating relative fluorescence units per cubic area for a defined area in cells.

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VITA

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