Interaction of artificial lipid bilayers (liposomes) with the purified transit peptide (SS-tp) of the precursor form of the small subunit for ribulose-2,5-bisphosphate carboxylase/oxygenase (prSSU) has been studied using a vesicle-disruption assay (calcein dye release) and electron microscopy. Employing purified forms of Escherichia coli-expressed prSSU, mature small subunit, glutathione S-transferase-transit peptide fusion protein, and SS-tp in dye release studies demonstrated that lipid interaction is mediated primarily through the transit peptide. Using chemically synthesized peptides (20-mers), the lipid-interacting domain of the transit peptide was partially mapped to the C-terminal 20 amino acids of the transit peptide. Peptides corresponding to other regions of the transit peptide and control peptides promoted significantly less calcein release. Interaction between the transit peptide and the bilayer was very rapid and could not be resolved by stopped-flow fluorometry with a mixing time of \(<50\) ms. Interaction between the peptides and bilayer was also lipid class-dependent. Disruption occurred only when the bilayer contained the galactolipid monogalactosyldiacylglycerol (MGDG). The extent of bilayer disruption directly correlated with the relative concentration of MGDG in the liposome, with maximum calcein release occurring in 20 mol % MGDG liposomes. Lipid bilayers with greater than 20 mol % MGDG could not be achieved as determined by calcein entrapment. Electron microscopy of the liposomes before and after addition of the transit peptide suggested that the transit peptide induced a dramatic reorganization of lipids. These results are discussed in light of a possible mechanism for the early steps in protein transport that may involve polymorphic changes in the envelope membrane organization to include localized non-bilayer \(H_{II}\) structures.
membrane, which together constitute the chloroplast envelope. Both membranes have an unusual lipid content, containing monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfolipid, and the negatively charged phosphatidylglycerol (PG) (19). In fact the chloroplast outer envelope membrane is the only cytoplasmically exposed membrane in the plant cell that contains the galactolipids, MGDG and DGDG. Moreover, the outer membrane has a very high lipid/protein ratio of 3.0, indicating that the lipid domains are largely exposed to the cytoplasm (20). It has been suggested that the unique lipids in the chloroplast envelope may play a direct or indirect role in the protein transport process (21). In fact, it has been shown that treatments which alter the lipid content of the outer envelope can cause a significant change in the protein transport activity (22). The potential involvement of lipids in protein transport is further suggested by the pronounced "membrane-active" nature of targeting sequences in bacterial and endoplasmic reticulum signal peptides (1, 23–25), mitochondrial presequences (26–29), and chloroplast transit peptides (30–32) are capable of interacting with artificial bilayers and monolayers which are devoid of protein components.

In this study we investigated the interactions between SS-tp and artificial bilayers whose composition mimics the chloroplast outer membrane. We utilized purified forms of prSSU, mSSU (the precursor and mature forms of ribulose bisphosphate carboxylase/oxygenase; mSSU, mature form; prSSU, precursor for small subunit; GST, glutathione S-transferase) from Sigma. All other chemicals were analytical grade.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plant MGDG and plant DGDG were purchased from Matreya, Inc. PG and PC were purchased from Boehringer Mannheim. Synthetic peptides corresponding to regions of the prSSU transit peptide were obtained from Multiple Peptide Systems and the purity was shown to be between 60 and 90% as determined by high performance liquid chromatography (data not shown). Glutathione-agarose, S-peptide, and reduced, carboxymethylated lactalbumin, were purchased from Sigma. All other chemicals were analytical grade.

**Preparation of Precursor Proteins and the Transit Peptide**—Overexpressed prSSU and mSSU proteins were isolated from BL21 strain of Escherichia coli as described by Klein and SalTCU (33). Radiolabeled prSSU was obtained by growing the cells for 3 h in methionine/cysteine-deficient media, followed by incubation with Trans35S-label metabolic labeling reagent from ICN. We routinely solubilize the inclusion bodies in the presence of a chemical denaturant such as 6 M guanidine HCl or 8 M urea.

GST-tp, GST, and SS-tp—The proteins/peptides were isolated from GST-gene fusion system.2 The protein of interest was fused to the C terminus of glutathione-S-transferase from Schistosoma japonicum (34) and purified from bacterial lysates by affinity chromatography using glutathione-Sepharose 4B. Fusion protein (GST-tp) was isolated under mild conditions by elution with 5 mM glutathione in phosphate-buffered saline. Transit peptide expressed in the vector pGEX-2T was obtained by cleavage with thrombin. GST was isolated by thrombin cleavage followed by glutathione elution.

**In Vitro Protein Import/Competition Assays**—Dwarf pea seedlings were grown in a EGC TC-30 growth chamber at 17.5°C with 165 lux for 10 days. Intact chloroplasts were isolated by continuous Percoll. Import assays were performed as described previously (35). BrieFLY, 35S-labeled prSSU (5 × 106 cpm/µg) was incubated with freshly prepared chloroplasts (1 mg/ml chlorophyll) in the presence of 3 mM MgATP for 30 min at room temperature. In competition experiments, 20–30 molar excess of the competing protein/peptide was added just before the radiolabeled precursor. Intact chloroplasts were pelleted over a 40% Percoll cushion. Chloroplast pellets were dissolved in 1 × SDS sample buffer and subjected to SDS-PAGE, followed by autoradiography (35).

**Thermolysin Treatment**—Post-imported chloroplasts were incubated at 4°C for 15 min with 200 µg/ml thermolysin in import buffer (35). Enzyme reactions were terminated by adding EDTA to 10 µM. Intact chloroplasts were reisolated over a 40% Percoll cushion. Chloroplast pellets were dissolved in SDS sample buffer and subjected to SDS-PAGE, followed by autoradiography (35).

**Liposome Preparation**—Lipid vesicles were prepared by mixing appropriate mixtures of diacyl lipids (10 µM) in chloroform. Solvent was evaporated under a stream of N2, and the samples were vacuum desiccated for no less than 3 h. The dried lipid film was then hydrated overnight in phosphate-buffered saline, pH 7.8, containing 1 mM EGTA, 0.02% azide, and 50 mM CaCl2. Small unilamellar vesicles were prepared by vortexing the lipid mixture and then sonicating in a bath sonicator (Laboratory Supplies Inc.) for 5 min. Two additional sonication cycles were performed with a 6–12-h interval. Free unincorporated caIein was removed by chromatography on a Bio-Gel A-0.5 column equilibrated in phosphate-buffered saline/EGTA/azide buffer. Liposomes eluted in the void volume fractions showed a calcein fluorescence quenching of >70–80% (see below). Liposomes were used at a lipid concentration of 1–2 µM.

**Fluorescence Quenching Measurements**—Fluorescence of liposomes was performed as described (36) using a Perkin-Elmer LS 50 Spectrofluorometer. Fluorescence excitation was at 490 nm. Fluorescence quenching was calculated from the formula below.

\[
\% \text{ Quenching} = \left( 1 - \frac{F}{F_0} \right) \times 100\%
\]

1 The abbreviations used are: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; prSSU, precursor for small subunit of ribulose-2,5-bisphosphate carboxylase/oxygenase; mSSU, mature form of ribulose-2,5-bisphosphate carboxylase/oxygenase; SS-tp, transit peptide for prSSU; GST, glutathione S-transferase; PC, phosphatidylcholine; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; I.B., chloroplast import buffer; DTT, dithiothreitol; S-peptide, ribonuclease-S-peptide from bovine pancreas; Lkt A16, synthetic peptide from P. hemolytica leucotxin A (amino acids 793–811); Lkt A16, synthetic peptide from P. hemolytica leucotxin A (amino acids 780–796); F1, C-terminal C-terminal 16 amino acids of ferritin heavy chain; PAGE, polyacrylamide gel electrophoresis; OM, outer membrane; prFd, chloroplastic precursor of ferrodoxin.
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proteins and peptides. Purity of the peptides/proteins was determined by SDS-PAGE, followed by Coomassie Brilliant Blue staining (Fig. 1A). Both prSSU and mSSU (tobacco) were expressed in *E. coli* using the pET expression system (Novagen), as described in Ref. 33. The full-length transit peptide of pea prSSU was expressed in *E. coli* as a C-terminal fusion protein to GST using the pGEX (Pharmacia) expression system. The transit peptide was removed from GST by cleavage with thrombin. These proteins were judged near homogeneous, based on Coomassie Brilliant Blue staining.

To verify the biological activity of the proteins produced in *E. coli*, we performed *in vitro* chloroplast import assays as described in Ref. 35. These assays test the import competence of prSSU as well as the ability of other proteins/peptides to competitively inhibit the import of *35S*-prSSU (Fig. 1B). The data show that prSSU competes with GST for import (translocation); however, the inhibition was not complete at the concentration in this experiment. Interestingly, prSSU appears to accumulate as a membrane-bound form in the presence of competing SS-tp, suggesting that SS-tp competitively blocks import and processing of the prSSU protein after binding to the chloroplast.

To confirm that the processed form of prSSU is imported and authentically processed in the chloroplast stroma, intact chloroplasts were treated with thermolysin to digest surface-exposed forms of prSSU or mSSU (Fig. 1, lanes 7–12). Thermolysin is unable to cross the outer envelope membrane (37) and, therefore, should digest only the proteins exposed or bound to the surface of the chloroplast. The majority of processed mSSU is resistant to thermolysin, indicating that mSSU is localized in the stroma (lanes 7–12). In the case where import was blocked with the SS-tp (Fig. 1A, lane 6), the form of prSSU that accumulated was still associated with the exterior of the chloroplast since it was removed by thermolysin digestion (Fig. 1A, lane 12). However, SS-tp does not block prSSU from binding to the chloroplast (lanes 6 and 12). These data indicate that SS-tp blocks prSSU translocation across the envelope membrane without affecting its ability to initially bind to the transport apparatus. Taken together, these results confirm the biological activity of the proteins/peptides used in this study.

### Table I

| Plastid membrane lipid composition | Lipid composition |
|-----------------------------------|------------------|
|                                  | MGDG | DGDG | SL | PC | PG | PI |
| Spinach chloroplasts              |      |      |    |    |    |    |
| Thylakoid                         | 57   | 27   | 7  | 0  | 7  | 1  |
| Total envelope                    | 32   | 30   | 6  | 20 | 9  | 4  |
| Inner membrane                    | 49   | 30   | 5  | 6  | 8  | 1  |
| Outer membrane                    | 17   | 29   | 6  | 32 | 10 |    |
| Pea etioplasts                    |      |      |    |    |    |    |
| Total envelope                    | 34   | 31   | 6  | 17 | 5  | 4  |
| Cauliflower proplastids           |      |      |    |    |    |    |
| Total envelope                    | 34   | 31   | 6  | 17 | 5  | 4  |
| OM liposomes                      | 20   | 30   | 40 | 10 |     |    |

**SL**, sulfolipid.

![Image](image.png)

**A** shows the data from an import assay using *35S*-prSSU in the presence or absence of a competing protein/peptide. A 20% excess of cold prSSU completely blocked the import and processing of *35S*-prSSU (Fig. 1B, lane 2). As expected, mSSU, which lacks the SS-tp, had no effect on the import and processing of prSSU. SS-tp competed with prSSU for import (translocation); however, the inhibition was not complete at the concentration used in this experiment. Interest-
Transit Peptide Interactions with Chloroplast Lipids

Precursor/Lipid Interaction Is Mediated through the C Terminus of SS-tp—Fig. 3 shows the amino acid sequence of the peptides used in the following experiments. The transit peptide of prSSU, which consists of 58 amino acids, is both necessary and sufficient for protein transport into chloroplasts (38). As shown in Fig. 4, SS-tp induced a rapid dye release from OM liposomes. Furthermore, liposome lysis was concentration-dependent, resulting in a maximum of ∼50% dye release at 6–8 μM peptide. Taken together with data in the previous section, these data indicate that the precursor-lipid interaction is mediated primarily through SS-tp. Furthermore, this peptide-liposome interaction was specific, since the control peptides (S-peptide, LktA18, LktA16, and F16 C-terminus) whose sequence is shown in Fig. 3, generated no significant dye release from the OM liposomes.

Next, we investigated the interaction between OM liposomes and four 20-mer peptides which correspond to different regions of SS-tp (Fig. 3). As indicated in Fig. 4, the C terminus of SS-tp (SS-tp-(41–60)) induced the greatest degree of liposome lysis. The N-terminal 20 amino acids also caused a significant dye release. In contrast, the more hydrophobic middle region of the transit peptide, SS-tp-(21–40) did not induce liposome destabilization since the amount of dye released was insignificant. Table II summarizes the data discussed thus far in order of decreasing activity: prSSU > SS-tp > SS-tp-(41–60) > SS-tp-(1–20) > mSSU − reduced, carboxymethylated lactalbumin ≥ SS-tp-(31–50) ≥ SS-tp-(21–40) > S-peptide > GST-tp − GST.

The finding that SS-tp-(41–60) was more disruptive to the OM liposomes than SS-tp-(31–50) suggests that the C-terminal 10 amino acids of the SS-tp are the most “membrane-active” region of the 60 amino acid SS-tp. Therefore, the precursor/lipid interaction was mediated primarily via the extreme C terminus of SS-tp. However, the fusion protein GST-tp caused very little vesicle disruption (Fig. 2B). This construct had no more vesicle disruption activity than GST alone. This result suggests that the transit peptide must exist either as a free peptide or as an N-terminal extension of prSSU to engage the bilayer. In both cases, the N-terminal domain would be free.

Precursor/Lipid Interaction Is Lipid Class-dependent—The chloroplast outer envelope membrane contains ∼20% MGDG, the only HII phase-forming lipid present in this membrane (19). To investigate whether the SS-tp/lipid interaction is mediated via this non-bilayer-forming lipid, we made calcein-encapsulated liposomes with varying lipid compositions. As shown in Fig. 3A, SS-tp interacted only with liposomes containing MGDG. Liposomes composed of 100% PC, PC/DGDG (7:3 molar ratio), or PC/PG (9:1 molar ratio) did not produce a significant calcein release when incubated with SS-tp. As expected (Fig. 3B), mSSU failed to promote dye release from any of the liposome compositions tested. Similar to what was seen with SS-tp, prSSU promoted dye release only from liposomes that contain MGDG.

To confirm that liposome destabilization, as measured by calcein release, was truly dependent on the presence of MGDG, we made liposomes containing varying molar percentages of MGDG. Mol % of PC was varied to compensate for the difference in MGDG. DGDG and PG concentrations were kept constant (5 and 10 mol %, respectively). As shown in Fig. 6, increasing MGDG content resulted in increased lysis of liposomes in the presence of increasing amounts of SS-tp. As the mol percentage of MGDG was increased, the percentage of calcein release also increased. Even at the maximum (20% MGDG), however, the degree of calcein release was peptide-dependent and increased linearly with SS-tp concentration.

The observation that maximum calcein release occurred at 20 mol % MGDG is interesting for two reasons. First, 20 mol %

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Fig. 2. Fluorescence of calcein containing liposomes. A, fluorescence emission spectra of MGDG/PC/PG liposomes containing entrapped calcein upon incubation with either buffer alone, Triton X-100, 0.45 M urea or increasing concentrations of prSSU. Liposomes were used at a level representing 1–2 μM total lipids. B, results from precursor protein-induced dye release of liposomes from A plotted as function of protein concentration. Fluorescence of liposomes was performed as described by Pinnaduwage and Huang (36) using a Perkin-Elmer LS50 Spectrofluorometer. Percent dye release was determined as described by Pinnaduwage and Huang (36).

ygenase (mSSU) did not promote any significant calcein release even at the highest concentration tested, indicating that only the precursor form of the protein interacts sufficiently with the liposomes to cause vesicle disruption. The control protein GST also failed to induce a significant release of dye from the liposomes.

Both prSSU and mSSU were synthesized in E. coli as inclusion bodies and solubilization in 8 M urea or 6 M guanadimine HCl yielding an unfolded form of the protein. To test whether other unfolded proteins could interact with the bilayer and produce a calcein release, we incubated the liposomes with reduced carboxymethylated α-lactalbumin, a protein that is permanently unfolded. This protein had little effect on the liposomes even at the highest concentrations tested. We also confirmed that urea alone had no effect on calcein release from the liposomes. These results indicate that only the transit peptide-containing prSSU interacts with OM liposomes in such a way that promotes calcein release.
wasthemaximumamountofMGDGthatwecouldincorporate
intoliposomeswhilemaintainingcalceinentrapment(datanot
shown). Second, the chloroplast outer envelope contains ap-
proximately 20 mol % MGDG in vivo. These observations sug-
gest that 20 mol % is the maximum amount of this MGDG that 
can be maintained in this bilayer either in vitro or in vivo. Fur-
thermore, our data indicate that this $H_2$ phase-forming lipid may mediate the in vitro interaction between the precur-
sor/transit peptide and the lipid bilayer, leading to calcein 
release. Collectively, these findings argue that the chloroplast 
outer envelope resides in a meta-stable state, which is suscep-
tible to additional destabilization by interacting with SS-tp 
alone or as part of prSSU.

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into liposomes while maintaining calcein entrapment (data not 
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tible to additional destabilization by interacting with SS-tp 
alone or as part of prSSU.

Kinetic Analysis of Peptide/Lipid Interactions—The previ-
ous experiments have demonstrated the ability of prSSU and 
SS-tp to interact with lipid bilayers in a way that causes loss of 
vesicle barrier function. However, these experiments reflect 
steady state measurements once the interaction has reached 
equilibrium. To investigate the rate of dye release from lipo-
somes upon interaction with SS-tp, we performed rapid 
stopped-flow fluorometry. This technique allows us to rapidly 
mix the liposomes and peptides, and monitor the fluorescence 
increase as a function of time. The mixing time of our instru-
ment was 50 ms. Nonetheless, the rate of interaction was too 
rapid to be resolved even by this stopped-flow device. The 
increase in calcein fluorescence had already reached its maxi-

TABLE II
Activity of proteins/peptides used in calcein release assay

| Protein/peptide used | Calcein release | [Peptide]$_{50\text{\% max. release}}$ |
|----------------------|----------------|-------------------------------------|
| prSSU                | 86             | 1.8                                 |
| mSSU                 | 16             | 2.5                                 |
| RCMLA$^a$            | 12             | 2.5                                 |
| GST                  | <4             | Not determined                      |
| GST-tp               | <5             | Not determined                      |
| Peptides             |                |                                     |
| SS-tp$_{1-58}$       | 48             | 3.0                                 |
| SS-tp$_{1-20}$       | 29             | 17.0                                |
| SS-tp$_{21-40}$      | 8              | Not determined                      |
| SS-tp$_{41-60}$      | 47             | 19.0                                |
| SS-tp$_{61-80}$      | 14             | 16.0                                |
| S-peptide            | 8              | Not determined                      |

$^a$ RCMLA, reduced, carboxymethylated lactalbumin.
tides derived from the transit peptide share the ability to cause a rapid release of calcein from MGDG-containing bilayers. The precise mechanism leading to this calcein release is not known. These experiments are not able to distinguish whether calcein release is the result of the formation of discrete peptide-induced channels or, alternatively, whether these sequences cause structural changes in the lipid bilayer that permit calcein release. To attempt to resolve these two opposing models, we stained the liposomes with the negative stain, uranyl acetate, and visualized peptide-induced morphological changes by electron microscopy. We examined two different liposome preparations: 1) the OM liposomes that mimic the composition of the chloroplast outer envelope, and 2) liposomes containing PC alone, to serve as a control. The PC-liposomes did not disrupt when incubated with the transit peptide (Fig. 8, A and B). This observation is consistent with fluorescence data, which showed little effect of the transit peptide on release of calcein from this
liposome preparation (Fig. 5, A–C). In contrast, when the OM liposomes were treated with the transit peptide at 10 μM, a marked structural change in vesicle morphology was apparent (Fig. 8, D–F). Although the kinetics of this transition have not been carefully studied, the morphological change occurred less than 2 min after peptide addition. This effect involved the conversion of spherical liposomes (Fig. 8C) into a stacked array of elliptical vesicles. Analysis of 456 particles indicated that >70% of the liposomes underwent a change in morphology from an initial spherical shape to an elliptical and/or stacked array after addition of the transit peptide (data not shown). Once formed, these elliptically shaped particles appeared to associate with one another and formed a stacked array of elliptical discs. Although the majority of the particles were found in stacked arrays with 2–5 discs, ~30% of the vesicles remained spherical that stained less electron dense than the aggregated vesicles. Although the significance of these morphological changes is not self-evident, they suggest that interaction with SS-tp may induce a dramatic local lipid reorganization.

DISCUSSION

In this study, we have investigated the interactions between SS-tp and artificial bilayers whose composition mimics the chloroplast outer membrane. We have utilized purified forms of prSSU, mSSU, SS-tp, and synthetic peptides which correspond to four domains of the transit peptide. We have demonstrated that the interaction of prSSU with artificial bilayers is mediated primarily via the transit peptide. The membrane interacting domain has been mapped to the C-terminal 10 amino acids of the transit peptide. The interaction is strongly dependent on the lipid content of the artificial bilayer, requiring the galactolipid, MGDG. Furthermore, the interaction is very rapid and leads to a drastic change in the morphology of the liposomes. This work represents only the second attempt to characterize the interaction of a full-length chloroplast transit peptide with artificial membranes and represents the longest transit peptide studied to date (31).

The C-terminal Domain of SS-tp Disrupts Bilayers—Fluorescence emission spectra of dye-filled liposomes in the presence of SS-tp indicate that the transit peptide region of the precursor was sufficient to destabilize membranes and promote calcein release, although the full-length precursor produced an even greater effect in the same assay. The magnitude of liposome destabilization by prSSU alone exceeded the additive effect of the transit peptide alone and mSSU alone (86% release versus 60% for SS-tp + mSSU), suggesting that the mature domain enhances the vesicle disruption activity of the transit peptide in cis, when it is physically fused at its C terminus. Recently, an analogous study of the chloroplastic precursor of ferredoxin (prFd) and its full-length transit peptide also demonstrated that the transit peptide was active in disrupting vesicles, but surprisingly, the full-length precursor was not (30). The authors suggest that the ferredoxin mature domain somehow prevents vesicle lysis. Unknown structural differences between prSSU and prFd probably account for the dramatic difference in the ability of these two chloroplast-destined precursors to disrupt bilayers. Nonetheless, finding that the mature domains of ferredoxin and small subunit appear to modulate the membrane-interactive properties of their respective precursors (decreased for prFd, increased for prSSU) suggests that the junction between the transit peptide and mature protein may significantly influence the structure and function of the transit peptide.

We have shown that the transit peptide is able to disrupt vesicle barrier function as a free peptide, fused to the N terminus of mSSU, but not as a C-terminal domain to GST. Our data strongly suggest that the N terminus must be free in order to engage the lipid bilayer. This finding is supported by in vitro competition studies that show that neither GST-tp nor His-tag-tp (a fusion protein with a His-tag and S-tag at the N terminus of SS-tp) are able to compete with prSSU for binding or import. The rationale behind the requirement for a free N terminus in lipid bilayer interaction is not entirely clear; however, it could involve a mechanism similar to the “loop out mechanism” that was initially proposed to describe the interaction of signal peptides with their target membranes (39).

Perry and co-workers (40) also found that SS-tp-(1–20) lysed chloroplasts. They speculated that perhaps part of the amino terminus of the transit peptide is involved in lipid-mediated binding to the chloroplast surface. To further investigate this possibility, we employed 20-residue synthetic peptides in the calcine release assay. Contrary to the proposal of Perry et al., however, we found that the C-terminal 20 amino acids of the transit peptide (SS-tp(41–60)) were most destabilizing to liposomes. Furthermore, the overlapping peptide, SS-tp-(31–50), was significantly less active than SS-tp-(41–60), suggesting that the maximum bilayer-disrupting activity may actually map to the C-terminal 10 amino acids of SS-tp(41–60), of which only 7 amino acids are in the transit peptide proper (Fig. 3). Because 3–5 fold more SS-tp(41–60) was required to induce the same amount of calcine release as was induced by the entire 60-amino acid transit peptide construct, the C terminus

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FIG. 8. Electron microscopy of liposomes. Electron micrographs of PC control liposomes (A and B) or MGDG/PC liposomes (C and D). Micrographs were taken before (A and C) or after (B and D) the addition of the purified transit peptide, SS-tp. Liposomes were treated with or without the peptide for 10 min at room temperature. The liposomes were then negatively stained with 0.1% uranyl acetate and viewed in a Hitachi 600 electron microscope at 75 kV. All photos (A–D) represent an equal magnification (×100,000). Panels E and F represent enlargements of regions of panel D.
Transit Peptide Interactions with Chloroplast Lipids

may require other regions of the transit peptide, perhaps cooperatively, to interact with the chloroplast outer envelope. Interestingly, we found that the central region of the transit peptide, SS-tp-(21–40) and SS-tp-(31–50), had little or no activity in the calcium release/liposome disruption assay. An earlier study utilizing synthetic peptides corresponding to the same 20 amino acid regions of SS-tp demonstrated with a monolayer-insertion assay that SS-tp-(41–60) was most interactive when the monolayer was made either from MGDG or from extracts of the outer envelope (41). Although their work indicated a much stronger lipid interaction for SS-tp-(21–40) in the presence of DG, it still supports our conclusion that SS-tp interacts with artificial membranes primarily via its C terminus.

Recent reports indicate that prFd and its purified transit peptide both are capable of inducing a change in conductivity across the chloroplast membranes (42). The authors suggest that the increase in conductivity results from a precursor-mediated opening of the translocation pores. Like our study, the ferredoxin report also implicates the transit peptide C terminus, since a mutant form of prFd (preFd-Δ7), which is deleted for the C-terminal 7 amino acids of the transit peptide, shows no ability to induce a change in conductivity, nor does the mutant precursor function as a competitive inhibitor of binding and import. Their findings are entirely consistent with our observation that the C terminus of the prSSU transit peptide is required to induce large changes in membrane permeability. Therefore, two independent laboratories have now established that the membrane-interacting activity associated with chloroplast transit peptides is mediated by a C-terminal domain that is only 7 amino acids long.

Extrapolation of these findings to the larger population of chloroplast-targeted proteins may have significant implications for protein targeting to the chloroplast and its various compartments. For example, it was previously established that the stromal-targeting domain of plastocyanin does not interact with artificial bilayers that are composed of either PC/PG (molar ratio 9:1) or MG/DO/PC/PG (molar ratio 15:35:40:10, representative of the chloroplast envelope), even at concentrations as high as 25 μM (43). This result may be explained by fact that the plastocyanin transit peptide contains not only a 43-amino acid stromal-targeting domain but also an additional 23-amino acid C-terminal domain in vitro, which is proposed to function as the thylakoid-transfer domain. By analogy to our work and the findings of Bulychev and co-workers (42), the membrane-interactive region of the plastocyanin bipartite transit peptide is predicted to lie exclusively in the adjacent thylakoid-targeting domain. Consistent with this hypothesis, the putative thylakoid-targeting domain is rich in hydrophobic amino acids and is believed to resemble the signal peptide for secreted proteins (44). Furthermore, chimeric proteins containing only the 1–43 region of the plastocyanin transit peptide fused to dihydrofolate reductase failed to import into chloroplasts in vitro; however, when the chimeric protein contained at least residues 1–53 of plastocyanin, import was restored (45). Thus, with plastocyanin, the presence of a membrane-active domain as small as 10 amino acids may be sufficient to restore transport across the chloroplast envelope. These observations provide some insight into the minimal size of the membrane-interacting domain needed for successful binding and import into chloroplasts. They also suggest that the requirement for a membrane-interacting domain is a general feature of chloroplast transport and is probably independent of subcompartmentalization. Further experimentation will be required to determine if proteins localized to the inner membrane, outer membrane, and inter-membrane space of the envelope share this same targeting requirement.

SS-tp/Lipid Interaction Requires a Non-bilayer-forming Lipid—Without exception, membranes that are active in protein translocation contain significant levels of lipids that strongly prefer to adopt a non-bilayer structure. Specific examples include phosphatidylethanolamine (PE) in the inner membrane of E. coli, cardiolipin (CL) in the endoplasmic reticulum and mitochondria, and MGDG in the chloroplast envelope and thylakoids (46). These unique lipids are believed to exhibit a wedge-like molecular shape and they prefer to form an H_{11} phase when isolated. Several reports have suggested that these non-bilayer-forming lipids are required for protein translocation. Consistent with this notion, our work has demonstrated that the interaction between the transit peptide and the artificial bilayers is dependent on lipid composition. More specifically, this interaction is only observed when the liposomes contain MGDG. Previous monolayer insertion experiments also observed an MGDG dependence for the ferredoxin transit peptide (31) and a synthetic peptide corresponding to the last 20 amino acids of SS-tp (41).

A similar lipid class dependence has been observed for interactions between mitochondrial presequences and artificial membranes (47, 48). It was first observed that the presequence to the mitochondrial cytochrome oxidase subunit IV would only interact with liposomes containing CL (47). More recently, mitochondrial presequences have been shown to induce contact sites between large unilamellar vesicles (49) and also between monolayers and large unilamellar vesicles (48). In both cases, this interaction was dependent on the presence of CL. Again, it was believed that a unique structural aspect of CL was important, since other anionic phospholipids failed to promote the interaction. Although the molecular architecture of these contact sites is not known, it was proposed that a local non-bilayer intermediate formed which CL could stabilize due to its H_{11} phase preference (46). In support of this model, contact sites isolated from both yeast mitochondria (50) and mouse liver mitochondria (51) are substantially enriched in CL.

Possibly the strongest evidence for the essential involvement of non-bilayer lipid structures in protein transport comes from the recent characterization an E. coli mutant that is devoid of PE (52). In this strain, growth is tightly coupled to increased synthesis of CL, which in the presence of specific divalent cations (Mg^{2+}) will adopt a preference for the non-bilayer phase (53). These cells grow only in the presence of specific cations, indicating that polymorphic regulation of membrane-lipid composition is essential for cell viability. In vitro studies with in vitro studies with inverted membrane vesicles from this mutant further indicate that protein translocation is dependent on either the presence of the cations listed above or by the incorporation of the non-bilayer lipid, PE. The authors conclude that non-bilayer lipids are essential for efficient protein transport across the plasma membrane of E. coli, and that cation-induced conversion of CL to a non-bilayer-forming lipid permits protein secretion and thus growth of this mutant.

The fact that the SS-tp/liposome interaction observed in this study is specific for MGDG supports a possible involvement of non-bilayer structures in chloroplast protein transport. Interestingly, the only other studies that have addressed the membrane interacting activity of a full-length higher plant transit peptide demonstrated that the ferredoxin transit peptide/lipid interaction is strongest with the anionic lipids, PG and sulfonolyminosyldiacylglycerol (31). However, even with this transit peptide, a significant interaction occurs with monolayers that contain MGDG.

SS-tp/Lipid Interaction Is Very Rapid, Resulting in Altered Liposome Morphology—The mechanism by which prSSU and
SS-tp promote dye release is not known. We have measured that calcein release is very rapid (<50 ms), in contrast to several other studies that have demonstrated that peptide/lipid interactions exhibit much slower kinetics, on the order of 2–10 min. Slow kinetics have been reported for mitochondrial presequences (29, 47, 49), signal peptides (24), and trans peptide (31). The basis of such disparate kinetics is unknown.

Electro-physiological studies on intact chloroplasts have also demonstrated a much slower change in envelope resistance, such as protein/lipid ratio, the potential buffering capacity of protein components, or the fact that the integrity of intact chloroplast is enhanced by two distinct bilayers.

Finally, we have observed a pronounced change in liposome morphology upon interaction with SS-tp. Mitochondrial presequences (29) and bacterial signal peptides (54) have also been shown to cause membrane aggregation. However, we are not aware of any other example in the literature where the morphology of individual liposomes changes so dramatically. Our OM liposomes were initially spherical and fairly homogeneous. After exposure to the SS-tp, however, the liposomes transformed into a clearly heterogeneous population containing a new component of elliptical and stacked vesicles. Although this type of electron microscopy cannot provide a three-dimensional image, it is possible that these new arrays represent a stack of disc-shaped vesicles. This observation evokes the obvious comparison to the appressed membrane stacks found in the thylakoid grana.

In summary, the results of this study provide new evidence for the potential involvement of specific precursor/lipid interactions that could be intimately involved in the chloroplastic protein-import process. Our work also suggests that chloroplastic pretransit peptides may universally contain a membrane interacting domain that is restricted to its C terminus and may even span into the N terminus of the mature domain. The precursor/lipid interaction reported in this paper is dependent on the presence of the non-bilayer-forming lipid, MGDG, providing additional evidence for the universal involvement of non-bilayer structures in the insertion and translocation of proteins across membranes. Clearly, a comprehensive model of protein translocation will require new experiments that simultaneously incorporate the role of membrane lipids with the recently identified proteinaceous components of the protein translocation apparatus.