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Insertion of Leader Peptidase into the Thylakoid Membrane during Synthesis in a Chloroplast Translation System

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The mechanisms of targeting and insertion of chloroplast-encoded thylakoid membrane proteins are poorly understood. In this study, we have used a translation system isolated from chloroplasts to begin to investigate these mechanisms. The bacterial membrane protein leader peptidase (Lep) was used as a model protein because its targeting and insertion mechanisms are well understood for *Escherichia coli* and for the endoplasmic reticulum. Lep could thus provide insight into the functional homologies between the different membrane systems. Lep was efficiently expressed in the chloroplast translation system, and the protein could be inserted into thylakoid membranes with the same topology as in *E. coli* cytoplasmic membranes, following the positive-inside rule. Insertion of Lep into the thylakoid membrane was stimulated by the *trans*-thylakoid proton gradient and was strongly inhibited by azide, suggesting a requirement for SecA activity. Insertion most likely occurred in a cotranslational manner, because insertion could only be observed if thylakoid membranes were present during translation reactions but not when thylakoid membranes were added after translation reactions were terminated. To halt the elongation process at different stages, we translated truncated Lep mRNAs without a stop codon, resulting in the formation of stable ribosome nascent chain complexes. These complexes showed a strong, salt-resistant affinity for the thylakoid membrane, implying a functional interaction of the ribosome with the membrane and supporting a cotranslational insertion mechanism for Lep. Our study supports a functional homology for the insertion of Lep into the thylakoid membrane and the *E. coli* cytoplasmic membrane.

INTRODUCTION

Little is known about the mechanisms of targeting and insertion of the chloroplast-encoded thylakoid membrane proteins, despite their importance in chloroplast function (Jagendorf and Michaels, 1990; Kuras et al., 1995; van Wijk et al., 1995; Rochaix, 1996; Choquet et al., 1998). Because chloroplasts were originally derived from prokaryotes, it can be postulated that targeting and insertion pathways of the chloroplast-encoded proteins are likely to show strong similarity to those in the bacterium *Escherichia coli*. Indeed, several chloroplast homologs of *E. coli* targeting and insertion components have been identified, such as the 54-kD subunit of the signal recognition particle (SRP) (Franklin and Hoffman, 1993) as well as the translocon components SecA (Nakai et al., 1994; Yuan et al., 1994) and SecY (Laidler at al., 1995). It is also possible that precursor mRNA is directly targeted to the thylakoid membrane via an interaction of the 5′ untranslated region (5′ UTR) with mRNA binding proteins, as has been observed in yeast mitochondria (Rochaix, 1996; Sanchirico et al., 1998).

Based on a number of observations in chloroplasts, such as run-off translations of thylakoids with bound ribosomes (rough thylakoids) and detection of translation intermediates in the membrane, it can be postulated that insertion of the polytopic chloroplast–encoded membrane proteins occurs cotranslationally (Margulies and Michaels, 1975; Herrin and Michaels, 1985; Klein et al., 1988; van Wijk et al., 1996). Thus, to reconstitute this targeting and insertion process, a chloroplast in vitro initiation/translation system is required in which exogenous transcripts can be accurately translated. The recent discovery of a translation system isolated from tobacco chloroplasts has opened up novel possibilities to address these important processes in more detail (Hirose and Sugiura, 1996).

We decided to study the mechanisms of protein insertion by expressing a model protein, the *E. coli* leader peptidase (Lep), in a chloroplast translation system and to attempt insertion of Lep into the thylakoid membrane. Lep is a 37-kD *E. coli* cytoplasmic membrane protein with two transmembrane domains and a long periplasmic C-terminal domain, the P2 domain. The cytoplasmic P1 loop connects the two transmembrane domains (Figure 1A). Lep has served as a model protein to study targeting and insertion of membrane proteins into the cytoplasmic membrane of *E. coli* (e.g., Wolfe et al., 1985; Dalbey and Wickner, 1987; Moore et al., 1988; Valent et al., 1998) as well as into microsomes

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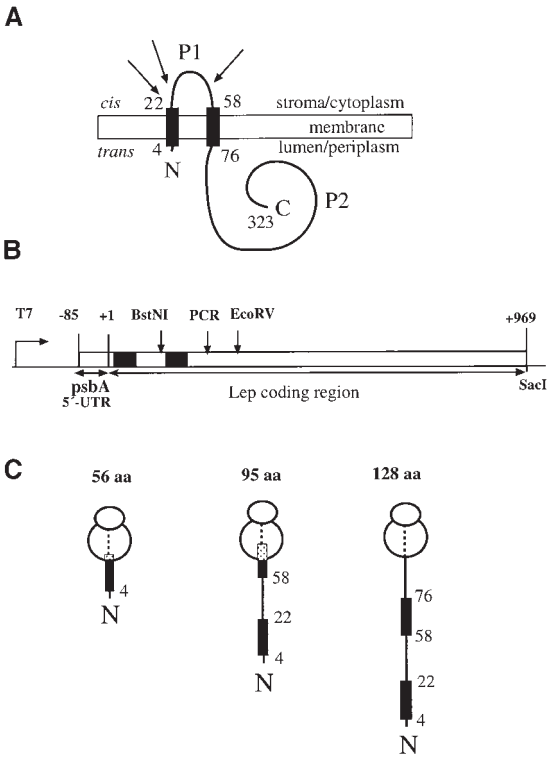

Figure 1. DNA Template, Predicted Topology, and Formation of Lep RNCs.

(A) Topology of Lep in the *E. coli* cytoplasmic membrane as well as the predicted topology of Lep in the thylakoid membrane. The P1 loop is located on the *cis*-side of the membrane (the stromal or cytoplasmic side), whereas the long P2 domain is translocated to the *trans*-side (the lumenal or periplasmic side). The transmembrane domains are indicated as black boxes, and the numbers indicate the amino acid residues at the beginning and end of the transmembrane domains and at the C terminus. N and C indicate the N and C termini of Lep, respectively. Arrows indicate the accessibility by proteases from the stromal or cytoplasmic side.

(B) Schematic representation of the DNA template used to synthesize full-length and truncated versions of Lep. The location of the restriction sites (EcoRV and BstNI), the annealing site of the reverse polymerase chain reaction (PCR) primer, and the transmembrane domains (black boxes) are indicated.

(C) Schematic predicted representation of truncated versions of Lep when attached to the ribosome, forming RNCs. The transmembrane domains are indicated as black boxes, and the amino acid residue numbering is indicated. The regions of the nascent chains buried in the ribosome (assumed to be 30 to 40 amino acids [aa]) are indicated by broken lines and stippled rectangles (for transmembrane domains).

(membranes of the endoplasmic reticulum [ER]) (Nilsson et al., 1994; Mothes et al., 1997). In *E. coli*, assembly of Lep is dependent on SRP, SecA, and SecY (Wolfe et al., 1985; de Gier et al., 1996; Valent et al., 1997, 1998). Assembly of Lep into microsomes also requires the SRP and the Sec61 translocon, and detailed in vitro studies indicate a cotranslational mode of insertion of Lep (Mothes et al., 1997).

In this study, we show that Lep could readily be translated and stably inserted into thylakoid membranes in the chloroplast translation system. Evidence is presented that insertion of Lep occurred cotranslationally but not post-translationally. Ribosome nascent chain complexes (RNCs) of truncated versions of Lep bound with high affinity to the thylakoid membrane, indicating that functional junctions between the ribosome nascent chains and the membrane (translocon) were made. Azide, a potent inhibitor of ATP-dependent activity of SecA, strongly decreased proper insertion of full-length Lep but not the strong association with the thylakoid membrane. This suggests that translocation of the long P2 domain required ATP-dependent SecA activity, analogous to the translocation of the P2 domain across the cytoplasmic membrane of *E. coli*. When azide was added together with an uncoupler of the proton gradient, insertion of Lep was completely eliminated, whereas the addition of the uncoupler alone reduced insertion only slightly. This indicates that the proton gradient plays an additional role in the translocation of the P2 domain.

In this study, we have expressed and inserted a polytopic membrane protein into thylakoid membranes by using a chloroplast translation system. We expect that this experimental setup will allow us to identify and characterize the mechanisms and components involved in targeting, insertion, and assembly of chloroplast-encoded thylakoid membrane proteins.

RESULTS

To express Lep in the chloroplast translation system, the gene encoding Lep was cloned behind the 5′ UTR of the chloroplast *psbA* gene, which encodes the D1 protein (Figure 1B). This construct was used as DNA template for mRNA synthesis. When the chloroplast translation system isolated from tobacco leaves was programmed with this mRNA, a high translation yield of full-length Lep was observed without the problem of premature termination (Figure 2A, left).

Pea plants have been the most popular source of plant material for chloroplast import and targeting experiments for many years, because pea plants can be grown rapidly and the isolation of large amounts of intact chloroplasts is relatively easy. Therefore, we made active translation extracts from isolated pea chloroplasts by using the same experimental protocols that were used for tobacco. Excellent translation rates, indistinguishable from those with tobacco extracts, were routinely obtained in the pea translation extracts (Figure 2A, left). The identity of the pea translated product was verified by immunoprecipitation, with antiserum directed against Lep (Figure 2A, right). No difference in expression of Lep or other substrates, such as the D1 protein

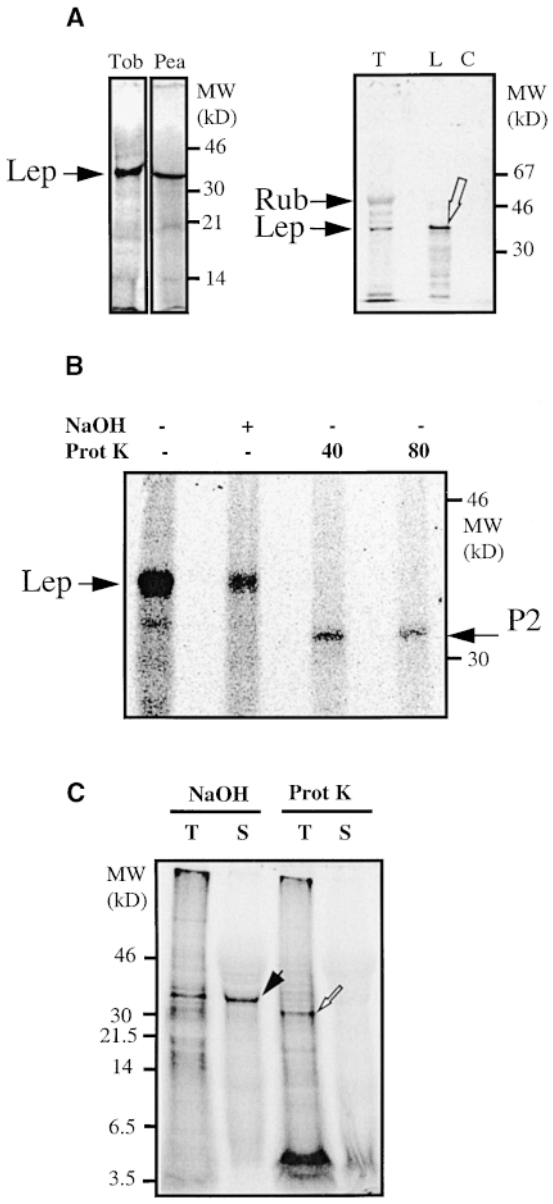

Figure 2. Translation, Immunoprecipitation, and Insertion of Full-Length Lep into Thylakoid Membranes Isolated from Purified Pea Chloroplasts.

(A) Radiolabeled, full-length Lep was synthesized in the tobacco (Tob) and pea (Pea) chloroplast translation system and separated by SDS-PAGE (left). The products of the pea translation were immunoprecipitated with antiserum directed against Lep (L) as well as with control serum (C) (right). Five percent of the total pea translation reaction performed for the immunoprecipitations (marked with T) was directly loaded onto the gel (right). Immunoprecipitated Lep is indicated by the open arrow. Molecular mass markers (MW) are indicated at right in kilodaltons. The background signal of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rub) is indicated.

(Nilsson et al., 1999), was observed when the pea or tobacco extracts were compared. In this study, we used both the chloroplast translation extracts isolated from tobacco and pea leaves and compared targeting and insertion efficiencies to the thylakoid membranes of peas (see below).

Insertion of Lep

To test whether Lep could be inserted into the thylakoid membrane, we developed a system for thylakoid targeting and insertion experiments optimized to maximize thylakoid membrane integrity, minimize background translation, and strongly reduce proteolysis, using the chloroplast translation system developed by Hirose and Sugiura (1996) as a basis (see Methods). Neither the alterations from the original recipe (Hirose and Sugiura, 1996) nor the addition of a fairly high concentration of thylakoid membranes (up to 0.5 mg mL^{-1} chlorophyll) affected the translation yield of Lep (data not shown).

Using our optimized system, full-length Lep was synthesized in the presence of thylakoid membranes. After translation, the thylakoid membranes were separated from the translation mixture, and different treatments were used to examine the association of Lep with the thylakoid membrane. After washing the thylakoid membranes with HMS100 buffer (see Methods), 50% of Lep remained associated with the membranes (Figure 2B). Approximately 20% of this membrane-associated Lep was resistant to alkali washing (Figure 2B), indicating that at least one transmembrane domain was inserted into the lipid bilayer. To monitor the insertion and topology of Lep, we incubated thylakoid

(B) Radiolabeled Lep was synthesized in the tobacco chloroplast translation system in the presence of 0.2 mg of chlorophyll per mL of thylakoid membranes isolated from purified pea chloroplasts. Membranes were subsequently separated from the translation mix, washed, and loaded on SDS gels either before (−) or after (+) treatment with 0.1 M NaOH or incubated with proteinase K (Prot K at 40 or 80 μg mL^{-1}), as indicated. Arrows indicate full-length Lep and the protease-protected Lep fragment (P2). Molecular mass markers (MW) are indicated at right in kilodaltons.

(C) Radiolabeled Lep was synthesized in the pea chloroplast translation system in the presence of 0.2 mg of chlorophyll per mL thylakoid membranes isolated from purified pea chloroplasts. Membranes were subsequently separated from the translation mix, washed, and loaded on SDS–polyacrylamide gels (T) after treatment with 0.1 M NaOH or incubated with proteinase K (Prot K) at 40 μg mL^{-1}, as indicated. The supernatants of the translation reactions (S) were also loaded on SDS gels and, where indicated, incubated with proteinase K. Arrows indicate full-length Lep and the protease-protected Lep fragment (P2) (closed and open arrows, respectively). Molecular mass markers (MW) are indicated at left in kilodaltons.

membranes with proteinase K, revealing a protease-protected band of ~30 kD (Figure 2B). Such a fragment is expected if the P1 loop of Lep is digested by proteinase K and if the second transmembrane domain and the P2 domain are not accessible to the protease (see Figure 1A). Figure 2C shows the results of a similar experiment, but in this case, the supernatant also was treated with proteinase K. Clearly, no protease protection was observed with soluble Lep, confirming that protease protection was not due to secondary structure or to protective interactions with soluble components. Figure 2C also shows the accumulation of lower molecular weight fragments. These fragments were not observed when Lep mRNA was omitted from the translation assays or when thylakoids were added after termination of translation (see next section). Thus, these low molecular weight fragments are derived from Lep and are mostly protease-protected membrane-inserted domains. Given that the low molecular weight fragments have a high intensity and that these fragments are not observed in the NaOH-washed membranes, we concluded that some of these fragments are not integrated into the membrane but most likely "stick" onto the thylakoid membrane in a protease-protected manner.

Thus, we conclude that Lep was inserted into the thylakoid membrane in the same orientation as in the cytoplasmic membrane of *E. coli*, with the C and N termini on the *trans*-side (in the lumen or periplasm, respectively). This demonstrates that insertion of Lep into the thylakoid membrane obeyed the "positive-inside rule" (von Heijne, 1989; Gavel et al., 1991). Comparing the alkali-resistant band with the protease-protected band and taking into account that the second transmembrane domain and the P2 domain contain six of the eight methionine residues present in Lep, we conclude that all of the alkali-resistant Lep was properly inserted. An overall insertion efficiency of ~10% was routinely observed, which is comparable with the efficiency of Lep insertion into the *E. coli* cytoplasmic membrane in a homologous *E. coli* in vitro translation system (van Klompenburg et al., 1997). No differences in insertion efficiencies of Lep were observed when comparing the pea and tobacco extracts.

Lep Is Inserted in a Cotranslational Manner

To investigate whether Lep insertion into the thylakoid membranes occurred cotranslationally or post-translationally, we performed translation of Lep in the absence of membranes. Subsequently, 2 mM puromycin and 15 μM lincomycin were added to dissociate the ribosomes and to prevent translation and initiation, respectively. After 5 min of incubation, thylakoid membranes were added together with protease inhibitors, and after a 15- or 30-min incubation, Lep insertion was analyzed by treatment with alkali and proteinase K (Figures 3A and 3B). Maximally, 2% of the total amount of synthesized Lep was associated with the membranes, but no proteinase K–protected fragment could be observed (Figures 3A and 3B). Apparently, once full-length Lep was synthesized, anchoring into the membrane was very inefficient, and post-translational translocation of the P2 domain did not occur. Thus, it can be concluded that Lep was inserted in a cotranslational manner or, less likely, that fully translated Lep is only insertion competent for a short period of time.

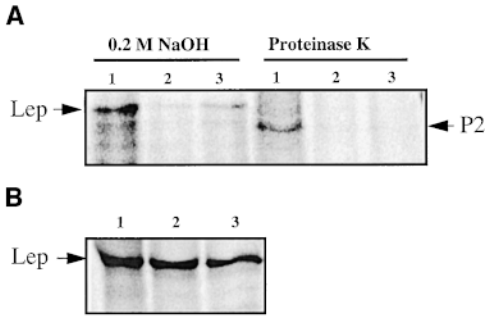

Figure 3. Cotranslational or Post-Translational Insertion of Lep.

(A) and **(B)** show Lep that was synthesized in the tobacco chloroplast translation system in the presence (lanes 1) or absence (lanes 2 and 3) of 0.4 mg of chlorophyll per mL thylakoid membranes isolated from purified pea chloroplasts. Subsequently, translation assays in the absence of membranes were treated with 2 mM puromycin and 15 μM lincomycin for 5 min at 30°C. Membranes (0.4 mg mL^{-1} chlorophyll) and protease inhibitors were added, and after a 15- or 30-min incubation at 30°C (lanes 2 and 3, respectively), membranes were separated from the translation mix, washed, and treated with 0.1 M NaOH or proteinase K, as indicated.

(A) Thylakoid membranes from translation assays after NaOH or proteinase K treatment, as indicated.

(B) Soluble fraction of the translation assays.

Association of Truncated Versions of Lep with the Thylakoid Membrane

To further characterize the cotranslational insertion process, we trapped elongation at different stages by translation of truncated transcripts of different length, resulting in truncated Lep nascent chains of 56, 95, or 128 amino acid residues (Figures 1C and 4A). Because of the lack of a stop codon at the 3′ end of the truncated mRNAs, the ribosome halts at the end of the transcript, and RNCs are generated. After synthesis of such truncated Lep nascent chains in the chloroplast translation system, 70 to 85% of the nascent chains could be purified as stable RNCs on sucrose cushions (for other substrates, see Nilsson et al., 1999).

To study the possible docking of Lep RNCs onto the thylakoid membrane, the three truncated Lep transcripts were translated in the presence of thylakoid membranes, and their affinity for the membrane was monitored using salt washings (50 to 500 mM KOAc) (Figure 4B). At the lowest

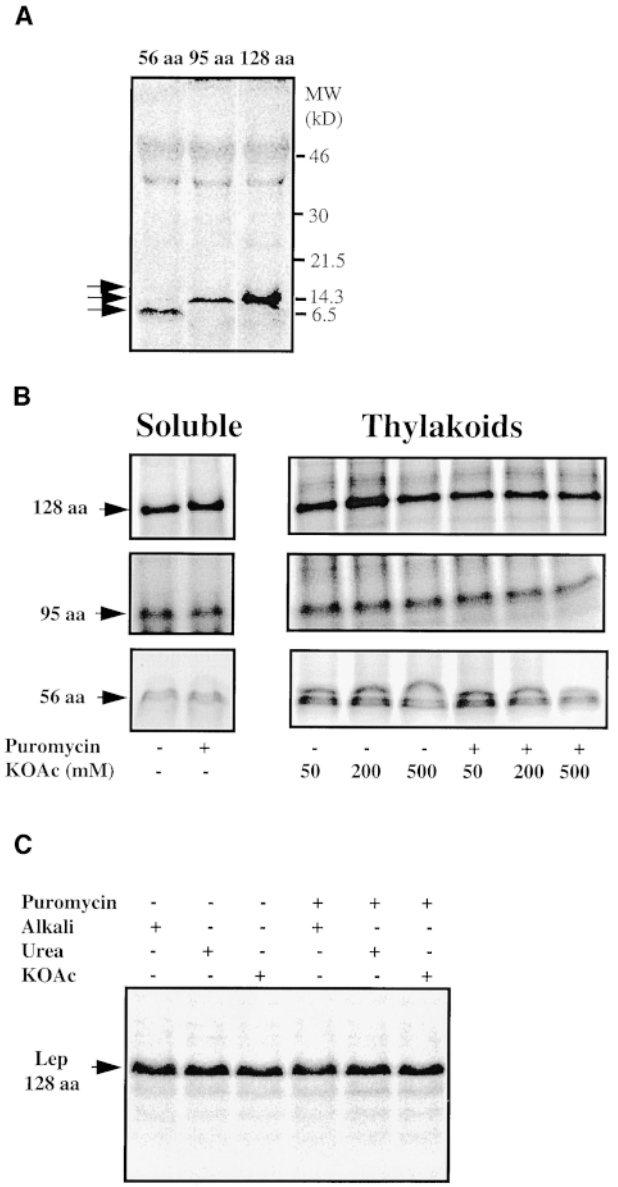

Figure 4. Binding of Truncated Versions of Lep to the Thylakoid Membranes of Pea.

(A) Translation of truncated mRNAs coding for Lep. The number of amino acid residues is indicated. Molecular mass (MW) markers are indicated at right in kilodaltons. Arrows indicate the three Lep translation products.

(B) Lep nascent chains of indicated length were synthesized in the tobacco chloroplast translation system in the presence of thylakoid membranes isolated from purified pea chloroplasts. Membranes were separated from the translation mix and washed with different salt concentrations (50, 200, or 500 mM potassium acetate [KOAc]). Where indicated, thylakoids were pretreated with puromycin before the salt washes. The presence or absence of the different treatments is indicated by (+) or (−), respectively.

(C) Lep nascent chains of 128 amino acid residues were synthesized in the tobacco chloroplast translation system in the presence of thylakoid membranes isolated from purified pea chloroplasts. Subsequently, samples were treated with 2 mM puromycin for 5 min at 30°C. Membranes were separated from the translation mix and then incubated and washed with either 500 mM potassium acetate, 4 M urea, or 0.1 M $NaHCO_3$/NaOH, pH 12.5. The absence or presence of the different treatments is indicated by (−) or (+), respectively.

aa, amino acid residues.

salt concentration, 60 to 80% of the nascent chains were found associated with the membrane. With increasing salt concentration, maximally 10% of the nascent chains could be removed, indicating that the RNCs were apparently making functional interactions with the membrane. When the membranes were incubated in the presence of the tRNA analog puromycin before the salt washes, 40% of the shortest (56 amino acids) nascent polypeptide was removed from the membrane by high salt, whereas nearly all the 128–amino acid polypeptides remained associated with the membrane. This indicates that the membrane association of the shortest nascent chain was partially dependent on the ribosome. The most likely explanation is that this short nascent chain was inserted into a translocon and did not make direct contact with the lipid bilayer. The 95–amino acid Lep nascent chain responded to these salt washes in a manner intermediate to the 56– and 128–amino acid nascent chains (Figure 4B).

To further investigate whether the 128–amino acid Lep nascent chain had been inserted into the lipid bilayer, we treated the membranes with alkali, urea, or high salt after translation in the presence of thylakoid membranes (Figure 4C). Such harsh treatments with chaotropic agents remove proteins peripherally bound to the membrane. Incubation with puromycin was done before these treatments to destabilize the ribosome. The 128–amino acid nascent chains remained tightly bound to the membrane during all three treatments, independent of preincubation with puromycin (Figure 4C). This implies that at least one transmembrane domain had stably integrated into the lipid bilayer, in agreement with studies of Lep insertion into microsomes (Mothes et al., 1997).

The Role of SecA and the *trans*-Thylakoid Proton Gradient during Lep Insertion

In vivo *E. coli* targeting studies have shown that the translocation of the P2 domain (Figure 1A) requires the translocon components SecY and SecA, whereas the ATP-dependent activity of SecA is not required to achieve binding of Lep to the cytoplasmic membrane (Wolfe et al., 1985). In chloroplasts, SecA was shown to be localized predominantly as a soluble protein in the stroma, with only 20% associated with the thylakoid membrane (Yuan et al., 1994). To investigate

the possible role of SecA in targeting and insertion of Lep, we added azide prior to translation of full-length Lep (Figures 5A and 5B). Azide interferes with the ATPase activity of SecA and has been used to examine the involvement of SecA in translocation, in both *E. coli* (Oliver et al., 1990) and chloroplasts (e.g., Knott and Robinson, 1994). In thylakoids, a light-induced *trans*-thylakoid proton gradient (up to 3.5 pH units) can be generated. This proton gradient is required for the post-translational translocation of a subset of nuclear-encoded lumenal proteins. In case of a number of other nuclear-encoded substrates, the proton gradient is believed to stimulate ATP- or GTP-dependent translocation (reviewed in Cline and Henry, 1996; Robinson and Mant, 1997). To assess the contribution of the proton gradient, we performed targeting experiments in the presence of the uncoupler nigericin.

We observed that azide as well as nigericin partially inhibited translation but that the amount of Lep, which was associated with the membrane in an alkali-resistant manner, was not negatively affected by either inhibitor (Figures 5A, 5B, and 6). In fact, when azide and nigericin were combined, the amount of Lep associated with the membrane increased

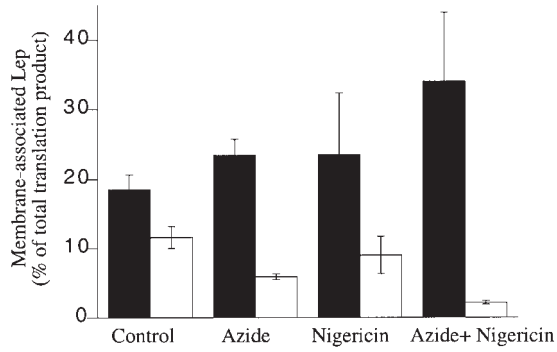

Figure 6. Quantification of the Effects of Azide (5 mM) and Nigericin (1.0 μM) on Lep Insertion, Shown in the Autoradiogram of Figure 5 and Other Experiments, Also Including the Pea Chloroplast Translation System.

The insertion efficiency is expressed as the amount of Lep in the thylakoid pellets as a percentage of total translation products. The filled columns represent Lep that is resistant to alkali washing, whereas the open columns represent the protease-protected Lep fragment (P2). Error bars indicate standard deviations.

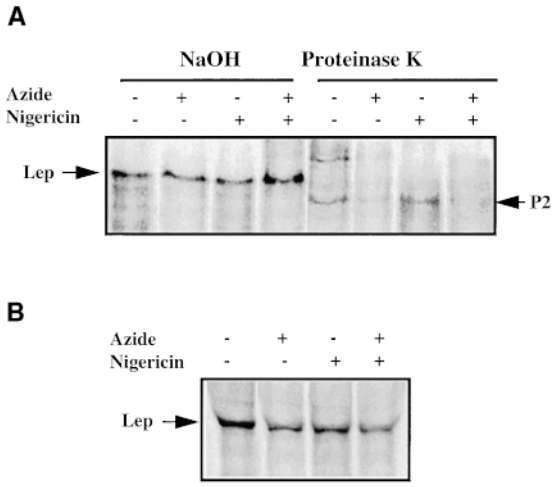

Figure 5. Effect of Azide and Nigericin on Insertion of Full-Length Lep.

Radiolabeled Lep was synthesized in the tobacco chloroplast translation system in the presence of thylakoid membranes isolated from purified pea chloroplasts. At the start of the translation, azide (final concentration 5 mM) or nigericin (final concentration 1.0 μM) or a combination of both was added. After translation, the membranes were separated from the translation mix, washed, and treated with 0.1 M NaOH or proteinase K (40 μg^{-1}). The absence or presence of the different treatments is indicated by (−) or (+), respectively. Arrows indicate full-length Lep and the protease-protected P2 domains.

(A) Lep associated with the thylakoid membranes.

(B) Lep accumulated in the soluble fraction.

when expressed as a percentage of the total translation product. A possible explanation is that Lep becomes locked into the translocon because SecA cannot cycle through its different (de)insertion states. However, further experimentation is needed to address this point in more detail. The amount of protease-protected Lep was significantly decreased by the addition of azide (~50%) and to a much lesser extent by the addition of nigericin (~20%) (Figure 6). Combining both nigericin and azide nearly eliminated the formation of protease-protected Lep (Figures 5A, 5B, and 6). As is schematically depicted in Figure 7, we propose that the translocation of the P2 domain of Lep is sensitive to azide, most likely indicating that this process is dependent on the ATP-dependent activity of SecA. The complementary effect of azide and nigericin could indicate that SecA and the proton gradient both play a role in Lep integration.

DISCUSSION

In this study, we have addressed the mechanisms of protein insertion into the thylakoid membrane, by using a chloroplast translation system. We used Lep in our study as a model protein because it has been used to study targeting to and insertion into the cytoplasmic membrane of *E. coli* (e.g., Wolfe et al., 1985; Dalbey and Wickner, 1987; Moore et al., 1988; Valent et al., 1998) as well as ER microsomes (e.g., Nilsson et al., 1994; Mothes et al., 1997). In addition, Lep has a number of experimental advantages over other substrates. We expected that thylakoid insertion studies

with Lep would also provide insight into the functional homologies between the different membrane systems.

Here, we show that Lep could be synthesized in the chloroplast translation system with high efficiency and without premature truncations, using the 5′ UTR of the *psbA* gene. We used this *psbA* leader because it expressed Lep but also because it is possible that the 5′ UTR is involved in the targeting process (see Rochaix, 1996; Cohen and Mayfield, 1997; Zerges and Rochaix, 1998), possibly similar to that observed for the integral membrane proteins Cox2p and Cox3p in mitochondria of *Saccharomyces cerevisiae* (Sanchirico et al., 1998). In the case of cytochrome *f*, the 5′ UTR has been implicated in autoregulation of its translation through an as yet unknown mechanism (Choquet et al., 1998).

Full-length Lep was inserted into the thylakoid membrane when synthesized directly in the chloroplast translation system in the presence of thylakoid membranes. All membrane-associated Lep, which was resistant to alkali washing, was inserted in the same orientation as into cytoplasmic membranes of *E. coli* (von Heijne, 1989) and ER microsomes (Nilsson et al., 1994), with the N and C termini on the *trans*-side of the membrane (into the thylakoid lumen). It was shown in *E. coli* and in the ER that this orientation of Lep was due to the distribution of positive charges in the P1 loop of Lep and that insertion of Lep thus followed the so-called positive-inside rule (von Heijne, 1989). Statistical analysis of the charge distribution among the known thylakoid membrane proteins confirmed this rule for thylakoid proteins (Gavel et al., 1991). Thus, control or selection mechanisms must have been operative in our targeting and insertion experiments with Lep, ensuring insertion of Lep in a single orientation and thereby following the positive-inside rule.

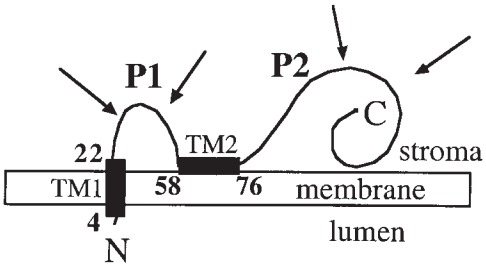

Figure 7. Schematic Representation of the Effect of Azide and Nigericin on the Insertion of Lep into the Thylakoid Membrane.

The addition of both inhibitors during translation prevents translocation of the P2 domain to the lumenal side of the thylakoid membrane. The first and second loops (P1 and P2) and the transmembrane domains (TM1 and TM2) are indicated. Numbers indicate the amino acid residue at the beginning and end of the first and second transmembrane domain. Arrows indicate domains sensitive to proteases added to the thylakoid membranes. The N and C termini of Lep are indicated (N and C, respectively).

Insertion of Lep into the thylakoid membranes was observed only when the membranes were present during translation. The addition of membranes after translation did not lead to proper insertion, indicating that the insertion process occurred cotranslationally. Further support for a cotranslational insertion mechanism came from the use of Lep nascent chains that were still attached to the ribosome. A large percentage of these RNCs was found associated with high affinity to the thylakoid membrane. This was possibly due to an active targeting mechanism, as observed for Lep RNCs in *E. coli* and the ER, or, less likely, to a high natural affinity of RNCs for the thylakoid membrane. Targeting of Lep to the *E. coli* cytoplasmic membrane and ER microsomes was shown to require a ribonucleoprotein complex, the SRP. Similar mechanisms might operate in the chloroplast because a chloroplast homolog to SRP54, known as cpSRP54, has been identified (Franklin and Hoffman, 1993). Interestingly, cpSRP54 could be cross-linked to different substrates when attached as nascent chains to 80S ribosomes from wheat germ translation extract (High et al., 1997) as well as to nascent chains of the chloroplast-encoded D1 protein attached to 70S ribosomes from the chloroplast translation system (Nilsson et al., 1999). A dominant negative cpSRP54 mutant of Arabidopsis showed pleiotropic effects on chloroplast development in young plants (Pilgrim et al., 1998). However, the absence of an RNA in the chloroplast SRP and the presence of an unusual second protein, which was assigned cpSRP43 (Schuenemann et al., 1998; Klimyuk et al., 1999), indicate that the role of the SRP in chloroplasts could be different than it is in bacteria, yeast, or mammalian cells.

It is also possible that the mRNA encoding Lep was targeted directly to the membrane through binding of the 5′ UTR to mRNA binding proteins already present on the membrane (Zerges and Rochaix, 1998). In this case, targeting of the nascent chain from the soluble phase to the membrane phase could possibly be avoided, although the translating protein would still need to be directed to the translocon.

Synthesis of truncated Lep polypeptide chains (56, 95, and 128 amino acid residues long) in the chloroplast translation system yielded stable RNCs. When these RNCs were synthesized in the presence of thylakoid membranes, most associated with the membrane in a high salt–resistant manner. Studies with lysed chloroplasts showed that high salt concentrations are able to remove endogenous RNCs from the membrane when they are nonspecifically bound through electrostatic interactions (Yamamoto et al., 1981). Thus, apparently, the Lep RNCs in our translation system were specifically bound to the thylakoid membrane.

For microsomes, it has been demonstrated that Lep RNCs make salt-resistant interactions with the microsomes once the RNCs are docked onto a proteinaceous translocon (reviewed in Matlack et al., 1998). Cross-linking experiments and cryoelectron microscopy further confirmed this docking process (Beckmann et al., 1997; Matlack et al., 1998). In microsomes as well as in the cytoplasmic membrane in *E. coli*,

this translocon is a large multisubunit proteinaceous pore complex, with the major subunit formed by a large membrane protein denoted Sec61α in the ER and SecY in prokaryotes (Andrews and Johnson, 1996; reviewed in Wickner and Leonard, 1996; Matlack et al., 1998). Also in chloroplasts of higher plants, a SecY homolog has been identified (Laidler et al., 1995); however, to date, no functional interaction with the 70S ribosomes and nascent chains has been demonstrated. Our observation of a strong interaction of Lep RNCs with the thylakoid membrane may represent a functional interaction with the SecY translocon.

When the thylakoid membranes with docked Lep RNCs of 56 amino acid residues were incubated with the tRNA analog puromycin and washed with high salt, a significant percentage of the nascent chains was released, indicating that the nascent chains had not inserted into the lipid bilayer. However, when these experiments were performed with a much longer nascent chain (128 amino acids) that contained both transmembrane domains, no loss of nascent chains from the membranes occurred, even after incubations with chaotropic agents. Thus, it seems most likely that in the case of these longer nascent chains, at least one transmembrane domain had inserted into the lipid bilayer. It is possible that during the synthesis of Lep, the first transmembrane domain is already laterally moving out of the translocon into the lipid bilayer. In fact, this movement was observed when Lep was expressed in the presence of microsomes (Mothes et al., 1997). An alternative explanation is that the transmembrane domains accumulate in the translocon until translation is completed. Thus, upon destabilization of the RNCs by puromycin, the lateral exit of one or both transmembrane domains from the translocon was stimulated, and integration into the lipid bilayer occurred (Borel and Simon, 1996). The successful insertion of full-length Lep and the high affinity of Lep RNCs in our system will enable us to characterize such events in chloroplasts for the first time.

The 60 to 80% efficiency for insertion of the truncated forms of Lep contrasts with the 10% translocation of the P2 domain. This indicates that the requirements for translocation of the long (247 amino acids) P2 domain were different than for insertion of one or two transmembrane domains in the case of the truncated 128–amino acid nascent chain. In addition, it is possible that the trapping of elongation by the use of truncated mRNA provided a larger time window for targeting and interaction of the nascent chains with soluble stromal components and subsequent targeting and insertion into the membrane.

Studies with *E. coli* have shown that insertion into and translocation across the cytoplasmic membrane of a number of proteins also require SecA, which is a large protein that is part of the translocon (e.g., Nishiyama et al., 1996; reviewed in Duong et al., 1997; Matlack et al., 1998). Insertion and deinsertion of SecA into the membrane driven by the energy liberated through ATP binding and hydrolysis translocate these proteins. In *E. coli*, Lep nascent chains could be cross-linked to SecA at the cytoplasmic membrane surface (Valent et al., 1998), and conditional mutants showed that translocation of the P2 domain required ATP-dependent SecA activity (Wolfe et al., 1985).

In chloroplasts, cpSecA was shown to exist mostly in a soluble form, and purified cpSecA supported post-translational translocation of a number of nuclear-encoded lumenal proteins (Yuan et al., 1994; Nohara et al., 1996). cpSecA could be cross-linked to translocating OEC33 (Haward et al., 1997), and antiserum directed against cpSecA inhibited translocation of OEC33 to the thylakoid lumen (Nakai et al., 1994). Thus, a role for cpSecA in post-translational translocation across the thylakoid membrane protein has clearly been established in vitro. A deletion mutant of cpSecA in maize (*tha1*) showed a loss of post-translational targeting of a specific class of nuclear-encoded proteins and, interestingly, also an effect on at least one chloroplast-encoded protein, cytochrome *f* (Voelker and Barkan, 1995; Voelker et al., 1997). Because it is likely that cytochrome *f* is cotranslationally inserted into the thylakoid membrane (because *petA* mRNA is located on thylakoid-bound ribosomes; see Friemann and Hachtel, 1988), cpSecA could be involved in cotranslational insertion of cytochrome *f*. Although cpSRP54 could be cross-linked to cytochrome *f* nascent chains attached to 80S ribosomes from wheat germ extracts (High et al., 1997), it remains unclear whether the chloroplast SRP particle also plays a role in the cotranslational targeting process of cytochrome *f* and other chloroplast-encoded thylakoid proteins, such as the D1 protein (Nilsson et al., 1999). Studies using chimeric constructs of cytochrome *f* expressed in a wheat germ translation system showed that when cytochrome *f* is post-translationally imported into the chloroplast, its insertion into the thylakoid membrane requires SecA and the N-terminal signal peptide (Nohara et al., 1996; Mould et al., 1997; Zak et al., 1997).

The stable integration of full-length Lep, as determined by the protease assay, was sensitive to azide and the uncoupler, suggesting that the insertion and translocation of the P2 domain of Lep required ATP-dependent activity of SecA and was somewhat stimulated by the *trans*-thylakoid proton gradient. However, the effect of azide was much stronger than the effect of uncoupling, whereas abolition of both components eliminated proper integration into the thylakoid membrane. Therefore, it is most likely that the proton gradient contributed in a complementary way to translocation of the P2 domain. It is important to emphasize that although the uncoupler and azide reduced the amount of protease-protected Lep, neither inhibitor affected the amount of membrane-bound Lep that resisted extraction by chaotropic reagents. Thus, we propose that at least one transmembrane domain can integrate into the lipid bilayer without the involvement of ATP-dependent activity of SecA or the proton gradient, as schematically depicted in Figure 7. A recent study on the integration of an artificial single-membrane-spanning protein in the *E. coli* cytoplasmic membrane also revealed that insertion and lateral movement of the transmembrane domain from the translocon out into the lipid bi-

layer did not require ATP-dependent SecA activity (Duong and Wickner, 1998).

We conclude from this study that there are several similarities between insertion of Lep in the chloroplast and in *E. coli*: (1) Lep is inserted in a cotranslational manner; (2) azide prevents translocation of the P2 domain but does not inhibit a tight association of Lep to the membrane; and (3) Lep insertion follows the positive-inside rule.

Finally, translation extracts were made not only from tobacco chloroplasts, as originally shown by Hirose and Sugiura (1996), but also from young pea leaves. Expression of Lep as well as targeting efficiencies were identical for both systems, indicating that the tobacco preparation can be extended to other higher plant systems. This might be of help in the study of species-specific differences.

In conclusion, we have developed a good experimental system in which we can synthesize both full-length and truncated versions of Lep and monitor membrane insertion. We expect that this will enable us to unravel the mechanisms of targeting, insertion, and assembly of thylakoid membrane proteins encoded by the chloroplast genome.

METHODS

DNA Constructs

pBSpsbA contains the full-length 5′ untranslated leader (−85 to +1) and coding region of *psbA* (the chloroplast gene encoding the polytopic D1 thylakoid membrane protein) under the control of the T7 promoter (Hirose and Sugiura, 1996). An NcoI site was introduced at the start position of the D1 coding region by site-directed mutagenesis (Quickchange site-directed mutagenesis kit; Strategene), yielding pBSpsbANcoI. The Lep coding region, from pGEM1Lep (Nilsson et al., 1994), was excised using NcoI and SacI and was swapped against the D1 coding region into pBSpsbANcoI, yielding pBSLep.

Preparation of mRNA Templates

For in vitro transcription of full-length *Escherichia coli* leader peptidase Lep, pBSLep was digested with SacI. DNA templates for the synthesis of truncated versions of Lep were produced either by digestion of the plasmid using internal restriction sites (BstNI and EcoRV for Lep nascent chains of 56 and 128 amino acid residues, respectively) or by polymerase chain reaction, according to Nilsson et al. (1994), for production of Lep with 95 amino acid residues (Figure 1B). For polymerase chain reaction production of Lep template (95 amino acids), we used the forward primer 5′-GGCTGCGCAACTGTTGGGAAGGGCG-3′, annealing to the plasmid ~120 bases before the start of the T7 promotor, and reverse primer 5′-CATCATCATCATCGAACCTGACGGG-3′, annealing to the Lep coding sequence; this added three additional methionine residues at the C terminus to enhance the signal on imaging screens (PhosphorImager; Fuji, Tokyo, Japan). Transcription with T7 polymerase was performed according to the Ribomax system (Promega). After transcription, DNA templates were digested by DNase I, and transcripts were subsequently purified by phenol–chloroform extraction and ethanol precipitation, according to standard procedures (Sambrook et al., 1989). Size and quality of the mRNA were routinely verified on gels.

Isolation of the Chloroplast Translation System and in Vitro Translations

The preparation of chloroplast translation extracts from tobacco or pea leaves was performed essentially according to Hirose and Sugiura (1996). Tobacco (*Nicotiana tabacum* var Bright Yellow) was grown in the greenhouse at 26 and 21°C day and night temperatures, respectively, under daylight with additional illumination; after 30 to 40 days, leaves between 5 and 10 cm in length were harvested. Pea (*Pisum sativum* var De Grace) was grown for 12 to 14 days in a growth chamber at 25 and 21°C day and night temperatures, respectively, with 12 hr of artificial light (~95 μmol photons m^{-2} sec^{-1}) per day. Leaves (~500 g for pea and 1200 g for tobacco) were ground in a commercial homogenizer in 2.5 liters of MCB1 (see Hirose and Sugiura, 1996) with 0.1% BSA and 0.6% polyvinylpyrrolidone (average molecular mass of 360,000 D; Sigma), filtered through Miracloth (22 μm), and the filtrate was spun for 3 min at 1300*g*. The pellet was resuspended in ~50 mL of MCB1 with 0.1% BSA and overlayed on four 15-mL Percoll step gradients (30%/70% Percoll in MCB2; see Hirose and Sugiura, 1996) and centrifuged for ~5 min at 4500*g*. Intact chloroplasts were collected from the Percoll interface and washed once in 100 mL of MCB2. Further lysis of the chloroplasts and preparation of the chloroplast S30 fractions were performed exactly as described by Hirose and Sugiura (1996).

Translation reactions were performed essentially according to Hirose and Sugiura (1996). However, a number of essential modifications were made for the insertion of Lep into the thylakoid membrane. Mannitol (100 mM) was added to the translation reactions to stabilize the thylakoid membranes, and NH_4Cl, present in the original recipe, was omitted because it is an uncoupler of the *trans*-thylakoid proton gradient. The protease inhibitors antipain and pepstatin were also added, on average increasing the amount of stable translation products by 30% (data not shown). In summary, the optimized translation mixture contained 100 mM mannitol, 30 mM Hepes-KOH, pH 7.7, 10 mM Mg acetate, 60 mM K acetate, 8 mM creatine phosphate, 1% polyethylene glycol 6000, 1 mM ATP, 0.1 mM GTP, 2 mM DTT, 0.4 μg μL^{-1} creatine phosphokinase (type I), 0.4 μg μL^{-1} *E. coli* tRNA, 2.6 units μL^{-1} RNasin (Promega), 0.45 μCi μL^{-1} ^{35}S-methionine (>1000 Ci $mmol^{-1}$), and 1 μg mL^{-1} leupeptin, antipain, and pepstatin. Translations (volumes 10 to 50 μL) were performed at 28 to 30°C for 25 to 30 min.

Isolation of Ribosome Nascent Chain Complexes

For isolation of ribosome nascent chain complexes (RNCs), 250 μg mL^{-1} chloramphenicol was added to the translation reaction, and translations were overlaid on 0.5 M sucrose cushions containing 50 mM Hepes-KOH, pH 7.7, 50 mM potassium acetate, 5 mM magnesium acetate, 2 mM DTT, 250 μg mL^{-1} chloramphenicol, and a cocktail of protease inhibitors (Pefablok [50 μg mL^{-1}], antipain, and leupeptin [2 μg mL^{-1}]). Sucrose cushions (150 to 500 μL) were spun at 200,000*g* for 50 min in a rotor (model TLA100.3; Beckman Instruments, Palo Alto, CA).

Preparation of Thylakoid Membranes

Pea seedlings were grown for 7 days, as described above. Intact chloroplasts were prepared from pea leaves essentially as described by Cline (1986). The chloroplasts were lysed at 0.5 mg mL^{-1} chlorophyll in 50 mM Hepes-KOH, pH 7.6, 10 mM $MgCl_2$, protease inhibitor cocktail, and 1 mM DTT for 5 min on ice. The thylakoid membranes (containing 200 to 300 μg of chlorophyll) were pelleted by a 1-min centrifugation at 10,500*g* and washed once in 500 μL of 50 mM Hepes-KOH, pH 8.0, 10 mM $MgCl_2$, 100 mM sorbitol, protease inhibitor cocktail, and 1 mM DTT (referred to as HMS100). After resuspension in HMS100 at 1 mg mL^{-1} chlorophyll, the membranes were treated for 15 min on ice with micrococcal nuclease (7 units μL^{-1}) in the presence of 10 mM $CaCl_2$, protease inhibitor cocktail, and 1 mM DTT. The digestion was stopped with 20 mM EGTA, and the membranes were collected by a 1-min centrifugation at 10,500*g*. The membranes were washed three times with HMS100 to remove the EGTA. After the washes, the membranes were resuspended in HMS100 at 4 mg mL^{-1} chlorophyll.

Insertion Assays

In cotranslational insertion experiments, translations were performed with supplemental illumination (~50 μmol photons m^{-2} sec^{-1}) in the presence of 0.2 to 0.5 mg of chlorophyll per mL of thylakoid membranes and 100 mM mannitol. In post-translational insertion experiments, translations were performed in the presence of 100 mM mannitol but in the absence of thylakoid membranes. After 30 min of translation, puromycin and lincomycin were added to a final concentration of 2 mM and 15 μM, respectively, and the samples were incubated for another 5 min at 30°C. Subsequently, membranes and protease inhibitors were added, and samples were kept for 15 or 30 min at 30°C. The membranes were separated from the translation mix by centrifugation at 10,500*g* for 1 min and were subsequently washed twice with HMS100.

To monitor the membrane insertion and the topology of Lep, we treated the membranes and supernatants with proteinase K at a final concentration of 40 or 80 μg mL^{-1}, and the samples were incubated for 30 min on ice. To stop proteolysis, we added phenylmethylsulfonyl fluoride to a final concentration of 0.5 mg mL^{-1}, and the membranes were spun down, whereas the supernatant was precipitated with 10% trichloroacetic acid. To further monitor integration of targeted proteins into the thylakoid membrane, we incubated membranes at 0.1 mg mL^{-1} chlorophyll in 0.1 M $NaHCO_3$-NaOH, pH 12.5, or 4 M urea in 50 mM potassium acetate, 100 mM mannitol, 50 mM Hepes-KOH, pH 7.7, 5 mM magnesium acetate, 2 mM DTT, and a protease inhibitor cocktail for 15 to 30 min on ice, after which the membranes were collected by a centrifugation at 15,800*g* for 3 min. The membranes were subsequently washed with HMS100. To monitor the membrane association of the RNCs, we incubated membranes at 0.1 mg mL^{-1} chlorophyll for 10 min on ice in 50, 200, or 500 mM potassium acetate in 100 mM mannitol, 50 mM Hepes-KOH, pH 7.7, 5 mM magnesium acetate, 2 mM DTT, and a protease inhibitor cocktail.

Protein Analysis and Chlorophyll Determination

For immunoprecipitation of translation reactions, the translation reactions or cells were precipitated in ice-cold 10% trichloroacetic acid, and the pellets were washed in ice-cold 70% acetone and subsequently solubilized in 2% SDS in 10 mM Tris-HCl, pH 8.0. Immunoprecipitations using polyclonal antiserum directed against overexpressed Lep were performed as described by Nilsson et al. (1999). Proteins were separated by linear (14% T; 2.6% C) or gradient SDS-PAGE (7 to 17% T; 2.6% C) (Laemmli, 1970) or by tricine–SDS-PAGE (14% T; 3% C) (Schägger and von Jagow, 1987). Gels were fixed and stained with Coomassie Brilliant Blue R 250, dried, and exposed to a PhosphorImager screen (Fuji). Chlorophyll concentrations were spectroscopically determined in 80% acetone (Porra et al., 1989).

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