

# Protein Targeting and Integration Signal for the Chloroplastic Outer Envelope Membrane

Hsou-min Li<sup>1</sup> and Lih-Jen Chen

Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 11529, Taiwan, Republic of China

**Most proteins in chloroplasts are encoded by the nuclear genome and synthesized in the cytosol. With the exception of most outer envelope membrane proteins, nuclear-encoded chloroplastic proteins are synthesized with N-terminal extensions that contain the chloroplast targeting information of these proteins. Most outer membrane proteins, however, are synthesized without extensions in the cytosol. Therefore, it is not clear where the chloroplastic outer membrane targeting information resides within these polypeptides. We have analyzed a chloroplastic outer membrane protein, OEP14 (outer envelope membrane protein of 14 kD, previously named OM14), and localized its outer membrane targeting and integration signal to the first 30 amino acids of the protein. This signal consists of a positively charged N-terminal portion followed by a hydrophobic core, bearing resemblance to the signal peptides of proteins targeted to the endoplasmic reticulum. However, a chimeric protein containing this signal fused to a passenger protein did not integrate into the endoplasmic reticulum membrane. Furthermore, membrane topology analysis indicated that the signal inserts into the chloroplastic outer membrane in an orientation opposite to that predicted by the “positive inside” rule.**

## INTRODUCTION

Most proteins in a cell are synthesized together in the cytosol. Correct sorting and transport of proteins during or after translation are vital to the survival of a cell. Protein sorting to most, if not all, organelles depends on a “targeting signal” that resides within the protein being transported and is specific for the destined organelle as well as transport machinery recognizing and interacting with the targeting signal.

Although chloroplasts have their own genomes, like other organelles in a cell, most proteins in chloroplasts are encoded by the nuclear genome and synthesized in the cytosol. There appear to be at least two classes of chloroplastic proteins, distinguished by the presence or absence of cleavable targeting signals and the use of different import pathways. The first class of proteins consists of proteins targeted to the interior of chloroplasts (the inner envelope membrane, the stroma, the thylakoid membrane, and the thylakoid lumen). These proteins are synthesized as higher molecular weight precursors with N-terminal extensions called transit peptides. Transit peptides are necessary and sufficient for targeting these precursor proteins to chloroplasts. No consensus sequences have been found for the transit peptides, except that they are generally devoid of acidic amino acids and have a high content of basic and hydroxylated amino acids (Keegstra et al., 1989; von Heijne et al., 1989).

The import of these transit peptide-bearing precursor proteins into chloroplasts is initiated by a binding step that involves

a specific interaction between the transit peptide and a thermolysin-sensitive receptor complex on the chloroplastic outer membrane. This step is followed by translocation of the precursor proteins across the chloroplastic envelope. Once in the stroma, the precursor proteins are either processed to their mature size by the removal of the transit peptides or further sorted to other internal compartments of chloroplasts. Both the binding and the translocation steps require energy in the form of ATP hydrolysis (Keegstra, 1989; Olsen et al., 1989).

The second class of proteins consists of most chloroplastic outer envelope membrane proteins. Among the seven outer envelope membrane proteins identified so far, five are synthesized in the cytosol at their mature size without a cleavable transit peptide (Salomon et al., 1990; Li et al., 1991; Ko et al., 1992; Fischer et al., 1994; Kessler et al., 1994; Seedorf et al., 1995). Their insertion into the outer envelope membrane does not require ATP. At least four of them also do not require thermolysin-sensitive components on the chloroplastic surface for their import. Most likely, these outer membrane proteins contain a different kind of targeting signal that recognizes a different set of transport machinery. However, because they are synthesized at their mature size, it is not clear where the targeting signals reside within the polypeptides and what the signals look like.

The other two known outer membrane proteins are synthesized with cleavable transit peptides such as the interior-targeted precursor proteins (Hirsch et al., 1994; Schnell et al., 1994; Tranel et al., 1995). They are components of the receptor complex mediating the import of interior-targeted precursor

<sup>1</sup> To whom correspondence should be addressed.

proteins. Current results indicate that the import pathway(s) that they use is more similar to the one used by interior-targeted precursor proteins than to the one used by other outer membrane proteins (Tranel et al., 1995). A third component of the receptor complex is one of the five outer membrane proteins that does not possess cleavable targeting sequences (Kessler et al., 1994; Seedorf et al., 1995).

In contrast to the considerable amount of information that we have about the import of interior-targeted precursor proteins, very little is known about the targeting mechanism of the group of outer membrane proteins that do not possess cleavable transit peptides. This knowledge is not only important for understanding the biogenesis of the chloroplastic outer membrane but also could be important for understanding how receptor proteins themselves are transported to chloroplasts. As a first step toward investigating this unique targeting pathway, we have identified the targeting and integration signal of one of these outer membrane proteins, OEP14 (outer envelope membrane protein of 14 kD, previously named OM14; Li et al., 1991). The biological function of OEP14 is not known. However, the protein has been shown to be present in a carotenoid-containing particle from the pea chloroplastic outer envelope membrane (Markwell et al., 1992). We report here that the outer membrane targeting and integration signal of OEP14 is within the first 30 amino acids of the protein. Interestingly, the structure of this signal resembles the signal peptides of secretory proteins. Nevertheless, this signal is specific for the chloroplastic outer envelope membrane.

## RESULTS

### Outer Envelope Membrane Targeting Signal of OEP14

To localize the outer membrane targeting information within OEP14, we made chimeric proteins with various lengths of

OEP14 fused to the passenger protein dihydrofolate reductase (DHFR). During the construction of fusion proteins, OEP14 was resequenced, and a mistake was found in the previously published sequence for OEP14 (Li et al., 1991). A cytosine residue was missed in the coding sequence for amino acid 41. This caused a frameshift from amino acid 41. This mistake has been corrected in the GenBank data base, and the polypeptide sequence deduced from the new sequence is shown in Figure 1. According to the new sequence, OEP14 contains 65 amino acids with a calculated molecular mass of 6.9 kD. When analyzed by Tris-glycine SDS-PAGE, which does not resolve small proteins very well, OEP14 ran as a protein of 14 kD (Li et al., 1991). However, when run on a Tricine-SDS gel (Schägger and von Jagow, 1987), OEP14 ran as a protein of ~10 kD (Figure 2A, lane 1), closer to its calculated molecular mass. The sequence of OEP14 predicts one major hydrophobic domain at the N terminus (Figure 1, underlined). Interestingly, it also has a row of six consecutive proline residues from amino acids 49 to 54 (Figure 1, italicized).

Five fusion constructs were initially made between various lengths of OEP14 and DHFR (Figure 1). OEP14×DHFR contains full-length OEP14 fused to DHFR. OEP14(1-44)×DHFR does not have the row of proline residues. OEP14(1-30)×DHFR contains the hydrophobic domain plus some flanking residues to preserve the charge distribution pattern around the hydrophobic domain. OEP14(1-15)×DHFR has half of the hydrophobic domain. OEP14(32-65)×DHFR contains the C-terminal half of OEP14 without the hydrophobic domain. In this last construct, amino acid 31 was replaced by a methionine for translation initiation.

Fusion proteins derived from each construct were synthesized by using in vitro transcription and in vitro translation systems and were tested for their import competency with isolated chloroplasts (Perry et al., 1991). As shown in Figure 2A, OEP14 was imported into chloroplasts (lane 2), and after thermolysin digestion, a 4-kD thermolysin-resistant fragment is visible (lane 3). As shown in Figure 2B, both the imported

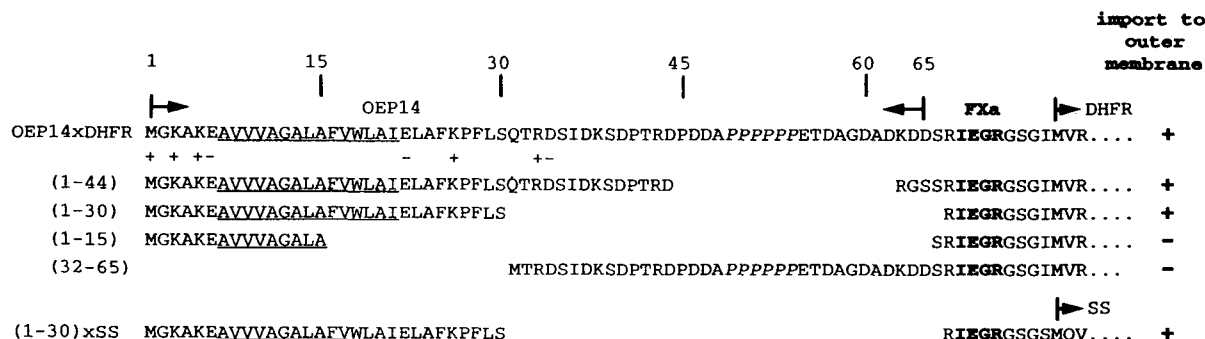
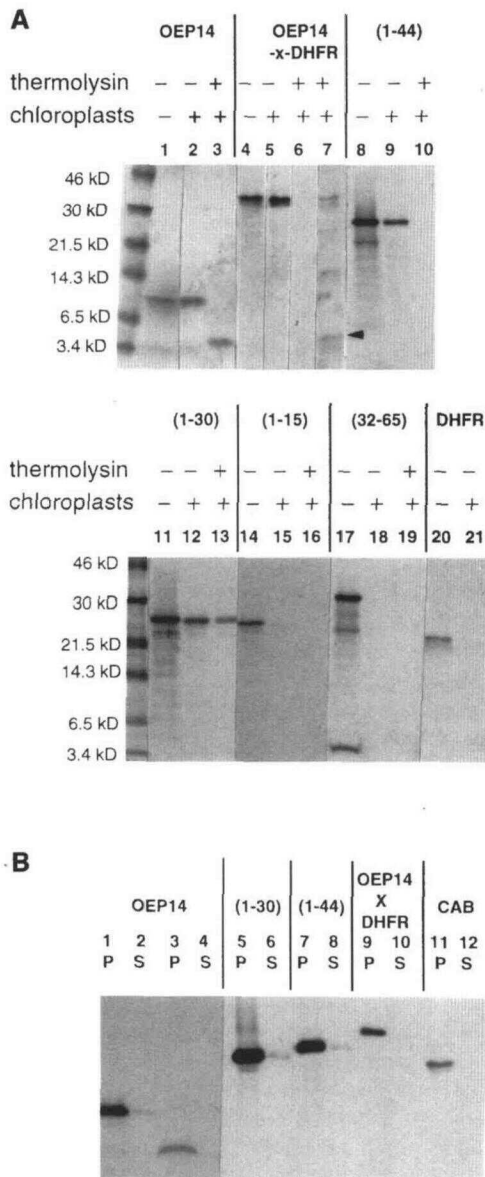


Figure 1. Amino Acid Sequence of the Fusion Proteins.

Amino acid numbering for OEP14 is indicated at the top. The hydrophobic domain is underlined. The row of proline residues from amino acids 49 to 54 is italicized. Charged residues within 15 amino acids of the hydrophobic domain are shown under the sequence of OEP14×DHFR. The N-terminal amino group is also given a positive charge, assuming it is not modified. The recognition site for factor Xa (FXa) is in bold. The (+) and (-) signs at right indicate whether a fusion protein can be targeted to chloroplasts. Arrows indicate the range of each protein. (1-44), OEP14(1-44)×DHFR; (1-30), OEP14(1-30)×DHFR; (1-15), OEP14(1-15)×DHFR; (32-65), OEP14(32-65)×DHFR; (1-30)×SS, OEP14(1-30)×SS.



**Figure 2.** Chloroplast Targeting and Integration Signal of OEP14 Is in Amino Acids 1 to 30.

**(A)** Amino acids 1 to 30 are necessary and sufficient to target a passenger protein to chloroplasts. The protein used for each set of samples is indicated at the top. In each set of samples, the first lane (lanes 1, 4, 8, 11, 14, 17, and 20) is the *in vitro* translation product obtained by sequential transcription and translation from chimeric or cDNA constructs. The second lane in each set (lanes 2, 5, 9, 12, 15, 18, and 21) shows chloroplasts after incubation with the respective *in vitro* translation product under import conditions. The third lane in each set (lanes 3, 6, 10, 13, 16, and 19) is the same as the second lane, except that the chloroplasts were treated with thermolysin after import. The (+) and (-) signs at top indicate the addition or omission, respectively, of chloroplasts or thermolysin in the reactions. Lane 7 is a longer exposure of lane 6. The arrowhead indicates the thermolysin-resistant fragment of OEP14 x DHFR that is the same size as the thermolysin-resistant fragment of OEP14 in lane 3. Samples were analyzed by using

OEP14 and the 4-kD thermolysin-digested fragment were resistant to alkaline extraction (Figure 2B, lanes 1 to 4), confirming that they are integral membrane proteins. The cleavage of imported OEP14 by thermolysin and the production of a 4-kD thermolysin-resistant fragment also suggest that part of OEP14 was exposed on the surface of chloroplasts and part of the molecule was inserted into the outer membrane.

DHFR by itself did not associate with chloroplasts (Figure 2A, lanes 20 and 21). When fused to full-length OEP14, DHFR was targeted to chloroplasts (Figure 2A, lanes 4 to 6). Like OEP14 itself, the OEP14 x DHFR fusion protein was thermolysin sensitive after import (lane 6). A thermolysin-resistant fragment the same size as the thermolysin-resistant 4-kD fragment of OEP14 can be seen in lane 6 containing thermolysin-treated OEP14 x DHFR after a longer exposure (Figure 2A, lane 7, arrowhead). This suggests that the OEP14 portion of the OEP14 x DHFR fusion protein assumed the same membrane topology as OEP14 by itself in the outer membrane. However, some full-length OEP14 x DHFR that was thermolysin resistant can also be seen after a longer exposure. This resistance could be due to incomplete digestion by thermolysin. It is also possible that the DHFR portion of the fusion protein may have folded into a protease-resistant conformation after import to chloroplasts. Protease resistance of DHFR has been observed when used as a passenger protein (van Loon et al., 1986; Verner and Schatz, 1987; Eilers et al., 1988; Endo et al., 1989). This protease-resistant conformation of DHFR could shield the OEP14 portion of the fusion protein from thermolysin digestion or even hinder some of the imported molecules from inserting into the outer membrane properly, resulting in thermolysin-resistant OEP14 x DHFR. This latter possibility may also explain why much less of the 4-kD thermolysin-resistant fragment was seen in the OEP14 x DHFR sample than in the OEP14 sample. Because much less of OEP14 x DHFR inserts into the outer membrane than does OEP14, we cannot exclude the possibility that some of the OEP14 x DHFR molecules that we saw after an import reaction could have been associating with the chloroplasts nonspecifically.

Similar to full-length OEP14, the first 44 or 30 amino acids of OEP14 could also target DHFR to chloroplasts (Figure 2A, lanes 8 to 13). Most of the imported fusion proteins had inserted into the membrane, as revealed by their resistance to alkaline extraction (Figure 2B, lanes 5 to 10). About 97% of OEP14(1-30) x DHFR, 94% of OEP14(1-44) x DHFR, and 88%

SDS-PAGE and autoradiography. Molecular mass markers in kilodaltons are shown at left.

**(B)** Most of imported OEP14 and the fusion proteins are integral membrane proteins. The protein used for each set of samples is indicated at top. After an import reaction, repurified chloroplasts were extracted with 100 mM sodium carbonate and separated into pellet (P) and supernatant (S) fractions. Lanes 3 and 4 are the same as lanes 1 and 2, except that the chloroplasts were treated with thermolysin after import. (1-30), OEP14(1-30) x DHFR; (1-44), OEP14(1-44) x DHFR; CAB, photosystem II chlorophyll *a/b* binding protein.

of OEP14×DHFR were found in the pellet fractions after alkaline extraction. These percentages are comparable to or only slightly lower than that of the photosystem II chlorophyll *a/b* binding protein (CAB; 94%), which is an integral thylakoid membrane protein, and to that of OEP14 itself (98%) or the 4-kD thermolysin-resistant fragment (no signals above background were obtained for the supernatant fraction). The portions that were extracted by the basic solution could represent the molecules that had not been fully inserted or had failed to insert properly into the outer membrane.

Fusion proteins with only the first 15 amino acids, or the C-terminal half without the first 30 amino acids of OEP14, did not associate with chloroplasts (Figure 2A, lanes 14 to 19). From these observations, we conclude that amino acids 1 to 30 of OEP14 are both necessary and sufficient to target proteins to chloroplasts.

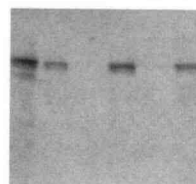
### Localization of the Fusion Proteins in Chloroplasts

Like OEP14 itself, fusion proteins with full-length OEP14 and the first 44 amino acids of the protein remained thermolysin sensitive after being targeted to chloroplasts (Figure 2A, lanes 3, 6, and 10). This result shows that they are located in the outer envelope membrane because only proteins exposed on the chloroplastic surface will be thermolysin sensitive (Cline et al., 1984).

Some of the OEP14(1–30)×DHFR fusion proteins were thermolysin resistant after being targeted to chloroplasts (Figure 2A, lane 13). One possible explanation is that the imported proteins were located internal to the outer membrane. Or it is possible that the imported proteins were still located in the outer envelope membrane but had assumed a particular conformation that was protease resistant. For example, it is possible that in the OEP14×DHFR and OEP14(1–44)×DHFR fusion proteins, thermolysin cleaves mainly at the OEP14 portion of the fusion proteins, resulting in their thermolysin sensitivity. For OEP14(1–30)×DHFR, the first 30 amino acids of OEP14 may be totally buried in the outer membrane, and thermolysin might only cleave at the DHFR portion with low efficiency due to the protease-resistant conformation of DHFR. This would result in a substantial population of thermolysin-resistant OEP14(1–30)×DHFR.

To localize imported OEP14(1–30)×DHFR, chloroplasts were fractionated after import. As shown in Figure 3, most of the imported OEP14(1–30)×DHFR is in the outer envelope membrane fraction. This fractionation pattern is similar to that of OEP14 (Li et al., 1992). The amount present in the inner envelope membrane fraction is most likely due to contamination by the outer membrane (Cline et al., 1981). Furthermore, OEP14(1–30)×DHFR is resistant to alkaline extraction after import (Figure 2B), indicating that OEP14(1–30)×DHFR is an integral membrane protein. Therefore, we conclude that amino acids 1 to 30 constitute the chloroplastic outer membrane targeting and integration signal of OEP14.

TR C S I T O



**Figure 3.** Imported OEP14(1–30)×DHFR is in the Outer Envelope Membrane.

After import, reisolated intact chloroplasts were fractionated into the stromal (S), inner envelope membrane (I), thylakoid (T), and outer envelope membrane (O) fractions. TR, in vitro translation product; C, total chloroplasts after import. One microgram of protein was loaded in lanes S, T, and I. Only 0.17  $\mu$ g of protein was loaded in lane O.

### Membrane Topology of OEP14

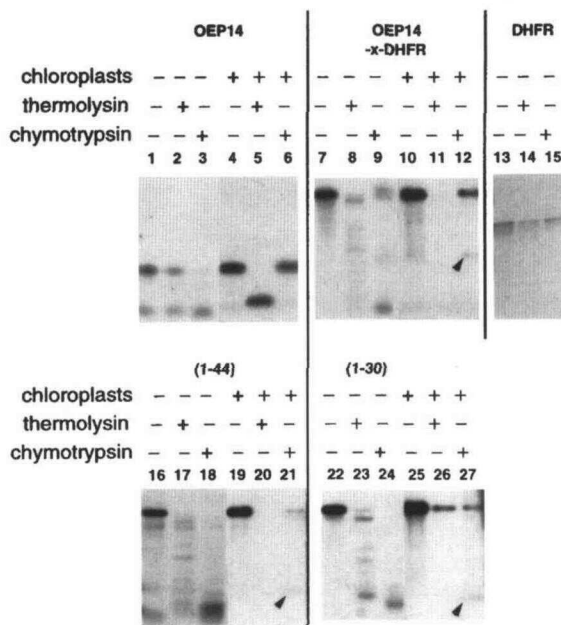
A thermolysin-resistant fragment was detected after the imported OEP14 protein was protease treated (Figure 2A, lane 3). OEP14 has only one methionine in its sequence, the N-terminal initiator methionine (Figure 1). The fact that a thermolysin-resistant fragment could be seen when the protein was labeled with  $^{35}$ S-methionine indicates that the N terminus of OEP14 is protected from the protease. The N terminus is followed by the only hydrophobic domain of the protein (Figure 1). Therefore, OEP14 most likely spans the outer membrane once with this hydrophobic domain, in an orientation with the N terminus facing the intermembrane space (ims) of the envelope. A plausible model for the membrane topology of OEP14 is therefore  $N_{\text{ims}}-C_{\text{cytosol}}$ , that is, the N terminus facing the intermembrane space and the C-terminal portion exposed in the cytosol.

To examine further whether the C-terminal end of OEP14 and the DHFR portion of fusion proteins were exposed on the cytosolic side of the outer membrane, we employed two other proteases. The first one was chymotrypsin, to which OEP14 has been shown to be resistant after being inserted into the outer membrane of chloroplasts (Li et al., 1991). Thus, any digestion of fusion proteins would only arise from digestion of the DHFR portion of the fusion proteins. As shown in Figure 4, OEP14 is chymotrypsin resistant after import (lane 6), confirming our previous observation (Li et al., 1991). Chymotrypsin preferentially cleaves peptide bonds at the C termini of phenylalanine, tyrosine, and tryptophan. The most likely cutting sites for chymotrypsin in OEP14 are the phenylalanines at amino acids 25 and 28. The fact that OEP14 is chymotrypsin resistant after import suggests that these two residues are probably buried in the membrane and therefore are not available for cleavage.

If the C-terminal end of OEP14 and the DHFR portion of fusion proteins were exposed on the cytosolic side of the outer membrane, the next most likely cutting site for chymotrypsin in the three fusion proteins would be the tryptophan residue

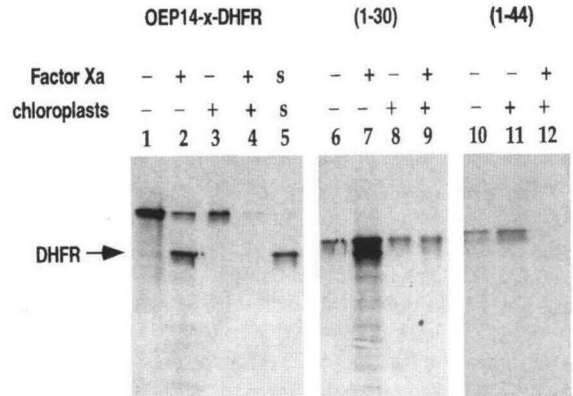
of amino acid 25 of DHFR. Cleavage at this residue would result in fragments slightly larger than the respective OEP14 portion in each fusion protein. Indeed, for all three fusion proteins, a chymotrypsin-cleaved fragment was detected after the chloroplasts were treated with the protease after import (Figure 4, lanes 12, 21, and 27, arrowheads). The fragment in the OEP14×DHFR sample (lane 12, arrowhead) is slightly larger than full-length OEP14. The fragments in the OEP14(1–44)×DHFR and the OEP14(1–30)×DHFR samples are sequentially smaller. Therefore, it is likely that chymotrypsin cleaved at the N-terminal portion of DHFR in each fusion protein. Cleavage of the imported fusion proteins by chymotrypsin also suggests that the DHFR part of each fusion protein is on the cytosolic side of the outer membrane, supporting an  $N_{\text{ims}}-C_{\text{cytosol}}$  membrane topology for OEP14.

A substantial proportion of the fusion proteins was chymotrypsin resistant after import to chloroplasts. One possible explanation for this result is that the DHFR portion in many of the imported fusion protein molecules had folded into a protease-resistant conformation. In agreement with this hypothesis, DHFR on its own was resistant to both thermolysin and chymotrypsin (Figure 4, lanes 13 to 15). However, we cannot exclude the possibility that some of the imported proteins had



**Figure 4.** Chymotrypsin and Thermolysin Digestion of Fusion Proteins.

Proteins obtained by *in vitro* translations were either treated directly with thermolysin or chymotrypsin (first three lanes of each set of sample) or incubated with chloroplasts under import conditions. After import, the chloroplasts were then treated with the two proteases as labeled at top. The arrowheads in lanes 12, 21, and 27 indicate the chymotrypsin-digested fragments of each fusion protein. The (+) and (–) signs at top indicate the addition or omission, respectively, of each component labeled at the left. (1–44), OEP14(1–44)×DHFR; (1–30), OEP14(1–30)×DHFR.



**Figure 5.** Factor Xa Digestion of Fusion Proteins.

Proteins obtained by *in vitro* translations were either treated directly with factor Xa (lanes 2 and 7) or incubated with chloroplasts under import conditions. After import, the chloroplasts were then treated with the protease (lanes 4, 9, and 12) as indicated by the (+) signs at top. Lane 5 is the supernatant fraction (s) of the digestion shown in lane 4. The position of DHFR is indicated by the arrow. The (+) and (–) signs at top indicate the addition or omission, respectively, of the components labeled at left. (1–44), OEP14(1–44)×DHFR; (1–30), OEP14(1–30)×DHFR.

assumed a different membrane topology and thus were chymotrypsin resistant.

Similar results were obtained by digestion of the three fusion proteins with another protease, factor Xa. The recognition site for this protease was engineered into the junctions between the various lengths of OEP14 and DHFR during the construction of fusion proteins (see Methods and Figure 1). As shown in Figure 5, both OEP14×DHFR and OEP14(1–44)×DHFR are factor Xa sensitive after being targeted to chloroplasts (lanes 4 and 12). A protein of the same size as DHFR was generated in the supernatant after digestion of imported OEP14×DHFR with factor Xa (Figure 5, lane 5), confirming that factor Xa cuts at the designated site at the end of OEP14. These results indicate that the C terminus of OEP14, at least from amino acids 44 to 65, was exposed on the cytosolic side of the outer membrane.

#### Fusion with a Different Passenger Protein

OEP14(1–30)×DHFR was factor Xa resistant and partly thermolysin resistant after import (Figure 5, lane 9, and Figure 2A, lane 13). One possible explanation for protease resistance is that amino acids 1 to 30 of OEP14 in the fusion protein are totally buried in the outer membrane, whereas the DHFR portion in most imported molecules has folded into a protease-resistant conformation or is even partially buried in the membrane. The DHFR portion, therefore, shields the factor Xa site from exogenous factor Xa. However, it is also possible that some of the imported OEP14(1–30)×DHFR have assumed

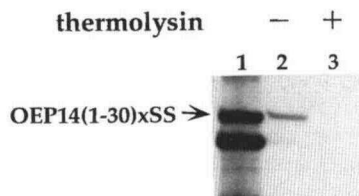


an  $N_{\text{cytosol}}-C_{\text{ims}}$  topology, thus placing the factor Xa site and DHFR passenger protein in the intermembrane space.

To test whether the protease-resistant results of OEP14(1–30)×DHFR were due to the protease resistance of DHFR or to the orientation of the imported fusion protein in the membrane, we made another fusion construct in which amino acids 1 to 30 of OEP14 were fused to a passenger protein that was less likely to fold into a protease-resistant conformation. The passenger protein chosen was the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SS). The fusion protein was named OEP14(1–30)×SS (Figure 1). As shown in Figure 6, OEP14(1–30)×SS was totally thermolysin sensitive after being targeted to chloroplasts. This indicates that the small subunit part of the fusion protein was exposed on the chloroplastic surface. Therefore, the thermolysin resistance of OEP14(1–30)×DHFR is probably due to the protease-resistant conformation of DHFR. This result further supports the idea that OEP14 spans the outer membrane in an  $N_{\text{ims}}-C_{\text{cytosol}}$  orientation. Interestingly, because the C-terminal flanking side of the OEP14 hydrophobic domain has fewer positive charges than the N-terminal flanking side (Figure 1), the topology of OEP14 is the opposite of what would be predicted by the “positive inside” rule (von Heijne and Gavel, 1988).

### Specificity of the Targeting Signal of OEP14

The structure of the OEP14 outer membrane targeting signal bears some resemblance to the signal peptides of proteins targeted to the endoplasmic reticulum (ER). First, it is localized at the N terminus of OEP14. Furthermore, it has a short, positively charged N-terminal portion followed by a hydrophobic core with a strong tendency to form an  $\alpha$ -helix (von Heijne, 1990). In fact, this targeting signal has 60 to 75% similarity to the signal peptides of various collagen precursor proteins



**Figure 6.** OEP14(1–30)×SS Is Thermolysin Sensitive after Import.

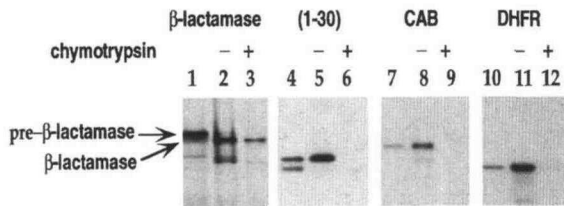
Lane 1 contains the *in vitro* translation product of OEP14(1–30)×SS. The lower molecular mass band is the same size as the mature small subunit (data not shown) and most likely arose from internal initiation from the first residue of the mature small subunit, which happens to be a methionine. Lane 2 contains chloroplasts after the import of OEP14(1–30)×SS. Lane 3 is the same as lane 2, except that the chloroplasts were treated with thermolysin after import. The (+) and (–) signs at top indicate the presence or absence, respectively, of thermolysin after import.

at the primary sequence level (data not shown). This likeness raised the question of whether the signal was really specific for the chloroplastic outer envelope membrane. We sought to answer this question by trying to import the OEP14(1–30)×DHFR fusion protein into microsomal membranes. If the fusion protein could insert into microsomal membranes in the same way that it inserted into the chloroplastic outer membrane, it should be chymotrypsin resistant or a specific chymotrypsin-cleaved fragment should be seen (Figure 4, lane 27) after insertion. As shown in Figure 7, the fusion protein associated with the microsome but remained totally sensitive to chymotrypsin (Figure 7, lanes 4 to 6). This result indicates that OEP14(1–30)×DHFR does not insert into the ER membrane. The association with the ER membrane (Figure 7, lane 5) is probably not due to a specific recognition of the ER membrane by the fusion protein because DHFR by itself (Figure 7, lane 11) and another chloroplastic membrane protein, CAB (Figure 7, lane 8), also stuck to the microsomal membranes under the same experimental condition. As a positive control, a signal peptide-bearing precursor protein, pre- $\beta$ -lactamase, was tested for import at the same time. It was processed to a lower molecular mass protein due to the removal of the signal peptide (Figure 7, lane 2). The processed mature  $\beta$ -lactamase was resistant to protease digestion (Figure 7, lane 3), indicating that it is inside the microsomal vesicles. However, pre- $\beta$ -lactamase did not associate with chloroplasts in an *in vitro* chloroplast import experiment (data not shown).

### DISCUSSION

The targeting and integration signal of OEP14 did not insert into the ER membrane, even though it resembled the signal peptides of secretory proteins. It has also been shown that OEP14 will not insert into mitochondrial membranes (Li et al., 1991). Our preliminary results suggest that OEP14 did not insert into the thylakoid membrane when directed into the stroma of chloroplasts (H.-m. Li, unpublished data). Therefore, even though import of OEP14 to chloroplasts does not require ATP or thermolysin-sensitive components on the chloroplastic surface, its insertion is specific to the chloroplastic outer membrane.

DHFR has been shown to be sometimes protease resistant when used as a passenger protein in fusion constructs. For example, a fusion protein with the presequence of the yeast mitochondrial cytochrome oxidase subunit IV fused to DHFR is resistant to proteinase K and trypsin when synthesized in an *in vitro* translation system or purified from *Escherichia coli* overexpressing the fusion protein (van Loon et al., 1986; Verner and Schatz, 1987; Eilers et al., 1988; Endo et al., 1989). The fusion protein becomes protease sensitive upon binding to the surface of mitochondria. This observation has been used as evidence for the unfolding of precursor proteins when they bind to mitochondria (Eilers et al., 1988; Endo et al., 1989). How-



**Figure 7.** OEP14(1-30)×DHFR Did Not Insert into Microsomal Membranes.

Rabbit reticulocyte lysate and *in vitro*-transcribed RNA encoding various proteins were incubated in the absence (lanes 1, 4, 7, and 10) or presence (lanes 2, 5, 8, and 11) of microsomal membranes under import conditions. RNA used for each set of samples is indicated at top. Half of the reactions containing the microsomes were further treated with chymotrypsin (lanes 3, 6, 9, and 12). Microsomes were then recovered by centrifugation and analyzed by using SDS-PAGE and autoradiography. Pre- $\beta$ -lactamase indicates the precursor form of  $\beta$ -lactamase containing the signal peptide. Arrows indicate the positions of pre- $\beta$ -lactamase and  $\beta$ -lactamase. The (+) and (−) signs at top indicate the presence or absence, respectively, of chymotrypsin after import. (1-30), OEP14(1-30)×DHFR.

ever, the same fusion protein is sensitive to thermolysin even before binding to mitochondria (Eilers and Schatz, 1986). Another DHFR fusion protein has also been shown to be thermolysin sensitive when synthesized in an *in vitro* translation system (America et al., 1994). For the three fusion proteins that we constructed, the DHFR portions were sensitive to both thermolysin and chymotrypsin when synthesized in the *in vitro* translation system before import to chloroplasts (Figure 4, lanes 8, 9, 17, 18, 23, and 24). They became protease resistant only after being targeted to chloroplasts. It is possible that fusion to the OEP14 sequences caused changes in DHFR conformation. It is also possible that there are some cytosolic factors that recognize and bind to the targeting signal of OEP14. These factors may help to keep the passenger protein in a loosely folded conformation, rendering it more protease sensitive. However, currently there is no evidence supporting either hypothesis.

The chloroplast targeting signal of a peripheral outer membrane protein, SCE/Com70, has been analyzed (Wu and Ko, 1993). This protein is localized to the periphery of chloroplasts and is hydrophilic in nature. Its sequence is similar to major soluble cytosolic heat shock protein 70 (Ko et al., 1992). It has been shown that the first 48 amino acids of this protein can direct the peripheral association of a passenger protein with chloroplasts (Wu and Ko, 1993). These 48 amino acids have no homology with the targeting signal of OEP14. However, because Com70 is a hydrophilic peripheral membrane protein, it is possible that it uses a different targeting mechanism than integral membrane proteins, such as OEP14.

The remaining three known chloroplastic outer membrane proteins that do not have cleavable transit peptides, OEP6,7, OEP24, and OEP34, are also predicted to have only one ma-

for membrane-spanning domain (Salomon et al., 1990; Fischer et al., 1994; Seedorf et al., 1995). It is interesting to speculate that similar to OEP14, these membrane-spanning domains are also the outer membrane targeting signals of these proteins. In the case of pea OEP34, deletion of 58 amino acids from the C terminus, which includes the membrane-spanning domain flanked by 15 amino acids on the amino side and 28 amino acids on the C-terminal side, abolishes the association of the protein with chloroplasts (Seedorf et al., 1995). It is not clear whether the information necessary for chloroplast association resides within the membrane-spanning domain or within any of the flanking portions. Our preliminary data indicate that a sequence containing only two-thirds of the membrane-spanning domain and the C-terminal flanking portion from an Arabidopsis homolog of pea OEP34 could still target a passenger protein to chloroplasts (H.-m. Li, unpublished data). More work is required before we know the consensus sequence or structure for the chloroplastic outer envelope membrane targeting signal. This information will facilitate the identification of the machinery recognizing these signals.

All of the mitochondrial outer membrane proteins that have been characterized also do not possess cleavable presequences. However, unlike chloroplasts, the import of most mitochondrial outer membrane proteins still uses the same receptor complex as the one used by the majority of interior-targeted precursor proteins—the Tom20–Tom22 complex (Söllner et al., 1989, 1990; Keil and Pfanner, 1993; Lill and Neupert, 1996). The other mitochondrial receptor complex, Tom70–Tom37, is used only by a few precursor proteins targeted to the inner membrane and intermembrane space and matrix (Lill and Neupert, 1996), for example, the ADP/ATP carrier protein of the inner membrane. However, in the absence of Tom70, the ADP/ATP carrier protein can still be imported into isolated mitochondria via the Tom20–Tom22 complex, although with a lower efficiency (Steger et al., 1990). Therefore, the phenomenon that a different import pathway exists for the majority of outer membrane proteins seems to be unique to chloroplasts.

The targeting signals of two yeast mitochondrial outer membrane proteins have been identified (Li and Shore, 1992; Nguyen et al., 1993). Interestingly, both signals are the membrane-anchoring sequences of the two proteins, similar to OEP14. Because these hydrophobic membrane-anchoring sequences are quite different from the usual cleavable mitochondria targeting presequences, it is not clear how the same mitochondrial receptor complex recognizes these different targeting sequences. No information is available on the targeting signals for plant mitochondrial outer membrane. If some plant mitochondrial outer membrane proteins also possess a targeting signal similar to that of the two yeast mitochondrial outer membrane proteins, a plant cell must have additional mechanisms to distinguish among the mitochondrial and chloroplastic outer membrane targeting signals and the ER targeting signal peptide.

Knowing the direction in which the targeting signal inserts into the outer membrane is essential for understanding the

targeting mechanism of OEP14. It may also be important for the future isolation and characterization of factors assisting the targeting and insertion of OEP14 into the outer membrane. Our analysis of OEP14 membrane topology indicates that it inserts into the outer membrane in an  $N_{\text{ims}}-C_{\text{cytosol}}$  orientation. Because the C-terminal flanking side of the hydrophobic domain has fewer positive charges than does the N-terminal side, this orientation is the opposite of what would be predicted by the "positive inside" rule (von Heijne and Gavel, 1988) or the "charge difference" rule (Hartmann et al., 1989). By analyzing membrane proteins of known topologies, these rules predict that the flanking side of the hydrophobic domain with more positive charges (positive inside), or with a net positive charge compared with the other flanking side (charge difference), will be retained on the cytosolic side of the membrane. The rules have been shown to apply to most proteins from the ER membrane, the mitochondrial inner and outer membranes, the chloroplastic thylakoid membrane, and the bacterial plasma membrane (von Heijne and Gavel, 1988; Gavel et al., 1991; Li and Shore, 1992). It is not clear whether chloroplastic outer membrane proteins follow a different rule for orientation in the membrane or whether OEP14 is an exception. It may be that OEP14 tends to insert into the outer membrane at certain regions that have a lower anionic lipid surface density or that the positive charges at the N terminus of OEP14 can insert into the outer membrane as ion pairs (Krishtalik and Cramer, 1995), resulting in reduced energy barrier and the translocation of the positively charged N terminus across the membrane. More research is required to unveil the determining factors for membrane orientations of chloroplastic outer envelope membrane proteins.

## METHODS

### Chimeric Constructs Encoding Fusion Proteins

The dihydrofolate reductase (DHFR) cDNA was excised from the plasmid PC-DHFR (Hageman et al., 1990) and subcloned into the BamHI and HindIII sites of pBluescript SK+. A linker, made of two oligonucleotides with the sequences 5'-CTAGAATCGAAGGTCGTG-3' and 5'-GATCCACGACCTTCGATT-3', which contains XbaI, factor Xa, and BamHI restriction sites, was then inserted into the XbaI and BamHI sites of the plasmid. The resulting plasmid was called pXDHFR. The XbaI-HindIII fragment of pXDHFR containing the factor Xa processing site and the DHFR cDNA was further subcloned into the XbaI and HindIII sites of pSP65. The resulting plasmid was named pSP65-XDHFR. Regions corresponding to amino acids 1 to 15, 1 to 30, 32 to 65, and the full length of OEP14 were amplified by polymerase chain reactions (PCRs) using primers specific for the desired regions plus the sequence for the EcoRI restriction site at the 5' end of the N-terminal primers and the sequence for the XbaI restriction site at the 5' end of the C-terminal primers. PCR products were digested with EcoRI and XbaI and subcloned into the EcoRI and XbaI sites of pSP65-XDHFR. The fusion construct pOEP14(1-44)×DHFR was made by excising the

coding region for amino acids 1 to 44 of OEP14 with EcoRI and an internal BamHI site located at amino acids 43 to 45. The BamHI site was blunt ended with the large fragment of DNA polymerase I (Klenow fragment), and the resulting fragment was cloned into the EcoRI and SmaI sites of pSP65-XDHFR.

The plasmid encoding OEP14(1-30)×SS was constructed as follows. The DNA encoding the mature region of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SS) was excised from the plasmid pRBCS65 (Cashmore, 1983) with SphI and PstI and subcloned into the SphI and PstI sites of pSP72. The resulting plasmid was named pSP72-mSS. The coding region for the first 30 amino acids of OEP14 and the factor Xa restriction site was amplified by PCR from the plasmid encoding the fusion protein OEP14(1-30)×DHFR. The sequence for the SphI restriction site was added to the 5' end of the C-terminal primer. The amplified fragment was blunt ended with the Klenow fragment, digested with SphI, and subcloned into the PvuII and SphI sites of pSP72-mSS.

All constructs involving PCR-amplified fragments were confirmed by sequencing the entire amplified regions. Junctions between various fragments in all chimeric constructs were also sequenced. Sequencing was performed with the dideoxynucleotide chain termination method on double-stranded plasmids using the Sequenase II kit from United States Biochemical (Cleveland, OH).

### Protein Import and Postimport Treatments

<sup>35</sup>S-methionine-labeled proteins were synthesized through in vitro transcription (Perry et al., 1991) and in vitro translation with wheat germ extracts (Promega, Madison, WI) according to the manufacturer's specifications. Isolation of chloroplasts from 9-day-old pea (*Pisum sativum* cv Dark-Skinned Perfection) seedlings and import of proteins into chloroplasts were performed as described previously (Perry et al., 1991).

Thermolysin (Boehringer Mannheim) and chymotrypsin (sequencing grade; Boehringer Mannheim) treatments of chloroplasts after import were performed as described by Li et al. (1991). Factor Xa (restriction protease grade; Boehringer Mannheim) treatment was performed by incubating chloroplasts after import into an import buffer (330 mM sorbitol, 50 mM Hepes-KOH, pH 8.0) containing 400 µg/mL factor Xa and 1 mM CaCl<sub>2</sub> at room temperature in the dark for 2 hr. Intact chloroplasts after treatments were reisolated through a 40% Percoll solution as described previously (Perry et al., 1991). Alkaline extraction of chloroplasts after import was performed as described by Tranel et al. (1995). The extracted pellet and the soluble fractions were separated by centrifugation at 125,000g for 45 min in a Beckman (Palo Alto, CA) TLA 45 rotor.

Fractionation of chloroplasts was performed as described by Li et al. (1991), except that the step gradient was made of 1.5 mL of 1 M sucrose, 1.2 mL of 0.8 M sucrose, and 1 mL of 0.46 M sucrose solutions, and the gradient was centrifuged at 50,000 rpm for 1 hr in a Beckman SW60 rotor.

Dog pancreatic microsomes and rabbit reticulocyte lysates were purchased from Promega. Microsomal imports of proteins were performed as suggested by the manufacturer. After import and chymotrypsin digestion, the microsomes were recovered by centrifugation at 125,000g for 45 min.

Samples were analyzed by SDS-PAGE on 10 to 20% gradient Tricine gels purchased from Novex (San Diego, CA). Quantitation of samples was performed using PhosphorImager SP (Molecular Dynamics, Sunnyvale, CA) with dried gels.



## ACKNOWLEDGMENTS

We thank Jenny Dori and Drs. Ken Keegstra, Kathy Archer, and Laura Olsen for critical reading of the manuscript. This work was supported by grants to H.-m.L. from the National Science Council (Grant No. 85-2311-B-001-086) and the Academia Sinica of Taiwan, Republic of China.

Received May 10, 1996; accepted August 1, 1996.

## REFERENCES

- America, T., Hageman, J., Guera, A., Rook, F., Archer, K., Keegstra, K., and Weisbeek, P. (1994). Methotrexate does not block import of a DHFR fusion protein into chloroplasts. *Plant Mol. Biol.* **24**, 283–294.
- Cashmore, A.R. (1983). Nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. In *Genetic Engineering of Plants*, T. Kosuge, C.P. Meredith, and A. Hollaender, eds (New York: Plenum Publishing), pp. 29–38.
- Cline, K., Andrews, J., Mersey, B., Newcomb, E.H., and Keegstra, K. (1981). Separation and characterization of inner and outer envelope membranes of pea chloroplasts. *Proc. Natl. Acad. Sci. USA* **78**, 3593–3599.
- Cline, K., Werner-Washburne, M., Andrews, J., and Keegstra, K. (1984). Thermolysin is a suitable protease for probing the surface of intact pea chloroplasts. *Plant Physiol.* **75**, 675–678.
- Eilers, M., and Schatz, G. (1986). Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* **322**, 228–232.
- Eilers, M., Hwang, S., and Schatz, G. (1988). Unfolding and refolding of a purified precursor protein during import into isolated mitochondria. *EMBO J.* **7**, 1139–1145.
- Endo, T., Eilers, M., and Schatz, G. (1989). Binding of a tightly folded artificial mitochondrial precursor protein to the mitochondrial outer membrane involves a lipid-mediated conformational change. *J. Biol. Chem.* **264**, 2951–2956.
- Fischer, K., Weber, A., Arbinger, B., Brink, S., Eckerskorn, C., and Flügge, U.-I. (1994). The 24 kD outer envelope membrane protein from spinach chloroplasts: Molecular cloning, in vivo expression and import pathway of a protein with unusual properties. *Plant Mol. Biol.* **25**, 167–177.
- Gavel, Y., Steppuhn, J., Herrmann, R., and von Heijne, G. (1991). The “positive-inside rule” applies to thylakoid membrane proteins. *FEBS Lett.* **282**, 41–46.
- Hageman, J., Baecke, C., Ebskamp, M., Pilon, R., Smeekens, S., and Weisbeek, P. (1990). Protein import into and sorting inside the chloroplast are independent processes. *Plant Cell* **2**, 479–494.
- Hartmann, E., Rapoport, T.A., and Lodish, H.F. (1989). Predicting the orientation of eukaryotic membrane spanning proteins. *Proc. Natl. Acad. Sci. USA* **86**, 5786–5790.
- Hirsch, S., Muckel, E., Heemeyer, F., von Heijne, G., and Soll, J. (1994). A receptor component of the chloroplast protein translocation machinery. *Science* **266**, 1989–1992.
- Keegstra, K. (1989). Transport and routing of proteins into chloroplasts. *Cell* **56**, 247–253.
- Keegstra, K., Olsen, L., and Theg, S.M. (1989). Chloroplastic precursors and their transport across the envelope membranes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 471–501.
- Keil, P., and Pfanner, N. (1993). Insertion of MOM22 into the mitochondrial outer membrane strictly depends on surface receptors. *FEBS Lett.* **321**, 197–200.
- Kessler, F., Blobel, G., Patel, H.A., and Schnell, D.J. (1994). Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science* **266**, 1035–1039.
- Ko, K., Bornemisza, O., Kourtz, L., Ko, Z.W., Plaxton, W.C., and Cashmore, A.R. (1992). Isolation and characterization of a cDNA clone encoding a cognate 70-kD heat shock protein of the chloroplast envelope. *J. Biol. Chem.* **267**, 2986–2993.
- Krishtalik, L.I., and Cramer, W.A. (1995). On the physical basis for the *cis*-positive rule describing protein orientation in biological membranes. *FEBS Lett.* **369**, 140–143.
- Li, H.-m., Moore, T., and Keegstra, K. (1991). Targeting of proteins to the outer envelope membrane uses a different pathway than transport into chloroplasts. *Plant Cell* **3**, 709–717.
- Li, H.-m., Sullivan, T.D., and Keegstra, K. (1992). Information for targeting to the chloroplastic inner envelope membrane is contained in the mature region of the maize *Bt1*-encoded protein. *J. Biol. Chem.* **267**, 18999–19004.
- Li, J.-M., and Shore, G.C. (1992). Reversal of the orientation of an integral protein of the mitochondrial outer membrane. *Science* **256**, 1815–1817.
- Lill, R., and Neupert, W. (1996). Mechanisms of protein import across the mitochondrial outer membrane. *Trends Cell Biol.* **6**, 56–61.
- Markwell, J., Bruce, B.D., and Keegstra, K. (1992). Isolation of a carotenoid-containing sub-membrane particle from the chloroplastic envelope outer membrane of pea (*Pisum sativum*). *J. Biol. Chem.* **267**, 13933–13937.
- Nguyen, M., Millar, D.G., Yong, V.W., Korsmeyer, S.J., and Shore, G.C. (1993). Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. *J. Biol. Chem.* **268**, 25265–25268.
- Olsen, L.J., Theg, S.M., Selman, B.R., and Keegstra, K. (1989). ATP is required for the binding of precursor proteins to chloroplasts. *J. Biol. Chem.* **264**, 6724–6729.
- Perry, S.E., Li, H.-m., and Keegstra, K. (1991). *In vitro* reconstitution of protein transport into chloroplasts. *Methods Cell Biol.* **34**, 327–344.
- Salomon, M., Fischer, K., Flügge, U.I., and Soll, J. (1990). Sequence analysis and protein import studies of an outer chloroplast envelope polypeptide. *Proc. Natl. Acad. Sci. USA* **87**, 5778–5782.
- Schägger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368–379.
- Schnell, D.J., Kessler, F., and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. *Science* **266**, 1007–1012.
- Seedorf, M., Waagemann, K., and Soll, J. (1995). A constituent of the chloroplast import complex represents a new type of GTP-binding protein. *Plant J.* **7**, 401–411.

- Söllner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert, W.** (1989). MOM19, an import receptor for mitochondrial precursor proteins. *Cell* **59**, 1061–1070.
- Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W.** (1990). A mitochondrial import receptor for the ADP/ATP carrier. *Cell* **62**, 107–115.
- Steger, H.F., Söllner, T., Kiebler, M., Dietmeier, K.A., Pfaller, R., Trülsch, K.S., Tropschug, M., Neupert, W., and Pfanner, N.** (1990). Import of ADP/ATP carrier into mitochondria: Two receptors act in parallel. *J. Cell Biol.* **111**, 2353–2363.
- Tranel, P.J., Froehlich, J., Goyal, A., and Keegstra, K.** (1995). A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO J.* **14**, 2436–2446.
- van Loon, A.P.G.M., Brändil, A.W., and Schatz, G.** (1986). The presequences of two imported mitochondrial proteins contain information for intracellular and intramitochondrial sorting. *Cell* **44**, 801–812.
- Verner, K., and Schatz, G.** (1987). Import of an incompletely folded precursor protein into isolated mitochondria requires an energized inner membrane, but no added ATP. *EMBO J.* **6**, 2449–2456.
- von Heijne, G.** (1990). The signal peptide. *J. Membr. Biol.* **115**, 195–201.
- von Heijne, G., and Gavel, Y.** (1988). Topogenic signals in integral membrane proteins. *Eur. J. Biochem.* **174**, 671–678.
- von Heijne, G., Steppuhn, J., and Herrmann, R.G.** (1989). Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* **180**, 535–545.
- Wu, C., and Ko, K.** (1993). Identification of an uncleavable targeting signal in the 70-kilodalton spinach chloroplast outer envelope membrane protein. *J. Biol. Chem.* **268**, 19384–19391.

**Protein targeting and integration signal for the chloroplastic outer envelope membrane.**

H M Li and L J Chen

*Plant Cell* 1996;8;2117-2126

DOI 10.1105/tpc.8.11.2117

This information is current as of July 19, 2018

<b>Permissions</b>	<a href="https://www.copyright.com/ccc/openurl.do?sid=pd_hw1532298X&amp;issn=1532298X&amp;WT.mc_id=pd_hw1532298X">https://www.copyright.com/ccc/openurl.do?sid=pd_hw1532298X&amp;issn=1532298X&amp;WT.mc_id=pd_hw1532298X</a>
<b>eTOCs</b>	Sign up for eTOCs at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a>
<b>CiteTrack Alerts</b>	Sign up for CiteTrack Alerts at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a>
<b>Subscription Information</b>	Subscription Information for <i>The Plant Cell</i> and <i>Plant Physiology</i> is available at: <a href="http://www.aspb.org/publications/subscriptions.cfm">http://www.aspb.org/publications/subscriptions.cfm</a>