Many of the proteins in the chloroplast envelope play an important role in facilitating the biochemical and transport processes of the compartment. For the transport of proteins into the chloroplast, we have recently identified at least three different envelope proteins (Com44/Cim44, Com70, and Cim97) in close physical proximity to a partially translocated chimeric precursor protein (Wu, C., Seibert, F. S., and Ko, K. (1994) J. Biol. Chem. 269, 32264–32271). In this study we report the characterization of a cDNA clone encoding a member of the Com44/Cim44 envelope proteins. The combined data from nucleotide sequencing, and RNA and protein blot analyses indicate the existence of multiple forms of the 44-kDa envelope protein. Depending on the plant species examined, immunologically-related protein bands with molecular masses of 42 to 46 kDa were observed. Organelle subfractionation, protease treatment, and immunomicroscopic studies together provide an indication that the immunologically-related proteins may be present in both the outer and inner envelope membranes. Co-migration of the product synthesized from the cDNA insert with a 44-kDa immunoreactive band of the chloroplast envelope, and the in vitro import results, together suggest that the in vitro synthesized 44-kDa protein is targeted to the envelope membrane without any further processing.

Chloroplast envelope proteins play a major role in modulating the vectorial flow of molecules across the membrane, including large proteinaceous entities. The import of proteins into the chloroplast is a complex process requiring the close collaboration of both the outer envelope and the inner envelope membranes. Evidence for the possible existence of two distinct protein import complexes, one in each envelope membrane, is beginning to emerge from a number of recent investigations (Waegemann and Soll, 1991; Soll and Waegemann, 1992; Schnell and Blobel, 1993; Alefson et al., 1994; Schell et al., 1994; Kessler et al., 1994; Wu et al., 1994). An important step in the characterization of the protein translocating complexes is the identification of the components involved. The identification of outer and inner envelope polypeptides of these protein translocating complexes has been achieved using a variety of strategies (Cornwall and Keegstra, 1987; Kaderbhai et al., 1988; Pain et al., 1988; Schnell et al., 1990a, 1994; Hinz and Flugge, 1988; Soll and Waegemann, 1992; Waegemann et al., 1990; Perry and Keegstra, 1994; Alefson et al., 1994; Kessler et al., 1994; Wu et al., 1994; Hirsch et al., 1994; Seedorf et al., 1995; Seedorf and Soll, 1995; Gray and Row, 1995). So far these studies collectively indicate that envelope proteins with molecular masses of 30, 34, 36, 44, 45, 51, 66, 70, 75, 86, 97, and 100 kDa may be possible constituents of the chloroplast protein import apparatus; however, it is not obvious from the existing data whether some of the predicted similar sized components are identical to each other.

The complex nature of protein translocation mechanisms observed in other membranous systems, such as the mitochondrion and the endoplasmic reticulum, suggests that there is most likely a significant number of chloroplast envelope components that need to be identified and characterized in detail. Our major strategy for identifying and studying components of the protein translocation apparatus is to isolate cDNA clones that encode all types of chloroplast envelope proteins and then to systematically sort out the identity and/or function of the clones. This approach allows us to circumvent the technical problems and limitations of purifying small quantities of authentic proteins from the envelope. In this study, we report on the identification and molecular characterization of one of the cDNA clones that encodes a 44-kDa envelope protein with unusual features. The 44-kDa polypeptide encoded by this cDNA insert is a member of the Com44/Cim44 chloroplast envelope proteins recently found in close physical proximity to a partially translocated chimeric precursor protein (Wu et al., 1994). Specific antibodies raised against the 44-kDa protein were used to determine the location of the immunologically-related polypeptides in the chloroplast envelope. The implications of the potential locale of these immunologically-related proteins are discussed in relation to recent developments in our understanding of the uptake of proteins into the chloroplast.

**Materials and Methods**

Antibody Preparation—The polyclonal antiserum used in the identification of cDNA clones was raised against total pea chloroplast envelope proteins as described previously in Ko et al. (1992). Antibodies against the COOH terminus of the tomato 44-kDa envelope protein and both NH2 and COOH termini of the Brassica napus 44-kDa envelope protein (Bce44B) were generated as outlined in Wu et al. (1994). Pre-immune IgGs were collected prior to the injection of each rabbit.

Identification of cDNA Clones Encoding 44-kDa Proteins—Construction of the B. napus (cv. Topas) cDNA expression library and the strategy for immunoscreening the B. napus cDNA expression library was reported elsewhere (Ko et al., 1992, 1994). The cDNA inserts were retrieved by an EcoRI–NtI digestion and subcloned into pGEM11Z

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ EMBL Data Bank with accession number(s) X79091.

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chloroplast envelope proteins (Ko et al., 1994). Over 400 immuno-
positive cDNA clones representing different types of chloro-
plast envelope proteins were obtained. One of the cDNA clone-
specifying affinity purified antibodies immunoreacted with 42-
and 44-kDa pea envelope proteins. However, the cDNA insert
was only 760 base pairs in length (resulting plasmid designated
pBCE44A) and was confirmed by nucleotide sequencing to pos-
sess insufficient coding information (sequences marked A in
Fig. 1). A full-length cDNA clone was obtained by rescoring
the unamplified version of the cDNA library with antibodies
generated against the COOH terminus of the 44-kDa protein
(resulting plasmid designated pBCE44B). Positive immunore-
actions of the Bce44B recombinant phage plaque were obtained
with antibodies generated against the cDNA-encoded Bce44B
protein itself (IgGs made separately against the NH₂ or the
COOH terminus) or the IgGs against a tomato version of the
Bce44B protein. The latter antibodies were originally used to
identify the close physical proximity of Com44/Cim44 to par-
tially translocated chimeric precursor proteins, thus the
cDNA-encoded Bce44B represents a member of the same
Com44/Cim44 envelope components previously reported (Wu et
al., 1994).

Antibodies against the cDNA-encoded Bce44B (NH₂ and
COOH terminus) gave rise to identical chemical cross-linking/
immunoprecipitation results as reported by Wu et al. (1994)
(Fig. 2), providing a confirmation of Bce44B’s identity and its
predicted function. The membrane impermeable chemical
cross-linker DTSSP allowed the formation of cross-linked com-
pleses between Bce44B and partially translocated Oee1-Dhfr
precursors (Fig. 2, lane 1). These complexes were immunopre-
cipitated only with antibodies against Bce44B but not the
abundant 37-kDa inner membrane protein (Fig. 2, lane 4). Cross-
linked complexes were not immunoprecipitated with pre-
immune IgGs (Fig. 2, lane 5) or when the assays were con-
ducted with 1 mM ATP (Fig. 2, lane 2) or in the absence of
DTSSP (Fig. 2, lane 3). The ability of DTSSP to form cross-
linked complexes indicates accessibility from the cytosolic side
of the envelope.

The nucleotide sequence of the bce44B cDNA insert (se-
quences marked B in Fig. 1) was found to contain an open
reading frame of 969 nucleotides plus 122 and 102 nucleo-
tides of 5’- and 3’-untranslated sequences, respectively. The
total length of the cDNA insert is 1,193 nucleotides, compared
with an estimated mRNA size of 1,200 nucleotides (Fig. 3), sug-
gesting that the cDNA insert is probably full-length. The initi-
ating methionine codon was assigned to nucleotides 122–124
which results in a protein of 323 amino acids. The deduced protein
sequence was calculated to have a molecular mass of 36 kDa
which is substantially less than the value estimated by SDS-
PAGE. This difference may be attributed to some under-
determined structural feature of the protein that affects its migra-
tion in SDS-polyacrylamide gels. Similar discrepancies have
been noted before, especially for proteins originating from
membranous locations, e.g. Omp24 (Fisher et al., 1994). This
possibility is supported by the fact that the in vitro product
synthesized from the cDNA insert co-migrated with the native
B. napus envelope protein (discussed in the import results).

The hydropathy profile generated for the deduced bce44B
protein sequence did not show any clear discernable hydrophobic
or membrane-spanning features which could have given us a clue
as to its location in the chloroplast envelope membrane (data
not shown). This characteristic is reflected in the amino acid
composition: 48.6% for nonpolar and 47.7% for polar residues.
The amino-terminal 62 residues are completely devoid of acidic
residues, a feature characteristic of targeting sequences. Com-
parison of the bce44A nucleotide and deduced amino acid
sequences to entries in the gene data banks did not reveal any identity/function at the time of manuscript preparation. The deduced BCE44B protein sequence is identical to the partial sequence for the tomato version of this polypeptide (Tce44) (data not shown), providing sequence confirmation that BCE44B is identical to the protein used to generate IgGs for the previous chemical cross-linking study (Wu et al., 1994).

The cDNA sequence data indicate that the transcripts (bce44A and bce44B) are likely derived from different genes since the 3'-untranslated regions are different (Fig. 1). This possibility was confirmed by the distinct genomic DNA hybridization patterns displayed by the two cDNA clones. Differences were also observed at the steady state transcript level (Fig. 3). The amount of steady state bce44B transcripts was relatively low when compared to the highly abundant transcripts of rbcS. The bce44B transcripts were estimated to be approximately 1,200 nucleotides long. The steady state mRNA levels of bce44B were not significantly influenced by light. The bce44B mRNA level appears to remain constant despite dark treatment for 3 days, whereas steady state mRNA levels of the positive light-regulated rbcS gene decreased to a very low amount. Steady state transcripts were not detected with the bce44A probe (Fig. 3, lanes 1 and 2) and were not influenced by light such that the transcripts became detectable after a dark treatment. These results indicate that bce44B possesses an

2 K. Ko, unpublished data.
expression profile different from bce44A, further supporting the possibility that bce44A is distinct from bce44B.

Localization of the 44-kDa Envelope Proteins—The pattern of protein bands that immunoreacted with the anti-44 kDa IgGs was different in the three plant species examined (Fig. 4A, lanes 1–3). Two distinct bands were observed in pea, a major 44-kDa band and a minor 42-kDa band, whereas three discernible bands with relative molecular masses of 46, 44, and 42 kDa were observed in B. napus. Only one intense band of approximately 44 kDa was observed in tomato. The anti-44 kDa IgGs reacted specifically to the 42- and 44-kDa protein bands in the pea chloroplast envelope (Fig. 4B, lane 3) and showed very little cross-reactivity to stromal or thylakoid proteins (Fig. 4A, lanes 1 and 2). The immunoreactive bands were predominantly in the inner membrane fraction (Fig. 4B, lane 5). Less intense immunoreactive bands were also present in the outer membrane fraction (Fig. 3B, lane 4). These outer envelope bands were not due to contamination by small quantities of inner membrane as evident by the lack of immunoreaction with antibodies against the 37-kDa inner membrane protein, a lower abundant polypeptide (Dreses-Werringloer et al., 1991) (Fig. 4B). Anti-Com70 IgGs, antibodies against an outer membrane polypeptide (Wu et al., 1994; formerly designated Sce70 in Ko et al. (1992)), reacted with a protein in both outer and inner membrane fractions (Fig. 4B). The preimmune IgGs did not cross-react to any chloroplastic proteins (Fig. 4B). The resulting immunoreactive protein patterns were not due to degradation since tissues homogenized in 10% trichloroacetic acid gave the same result (Fig. 4A, lanes 4 and 5), indicating that multiple immunologically-related forms of the 44-kDa protein exist in the envelope membrane. One possibility is that the multiple forms observed are different from each other and are related by virtue of common antigenic moieties. Alternatively, the proteins may be similar but possess different mobilities due to differences in size and/or amino acid composition. The relationship between the immunologically-related forms and the different cDNAs remains to be elucidated. Since these immunologically-related proteins display sizes ranging from 42 to 46 kDa in the three plant species examined, we collectively refer to the whole set of bands as 44-kDa proteins to simplify presentation of some of the results.

Thermolysin and trypsin treatments of pea chloroplasts were conducted to further examine the nature of the 44-kDa proteins' association with the envelope. Thermolysin is a protease that cannot penetrate the outer envelope hence it is useful for probing polypeptides accessible on the surface of the outer membrane. Trypsin can penetrate the outer envelope and can thus be used to probe accessible protein moieties external to the inner envelope membrane (Joyard et al., 1983; Cline et al., 1985). Identical results were obtained with whole organelles and with envelopes prepared from treated plastids as well as with B. napus chloroplasts, therefore only the results of the pea chloroplast envelope experiment are presented (Fig. 5A). The majority of the immunorelated 44-kDa proteins are resistant to proteases (both thermolysin and trypsin) and are probably protected by the inner envelope membrane. However, thermolysin treatment gave rise to small amounts of a polypeptide with a relative molecular mass of approximately 42 kDa (Fig. 5A, lane 2). The thermolysin-generated 42-kDa band was, however, degraded with a subsequent trypsin treatment or by using trypsin in place of thermolysin (Fig. 5A, lane 3). A distinct smaller sized trypsin-generated immunoreactive product of approximately 30 kDa was observed (Fig. 5A, lane 3). The thermolysin-generated 42-kDa band is distinct from the native 42-kDa immunoreactive protein since the native band is still

Fig. 3. RNA blot analysis of B. napus. RNA blot analysis of steady-state bce44A and bce44B transcript levels in response to light-dark treatments. Hybridization experiments were carried out using cDNA probes for bce44A (lanes 1 and 2) and bce44B (lanes 3 and 4). Total RNA from dark treated and light grown plants is indicated by D and L, respectively. A control experiment using the rbcS probe is also presented (second row marked RBCS). The estimated size of the bce44B transcript is indicated in nucleotides.

Fig. 4. Immunoblot analysis of the 44-kDa chloroplast envelope proteins. A, a comparison of immunoreactive 44-kDa envelope bands in total chloroplast samples prepared from pea, tomato, and B. napus is presented in lanes 1–3, respectively. The anti-44-kDa IgGs were also reacted to total pea chloroplast proteins (lane 4) and total pea plant cell proteins (lane 5) that were extracted with 10% trichloroacetic acid. B, in the first row, anti-44-kDa IgGs were reacted to total stromal proteins (lane 1), total thylakoid proteins (lane 2), total envelope fraction (lane 3), outer (lane 4) and inner (lane 5) envelope fractions. For comparison, the same samples were reacted with anti-37-kDa IgGs (second row marked 37 kDa) and anti-Com70 IgGs (third row marked 70 kDa), representing inner and outer envelope proteins, respectively. The same fractions were also probed with preimmune IgGs (fourth row marked CON).

Fig. 5. Analysis of the 44-kDa proteins by protease treatment. A, the 44-kDa proteins were analyzed using pea total envelope fractions purified after the prescribed treatment scheme. Lanes 1–3 represent isolated mixed envelopes with no treatment, thermolysin (100 μg/ml) and trypsin (75 μg/ml)-treated total envelopes, respectively. Chloroplast samples given the same treatments as in the first row were probed with anti-37-kDa IgGs in the second row marked 37 kDa. B, total pea envelopes were treated with 1% Triton X-100 and thermolysin (100 μg/ml) (lane 1) or trypsin (75 μg/ml) (lane 2). C, purified inner envelopes (equivalent to 10 μg of protein) were treated with thermolysin (100 μg/ml). Lanes 1 and 2 represent treatments with 0 and 100 μg/ml thermolysin, respectively. The samples were probed with anti-44-kDa (first row marked 44 kDa) and with anti-37 kDa IgGs (second row marked 37 kDa).
present in the same amount after the trypsin treatment (Fig. 5A, lane 3), indicating that the native 42-kDa band is resistant to trypsin. The densitometer scans indicate that the decrease in the amount of native 44-kDa protein after a protease treatment was similar for the two types of proteases used, suggesting that the protease-sensitive 44-kDa form is present in a finite amount and is distinct from the protease-resistant 44-kDa forms. The protease degradation characteristics displayed by the protease-sensitive 44-kDa form suggests that this protein was accessible from the external side of the outer envelope. The 37-kDa inner envelope protein was unaffected by both types of proteases and was used as an internal control and for normalization purposes (Fig. 5A). All immunorelated 44-kDa forms were susceptible to proteases in the presence of 0.1% Triton X-100 (Fig. 5B).

The major protease-resistant, immunorelated 44-kDa forms of the inner envelope were sensitive to thermolysin only after isolated inner membranes were given a post-fractionation thermodysin treatment (Fig. 5C). Thermolysin cleaved all inner envelope immunorelated 44-kDa forms into 42-kDa products, indicating that these proteins were partly protected by the inner membrane. The 37-kDa inner membrane protein also exhibited the same characteristics, giving rise to a 35-kDa band. Since treatment of chloroplasts with trypsin (Fig. 5C) did not give rise to a similar pattern of protease-degraded products as with fractionated inner envelopes, the evidence suggests that the thermolysin-sensitive 44-kDa form in the outer envelope is distinct from the inner membrane 44-kDa forms.

Two immunomicroscopic techniques were used to further examine the possibility of immunorelated 44-kDa proteins in the outer and inner envelope. Isolated intact chloroplasts were subjected to immunofluorescence labeling as described under "Materials and Methods." Incubation of intact chloroplasts with buffer or preimmune IgGs followed by a secondary labeling reaction with fluorescent isothiocyanate-conjugated goat anti-rabbit IgGs did not result in fluorescent labeling of the plastids (Fig. 6, A and B). A primary incubation with anti-44-kDa antibodies yielded a patched pattern of immunofluorescence (Fig. 6C), indicating accessibility from the external side of the outer envelope.

The immunoelectron microscopy results also suggest the same possibility as that found in the above experiments. Intact chloroplasts were decorated with gold in discrete areas of the envelope membrane (Fig. 7), a pattern similar to the one obtained by immunofluorescence microscopy. The majority of the labeling was observed to be in clusters suggesting that the antigenic moieties were accessible from the outside. Immunogold labeling was not observed with chloroplasts incubated with gold-conjugated IgGs alone or with preimmune IgGs (data not shown).

Import Studies of Bce44B—In vitro chloroplast import assays were conducted for Bce44B to determine its association with the organelle. In vitro translation of bce44B mRNA resulted in a 44-kDa protein that co-migrated with a 44-kDa immunoreactive polypeptide band of the B. napus chloroplast envelope (Fig. 8C), suggesting a lack of processing upon targeting to the envelope membrane. The same possibility was drawn from the results of the following in vitro import experiments.

Radiolabeled Bce44B precursors were used directly for assaying import into pea chloroplasts. Thermolysin or trypsin treatment of reisolated intact chloroplasts was employed to determine the location of the imported products. Bce44B is targeted to the chloroplast independent of ATP or light (Fig. 8A, lanes 2–9). The presence of nigericin had no obvious effect on its targeting. Approximately 50–60% of the added radiolabeled precursors associated with the plastids, however, most of the associated precursors were susceptible to a subsequent thermolysin treatment. Approximately 5% of the associated Bce44B were targeted to a site that was inaccessible to thermolysin or trypsin (Fig. 7A, lanes 4–6). The low levels of Bce44B targeted to the envelope in a protease-resistant manner relative to precursors destined for internal compartments is most likely reflective of the limited capacity of the envelope membrane for incorporating additional proteins. Control import experiments conducted with cytoplasmic proteins such as pyruvate kinase (Wan et al., 1995) and chloroplast proteins without transit peptides (e.g., Oee1) (Ko and Cashmore, 1989), do not import nor do they bind at any level to the chloroplast. The affinity Bce44B possesses for chloroplasts is therefore genuine and did not arise from nonspecific associations. Bce44B lacks affinity for thylakoid membranes when tested with isolated thylakoids or when redirected into the chloroplast. 

Recent import studies with deletions of Bce44B confirm the specificity of its targeting and the existence of the protease-resistant form. The results of these experiments will be reported separately.

The suborganellar location of imported protease-resistant Bce44B form was first determined by crude subfractionation (Fig. 8B). Bce44B was found predominantly in the membranous fractions such as crude envelope and thylakoid membranes. The imported protease-resistant Bce44B proteins co-fractionated with both outer and inner envelope membrane fractions (Fig. 8B, lanes 3 and 4), a pattern similar to the one observed for the outer envelope protein Com70 (Wu and Ko, 1993; formerly designated as Sce70). The separation of outer and inner membrane fractions was confirmed by immunoblotting analysis of the same samples with the anti-37-kDa inner membrane protein. The same immunoreactive pattern as that discussed above was observed in these import fractionation experiments (data not shown). Thus like Com70, Bce44B may

\[ \text{K. Ko, unpublished observations.} \]
also be located in the outer envelope membrane. However, the possibility that the same Bce44B may be located in both outer and inner envelopes cannot be ruled out at this point, since unlike the protease sensitivity displayed by imported Com70, a population of Bce44B is protease resistant. Imported Bce44B co-migrated with an authentic 44-kDa B. napus envelope polypeptide and the in vitro translation product, strongly indicating that this protein did not contain a cleavable targeting signal (Fig. 8, lanes 1, 2, and 4).

**DISCUSSION**

We have isolated and characterized a full-length cDNA clone encoding a 44-kDa protein of the B. napus chloroplast envelope (Bce44B) that possesses a number of unusual characteristics. One noteworthy feature is that the native 44-kDa envelope proteins appear as prominent immunostaining bands of total envelope protein profiles, but stains very poorly with Coomassie Blue, suggesting that these proteins do not possess a high affinity for the dye molecule. Another interesting characteristic is that Bce44B is an integral component present in both envelope membranes and yet the deduced protein sequence does not indicate a typical membrane polypeptide. The hydropathy plot of the deduced Bce44B protein did not show any obvious structural characteristics common to membrane polypeptides such as membrane spanning regions, instead the plot indicates that Bce44B is hydrophilic in nature. Interestingly the same types of features were found in ISP42 and MPI1/ISP45, integral components of the mitochondrial protein translocation apparatus of the outer and inner membranes (Vestweber et al., 1989; Baker et al., 1990; Maarse et al., 1992). The amino acid sequences deduced from the nucleotide sequences available for the two B. napus cDNA inserts (full and partial length) did exhibit a high degree of similarity to each other indicating that the encoded proteins are indeed related. Even though they are related at the protein level, the 3' untranslated region of the two B. napus cDNA clones are different indicating that there are at least two distinct genes in the B. napus cultivar used. The distinct blot hybridization patterns obtained for the genomic DNA and for total plant cell RNA support the presence of at least two different genes encoding 44-kDa envelope proteins in B. napus. It is possible that the two different genes were derived from the two parental lines used to create the B. napus line employed in our study but the RNA blot analysis results clearly show that the steady state transcript levels of the two genes are different and hence they have different expression profiles. Due to the presence of immunorelated forms of the 44-kDa protein, the relationship between the steady state transcript level for the two different cDNA clones and the protein levels observed in the chloroplast envelope is not known at this point and remains to be determined.

The possibility of multiple related forms of the 44-kDa envelope protein was not limited to the nucleic acid level. Further analysis of the native 44-kDa proteins in pea envelopes provided several lines of evidence that at least one immunorelated form resides in the outer envelope and more abundant immunorelated forms are present in the inner membrane. The resulting immunoblots of outer and inner subfractions of pea envelopes indicate that immunologically-related 44-kDa proteins were present in both membrane locations. The inner
membrane fraction appeared to contain the majority of the immunorelated 44-kDa proteins observed in the envelope. Further indication for the dual location of the immunorelated 44-kDa proteins can be found in the protease treatment results. At least one immunorelated 44-kDa form is sensitive to thermolysin and gave rise to a distinct protease-generated 42-kDa band. This 42-kDa thermolysin-generated product appeared to be protected by the outer envelope, but was susceptible to trypsin, indicating that at least a part of an immunorelated 44-kDa protein was accessible to trypsin in the intermembrane space. The inner membrane immunorelated 44-kDa forms were resistant to both proteases and were likely protected against trypsin by the inner envelope. The inner membrane immunorelated 44-kDa forms were susceptible to thermolysin only when inner membrane fractions were treated with proteases, subsequent to the purification of the envelope membranes. Even though the inner immunorelated 44-kDa forms were accessible to proteases in isolated inner envelopes, protease treatment gave rise to a distinct proteolytic product. This suggests that the stromal facing part of the inner proteins was protected to a large extent by the inner membrane, perhaps by being embedded in the membrane. Interestingly, trypsin-treated chloroplasts gave rise to a 30-kDa product that is distinct from the thermolysin-generated 42-kDa product observed with isolated inner membranes. Protease digestion in the presence of detergent abolished all forms of the immunorelated 44-kDa proteins, confirming that the proteins themselves were completely susceptible in the absence of membrane association. Immunofluorescence and immunoelectron microscopy provided two additional lines of evidence that there are multiple immunorelated 44-kDa forms. The immunofluorescence pattern indicates that at least one form of the immunorelated 44-kDa envelope protein was accessible from the outside and was distributed in an uneven fashion in distinct patches on the surface of the organelle. This interpretation was reinforced by the immunoelectron microscopy data. The two sets of microscopy results were consistent with each other as well as with the immunoblotting and proteolysis experiments. Furthermore, previous cross-linking and co-immunoprecipitation results indicate that a translocating chimeric precursor protein can be cross-linked to outer membrane forms (Com44) as well as to inner membrane forms (Cim44) (Wu et al., 1994). The amount of translocating precursors cross-linked to outer membrane forms (Com44) versus inner membrane forms (Cim44) was consistent with the ratios observed in the subfractionation and immunoblotting experiments. Partial impairment of Oee1 import by anti-44-kDa IgGs also gave a further indication that at least one form is potentially accessible from the cytoplasmic side of the chloroplast and that its limited external location may have a functional significance. 2 On the whole, the combined data support the possibility that there are multiple immunorelated forms of the 44-kDa envelope protein and that at least one immunorelated form is accessible from the external side of the outer envelope.

The import results of one of these immunorelated 44-kDa proteins, BCE44B, indicate that it is targeted to the chloroplast and the majority of the associated proteins are sensitive to proteases. Only approximately 5–10% of the associated polypeptides were protease-resistant. The lower level of import achieved relative to other well studied chloroplastic precursor proteins most likely reflects the limited capacity of the envelope membrane for accommodating additional new proteins. The similarity of the subfractionation pattern of imported protease-resistant BCE44B to that reported for Com70, an outer membrane protein (Wu and Ko, 1993), suggests that BCE44B may also be an outer envelope protein. However, unlike Com70, the protease-resistant pattern of a population of BCE44B suggests an alternative possibility that the same BCE44B protein is targeted to the organelle and subsequently distributed to both the outer and inner membranes. The dual distribution of the imported BCE44B protein does not appear to be a result of the subfractionation technique since the antibodies against the 37-kDa inner membrane protein immunoreact with a protein band exclusively in the inner membrane fraction. Therefore the BCE44B found in the outer fraction is not due to contamination of the outer membranes with inner membranes. The targeting mechanism of BCE44B is currently the subject of another study and will be reported separately.

The translocation characteristics of BCE44B appear to be distinct from other chloroplast precursors, but are more similar to Omp24, Soe1, Com70, Oep34, and Oml4 (Salomon et al., 1992; Li et al., 1992; Wu and Ko, 1993; Fischer et al., 1994; Seedorf et al., 1995). Like these other outer envelope proteins, BCE44B does not appear to contain a cleavable transit sequence. The imported protein co-migrates with the authentic 44-kDa band in Brassica chloroplast envelopes and in vitro translation product. The BCE44B cDNA clone will allow us to further study the translocation pathway of another envelope protein.

Similarly sized polypeptides immunologically-related to BCE44B have been found in the outer and the inner chloroplast envelopes and have been implicated to play a role(s) in the translocation of precursor proteins across the chloroplast envelope membrane (Wu et al., 1994). These envelope proteins, designated collectively as Com44 (for the outer membrane forms) and Cim44 (for the inner membrane forms), were previously demonstrated to be in close physical proximity to translocation intermediates (Wu et al., 1994). There are several lines of evidence strongly indicating that BCE44B is a member of the Com44/Cim44 envelope components of the chloroplast protein translocation apparatus. First, the antibodies used in the previous study immunoreact strongly with the BCE44B protein and its corresponding recombinant phase plaque. Second, the deduced BCE44B protein sequence is identical to the COOH-terminal polypeptide used to generate the antibodies employed in the previous chemical cross-linking study. Third, antibodies generated against the cDNA-encoded BCE44B protein itself (IgGs made separately against the NH2 or the COOH terminus) gave rise to identical chemical cross-linking immunoprecipitation results as reported by Wu et al. (1994). In addition, the developmental profile of the immunorelated 44-kDa envelope proteins also appears to correlate with the predicted protein import activity of the various plant tissues. 4

Due to the multiple characteristics of the 44-kDa chloroplast envelope protein, we are continuing to isolate and characterize more cDNA clones for these proteins from a variety of plants and tissues to gain further insight into these intriguing immunorelated proteins and to determine the relationship between specific proteins and their cDNA clones. The possibility of immunorelated 44-kDa proteins being located in both outer and inner envelope membranes is especially interesting in relation to their potential role in protein import. In the mitochondrial system, the ISP42 component is part of the outer membrane import system and the inner membrane translocation system involves ISP45. However, ISP45 bears no resemblance to ISP42. It appears that in the chloroplast import system immunologically-related proteins may play a role in both the outer and inner import machineries. The potential locale of the 44-kDa immunorelated proteins presented in this study appears to fit into the chloroplast protein import model recently suggested by Schnell and Blobel (1993) featuring two distinct

* F. Seibert, unpublished observations.
protein conducting channels, one in the outer membrane and one in the inner envelope membrane.

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