The EMBO Journal vol.15 no.16 pp.4230–4238, 1996

Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane

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Proteins from both the inner and outer envelope membranes are engaged in the recognition and translocation of precursor proteins into chloroplasts. A 110 kDa protein of the chloroplastic inner envelope membrane was identified as a component of the protein import apparatus by two methods. First, this protein was part of a 600 kDa complex generated by cross-linking of precursors trapped in the translocation process. Second, solubilization with detergents of chloroplasts containing trapped precursors resulted in the identification of a complex containing both radiolabeled precursor and IEP110. Trypsin treatment of intact purified chloroplasts was used to study the topology of IEP110. The protease treatment left the inner membrane intact while simultaneously degrading domains of inner envelope proteins exposed to the intermembrane space. About 90 kDa of IEP110 was proteolitically removed, indicating that large portions protrude into the intermembrane space. Hydropathy analysis of the protein sequence deduced from the isolated cDNA clone in addition to Western blot analysis using an antiserum of IEP110 specific to the N-terminal 20 kDa, suggests that the N-terminus serves to anchor the protein in the membrane. We speculate that IEP110 could be involved in the formation of translocation contact sites due to its specific topology.

Keywords: chloroplast/envelope/import/*Pisum sativum* L./precursor protein

Introduction

Most chloroplastic proteins are encoded by nuclear genes, synthesized in the cytosol as precursors and translocated into the organelle via a post-translational process (Chua and Schmidt, 1979; Grossmann *et al.*, 1980; Joyard *et al.*, 1991). Recognition and transport of chloroplast-destined precursors is accomplished by an apparatus that includes proteins from both the outer and inner membranes of the envelope that surrounds chloroplasts. Recent efforts to identify the components of the chloroplastic protein import apparatus have employed the use of precursor proteins that are trapped during some stage of the transport process (Waegemann and Soll, 1991; Perry and Keegstra, 1994; Schnell *et al.*, 1994; Wu *et al.*, 1994; Ko *et al.*, 1995). The trapped translocation intermediates were used as a marker to isolate protein complexes from purified outer envelope membranes (Waegemann and Soll, 1991; Soll and Waegemann, 1992) or from intact chloroplasts (Schnell *et al.*, 1994). These 'import complexes' contained the Outer Envelope Proteins OEP86 and OEP70 (an hsc70 homologue), OEP34 (Waegemann and Soll, 1991, 1993; Schnell *et al.*, 1994) and the inner envelope components IAP100 and IAP36 (Schnell *et al.*, 1994). Chemical cross-linkers were used to demonstrate the close interaction between precursor proteins and either OEP86 and OEP75 (Perry and Keegstra, 1994), Com/Cim 44 and Cim 97 (most likely identical to IAP100) (Wu *et al.*, 1994). cDNA clones have been obtained for OEP86 (Hirsch *et al.*, 1994; Kessler *et al.*, 1994), OEP75 (Schnell *et al.*, 1994; Tranel *et al.*, 1995) and OEP34 (Kessler *et al.*, 1994; Seedorf *et al.*, 1995). Taken together, these studies provide evidence for the involvement of these envelope polypeptides in protein transport into chloroplasts.

Biochemical data support the hypothesis that OEP86 might function as a receptor for precursors (Hirsch *et al.*, 1994; Perry and Keegstra, 1994), while OEP75 forms the core of the translocation channel across the outer membrane (Perry and Keegstra, 1994; Schnell *et al.*, 1994; Tranel *et al.*, 1995). The GTP binding protein OEP34 was found in close physical proximity with OEP75 and might have a regulatory role (Seedorf and Soll, 1995; Seedorf *et al.*, 1995; Soll, 1995). Precursor translocation seems to occur simultaneously through both the outer and inner envelope membrane translocation complexes (Schnell and Blobel, 1993; Alefsen *et al.*, 1994). Joint translocation sites are most likely formed transiently by the coordinate action of the import machineries of both the outer and inner envelope membranes. If such a mechanism occurs, based on analogy to other systems (Glick *et al.*, 1991; Stuart *et al.*, 1994; Horst *et al.*, 1995) the import complex in the inner envelope membrane should contain polypeptides, which (i) are involved in the formation of such joint translocation sites, (ii) act as a translocation channel and (iii) interact with stromal chaperones in order to facilitate the movement of precursors across membranes. Identification and characterization of the components involved in protein translocation across the chloroplastic envelope membranes will help us to understand fundamental aspects of membrane transport and organelle biogenesis. Our continuous efforts to characterize chloroplastic envelope proteins by describing their structure and function together with recent reports on a high molecular weight chloroplastic inner envelope membrane protein involved in protein import (Schnell *et al.*, 1994; Wu *et al.*, 1994) caused us to investigate IEP110.

In this report we identify IEP110 as a component of the translocation complex in the chloroplastic envelope membranes. A cDNA encoding this protein was isolated and characterized, providing sequence information about

the protein. Its sequence is identical to IAP100 (F.Kessler and G.Blobel, personal communication), but it has no similarity to other known proteins. IEP110 is anchored into the inner envelope membrane by a short hydrophobic sequence near the N-terminus with the large majority of the protein exposed in the intermembrane space between the two envelope membranes.

Results

Isolation of protein import complexes from chloroplastic envelopes

IEP110 is a prominent protein in the inner envelopes from pea and spinach chloroplasts (Cline *et al.*, 1981; Block *et al.*, 1983). It has been used as a marker protein for the chloroplastic inner envelope membranes (Werner-Washburne *et al.*, 1983). Antibodies were raised against this protein during studies to elucidate the biological properties of the chloroplastic envelope membranes (Waegemann *et al.*, 1992). Recent evidence that a protein with the properties of IEP110 might be a component of the import apparatus (Schnell *et al.*, 1994; Wu *et al.*, 1994) caused us to investigate IEP110 in detail.

The import of precursor proteins into isolated chloroplasts can be controlled by varying the ATP concentration in the reaction mixture. Low concentrations of ATP (5–100 μM) support the stable interaction of precursor proteins with the import machinery (Olsen *et al.*, 1989; Olsen and Keegstra, 1992). However, completion of import into chloroplasts occurs only in the presence of higher ATP concentrations (>100 μM ATP) (Theg *et al.*, 1989). Thus, under low ATP conditions precursor proteins are trapped at a discrete stage of the transport process and yield defined translocation intermediates (Waegemann and Soll, 1991; Olsen and Keegstra, 1992). These translocation intermediates were shown to be on the productive import route, i.e. upon raising the ATP concentrations, the mature form appeared inside chloroplasts (Theg *et al.*, 1989; Waegemann and Soll, 1991). In order to identify additional components of the chloroplast protein import machinery, radiolabeled precursor of the small subunit of ribulose bisphosphate carboxylase (preSSU) was incubated with intact pea chloroplasts in the presence of 75 μM ATP to form these translocation intermediates. Chloroplasts were subsequently treated with the cross-linker dithiobis(bis-succinimidyl propionate) (DSP) to covalently connect preSSU to nearby proteins and to connect adjacent proteins to each other. DSP-treated chloroplasts were solubilized by LDS and proteins separated by SDS–PAGE. Radiolabeled preSSU was found primarily in a complex that migrated with an apparent molecular mass of 600 kDa (Figure 1, lane 1). The molecular weight of 600 kDa was estimated after drawing the calibration curve from the standards shown in Figure 1. In this gel, molecular standards migrated almost linear versus log(MW). The solubilized complex could be immunoprecipitated by an antiserum to IEP110 (Figure 1, lane 2), but not by pre-immune serum (Figure 1, lane 3). Thus, IEP110 is present in a DSP-cross-linked complex containing a chloroplast-destined precursor protein. This complex was also immunoprecipitated by antibodies raised against the outer envelope proteins, OEP86, OEP75 and OEP34, which are known components of the protein transport apparatus (M.Akita and K.Keegstra, manuscript in preparation).

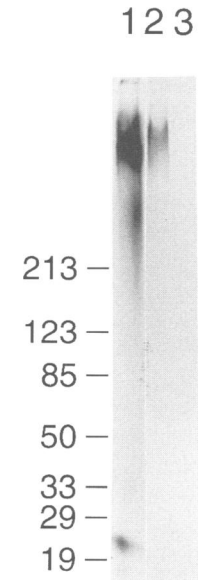

Fig. 1. IEP110 is present in a preSSU-envelope membrane protein complex cross-linked with DSP. Intact pea chloroplasts were incubated with [^{35}S]preSSU in the presence of 75 μM ATP. Chloroplasts were re-isolated and treated with 2.5 mM DSP. Total membranes were recovered after lysis of chloroplasts, then solubilized with LDS. Solubilized membranes (equivalent to 25 μg chlorophyll) were immunoprecipitated with αIEP110 (lane 2) or its pre-immune serum (lane 3). Lane 1 shows 20% of the sample subjected to immunoprecipitation. All samples were analyzed by SDS–PAGE under non-reducing conditions, followed by fluorography.

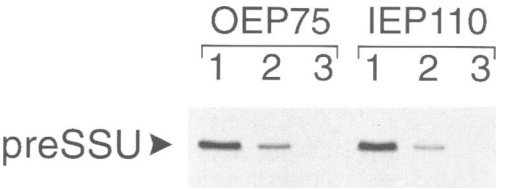

Fig. 2. IEP110 associates with precursor under binding conditions. ^{35}S-labeled, wheat-germ-translated preSSU was bound to isolated chloroplasts in the presence of 100 μM ATP. After re-isolation of intact chloroplasts, the chloroplast pellet was solubilized in decylmaltoside buffer and insoluble material was removed by centrifugation. Ten percent of this supernatant was removed before immunoprecipitation (lane 1). The remaining 90% was split into equal portions to which antiserum (lane 2) or pre-immune serum (lane 3) to either OEP75 or IEP110 and protein A–Sepharose were added. After immunoprecipitation the fractions were separated by SDS–PAGE and visualized by fluorography.

To obtain further evidence for the presence of IEP110 in a protein translocation complex, a different solubilization strategy was used. Chloroplasts were incubated with radiolabeled preSSU in the presence of low levels of ATP to form the same translocation intermediate that was used above. The chloroplasts were then solubilized by decylmaltoside in the absence of a cross-linking reagent (Figure 2). The solubilized proteins and complexes were then subjected to immunoprecipitation by an antiserum to OEP75, a component of the chloroplast outer envelope import machinery (Schnell *et al.*, 1994; Tranel *et al.*, 1995). The antiserum to OEP75, but not the pre-immune serum, was able to coimmunoprecipitate preSSU, demonstrating the presence of a known component of the

chloroplast import machinery in a complex with the radiolabeled precursor (Figure 2, lanes 1–3). The IEP110 antiserum was also able to coimmunoprecipitate labeled preSSU (Figure 2, lanes 4–6). The amount of preSSU coimmunoprecipitated could be correlated with the concentration of IEP110 antiserum used in the immunoprecipitation (data not shown). These results provide further evidence that IEP110 is present in a complex containing a precursor protein; this complex is stable even in the absence of a chemical cross-linking reagent. Several laboratories (Waegemann and Soll, 1991; Schnell *et al.*, 1994; Wu *et al.*, 1994) have observed a complex of OEP75, OEP86 and OEP34 in association with precursor under binding conditions. We wanted to know whether these other translocation components were also contained in our immunoprecipitated complexes. Using immunoblotting techniques to analyze the immunoprecipitated protein complexes, we detected IEP110 as well as OEP86 and OEP34 in association with immunoprecipitated OEP75 (E.Nielsen and K.Keegstra, manuscript in preparation). These observations support the conclusion that IEP110 is part of the protein import machinery of the chloroplastic inner envelope membrane and forms a stable complex with the components of the outer envelope import machinery.

Molecular analysis of IEP110

A cDNA clone encoding IEP110 was isolated from an expression library made from 5-day-old, light-grown pea seedlings by screening with an antiserum to IEP110. The amino acid sequence deduced from the cDNA clones coded for a protein of 996 amino acids with a calculated molecular mass of 110 kDa (Figure 3). Peptide sequences obtained either from the N-terminus or from proteolytic fragments of IEP110 (see below) demonstrated identity between the isolated cDNA clones and the polypeptide (Figure 3A). The N-terminal sequence data (SSDTNNPAS) also indicate that IEP110 is made as a larger precursor protein (preIEP110) with a transit peptide containing 37 amino acid residues (Figure 3A). A search of the sequence databases revealed no significant similarities to any other known polypeptide, except to a cDNA clone from a rice EST (GenBank accession No. D24428) (Figure 3B). This rice sequence shows strong similarity to the pea protein and we conclude that it represents the rice homolog of IEP110.

Analysis of the deduced amino acid sequence of IEP110 revealed that it contained ~30% charged amino acids (see also Figure 3C), which seems unusually high for a membrane protein. Hydropathy analysis identified one potential membrane-spanning region at position 101–139 (Figure 3A and C). This region shows potential to form a hydrophobic alpha helix and might anchor the protein in the envelope membrane. Indeed, this hypothesis is consistent with the topological studies presented below.

To evaluate the hypothesis that IEP110 was sythesized as a larger precursor, preIEP110 was synthesized by *in vitro* transcription and translation (see Materials and methods). The import of preIEP110 into isolated intact chloroplasts was examined. In the presence of low levels of ATP (50 μM), preIEP110 (Figure 4, lane 5) binds to chloroplasts, but remains in a protease-accessible location at the organellar surface (Figure 4, lanes 1 and 2). The lower molecular weight (mature) form is detected only in

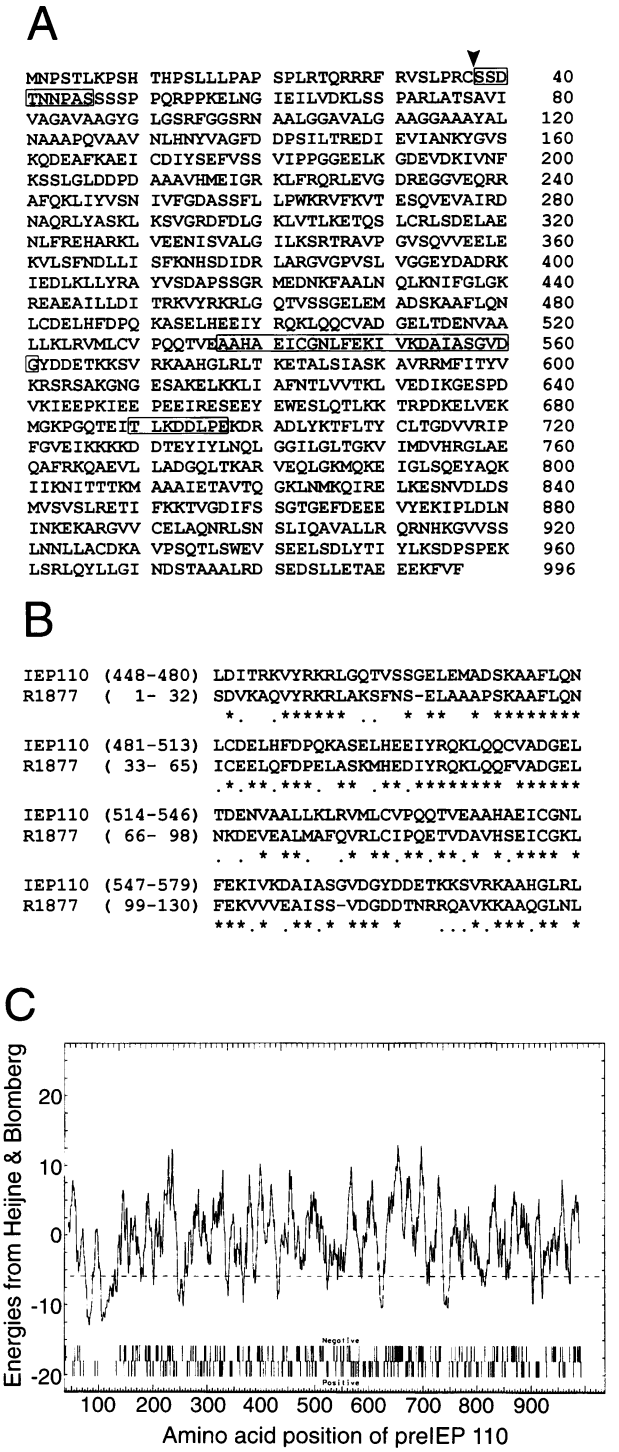

Fig. 3. Amino acid sequence and hydropathy profile for IEP110. A cDNA clone for IEP110 (accession No. Z68506) was isolated by immunoscreening. (**A**) The deduced amino acid sequence for preIEP110 is shown. Peptide sequencing of endogenous IEP110 revealed that the mature protein begins at amino acid position 37 (indicated by an arrowhead). Further internal peptide sequences were obtained after endoprotease gluc-C digestion and are boxed. (**B**) Sequence comparison between IEP110 and a rice expressed sequence tag clone (R1877, accession No. D24428). Stars indicate identical amino acids, dots indicate conservative changes. (**C**) A hydropathy analysis was carried out according to von Heijne and Blomberg (1979) using a window of 11 amino acids. The position of charged amino acids is indicated.

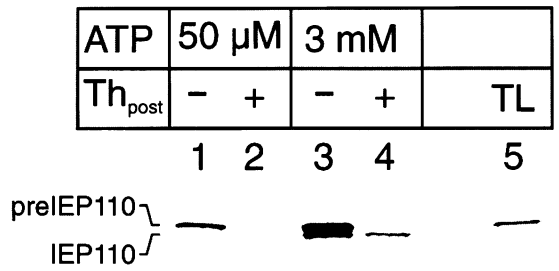

Fig. 4. Import of preIEP110 into chloroplasts. Intact purified pea chloroplasts (equivalent to 20 µg chlorophyll) were incubated with ^{35}S-labeled preIEP110 for 30 min in the presence of 50 µM (lanes 1 and 2) or 3 mM ATP (lanes 3 and 4) at 25°C, respectively. Chloroplasts were isolated and were either untreated or treated (lanes 2 and 4) with the protease thermolysin (400 µg/mg chlorophyll) after completion of the import reaction. Further experimental conditions were exactly as described previously (Waegemann and Soll, 1991). Lane 5, translation product of preIEP110, 5% of which was added to each import experiment.

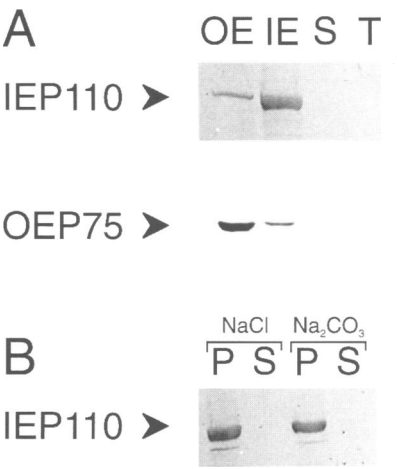

Fig. 5. IEP110 is an integral protein in the inner envelope membrane of pea chloroplasts. (**A**) Immunoblot analysis of IEP110 and OEP75 distribution in the pea chloroplast subcompartments. Lane 1, outer envelope; 2, inner envelope; 3, stroma; 4, thylakoid, 8 µg of each protein fraction was used. (**B**) Inner envelope membranes (20 µg of protein) were washed with 1 M NaCl (lanes 1 and 2) or at pH 11 (lanes 3 and 4) and separated into an insoluble pellet (lanes 1 and 3) or a soluble fraction (lanes 2 and 4) prior to SDS–PAGE and immunoblotting with αIEP110.

the presence of 3 mM ATP (Figure 4, lanes 3 and 4). The mature form of IEP110 is inside chloroplasts as evidenced by the observation that it is no longer sensitive to protease digestion (Figure 4, lane 4). The mature form of IEP110 is found in the chloroplastic envelopes, when chloroplasts were fractionated after an import experiment (not shown).

Localization and orientation of IEP110

Methods to separate the outer and inner envelope membranes (Cline *et al.*, 1981; Block *et al.*, 1983) were used to determine the localization of endogenous IEP110 within the chloroplastic envelope. Immunoblot analysis revealed that IEP110 is located in fractions enriched in inner envelope membranes (Figure 5A, lane 2), whereas the stroma and the thylakoids did not contain significant amounts of IEP110 (Figure 5A, lanes 3 and 4). Low amounts of IEP110 were also detected in the outer envelope membrane fractions (Figure 5A, lane 1). The outer envelope marker protein OEP75 (Cline *et al.*, 1981; Block *et al.*, 1983) was largely present in the outer envelope membrane fractions (Figure 5A), indicating that the purity of each membrane fraction was >90% (Joyard *et al.*, 1991). We conclude that IEP110 is localized in the inner envelope membrane of pea chloroplasts corroborating earlier results (Werner-Washburne *et al.*, 1983; Waegemann *et al.*, 1992). IEP110 was recovered in the insoluble fraction, when purified inner envelope membranes were extracted either with high salt (1 M NaCl) or at high pH (pH 11, 0.1 M Na_2CO_3), demonstrating that it behaves as an integral membrane protein (Figure 5B, lanes 1–4).

Selective proteolytic digestions were used to investigate how IEP110 was oriented in the inner envelope membrane. In contrast to mitochondria, techniques have not yet been developed to selectively remove the outer envelope membrane from isolated chloroplasts. However, it has been demonstrated that certain proteases have the ability to destroy the permeability barrier of the outer envelope membrane, but do not destroy the integrity of the inner envelope membrane (Marshall *et al.*, 1990). This strategy has been adapted for our studies (see Materials and methods) using trypsin to penetrate the outer envelope and attack inner envelope proteins that are exposed to the intermembrane space. One critical control in such studies is the demonstration that the chloroplasts remained intact during the protease treatment, i.e. that trypsin does not penetrate the inner envelope membrane and lyse the chloroplasts. Two criteria were used to evaluate this point. First, chloroplasts were re-isolated after trypsin treatment through a 40% Percoll cushion and only the intact organelles recovered in the pellet fraction were used for further analysis. Second, the intactness of the recovered chloroplasts was established using the latency of the Hill reaction (Figure 6). The Hill reaction determines the light-dependent reduction of $K_3[Fe(CN)_6]$ at the thylakoid membranes. $K_3[Fe(CN)_6]$ cannot enter intact chloroplasts and its rate of reduction can thus be used as a measure of chloroplast intactness. At the highest trypsin concentration used (1000 µg/mg Chl) ~25% of the chloroplasts passed through the Percoll cushion. According to the Hill reaction these chloroplasts were at least 75% intact. As a control, chloroplast samples that had been trypsin treated and repurified through Percoll gradients were resuspended in hypotonic buffer in order to lyse them. The lysed chloroplasts showed maximal Hill reaction activity (Figure 6, columns 10 and 11). Experimental conditions were established to ensure that the Hill reaction was measured in the linear range (see Materials and methods; data not shown).

Once it was established that trypsin treatment left the chloroplasts intact, the proteolytic fragmentation of IEP110 was analyzed by SDS–PAGE and immunoblotting. Increasing concentrations of trypsin (10–1000 µg/mg Chl) resulted in a progressive degradation of IEP110; a typical fragmentation pattern is presented in Figure 7 (Figure 7A, lanes 6–10). At the highest trypsin concentrations (Figure 7A, lane 10) only two peptides at ~29 and 34 kDa were detectable. The data support the conclusion that most of IEP110 is exposed to the outside of the inner envelope and faces the intermembrane space between the two envelope membranes. The trypsin fragmentation pattern

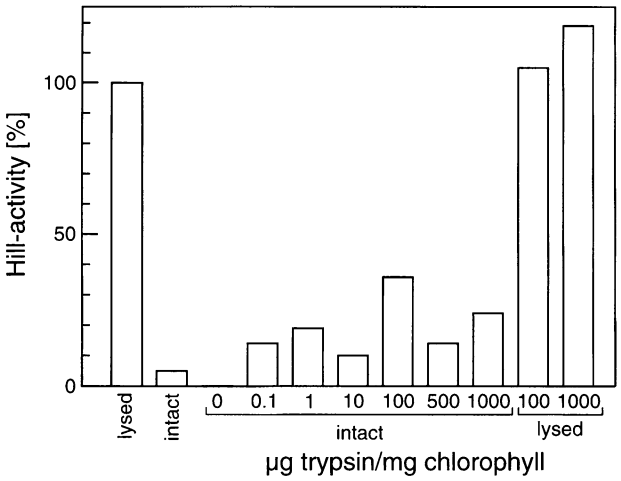

Fig. 6. Hill reaction of intact and lysed trypsin-treated chloroplasts. Chloroplasts equivalent to 40 µg chlorophyll were used in each assay and $K_3[Fe(CN_6]$ reduction was measured at 0, 30, 60, 90 and 180 s. Columns 1 and 2 show the Hill activity of freshly prepared, lysed and intact pea chloroplasts, respectively. Columns 3–9 show the Hill activity of pea chloroplasts, which were either untreated (0 µg protease) or treated with the indicated amounts of protease, purified through a 40% Percoll cushion and washed (see Materials and methods). Columns 10 and 11 show the Hill activity of trypsin-treated chloroplasts, but which were lysed prior to the Hill reaction. The 100% value of column 1 corresponds to 2.3 µmol Fe^{3+} (reduced) per min per mg chlorophyll.

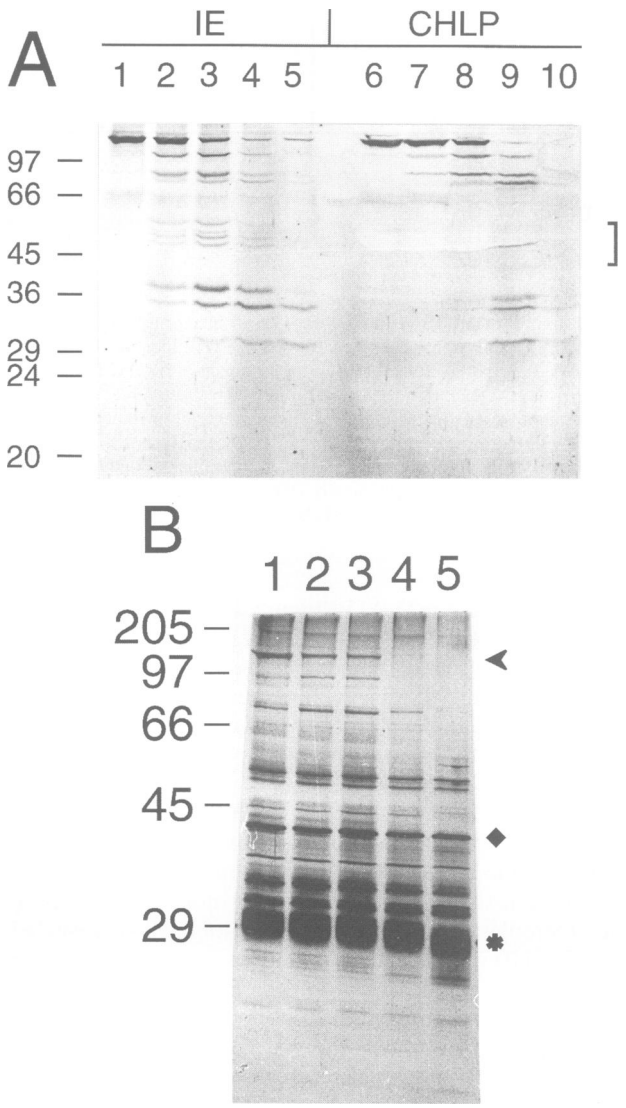

Fig. 7. Migration pattern of IEP110 tryptic peptides derived from intact chloroplasts (CHLP) and inner envelope membranes (IE). (**A**) Intact chloroplasts (200 µg chlorophyll) and inner envelope membranes (20 µg protein) were treated without (lanes 1 and 6) or with trypsin (lanes 2–5 and 7–10) as outlined in Materials and methods. Trypsin concentrations used were: 1, 10, 100 and 1000 ng per mg envelope protein in lanes 2, 3, 4 and 5, respectively and 10, 100, 500 and 1000 µg per mg chlorophyll in lanes 7, 8, 9 and 10, respectively. Chloroplasts equivalent to 50 µg chlorophyll were lysed and a total membrane fraction prepared and loaded onto the gel. Proteins were separated by SDS–PAGE, transferred to nitrocellulose filters and immunodecorated with an antiserum against IEP110. An immunostain is shown. (**B**) Inner envelope membranes were treated with trypsin as in A, separated by SDS–PAGE and detected by silver staining. The positions of IEP110, the phosphate-triose-phosphate-translocator and an unknown trypsin resistant protein are indicated by ◄, ◆ and ✱, respectively.

obtained from IEP110 in intact chloroplasts was compared with the fragmentation pattern of IEP110 obtained when isolated vesicles of purified inner envelope membrane were treated with trypsin. Less protease and shorter incubation times were used for the protease treatment of isolated envelope vesicles, since the vesicle exposed polypeptides are directly accessible to the enzyme and the protease does not have to penetrate the outer membrane (see Materials and methods for details). Trypsin treatment of isolated inner envelope vesicles yielded nearly identical proteolytic fragments of IEP110 (Figure 7A, lanes 1–5) as treatment of intact chloroplasts (Figure 7A, lanes 6–10). One important conclusion from these results is that inner envelope vesicles possess mostly a right-side-out orientation, i.e. the same orientation as in intact chloroplasts. A number of proteolytic fragments of IEP110 in the range between 48 and 55 kDa (Figure 7A, lanes 2–4) were not as clearly visible in the experiments with intact chloroplasts. Although they were detectable (Figure 7A, lanes 8 and 9, indicated by a bracket), their migration was disturbed and compressed due to the high amounts of ribulose-1,5-bisphosphate carboxylase large subunit still present in the total membrane fraction used for analysis.

Among the major proteins of the inner envelope membrane, IEP110 is one of the most sensitive to proteolytic digestion (indicated by an arrowhead in Figure 7B). At higher protease concentrations other inner membrane polypeptides were also digested, e.g. the phosphate-triose-phosphate translocator at 29 kDa.

In order to determine if the N-terminal hydrophobic region of IEP110 (see Figure 3C) serves to anchor the protein in the inner envelope membrane, additional studies were performed. A deletion of the 5′-end of the cDNA clone encoding for IEP110 was constructed. The modified cDNA clone encoded IEP110ΔN, which starts at residue 216 (Figure 3A). When the truncated protein was expressed in *Escherichia coli*, it accumulated in inclusion bodies. The inclusion bodies were purified and the truncated protein was solubilized and coupled to CNBr-activated Sepharose. An aliquot of the antiserum to full-length IEP110 was incubated with IEP110ΔN–Sepharose. Unbound antibodies were recovered and tested for specificity. The affinity-depleted antiserum recognized only mature IEP110, but not IEP110ΔN (Figure 8, lanes 1–4).

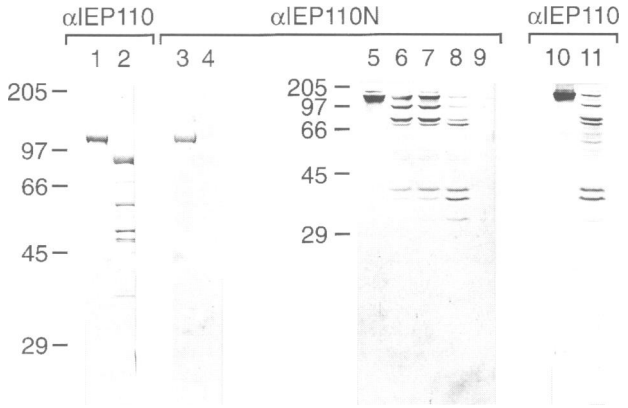

Fig. 8. IEP110 is anchored by its N-terminus in the chloroplastic inner envelope. IEP110ΔN (amino acids 216–996) was expressed in *E.coli* and used to isolate a subpopulation of IEP110 IgGs which recognizes only the N-terminus of IEP110. The antiserum was named αIEP110N. Lanes 1 and 2 show an immunoblot analysis using crude IEP110 serum against inner envelopes (lane 1) or overexpressed IEP110ΔN. Lanes 3 and 4 as lanes 1 and 2, with the exception that affinity-purified αIEP110N was used. Lanes 5–9, purified inner envelope membranes were treated with trypsin as in Figure 4 and the proteolytic fragmentation pattern of IEP110 analyzed by SDS–PAGE and immunoblotting using αIEP110N. Lanes 10 and 11, inner envelope membranes were extracted at pH 11 either untreated (lane 10) or treated with trypsin (lane 11). An immunoblot analysis is shown.

Thus we conclude that the depleted antiserum (termed αIEP110N) was specific for epitopes located in the N-terminal region of IEP110. Vesicles of inner envelope membranes were treated with low amounts of trypsin (compare also Figure 7) and the degradation products were analyzed with αIEP110N (Figure 8, lanes 5–9). The typical proteolytic fragments of IEP110 (Figure 7, lanes 2–4) were also all detectable by αIEP110N. In addition these fragments were all resistant to extraction at pH 11 (Figure 8, lanes 10 and 11). From these data we conclude that interaction of IEP110 with the inner envelope membrane occurs via the hydrophobic domain located at the N-terminus of the protein.

Discussion

Two approaches were used to identify IEP110 as a component of the chloroplastic protein import machinery. First, a radiolabled precursor protein, trapped in the transport process by limiting the level of ATP, was chemically cross-linked to nearby components of the transport apparatus. The cross-linked complex was solubilized with LDS and resolved by SDS–PAGE whereby it migrated with an apparent molecular mass of 600 kDa. The 600 kDa complex could be specifically immunoprecipitated with antibodies against IEP110, providing evidence that IEP110 was part of the translocation complex. Second, the same radiolabeled precursor protein, trapped in the transport process by limiting the level of ATP, was solubilized with a mild detergent, without cross-linking. Some of the precursors were present in complexes that could be immunoprecipitated with antibodies against envelope membrane proteins. Specifically, some of the precursor could be immunoprecipitated by antibodies to OEP75 (Figure 2), a protein of the outer envelope membrane that has previously been identified as a component of the transport apparatus (Perry and Keegstra, 1994; Schnell *et al.*, 1994; Tranel *et al.*, 1995). More importantly, some of these native complexes could also be immunoprecipitated with antibodies to IEP110, again providing evidence that IEP110 is a component of the protein import apparatus. These experiments did not determine whether OEP75 and IEP110 are in the same complex or in different complexes, but we favor the hypothesis that they are in the same complex and are currently investigating this point. Solubilization with mild detergents and coimmnoprecipitation have been used successfully with translocation complexes from mitochondrial membranes, both to identify new transport components (Kiebler *et al.*, 1990) and to investigate interactions between previously identified components (Hachiya *et al.*, 1995).

Antibodies against IEP110 were used to isolate a cDNA clone encoding this protein. Sequence comparisons indicate that IEP110 is the same protein as IAP100 identified in earlier studies (Schnell *et al.*, 1994; G.Blobel, personal communication). IEP110 is also most likely identical to CIM97 (Wu *et al.*, 1994), a protein that can be cross-linked to precursors trapped during transport. However, the identity between CIM97 and IEP110 remains to be established. Collectively, these findings provide strong evidence that IEP110 from pea is indeed part of the chloroplastic import machinery.

Limited proteolysis of intact chloroplasts demonstrates that large portions of IEP110 are exposed to the intermembrane space. Using an immunodepleted antiserum, αIEP110N, which recognized the N-terminal 20 kDa of the protein, it was possible to demonstrate that the C-terminus projects into the intermembrane space. Furthermore the N-terminus is sufficient to anchor IEP110 into the inner envelope, as deduced from the fact that the proteolytic fragments are resistant to extraction at pH 11. Proteolytic degradation products of IEP110 <29 kDa were not detected. This could be due to two reasons: (i) the N-terminus traverses the inner envelope twice, i.e. the N- and C-termini are both exposed to the intermembrane space and the final proteolytic product is either too small or not sufficiently antigenic to be detected, or (ii) IEP110 is a monotopic membrane protein (Blobel, 1980), which has no transmembrane segments but which is only anchored in the outer leaflet of the inner envelope lipid bilayer. IEP110 was almost completely degraded under conditions where chloroplasts were still intact as indicated by the latency of the Hill reaction. Furthermore trypsin treatment of inner envelope vesicles using protease concentrations which left most polypeptides intact (Figure 7B) resulted in no detectable peptides below the size of 29 kDa (Figure 8) indicating that only very short (<5–10 kDa) transmembrane segments of IEP110 remained protease-protected, though they were too small to be detected. Therefore we can not distinguish between these two possible membrane arrangements of IEP110.

The proteolytic fragmentation pattern of IEP110 in intact chloroplasts and isolated inner envelope membrane vesicles indicate that the envelope vesicles are isolated in a right-side-out orientation. Labeling experiments of the phosphate-triose-phosphate translocator by the specific reagents pyridoxalphosphate and 1,2-dehydro-1,2-(2,2′-disulfo-4,4-diisothiocyano)diphenyl ethane support this conclusion (J.Lübeck and J.Soll, unpublished). The lysis procedure of intact chloroplasts seems critical for the

orientation of the envelope vesicles. In our work, inner envelope membranes were isolated from hypertonically shrunken chloroplasts which were ruptured by 50 strokes in a tight-fitting Dounce homogenizer (Keegstra and Youssif, 1986). Other studies (Cline *et al.*, 1985) used a freeze–thaw cycle to rupture hypertonically-shrunken chloroplasts and concluded, from electron microscopic studies, that the vesicles were largely inside-out.

Translocation of precursor proteins into chloroplasts is thought to occur at contact sites where the two envelope membranes are held in close physical proximity (Schnell and Blobel, 1993; Alefsen *et al.*, 1994). Many questions regarding the structure and formation of these translocation sites remain unanswered. For example, it is unclear whether they are permanent structures or whether they are formed only during the translocation of precursor proteins. It is unclear whether the two membranes are held together by the protein being transported or whether each membrane contains proteins that interact with partners from the other membrane. Given the location of IEP110 as an integral protein of the inner envelope membrane with most of the protein exposed to the intermembrane space, it is tempting to speculate that IEP110 might be involved in the interactions between the import components in the inner and outer envelope membranes, respectively.

While chloroplastic outer envelope membranes can also be used as a bona fide system to study early events in protein import (Waegemann and Soll, 1991) binding of different chloroplast-destined percursor proteins to inner envelope membrane vesicles did not show a specific interaction, e.g. dependency on ATP or a transit sequence (J.Lübeck and J.Soll, unpublished observation), indicating that the inner envelope membrane import machinery can not act independently in chloroplasts. These preliminary studies could suggest that a tight cooperation of both envelope membranes is obligatory for protein transport into chloroplasts. IEP110 might play a pivotal role in this cooperation.

Materials and methods

Isolation of chloroplasts and inner envelope vesicles

Pea plants (*Pisum sativum* L. var. Golf) were grown for 12–14 days in a growth chamber under a 14/10 h light/dark regime. Intact chloroplasts were isolated from leaves and purified further by silica–sol gradients by standard procedures (Waegemann and Soll, 1991).

Chloroplasts equivalent to 200 mg of chlorophyll were used to purify chloroplast inner envelope membranes (Keegstra and Youssif, 1986; Waegemann *et al.*, 1992) after rupturing intact organelles by 50 strokes with a Dounce homogenizer (Kontes Instruments, Veneland).

Solubilization of chloroplasts by decylmaltoside

Chloroplasts were pretreated with 5 μM nigericin to inhibit photophosphorylation prior to the binding reaction. ^{35}S-labeled, wheat-germ-translated preSSU that had been gel-filtered to remove residual nucleotides was bound to isolated chloroplasts (50 μg of chlorophyll) in the presence of 100 μM ATP for 10 min in the dark at room temperature. After re-isolation of intact chloroplasts by sedimentation through a 40% Percoll cushion, the intact chloroplast pellet was solubilized in decylmaltoside buffer [1% decylmaltoside, 25 mM HEPES pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After a 10 min incubation at room temperature the solubilized chloroplasts were spun for 5 min at 100.000 *g* to remove insoluble material. Ten percent of this supernatant was removed before immunoprecipitation for direct analysis on SDS–PAGE. The remaining 90% was split into equal portions to which 10 μl of antiserum or preimmune serum and 100 μl of a 50:50 slurry of protein A–Sepharose were added. Immunoprecipitations were rocked in the dark at 4°C for 2 h and then washed extensively with decylmaltoside buffer. Immunoprecipitates were released from the protein A–Sepharose beads by boiling in SDS–PAGE buffer containing 2-mercaptoethanol. Fractions were separated by SDS–PAGE and visualized by fluorography.

Trypsin treatment of intact chloroplasts and isolated inner envelope membranes

Purified intact pea chloroplasts were suspended in 50 mM HEPES–KOH pH 8, 330 mM sorbitol (buffer A) with the addition of 0.1 mM $CaCl_2$ at a chlorophyll concentration of 1 mg/ml. Various amounts of trypsin (Sigma, bovine pancreas 10700 BAEE U/mg) were added in a final reaction volume of 200 μl and the reaction was allowed to continue for 1 h at 20°C. The trypsin treatment was stopped by the addition of either 1 mM PMSF or a 5-fold molar excess of soybean trypsin inhibitor. Either method is suitable if the inhibitor solution is prepared fresh every time. The subsequent steps were carried out in the presence of inhibitor. Intact chloroplasts were separated from broken organelles by centrifugation through a 40% Percoll cushion in buffer A. The chloroplast pellet was washed twice with 1 ml of 50 mM HEPES–KOH pH 7.6, 330 mM sorbitol and 3 mM $MgCl_2$. The final chloroplast pellet was resuspended in 100 μl washing buffer and the chlorophyll concentration was determined (Arnon, 1949). Chloroplasts equivalent to 50 μg chlorophyll were lysed in 200 μl HEPES–KOH pH 7.6 for 60 min at 4°C. A total membrane fraction was recovered by centrifugation (10 min, 165 000 *g*). The membrane pellet was dissolved directly in SDS–PAGE sample buffer (Laemmli, 1970) and boiled for 3 min.

Inner envelope membranes (equivalent to 20 μg protein) were treated with different amounts of trypsin in 50 mM Tricine–KOH pH 8.5 and 0.1 mM $CaCl_2$ for 90 s at 20°C in a final volume of 20 μl. The trypsin treatment was stopped by the addition of PMSF or soybean trypsin inhibitor as above. Membranes were recovered by centrifugation as above and washed once in 10 mM tricine–KOH pH 8 at 0.5 mg protein per ml. Membranes were solubilized in SDS–PAGE sample buffer in the presence of trypsin inhibitor and subjected to electrophoresis without additional heating. The phosphate-triose-phosphate translocator aggregates upon heating in SDS–PAGE sample buffer and this treatment must be avoided whenever this protein is analyzed.

SDS–PAGE and Western blotting

SDS–PAGE was done essentially as described (Laemmli, 1970). Slab gels were stained in Coomassie Brilliant Blue or prepared for fluorography as in Bonner and Laskey (1974). Proteins were transferred to nitrocellulose sheets using a semi-dry blotting procedure (Kyhse-Andersen, 1984). The filters were treated and immunodecorated with antiserum as described (Towbin *et al.*, 1979). An alkaline phosphatase stain in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium was used for detection. The antiserum against IEP110 is described (Waegemann *et al.*, 1992). Briefly, it was raised in a rabbit using the SDS–PAGE purified IEP110 blotted onto nitrocellulose filters. The nitrocellulose sheets were dissolved in DMSO and injected into the animal. The resulting antiserum was specific for IEP110 (see below and Waegemann *et al.*, 1992).

Hill reaction

The Hill reaction (Trebst, 1972) was carried out in a medium containing 330 mM sorbitol, 50 mM HEPES–KOH pH 7.6, 1.5 mM $MgCl_2$, 1 mM K_2HPO_4, 1 mM ADP and 2 mM $K_3[Fe(CN)_6]$. The reaction was started by the addition of chloroplasts (40 μg chlorophyll) either untreated or treated with various amounts of trypsin. The reaction was illuminated with a slide projector in a temperature-regulated water bath (20°C). Aliquots were taken from the reaction mixture at: 0, 30, 60, 90 and 180 s, protein was precipitated with 10% trichloracetic acid and after 30 min at 4°C, samples were centrifuged for 10 min at 15 000 *g*. The supernatant was diluted 10-fold with H_2O and the optical density determined at 405 nm. Lysed chloroplasts were prepared as above.

Isolation of a cDNA clone for IEP110

A cDNA expression library (Uni Zap XR, Stratagene, USA) made from poly(A)$^+$ RNA of 5-day-old light grown pea seedlings (*P.sativum* var. Golf) was screened using a polyclonal antiserum to IEP110. Three isolates (*IEP110/1.1*, *1.5* and *1.6*) were obtained, all of which coded for IEP110. None of the clones were full-length as deduced from the missing N-terminal protein sequence. A 5′ probe of 340 nucleotides was synthesized by PCR™ from clone *IEP110/1.1* in the presence of digoxigenin-labeled dUTP (Boehringer, according to the manufacturer's

recommendation). The second round of screening resulted in one positive isolate (*IEP110/2.12*) of 2024 nucleotides, the coding region of which extended 5′ of the coding sequence for the N-terminal peptide sequence of IEP110 (see below). However, *IEP110/2.12* was not full-length because it did not contain the 3′ reading frames present in *IEP110/1.1*, *1.5* and *1.6*. The coding sequence of IEP110 deposited at the EMBL databank (accession No. Z68506) represents an in-frame fusion of *IEP110/1.1* and *IEP110/2.12*.

We used the following procedure to obtain a full-length coding sequence for preIEP110 for *in vitro* transcription–translation and expression in *E.coli*. A 2360 nucleotide fragment of *IEP110/1.1* was obtained by PCR™ using the primers 5′-GGGGGCCATGGAGATTGGTAGGA-AGC-3′ and 5′-GGGGGCTCGAGGAATACAAACTTCTCTTCC-3′ for the 5′- and 3′-ends, respectively. The fragment was subcloned into the vector pET21d (Novagen, Madison, USA) using *Nco*I/*Xho*I restriction, resulting in *IEP110/P1*. A second 827 nucleotide fragment was obtained by PCR™ from *IEP110/2.12* using the primers 5′-GGGGGGATGG-ACCCTTCCACGCTAA-3′ and 5′-GGGGGGCTCGAGGACCTTGAA-GACACGCTTCC-3′ for the 5′- and 3′-ends, respectively. The PCR™ product was digested with *Nco*I/*Nsi*I and cloned into *IEP110/P1* using *Nco*I/*Nsi*I to linearize the vector, resulting in *IEP110/P2*. *IEP110/P2* was sequenced to verify its identity with the original cDNA clones. The cloning strategy resulted in the exchange of an asparagine for an aspartate at position two of preIEP110. In addition a C-terminal His_6-tag was present in-frame with the coding region of preIEP110. *In vitro* transcription–translation was done in a reticulocyte lysate system as described earlier (Salomon *et al.*, 1990). Synthesis of preIEP110 from *IEP110/P2* or IEP110ΔN from *IEP110/P1* in *E.coli* BL21De3 cell was done as in Waegemann and Soll (1995).

Purification of an antiserum specific for the N-terminus of IEP110

IEP110/P1 was overexpressed in *E.coli* and the IEP110ΔN protein recovered from inclusion bodies. IEP110ΔN was dissolved in SDS and coupled to CNBr-activated Sepharose according to the manufacturer's recommendations (Pharmacia, Sweden). One hundred microlitres of αIEP110 were incubated with the affinity matrix overnight (1 ml). The antiserum specific for the N-terminus of IEP110, namely αIEP110N, was recovered in the supernatant.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to J.S. and by grants to K.K. from the US National Science Foundation and from the Energy Biosciences Program at the US Department of Energy.

References

Alefsen,H., Waegemann,K. and Soll,J. (1994) Analysis of the chloroplast protein import machinery. *J. Plant Physiol.*, **144**, 339–345.

Arnon,D.J. (1949) Copper enzymes in isolated chloroplasts. Phenoloxidase in Beta vulgaris. *Plant Physiol.*, **24**, 1–15.

Blobel,G. (1980) Intracellular protein topogenesis. *Proc. Natl Acad Sci. USA*, **77**, 1496–1499.

Block,M.A., Dorne,A.-J., Joyard,J. and Douce,R. (1983) Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. II. Biochemical characterization. *J. Biol. Chem.*, **258**, 13281–13286.

Bonner,W.M. and Laskey,R.A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.*, **46**, 83–88.

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**, 3595–3599.

Cline,K., Keegstra,K. and Staehelin,L.A. (1985) Freeze-fracture electron microscopic analysis of ultrarapidly frozen envelope membranes on intact chloroplasts and after purification. *Protoplasma*, **125**, 111–123.

Chua,N.-H. and Schmidt,G.W. (1979) Transport of proteins into mitochondria and chloroplasts. *J. Cell Biol.*, **81**, 461–483.

Glick,B.S., Wachter,C. and Schatz,G. (1991) Protein import into mitochondria: two systems acting in tandem? *Trends Cell Biol.*, **1**, 99–103.

Grossmann,A., Bartlett,S. and Chua,N.-H. (1980) Energy-dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts. *Nature*, **285**, 625–628.

Hachiya,N., Mihara,K., Suda,K., Horst,M., Schatz,G. and Lithgow,T. (1995) Reconstitution of the initial steps of mitochondrial protein import. *Nature*, **376**, 705–709.

Hirsch,St., Muckel,E., Heemeyer,F., von Heijne,G.V. and Soll,J. (1994) A receptor component of the chloroplast protein translocation machinery. *Science*, **266**, 1989–1992.

Horst,M., Hilfiker-Rothenfluh,S., Oppliger,W. and Schatz,G. (1995) Dynamic interaction of the protein translocation systems in the inner and outer membranes of yeast mitochondria. *EMBO J.*, **14**, 2293–2297.

Joyard,J., Block,M.A. and Douce,R. (1991) Molecular aspects of plastid envelope biochemistry. *Eur. J. Biochem.*, **199**, 489–509.

Keegstra,K. and Yousif,A.E. (1986) Isolation and characterization of chloroplast envelope membranes. *Methods Enzymol.*, **118**, 316–325.

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.

Kiebler,M., Pfaller,R., Söllner,Th., Griffiths,G., Horstmann,H., Pfanner,N. and Neupert,W. (1990) Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. *Nature*, **348**, 610–616.

Ko,K., Budd,D., Wu,C., Seibert,F., Kourtz,L. and Ko,Z.W. (1995) Isolation and characterization of a cDNA clone encoding a member of the Com44/Cim44 envelope components of the chloroplast protein import apparatus. *J. Biol. Chem.*, **270**, 28601–28608.

Kyhse-Andersen,J. (1984) Electroblotting of multiple gels - a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods*, **10**, 203–209.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Marshall,J.S., DeRocher,A.E., Keegstra,K. and Vierling,E. (1990) Identification of heat shock protein hsp70 homologues in chloroplasts. *Proc. Natl Acad. Sci. USA*, **87**, 374–378.

Olsen,L.J. and Keegstra,K. (1992) The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. *J. Biol. Chem.*, **267**, 433–439.

Olsen,L.J., Theg,St.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. and Keegstra,K. (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. *Plant Cell*, **6**, 93–105.

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.

Schnell,D.J. and Blobel,G. (1993) Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. *J. Cell Biol.*, **120**, 103–115.

Schnell,D.J., Kessler,F. and Blobel,G. (1994) Isolation of components of the chloroplast protein import machinery. *Science*, **266**, 1007–1012.

Seedorf,M. and Soll,J. (1995) Copper chloride, an inhibitor of protein import into chloroplasts. *FEBS Lett.*, **367**, 19–22.

Seedorf,M., Waegemann,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.

Soll,J. (1995) New insights into the protein import machinery of the chloroplast's outer envelope. *Bot. Acta*, **108**, 277–282.

Soll,J. and Waegemann,K. (1992) A functionally active protein import complex from chloroplasts. *Plant J.*, **2**, 253–256.

Stuart,R.A., Cyr,D.M., Craig,E.A. and Neupert,W. (1994) Mitochondrial molecular chaperones: their role in protein translocation. *Trends Biochem. Sci.*, **19**, 87–92.

Theg,St.M., Bauerle,C., Olsen,L.J., Selman,B.R. and Keegstra,K. (1989) Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. *J. Biol. Chem.*, **264**, 6730–6736.

Towbin,A., Staehlin,T. and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA*, **76**, 4350–5354.

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.

Trebst,A. (1972) Measurement of Hill reactions and photoreduction. *Methods Enzymol.*, **23B**, 146–165.

von Heijne,G. and Blomberg,C. (1979) Trans-membrane translocation of proteins. *Eur. J. Biochem.*, **97**, 175–181.

Waegemann,K. and Soll,J. (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. *Plant J.*, **1**, 149–158.

Waegemann,K. and Soll,J. (1993) Isolation and characterization of a functionally active protein translocation apparatus from chloroplast envelopes. In Morré,D.J., Howell,E. and Bergeron,J.J.M. (eds), *Molecular Mechanisms of Membrane Traffic. NATO ASI Series*. Springer-Verlag, Berlin, Vol. H74, pp. 101–104.

Waegemann,K. and Soll,J. (1995) Characterization and isolation of the chloroplast protein import machinery. *Methods Cell Biol.*, **50**, 255–267.

Waegemann,K., Eichacker,S. and Soll,J. (1992) Outer envelope membranes from chloroplasts are isolated as rightside-out vesicles. *Planta*, **187**, 89–94.

Werner-Washburne,M., Cline,K. and Keegstra,K. (1983) Analysis of pea chloroplast inner and outer envelope membrane proteins by two-dimensional gel electrophoresis and their comparison with stromal proteins. *Plant Physiol.*, **73**, 569–575.

Wu,C., Seibert,F.S. and Ko,K. (1994) Identification of chloroplast envelope proteins in close physical proximity to a partially translocated chimeric precursor protein. *J. Biol. Chem.*, **269**, 32264–32271.

Received on February 15, 1996; revised on April 23, 1996