Two Components of the Chloroplast Protein Import Apparatus, IAP86 and IAP75, Interact with the Transit Sequence during the Recognition and Translocation of Precursor Proteins at the Outer Envelope

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Abstract. The interactions of precursor proteins with components of the chloroplast envelope were investigated during the early stages of protein import using a chemical cross-linking strategy. In the absence of energy, two components of the outer envelope import machinery, IAP86 and IAP75, cross-linked to the transit sequence of the precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (pS) in a precursor binding assay. In the presence of concentrations of ATP or GTP that support maximal precursor binding to the envelope, cross-linking to the transit sequence occurred predominantly with IAP75 and a previously unidentified 21-kD polypeptide of the inner membrane, indicating that the transit sequence had inserted across the outer membrane. Cross-linking of envelope components to sequences in the mature portion of a second precursor, preferedoxin, was detected in the presence of ATP or GTP, suggesting that sequences distant from the transit sequence were brought into the vicinity of the outer membrane under these conditions. IAP75 and a third import component, IAP34, were coimmunoprecipitated with IAP86 antibodies from solubilized envelope membranes, indicating that these three proteins form a stable complex in the outer membrane. On the basis of these observations, we propose that IAP86 and IAP75 act as components of a multisubunit complex to mediate energy-independent recognition of the transit sequence and subsequent nucleoside triphosphate-induced insertion of the transit sequence across the outer membrane.

The vast majority of chloroplast polypeptides are encoded in the nucleus and synthesized on cytoplasmic ribosomes. These polypeptides are posttranslationally imported into the organelle, sorted to one of at least six suborganellar compartments, and assembled into their functional native forms. The initial, common event in this cascade of protein targeting and assembly reactions is the translocation of precursor proteins across the double membrane of the chloroplast envelope (for review see de Boer and Weisbeek, 1991; Theg and Varian, 1993; Schnell, 1995).

Protein import across the chloroplast envelope can be divided into two general stages. The first stage corresponds to the specific binding of precursor at the outer envelope (Cline et al., 1985; Friedman and Keegstra, 1989). Envelope binding requires ATP and GTP hydrolysis (Olsen et al., 1989; Olsen and Keegstra, 1992; Kessler et al., 1994) and is mediated by the amino-terminal transit sequence of the precursor protein (Cline et al., 1985). The second stage of import is the translocation of the polypeptide precursor across the double membrane of the envelope into the stromal compartment. Translocation requires ATP hydrolysis in the stromal compartment at concentrations approximately 5-10-fold higher than those required for envelope binding (Grossman et al., 1980; Pain and Blobel, 1987; Olsen et al., 1989; Theg et al., 1989). Translocation is localized to contact zones between the outer and inner membrane, thereby facilitating the simultaneous transport of polypeptides across the two membranes (Schnell and Blobel, 1993).

Precursor binding at the chloroplast surface has been the focus of extensive investigation because this event presumably represents recognition of the transit sequence by chloroplast receptors and the initial engagement of the envelope translocation machinery. Although the association of precursors with the envelope exhibits dissociation constants of 10-100 nM, it is irreversible, suggesting that binding is not a simple receptor–ligand interaction but is composed of a more complex set of recognition and binding reactions that result in the stable association of the precursor with the envelope (Olsen et al., 1989; Schnell and Blobel, 1993; Perry and Keegstra, 1994). This hypothesis is supported by the rather complicated energetics of the re-
mediate. Corn70 is peripherally associated with the cytosol.

Schnell et al., 1994), are also associated with an early import intermediate. Predictions of the secondary structure of IAP75 suggest that it may possess extensive β-sheet structure similar to the bacterial porins, prompting Schnell et al. (1994) and Tranel et al. (1995) to propose that IAP75 functions as a component of the protein-conducting channel through a GTP-binding and hydrolysis cycle (Kessler et al., 1994).

A third component, IAP75, is an integral membrane protein whose primary structure reveals no similarity to proteins of known function (Schnell et al., 1994; Tranel et al., 1995). IAP75 is resistant to alkaline extraction and protease digestion in intact envelope membranes, indicating that it is deeply embedded in the outer membrane bilayer (Schnell et al., 1994; Tranel et al., 1995). Predictions of IAP75 suggest that it may possess extensive β-sheet structure similar to the bacterial porins, prompting Schnell et al. (1994) and Tranel et al. (1995) to propose that IAP75 functions as a component of the protein-conducting channel or pore for outer membrane translocation.

Two members of the hsp70 family of molecular chaperones, Com70 (Wu et al., 1994) and the hsp70-IAP (Schnell et al., 1994), are also associated with an early import intermediate. Com70 is peripherally associated with the cytosolic surface of the outer membrane (Wu et al., 1994). The hsp70 IAP is resistant to alkaline extraction from the envelope and is not susceptible to externally added protease in intact chloroplasts (Schnell et al., 1994). These observations suggest that the bulk of the hsp70 IAP molecule faces the intermembrane space. The localization of these

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**Preparation of the pS-1 and pFd-protA Precursor Proteins**

Plasmid pET21d-pS-1 encoding the pS-1 precursor was constructed from two DNA fragments. The first fragment, corresponding to amino acids −57 to +41, was generated by amplification of the pS-coding sequence using the PCR with primers that incorporated inframe NcoI and BamH1 sites at the 5' and 3' ends, respectively. The second fragment, corresponding to residues +42 to +112 of pS, was generated by amplification using the PCR with primers that incorporated inframe BamH1 and EcoR1 sites at the 5' and 3' ends, respectively. The 5'-BamH1 primer resulted in a change of the cysteine at position +41 to a proline, and the 3'-EcoR1 primer resulted in a change of cysteine +112 to a serine with respect to authentic pS. Both fragments were amplified using pT7-pS (Pain and Blobel, 1987) as the template. The amplification products were digested with BamH1, ligated, and the resulting ligation product was digested with NcoI and EcoR1 and inserted into the NcoI and EcoR1 sites of pET21d (Novagen, Inc., Madison, WI). The resulting plasmid, pET21d-pS-1, encoded residues corresponding to amino acids −57 to +112 of authentic pS with a cysteine to proline change at position +41 and a cysteine to serine change at position +112. Plasmid pET21b-pF3-protA encoding the pF3-
Modification of pS-1 and pFd-protA with [125I]APDP

The heterobifunctional cross-linking reagent, N-[4-(p-azidosalicylamido)butyl]-3-(2-pyridyldithio)propionamide (APDP), was purchased from Pierce, Inc. (Rockford, IL). All iodination and precursor modification reactions were performed in complete darkness. APDP (50 nmole) was iodinated in the presence of 1 nCi of carrier-free Na[125I] in a micro-

centrifuge and subfractionated as described above. The amount of radiolabel incorporated into envelope proteins that is recovered in the outer membrane fractions following cross-linking to pS-l-[125I]ADP is ~10% of the amount recovered in the mixed membrane fractions. In contrast, the amount of radiolabel recovered in outer membrane fractions following pFd-protA-[125I]ADP is typically <1% of that recovered in the mixed membrane fractions.

Antibody Production and Immunoprecipitation

Antibodies to IAP34 were prepared as previously described (Kessler et al., 1994). Antibodies to E. coli expressed IAP75 and partial IAP86 were generated in rabbits. The proteins for immunization were prepared as follows. The complete IAP75 coding region of pBSIAP75 (nucleotides 8-2344) was amplified by PCR with primers that incorporated NdeI and BamHI sites at the 5' and 3' ends of the DNA, respectively. The DNA was inserted into the NdeI and BamHI sites of pTE21b (Novagen, Inc.) to generate pTE21b-IAP75. A part of the IAP86 coding region of pBS-IAP86 (nucleotides 624-2699 corresponding to amino acids 190-879) (Kessler et al., 1994) was amplified by PCR with primers that incorporated NdeI and BamHI sites at the 5' and 3' ends of the DNA, respectively. The DNA was inserted into the NdeI and BamHI sites of pTE21b (Novagen, Inc.) to generate pTE21b-IAP86p. The pTE21b-IAP75 and pTE21b-IAP86p were transformed into E. coli BL21 (DE3), expressed, and affinity chromatography on a nickel-chelate matrix according to the supplier's recommendations (Novagen, Inc.). Expression resulted in complete IAP75 or partial IAP86 polypeptides fused to a sequence of six histidine residues at their COOH termini.

For the preparation of antibodies to IAP21, total inner and mixed inner and outer envelope polypeptides were resolved by SDS-PAGE and the polypeptide band corresponding to IAP21 was excised and injected into rabbits. The envelope membranes were purified from chloroplasts that were treated with 100 µg/ml thermolysin for 30 min on ice (Cline et al., 1984). The protease treatment degrades the outer envelope 21-kD polypeptide, thereby minimizing the possibility that it is a major contaminant of the IAP21 preparation.

Sample Analysis and Quantitation

Isolated intact chloroplasts were incubated in HS buffer that contains 50 mM KOAc, 4 mM MgOAc (import buffer) in the presence of 400 nM nigericin for 15 min at 26°C, to deplete them of endogenous ATP. All binding reactions were performed in the dark. For the binding of precursor in the absence of ATP (early import intermediate), ATP-depleted chloroplasts (equivalent to 2 mg of chlorophyll) in 1 ml of import buffer were incubated in the presence of 25 µM MgATP for 5 min at 26°C. For the binding of precursor in the absence of ATP, ATP-depleted chloroplasts (equivalent to 2 mg of chlorophyll) in 1 ml of import buffer were incubated in the presence of 20 µM of apyrase (Sigma Chemical Co., St. Louis, MO) for 5 min at 26°C. After the pretreatment, una-denatured pS-l-[125I]ADP or pFd-protA-[125I]ADP was added to a final concentration of 200 nM and the incubation was continued for 10 min at 26°C. For the binding reaction, the chloroplasts were resuspended in Percoll silica gel and resuspended in 1.5 ml of HS buffer. The chloroplasts were transferred to the bottom half of a glass petri dish on ice and irradiated from above with a Chromato-Vu transilluminator (UVP, Inc., Upland, CA) at 312 nm at a distance of 5 cm for 20 min with constant shaking. The chloroplasts were collected by centrifugation for 30 s at 6,000 g in a micro-

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Immunoprecipitation following solubilization of membranes under non-denaturing conditions was performed with the method of Anderson and Blobel (1983). Before immunoprecipitation reactions, total envelope membranes (25 µg protein) were incubated in 100 µl of 50 mM tricine, pH 7.5, 2 mM EDTA (TE buffer) containing 2% vol/vol β-mercaptoethanol at 35°C for 15 min to remove the cross-linker from the precursor, and membranes were recovered by centrifugation at 40,000 g for 30 min in a TLA 100.3 rotor and washed with TE buffer to remove residual β-mercaptoethanol.

Immunoprecipitation following solubilization of membranes under denaturing conditions was performed with anti-21-kD serum or IAP86 IgG coupled directly to Sepharose. Before solubilization, total envelope membranes were incubated in TE buffer containing 2% vol/vol β-mercaptoethanol at 26°C for 5 min to release the cross-linker from the precursor. The membranes were recovered by centrifugation at 40,000 g for 30 min in a TLA 100.3 rotor and washed with TE buffer to remove residual β-mercaptoethanol. The membrane pellet was resuspended in TE buffer containing 250 mM NaCl and 10% glycerol (TEGS buffer) to a concentration of 1 mg protein/ml. The suspension was diluted with an equal volume of 4% Triton X-100 in TEGS buffer and incubated on ice for 10 min. The sample was centrifuged for 15 min at 40,000 g to remove insoluble aggregates. The supernatant was incubated with 20 µl of anti-21-kD serum or anti-IAP86 IgG-Sepharose for 4 h at 4°C. Anti-21-kD immunoprecipitates were incubated for 1 h at 4°C with 25 µl (packed volume) of protein A-Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). After the incubations, the protein A-Sepharose and anti-IAP86-Sepharose were washed extensively with TEGS buffer. The protein A-Sepharose-bound fraction was eluted directly into SDS-PAGE sample buffer. The anti-IAP86-Sepharose bound fraction was eluted with 0.2 M glycine, pH 2.2, containing 1% Triton X-100. The eluates were concentrated by precipitation with 10% (wt/vol) trichloroacetic acid and analyzed by SDS-PAGE.

Sample Analysis and Quantitation

All cross-linked samples were resolved by SDS-PAGE on 12% polyacrylamide gels. The radioactive signals in dried gels were captured and quantitated using a PhosphorImager SI (Molecular Dynamics, Inc., Sunnyvale, CA) with the IPLab Gel Scientific Image Processing program (version

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This construct, designated pS-1, resulted in a precursor A). The second construct was a hybrid precursor protein. To this end, we performed precursor binding and chemical cross-linking. They were expressed in E. coli protein (Fig. 1 A). Both proteins also contained a COOH-terminal region (position -1) of the transit sequence (Fig. 1). The transit sequence and residues +1 to +112 of the mature sequence is devoid of cysteines. Therefore, the transit sequences of the precursors did not require mutagenesis to generate or eliminate specific sites for APDP incorporation.

The first construct consisted of pS containing the entire transit sequence and residues +1 to +112 of the mature sequence. The mature sequence was modified by site-directed mutagenesis to eliminate all cysteine residues. This construct, designated pS-1, resulted in a precursor containing a single cysteine residue located at the carboxyterminal region (position -1) of the transit sequence (Fig. 1 A). The second construct was a hybrid precursor protein consisting of pFd fused to the IgG-binding domains of staphylococcal protein A (pFd-protA). The transit sequence of the pFd domain of this hybrid is devoid of cysteine residues, whereas the pFd transit sequence is devoid of cysteine residues. Therefore, the transit sequences of the precursors did not require mutagenesis to generate or eliminate specific sites for APDP incorporation.

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We wished to map the interactions of the envelope import apparatus with chloroplast precursor proteins during the early stages of import to assess which components function in transit sequence recognition and/or membrane transport. To this end, we performed precursor binding and chemical cross-linking studies using two precursor proteins that contained the cleavable, photoactivatable reagent, [125I]APDP, linked by disulfide bonds to specific cysteine residues within their transit sequence or mature sequence. Chemical reduction of reactions following photo-induced cross-linking with APDP-modified substrates cleaves the disulfide linkage and leaves the radiolabeled cross-linker covalently attached to the chloroplast target protein. The two substrates for the cross-linking studies were based on pS and preferredoxin (pFd). These two precursor proteins were chosen for two reasons. First, previous studies have established that pS and pFd use a common pathway for envelope binding and translocation that involves IAP34, IAP75, IAP96, and the hsp70 IAP (Schnell et al. 1991; Schnell et al., 1994). Therefore, their interactions with envelope components during the import process are predicted to be similar. Second, the transit sequence of pS contains a single cysteine, whereas the pFd transit sequence is devoid of cysteines. Therefore, the transit sequences of the precursors did not require mutagenesis to generate or eliminate specific sites for APDP incorporation.

The pS-1 and pFd-protA proteins were modified by disulfide exchange reactions with [125I]APDP. This resulted in the incorporation of a photoactivatable, radiolabeled phenyl azide group at cysteine residues in each polypeptide. The [125I]APDP-modified precursors were referred to as pS-1-[125I]ADP (Fig. 1 B, lane 2) and pFd-protA-[125I]ADP (Fig. 1 B, lane 1). Fig. 1 B shows that the reactivity of pS-1 and pFd-protA with [125I]APDP resulted in incorporation of radiolabeled cross-linker into the poly-
of light induced cross-linking. Fig. 2 shows that pFd-protA-[\(^{125}\)I]ADP and pS-1-[\(^{125}\)I]ADP bind to the chloroplast surface in an energy-dependent manner that is characteristic of the early import intermediate. Binding of both precursors is stimulated most effectively by 75 \(\mu\)M ATP (Fig. 2, A and B, compare lanes 1 and 3) and to a lesser degree by 200 \(\mu\)M GTP (Fig. 2, A and B, compare lanes 1 and 4). The energy requirements for the formation of the early intermediate using pS-1-[\(^{125}\)I]ADP and pFd-protA-[\(^{125}\)I]ADP are indistinguishable from those previously observed for authentic pS (Olsen et al., 1989; Olsen and Keegstra, 1992) and pFd (Pilon et al., 1990). The pS-1-[\(^{125}\)I]ADP and pFd-protA-[\(^{125}\)I]ADP proteins were fully imported into chloroplasts when the ATP concentration was raised above 1 mM, although incomplete processing of pS-1-[\(^{125}\)I]ADP to its mature form was observed due to the position of the APDP modification at the transit peptidase cleavage site (data not shown).

### Chemical Cross-linking of Envelope Polypeptides to the Transit Sequence and Mature Sequence of Early Import Intermediates

To investigate the interaction of envelope polypeptides with the early import intermediate, pS-1-[\(^{125}\)I]ADP and pFd-protA-[\(^{125}\)I]ADP were bound to chloroplasts in the presence of 75 \(\mu\)M ATP, and cross-linking to nearby proteins was induced with UV light. After the cross-linking reaction, the chloroplasts were lysed, subfractionated by sucrose density gradient centrifugation, and fractions corresponding to total envelope membranes were analyzed by SDS-PAGE. Four cross-linked products of 86, 75, 24, and 21 kD were observed with the pS-1-[\(^{125}\)I]ADP substrate (Fig. 3, lane 2). A faint labeled band at 44 kD also was present (Fig. 3, lane 2). The 24- and 44-kD bands were immunoprecipitated with anti-pS serum, indicating that they are derivatives of pS-1-[\(^{125}\)I]ADP (data not shown). It is most likely that they correspond to monomeric and dimeric pS that are labeled by intramolecular cross-linking. Both bands were present in the pS-1-[\(^{125}\)I]ADP preparation used for cross-linking (Fig. 1 B, lane 2).

Cross-linking to the mature sequence of pFd-protA-[\(^{125}\)I]ADP results in one major labeled product of 86 kD and a minor labeled product of 75 kD (Fig. 3, lane 4). A band at 52 kD corresponding to pFd-protA is also visible (Fig. 3, lane 4). In the case of both pS-1-[\(^{125}\)I]ADP and pFd-protA-[\(^{125}\)I]ADP, cross-linking was dependent upon UV irradiation (Fig. 3, compare lanes 1 and 2; 3 and 4), and discrete cross-linked products were observed only when irradiated samples were treated with reducing agents prior to SDS-PAGE analysis (Fig. 3, compare lanes 2 and 5; 4 and 6).

The 75- and 86-kD polypeptides that cross-linked to pS-1-[\(^{125}\)I]ADP and pFd-protA-[\(^{125}\)I]ADP were coincident with IAP75 and IAP86, respectively. Fig. 4 shows that anti-IAP75 (Fig. 4, A and B, lane 4) and anti-IAP86 (Fig. 4, A and B, lane 2) sera immunoprecipitate the cross-linked products confirming that these polypeptides are IAP86 and IAP75. These results are consistent with the previous results of Perry and Keegstra (1994) that indicate that IAP75 and IAP86 are in close proximity to early import intermediates.

The 21-kD polypeptide that cross-links to pS-1-[\(^{125}\)I]ADP (Fig. 3, lane 2) is a previously unidentified candidate for a component of the import machinery. Although the mobility of this polypeptide is similar to that predicted for the mature form of the pS-1-[\(^{125}\)I]ADP substrate, it is not immunoprecipitated with anti-pS serum (data not shown). This eliminates the possibility that the 21-kD polypeptide represents a fraction of the precursor.
Figure 3. Cross-linking of envelope-bound pFd-protA-[125I]ADP and pS-1-[125I]ADP to envelope polypeptides. Isolated chloroplasts equivalent to 2 mg of chlorophyll were incubated with 200 nM urea-denatured pFd-protA-[125I]ADP (+pFd-protA-ADP) or pS-1-[125I]ADP (+pS-1-ADP) in a standard binding assay containing 75 μM ATP for 10 min at 26°C in the dark. After the binding reaction, chloroplasts were reisolated over Percoll silica gel, resuspended in binding buffer in the absence of ATP, and irradiated at 312 nM for 20 min on ice with a transilluminator at a distance of 5 cm (+UV light) or incubated in the dark (-UV light) on ice. The chloroplasts were recovered, lysed, and fractionated to yield a total envelope membrane fraction. Envelope membranes (25 μg of protein) were analyzed by SDS-PAGE in the presence (+DTT) or absence (-DTT) of 100 mM dithiothreitol. The positions of pFd-protA-[125I]ADP (pFd-protA-ADP) or pS-1-[125I]ADP (pS-1-ADP) are indicated to the left of the figure. The molecular masses of standard proteins are indicated to the right of the figure. Image capture and analysis were performed on a Phosphorimager (Molecular Dynamics, Inc.).

Figure 4. Immunoprecipitation of cross-linked envelope polypeptides with antibodies specific to IAP75 and IAP86. Chloroplast envelope membranes (25 μg protein) from pFd-protA-[125I]ADP (A) and pS-1-[125I]ADP (B) cross-linking experiments were dissolved under denaturing conditions and immunoprecipitated with anti-IAP86 serum (+ anti-86), anti-IAP75 serum (+ anti-75), or the corresponding preimmune serum (+ anti-75 PI or + anti-86 PI) (see Materials and Methods). The immunoprecipitated products were analyzed by SDS-PAGE. Lane 1 of each panel contains the envelope membranes (10 μg protein) used for the immunoprecipitation reactions. The position of pFd-protA-[125I]ADP that precipitates in all reactions due to its IgG-binding domain is indicated to the right of A. The molecular masses of standard proteins are indicated to the left of the figures. Image capture and analysis were performed on a Phosphorimager (Molecular Dynamics, Inc.).

that was proteolytically processed during the binding reaction or subsequent fractionation of the chloroplasts. We refer to this protein as IAP21 (import intermediate associated protein of 21 kD). The nature of this polypeptide is investigated in more detail in a following section.

Two-dimensional gel electrophoresis of envelope membranes from the pS-1-[125I]ADP and pFd-protA-[125I]ADP cross-linking experiments did not reveal detectable cross-linking to envelope polypeptides other than IAP75, IAP86, and the 21-kD polypeptide (data not shown). In addition, cross-linking to polypeptides in the stromal fraction of the chloroplasts was not detected with either substrate (data not shown).

Interactions of the Transit Sequence with the Import Machinery

On the basis of results in Fig. 3, it is apparent that the topology of the stable early import intermediate results in an intimate association of IAP75 and IAP86 with amino acids within the transit sequence and mature sequence of the bound precursor. We and others have suggested that the early import intermediate represents a stage in import that is subsequent to the recognition of the precursor by its initial receptor (Schnell and Blobel, 1993; Perry and Keegstra, 1994). To detect interactions of envelope proteins
with the transit sequence prior to the energy-dependent formation of the stable early intermediate, we performed cross-linking to pS-1-[\textsuperscript{125}I]ADP in the presence and absence of ATP and GTP and their nonhydrolyzable analogs. Prior to the cross-linking reactions, the chloroplasts were depleted of endogenous and exogenous NTP by incubation in the dark at 26°C in the presence of apyrase. In addition, the proton ionophore, nigericin, was added to prevent any subsequent light-driven ATP synthesis.

To determine the site of the interaction between the precursor and the import components, the chloroplast envelopes were subfractionated following the cross-linking reactions into outer membrane and mixed outer and inner membrane preparations containing contact zones. Although early import intermediates previously have been localized to contact zones (Schnell and Blobel, 1993), Perry and Keegstra (1994) detected cross-linking of IAP86 in free outer membrane, suggesting an initial interaction of the precursor with the import machinery in this envelope subdomain.

Fig. 5 shows that cross-linking of the transit sequence of pS-1-[\textsuperscript{125}I]ADP to IAP86 and IAP75 is detected in the absence of exogenous ATP or GTP (Fig. 5, A and B, lane 1). Quantitative analysis of cross-linking indicates that labeling of these two proteins is detected primarily in the outer membrane (Fig. 5 A, lane 1, and C). IAP86 is preferentially cross-linked approximately 2:1 over IAP75 in the outer membrane (Fig. 5 C). In the mixed outer–inner membrane fraction, IAP75 and IAP86 are labeled at lower levels than in the outer membrane, but both proteins are cross-linked at approximately equal levels (Fig. 5 B, lane 1, and D). These data suggest that IAP75 and IAP86 form a receptor site for the transit sequence at the outer membrane and that the initial interaction between these two import components and the transit sequence occurs without the input of energy.

The addition of ATP to the pS-1-[\textsuperscript{125}I]ADP-binding reaction has significant effects on the degree of cross-linking to IAP75 and IAP86 and on the distribution of these cross-linked products between the outer membrane and mem-

**Figure 5.** Energy dependence of pS-1-[\textsuperscript{125}I]ADP cross-linking to chloroplast envelope polypeptides and distribution of cross-linked products in envelope membrane fractions. Isolated chloroplasts (2 mg chlorophyll) were incubated with 200 nM urea-denatured pS-1-[\textsuperscript{125}I]ADP in a standard binding assay containing apyrase (+ Apyrase), 75 μM ATP (+ ATP), 200 mM GTP (+ GTP), 100 μM GMPPNP (+ GMPPNP), or 100 μM AMP-PNP (+ AMP-PNP) for 10 min at 26°C in the dark. The chloroplasts were resolubilized through Percoll silica gel, irradiated with UV light, recovered, lysed under hypertonic conditions, and separated into stroma and membrane fractions. The total membrane fraction was subfractionated by flotation into linear 20–40% wt/vol sucrose gradients. Sucrose gradient fractions corresponding to outer envelope membrane (Outer Membrane) and mixed outer and inner envelope membrane (Outer-Inner Membrane) were analyzed by SDS-PAGE. (A and B) Representative fluorographs of SDS-PAGE resolved outer (A) and mixed outer and inner (B) membrane fractions. Each lane contains 5 μg of protein. The positions of IAP86, IAP75, IAP21, and pS-1-[\textsuperscript{125}I]ADP are indicated to the right of B. The molecular masses of standard proteins are indicated to the left of both figures. (C and D) Quantitation of the radioactivity incorporated into IAP75 and IAP86 in outer (C) and mixed outer-inner envelope membranes (D). The quantitative data in C and D are the average of triplicate experiments. Image capture and quantitation were performed on a Phosphorimager (Molecular Dynamics, Inc.).
In the presence of ATP, the labeling of IAP86 and IAP75 in the outer membrane is approximately equal (Fig. 5 A, lane 2, and B), but the overall levels of cross-linking to both proteins are less than in the absence of energy (Fig. 5 A, compare lanes 1 and 2, and C). The labeling of IAP75 increases fivefold in the mixed membrane fraction compared to the energy-depleted sample, and the relative cross-linking to IAP75 and IAP86 increases from a 1:1 to a 4:1 ratio (Fig. 5 B, compare lanes 1 and 2, and D). These data suggest that ATP hydrolysis induces a dramatic change in the topology of the bound precursor such that the near-neighbor interactions with the transit sequence change from a principle interaction with IAP86 in free outer membrane to one that primarily involves IAP75 at envelope contact sites. The high levels of cross-linking detected in the mixed outer and inner membrane fraction is consistent with previous data that localize the stable early import intermediate to contact zones (Schnell and Blobel, 1993).

GTP partially mimics the effects of ATP on the cross-linking pattern observed with pS-1-[\(^{125}\)I]ADP. The pattern and levels of cross-linking to IAP86 and IAP75 in the outer membrane are similar to those of the ATP-treated samples (Fig. 5 A, compare lanes 2 and 3, and C), however, the overall level of cross-linking to IAP75 in the mixed outer and inner membrane fraction is less than with ATP (Fig. 5, B and D, lane 3). This result is consistent with the data demonstrating that GTP is less effective in supporting formation of the early import intermediate (Olsen et al., 1992; Schnell et al., 1994).

The cross-linking patterns of IAP75 and IAP86 in the presence of the nonhydrolyzable ATP and GTP analogs adenosyl-5'-[\(\beta,\gamma\text{imido}\)]triphosphate (AMP-PNP) (Fig. 5, A and B, lane 5) and guanosyl-5'-[\(\beta,\gamma\text{imido}\)]triphosphate (GMP-PNP) are indistinguishable from those observed in the absence of NTP (Fig. 5, A and B, lane 4). The slowly hydrolyzable analogs of ADP and GDP, adenosine-5'-[\(\beta,\gamma\text{imido}\)]triphosphate (AMP-PNP) and guanosine-5'-[\(\beta,\gamma\text{imido}\)]triphosphate (GMP-PNP) had similar effects (data not shown). These results are consistent with the previous observation that hydrolysis of ATP and GTP are necessary for formation of the early import intermediate.

Radiolabeling of the 21-kD protein with pS-1-[\(^{125}\)I]ADP is observed in the presence of NTP (Fig. 5 B, compare lanes 1, 2, and 3). Labeling is highest in the presence of ATP (Fig. 5 B, lane 2), but labeling is also detected in the presence of GTP (Fig. 5 B, lane 3). No detectable cross-linking is observed in the absence of ATP or GTP (Fig. 5 B, lane 1) or in the presence of GMP-PNP (Fig. 5 B, lane 4) or AMP-PNP (Fig. 5 B, lane 5). Radiolabeled IAP21 protein is detected in mixed outer and inner membrane fractions (Fig. 5 B), but not in outer membrane fractions (Fig. 5 A). These observations suggest that the 21-kD protein interacts with the transit sequence only under conditions that promote formation of the stable early import intermediate that is localized at envelope contact zones.

The cross-linking of pS-1-[\(^{125}\)I]ADP to IAP75 and IAP86 in the absence of energy may represent an initial recognition of the transit sequence by the outer envelope import machinery prior to the energy-dependent promotion of the precursor to the stable early intermediate. To test if the energy-independent cross-linking to pS-1-[\(^{125}\)I]ADP is dependent on the transit sequence and not due to a nonspecific interaction, cross-linking to energy-depleted chloroplasts was performed in the absence or presence of increasing concentrations of unlabeled authentic pS and mature small subunit (S). Fig. 6 A shows that pS (compare lanes 1 to 3), inhibits cross-linking to IAP75 and IAP86 in the absence of NTP, but S does not (compare lanes 1 and 4). Fig. 6 B shows that cross-linking to IAP86, IAP75, and IAP21 in the presence of ATP can also be competed by pS (compare lanes 1 to 3), but not by mature S (compare lanes 1 and 4). These results demonstrate that the interactions of the precursor with these import components are transit sequence dependent.

**Interactions among the Outer Envelope Import Components**

IAP75 and IAP86 previously have been shown to comiunoprecipitate (Schnell et al., 1994) and cofractionate on sucrose gradients (Waegemann and Soll, 1991) with an early import intermediate. However, it was not clear from these experiments whether these two import components interact with precursors independently or as components of an import complex. To distinguish between these two

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Figure 6. Competition of pS-1-[\(^{125}\)I]ADP cross-linking to envelope polypeptides with authentic pS. Isolated chloroplasts equivalent to 2 mg of chlorophyll were incubated with increasing concentrations of urea-denatured, unlabeled pS (pS) or mature S (S) as indicated in the presence of 75 \(\mu\text{M}\) ATP (+ATP) or 20 U of apyrase (-ATP) for 5 min at 26°C. Urea-denatured pS-1-[\(^{125}\)I]ADP (pS-1-ADP) was added to 50 nM and the incubation was continued for 10 min at 26°C in the dark. The chloroplasts were resolubilized through Percoll silica gel, irradiated with UV light, lysed, and subfractionated by flotation into sucrose gradients (see Fig. 5). Fractions corresponding to envelope membranes were pooled and analyzed by SDS-PAGE. Each lane contains 5 \(\mu\text{g}\) of protein. (A) Fluorograph of envelope polypeptides from competition of cross-linking in the absence of ATP. (B) Fluorograph of envelope polypeptides from competition of cross-linking in the presence of ATP. The positions of IAP86, IAP75, IAP21, and pS-1-[\(^{125}\)I]ADP are indicated between the two figures. Image capture and quantitation were performed on a Phosphorimager (Molecular Dynamics, Inc.).
possibilities, envelope membranes from pS-1-[\(^{125}\)I]ADP cross-linking experiments performed in the presence or absence of ATP were treated with reducing agent, dissolved under nondenaturing conditions in detergent containing buffers, and immunoprecipitated with anti-IAP86 specific serum. Fig. 7 A shows that labeled IAP75 is coprecipitated with IAP86 in both ATP treated (lane 4) and ATP depleted (lane 2) samples. The preimmune serum of anti-IAP86 does not precipitate either protein (Fig. 7 A, lane 5). Anti-IAP86 serum does not cross-react with IAP75 on immunoblots discounting the possibility that IAP75 is directly precipitated in these experiments (data not shown). The results presented in Fig. 7 A suggest that IAP75 and IAP86 interact with pS-1-[\(^{125}\)I]ADP in concert and not as independent polypeptides.

The ability of anti-IAP86 serum to coprecipitate IAP75 raises the possibility that these two components function as a stable complex in the outer membrane. To explore this possibility, we used anti-IAP86 IgG to immuno-affinity purify IAP86 and any associated proteins from detergent solubilized envelope membranes. The envelope membranes were derived from chloroplasts that had not been incubated with a precursor protein. Fig. 7 demonstrates that anti–IAP86 IgG is capable of coimmunoprecipitating IAP75 and IAP34 from solubilized outer membranes (Fig. 7, B and C, lane 2) or mixed outer and inner membranes (Fig. 7, B and C, lane 4) even in the absence of bound precursor. IgG from preimmune serum does not precipitate these envelope polypeptides (Fig. 7, B and C, lane 5). These data strongly argue that these three outer membrane import components act as members of a multisubunit import complex.

**Topology of Early Import Intermediates**

On the basis of the data in Fig. 5, it is apparent that ATP and to a lesser degree GTP induce a major reorganization of the interactions between the transit sequence of the precursor and components of the import machinery. The 21-kD polypeptide that is cross-linked to the pS-1-[\(^{125}\)I]ADP in the presence of NTP is detected only in mixed outer and inner membrane fractions, suggesting that it may be an inner membrane protein (Fig. 5 B). This observation implies that the early import intermediate has inserted across the outer membrane. To explore the membrane topology of the early import intermediate in more detail, we investigated the localization of cross-linked IAP21. After cross-linking to pS-1-[\(^{125}\)I]ADP in the presence of ATP, the chloroplasts were reisolated and treated with exogenous thermolysin. Thermolysin has been shown to digest surface-exposed components of the chloroplast, but it does not digest inner membrane proteins (Cline et al., 1984).
Fig. 8A shows that the 21-kD, cross-linked product is not susceptible to exogenous thermolysin (compare lanes 1 and 2). In contrast, the bound precursor and labeled IAP86 are degraded (Fig. 8A, compare lanes 1 and 2). Consistent with previous observations (Schnell et al., 1994), IAP75 is not degraded by exogenous thermolysin (Fig. 8A, compare lanes 1 and 2). Thermolysin treatment reveals an additional protease-insensitive, cross-linked product of 25 kD (Fig. 8A, lane 2) that was not detected previously due to its close migration to pS-1-[125I]ADP. We refer to this protein as IAP25. The nature of this product has not been investigated in detail.

The outer and inner membranes each contain polypeptide bands of 21 kD that are coincident with the labeled polypeptide on SDS-PAGE (Fig. 8B, lanes 1 and 3). Thermolysin treatment of intact chloroplasts completely degrades the outer membrane 21-kD polypeptide (Fig. 8B, compare lanes 3 and 4) but does not digest the inner membrane polypeptide (Fig. 8B, compare lanes 1 and 2) as assessed by SDS-PAGE and Coomassie blue staining. The results in Fig. 8 argue against the localization of the cross-linked 21-kD polypeptide to the outer membrane and suggest it is a component of the inner membrane. To test this directly, antibodies were raised to the inner membrane protein that had been resolved by SDS-PAGE. The inner membrane fractions used for antibody production were derived from chloroplasts that were previously treated with exogenous thermolysin to minimize the possibility of contamination of the 21-kD band with the outer membrane protein. Fig. 9A shows that the anti-21 kD serum reacts with the inner membrane protein (lane 1). No cross-reactivity is detected with polypeptides in the stroma or outer membrane (Fig. 9A, lanes 2 and 4). A small degree of reactivity is detected in thylakoid membranes (Fig. 9A, lane 3) and is most likely due to contamination of the thylakoids with envelope membranes. The anti-21-kD serum is able to immunoprecipitate the 21-kD polypeptide that was labeled by cross-linking to pS-1-[125I]ADP (Fig. 9B, lane 2). These results provide convincing evidence that the 21-kD cross-linking target is a protein that is associated with the inner membrane. Furthermore, they indicate that the transit sequence of pS-1-[125I]ADP has inserted across the outer membrane in the presence of ATP or GTP and is in intimate contact with the inner membrane polypeptide.

**Figure 8.** Protease sensitivity of pS-1-[125I]ADP cross-linked products. Chloroplasts equivalent to 2 mg of chlorophyll that had been cross-linked to pS-1-[125I]ADP in the presence of 75 μM ATP were treated with 100 μg/ml thermolysin on ice for 30 min. The chloroplasts were reisolated through Percoll silica gel, lysed, and subfractionated by flotation into sucrose gradients (see Fig. 5). Fractions corresponding to outer (OM) and mixed outer and inner (OM-IM) envelope membranes were pooled and analyzed by SDS-PAGE. (A) Fluorograph of SDS-PAGE resolved mixed outer and inner membrane polypeptides from chloroplasts incubated in the absence (− T-lysin) or presence (+ T-lysin) of thermolysin. Each lane contains 5 μg of protein. (B) Coomassie blue stain of SDS-PAGE resolved outer (OM) and mixed outer and inner (OM-IM) membrane polypeptides from chloroplasts incubated in the absence (− T-lysin) or presence (+ T-lysin) of thermolysin. Lanes 1 and 2 contain 15 μg of protein; lanes 3 and 4 contain 10 μg of protein. The molecular masses of standard proteins are indicated to the left of both panels.

**Figure 9.** Localization of the 21-kD pS-1-[125I]ADP cross-linked product with anti-21-kD antibodies. (A) The proteins of inner membrane (IM), outer membrane (OM), thylakoid membrane (Th), and stromal extract (St) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-21-kD serum (anti-21) or corresponding preimmune serum (PI). Each lane contains 5 μg of protein. (B) Mixed outer and inner envelope membranes (OM-IM) (10 μg protein) from pS-1-[125I]ADP cross-linking in the presence of 75 μM ATP (see Fig. 5) were reduced, dissolved in TEGS buffer containing 2% Triton X-100, and immunoprecipitated with anti-21-kD serum (anti-21) or preimmune serum (PI) (see Materials and Methods). The samples were analyzed by SDS-PAGE and fluorography. Lane 1 contains envelope membranes equivalent to 25% of the material used for immunoprecipitation. The positions of pS-1-[125I]ADP (pS-1) and IAP21 (IAP21) are indicated to the right of B. The molecular masses of standard proteins are indicated to the left of both panels.

**Interactions of Mature Sequence of a Precursor with the Import Machinery**

To further explore the energy-induced changes in precursor
binding, the interactions of sequences within the mature portion of the precursor that are of a significant distance from the transit sequence were mapped using pFd-protA-[125I]ADP. In contrast to the cross-linking observed with pS-1-[125I]ADP, the cross-linking of pFd-protA-[125I]ADP to IAP75 and IAP86 is at very low levels in outer membrane fractions regardless of the energy state of the chloroplasts (Fig. 10 A, lanes 1-5, and C). Only a slight stimulation of cross-linking to IAP86 is observed in the outer membrane even in the presence of ATP (Fig. 10 A, lane 2, and C). Cross-linking to IAP75 in outer membrane fractions is nearly indetectable under all energy conditions (Fig. 10 A, lanes 1-5, and C). However, ATP dramatically increases the levels of cross-linking of IAP86 to the mature sequences of pFd-protA-[125I]ADP observed in mixed outer and inner membrane fractions (Fig. 10 B, lane 2, and D). GTP also stimulates labeling of IAP86, but to a much lesser degree than ATP (Fig. 10 B, compare lanes 2 and 3, and D). The levels and pattern of cross-linking in the presence of GMP-PNP (Fig. 10 A, lane 4, and B) or AMP-PNP (Fig. 10 A, lane 5, and B) are indistinguishable from those observed in the presence of apyrase. Taken together, these results demonstrate that the energy-induced insertion of the precursor across the outer membrane brings sequences of the precursor that are distant from the transit sequence (>20 amino acids from the transit sequence cleavage site) into the close vicinity of IAP86.

Discussion

We have used a covalent cross-linking strategy to map the interactions of the outer envelope import machinery with chloroplast precursors during the early stages of protein import. Our results indicate that two components of an outer membrane import complex, IAP86 and IAP75, are in close physical proximity to the transit sequence of a chloroplast precursor during an initial energy-independent interaction of the precursor with the outer membrane. ATP and/or GTP hydrolysis result in formation of a stably bound early import intermediate that has inserted across the outer membrane at contact zones. The transit sequence of the early intermediate cross-links to an inner

![Figure 10. Energy dependence of pFd-protA-[125I]ADP cross-linking to chloroplast envelope polypeptides and distribution of cross-linked products in envelope membrane fractions.](image-url)

Isolated chloroplasts (2 mg chlorophyll) were incubated with 200 nM urea denatured pFd-protA-[125I]ADP in a standard binding assay containing apyrase (+ Apyrase), 75 μM ATP (+ ATP), 200 μM GTP (+ GTP), 100 μM GMP-PNP (+ GMP-PNP), or 100 μM AMP-PNP (+ AMP-PNP) for 10 min at 26°C in the dark. The chloroplasts were reisolated through Percoll silica gel, irradiated with UV light, lysed under hypertonic conditions, and subfractionated by flotation into linear 20-40% wt/vol sucrose gradients (see Fig. 5). Sucrose gradient fractions corresponding to outer envelope membrane (Outer Membrane) and mixed outer and inner envelope membrane (Outer-Inner Membrane) were analyzed by SDS-PAGE. Each lane contains 5 μg of protein. (A and B) Representative fluorographs of SDS-PAGE resolved outer (A) and mixed outer and inner (B) membrane fractions. The positions of IAP86 (IAP86), IAP75 (IAP75), and pFd-protA-[125I]ADP (pFd-protA) are indicated to the right of B. The molecular masses of standard proteins are indicated to the left of both figures. (C and D) Quantitation of the radioactivity incorporated into IAP75 and IAP86 in outer (C) and mixed outer-inner envelope membranes (D). The quantitative data in C and D are the average of triplicate experiments. Image capture and quantitation were performed on a Phosphorimager (Molecular Dynamics, Inc.).
membrane protein, indicating that it is in close proximity to the protein conducting machinery of this membrane.

The results presented here support and extend a previously proposed model for the function of the outer envelope import components (Schnell, 1995). In the updated model, recognition of the precursor and translocation across the outer envelope membrane are mediated by a multisubunit complex of proteins that includes IAP86, IAP75, and IAP34. The initial recognition of cytoplasmic chloroplast precursor proteins would take place across the surface of the outer membrane. Binding of the transit sequence at the import complex would occur at a receptor site that is composed of IAP86 and IAP75. The initial binding is energy independent and readily reversible. Productive binding of the transit sequence to the receptor site would trigger insertion of the precursor into the protein-conducting machinery of the outer membrane in a set of reactions that require ATP and GTP hydrolysis. IAP75 would constitute part or all of the protein-conducting channel. Insertion of the precursor across the outer membrane would be concomitant with the engagement of envelope contact sites and the interaction of the transit sequence with the inner envelope import machinery. The association of the transit sequence with the inner membrane would be mediated at least in part by IAP21. At this point, the precursor would be poised for insertion in the protein-conducting machinery of the inner membrane at contact sites. This stably bound precursor represents the early import intermediate that has been previously described (Schnell and Blobel, 1993; Perry and Keegstra, 1994).

There is now compelling evidence to support the major elements of this model. First, Perry and Keegstra (1994) demonstrated that IAP86 could be cross-linked to pS in a binding assay even in the absence of energy, suggesting that it may be involved in a primary precursor recognition event. In further support of this assignment, the $F_{ab}$ of anti-IAP86 antibodies have been shown to inhibit formation of the stable early import intermediate (Hirsch et al., 1994). Our results demonstrate that IAP86 is a major target of cross-linking to the transit sequence of pS during the early stages of import (Fig. 5). These results support the role for IAP86 in precursor recognition by providing evidence that IAP86 interacts with the transit sequence of the precursor. However, we detect significant cross-linking of the transit sequence to IAP75 (Fig. 5), and therefore, it is equally possible that IAP86 and IAP75 together form a receptor site on the outer membrane.

Second, the predicted porin-like structure of IAP75 and its deeply embedded membrane topology suggest that it is a component of the protein-conducting machinery of the outer membrane (Schnell et al., 1994; Tranel et al., 1995). Our results demonstrate that IAP75 is the major cross-linked product in reactions in which the transit sequence is inserted across the outer membrane (Fig. 5). These results indicate that this component remains in close physical proximity to the transit sequence during precursor translocation across the membrane and supports its role as a channel component of the import apparatus. Further support for this function is provided by the observation that anti-IAP75 $F_{ab}$ inhibits pS import (Tranel et al., 1995).

Third, formation of the stably bound early import intermediate appears to consist of a two-stage process involving transit sequence recognition and insertion of the precursor across the outer membrane. The first stage is represented by the cross-linking of the transit sequence to IAP86 and IAP75 in the absence of NTP (Fig. 5). This observation demonstrates that the initial interaction of the transit sequence with the receptor site does not require energy. Low levels of binding of precursors to chloroplasts in the absence of NTP have been observed previously using precursor binding assays (Fig. 2) (Cline et al., 1985; Olsen et al., 1989; Olsen and Keegstra, 1992), but the nature of this binding was not clear. The cross-linking results presented here suggest that the low level of energy-independent binding corresponds to a productive step in precursor recognition. In the second stage of envelope binding, NTP hydrolysis promotes insertion of the precursor across the outer membrane and the engagement of contact sites resulting in a stable association of the precursor with the envelope. Our results clearly show that hydrolysis and not simply binding of these NTPs is required for generation of this early import intermediate (Figs. 5 and 10).

A fourth outer membrane component, the hsp70-IAP, has been found in import complexes containing early import intermediates (Waegemann and Soll, 1991; Schnell et al., 1994). We previously proposed that binding of the precursor to the hsp70-IAP in the intermembrane space following insertion of the precursor across the membrane stabilizes the early intermediate and accounts for the ATP requirement of stable binding (Schnell et al., 1994). Surprisingly, we did not detect cross-linking of the hsp70-IAP to precursor when envelope membrane proteins were analyzed by two-dimensional gel electrophoresis, although the hsp70-IAP was detectable by immunoblotting (data not shown). The lack of detectable cross-linking to the hsp70-IAP may be due to the insufficient sensitivity of the assay. Alternatively, the hsp70-IAP may not significantly interact with the transit sequence of the precursor and therefore is not labeled with the pS-1-[$^{125}$I]ADP at detectable levels. A direct association of the hsp70-IAP with bound precursor has yet to be established, and its role in anchoring the early intermediates will require further investigation.

Our results with cross-linking of pFd-protA-[$^{125}$I]ADP are consistent with a significant reorientation in the topology of bound precursor upon NTP hydrolysis. NTP-induced insertion of the precursor across the outer membrane brings sequences of pFd-protA-[$^{125}$I]ADP that are at least 20 amino acids from the transit sequence cleavage site within close proximity to IAP86 (Fig. 10). These results agree with a model in which the amino-terminal region of the precursor is inserted across the outer membrane upon NTP hydrolysis.

Fourth, the communoprecipitation of IAP75 and IAP34 with IAP86 using anti-IAP86 serum (Fig. 7) indicates that these three polypeptides constitute all or part of a protein import complex in the outer membrane. It has been demonstrated previously that these three components are present in stable association with early import intermediates (Waegemann and Soll, 1991; Schnell et al., 1994), but the nature of these associations was unclear. Perry and Keegstra (1994) suggested that IAP86 and IAP75 bind sequentially to precursors. In contrast, our data suggest that these two components act as parts of a multisubunit complex. The dynamics of this complex have yet to be investi-
gated, but the ability to coprecipitate all three of the proteins from outer or mixed outer and inner membranes in the absence of import intermediates (Fig. 7 B) suggests that their association is stable and not dependent on the presence of bound precursor. Covalent cross-linking between IAP75 and IAP34 has recently been reported by treating chloroplasts with chemical oxidants, providing further evidence of a physical association of at least two of the outer membrane components (Seedorf and Soil 1995). Although IAP86 and IAP75 appear to interact with the precursor during import, we did not detect cross-linking of IAP34 to bound precursors. This observation suggests that IAP34 plays a regulatory role in the outer membrane, perhaps by modulating the activities of other membrane or soluble components of the import apparatus.

Finally, the NTP-induced changes in precursor binding that result in the insertion of the transit sequence across the outer membrane are accompanied by the engagement of envelope contact zones. Our results demonstrate that cross-linking of the transit sequence to an inner membrane component, IAP21, is energy dependent and is detected only in membrane fractions containing contact zones (Fig. 5 B). Contact zones previously have been shown to function in the simultaneous translocation of precursors across the two envelope membranes (Schnell and Blobel, 1993). The nature of contact zones is a mystery, but it will be interesting to determine whether these zones form as a consequence of import or whether they are permanent structures that facilitate the cooperation between the import machineries of the outer and inner membranes.

The IAP21 and IAP25 polypeptides represent new candidates for components of the import apparatus. The localization of IAP25 has not been determined. The observation that IAP21 cross-links to the pS transit sequence (Fig. 5 B) suggests that it may function as a transit sequence receptor in the inner membrane. Three other inner membrane components, Com44 (Wu et al., 1994), IAP36, and IAP100 (Schnell et al., 1994), have been identified as candidates for components of the inner membrane import machinery. The relationship of these proteins to IAP21 remains to be determined, but their relationships should aid in defining the mechanism of translocation across the inner chloroplast membrane.

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