

# The Chloroplast Import Receptor Is an Integral Membrane Protein of Chloroplast Envelope Contact Sites

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**Abstract.** A chloroplast import receptor from pea, previously identified by antiidiotypic antibodies was purified and its primary structure deduced from its cDNA sequence. The protein is a 36-kD integral membrane protein (p36) with eight potential transmembrane segments. Fab prepared from monospecific anti-p36 IgG inhibits the import of the ribulose-1,5-bisphosphate carboxylase small subunit precursor (pS) by interfering with pS binding at the chloroplast surface. Anti-p36 IgGs are able to immunoprecipitate a Triton X-100 soluble p36-pS complex, suggesting a direct interaction between p36 and pS. This immunoprecipitation was specific as it was abolished by a pS synthetic transit peptide, consistent with the transit sequence receptor function of p36. Immunoelectron microscopy localized p36 to regions of the outer chloroplast membrane that are in close contact with the inner chloroplast membrane. Comparison of the deduced sequence of pea p36 to that of other known

proteins indicates a striking homology to a protein from spinach chloroplasts that was previously suggested to be the triose phosphate-3-phosphoglycerate-phosphate translocator (phosphate translocator) (Flügge, U. I., K. Fischer, A. Gross, W. Sebald, F. Lottspeich, and C. Eckerskorn. 1989. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:39-46). However, incubation of Triton X-100 solubilized chloroplast envelope material with hydroxylapatite indicated that p36 was quantitatively adsorbed, whereas previous reports have shown that phosphate translocator activity does not bind to hydroxylapatite (Flügge, U. I., and H. W. Heldt. 1981. *Biochim. Biophys. Acta.* 638:296-304). These data, in addition to the topology and import inhibition data presented in this report support the assignment of p36 as a receptor for chloroplast protein import, and argue against the assignment of the spinach homologue of this protein as the chloroplast phosphate translocator.

CHLOROPLAST biogenesis requires a system by which hundreds of different precursor polypeptides are imported into the organelle from their site of synthesis on cytoplasmic ribosomes. The translocation of chloroplast proteins takes place posttranslationally and is an energy-dependent process (Grossman et al., 1980; Schmidt and Mishkind, 1986; Keegstra, 1989), requiring the hydrolysis of ATP within the organelle (Pain and Blobel, 1987; Theg et al., 1989). A critical step in the targeting of all chloroplastic precursors is the recognition of these proteins at the chloroplast surface. This recognition event is mediated by the NH<sub>2</sub>-terminal transit sequence of the precursor protein (Dobberstein et al., 1977). Upon translocation of the protein, the transit sequence is removed by a specific stromal protease (Robinson and Ellis, 1984).

Although it is well established that the transit sequence serves as the topogenic signal for chloroplast import, little information is available on the components involved in transit sequence recognition or the mechanism of uptake. Mild proteolytic treatment of intact chloroplasts eliminates their ability to import (Chua and Schmidt, 1979; Cline et al., 1985), suggesting that the envelope contains proteinaceous

receptors necessary for the recognition and binding of precursor polypeptides.

Recently, we described the generation of antiidiotypic antibodies from an antiserum raised against a synthetic peptide corresponding to a region of the transit sequence of the ribulose-1,5-bisphosphate carboxylase small subunit precursor (pS) (Pain et al., 1988). The antiidiotypic antibodies, which presumably contain antibodies that mimic the transit sequence, were able to inhibit the import of pS into intact chloroplasts. Immunoprecipitation, immunoblotting, and chloroplast subfractionation experiments suggested that the chloroplast import receptor was an integral membrane protein of the chloroplast envelope. The immunoreactive protein migrated in SDS-PAGE as a polypeptide with an apparent molecular mass of 30 kD. Immunoelectron microscopy localized this protein to zones of contact between the inner and outer membrane that have previously been proposed to contain the sites of protein translocation into the organelle (Dobberstein et al., 1977). Here we report the purification and the complete cDNA deduced amino acid sequence for this protein. As its molecular mass has been calculated to be 36 kD, it will be referred to as p36. We present evidence to

support a direct role for p36 in the transit peptide-mediated recognition and binding of chloroplast precursors at the chloroplast surface.

## Materials and Methods

### Materials

Pea seeds (progress No. 9) were from W. Atlee Burpee Co. (Warminster, PA). [<sup>35</sup>S]methionine (1,000 Ci/mmol) and [<sup>125</sup>I]protein A (low specific activity) were from Dupont New England Nuclear (Wilmington, DE). Fluorescein-conjugated anti-rabbit IgG was from Cappel Laboratories (Malvern, PA). Glutaraldehyde, paraformaldehyde, and poly(lysine hydrobromide) for immunofluorescence and immunoelectron microscopy were from Polysciences, Inc. (Warrington, PA). Immunogold products for EM were from Janssen Life Sciences Products (Olen, Belgium). Protein A-Sepharose and Percoll were from Pharmacia Fine Chemicals (Piscataway, NJ). Chloroform and methanol were from EM Science (Cherry Hill, NJ). SDS and HTP hydroxylapatite were from Bio-Rad Laboratories (Richmond, CA). Restriction enzymes and Triton X-100 were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nigericin was from Calbiochem Corp. (La Jolla, CA). Cloning and sequencing vectors were from Stratagene Cloning Systems (La Jolla, CA). Peptide SC30 corresponding to the COOH-terminal 30 amino acids of the pS transit sequence was synthesized as previously described (Pain et al., 1988).

### Isolation of Chloroplasts and Purification of p36

Intact chloroplasts were isolated from 2-wk-old pea seedlings (*Pisum sativum*, progress No. 9) or from spinach leaves by homogenization and Percoll gradient centrifugation as previously described (Pain and Blobel, 1987). If chloroplasts were to be used in import assays, they were resuspended in 50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol to a concentration of 2 mg chlorophyll per ml and treated as for the import assay described below. For preparation of chloroplast envelope membranes, chloroplasts were treated essentially as previously described (Pain et al., 1988). Briefly, chloroplasts were resuspended in ice-cold lysis buffer (20 mM Hepes-KOH, pH 7.7, 0.25 M KCl, 5 mM EDTA, 1 mM para-aminobenzamide, 5 mM epsilon-aminocaproic acid, 50 U/ml trypsin, 0.5 mM PMSF, 2 mM DTT) to a concentration of 1.5 mg chlorophyll per ml by 20 strokes in a Dounce homogenizer. Membranes were pelleted by centrifugation at 27,000 g for 30 min. The membranes were resuspended by homogenization in lysis buffer containing 42% wt/vol sucrose to a concentration of 1–1.2 mg chlorophyll per ml. Resuspended membranes (10 ml) were transferred to an SW28 centrifuge tube and overlaid with a 28-ml linear gradient of 20–38% wt/vol sucrose in lysis buffer. The tubes were spun at 96,000 g for 17 h. The fractions corresponding to 25–32% wt/vol sucrose containing chloroplast envelope membranes were collected from the gradients, diluted with 2 vol of 20 mM Hepes-KOH, pH 7.7, 4 mM MgCl<sub>2</sub>, and spun at 200,000 g for 90 min. The pelleted chloroplast envelope membranes were resuspended in 20 mM Hepes-KOH, pH 7.7, 0.25 M sucrose, 50 mM KCl, 4 mM MgCl<sub>2</sub> to a concentration of 400 µg protein per ml and stored at –80°C.

For purification of p36, chloroplast envelope membranes prepared as described above were spun at 356,000 g for 30 min and resuspended to 7 mg protein per ml in the same buffer. The resuspended envelopes were extracted with 20 vol of ice-cold chloroform/methanol (2:1 vol/vol) by vortex mixing, followed by incubation on ice for 15 min. The mixture was spun at 15,000 g for 20 min, and the organic phase was collected, carefully avoiding the interface and pelleted material. The chloroform/methanol supernatant was dried by evaporation under reduced pressure, and the pellet was dissolved in 5% wt/vol SDS, 10 mM NaPO<sub>4</sub>, pH 6.8, 50 mM DTT to 1/10 vol of the original extract. This solution was diluted to 0.1% wt/vol SDS, 10 mM NaPO<sub>4</sub>, pH 6.8, 1 mM DTT and loaded onto a 0.9 × 12 cm HTP hydroxylapatite column equilibrated in the same buffer. The amount of protein loaded never exceeded 1 mg total. The column was washed with three column volumes of the loading buffer and was eluted stepwise with 20 ml each of 350 mM NaPO<sub>4</sub>, pH 6.8, and 700 mM NaPO<sub>4</sub>, pH 6.8, in 0.1% SDS, 1 mM DTT. The flow rate was 17 ml/h. Fractions of 1 ml were collected and analyzed by SDS-PAGE in 12% polyacrylamide and p36 was identified by immunoblotting with antiidiotypic antibodies as previously described (Pain et al., 1988).

### CNBr Cleavage and Amino Acid Sequencing of p36

SDS/hydroxylapatite fractions containing p36 were concentrated by precipitation with 10% wt/vol TCA and resolved on SDS-PAGE in 12% wt/vol polyacrylamide. After electrophoresis, the resolved polypeptides were stained with Coomassie blue or transferred to polyvinylidene difluoride membrane (immobilon PVDF; Millipore Continental Water Systems, Bedford, MA) overnight at 4°C in 15 mM NaPO<sub>4</sub>, pH 6.8, 20% vol/vol methanol, 0.05% SDS at 15 mA. The PVDF membrane was subsequently stained with 0.1% wt/vol Coomassie blue in 50% vol/vol methanol and destained in 40% vol/vol methanol, 10% vol/vol acetic acid. After extensive washing with H<sub>2</sub>O, the membrane was dried. The region containing p36 was excised and subjected to sequencing on a gas-phase sequenator (Applied Biosystems, Inc., Foster City, CA).

Cyanogen bromide fragments of p36 were generated by a modification of a previously described method (Nikodem and Fresco, 1979). SDS/hydroxylapatite fractions containing p36 were resolved by SDS-PAGE. The gel was stained for 10 min at room temperature in 0.1% wt/vol Coomassie blue in 45% vol/vol methanol, 10% acetic acid and destained for 15 min at room temperature in 50% vol/vol methanol. The region containing p36 was excised from the gel and placed in a 1.5-ml microcentrifuge tube. Cyanogen bromide was dissolved to 2.5 g/ml in acetonitrile and 40 µl was added to each gel slice. The volume of an average gel slice was ~100 µl. After addition of the cyanogen bromide solution, 400 µl of 0.125 M Tris-HCl, pH 6.8, and 400 µl of 0.6 N HCl were added. The tube was sealed and incubated overnight at room temperature in the dark with gentle shaking. After incubation, the gel slice was washed for 1 h with two 20-ml changes of 0.125 M Tris-HCl, pH 6.8, 1% wt/vol SDS. The gel slice was inserted into the well of a 15% wt/vol polyacrylamide gel and electrophoresed. The resolved cleavage products were transferred to PVDF membrane and subjected to sequencing as given above. Each gel slice used for cyanogen bromide digestion contained 3–5 µg of protein.

### Construction and Screening of cDNA Libraries

Total RNA was isolated by coprecipitation with cetyltrimethylammonium bromide (Taylor and Powell, 1982) from the stems and leaves of 2-wk-old pea seedlings. Poly A RNA was isolated by chromatography on oligo-dT cellulose (Aviv and Leder, 1972). Total poly A+ RNA was used to construct double-stranded cDNA libraries (Gubler and Hoffman, 1983). Two separate libraries were constructed, one utilizing oligo-dT as the primer for cDNA synthesis and the second utilizing random hexanucleotides as primers. The cDNA libraries were ligated into the lambda gt11 vector using Eco RI linkers. Both libraries were constructed from 10 µg of poly A RNA and yielded ~3 × 10<sup>7</sup> recombinant plaques.

A 61-bp probe specific for p36 was synthesized by the polymerase chain reaction (PCR)<sup>1</sup> (Mullis and Fagona, 1987) using oligo-dT primed pea cDNA as the template. The degenerate primers for the PCR reaction represented the sense strand corresponding to amino acids 6–11 and the antisense strand corresponding to amino acids 16–20 of the amino-terminal amino acid sequence determined for p36 (Fig. 4B). In addition, eight nucleotide 5' extensions carrying Sal I and Eco RI restriction sites were added to the sense and antisense primers, respectively. The PCR was carried out using 5 µM primer and 50 ng of pea cDNA in a 100-µl reaction volume for 25 cycles of denaturation (95°C for 1.5 min), annealing (42°C for 2.5 min) and polymerization (72°C for 3.0 min) as described previously (Lee et al., 1988). A 61-bp PCR product was isolated by agarose gel electrophoresis and subcloned into pBluescript II. The PCR product was designated oligo-61 and its authenticity was confirmed by dideoxy sequencing (Sanger et al., 1977).

The unamplified lambda gt11/pea cDNA library was replica plated and the filters were hybridized to oligo-61 using standard methods (Wood, 1987). Oligo-61 was excised from the vector, isolated by agarose gel electrophoresis, and labeled by [<sup>32</sup>P]ATP and T4 polynucleotide kinase. The [<sup>32</sup>P]oligo-61 was added to the filters at a concentration of 1 × 10<sup>6</sup> cpm/ml and the mixture was incubated overnight at 42°C. After hybridization, the filters were exposed to Kodak XAR-5 film. Positive clones were isolated and analyzed by restriction enzyme digestion. Positively hybridizing insert cDNAs were subcloned into the pBluescript II vector as described by the supplier.

1. **Abbreviations used in this paper:** DIDS, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene; PCR, polymerase chain reaction; PLP, pyridoxal 5'-phosphate; PRS, postribosomal supernatant.

## Northern Blots

Poly A RNA from pea seedlings was electrophoresed in a 1% wt/vol agarose/2.2 M formaldehyde gel and transferred to nitrocellulose as described (Lehrach et al., 1977). The filter was hybridized to the p36 cDNA by standard methods (Maniatis et al., 1982). The cDNA clones were radio-labeled with [<sup>32</sup>P]dCTP using the random primer method (Feinberg and Vogelstein, 1983). The blots were washed and exposed as given above.

## cDNA Sequencing and Sequence Analysis

Double-stranded sequencing of p36 cDNAs was carried out by the dideoxy method (Sanger et al., 1977). The sequencing strategy for the full-length pPCR301 clone is shown in Fig. 5. DNA sequence analysis, protein secondary structure predictions, and protein sequence homology searches were performed using the DNASTAR software program (DNASTAR, Inc., Madison, WI). Hydropathy analysis was carried out using standard algorithms (Eisenberg et al., 1984; Kyte and Doolittle, 1982).

## Preparation of Anti-p36 Serum

The SDS/hydroxylapatite fractions containing p36 were pooled and the proteins resolved by SDS-PAGE. The region containing p36 (i.e., reacting with antiidiotypic antibodies) was excised from the gel and used to raise antibodies in rabbits. The IgG purification and Fab preparation were performed according to standard methods (Chua et al., 1982; Garvey et al., 1977).

## Cell-free Transcription and Translation

Plasmid pT7-pS carrying the entire coding region of pS was prepared as previously described (Pain and Blobel, 1987). In vitro transcription of pT7-pS was carried out as described (Pain and Blobel, 1987). Approximately 2 µg of transcript were obtained per microgram of DNA template.

The pS transcript was translated in a wheat germ cell-free translation system in the presence of [<sup>35</sup>S]methionine for 1 h at 25°C as previously described (Pain and Blobel, 1987). After incubation, the translation mixture was centrifuged for 22 min at 4°C in an airfuge at 30 psi (140,000 g) (Beckman Instruments, Inc., Palo Alto, CA). This postribosomal supernatant (PRS) containing the newly synthesized pS was used either directly in the chloroplast import assay or was stored at -80°C for future use.

## Chloroplast Import Assay

The import of pS into chloroplasts was assayed essentially by a standard method (Pain and Blobel, 1987) using isolated intact chloroplasts equivalent to 25 µg of chlorophyll per assay. In assays containing Fab, the chloroplasts were diluted to a final volume of 135 µl in 50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol (HS buffer) or the same buffer containing the appropriate amount of Fab. Diluted chloroplasts containing Fab were incubated on ice for 45 min before initiating the import assay. The levels of import were quantitated by scanning densitometry of the fluorographs or by direct quantitation of radioactivity using radioanalytic imaging system (AMBIS Systems, Inc., San Diego, CA).

## Chloroplast Binding Assay

The precursor binding assays were carried out by a modification of the procedures described previously (Cline, et al., 1985). Isolated chloroplasts equivalent to 25 µg of chlorophyll were incubated in the dark for 15 min at room temperature to deplete them of endogenous ATP. The chloroplasts were chilled on ice and mixed with the appropriate immune or preimmune Fab to a final volume of 135 µl in HS buffer containing 400 nM nigericin. The samples were incubated on ice for 45 min. Each sample was mixed with a 10× concentrated binding buffer to yield a binding assay mixture with a final volume of 150 µl containing 50 mM Hepes-KOH, pH 7.7, 2 mM Mg(OAc)<sub>2</sub>, 40 mM KOAc, 0.025 mM DTT, 0.33 M sorbitol, 100 µg/ml BSA, 10 µM ATP, and 1 µl of PRS containing pS. The binding assay mixture was incubated at room temperature in the dark for 30 min. After incubation, the samples were chilled on ice and layered on a 1-ml cushion of 50 mM Hepes-KOH, 0.45 M sorbitol, 5 mM EDTA, 2 mM DTT and centrifuged for 30 s at 3,500 g at 4°C. To assay for the import of the bound pS, the chloroplast pellet was resuspended directly in 150 µl of 1× binding buffer containing 5 mM ATP but no PRS. The reaction was incubated for 30 min at room temperature. After import, the chloroplasts were reisolated. Precursor binding to or import into chloroplasts was analyzed by SDS-PAGE and quantitated as given above for the import assays.

## Immunoprecipitation of p36-bound pS

Chloroplast envelope membranes (see above) equivalent to ~50 µg of protein were solubilized on ice for 20 min in 150 µl of buffer containing 20 mM Hepes-KOH, pH 7.7, 0.25 M sucrose, 50 mM KCl, 100 mM NaCl, 4 mM MgCl<sub>2</sub>, 8 mM EDTA, 2 mM DTT, 1 mM PMSF, and 1% Triton X-100. The mixture was centrifuged at 15,000 g in a microcentrifuge for 20 min at 4°C and the supernatant was recovered for further use. The solubilized membranes equivalent to 1.5 µg of protein were incubated at 25°C for 30 min in a pS binding buffer that contained 20 mM Hepes-KOH, pH 7.7, 15 mM sucrose, 35 mM KCl, 15 mM NaCl, 4 mM MgCl<sub>2</sub>, 0.2 mM DTT, 0.5 mM EDTA, 0.1% Triton X-100, 10 µM ATP, and 0.5 µl of PRS containing pS, and where indicated, the appropriate amount of synthetic peptide to a final volume of 50 µl. Thereafter, an appropriate amount of anti-p36 IgG or preimmune IgG in binding buffer minus PRS was added to each reaction to give a final volume of 110 µl. This mixture was incubated at 4°C for 1 h. The reactions were centrifuged at 4°C for 2 min at 15,000 g to remove aggregates, and the supernatants were removed and incubated for 1 h at 25°C with 10 mg of protein A-Sepharose in binding buffer minus PRS to yield a final volume of 500 µl. The protein A-Sepharose was pelleted for 30 s at 15,000 g, and the supernatants were discarded. The pellets were washed four times in binding buffer minus PRS and two times in the same buffer minus Triton X-100. The samples were analyzed by SDS-PAGE and fluorography. The radioactive signals were quantitated as given above for the import assay.

## Immunofluorescence and Immunoelectron Microscopy

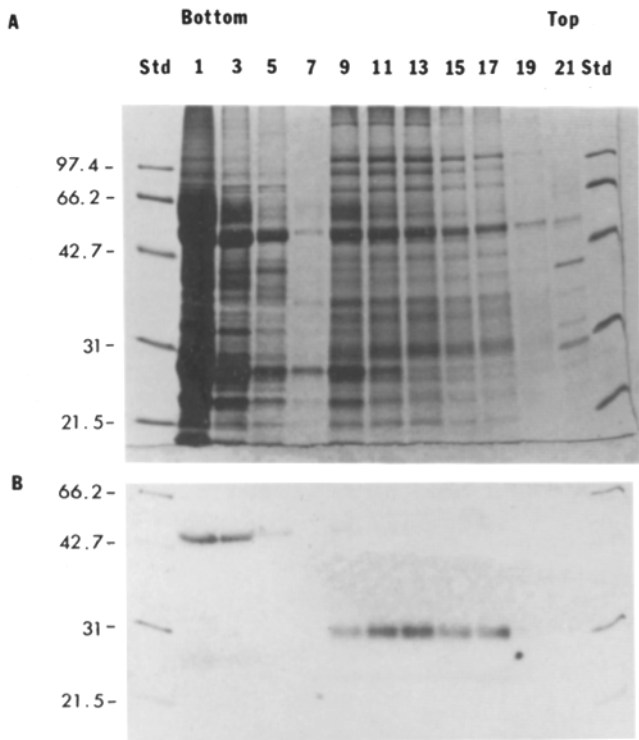
The immunofluorescence microscopy of immobilized intact chloroplasts was carried out as previously described (Pain et al., 1988) with minor modifications. Chloroplasts were prefixed in ice-cold 0.05% glutaraldehyde, 0.5% paraformaldehyde rather than the lower concentration of aldehyde fixative described earlier. Incubation with the decomplemented immune or nonimmune antiserum was carried out at a 1:500 dilution.

Competition of peptide SC30 with the antiidiotypic antibodies for binding to the chloroplast surface was carried out as follows. Isolated chloroplasts were depleted of endogenous ATP by incubating in the dark at room temperature for 15 min. Samples equivalent to 10 µg of chlorophyll were diluted to 500 µl in 50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol, 40 mM KOAc, 2 mM Mg(OAc)<sub>2</sub> (buffer A) containing 400 nM nigericin, 10 µM ATP, and 10 µM of the peptide and incubated 45 min at room temperature in the dark. The antiserum was then added at a final dilution of 1:250, and the incubation was continued at room temperature for an additional 45 min. Each sample was diluted to 10 ml with ice cold buffer A and centrifuged at 2,500 g for 5 min at 4°C. The supernatant was aspirated and the chloroplasts were gently resuspended in 200 µl buffer A containing 1:200 diluted fluorescein-conjugated goat anti-rabbit IgG and incubated at room temperature for 30 min. The chloroplasts were diluted and collected by centrifugation as given above. Each sample was fixed in buffer A containing 0.5% paraformaldehyde and 0.5% glutaraldehyde and incubated on ice for 30 min. After fixation, the samples were diluted and collected by centrifugation as above, washed with 10 mM NH<sub>2</sub>Cl and viewed on a Zeiss axiophot fluorescence microscope.

The immunoelectron microscopy of intact chloroplasts was performed essentially as described (Pain et al., 1988) with the following modifications. The immobilized chloroplasts were fixed for 30 min at 4°C in 0.5% paraformaldehyde and 0.05% glutaraldehyde before incubation with anti-p36 immune or preimmune serum. Incubation with the appropriate decomplemented serum was performed at a 1:500 dilution.

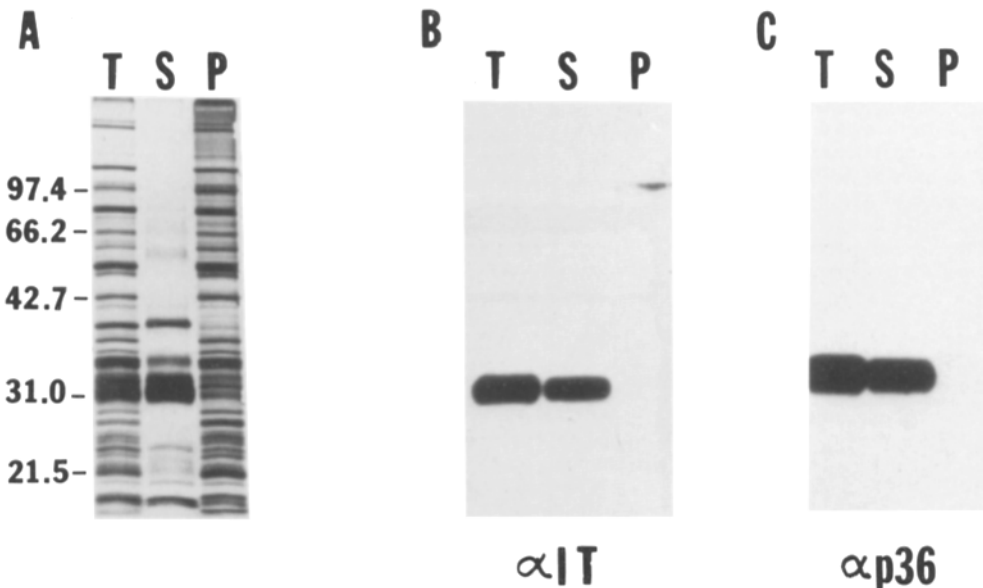
## Hydroxylapatite Adsorption of Solubilized Chloroplast Envelopes

The adsorption of chloroplast envelope polypeptides was carried out essentially as described (Flügge and Heldt, 1981). Chloroplast envelope membranes corresponding to 25–32% wt/vol sucrose gradient fractions (see above) were resuspended in 10 mM Hepes-KOH, pH 7.4, and 2% vol/vol Triton X-100 (HT buffer) to give a final protein concentration of 200 µg/ml in 1 ml vol. The sample was incubated on ice for 5 min and then centrifuged at 346,000 g for 5 min to remove any insoluble material. The supernatant (1 ml) was added to 2 ml (packed volume) HTP hydroxylapatite that had been equilibrated in HT buffer. The mixture was incubated at room temperature for 30 min followed by centrifugation at 2,000 g for 1 min. The supernatant was collected and the hydroxylapatite was washed four times with 2 ml of HT buffer. The hydroxylapatite-bound proteins were eluted by wash-



**Figure 1.** Isolation of chloroplast envelope membranes and identification of antiidiotypic antibody reactive polypeptides. Total chloroplast membranes (equivalent to 10 mg chlorophyll) were fractionated by flotation in a 20–38% wt/vol sucrose gradient (see Materials and Methods). Fractions of 1.5 ml were collected, analyzed by SDS-PAGE, and stained with Coomassie blue (*A*) or transferred to nitrocellulose and immunoblotted with the antiidiotypic antiserum (*B*). One-tenth the volume of fractions 1–7 (25  $\mu$ l) relative to fractions 9–21 (250  $\mu$ l) was loaded to avoid overloading of the gels. The molecular masses of standard proteins (*Std*) are indicated at the left of each panel in kilodaltons.

ing the hydroxylapatite with 3 ml of 0.8 M  $\text{NaPO}_4$ , pH 6.8, 0.1% SDS. The elution step was repeated three more times. 10% of each fraction was precipitated with 10% TCA and analyzed by SDS-PAGE and immunoblotting as described above.



**Figure 2.** Chloroform/methanol extraction of chloroplast envelope membranes. Total chloroplast envelope membranes (*T*) (equivalent to 10  $\mu$ g of protein) were extracted with 20 vol of ice-cold chloroform/methanol (2:1 vol/vol). The soluble (*S*) and pellet (*P*) fractions were separated by centrifugation, evaporated to dryness, and analyzed by SDS-PAGE. (*A*) Silver-stained gel. (*B*) Immunoblot with the antiidiotypic ( $\alpha IT$ ) serum. (*C*) Immunoblot with anti-p36 ( $\alpha p36$ ) serum. The molecular masses of standard proteins are indicated at the left of *A* in kilodaltons.

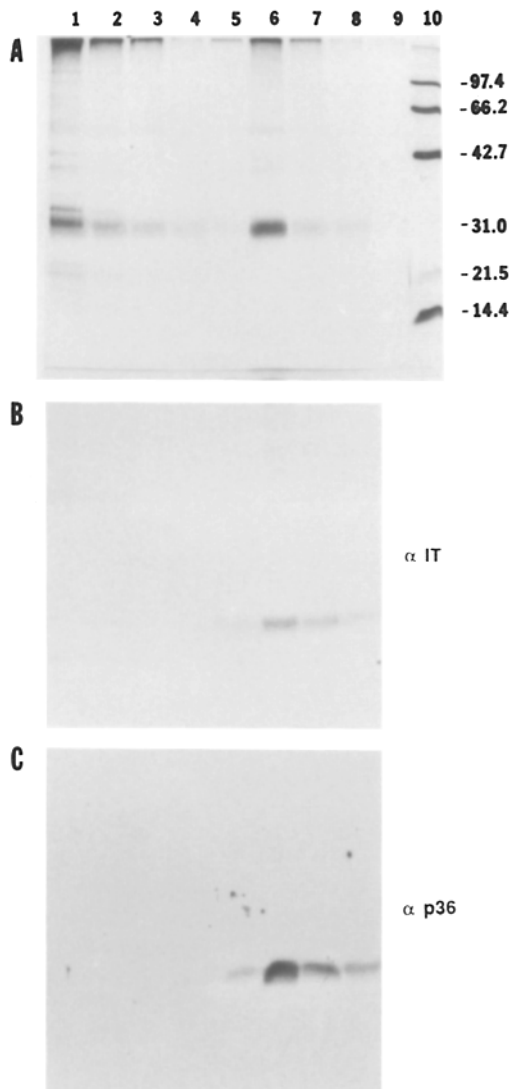
## Results

### Isolation of p36 and Preparation of Anti-p36 Serum

The chloroplast envelope membrane fraction which contained p36 was prepared by flotation of total chloroplast membranes in a linear gradient of 20–38% sucrose. Fig. 1 *A* shows the profile of SDS-PAGE resolved and Coomassie blue-stained membrane polypeptides of the sucrose gradient fractions. Three major subfractions of distinct polypeptide composition could be discerned. Fractions 1–7 which represent the 42% sucrose load zone consisted of thylakoid and, presumably, some inner membranes. The intermediate density fraction from 32–25% sucrose (fraction 9–19) contained a protein profile that represents the bulk of the chloroplast envelope (i.e., primarily inner chloroplast membrane and, presumably, regions of outer chloroplast membrane that are attached via contact sites). The least dense portion of the gradient (fraction 21) likely represents vesicles derived from outer membrane regions that are not associated with the inner membrane via contact sites (Keegstra and Yousif, 1986). The p36 that reacts with antiidiotypic antibodies cofractionated with the intermediate density membranes indicating that it is a component of the chloroplast envelope (Fig. 1 *B*). It should be noted that this fraction was previously shown to also contain chloroplast-bound pS (Pain et al., 1988). The other major antiidiotypic reactive polypeptide, the ribulose-1,5-bisphosphate carboxylase large subunit (Pain et al., 1988), remains largely, as expected, in the load zone (Fig. 1 *B*).

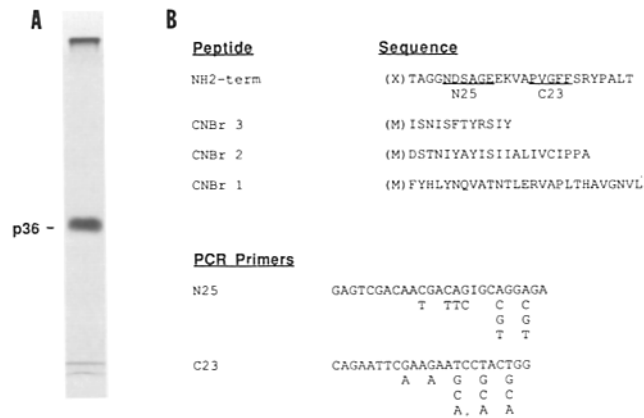
A three step fractionation procedure was utilized to separate p36 from other chloroplast envelope proteins. First, chloroplast envelopes were extracted with an ice-cold mixture of chloroform/methanol (2:1 vol/vol). This procedure resulted in the quantitative extraction of p36 into the organic phase (Fig. 2 *B*). The majority of envelope proteins was precipitated by the organic solvents and was recovered in the pellet fraction. The extraction procedure resulted in ~20-fold purification of p36, as judged by SDS-PAGE and silver staining (Fig. 2 *A*).

To separate p36 from the remaining contaminating polypeptides, the organic extract was further fractionated by



**Figure 3.** SDS/hydroxylapatite separation of chloroform/methanol-soluble envelope polypeptides. Chloroform/methanol-soluble envelope polypeptides were resuspended in 10 mM NaPO<sub>4</sub>, pH 6.8, 0.1% SDS, and 1mM DTT, chromatographed on hydroxylapatite, and eluted stepwise with 350 mM NaPO<sub>4</sub> (lanes 1–5) and 700 mM NaPO<sub>4</sub> (lanes 6–9). (A) Silver-stained gel. (B) Immunoblot of SDS/hydroxylapatite fractions probed with antiidiotypic serum ( $\alpha IT$ ). (C) Immunoblot with anti-p36 serum ( $\alpha p36$ ). A lane 10 contains protein standards with their molecular masses indicated at the right in kilodaltons.

**SDS-hydroxylapatite chromatography.** After evaporation of the organic solvents, the extract was resuspended in 0.1% SDS and 10 mM NaPO<sub>4</sub>, pH 6.8, and loaded onto an SDS/hydroxylapatite column. The proteins were eluted from the resin stepwise with 350 mM NaPO<sub>4</sub>, pH 6.8, 0.1% SDS followed by 700 mM NaPO<sub>4</sub>, pH 6.8, 0.1% SDS. Note that at both 350 and 700 mM NaPO<sub>4</sub>, a polypeptide migrating in the 30-kD region was among the major proteins eluted (Fig. 3 A, lanes 1–5 and 6–9, respectively). However, only the portion eluting at 700 mM NaPO<sub>4</sub>, but not that eluting at 350 mM NaPO<sub>4</sub>, reacted with the antiidiotypic antibodies (Fig. 3 B) or antibodies specifically raised against p36

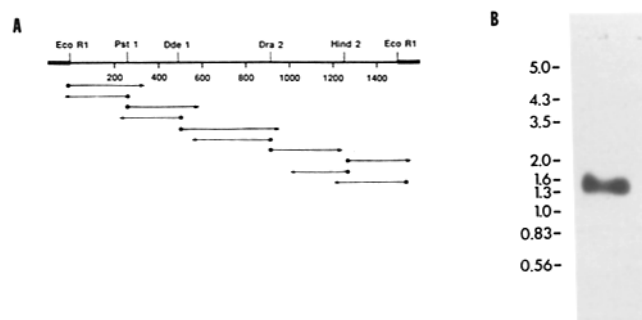


**Figure 4.** Amino acid sequence determined from purified p36, and nucleotide sequence of the primers used in the PCR. (A) Coomassie blue-stained gel of resolved polypeptides eluted with 700 mM NaPO<sub>4</sub> from hydroxylapatite (see Fig. 3, lanes 6–9). (B) Amino acid sequence obtained from the amino-terminus (NH<sub>2</sub>-term) and cyanogen bromide fragments (CNBr 1–3) of the p36, and the nucleotide sequences of the degenerate primers (N25 and C23) used to generate a 61-bp product that was in turn used to isolate the PCR301 cDNA from a pea leaf lambda gtl1 library. The underlined regions of the NH<sub>2</sub>-term peptide represent the regions from which the primers for PCR amplification were derived.

(Fig. 3 C). This observation indicates that there are at least two different proteins of this molecular mass in the chloroform/methanol extract of chloroplast envelopes, and that only one of them qualifies as p36 (i.e., reacts with antiidiotypic antibodies).

#### Cloning and Sequencing of p36 cDNA

Purified p36 (Fig. 4 A), as well as peptide fragments generated from cyanogen bromide cleavage of p36 were subjected to amino-terminal sequence analysis (Fig. 4 B). A 61 nucleotide product was generated by the PCR using two de-



**Figure 5.** Physical map of pPCR301 insert and Northern blot of pea poly A RNA with pPCR301. (A) Strategy for the dideoxy sequencing of pPCR301. The scale is in hundreds of nucleotide base pairs from the 5' end of the coding strand. Dots indicate the sites at which primers were annealed, and the arrows indicate the length of the sequence and the direction of sequencing. (B) Hybridization of PCR301 to pea poly A RNA. Poly A RNA (15  $\mu$ g) from light grown pea seedlings was separated on a 1% wt/vol agarose/2.2 M formaldehyde gel, transferred to nitrocellulose paper, and hybridized to [<sup>32</sup>P]pPCR301 cDNA. The molecular weights of single-stranded DNA markers are given in kilobases.

CGGCCGCTGTTTATCGTCTTTCAACATTTGATTCTC ATG GAG TCA CGA GTG CTG TCA CGC GCC ACC ACT CTC  
met-glu-ser-arg-val-leu-ser-arg-ala-thr-thr-leu-  
-70

TCC TCC CTC CCT ACC CTC AAC AAA CTG CAT CGG CTA CCC CTC GCC AAT GCT TCT CTC CCC TCC  
ser-ser-leu-pro-thr-leu-asn-lys-leu-his-arg-leu-pro-leu-ala-asn-ala-ser-leu-pro-ser-  
-60 -50 -40

GTG AAA TCG TTC GGA TCA GTC TCC GAT GGC GGG AAC CTC GTA TGG GGA AGA CAA CTT CGT CCG  
val-lys-ser-phe-gly-ser-val-ser-asp-gly-gly-asn-leu-val-trp-gly-arg-gln-leu-arg-pro-  
-30 -20

GAG CTC TGT TCT CCA GTG CTT AAG AAA GGT GCT TCA CTT CTC CGT CCT TGT CCC GCC ACT GCA  
glu-leu-cys-ser-pro-val-leu-lys-lys-gly-ala-ser-leu-leu-arg-pro-cys-pro-ala-thr-ala-  
-10 1

GGA GGA AAC GAT TCC GCT GGT GAA GAG AAG GTA GCT CCG GTT GGA TTC TTC AGC CGG TAT CCA  
gly-gly-asn-asp-ser-ala-gly-glu-glu-lys-val-ala-pro-val-gly-phe-phe-ser-arg-tyr-pro-  
10 20

GCT CTT ACT ACC GGT TTT TTC TTC ACT TGG TAC TTC TTG AAT GTG ATT TTC AAC ATC CTC  
ala-leu-thr-thr-gly-phe-phe-phe-phe-thr-trp-tyr-phe-leu-asn-val-ile-phe-asn-ile-leu-  
30 40

AAC AAG AAG ATC TAT AAC TAT TTC CCA TAT CCT TAC TTC GTG TCG GTA ATT CAT TTA GCT GTG  
asn-lys-lys-ile-tyr-asn-tyr-phe-pro-tyr-pro-tyr-phe-val-ser-val-ile-his-leu-ala-val-  
50 60

GGA GTG GTG TAT TGT TTG GTG AGC TGG ACC GTG GGC CTC CCT AAG CGC GCT CCT ATT GAC GGT  
gly-val-val-tyr-cys-leu-val-ser-trp-thr-val-gly-leu-pro-lys-arg-ala-pro-ile-asp-gly-  
70 80

AAC CTG CTG AAG TTG CTG ATT CCA GTA GCT GTG TGT CAT GCA TTA GGC CAT GTC ACT AGC AAT  
asn-leu-leu-lys-leu-leu-ile-pro-val-ala-val-cys-his-ala-leu-gly-his-val-thr-ser-asn-  
90 100

GTC TCA TTT GCA GCC GTT GCT GTT TCT TTC ACA CAT ACC GTT AAA GCT CTT GAG CCA TTC TTT  
val-ser-phe-ala-ala-val-ala-val-ser-phe-thr-his-thr-val-lys-ala-leu-glu-pro-phe-phe-  
110 120

AAT GCT GCT GCT TCA CAA TTT ATA CTT GGA CAA TCA ATT CCC ATC ACT CTA TGG CTC TCG TTG  
asn-ala-ala-ala-ser-gln-phe-ile-leu-gly-gln-ser-ile-pro-ile-thr-leu-trp-leu-ser-leu  
130 140 150

GCT CCT GTT GTT ATT GGT GTG TCA ATG GCA TCA TTG ACT GAA CTC TCA TTC AAC TGG CTT GGC  
ala-pro-val-val-ile-gly-val-ser-met-ala-ser-leu-thr-glu-leu-ser-phe-asn-trp-leu-gly-  
160 170

TTC ATA AGT GCT ATG ATT TCA AAC ATT TCC TTT ACA TAC AGG AGT ATC TAC TCA AAG AAA GCC  
phe-ile-ser-ala-met-ile-ser-asn-ile-ser-phe-thr-tyr-arg-ser-ile-tyr-ser-lys-lys-ala-  
180 190

ATG ACT GAT ATG GAT AGT ACC AAT ATC TAT GCC TAC ATT TCA ATC ATT GCT CTA ATT GTC TGC  
met-thr-asp-met-asp-ser-thr-asn-ile-tyr-ala-tyr-ile-ser-ile-ile-ala-leu-ile-val-cys-  
200 210

ATA CCG CCG GCC TTA ATT ATT GAA GGG CCT ACA CTG TTG AAG ACC GGT TTC AAT GAC GCA ATT  
ile-pro-pro-ala-leu-ile-ile-glu-gly-pro-thr-leu-leu-lys-thr-gly-phe-asn-asp-ala-ile-  
220 230

GCT AAA GTA GGT TTG GTT AAG TTT GTA TCA GAT CTC TTC TGG GTT GGT ATG TTT TAC CAT CTC  
ala-lys-val-gly-leu-val-lys-phe-val-ser-asp-leu-phe-trp-val-gly-met-phe-tyr-his-leu-  
240 250

TAC AAC CAG GTG GCA ACT AAC ACA TTG GAG AGA GTG GCT CCT CTT ACT CAT GCA GTT GGC AAC  
tyr-asn-gln-val-ala-thr-asn-thr-leu-glu-arg-val-ala-pro-leu-thr-his-ala-val-gly-asn-  
260 270

GTA CTC AAG CGT GTA TTC GTC ATT GGA TTT TCG ATC ATT ATC TTC GGC AAC AAG ATT TCC ACT  
val-leu-lys-arg-val-phe-val-ile-gly-phe-ser-ile-ile-ile-phe-gly-asn-lys-ile-ser-thr-  
280 290

CAA ACT GGC ATA GGA ACT GGC ATT GCA ATT GCT GGA GTA GCA CTC TAC TCT TTC ATC AAA GCC  
gln-thr-gly-ile-gly-thr-gly-ile-ala-ile-ala-gly-val-ala-leu-tyr-ser-phe-ile-lys-ala-  
300 310

CAG ATT GAA GAA GAG AAG CGA CAA GCA AAG GCA GCT TAA GGTGCAACTTGAGGATGCCAGGCAACTGTAA  
gln-ile-glu-glu-glu-lys-arg-gln-ala-lys-ala-ala-STOP  
320 330

ATCATCACAAATTTCTTTCTAAATAATTGACTTTGTTTCAGTATTGTAATCTTTGTGATAGCAGGCATTTTCATTTTCGTTCTTT  
ATTTTGTCAAATATTTCCCTTGTTCATTTCAGAAAACAAGCACTTGCTGATTTGCAGTCTCTTTTGACGACTGAAAAATGATGT  
ATCCATTATGGCATAAATAGCTCTTAAACCATTATCA

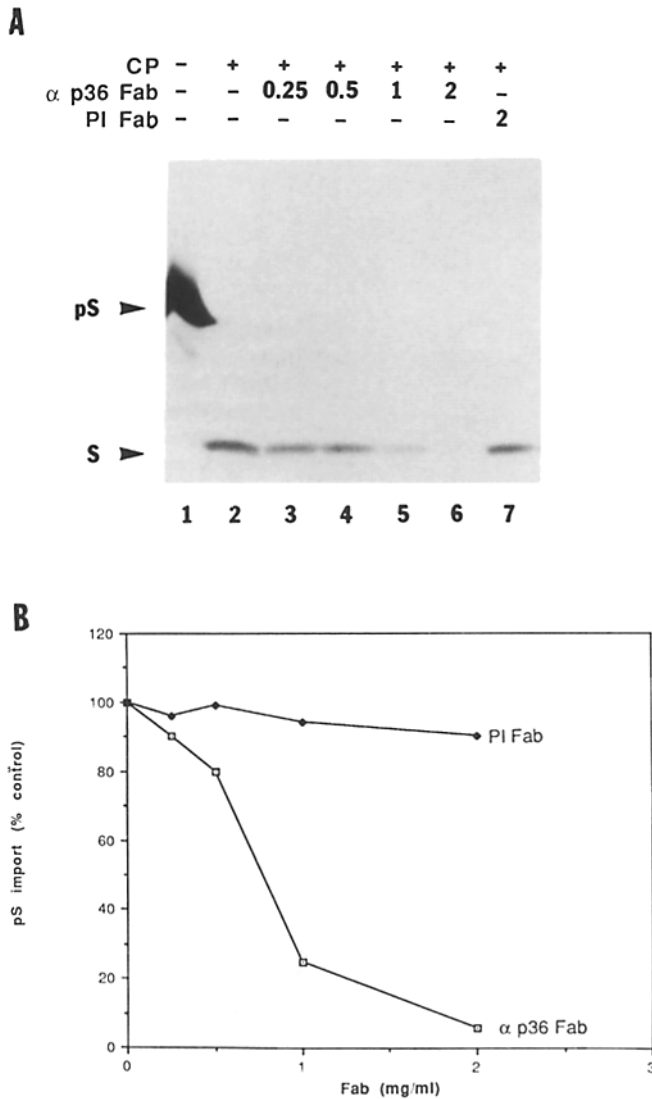
Figure 6. Nucleotide sequence of pPCR301 cDNA and the deduced amino acid sequence for p36. The arrow indicates the putative transit peptidase cleavage site. Residues corresponding to peptide sequence obtained from p36 are underlined. These sequence data are available from EMBL/GenBank/DBJ under accession number X54639.

generate oligonucleotides as primers (Fig. 4 B) and oligo-dT-primed pea cDNA as a template. The resulting PCR product corresponded to positions 6–20 of the amino-terminal amino acid sequence determined for p36 (Fig. 4 B).

Two cDNA libraries were constructed in the lambda gt11 vector from poly A RNA isolated from the stems and leaves of 2-wk-old pea seedlings. One library was prepared using oligodeoxythymidilic acid (oligo-dT) as the primer for cDNA synthesis, and the second was synthesized using random hexanucleotide primers to insure that all mRNA se-

quences would be represented. Both libraries were screened for p36 cDNA by hybridization to the p36-specific 61 nucleotide probe. Six independent positive clones from the oligo-dT primed library and three independent positive clones from the randomly primed library were obtained from  $6 \times 10^5$  plaques of each library screened. Each of these cDNA inserts were excised, subcloned into pBlue-script II, and their nucleotide sequences determined by the dideoxy chain terminating method. Two overlapping cDNA clones (pPCR3015 and pPCR301R2) were used to construct a





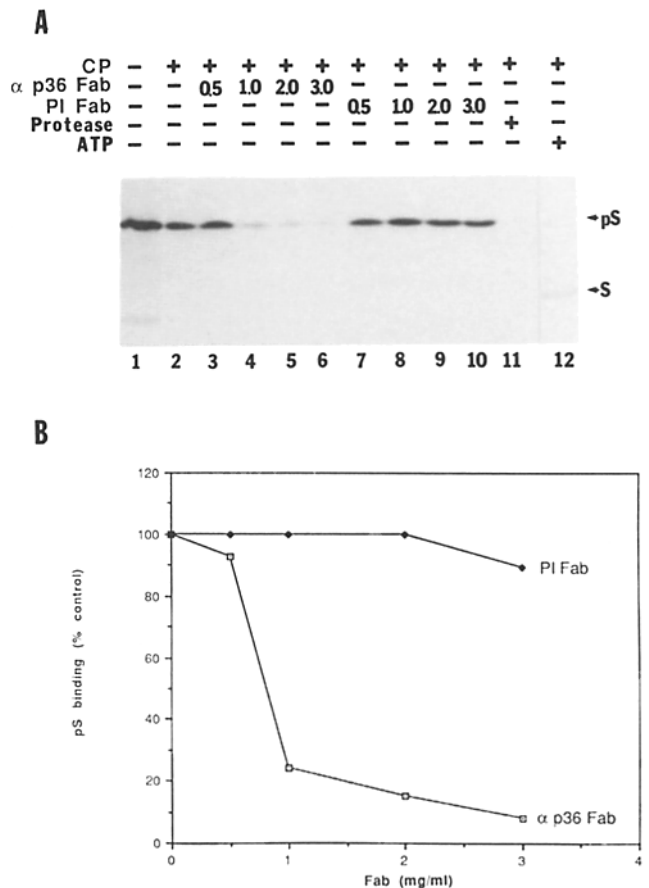
**Figure 9.** Inhibition of pS import into chloroplasts by anti-p36 Fab. Isolated chloroplasts (equivalent to 25  $\mu$ g chlorophyll) were incubated with the indicated concentration of Fab (in mg/ml) from anti-p36 IgG ( $\alpha$ p36 Fab) or preimmune IgG (PI Fab) on ice for 45 min before the import reaction (see Materials and Methods). (A) Import of pS. CP (+ or -) indicates the presence or absence, respectively, of chloroplasts in the assay. The positions of precursor (pS) or mature form (S) of the small subunit of ribulose-1,5-bisphosphate carboxylase are indicated by the arrows. Lane 1 contains 100% of the pS added to each fraction. (B) Quantitation of pS import as determined by densitometric scanning of the fluorogram shown in A.

### Analysis of the p36 Amino Acid Sequence

The pPCR301 cDNA encodes a polypeptide of 43,654 D. Comparison of the deduced amino acid sequence (Fig. 6) to the amino-terminal amino acid sequence determined for p36 (Fig. 4 B) indicates that the primary translation product contains an amino-terminal extension of 72 amino acids. The length and basic nature of the amino-terminal extension suggest that it serves as a transit sequence for targeting of the receptor protein to the chloroplast. After subtraction of the 72 residue long transit sequence, the molecular mass of p36

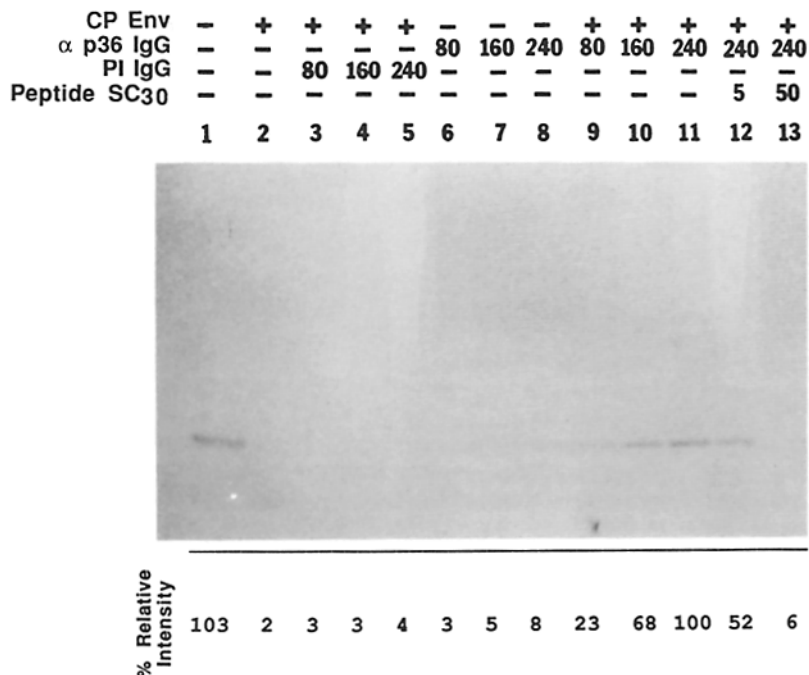
is 35,961 D. The amino acid sequences obtained for three cyanogen bromide peptides derived from p36 (Fig. 4 B) correspond to residues 177-188, 197-217, and 252-278 of the deduced amino acid sequence. These results verify that pPCR301 carries the p36 cDNA.

Analysis of the hydrophobic nature of the sequence excluding the proposed transit sequence indicates that the mature portion of the sequence contains a high proportion of hydrophobic amino acids. This observation is consistent with the solubility of the protein in chloroform/methanol and may account for the aberrant molecular weight observed for the protein on SDS-PAGE. Calculations of the hydrophobic moments for the sequence indicate eight potential transmem-



**Figure 10.** Inhibition of pS binding to isolated chloroplasts by anti-p36 Fab. Isolated chloroplasts (equivalent to 25  $\mu$ g chlorophyll) were incubated with the indicated concentrations of Fab (in mg/ml) from anti-p36 IgG ( $\alpha$ p36 Fab) or preimmune IgG (PI Fab) before the addition of a binding mixture containing radiolabeled pS. After the binding reaction, chloroplasts were reisolated and bound pS was analyzed by SDS-PAGE and fluorography. (A) Binding of pS in the presence or absence of Fab. CP (+ or -) indicates the presence or absence, respectively, of chloroplasts in the assay. The positions of the precursor (pS) or mature form (S) of the small subunit are indicated by the arrows. Lane 1 contains 25% of the radiolabeled pS added to each reaction. Lane 12 is the same as lane 2 except the chloroplasts carrying bound pS were resuspended in 5 mM ATP and incubated at room temperature after reisolation. (B) Quantitation of pS binding in the presence or absence of Fab by direct detection of radioactivity using an AMBIS radioanalytic imaging system.





**Figure 11.** Immunoprecipitation of a p36-pS complex with anti-p36 antibodies. Chloroplast envelope membranes (*CP Env*) were solubilized in 1% (vol/vol) Triton X-100 (see Materials and Methods). The soluble membranes were diluted to 0.1% (vol/vol) Triton X-100 and incubated with radiolabeled pS. After the incubation with pS, the mixtures were immunoprecipitated with anti-p36 IgGs (*ap36 IgG*) or IgGs derived from preimmune serum (*PI IgG*). The numbers indicate the amount of IgG added to each reaction in micrograms. In lanes 12 and 13, the binding reaction contained a synthetic transit peptide representing the carboxyl-terminal 30 amino acids of pS (*Peptide SC30*). The concentration of peptide in each reaction is given in micromoles per liter. (lane 1) 5% of the radiolabeled pS added to each reaction. The quantitation of the pS precipitated in each reaction is given below each lane relative to the amount in lane 11. The amount of radiolabeled pS was determined by direct detection of radioactivity using an AMBIS radioanalytic imaging system.

brane domains, the positions of which are indicated in Fig. 7. All eight positions conform to the rules set forth to predict transmembrane domains based on the hydrophobic nature of each residue (Eisenberg, 1984) with each domain carrying a hydrophobic index of at least 1.6 (Kyte and Doolittle, 1982). The mature portion of p36 contains only 14% charged amino acids, the major proportion of which are basic in nature, giving the protein a calculated isoelectric point of 9.17. Interestingly, the longest regions of high charge density are found at the amino and carboxyl terminal ends of the mature sequence (Fig. 7). It is likely that these domains are exposed to the aqueous environments of the cytosol or the intermembrane space.

Comparison of the p36 sequence to all known and predicted protein sequences in the GenBank or PIR libraries indicated striking homology of the p36 to a membrane protein of similar molecular mass from spinach chloroplasts that has been proposed to be the triose phosphate-3-phosphoglycerate-phosphate translocator (phosphate translocator) (Flügge et al., 1989) (Fig. 8). These two proteins show 44% identity in their predicted transit sequences and 84% identity in their mature polypeptides. This high degree of identity suggests that these two proteins are homologues, and that the small sequence differences are due to species diversity. No other significant identity was detected by analysis of the GenBank or PIR libraries.

### Effect of Anti-p36 Fab on Chloroplast Import

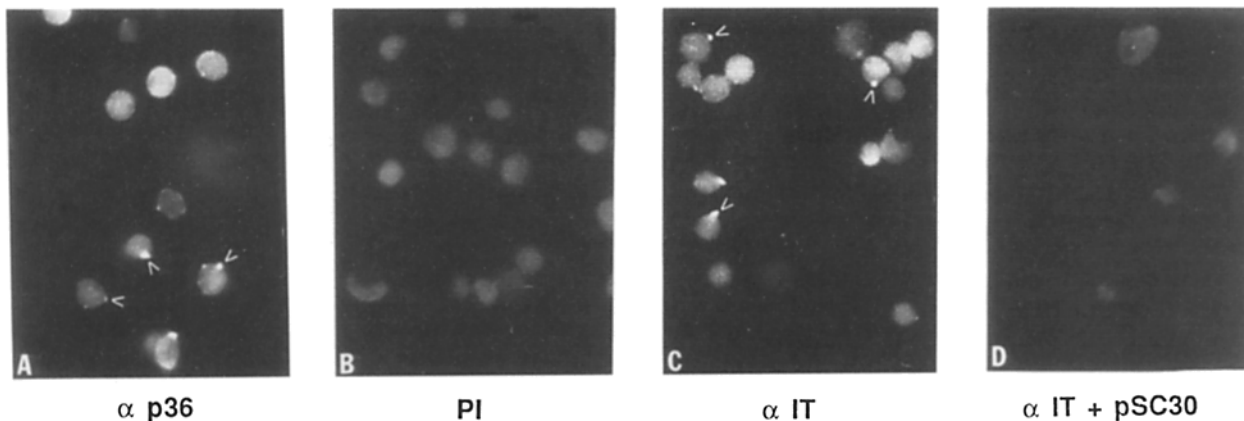
A key distinguishing feature between a chloroplast import receptor and a chloroplast phosphate translocator is the localization of the protein in the chloroplast envelope. The phosphate translocator would be expected to reside in the metabolite impermeable inner membrane where it would function to translocate phosphate and triose phosphates into the stroma (Flügge and Heldt, 1984). The outer membrane is freely permeable to small molecules (Heldt, 1980), and

thus metabolite transporters would not be required in this membrane. In contrast, a chloroplast import receptor would be expected to reside on the outer surface of the chloroplast, presumably in the outer membrane or at membrane contact sites where it could interact with cytosolically localized precursors.

To assess the function and topology of p36, the purified protein (see Fig. 4 A) was used to generate a rabbit antiserum. This antiserum reacted specifically with p36 and not with other proteins in the chloroplast envelope (Fig. 2 C). More importantly, the anti-p36 antibodies did not react with other polypeptides in the 30-kD region of either the chloroform/methanol insoluble material (Fig. 2 C) or polypeptides eluting early in SDS/hydroxylapatite chromatography of the chloroform/methanol extract of envelope membranes (Fig. 3 C). The monospecificity of the anti-p36 serum was confirmed with immunoblots of whole chloroplasts (data not shown). The anti-p36 antibodies were tested for their ability to inhibit the import of pS into isolated intact chloroplasts. Monovalent Fab was prepared from a purified anti-p36 IgG fraction for use in the assay to avoid artifacts imposed by the agglutinating properties of the divalent IgGs on intact chloroplasts. Chloroplast import activity was assayed as the conversion of pS ( $M_r = 20,000$ ) to its mature form ( $M_r = 14,000$ ) by the action of the specific stromal transit peptide protease.

Intact chloroplasts were preincubated with anti-p36 Fab or corresponding preimmune Fab before the addition of [ $^{35}$ S]pS in a standard import assay system. The results of this experiment are shown in Fig. 9. The anti-p36 Fab inhibit the import of pS in a concentration-dependent manner. At the highest concentration of Fab included in the assay (2 mg/ml), the import of pS was inhibited by >90%. Preimmune Fab at the same concentration had no significant effect on import (Fig. 9).

The initial identification of p36 via antiidiotypic antibodies (Pain et al., 1988) suggested that this protein functions



**Figure 12.** Immunofluorescence microscopy of isolated pea chloroplasts. Isolated chloroplasts were immobilized on polylysine-coated polystyrene dishes and incubated with 1:500 diluted anti-p36 serum (*A*), preimmune serum (*B*), or antiidiotypic serum (*C*). *D* was incubated with 10  $\mu$ M peptide SC30 (see Materials and Methods) before incubation with the antiidiotypic serum. The primary antibody was detected with fluorescein-conjugated goat anti-rabbit IgG. Arrows indicate patches of immunofluorescence at the chloroplast surface. Magnification, 1,068 $\times$ .

as a signal sequence receptor in the binding of precursors to the chloroplast surface before their import into the organelle. Therefore, anti-p36 Fab would be expected to interfere with pS binding to chloroplasts in a precursor binding assay (Cline et al., 1985). In the absence of anti-p36 Fab, a given quantity of chloroplasts was able to bind 28% of the radiolabeled pS added to the binding reaction (Fig. 10, lane 2). The surface localization of the bound pS was established by its susceptibility to exogenously added protease (Fig. 10, lane 11). Moreover, the observed binding was productive rather than nonspecific, as chloroplasts carrying bound pS and subsequently reisolated and incubated with a level of ATP sufficient to promote import, were able to import 60% of the bound pS (Fig. 10, lane 12) (the low level of signal present in lane 12 was due to the removal of three of the six radiolabeled methionines present in pS upon cleavage of the transit sequence by the transit peptide peptidase). With increasing concentrations of anti-p36 Fab added to the reactions (Fig. 10, lanes 3–6), the binding of pS was increasingly inhibited. At the highest concentration of Fab added (2 mg/ml) the level of bound pS fell to 8% of the control level. The concentrations of Fab necessary to inhibit pS binding were nearly identical to those necessary to block pS import (Fig. 9) indicating that pS binding may be the step at which the Fab inhibits the translocation reaction. Comparable levels of preimmune Fab had little effect on pS binding to chloroplasts (<10% inhibition at the highest concentration of preimmune Fab). It is interesting to note that the anti-p36 Fab had no effect on phosphate uptake into isolated chloroplasts (data not shown). These data strongly suggest that p36 functions as a chloroplast import receptor rather than as a phosphate translocator, since a phosphate translocator located in the inner membrane would not be accessible to the anti-p36 Fab.

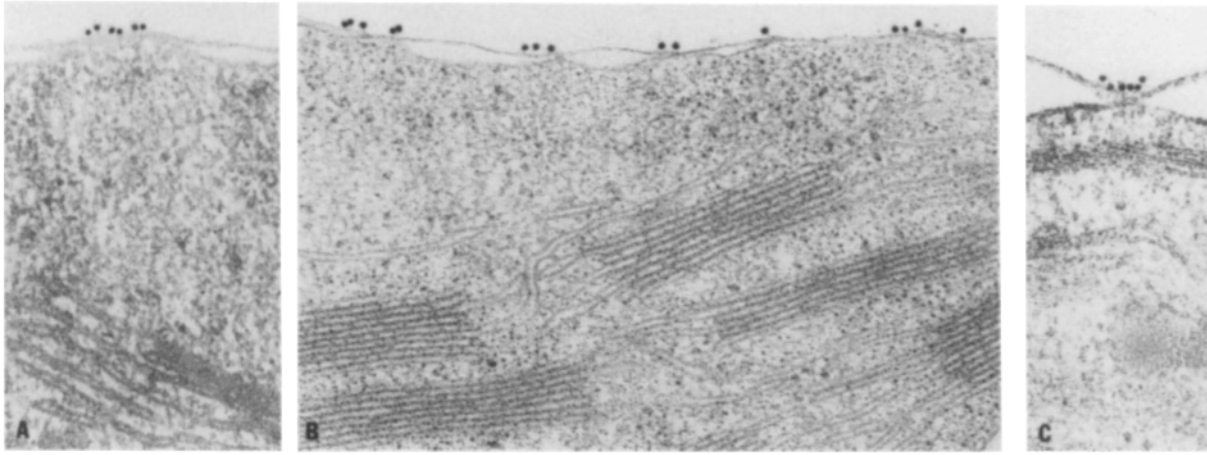
#### ***Immunoprecipitation of p36-bound pS with Anti-p36 IgGs***

The binding of pS to its chloroplast surface receptor has been shown to be a high affinity interaction with a dissociation constant of 6–10 nM (Friedman and Keegstra, 1989). This observation suggested that we could detect a physical associ-

ation between p36 and pS in a soluble precursor binding assay. The assay consisted of chloroplast envelope membranes that were solubilized with Triton X-100 and subsequently incubated with radiolabeled pS under conditions that promote the binding of pS to the chloroplast surface. After the binding reaction, the mixture was immunoprecipitated with the anti-p36 IgGs. Any pS bound to the solubilized p36 would coprecipitate with the p36-anti-p36 complex and could be detected by SDS-PAGE and fluorography.

The results of this experiment are shown in Fig. 11. Lanes 9–11 show that the anti-p36 IgGs are able to indirectly precipitate radiolabeled pS from a mixture of solubilized chloroplast envelopes and pS. This precipitation is dependent upon the amount of IgG added to the reaction with ~5% of the added pS precipitated with the maximum amount of IgG added (Fig. 11, lane 11). The specificity of this precipitation is established by the inability of comparable amounts of preimmune IgG to precipitate pS (Fig. 11, lanes 3–5). Lanes 6–8 of Fig. 11 demonstrate that the precipitation of pS is dependent upon the presence of chloroplast envelope membranes and is not due to a nonspecific precipitation of pS by the anti-p36 IgGs. In the absence of solubilized membranes, <8% of the amount of pS precipitated in the presence of membranes is precipitated at the highest concentration of anti-p36 IgG added (compare lanes 8 and 11 of Fig. 11). This background level is not significantly higher than that observed with the highest amount of preimmune IgG tested (Fig. 11, lane 5). The amount of pS precipitated by the preimmune IgG or by the anti-p36 IgG in the absence of soluble envelope material varies from 4–10% of the amount precipitated by the highest levels of anti-p36 IgG in the presence of envelope membrane material depending on the experiment. This background level of pS precipitation is most likely due to nonspecific adsorption of the precursor.

The specificity of the association of pS with p36 is further demonstrated in lanes 12 and 13 of Fig. 11. In these samples, the pS binding reaction was carried out in the presence of a synthetic transit peptide analog (peptide SC30). This peptide corresponds to the carboxyl-terminal 30 amino acids of the pS transit sequence. This peptide is able to eliminate the



**Figure 13.** Immunoelectron microscopy of isolated pea chloroplasts. Chloroplasts were immobilized, fixed, and successively incubated with anti-p36 serum and goat anti-rabbit IgG conjugated to colloidal gold. The panels represent different examples of p36 labeling at the chloroplast outer membrane at sites of contact with the chloroplast inner membrane. *A* and *B*, 65,000 $\times$  magnification; *C*, 75,000 $\times$  magnification.

precipitation of pS by the anti-p36 IgGs, presumably by competing for the transit sequence-mediated association of pS with p36. Peptide SC30 blocks pS import into isolated chloroplasts at the level of precursor binding (Schnell, D. J., D. Pain, and G. Blobel, unpublished observations). This peptide served as the transit peptide analog from which the antiidiotypic antibodies were generated (Pain et al., 1988). Taken together, these data strongly support a direct interaction between pS and p36 via the pS transit sequence.

#### Localization of p36

Previously, we have used indirect immunofluorescence microscopy to visualize the binding of the antiidiotypic antibodies to the chloroplast surface (Pain et al., 1988). As is shown in Fig. 12 *C*, the antiidiotypic antibodies give a marked punctate immunofluorescence pattern at the periphery of the chloroplasts. The binding of the antiidiotypic antibodies to the chloroplast surface apparently is due to their ability to mimic transit peptide structure as the immunofluorescence signal can be competed by the transit peptide analog (SC30) from which the antibodies were originally generated (Fig. 12 *D*).

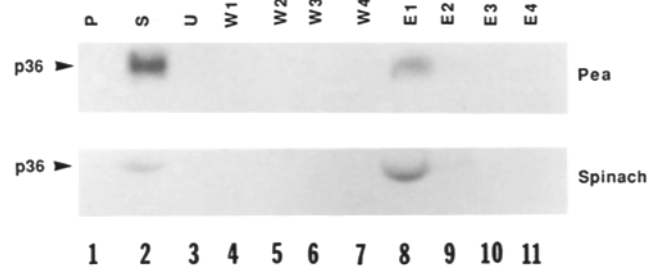
To assess the membrane localization of p36 more directly, the anti-p36 IgGs were used in immunofluorescence experiments with intact chloroplasts. The results of these experiments are shown in Fig. 12. Bright patches of fluorescence are visible at the periphery of the chloroplasts, indicating an uneven distribution of the polypeptide on the chloroplast surface (Fig. 12 *A*). This pattern is indistinguishable from that exhibited by the antiidiotypic serum (Fig. 12 *C*). The surface localization of p36 was supported by the observation that the anti-p36 serum agglutinated isolated chloroplasts in suspension (data not shown). The corresponding preimmune serum gave no immunofluorescence pattern (Fig. 12 *B*).

Immunoelectron microscopy of intact chloroplasts using anti-p36 IgGs confirmed the surface localization of the receptor and indicated an enrichment of the protein at membrane contact sites. These results are shown in Fig. 13. Indirect immunogold labeling of p36 was observed primarily at sites of contact between the inner and outer membrane of

the chloroplast envelope. Labeling was less frequently observed on the outer side of the outer envelope membrane at sites where the inner and outer membranes are not closely apposed. These data are consistent with the immunoelectron microscopic localization previously observed with the transit peptide antiidiotypic antibodies (Pain et al., 1988).

#### Adsorption of p36 to Hydroxylapatite

It has previously been reported that the phosphate translocating activity of the chloroplast envelope does not bind to hydroxylapatite after solubilization of the envelope membranes in Triton X-100 (Flügge and Heldt, 1981). The fraction that remained unbound to hydroxylapatite was reconstituted into phospholipid vesicles, and these vesicles were shown to contain 3.5–6% of the phosphate translocation efficiency of intact chloroplasts. To determine whether p36 cofractionated with phosphate translocator activity, isolated



**Figure 14.** Adsorption of p36 to hydroxylapatite. Chloroplast envelope membranes from pea (*Pea*) or spinach (*Spinach*) were treated with 10 mM HEPES-KOH, pH 7.4, 2% Triton X-100 (HT buffer), and the soluble (*S*) and pellet (*P*) fractions were separated by centrifugation. The soluble fraction was incubated with HTP hydroxylapatite and the unbound fraction was collected (*U*). The hydroxylapatite was washed four times with HT buffer (*W1-W4*). The bound fraction (*E1*) was eluted from the hydroxylapatite with 0.8 M NaPO<sub>4</sub>, pH 6.8, 0.1% SDS (elution buffer). The hydroxylapatite was subsequently washed with three consecutive aliquots of elution buffer (*E2-E4*). The samples were analyzed by SDS-PAGE and immunoblotting with anti-p36 serum.

envelope membranes were solubilized in 10 mM Hepes-KOH, 2% vol/vol Triton X-100 (Fig. 14, lanes 1 and 2) and incubated with hydroxylapatite that had been equilibrated in the same buffer. The hydroxylapatite was washed in the same buffer and then eluted with 0.8 M NaPO<sub>4</sub>, pH 6.8, 0.1% SDS. As the original experiments of Flügge and Heldt were carried out with envelopes isolated from spinach chloroplasts, we carried out the hydroxylapatite adsorption with envelope membrane material isolated from spinach chloroplasts and pea chloroplasts. Immunoblots of the SDS-PAGE-resolved fractions with anti-p36 antibodies showed that p36 from both spinach and peas was quantitatively adsorbed by the hydroxylapatite (Fig. 14, lanes 3–7), and that p36 could be eluted from the hydroxylapatite by 0.8 M NaPO<sub>4</sub>, pH 6.8, 0.1% SDS (Fig. 14, lanes 8–11). As previous results have shown that phosphate translocator activity remains in the supernatant after hydroxylapatite adsorption of Triton X-100-solubilized chloroplast envelope polypeptides (Flügge and Heldt, 1981), it is unlikely that p36 is the phosphate translocator.

## Discussion

By an antiidiotypic antibody approach, we have previously identified a 30-kD integral membrane portion of pea chloroplast envelopes as a candidate for a protein import receptor (Pain et al., 1988). We have now purified this protein and deduced its complete primary structure from cDNA clones. We refer to this protein as p36, as its calculated molecular mass is 36 kD. To further characterize the protein, we have raised an antiserum against purified p36 and have used these antibodies to provide further evidence that p36 functions as a chloroplast import receptor.

The complete amino acid sequence of p36 was deduced from several overlapping cDNA clones that encompass the complete p36 mRNA coding region. A surprising result of our studies (Fig. 8) was the unexpected amino acid sequence homology (84% identity) of the pea p36 with an integral membrane protein of similar molecular weight from spinach chloroplasts. This protein has been suggested to be the chloroplast phosphate translocator (Flügge et al., 1989). The high degree of identity indicated that these two proteins were most likely homologues, and that the small amount of sequence variation is due to species diversity.

Although it is possible that p36 serves a dual function in the chloroplast, our data make it unlikely that one and the same protein functions as a chloroplast import receptor and as a phosphate translocator. By necessity, a phosphate translocator should be located exclusively in the inner chloroplast envelope membrane, as the outer chloroplast membrane is permeable to small metabolites (presumably through porin-like molecules) (Heldt, 1980). In contrast, a transit sequence receptor should be located in the outer membrane, as the outer membrane is likely to be impermeable to proteins (Heldt, 1980). By several criteria, p36 localized to the outer membrane of the chloroplast envelope. Antiidiotypic antibodies and monospecific antibodies against p36 (Fig. 3 C), when used in immunofluorescence microscopy (Fig. 12), decorated patches on the surface of the chloroplasts. The antiidiotypic immunofluorescence signal could be eliminated by the inclusion of a pS transit peptide analogue (Fig. 12 D). The immunofluorescence patches observed with both anti-

bodies are likely to correspond to the immunogold-decorated patches observed at sites in which the outer membrane is closely associated with the inner membrane in contact sites as seen in immunoelectron microscopy (Fig. 13). Moreover, the monospecific anti-p36 antibodies agglutinated chloroplasts from suspension (data not shown). Most importantly, anti-p36 antibodies block binding of pS to the chloroplast surface (Fig. 10). If p36 were the phosphate translocator in the inner membrane and were accessible to anti-p36 Fab through a broken outer membrane, it is unlikely that such an event would interfere with the binding of pS to the outer chloroplast membrane.

Two laboratories have raised antibodies against polypeptides of the 30-kD region excised from SDS-PAGE gels of the spinach chloroplast envelope and used these antibodies in indirect immunofluorescence microscopy. One group was unable to detect reactivity of their antibodies with intact chloroplasts (Joyard et al., 1983), and they concluded, therefore, that the polypeptides of the 30-kD region are located in the inner membrane of the chloroplast envelope. Using a similarly generated antibody preparation, a second group was able to detect immunoreactivity with the chloroplast periphery in cryostat sections of leaf slices, but no immunofluorescence was detected with isolated intact chloroplasts (van Berkel et al., 1986). Again, the authors concluded that the polypeptides of the 30-kD region of the spinach chloroplast envelope resided in the inner membrane. Considering the heterogeneity of the polypeptides that comigrate at the 30-kD region in SDS-PAGE (see Figs. 2 A and 3 A), it is not clear whether any of the observed immunoreactivity in these studies was contributed by anti-p36 antibodies.

While the topology data argue against p36 being a phosphate translocator, the location of p36 in the outer membrane of the chloroplast envelope is consistent with its function as the import receptor. Its apparent enrichment at membrane contact sites is noteworthy in lieu of the strong evidence for contact sites containing the points of membrane translocation in mitochondria (Schleyer and Neupert, 1985; Schwaiger et al., 1987).

The outer chloroplast membrane vesicles that float to the top of 20–38% wt/vol sucrose gradients do not contain detectable quantities of p36 (see Fig. 1 A and B, lane 21). These low density membranes are most likely derived from regions of the outer membrane that are not part of the envelope contact sites. The absence of p36 in this envelope component is consistent with the absence of pS binding sites in this fraction. Our previous data showed that pS bound to chloroplasts in an import competent state fractionates with the p36 containing intermediate density envelope fraction (Fig. 1 A, fractions 9–19), but is absent from the low density membranes (Pain et al., 1988).

More direct evidence for the assignment of p36 as a chloroplast import receptor is provided by data obtained using the anti-p36 antibodies. Fab of anti-p36 IgGs inhibited import of pS (Fig. 9) by inhibiting the binding of precursor to intact chloroplasts (Fig. 10). Considering that p36 was originally identified with antiidiotypic antibodies and, therefore, is likely to contain a transit sequence binding site, the observed inhibition of precursor binding (Fig. 10) by anti-p36 Fab is likely to reflect direct interference at or near the transit sequence binding site of p36.

Additional evidence for p36 being the import receptor

comes from the observation that pS, when incubated with detergent-solubilized chloroplast envelopes, can be immunoprecipitated with anti-p36 antibodies. Immunoprecipitation of pS is abolished when a synthetic pS transit peptide (the same that was used to generate the antiidiotypic antibodies) was included in the precipitation reaction (Fig. 11, lanes 12 and 13). These data support the notion that p36 contains a transit sequence binding site, and that the transit sequence of pS is able to interact with this site. However, these data do not rule out the possibility that the transit sequence of pS interacts with another protein that is in association with p36, and by virtue of this association, pS and p36 are coprecipitated.

Another group has recently shown that a 24 amino acid synthetic peptide derived from the pS transit sequence could be covalently crosslinked to a 30-kD polypeptide when incubated with pea chloroplast envelope membranes (Kaderbhai et al., 1988). The identity of this protein remains to be determined, but it is possible that this protein is p36. A second group carried out similar crosslinking experiments using a photoactivatable form of the entire pS polypeptide (Cornwell and Keegstra, 1987). This construct specifically crosslinked to a polypeptide of 66 kD. The role of this polypeptide in import has not been determined, but it may be that this protein constitutes another component of the translocation machinery.

In view of the data presented here in support of p36 as the import receptor, it is useful to review the evidence that has led to the identification of p36 as the phosphate translocator. The most detailed studies of the phosphate translocator have been made using the nonspecific lysine modifying reagents pyridoxal 5'-phosphate (PLP) and 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene (DIDS). PLP has been used to inhibit chloroplast phosphate translocation in spinach, and its site of action was mapped by reduction of PLP-modified chloroplasts with [<sup>3</sup>H]sodium borohydride (Flügge and Heldt, 1977). Although incorporation of tritium into a 30-kD envelope polypeptide could be partially competed by the presence of phosphate translocator substrates, a separate study showed that at least nine other envelope proteins could be labeled by this procedure (Rumpho et al., 1988), prompting these authors to call into question the specificity of this reagent for identifying the phosphate translocator. In agreement with this nonspecificity argument, it was recently demonstrated that at the concentrations of PLP used to inhibit phosphate translocation, protein import is also strongly inhibited (Hinz and Flügge, 1988). In experiments utilizing DIDS, a correlation was observed between DIDS concentration and the inhibition of phosphate translocator function (Rumpho and Edwards, 1985). However, when [<sup>3</sup>H]DIDS was used to identify the translocator, the specificity of tritium incorporation into the 30-kD polypeptide was not demonstrated by competition with phosphate translocator substrates (Rumpho et al., 1988). Moreover, it could not be ruled out in these studies that DIDS inactivated other proteins by noncovalent interactions, as has been demonstrated in other systems (Bartel et al., 1989; Gratzer, 1990). In fact, protein import is strongly inhibited when isolated chloroplasts are treated with concentrations of DIDS similar to those used to inhibit phosphate uptake (Schnell, D. J., D. Pain, and G. Blobel, unpublished observations).

In cloning the putative spinach phosphate translocator, the hybridization probes used in screening spinach cDNA libraries were generated from the amino acid sequences of

tryptic peptides of material electroeluted from SDS-PAGE resolved total envelope proteins migrating at 30 kD (Flügge et al., 1989). The unfractionated material from which the tryptic peptides were generated most likely contained several polypeptides, as evidenced by our demonstration that the 30-kD polypeptide region consists of several distinct polypeptides (Figs. 2 and 3). In addition, the antiserum used to confirm the identity of the putative phosphate translocator clone was raised against the same unfractionated 30-kD region of spinach chloroplast envelopes (Flügge et al., 1989). Therefore, the relationship of the 30-kD protein identified by PLP and DIDS labeling to the protein that was sequenced (Flügge et al., 1989) remains to be clarified.

A previous study has shown that the chloroplast envelope phosphate translocator activity of spinach chloroplasts remained unbound when Triton X-100-solubilized envelopes were incubated with hydroxylapatite (Flügge and Heldt, 1981). These investigators were able to reconstitute 3.5–6% of the chloroplast phosphate translocation efficiency into liposomes from this unbound fraction. Our results showing that p36 from both spinach and peas is bound by hydroxylapatite using this procedure (Fig. 14, lanes 8–11) strongly suggest that p36 is not responsible for chloroplast phosphate translocator activity.

Taken together, the data presented here substantiate our identification of p36 as a chloroplast import receptor. The immunofluorescence and immunoelectron microscopy studies indicate that the receptor is localized at contact zones between the inner and outer membranes of the chloroplast envelope. These contact zones had originally been proposed to contain the sites of protein translocation into the organelle (Dobberstein et al., 1977). It is likely that the chloroplast import receptor is associated with other proteins of the outer and inner membrane that form the contact site for protein import, and that these associations remain intact under defined solubilization conditions. The availability of antibodies that immunoprecipitate p36 offers the possibility to identify associated proteins that may constitute other components of the import machinery.

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