

Tic20 and Tic22 Are New Components of the Protein Import Apparatus at the Chloroplast Inner Envelope Membrane

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Abstract. Two components of the chloroplast envelope, Tic20 and Tic22, were previously identified as candidates for components of the general protein import machinery by their ability to covalently cross-link to nuclear-encoded preproteins trapped at an intermediate stage in import across the envelope (Kouranov, A., and D.J. Schnell. 1997. *J. Cell Biol.* 139:1677–1685). We have determined the primary structures of Tic20 and Tic22 and investigated their localization and association within the chloroplast envelope. Tic20 is a 20-kD integral membrane component of the inner envelope membrane. In contrast, Tic22 is a 22-kD protein that is located in the intermembrane space between the outer and inner envelope membranes and is peripherally associated with the outer face of the inner membrane.

Tic20, Tic22, and a third inner membrane import component, Tic110, associate with import components of the outer envelope membrane. Preprotein import intermediates quantitatively associate with this outer/inner membrane supercomplex, providing evidence that the complex corresponds to envelope contact sites that mediate direct transport of preproteins from the cytoplasm to the stromal compartment. On the basis of these results, we propose that Tic20 and Tic22 are core components of the protein translocon of the inner envelope membrane of chloroplasts.

Key words: chloroplast • protein import • membrane translocation • protein targeting

NUCLEAR-ENCODED chloroplast proteins are imported into the organelle across the double membrane of the chloroplast envelope after synthesis on free polysomes in the cytoplasm (Cline and Henry, 1996; Fuks and Schnell, 1997; Lübeck, et al., 1997). The targeting signal for envelope translocation resides within a cleavable amino-terminal extension of the preprotein, designated the transit sequence. After import, the transit sequence is cleaved from the preprotein by the stromal processing peptidase, and the protein folds in the stroma with the assistance of molecular chaperones or is directed to the thylakoid or inner membrane by secondary targeting signals.

Three components of the translocon at the outer envelope membrane of chloroplasts (Toc¹ components), Toc34,

Toc86, and Toc75 form a stable complex in the outer membrane (Ma et al., 1996). Their roles in import were first established by the demonstration that they copurify with preprotein import intermediates from membrane detergent extracts (Waegemann and Soll, 1991; Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995). Antibodies to Toc86 and Toc75 inhibit protein import, providing additional evidence for their participation in the import reaction (Hirsch et al., 1994; Tranel et al., 1995). All three components of the Toc complex have been shown to associate with preproteins at the early stages of preprotein import by covalent cross-linking, suggesting that they form an integrated recognition site for the preprotein transit sequence (Perry and Keegstra, 1994; Ma et al., 1996; Kouranov and Schnell, 1997). The initial interaction of preproteins with the Toc complex does not require energy (Ma et al., 1996; Kouranov and Schnell, 1997), but subsequent translocation across the outer membrane requires the hydrolysis of ATP and GTP (Olsen and Keegstra, 1992). Although the role of ATP hydrolysis is not clear, the requirement for GTP hydrolysis has been attributed to Toc34 and/or Toc86 as a prerequisite for membrane translocation (Kouranov and Schnell, 1997). Toc75 and Toc86 appear to form at least part of the translocation channel at the outer membrane translocon because they are efficiently cross-linked to pre-

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1. *Abbreviations used in this paper:* EST, expressed sequence tag; protA, IgG-binding domain of staphylococcal protein A; pS, precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase; Tic, translocon at the inner envelope membrane of chloroplasts; Toc, translocon at the outer envelope membrane of chloroplasts.

proteins trapped in transit across the outer membrane (Kouranov and Schnell, 1997), and Toc75 exhibits ion channel activity upon reconstitution in artificial membrane bilayers (Hinnah et al., 1997).

Upon insertion across the outer membrane, the preprotein associates with components at the translocon at the inner envelope membrane (Tic components). The best studied of the Tic components, Tic110, is a 110-kD integral membrane protein of the inner envelope (Kessler and Blobel, 1996; Lübeck et al., 1996). Tic110 contains one or two amino-terminal transmembrane domains with the bulk of its mass (>90 kD) protruding into the stromal compartment (Kessler and Blobel, 1996; Jackson et al., 1998). Tic110 coimmunoprecipitates with two stromal chaperones, ClpC (Akita et al., 1997; Nielsen et al., 1997) and cpn60 (Kessler and Blobel, 1996). These observations have led to the hypothesis that the carboxyl-terminal domain acts as a stromal chaperone-docking site, thereby facilitating translocation and folding of preproteins (Kessler and Blobel, 1996; Nielsen et al., 1997). An additional inner membrane protein, designated Tic55, was shown to cofractionate with Tic110 on blue native gel electrophoresis (Caliebe et al., 1998), but the role of this protein in import remains to be established.

Several lines of evidence indicate that the Toc and Tic components interact at envelope contact sites to facilitate direct transport of the preprotein from the cytoplasm to the stroma. Early preprotein import intermediates that are in transit across the envelope are coimmunoprecipitated with Toc34, Toc75, Toc86, and Tic110 (Schnell et al., 1994; Akita et al., 1997; Nielsen et al., 1997). Furthermore, anti-Toc75 coimmunoprecipitates detectable amounts of Tic110 and ClpC from membrane detergent extracts under native conditions or conditions in which membrane complexes have been stabilized by covalent cross-links (Akita et al., 1997; Nielsen et al., 1997). The association of Toc and Tic components is not dependent upon the presence of a preprotein, indicating that the interaction does not require a translocating polypeptide, but may be mediated by direct binding between translocon components from the two membranes.

Recently, we demonstrated by covalent cross-linking that two novel envelope polypeptides, Tic(21) and Tic22, associate with two different chimeric preproteins during envelope translocation (Kouranov and Schnell, 1997). Cross-linking to Tic(21) and Tic22 occurred at an early intermediate stage in envelope translocation when the preproteins had inserted across the outer membrane, but had not accessed the stromal compartment. Cross-linking also was detected at late stages in import when the preproteins were inserted across both outer and inner envelope membranes. On the basis of these results, we hypothesized that Tic(21) and Tic22 represent new components of the inner membrane translocon. In this article, we report the deduced primary structures of Tic(21) and Tic22 and define their localizations within the chloroplast envelope. On the basis of its deduced sequence, we change the designation of Tic(21) to Tic20 in accordance with the uniform nomenclature for chloroplast import components (Schnell et al., 1997). Both Tic20 and Tic22 are associated with the inner envelope membrane, but Tic22 is peripherally bound to the outer face of the inner membrane, whereas Tic20 is in-

tegral to the inner membrane. Tic20 and Tic22 associate with other Toc and Tic components to form an active import supercomplex in the chloroplast envelope. These results suggest that Tic20 and Tic22 serve as a functional link between the translocon complexes in the outer and inner envelope of chloroplasts.

Materials and Methods

Chloroplast Isolation and Preprotein Cross-linking Reactions

Intact chloroplasts were isolated from 10–14-d-old pea seedlings (*Pisum sativum* var. Green Arrow) by homogenization and Percoll silica gel gradient centrifugation as previously described (Pain and Blobel, 1987). Isolated chloroplasts were resuspended in 50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol (HS buffer) to a concentration equivalent to 2–3 mg chlorophyll/ml. The modification of pS-protA and pS-1 with [¹²⁵I]APDP and covalent cross-linking reactions with intact chloroplasts were performed as previously described (Ma et al., 1996; Kouranov and Schnell, 1997). Thermolysin and trypsin treatment of intact chloroplasts were performed as described previously (Schnell et al., 1994) using 0.2 mg protease/ml for 30 min on ice.

To prepare chloroplast envelope membranes, intact chloroplasts were lysed under hypertonic conditions and separated into soluble and membrane fractions by differential centrifugation as described by Keegstra and Yousif (1986). The total membrane fraction was separated into envelope and thylakoid membrane fractions by flotation into linear sucrose gradients as previously described (Schnell et al., 1994). The envelope fractions were analyzed by SDS-PAGE. The radioactive signals in dried gels were captured and quantitated using a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA) with the IPLab Gel Scientific Image Processing version 1.5c program (Signal Analytics, Vienna, VA).

Reverse Phase Chromatography and Peptide Sequencing

Chloroplast envelope membranes (300 µg of protein) were incubated in the presence of 20 mM dithiothreitol at 23°C for 5 min to cleave the cross-linker. Envelope proteins were precipitated with 80% (vol/vol) acetone on ice for 1 h. The acetone precipitate was dissolved in 0.5 ml of 90% formic acid and applied directly to a 3-ml Resource RPC column using an FPLC system (Pharmacia Biotech, Piscataway, NJ). The proteins were eluted with a 45-ml linear gradient of 0–100% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid. Fractions of 1 ml in volume were collected and dried in a Speed-Vac concentrator (Savant Inc., Hicksville, NY). The fractions were analyzed directly by SDS-PAGE. Fractions containing radiolabeled Tic20 and Tic22 were pooled, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Polypeptide bands corresponding to Tic20 and Tic22 were excised and subjected directly to amino-terminal and internal peptide sequence determination (Fernandez et al., 1992).

Isolation of Tic20 and Tic22 cDNAs

Two degenerate oligonucleotide primers were used to amplify a 37-bp partial Tic20 cDNA from total pea cDNA by reverse transcription PCR. The first primer, NT1 (5' GGITTYMGITTYCCICCIATGAC 3'), was generated from the amino-terminal peptide sequence (residues 89–96) of pea Tic20. The second primer, A2 (5' AARTGCATICCRAAYTTNCCCC 3'), was based on the nucleotide sequence of an Arabidopsis-expressed sequence tag (EST) (GenBank/EMBL/DBJ accession number N96442, nucleotides 714–735) that showed extensive identity to Tic20. Primers based on the sequence of the 371-bp Tic20 cDNA fragment were used to isolate a full-length Tic20 cDNA by 5' and 3' rapid amplification of cDNA ends (Clontech, Palo Alto, CA).

Two oligonucleotide primers based on the sequence of rice and Arabidopsis EST cDNAs whose deduced amino acid showed extensive similarity to the pea Tic22 peptide sequences were used to amplify a 337-bp fragment of Tic22 cDNA from total pea cDNA by reverse transcription PCR. The first degenerate primer, P25.S (ACGCTTGAYCARGTTTAY-

ATG), was generated from a rice EST cDNA (GenBank/EMBL/DBJ accession number D23157, nucleotides 148–168). The second primer, P25.A (CCCAACCTGAATTCTTCTCGCTCATCTCC), was generated from an Arabidopsis EST cDNA (GenBank/EMBL/DBJ accession number T75737, nucleotides 726–755). The sequence of the 337-bp partial Tic20 cDNA was used to generate primers for the amplification of the complete pea Tic20 cDNA by 5' and 3' rapid amplification of cDNA ends (RACE) (Clontech).

Gel Filtration Chromatography

Chloroplast envelope membranes corresponding to a mixed outer and inner membrane population (OM/IM) containing envelope contact sites were purified from chloroplasts and solubilized in 50 mM Tricine-KOH, 2 mM EDTA, 150 mM NaCl, pH 7.5 (TES buffer) containing 1% (wt/vol) Triton X-100 (Sigma Chemical Co., St. Louis, MO), followed by a clarifying centrifugation at 100,000 g for 30 min. The membrane detergent extracts containing 200 µg protein were resolved by FPLC on a Superose 6HR column (Pharmacia Biotech) equilibrated in TES buffer containing 0.1% (wt/vol) Triton X-100. Fractions of 1 ml in volume were collected and the proteins in each fraction were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting.

Antibodies and Immunoblotting

Antibodies to Toc34, Toc75, Toc86, the small and large subunits of rubisco, and the phosphate-triose phosphate translocator were generated as previously described (Ma et al., 1996; Schnell et al., 1990). Tic110 and cpn60 antibodies were a generous gift of F. Kessler and G. Blobel (both from Rockefeller University, New York, NY). Tic55 antibodies were a generous gift of the laboratory of J. Soll (Christian-Albrechts University, Kiel, Germany). Anti-ClpC serum was a generous gift of the laboratory of K. Keegstra (Michigan State University, East Lansing, MI). The anti-IEP21 serum was prepared to a major 21-kD integral inner envelope membrane protein that had been resolved from the bulk of envelope proteins by sequential reverse phase FPLC and one-dimensional SDS-PAGE. The position of IEP21 is indicated with an *asterisk* in Fig. 1 A. The anti-Tic20 serum was generated to a 13-amino acid synthetic peptide corresponding to residues 84–96 of the deduced sequence of Toc20 (see Fig. 6 B). The anti-Tic22 serum was generated to full-length recombinant Tic22 that was expressed in *Escherichia coli* BL21 (DE3) as a fusion to a hexahistidine tag using the pET21d vector system (Novagen, Madison, WI). The recombinant Tic22 was purified on a nickel-chelate matrix according to the supplier's recommendations (Novagen) and was used directly for immunization of rabbits. Immunoblotting with all sera was performed as previously described (Ma et al., 1996) using chemiluminescence detection.

Immunoaffinity Chromatography

Envelope membranes corresponding to a mixed OM/IM population were used for all immunoaffinity chromatography reactions. Immunoaffinity purification of envelope components after membrane solubilization under denaturing conditions was performed by the method of Anderson and Blobel (1983). Before immunoaffinity purification, envelope membranes (80 µg of protein) were incubated in the presence of 20 mM dithiothreitol at room temperature to cleave the cross-linker, and membranes were recovered by centrifugation at 40,000 g for 30 min and washed with TE buffer to remove residual dithiothreitol. Immunoaffinity chromatography was performed on anti-Tic22 IgG Sepharose or preimmune IgG Sepharose as described below for chromatography under native conditions.

For immunoaffinity chromatography under native conditions, the membranes (100 µg of protein) were solubilized in TE buffer containing 150 mM NaCl (TES buffer) and 1% (wt/vol) Triton X-100 for 10 min on ice. The extract was clarified by centrifugation at 100,000 g for 30 min to remove insoluble aggregates. The supernatant was applied to IgG-Sepharose from preimmune sera, or sequentially to anti-Toc34 IgG-Sepharose, anti-Toc86 IgG-Sepharose, and anti-Toc110 IgG-Sepharose (1 ml of packed matrix containing 5 mg of bound IgG). The Sepharose was washed with 10 vol of TES buffer containing 0.2% (wt/vol) Triton X-100, and eluted with 0.2 M glycine, pH 2.2 containing 0.2% (wt/vol) decyl maltoside. The eluates and unbound fractions were analyzed by SDS-PAGE, phosphorimaging, and then immunoblotting.

Results

Tic20 and Tic22 Are Associated with the Inner Envelope Membrane

Tic20 and Tic22 were identified in label-transfer cross-linking reactions using two chimeric preproteins, pS-protA and pFd-protA (Kouranov and Schnell, 1997), and a modified version of pS (Ma et al., 1996) that were trapped at an intermediate stage in import across the chloroplast envelope. To investigate the characteristics of Tic20 and Tic22 in more detail, we identified the envelope proteins corresponding to these cross-linked products from the pS-protA cross-linking reaction. The radiolabeled polypeptides corresponding to Tic20 and Tic22 were separated from the bulk of envelope polypeptides by reverse phase

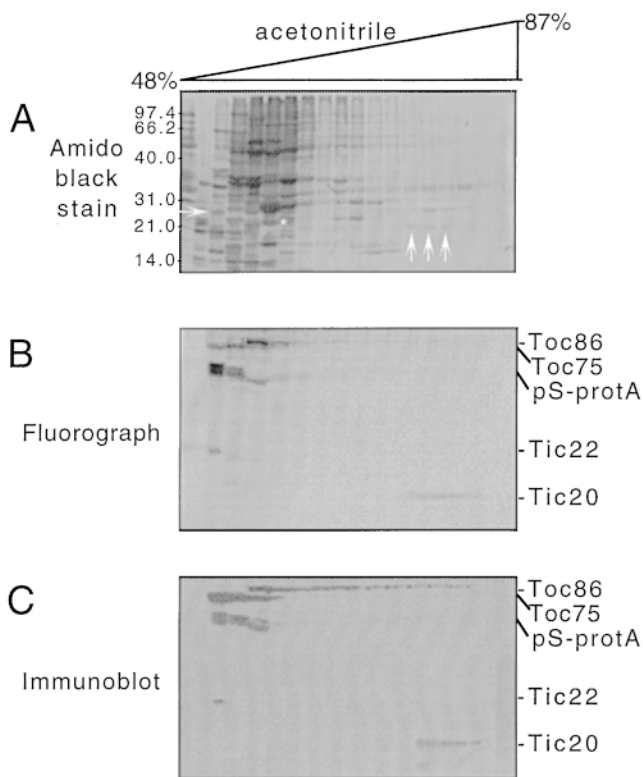


Figure 1. Resolution of Tic20 and Tic22 by reverse phase chromatography of pS-protA cross-linked envelope membranes. Chloroplast envelope membranes (300 µg of protein) from pS-protA cross-linked chloroplasts were dissolved in 90% formic acid and separated by reverse phase chromatography using a polystyrene matrix and a solvent gradient of 0 to 100% acetonitrile in 0.1% trifluoroacetic acid. Chromatography fractions were resolved by SDS-PAGE and transferred to nitrocellulose membrane. (A) Imido black stain of reverse phase profile of envelope membranes. The positions of Tic22, Tic20, and IEP21 are indicated by the *horizontal arrow*, *vertical arrows*, and *asterisk*, respectively. *Left*, positions of molecular weight standards. (B) Fluorograph of the reverse phase profile shown in A. (C) Immunoblot of the reverse phase profile shown in A with antisera to Toc75, Toc86, Tic22, and Tic20. The positions of the Toc and Tic components and pS-protA are shown at the right of B and C.

chromatography. The SDS-PAGE profile of the reverse phase separation of Tic20 and Tic22 from pS-protA cross-linked membranes is shown in Fig. 1 A. Tic22 elutes from the reverse phase column at ~50% acetonitrile (Fig. 1 A, horizontal arrow), whereas Tic20 elutes as a broad peak at 75–85% acetonitrile (Fig. 1 A, vertical arrows) suggesting that Tic20 is very hydrophobic in nature.

Both polypeptides were subjected to partial amino acid sequence determination. A single amino-terminal peptide was obtained from Tic20. Amino-terminal and two internal peptide sequences were obtained from Tic22. Tic20-specific antibodies were prepared against a synthetic peptide generated from the amino-terminal peptide sequence. Tic22 polyclonal antibodies were raised against the full-length recombinant protein expressed in *Escherichia coli*. Fig. 1 B shows that the anti-Tic20 and anti-Tic22 sera react selectively with 20- and 22-kD bands on the reverse phase profile of envelope polypeptides. The profiles of anti-Tic20 and anti-Tic22 reactivity are coincident with the fluorographic profile of Tic20 and Tic22 from cross-linked envelope membranes (Fig. 1, compare B with C), strongly suggesting that the antisera are monospecific for their respective envelope polypeptides.

To confirm the reactivity of the anti-Tic22 serum, we tested the ability of the antibodies to recognize the 22-kD cross-linked product. To avoid complications due to the presence of the protein A IgG-binding domains on the pS-protA cross-linking substrate, cross-linking was performed with pS-1 which lacks the IgG binding domain (Ma et al., 1996). The position of cross-linked Tic22 is largely obscured by [¹²⁵I]pS-1 on SDS-PAGE gels of envelope membranes because of their similar mobility (Fig. 2 A, lane 1). However, cross-linked Tic22 is visible if chloroplasts are treated with exogenous thermolysin after the cross-linking reaction (Fig. 2 A, lane 2). Thermolysin degrades envelope-bound [¹²⁵I]pS-1, but does not degrade Tic22 (see Fig. 5). Total envelope membranes from a pS-1 cross-linking experiment performed in the presence of 0.1 mM ATP and GTP were treated with reducing agent, dissolved under denaturing conditions, and then applied to anti-Tic22 IgG Sepharose. Fig. 2 B, lane 1 shows that the radioactive 22-kD polypeptide band corresponding to cross-linked-labeled Tic22 bound to the anti-Tic22 IgGs. The corresponding preimmune IgGs of anti-Tic22 did not bind any radioactively labeled proteins (Fig. 2 B, lane 2). These data confirm the reactivity of the antiserum with Tic22.

Although the Tic20 antipeptide sera was able to detect Tic20 on immunoblots, it was unable to immunoprecipitate the polypeptide from detergent-solubilized envelope membranes under a variety of conditions (data not shown). However, the fact that the cross-linked 20-kD polypeptide is effectively resolved from the majority of envelope polypeptides by reverse phase chromatography suggests that it contains only Tic20. This is supported by the fact that a single amino-terminal peptide sequence was obtained from the cross-link-labeled 20-kD region. On the basis of these results, we conclude that the anti-Tic20 serum is specific for Tic20.

To define the suborganellar localization of Tic20 and Tic22, chloroplast subfractions enriched in outer membranes, inner membranes, thylakoid membranes, and stroma were resolved by SDS-PAGE and immunoblotted

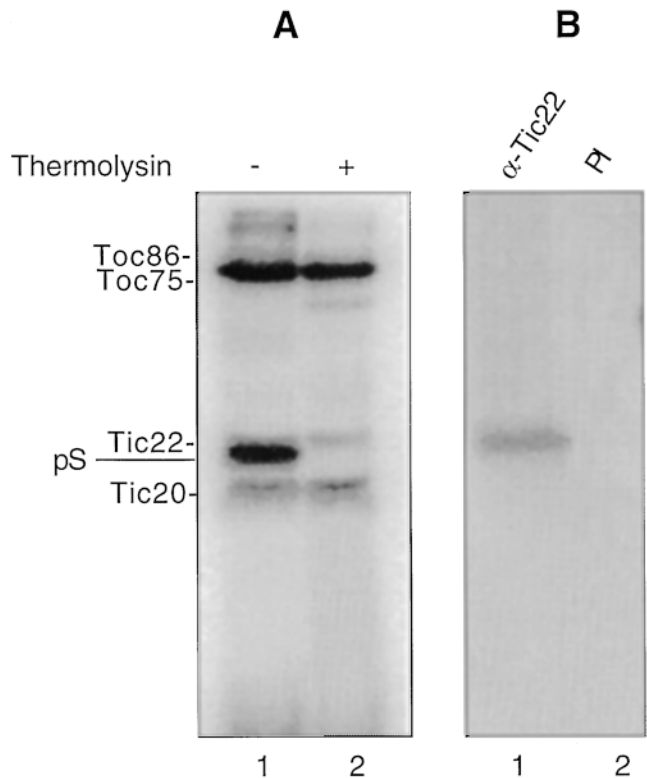


Figure 2. Immunoaffinity purification of [¹²⁵I]Tic22 with anti-Tic22 IgG Sepharose. Intact chloroplasts were cross-linked to pS-1 in the presence of 0.1 mM ATP and GTP. After import, the chloroplasts were treated in the absence or presence of 100 μg/ml thermolysin for 30 min on ice to remove bound pS-1, and chloroplast were subfractionated to yield a total envelope membrane fraction. Envelope membranes (80 μg of protein) from cross-linked chloroplasts were dissolved under denaturing conditions, and applied to anti-Tic22 Sepharose (α -Tic22) or preimmune IgG Sepharose (PI) as indicated. (A) Fluorograph of SDS-PAGE resolved thermolysin-treated or -untreated envelope membranes (50 μg of protein). The position of Tic and Toc components and pS-1 are indicated to the left of the figure. (B) Fluorograph of SDS-PAGE resolved immunoaffinity eluates of anti-Tic22 IgG Sepharose and preimmune IgG Sepharose.

with anti-Tic20 and anti-Tic22 sera. The distribution of Tic20 and Tic22 in chloroplast subfractions (Fig. 3, A and B) is similar to that of the inner membrane protein, Tic110 (Fig. 3 D), but distinct from the outer membrane protein, Toc75 (Fig. 3 D). Both proteins were detected only in fractions containing inner membrane proteins consistent with their association with this membrane (Fig. 3, A and B). The preimmune sera of anti-Tic20 and anti-Tic22 showed no reactivity with proteins in the chloroplast extracts (Fig. 3 C).

To investigate the association of Tic20 and Tic22 with the inner membrane, we tested the sensitivity of both proteins to extraction with alkaline buffer by treating envelope membrane fractions with 0.1 M Na₂CO₃, pH 11.5. Envelope membranes from a pS-protA cross-linking reaction were used in the extractions to follow both cross-link-labeled (Fig. 4 A) and total immunoreactive (Fig. 4 B)

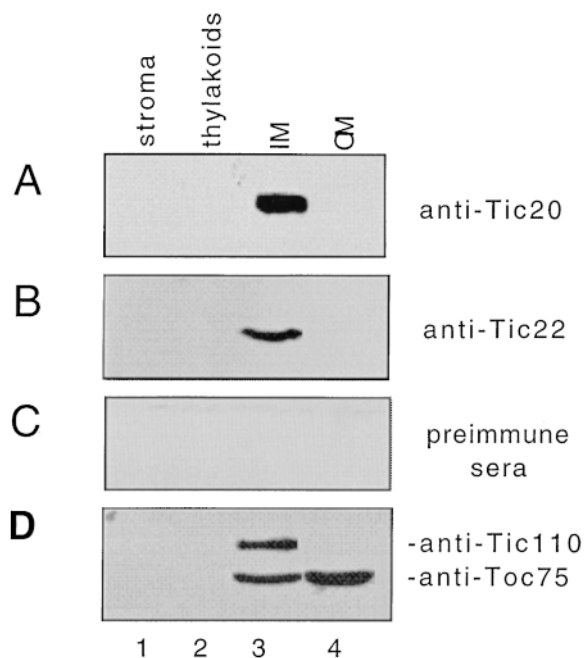


Figure 3. Identification of Tic20 and Tic22 as components of the inner envelope membrane. Samples (50 μ g of protein) from stromal extract (*stroma*), thylakoid membranes (*thylakoids*), enriched inner envelope membrane vesicles (*IM*), and outer envelope membrane vesicles (*OM*) were resolved by SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with (A) anti-Tic20, (B) anti-Tic22, (C) a mixture of anti-Tic20 and anti-Tic22 preimmune sera, or (D) a mixture of anti-Tic110 and anti-Toc75 sera.

Tic20 and Tic22. Before alkaline extraction, the envelope membranes were treated with dithiothreitol to cleave cross-links that could affect their membrane association. As a control for extraction, Toc75, a known integral envelope membrane protein (Schnell et al., 1994) also was assayed. As expected, Toc75 remains associated with the envelope membranes after alkali extraction (Fig. 4 B). Likewise, Tic20 is completely resistant to extraction from the envelope fraction (Fig. 4, A and B, compare lanes 2 with 3) consistent with the characteristics of an integral membrane protein. In contrast, 40–50% of cross-link-labeled Tic22 is extractable from envelope membranes with alkaline carbonate (Fig. 4, A and B, compare lane 1 with lanes 2 and 3). Based on this analysis, Tic22 appears to be a peripherally associated component of the inner membrane.

The topology of the Tic proteins in the inner membrane was investigated by exploring the sensitivity of both proteins to exogenous protease treatments in intact chloroplasts. We selected thermolysin and trypsin for these studies. Thermolysin is an outer membrane impermeable protease that selectively digests chloroplast surface-exposed proteins, whereas trypsin is capable of permeating the outer membrane and partially digesting several proteins at the outer face of the inner membrane (Cline et al., 1984; Jackson et al., 1998). Fig. 5 A shows that Tic20 was not depleted by either protease treatment (compare lane 1 with lanes 2 and 3) as measured by detection of

the immunoreactive protein, suggesting that it is not exposed to the intermembrane space between the outer and inner membranes. Tic22 was resistant to thermolysin treatment (Fig. 5 A, compare lanes 1 with 2), but almost completely degraded with trypsin treatment (Fig. 5 A, compare lanes 1 with 3). As controls for proteolysis, we monitored the degradation of Toc86 in the outer membrane and Tic110 in the inner membrane. Toc86 is degraded to a 52-kD fragment in thermolysin and trypsin treatments, indicating that the proteases were active in both reactions (Fig. 5 A) (Kessler et al., 1994). Tic110 was not degraded by either treatment (Fig. 5 A) consistent with the fact that the bulk of its mass is oriented to the stroma (Jackson et al., 1998). Tic20 and Tic22 were digested by the proteases if the membrane barrier was disrupted by detergent treatment (Fig. 5 B), confirming that their insensitivity to protease treatment in intact chloroplasts was due to their membrane localization and not to inherent protease insensitivity. On the basis of these results, we conclude that Tic20 is an integral inner membrane protein with little or no exposure to the intermembrane space. In contrast, Tic22 is peripherally associated with the outer face of the inner envelope membrane.

Primary Structures of Tic20 and Tic22

Comparison of the sequences of peptides derived from Tic22 and Tic20 to the database of ESTs identified cDNA clones from rice and Arabidopsis with a high degree of

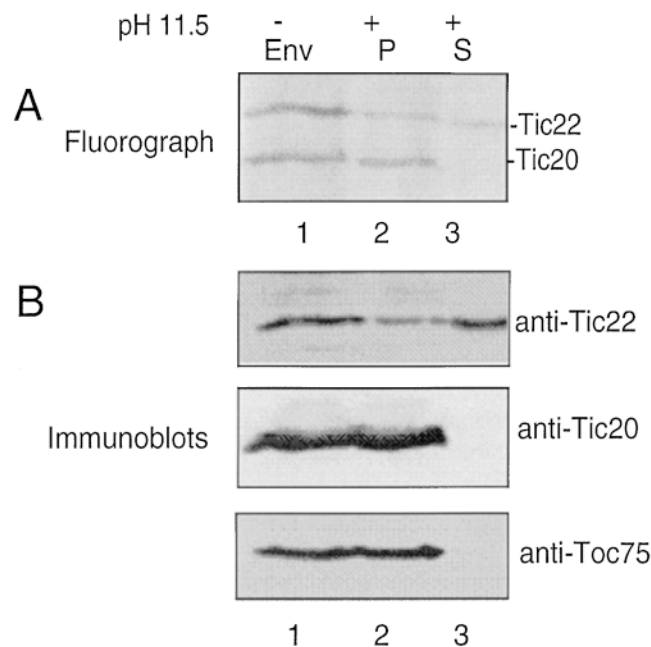


Figure 4. Sensitivity of Tic20 and Tic22 to extraction of envelope membranes with alkaline buffers. Total envelope membranes (*Env*) (50 μ g of protein) were incubated in the presence (+*pH 11.5*) or absence (*-pH 11.5*) of 0.1 M Na_2CO_3 , pH 11.5, on ice for 10 min. The insoluble (*P*) and soluble (*S*) fractions were separated by differential centrifugation and analyzed by (A) fluorography or (B) immunoblotting with anti-Tic22, anti-Tic20, or anti-Toc75 sera as indicated.

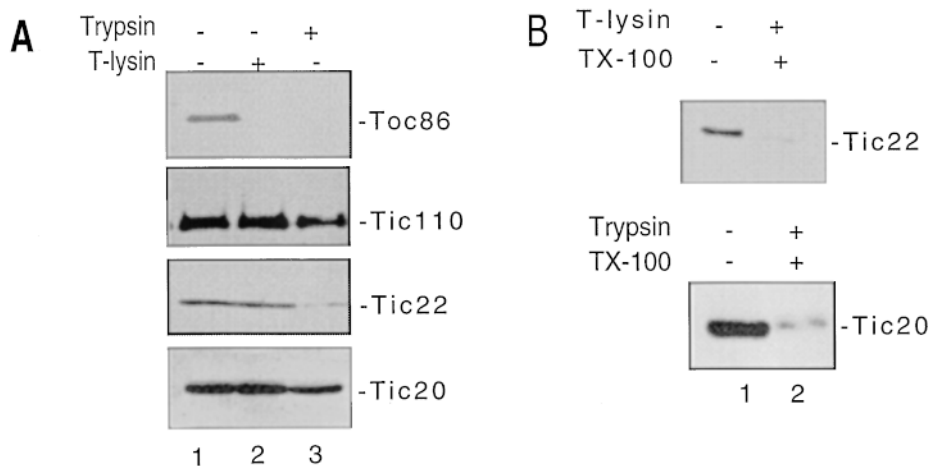


Figure 5. Protease sensitivity of Tic20 and Tic22 in intact chloroplasts. Chloroplasts equivalent to 2 mg of chlorophyll were treated with 200 μ g/ml thermolysin or trypsin on ice for 30 min. The chloroplasts were reisolated through Percoll silica gel, lysed, and then subfractionated by flotation into sucrose gradients. Fractions corresponding to envelope membranes were pooled and analyzed by SDS/PAGE. (A) Immunoblot of SDS-PAGE resolved envelope polypeptides from chloroplasts incubated in the absence ($-T$ -lysin) or presence ($+T$ -lysin) of thermolysin or the absence ($-Trypsin$) or presence ($+Trypsin$) of trypsin with anti-

Toc86, anti-Tic110, anti-Tic20, and anti-Tic22 sera. Each lane contains 50 μ g of protein. (B) Immunoblot of chloroplast envelopes with anti-Tic20 or Tic22 serum after treatment of membranes in the absence or presence of trypsin or thermolysin and the absence ($-TX$ -100) or presence ($+TX$ -100) of Triton X-100. Right, positions of the Toc and Tic components.

identity to Tic22 and Tic20 from pea. The Arabidopsis and rice cDNAs were sequenced and overlapping DNA sequences were used to design conservative oligonucleotide primers for the amplification of pea Tic22 and Tic20 cDNAs using reverse transcription PCR. Full-length Tic22 and Tic20 cDNAs were obtained by 5'- and 3'-RACE PCR.

The complete nucleotide sequence of pea Tic22 cDNA and its deduced amino acid sequence are shown in Fig. 6 A. The cDNA contains an open reading frame of 252 amino acids encoding a polypeptide of 28,252 Da. All three peptides obtained from partial amino acid sequencing of Tic22 align to corresponding sequences within the deduced cDNA sequence. The sequence of the amino-terminal peptide from Tic22 aligns to positions 51–70 of the deduced sequence indicating that the primary translation product of the mRNA is a preprotein containing a 50-amino acid presequence. The presequence is enriched in hydroxylated amino acids and contains a cleavage site motif Ala-Phe-Ala₂Ala at position 48–51 that closely resembles the consensus cleavage site for the stromal processing protease (Gavel and von Heijne, 1990). These characteristics suggest that the presequence may serve as a transit sequence for targeting Tic22 to the chloroplast. Tic22 is the first example of a protein localized to this chloroplast subcompartment, and therefore, the nature of the presequence and its possible role in targeting remain to be established.

The deduced sequence of mature Tic22 is overall hydrophilic in nature with no extensive regions of hydrophobic amino acids that could form potential transmembrane domains. This observation is consistent with our biochemical data demonstrating that Tic22 is not integrated into a membrane. Comparison of the sequence to protein sequence data banks reveals no similarity to known proteins other than the rice and Arabidopsis EST cDNAs. Likewise, the Tic22 sequence does not exhibit any readily identifiable sequence motifs or patterns, such as nucleotide-binding sites, that are indicative of enzymatic activity. Thus, Tic22 is a unique component of the chloroplast im-

port machinery without detectable homology to components of other known translocation systems.

The deduced amino acid sequence of the pea Tic20 cDNA corresponds to a polypeptide of 29,174 Da (Fig. 6 B). Alignment of the amino-terminal peptide sequence to the deduced Tic20 sequence indicates that mature Tic20 begins at amino acid 83 and represents a polypeptide of 20,419 Da. The 82-amino acid presequence has characteristics of a typical chloroplast transit sequence. Other integral inner membrane proteins, including Tic110 and the triose phosphate-phosphate translocator, are targeted to the chloroplast by typical transit sequences (Lübeck et al., 1996, 1997; Knight and Gray, 1995). Therefore, the presence of a putative transit sequence on Tic20 is consistent with its localization to the inner membrane.

Analysis of the deduced primary structure of preTic20 reveals the presence of three putative alpha-helical transmembrane domains at positions 144–166, 179–196, and 210–227 (Fig. 6 B). On the basis of the predicted mature sequence, the positions of the transmembrane domains would generate a 7,524-Da amino-terminal soluble domain and a carboxyl-terminal tail of 3,127 Da. The amino-terminal soluble domain is very basic in nature, with a predicted isoelectric point (pI) of 10.21. The two intervening sequences between the transmembrane domains contain only 12 and 13 amino acids, and therefore are predicted to provide sufficient length to allow a turn in the polypeptide, but not to extend significantly from the surface of the membrane. The results of proteolytic treatments in Fig. 5 are consistent with a topology for Tic20 in which the amino-terminal domain extends into the stroma and the carboxyl-terminal tail resides in the intermembrane space. This interpretation is based on the observation that the immunoreactivity of Tic20 with the anti-amino-terminal-specific antibody is not sensitive to trypsin treatments in intact chloroplasts (Fig. 5 A). In addition, the predicted topology would place the high concentration of positively charged amino acids in the loop between the first and second transmembrane domains in the intermembrane

space consistent with the predicted topogenic signals for integral membrane proteins (von Heijne and Gavel, 1988).

Tic20 and Tic22 Associate with Other Components of the Chloroplast Import Machinery

The ability of Tic20 and Tic22 to cross-link to translocating polypeptides (Ma et al., 1996; Kouranov and Schnell, 1997) raises the possibility that they may form part of a stable membrane complex at the core of a translocon in the inner membrane. As a first step to explore the nature of the inner membrane translocon, we investigated the interactions of Tic22 and Tic20 with each other. Total chloroplast envelope membranes were dissolved under mild conditions with Triton X-100 to maintain the native structure of import complexes and proteins were immunoaffinity purified with anti-Tic22 IgG Sepharose or preimmune IgG Sepharose. The IgG-bound fractions were resolved by SDS-PAGE and immunoblotted with anti-Tic20 and anti-Tic22. As expected, Tic22 was bound by anti-Tic22, but not by IgG from its corresponding preimmune sera (Fig. 7 A, compare lanes 3 with 4). Tic20 also is detected in the anti-Tic22-bound fraction (Fig. 7 A, lane 3), suggesting that these two components form a stable association in the inner membrane. As controls for the specificity of the immunoaffinity reaction, the anti-Tic22 Sepharose chromatography fractions were immunoblotted with antisera to the triose phosphate-phosphate translocator, an inner membrane metabolite transporter (Fliege et al., 1978), and IEP21, an integral inner membrane protein (Materials and Methods). In addition, the immunoblot was probed with antiserum to the large subunit of rubisco, a common stromal contaminant of the envelope membranes. None of the three control proteins were detected in the bound fraction confirming the selectivity of the anti-Tic22 Sepharose (Fig. 7 A).

To assess the degree of association of Tic20 and Tic22, detergent-solubilized envelope membranes were fractionated by gel filtration chromatography and the profile was immunoblotted with the anti-Tic antibodies (Fig. 7 B). The bulk of Tic20 and Tic22 do not cofractionate on gel filtration. However, a minor fraction of Tic22 does coelute with Tic20 consistent with the immunoprecipitation results. These results suggest that a minor fraction of the total Tic20 and Tic22 form a stable association in the chloroplast inner membrane, but that the bulk of the two proteins are not bound to the same complex.

Recently, Nielsen et al. (1997) demonstrated that Tic110 could be detected in anti-Toc75 immunoprecipitates of chloroplast membranes suggesting that the outer and inner membrane translocons associate. To investigate the possibility that Tic20 and Tic22 participate in this association and investigate the extent of association of import components in this supercomplex, we performed sequential immunoprecipitation of detergent-solubilized envelope membranes with antibodies to both Toc and Tic components. The membranes used in these experiments were derived from untreated chloroplasts or chloroplasts that had been cross-linked to saturating concentrations of an early pS-1 import intermediate in contact with Tic20 and Tic22 (Ma et al., 1996). The saturating amount of cross-

A

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CTAGAATCAAAA ATGGAGTCTCAGGGACAGTGGAACTCTCTATCATTTTCAAGGTTT 60
M E S Q G Q W N P L L S F S R F 16
ATCAATCACCACCTTAACCACCTTGCAACTCGTTTAGAAGAAACGAAACGTCTCGCCGGA 120
I N H H S N H L A T R L E E T K R L A G 36
ACCCTAATTCAACTCTCACACTCGCACCAACACAGCCCTCGCCGCTACTTTGACTCCCAAC 180
T L I Q S H T R T K P A F A A P L T P N 56
CACGTCGCCAAGTCCCTCGCCGGCACCTCCGTTTACACCCGTCAGCAANTTCGAGCAATGAG 240
H V A K S L A G T S V Y T V S N S D N E 76
TTTGTCTTATGTCTGACCGCGAAGGTGCTAAGTCCATTGGATTGCTCTGCTTTCGCCAA 300
F V L M S D A E G A K S I G L L C F R Q 96
GAAGACGAGAAGCATTCTGGCTCAGGTTTCGATCGCGGAAAAAGGAATTCGAGGCGGC 360
E D A E A P L A Q V R S R K K E F R G G 116
GCTAAGTCTTCTCTATAACACTTGATCAGGTTTACATTTGTAAGGTTGAAGTATTGCA 420
A K V V P I T L D Q V Y M L K V E G I A 136
TTCCGATTTTACCAGTCCAGTTCAAATAAGAAATGACTAGAGCTCAGAGCAAC 480
F R F L P D P V Q I K N A L E L R A A N 156
AGGGGAAGTTTTCAGCGAGTTCCTGTTTCCAGTCAGACCTTTTGGTGGTGAAGAAAG 540
R G S F T D G V P V F Q S D L L V V K K K 176
AACAAAGCTTATGCCCCTGTATATTTTCTAAGGAAGATCTTGAAGTAACTATCTAAG 600
N K R Y C P V Y F S K E D L E Y E L S K 196
GTTCCAGGTCATCAAGGTTTGGAGTTTCTCAGCATATAAGGTTGGTAGCTTTGAA 660
V S R S S K G V G V S Q H I M V G S F E 216
GATGTACTGAAAAAATGGAGTTGAGTGAAGAAAGTTCCAGGTCGGAGGATTTGTTT 720
D V L K K M E L S E K S G W E D L V F 236
ATTCCCCAGGAAAAAGCACCTCTCAACATATGCAAGAAGTTATGCTTAA TTAGCTCG 779
I P P G K K H S Q H M Q E V I A stop 252
GCCCAAGAGCTTACCATAATTTTGTGGTTTCTTGAGCAAAATGGATGTATTACTGT 839
CAAATCTAGTATGCTGATTCCTGCTTAAAGGATCTCTATCGATCCAAAGGTTGCTATAG 899
GTGAGAGCATAGGCATATCTGCCATCATCCATTTAAGGTAAGGTCACATGGATTAC 959
CATGTTGAGTATTTAAATCTAAATGTAAGGATTTTCCAAATACCATGTTTCAGTT 1019
TTTCATTTGACCCAGCTGTAACTTATTAATCTTCCGATTCATCAGTTAAGG 1079
GAGGCTTTGGACATGGGTTTGGAAATTAAGTTAAATACCGTCGAGGATTTTAAACTA 1168

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B

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AACAAAGCAACAACAACACTGACTAACTTCAAAATCAAAATCAACAACACTGACTAAA 60
CTTCCGAGTTAATCAACC ATGATTCAAAATGGTGGCAGCTGTATCCCAAGGTTCTGCTT 120
M I Q N G G T V S Q G S V L 14
TGTTATGCTTGTCAAAATCCCTGCTAAGGTTGCTCTCTCAAGTATCCGAAGTTTCTGGGA 180
C Y A C Q I P A K V A V S S I R S F W G 34
CATAGTTTGGAAAAAATAGCCAGAGGAATGACATTCACAGACATGCTCTGATCATCAAGC 240
H S L E N K P R G M T F T D M S A T S S 54
CTACTTTTAAAGGGGGTTCAGAAATTTCTGCTCTGCTACTTCCCGGTGTTACTACGCTG 300
L L L S G G Q N F L S R T I P V L P T L 74
CACAACTTCTTACCACCCCTCGGGCAACAAGATCTCCAGGCTTCCGATTTCCCTCCG 360
H K S S T T P R A T K D S S G F R F P P 94
ATGACAAAAAACCAAGATGGTGGTGGAGAACATATCATGCATCCCTTACCCTTACCT 420
M T K K P R W W R T L S C I P Y L L P 114
TTCCATCAGGCATGGATGATGCTCGGACAGCATACCATCTTCCACCCGTTTATCCCGTAT 480
F H Q A W M Y A R T A Y H L H P F I P Y 134
TTCCAGCCCATGACGTACCCTTTTCTCATGGCAATGGAACCTTACCTAGATGGRCTCTC 540
F Q P M T Y P F L M A I G T L P E R W S L 154
ATAGCATACTTCTTGTATGCATATCTTACAATTTGTGAGGAGAAAGAATGGCCCTATTTC 600
I A Y E L I A Y L T I A Y L K E W P H F 174
TTCCGATTCATGTCGCCCTCGGAATGTTAATCGAGATCGCCCTACAGGTTACCAGGAT 660
F R F H V A V G M L I E I A L Q V T G I 194
GTGAGCCGTTGGATGCCAGCTTCGTTTATTTGGGGTAAACTTGGAAATGCATTTCTGGACT 720
V S R W M P R S F Y W G K L G M H E M T 214
ACTGCATTTTTTGTCTTTTCTTTACTACCATAGAGTGCATATAGATGTCGCCCTTGTGGT 780
T A E E V E L E T T I E C I R C A L V G 234
ATGATGCCGATGTCCTTTTGTCTGTGATGAGCAGATCAATCAAAATACCACAGGAT 840
M Y A D V P P V C D A A Y I Q I P H E stop 253
GGTGAATGTGTAACAAAGAAACACCTTTACATTTCTGTTACTCTTGTGTTGTTACTAC 900
TTTTCTTTAGCAGAAACAGAAATGACTAATCATATAATCAGTTGTTGTTGTTGTTATA 960
TCGAAATACTACGAATTTGATGTTTCCCTTGAGTA 1010

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Figure 6. Nucleotide sequence of the Tic22 (A) and Tic20 (B) cDNAs and their deduced amino acid sequences. *Right*, nucleotides and amino acids. *Solid underline*, amino acids corresponding to peptide sequences obtained from Tic20 and Tic22. *Broken underline*, positions of the putative transmembrane segments of Toc20. The sequence of mature Tic20 begins at residue 83. The sequence of mature Tic22 begins at residue 51. The GenBank/EMBL/DBJ accession numbers for the Tic20 and Tic22 cDNAs are AF095285 and AF095284, respectively.

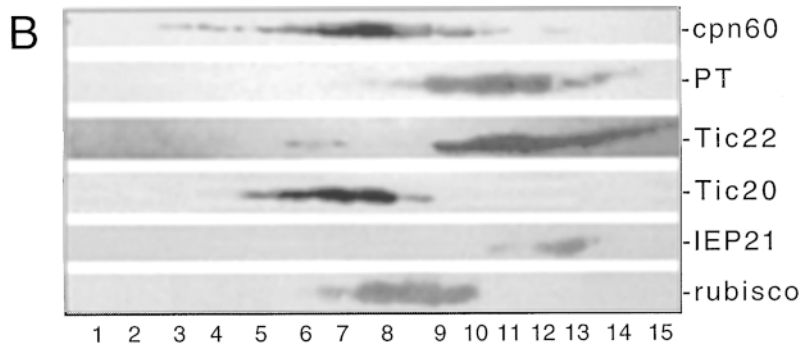
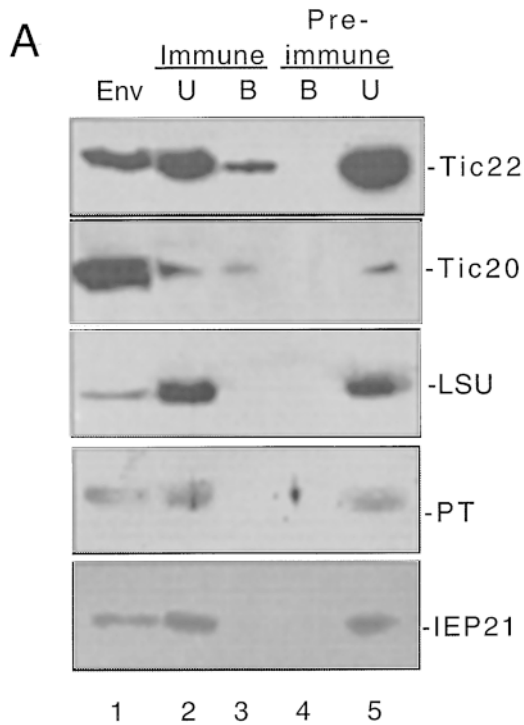


Figure 7. Association of Tic20 and Tic22 in the inner envelope membrane. Isolated mixed outer and inner envelope membrane vesicles (*Env*) were dissolved in TES buffer containing 1% (wt/vol) Triton X-100. (A) A portion of the soluble detergent extract (80 μ g of protein) was subjected to immunoaffinity chromatography on anti-Tic22 Sepharose (*anti-Tic22*) or preimmune IgG Sepharose (*PI*). The unbound (*U*) and bound (*B*) fractions from the chromatography runs were analyzed by SDS-PAGE and immunoblotting with antisera to chloroplast proteins as indicated. Lane 1 contains one-fourth of the total envelope membrane protein (20 μ g) applied to the Sepharose columns. (B) The soluble detergent extract (200 μ g of protein) was resolved by FPLC on a Superose 6HR column (Pharmacia) equilibrated in a buffer containing 0.1% Triton X-100. Fractions of 1 ml in volume were collected and analyzed by SDS-PAGE and immunoblotting with antisera against Tic20, Tic22, cpn60, triose phosphate-phosphate translocator (*PT*), a 21-kD inner envelope membrane protein (*IEP21*), and the small subunit of rubisco (*rubisco*).

linked pS-1 presumably would occupy all potential import sites. This allowed us to test the effects of a stably bound preprotein on the association of the import components.

The membranes corresponding to a population of mixed outer and inner membrane vesicles bound by envelope contact sites were dissolved in buffer containing Triton X-100 and insoluble material was removed by centrifugation. Although Nielsen et al. (1997) used decyl maltoside to dissolve the envelope membranes, we chose Triton X-100 for our experiments because we found this detergent to be more effective in overall membrane solubilization as measured by the fraction of Toc and Tic components that were recovered in the soluble fraction after differential centrifugation of the detergent extract. The solubilization conditions were optimized to ensure that at least 80% of the Tic and Toc components and cross-linked pS-1 were retained in the soluble fraction as determined by quantitative immunoblotting and fluorography (data not shown). The soluble detergent extract was applied sequentially to immunoaffinity columns composed of anti-Toc34 Sepharose,

anti-Toc86 Sepharose, and anti-Tic110 Sepharose. The ratio of membrane protein to affinity matrix was chosen to assure that each chromatography achieved quantitative depletion of the corresponding antigen.

The eluates and unbound fraction from the chromatographic runs were resolved by SDS-PAGE and analyzed by Coomassie blue staining and immunoblotting as shown in Fig. 8. As controls for the selectivity of the immunoaffinity chromatography presented, we probed the immunoblots with antisera to the triose phosphate-phosphate translocator, the large or small subunits of rubisco, and IEP21. None of these control proteins were detected in eluates from the three sequential immunoaffinity columns indicating the selectivity of their respective antigens (Fig. 8, A2 and B2). In addition, IgG Sepharose prepared from a mixture of anti-Toc34, anti-Toc86, and anti-Tic110 pre-immune sera did not bind any of the chloroplast proteins as assayed by immunoblotting (data not shown).

Anti-Toc34 immunodepletes Toc34, Toc86, and Toc75 in the presence or absence of bound pS-1, consistent with

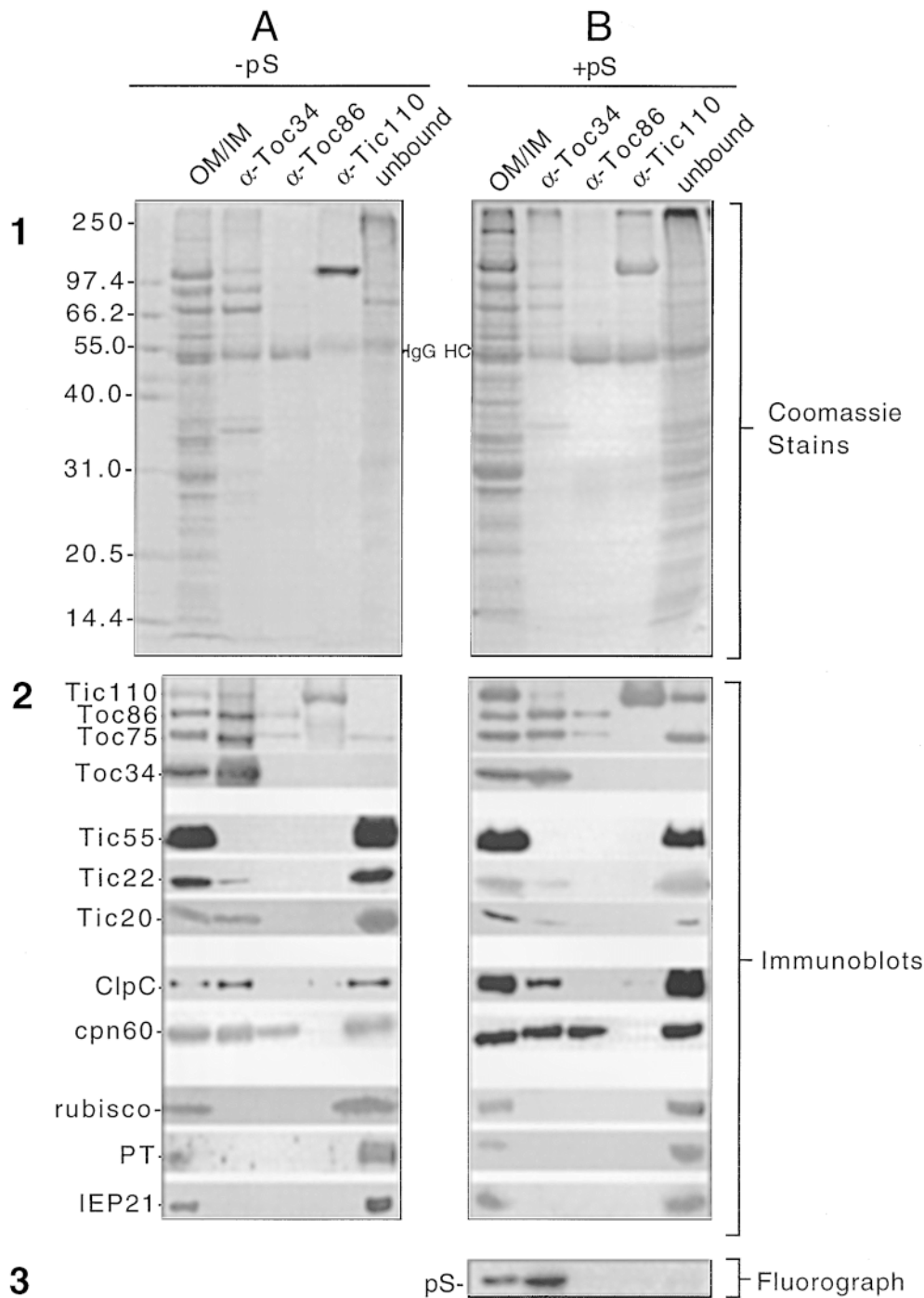


Figure 8. Sequential immunofluorescence chromatography of solubilized chloroplast envelope membranes. Isolated mixed outer and inner chloroplast envelope membranes (*OM/IM*) (100 μ g of protein) from untreated chloroplasts or chloroplasts that had been cross-linked to 400 nM pS-1 in the presence of 0.1 mM ATP and GTP were dissolved in TES buffer containing 1% Triton X-100, clarified by centrifugation, and sequentially passed over anti-Toc34 Sepharose (α -Toc34), anti-Toc86 Sepharose (α -Toc86), and anti-Tic110 Sepharose (α -Tic110). The eluates and unbound (*unbound*) fractions were resolved by SDS-PAGE. The resolved polypeptides were visualized directly by Coomassie blue staining (*A1* and *B1*), immunoblotting with antisera to chloroplast proteins as indicated (*A2* and *B2*), or analyzed by phosphorimaging (*B3*). The immunoblots in *A2* and *B2* were performed with Toc or Tic antisera as indicated and with antisera to ClpC, cpn60, triose phosphate-phosphate translocator (*PT*), a 21-kD inner envelope membrane protein (*IEP21*), and the small subunit of rubisco (*rubisco*). The position of the heavy chain of IgG (*IgG HC*) that dissociates from IgG Sepharose during chromatography is indicated between *A1* and *B1*.

the previous observation that these three components stably associate (Ma et al., 1996). The immunodepletion of Toc34 is quantitative, and the vast majority of Toc86 is immunodepleted with anti-Toc34 as judged by Coomassie staining (Fig. 8, *A1* and *B1*) and immunostaining of the anti-Toc34 eluate (Fig. 8, *A2* and *B2*). A minor fraction of Toc86 is recovered in the eluate of the subsequent anti-Toc86 chromatography, but this fraction represents less than 10% of the total Toc86. These results suggest that these two GTP-binding proteins are nearly quantitatively associated in the Toc complex. Approximately 50% of

Toc75 is stably associated with Toc34 and Toc86 (Fig. 8, *A2* and *B2*). This observation is consistent with the fact that the molar abundance of Toc75 in the outer membrane exceeds both Toc34 and Toc86 and that a fraction of Toc75 is not stably bound to the Toc34–Toc86 complex in outer membranes (Chen, X., and D.J. Schnell, unpublished observations). The anti-Toc34 Sepharose quantitatively depletes pS-1 from the membrane extracts (Fig. 8, *B3*) indicating that the envelope-bound pS-1 is engaged in import and that the Toc34 immunoprecipitated complex corresponds to functional protein import sites.

As expected, Tic110 is detected in the anti-Toc34 eluate consistent with the results of Nielsen et al. (1997) (Fig. 8, *A2* and *B2*). However, densitometric analysis of the Coomassie-stained profile of the chromatography fractions indicates that less than 5% of the total Tic110 associates with the Toc complex (Fig. 8, *A1* and *B1*). Tic20 and Tic22 also are detected in the anti-Toc34 eluate indicating that a fraction of both proteins are associated with the Toc complex (Fig. 8, *A2* and *B2*). The amount of Tic20 associated with the Toc complex is similar to the amount coimmunoprecipitated with anti-Tic22 (Fig. 7 *A*), suggesting that the cofractionation of Tic22 and Tic20 is due to their association with the Toc-Tic supercomplex. In fact, Tic20 was not detected in anti-Tic22 immunoprecipitates of the unbound fraction from the sequential immunofluorescence chromatography steps supporting this conclusion (data not shown). Remarkably, Tic20 and Tic22 are not detected in the anti-Tic110 eluate that has been depleted of the Toc components (Fig. 8, *A2* and *B2*, lane 4), indicating that Tic20, Tic22, and Tic110 do not form a stable association in the absence of the Toc complex. The presence of saturating amounts of bound pS-1 does not detectably alter the composition or abundance of the Tic components associated with the Toc complex (Fig. 8 *B*). These data strongly support the hypothesis that a fraction of Tic110, Tic22, and Tic20 form a stable Toc-Tic supercomplex with the translocon of the outer envelope membrane in the presence or absence of bound preprotein (Nielsen et al., 1997).

We also assayed for the presence of additional chloroplast proteins in the Toc-Tic complex that previously have been shown to associate with the import machinery. Two stromal chaperones, cpn60 and ClpC, previously were shown to coimmunoprecipitate with Tic110 (Kessler and Blobel, 1996; Akita et al., 1997; Nielsen et al., 1997). Cpn60 is detected in the Toc-Tic supercomplex, but is not coprecipitated with the bulk of uncomplexed Tic110. ClpC is detected in both the supercomplex and in the anti-Tic110 eluate that contains no associated Toc components. The detection of both chaperones in association with the Toc-Tic supercomplex is consistent with their participation in the import process.

We also determined the distribution of Tic55 in the immunofluorescence eluates. This inner membrane protein has been proposed as a candidate for a component of the inner membrane translocon by virtue of its association with Tic110, and its coprecipitation with a trapped preprotein import intermediate (Caliebe et al., 1998). We did not detect Tic55 in eluates of either anti-Toc34 or anti-Toc86 Sepharose or in the anti-Tic110 eluate (Fig. 8, *A2* and *B2*). We repeated the immunofluorescence chromatography using decyl maltoside extracts of envelope membranes under conditions reported by Caliebe et al. (1998). The patterns of immunoblots obtained with decyl maltoside envelope extracts were indistinguishable from those presented in Fig. 8. As with Triton X-100 extracts, Tic55 was not detected in the eluates from any of the Toc or Tic immunofluorescence columns (data not shown).

Discussion

The localization and primary structures of Tic20 and Tic22 in conjunction with previous covalent cross-linking results

shed new light on the possible functions of these two proteins in preprotein import into chloroplasts. The site-specific cross-linking data demonstrated that Tic22 is in contact with regions of a trapped import intermediate that have not yet engaged the integral membrane protein Tic20 (Kouranov and Schnell, 1997). Therefore, it would appear that Tic22 makes contact with the preprotein before Tic20. These results are consistent with a component that may serve as a link between the outer and inner membrane translocons. In this scenario, Tic22 would contact the preprotein in the intermembrane space as it emerges from the outer membrane translocon and thereby direct the preprotein to the inner membrane translocon. The predicted topology of Tic20 makes it an ideal candidate for a component of the protein-conducting machinery of the inner membrane translocon. This proposed function is consistent with the previous observation that cross-linking of preproteins to Tic20 increases at the later stages of protein import when the translocating chain has inserted across both envelope membranes (Kouranov and Schnell, 1997).

The bulk of Tic20, Tic22, and Tic110 do not appear to interact in a complex comparable to that formed by Toc34, Toc75, and Toc86 in the outer membrane translocon (Figs. 7 and 8). Based on the data presented in Figs. 7 and 8, the assembly of Tic20, Tic22, and Tic110 into a functional inner membrane translocon appears to be mediated by their direct or indirect association with the outer membrane translocon. This observation is consistent with previous reports demonstrating that Tic110 is detectable in immunoprecipitates of Toc75 under native conditions (Nielsen et al., 1997). Our data extend these results by demonstrating that Tic components that directly interact with preproteins (i.e., Tic20 and Tic22) also are associated in a supercomplex with the outer membrane translocon. An early translocation intermediate is associated with the Toc-Tic complex, indicating that this supercomplex represents the functional preprotein translocation apparatus of the chloroplast envelope.

It is likely that the Toc-Tic supercomplex corresponds to envelope contact sites that are observed by electron microscopy as zones of close contact between the outer and inner membranes (Cremers et al., 1988). Early import intermediates, including those that efficiently cross-link to Tic20 and Tic22, biochemically fractionate with mixed outer and inner membrane vesicles linked by contact sites (Schnell and Blobel, 1993; Perry and Keegstra, 1994; Ma et al., 1996). Furthermore, import intermediates have been localized to envelope contact sites by immunoelectron microscopy (Schnell and Blobel, 1993). In mitochondria, the formation of contact sites between the preprotein translocases in the outer and inner membrane appears to be directly mediated by the translocating preprotein (Segui-Real et al., 1993; Horst et al., 1995). In contrast, the presence of saturating amounts of envelope-bound preprotein did not detectably alter the amount or pattern of proteins in the Toc-Tic supercomplex (Fig. 8). These observations suggest that functional import sites in chloroplasts are not directly mediated by the preprotein, but arise from direct interactions between Toc and Tic components. The fact that major fractions of the Tic components are not associated with functional import sites implies that the Toc-Tic supercomplex is dynamic. Identification of factors (e.g.,

preprotein, ATP, or GTP) that regulate turnover of contact sites will be an important challenge for future investigations.

In addition to an association of the Toc and Tic components at contact sites, our data also support the participation of at least two stromal chaperones in the formation of the functional import supercomplex. Both the stromal hsp60 homologue, cpn60, and the hsp100 homologue, ClpC, are detected in the Toc-Tic complex (Fig. 8). Kessler and Blobel (1996) demonstrated that cpn60 was coimmunoprecipitated with anti-Tic110, and they hypothesized that Tic110 may serve as a docking site for the stromal chaperone at the inner membrane translocon. Our results suggest that cpn60 associates exclusively with the functional supercomplex, but is not detected in association with the bulk fraction of Tic110 (Fig. 8). These data strongly support the hypothesis that the stromal chaperone is specifically concentrated at the sites of protein import to facilitate the folding of newly imported proteins. Whether cpn60 and Tic110 directly interact at the import sites remains to be determined. Nielsen et al. (1997) demonstrated that Tic110 also could be coimmunoprecipitated with ClpC under native conditions. Although the bulk of envelope-bound ClpC associated with the Toc-Tic supercomplex in our experiments, a significant portion of ClpC also was detected in the anti-Tic110 eluate that previously had been depleted of Toc components (Fig. 8). These data support the conclusions of Nielsen et al. (1997) that Tic110 serves as a docking site for the ClpC chaperone at the stromal face of the inner membrane, but suggest that this interaction is not a priori linked to preprotein translocation.

One noticeable difference between the composition of the Toc-Tic complex isolated by immunoaffinity chromatography and previous reports of associated import components was the absence of Tic55. Caliebe et al. (1998) recently identified Tic55 as a candidate for a component of the inner membrane translocon. The 55-kD protein was identified by virtue of its cofractionation with Tic110 and ClpC on blue native gel electrophoresis of decyl maltoside-solubilized envelope membranes. This association was not dependent on the presence of a bound preprotein import intermediate. Tic55 also was shown to copurify with a pS import intermediate containing a polyhistidine tag by affinity chromatography on a nickel-chelate matrix. We did not detect Tic55 in association with other import components in Triton X-100 or decyl maltoside extracts of envelope membranes, regardless of the presence or absence of bound pS (Fig. 8 and data not shown). Although one difference in the experimental protocols was the method used to generate the early import intermediate, we cannot explain the absence of Tic55 from import complexes in the absence of a bound precursor. Resolution of this discrepancy must await further studies to define the structure of the outer and inner membrane translocons.

In conclusion, we have identified Tic20 and Tic22 as new candidates for components of the protein import apparatus of the chloroplast inner membrane. Previous data demonstrating covalent cross-linking of import intermediates to Tic20 and Tic22 in conjunction with the current data establishing an association of these two proteins with functional import complexes provides compelling evidence to support their roles in preprotein import into chloroplasts.

These two proteins are in close association with translocating preproteins, and therefore are likely to represent central components of the inner membrane translocon. Future studies should provide additional insight into their association with other components of the outer and inner membrane translocons and define the nature of their interactions with preproteins. Furthermore, Tic22 should provide a much-needed marker for the intermembrane space of the chloroplast envelope. A soluble resident of this compartment has not previously been identified, and investigation of the mechanism by which Tic22 is localized to the intermembrane space will be useful in defining the mechanism of protein targeting to this chloroplast sub-compartment.

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