

Inner envelope protein 32 is imported into chloroplasts by a novel pathway

Ahmed Nada and Jürgen Soll*

Department of Biology I, Botany, University of Munich, Menzinger Str. 67, Munich 80638, Germany

*Author for correspondence (e-mail: soll@uni-muenchen.de)

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Summary

The 32 kDa chloroplast inner envelope protein (IEP32) is imported into the organelle in the absence of a cleavable N-terminal pre-sequence. The ten N-terminal amino acids form an essential portion of this targeting information as deduced from deletion mutants. Recognition and translocation of IEP32 is not catalysed by the general chloroplast outer envelope translocon subunits Toc159, Toc75III and Toc34, because IEP32 import is neither inhibited by proteolytic removal of Toc34 and Toc159 nor by inhibition of the Toc75 import channel by CuCl₂ or spermine. Import of IEP32 only requires ATP

concentrations of below 20 μ M indicating that stromal chaperones are not involved in the process, but that IEP32 might be directly inserted from the intermembrane space into the inner envelope by a so far unidentified pathway. IEP32 may require the assistance of Tic22, an intermembrane space translocon subunit for import as indicated by the presence of a chemical crosslinked product between both polypeptides.

Key words: Protein import, Targeting signal, Chloroplast, Envelope membranes

Introduction

Chloroplasts are organelles of endosymbiotic origin. During evolution most of the genes from the endosymbiont were transferred to the host nucleus. Today more than 95% of the chloroplast proteome is nuclear encoded. Pre-proteins are synthesized in the cytosol and post translationally imported into the organelle (Chen and Schnell, 1999; Keegstra and Froehlich, 1999; Bauer et al., 2001; Soll, 2002). In most cases pre-proteins are synthesized with an NH₂-terminal pre-sequence also called targeting signal. The pre-sequence is both necessary and sufficient for organellar targeting and translocation initiation. Upon import the pre-sequence is proteolytically removed by the stromal processing peptidase (Oblong and Lippa, 1992) and the protein can attain its native conformation. Chloroplast pre-sequences seem to share little common sequence motifs or secondary structure (v. Heijne and Nishikawa, 1991). The NH₂-proximal part is normally devoid of negatively charged amino acids and the central domain is rich in the hydroxylated amino acids serine and threonine. The C-proximal region can form a β -sheet structure, which includes the processing site. The pre-sequence is recognized at the chloroplast surface by receptors, which are integral subunits of the Toc-complex (translocon at the outer envelope of chloroplast) (Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Seedorf et al., 1995). The Toc complex has three distinct core subunits, the GTP-dependent Toc34 receptor (Hirsch et al., 1994; Kessler et al., 1994; Seedorf et al., 1995), a β -barrel type import channel Toc75 (Schnell et al., 1994; Tranel et al., 1995; Hinnah et al., 1997) and a GTP-dependent receptor and motor protein Toc159 (Hirsch et al., 1994; Kessler et al., 1994; Ma et al., 1996; Schleiff et al., 2003). Binding to and translocation across the

Toc-complex is GTP dependent (Schleiff et al., 2003). Import across the inner envelope is facilitated by the Tic-complex and requires ATP in the stroma, most likely for the action of molecular chaperones (Flügge and Hinz, 1986; Schindler et al., 1987; Theg et al., 1989). The Tic complex is composed of several subunits: Tic110 and Tic20, which may form an import channel (Kouranov et al., 1998; Heins et al., 2002); Tic40 as a chaperone coordinating factor on the stromal site of the envelope (Stahl et al., 1999; Chou et al., 2003); Tic22 as an intermembrane space factor (Kouranov, 1998) and finally the redox proteins Tic62 and Tic55 (Caliebe et al., 1997; Kuchler et al., 2002). ATP concentrations above 50 μ M are generally required to complete import of a standard precursor protein into chloroplasts (Flügge and Hinz, 1986; Schindler et al., 1987; Theg et al., 1989). ATP hydrolysis by molecular chaperones such as HSP93 or HSP70 is thought to provide the driving force for the final membrane passage (Nielsen et al., 1997). Proteins of the outer envelope are generally targeted and inserted into the membrane by internal sequence information and they therefore do not contain a cleavable pre-sequence (Schleiff and Klösgen, 2001). No auxiliary factor or helper protein has been identified so far that facilitates insertion into the outer envelope. Indeed, in vitro insertion can occur spontaneously into protein-free liposomes. The only known exception is Toc75, which is synthesized with a cleavable pre-sequence and which uses the Toc and the Tic translocon for import (Tranel and Keegstra, 1996).

Several reports indicate that specialised import routes into chloroplasts might exist. A nuclear encoded heat-shock-induced protein of *Chlamydomonas* thylakoids did not contain a cleavable pre-sequence as deduced from sequence comparison. However no in vitro imports were conducted

(Grimm et al., 1989). From in vivo studies using a GFP-fusion of the chloroplast inner envelope localized quinone oxidoreductase (QORH) it was shown that internal sequence information was required for correct targeting and that neither N- or C-terminal transit peptides were required (Miras et al., 2002). Tic22 takes a different route again. Whereas the pre-protein contains a cleavable pre-sequence and requires protease-sensitive receptors, its import needed only low concentrations of ATP, consistent with the idea that stromal chaperones are not involved in Tic22 import (Kouranov et al., 1999).

In this paper we provide evidence for a distinct import pathway into the inner envelope of chloroplasts. The inner envelope protein IEP32, also named HP32, is targeted to chloroplasts independent of a cleavable pre-sequence and any protease-sensitive surface-exposed receptor protein. Import of IEP32 does not seem to require the Toc75 import channel as deduced from inhibitor studies. The involvement of stromal chaperones is also unlikely because ATP concentrations below 20 μ M are sufficient for import.

Materials and Methods

Transcription and translation

The coding region for IEP32 from pea (GenBank accession no. AY488758) was cloned into the vector PSP65 (Promega, Madison, USA) under the control of the SP6 promoter. Deletion mutants were constructed in the same vector using standard PCR protocols. All constructs were controlled by DNA sequencing. Transcription was performed in the presence of SP6 RNA polymerase and the resulting mRNA was translated in a reticulocyte lysate system (FlexiSystem, Promega, Madison, USA) in the presence of [³⁵S]methionine (Waegemann and Soll, 1995) at 25°C for 45 minutes. The translation mixture was then centrifuged at 250,000 g for 10 minutes at 4°C and the post-ribosomal supernatant was used for all import studies.

Chloroplast isolation and protein import

Chloroplasts were isolated from leaves of 10- to 12-day-old pea plants (*Pisum sativum*, var. Golf) and purified through Percoll density gradients as described (Waegemann and Soll, 1991). A standard import reaction contained chloroplasts equivalent to 15 μ g chlorophyll in 100 μ l import buffer (10 mM methionine, 10 mM cysteine, 20 mM potassium gluconate, 10 mM NaHCO₃, 330 mM sorbitol, 50 mM HEPES-KOH, pH 7.6, 5 mM MgCl₂) and 1-5% in vitro translation product. Import reactions were initiated by the addition of translation product and carried out for 15 minutes at 25°C unless indicated otherwise. Reactions were terminated by separation of chloroplasts from the reaction mixture by centrifugation through a 40% (v/v) Percoll cushion in import buffer. Chloroplasts were washed once and import products separated by SDS-PAGE and radiolabelled proteins analysed by a phosphor-imager.

Chloroplasts were treated with the protease thermolysin either before or after import at a protease concentration of 20 μ g/ml for 20 minutes on ice (Waegemann and Soll, 1995). Chloroplasts were repurified through Percoll density gradients before further use (Waegemann and Soll, 1995). Inhibitors like CuCl₂ (1 mM) (Seedorf and Soll, 1995) or spermine (5 mM) (Hinnah et al., 2002) were incubated with chloroplasts 20 minutes prior to import.

In some cases chloroplasts were treated with 6 M urea after import to separate bound pre-proteins from membrane-integrated polypeptides. Urea (6 M) treatment in 50 mM HEPES-KOH, pH 7.6 was carried out for 15 minutes at 25°C. Insoluble proteins were collected by centrifugation at 250,000 g for 10 minutes.

Chemical crosslinking was performed after separation of

chloroplasts from the import mixture by centrifugation as above in the presence of 0.5 mM dithiobis-succinimidyl-propionate (DSP) for 30 minutes at 4°C. The reaction was stopped by the addition of lysine (125 mM) and further incubation for 15 minutes. Chloroplasts were washed twice in 330 mM sorbitol, 50 HEPES-KOH, pH 7.6, 3 mM MgCl₂ and finally lysed in hypotonic buffer 20 mM HEPES-KOH, pH 7.0, 5 mM EDTA. A total-membrane fraction was recovered by centrifugation at 125,000 g for 30 minutes. Membranes were solubilized in 1% SDS (w/v), 25 mM HEPES-KOH pH 7.6, 150 mM NaCl for 10 minutes at 25°C, diluted tenfold in the above buffer in the absence of SDS and used for immunoprecipitation experiments with antisera to Tic110, Toc75 V, Toc75III, Tic22 and OEP21. Antisera incubations were continued for 1 hour followed by purification by Protein A-Sepharose. The affinity matrix was washed with 50 bed-volumes of the above buffer before elution with Laemmli sample buffer in the presence of mercaptoethanol to split the crosslink products.

Results

In a proteome analysis of inner envelope membranes from spinach chloroplasts Ferro and colleagues identified a protein HP32 that showed strong homologies to the *Arabidopsis* gene product At4g23430 (Ferro et al., 2003). A similar protein was identified in our studies using pea envelope membranes. We sequenced three tryptic fragments by Edman degradation and used this information to screen a pea cDNA library for a full-length clone (GenBank accession no. AY488758). The deduced amino acid sequence showed strong homology to At4g23430 (D. Sveshnikov and J.S., unpublished). The inner envelope localisation of IEP32 in pea chloroplasts was confirmed using an antiserum raised against the heterologously expressed protein (not shown). Owing to its apparent molecular weight on SDS-PAGE we named the protein IEP32. Surprisingly, the most N-terminal peptide that we sequenced comprised amino acids 10-23 and 9-22 of the pea or the *Arabidopsis* cDNA respectively, indicating that IEP32 has a very short or no cleavable targeting signal. In order to test this idea IEP32 was synthesized in vitro in a rabbit reticulocyte lysate system and the [³⁵S]-labelled pre-protein was incubated with isolated pea chloroplasts in the presence or absence of ATP. As an endogenous control we included the radiolabelled precursor of the Rubisco small subunit (pSSU) into each import reaction. In the presence of 3 mM ATP the pSSU precursor bound to the chloroplast surface and was imported as deduced from the presence of the processed lower molecular weight form mSSU (Fig. 1A, lane 2). The surface-bound precursor form, but not mSSU, was sensitive to the protease, indicating that mSSU had reached the inside of the chloroplast, i.e. the stroma (Fig. 1A, lane 3). IEP32 translation product also bound to the chloroplast surface under these conditions, however no processed lower molecular weight form could be detected. Upon protease treatment most of the chloroplast-bound IEP32 remained intact, indicating that it was either not protease sensitive or that it had entered the organelle and was in a protease-protected localisation following its import. In an attempt to verify if IEP32 had actually been imported we tested if the protein was protease sensitive. Radiolabelled IEP32 translation product was centrifuged at 200,000 g for 10 minutes and the soluble as well as the aggregated protein, which was recovered in the pellet, were treated with thermolysin. In both cases IEP32 was completely proteolyzed (Fig. 1B, lanes 4-7), indicating that the protein is not protease resistant per se. IEP32

behaves as an integral membrane protein (F. Hörmann and J.S., unpublished). We therefore used extraction by 6 M urea after import to test the membrane insertion of IEP32. About 80% of IEP32 was not extractable by urea, whereas all of mSSU was recovered in the soluble urea extract (Fig. 1C). In contrast IEP32 translation product is recovered largely in the soluble phase (Fig. 1B, lanes 1-3).

In the presence of $<20 \mu\text{M}$ ATP, which is carried into the import reaction from the translation mixture, binding of pSSU still occurred but in accordance with published data (Olsen et al., 1989; Theg et al., 1989) import was greatly reduced (Fig. 1A, lanes 4,5). Surprisingly the import of IEP32 in the presence of $<20 \mu\text{M}$ ATP was as efficient as in the presence of 3 mM ATP (Fig. 1A, compare lanes 3 and 5). In order to examine if

IEP32 import required ATP the translation products were treated with the ATP hydrolysing enzyme apyrase to remove exogenous ATP and the subsequent import reaction was carried out in the dark. In the absence of ATP the import of IEP32 is largely diminished. In general a 4- to 5-fold stimulation in the presence of ATP can be seen. Residual IEP32 bound to the chloroplast surface might be caused by partial import or aggregation of the protein at the chloroplast surface. PSSU neither bound nor imported in the absence of ATP. We conclude that the import of IEP32 is dependent on ATP but the ATP concentration required is much lower than for proteins that enter the stroma like pSSU (Fig. 1D, lanes 4,5).

In order to verify that IEP32 had actually reached the inner envelope, chloroplasts were fractionated into the soluble stroma, which contained processed mSSU as expected. Most IEP32 was recovered with the inner envelope, whereas only a little IEP32 co-fractionated with the outer envelope membrane (Fig. 1E). As outer envelope preparations always contain some inner envelope membrane contamination, we conclude that IEP32 has successfully reached its target membrane. The results from urea extraction, ATP dependence and chloroplast fractionation indicate that most IEP32 is imported into the inner envelope and that only about 20% of chloroplast-recovered IEP32 is on a non-productive pathway.

The data indicated that IEP32 could be imported without a cleavable pre-sequence, we therefore wanted to know how IEP32 is targeted to chloroplasts. Amino-terminal deletion mutants of IEP32 were constructed, in which we progressively

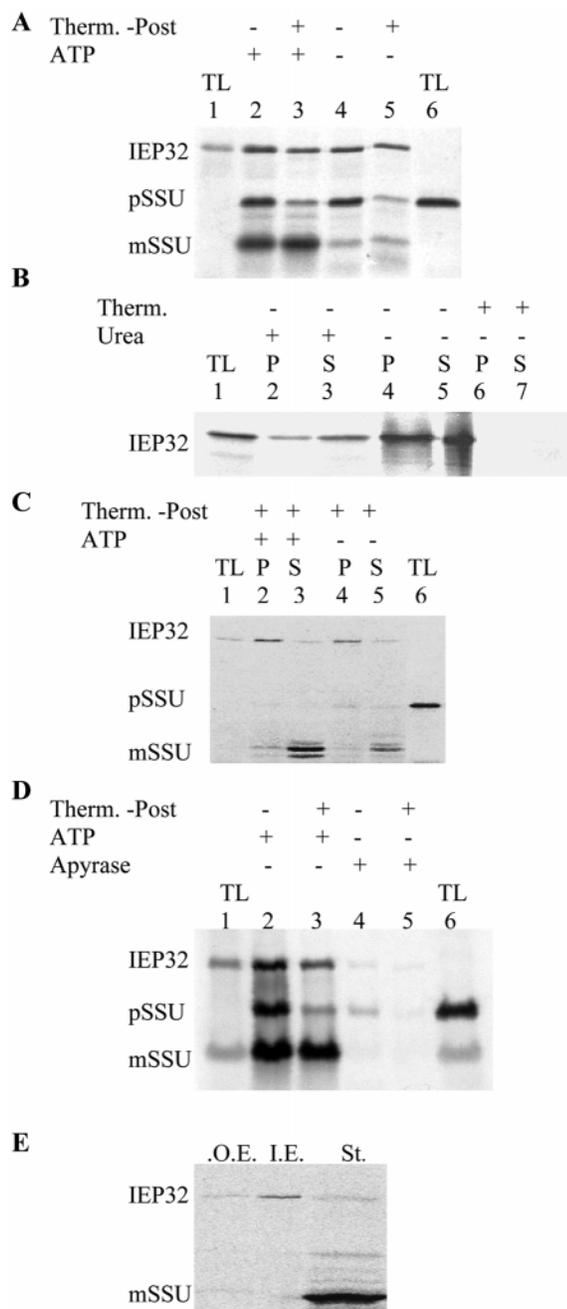


Fig. 1. Import of inner envelope protein 32 (IEP32) into chloroplasts requires ATP. (A) Purified pea chloroplasts equivalent to 15 μg chlorophyll were incubated simultaneously with in vitro translated [^{35}S]-labelled pre-proteins (TL) Rubisco small subunit pSSU and IEP32 in the presence of 3 mM ATP (lanes 2,3) or $<20 \mu\text{M}$ ATP (lanes 4,5), which was carried over from addition of the translation mixture. A post-ribosomal supernatant was used (see Materials and Methods). After incubation for 15 minutes at 25°C chloroplasts were repurified by centrifugation through a Percoll cushion at 4°C. Chloroplasts were then either treated or not treated with the protease thermolysin (Therm.-Post) as indicated. Intact chloroplasts were recovered by centrifugation and products analysed by SDS-PAGE and phospho-imager. Lanes 1 and 6 show 1/10 of the translation product (TL) added to each import reaction. pSSU was used as an endogenous control and was imported and processed as determined by the presence of the lower molecular weight form (mSSU). (B) IEP32 translation product (TL lane 1) was incubated with 6 M urea and separated into a soluble (S) and pellet (P) fraction by centrifugation for 10 minutes at 250,000 g . A post-ribosomal supernatant was obtained from the IEP32 translation product by centrifugation for 10 minutes at 250,000 g . Aggregated IEP32 recovered from the pellet (P) or soluble IEP32 from the supernatant (S) (lanes 2,3) was either not treated (lanes 4,5) or treated (lanes 6,7) with the protease thermolysin (Therm). (C) [^{35}S]-labelled IEP32 translation product was imported into chloroplasts as outlined in (A). Chloroplasts were then extracted by 6 M urea for 15 minutes at 25°C. Urea-insoluble proteins in the pellet (P) were separated from soluble proteins in the supernatant (S) by centrifugation and products analysed as outlined above. (D) Translation products were either treated (lanes 4,5) or not treated (lanes 2,3) with the ATP hydrolysing enzyme apyrase prior to the import reaction. All other conditions were as outlined in (A). (E) Chloroplasts were fractionated after import into outer (O.E) and inner envelope (I.E) membranes and the stroma (St).

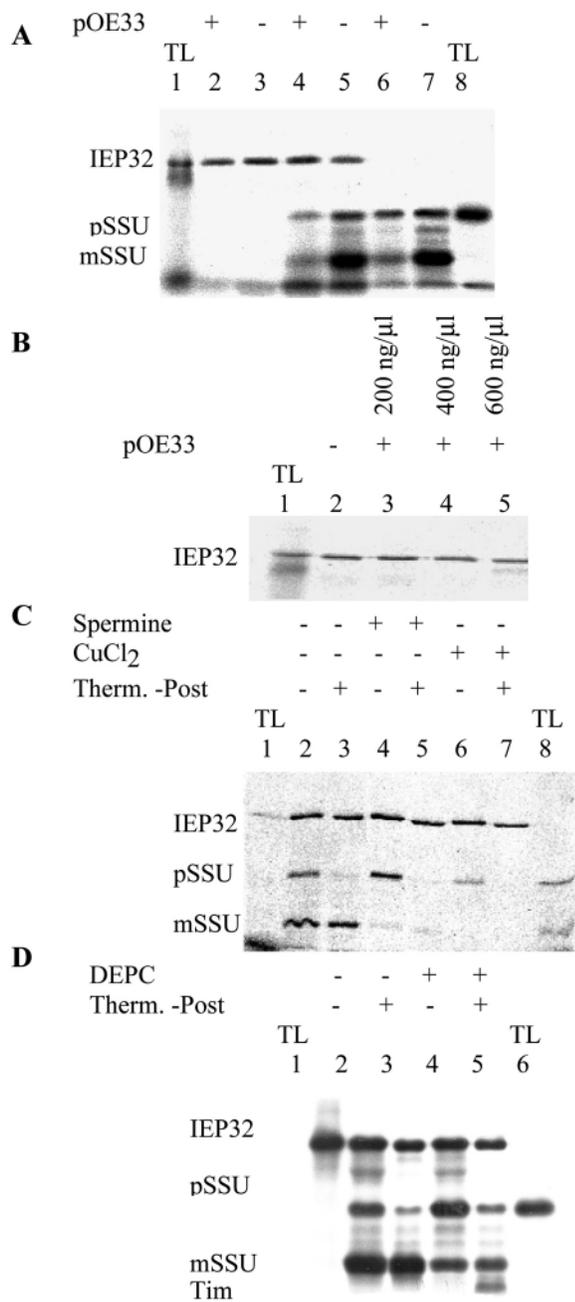


Fig. 4. Inner envelope protein 32 (IEP32) import into chloroplasts is not affected in the presence of different Toc translocon inhibitors. (A) Import of IEP32 and Rubisco small subunit pSSU was carried out in the presence of the chloroplast precursor pOE33 at a final concentration of 200 ng/ μ l as outlined in the Materials and Methods. Lanes 2,3 and lanes 6,7 show import reactions in the presence of a single pre-protein IEP32 and pSSU, respectively. Lanes 4 and 5 show a competition experiment in the presence of both pre-proteins. (B) Import of IEP32 was assayed in the presence of increasing pOE33 competitive concentrations as indicated. (C) Import of IEP32 and pSSU was conducted in the absence (lanes 2,3) or presence (lanes 4,5) of 5 mM spermine. In lanes 6 and 7 chloroplasts were pretreated with 1 mM CuCl₂ for 20 minutes at 4°C and used for import under standard conditions. All other conditions were as outlined and as described in Fig. 1A. (D) Chloroplasts were treated with 1 mM DEPC prior to carrying out the import reaction. All other conditions were as before. TIM: translocation intermediate.

In a second approach we used the inhibitors spermine and CuCl₂. CuCl₂ inhibits import because it catalyses the formation of disulfide bonds between Toc subunits and therefore inactivates its translocation activity (Seedorf and Soll, 1995). The positively charged spermine binds to Toc75 and blocks the import channel (Hinnah et al., 2002). In the presence of 2 mM spermine import of pSSU was strongly reduced (Fig. 4C, compare lanes 3 and 5), whereas binding is slightly increased. The data corroborate earlier findings that the effect of spermine on import is at a step after recognition but before translocation (Hinnah et al., 2002). In the presence of CuCl₂, translocation of pSSU is again completely inhibited and binding is reduced (Fig. 4B, lanes 6,7). IEP32 import was however influenced neither by spermine nor by CuCl₂ (Fig. 4B, lanes 2-7). We conclude from the competitor and inhibitor experiments that Toc75III is not involved in translocation of IEP32, but that IEP32 uses a so far unidentified pathway across the outer envelope. Diethylpyrocarbonate (DEPC) has been shown to inhibit import of pSSU at the level of the inner envelope (Caliebe et al., 1997). The import of pSSU into chloroplasts in the presence of DEPC yields a typical translocation intermediate, indicating that the Toc complex is still functional (Caliebe et al., 1997). IEP32 import was hardly affected in the presence of 1 mM DEPC, whereas pSSU import was drastically reduced (Fig. 4D). More pSSU remained in the precursor form, which could be converted into a lower molecular weight translocation intermediate upon thermolysin treatment (Fig. 4D, lane 5/TIM). These data indicate that IEP32 might insert into the inner envelope independent of the standard Tic pathway.

Chemical crosslinking can be used to identify proteins that are in close proximity to each other. In order to identify potential factors that are involved in the translocation of IEP32, we used a crosslinking approach under different import conditions, which should result in the formation of distinct translocation intermediates (Waegemann and Soll, 1991; Ma et al., 1996). Chloroplasts were incubated in the presence of the pSSU and IEP32 pre-proteins in the presence of the crosslinker DSP. Each pre-protein gave rise to a distinct set of labelled crosslinked products in the presence of chloroplasts (Fig. 5A). Though IEP32 gave rise to fewer crosslinked products than pSSU (Fig. 5A, lanes 3,4) they were clearly visible and distinct from the crosslinking profile of pSSU. In order to identify at least some of the crosslinked products they were co-immunoprecipitated by antibodies against the receptor polypeptides Toc159, Toc64 and Toc34 (Fig. 5B) as well as the import channel Toc75III, its isoforms Toc75V, the intermembrane space subunit Tic22 and Tic110. As a control we used an antibody raised against the outer envelope solute channel OEP21. Binding to the chloroplast receptors Toc159 and Toc34 is GTP dependent (Kessler et al., 1994; Schleiff et al., 2003). We therefore investigated the role of these two GTP receptors for IEP32 recognition in the presence of the non-hydrolyzable GTP analog GMP-PNP, which leads to an accumulation of pre-proteins at this very early import step. The pre-protein pSSU was crosslinked efficiently to Toc34, Toc64 and Toc159 (Fig. 5B), whereas IEP32 shows no detectable interaction. These results corroborate the data shown in Fig. 3, which also indicate that protease-sensitive Toc subunits are not involved in the import of IEP32. Under conditions that allow binding as well as partial translocation but not complete

import, i.e. incubation for 5 minutes at $<20 \mu\text{M}$ ATP and 4°C , crosslinking of pSSU was observed predominantly to Toc75III, Tic22 and to a lesser extent Tic110. No crosslinked products of pSSU could be observed to Toc75V and OEP21 (Fig. 5C). IEP32 did not yield any significant crosslink products under these conditions. When import reactions were carried out at 25°C for 5 minutes in the presence of 3 mM ATP further crosslinked products were formed. Although the pSSU-Toc75III, -Tic22, -Tic110 crosslinked products were still prominent, crosslinking of Tic22 and Tic110 to processed mature mSSU started to appear, supporting their role in the later stages of translocation (Kessler and Blobel, 1996; Kouranov and Schnell, 1997). In addition, low but reproducible amounts of IEP32 were recovered together with Tic110

and Tic22, but not with Toc75III or Toc75V (Fig. 5D), corroborating our earlier finding that none of the well-documented Toc subunits is involved in IEP32 translocation (compare Figs 3 and 4).

When crosslinking was carried out after 15 minutes' import at 25°C in the presence of 3 mM ATP the detectable products changed quite significantly. In comparison to the 5-minute time point much less pSSU was crosslinked to Toc75, Tic22 or Tic110 (Fig. 5E). Instead mSSU was the most prominent crosslinked product associated with Tic110 and Tic22 indicating that the import reaction was near completion. Under these conditions IEP32 was still crosslinked only to Tic22 and Tic110, indicating that these two proteins could be involved in IEP32 import. The association of IEP32 with Tic110 might

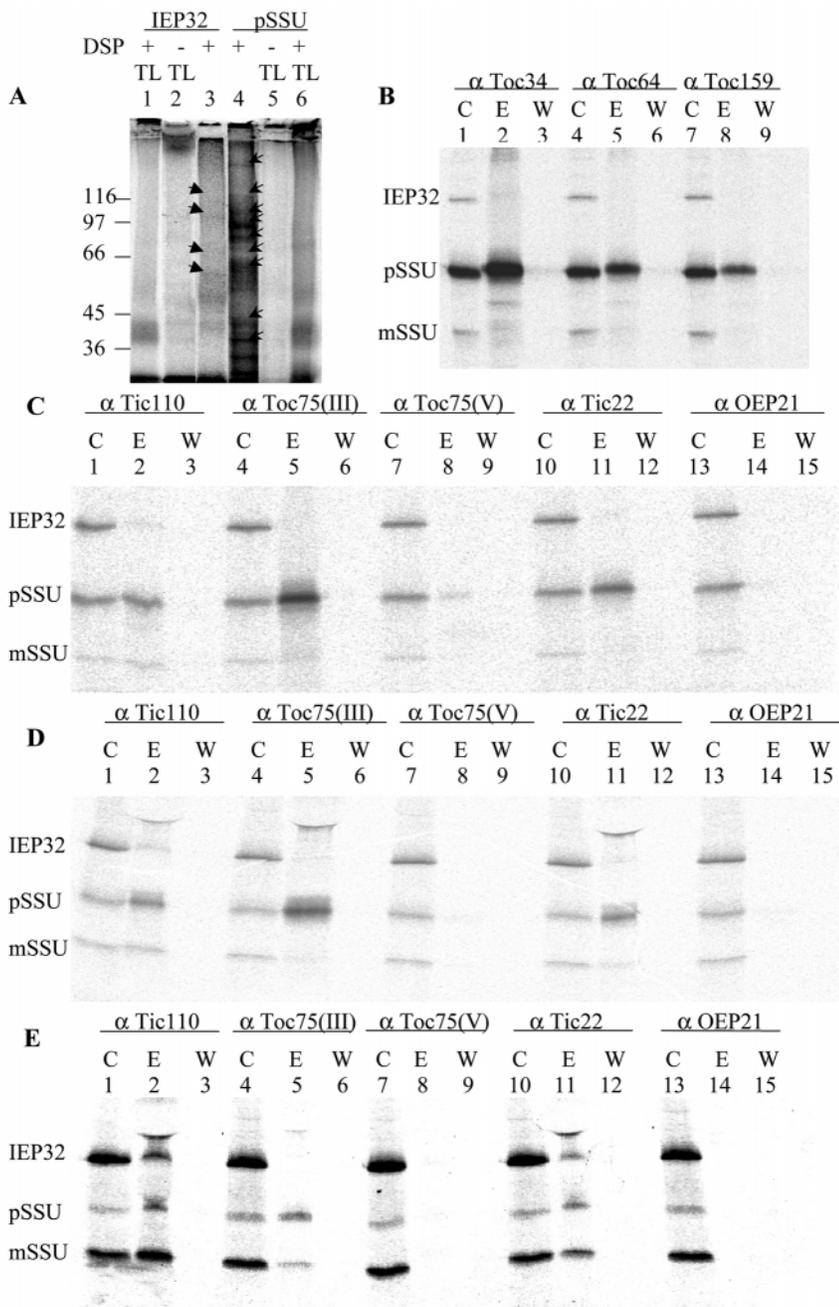


Fig. 5. Chemical crosslinking of inner envelope protein 32 (IEP32) and Rubisco small subunit pSSU pre-proteins to chloroplast translocon subunits. (A) IEP32 or pSSU [^{35}S]-labelled translation products (lanes 2,5) were incubated with 0.5 mM DSP either in the absence (lanes 1,6) or presence (lanes 3,4) of purified pea chloroplasts for 10 minutes at 3 mM ATP and separated by SDS-PAGE. Arrows indicate crosslinked protein products of IEP32 (lane 3) and Rubisco small subunit pSSU (lane 4). (B-E) Chloroplasts were incubated with [^{35}S]-labelled translation products under different conditions. Crosslinking was carried out for 30 minutes at 4°C in the presence of 0.5 mM DSP. Chloroplasts were then solubilized by 1% SDS and immunoprecipitation carried out after dilution to 0.1% SDS with antisera to various receptor/membrane proteins as indicated. (B) Chloroplasts were incubated for 5 minutes at 4°C in the presence of $<20 \mu\text{M}$ ATP and $<100 \mu\text{M}$ GMP-PNP. (C) Chloroplasts were incubated with pre-proteins for 5 minutes at 4°C in the presence of $<20 \mu\text{M}$ ATP. (D) Chloroplasts were incubated with pre-proteins for 5 minutes at 25°C and 3 mM ATP. (E) Chloroplasts were incubated with pre-proteins for 15 minutes at 25°C and 3 mM ATP. C, total chloroplasts; E, eluate from protein A agarose by SDS-sample buffer in the presence of mercaptoethanol to split the crosslink product; W, wash of protein A Sepharose with buffer.

however also represent the endpoint of IEP32 import, because it is closely associated with Tic110 in situ (F. Hörmann and J.S., unpublished).

Discussion

Most of the known nuclear-encoded chloroplast proteins are synthesized in the cytosol with an N-terminal pre-sequence that is cleaved upon import inside the organelle by the stromal processing peptidase. QORH (Miras et al., 2002) and IEP32 (this study) are the only documented examples to date of chloroplast proteins that import without a cleavable transit peptide. QORH was identified in a proteome approach as an inner envelope protein. Biochemical and computational analysis suggested that the protein did not contain a cleavable transit peptide. In vitro protein import studies were not conducted, however transient expression of a GFP-tagged QORH in *Arabidopsis* protoplasts suggested that the protein targets to chloroplasts without a cleavable pre-sequence, because the first 59 amino acids could be deleted without disturbing chloroplast targeting (Miras et al., 2002). This observation is different from the findings of this study that indicate that the most N-proximal amino acids are essential for targeting and import of IEP32. It seems that chloroplast targeting signals can be spread over a certain range within the pre-protein.

Tic22 is localized to the intermembrane space between the inner and the outer envelope (Kouranov et al., 1998). Its import characteristics are also different from pre-proteins that normally traverse both envelope membranes and import into the stroma (Kouranov et al., 1999). Tic22 is made in the cytosol with a cleavable pre-sequence, binding and import is stimulated by protease-sensitive chloroplast surface components, however import is not competed for by excess of a 'typical' pre-protein like pSSU and it requires ATP concentrations below 100 μ M for maximum import yields (Kouranov et al., 1999). These characteristics again differ in some respect from IEP32 import as IEP32 is not synthesized with a cleavable pre-sequence and does not require protease-sensitive receptors. Similarities do exist however; for example import of both pre-proteins requires only low ATP concentration and is not competed for by an excess of precursor protein. Together the data indicate that alternative import routes into chloroplasts exist, which can be distinguished from the general route into chloroplasts (Chen and Schnell, 1999; Keegstra and Froehlich, 1999; Bauer et al., 2001; Soll, 2002) by the following criteria: (i) energy requirement, (ii) presence of a cleavable pre-sequence, (iii) involvement of protease-sensitive surface components; (iv) competition by stroma-targeted pre-proteins; (v) usage of a different import channel than Toc75III. It should be noted however that the import characteristics found here and in other published studies all describe pathway(s) for polypeptides that are affiliated with the inner envelope membrane (Kouranov et al., 1999; Miras et al., 2002). These proteins most likely do not traverse the inner envelope completely, but are sorted to their final destination by as yet unidentified signals. This is also supported by the missing effect of DEPC on IEP32 translocation. This system could resemble a stop-transfer mechanism described for mitochondrial inner membrane protein import (Pfanner and Geissler, 2001).

Chemical crosslinking studies as well as competition experiments indicate that IEP32 does not use a Toc75 import channel, but that the outer envelope must contain further channel proteins that allow the passage of polypeptides. The outer envelope is known to contain several solute channels (Bölter et al., 1999), however none of them seem to be involved in IEP32 import. CuCl_2 treatment inhibits not only the Toc translocon but also the amino acid-selective ion channel OEP16 (Pohlmeyer et al., 1998). Furthermore, no crosslinks between the anion-selective channels OEP21 and OEP24 could be observed (Fig. 5 and not shown). Although this is negative evidence it supports our idea that additional ion channels have to be present in the outer envelope membrane.

IEP32 behaves as an integral membrane protein. How these hydrophobic proteins cross the aqueous intermembrane space is unknown. Tic22, which is localized in the envelope space might be a cofactor in this process and might function in a way similar to the small Tim proteins (Pfanner and Geissler, 2001) in the space between the outer and the inner mitochondrial membrane. The small Tim proteins are required for the translocation of hydrophobic carrier proteins into the inner mitochondrial membrane (Pfanner and Geissler, 2001). We obtained a clear crosslink product between Tic22 and IEP32 indicating that Tic22 is involved in the import of IEP32. The other crosslinked product was found to be Tic110. Whether Tic110 is involved in the actual import process of IEP32 cannot be resolved, because IEP32 and Tic110 are closely associated with each other in situ (F. Hörmann and J.S., unpublished). Therefore the observed crosslink could represent only the end point of import and indicate that IEP32 assembled correctly.

We conclude that IEP32 follows a distinct import pathway into chloroplasts, which shows many special features not observed by precursor proteins that are translocated across both envelope membranes. The major challenge for the future will be to identify the outer envelope components involved in recognition and translocation of IEP32.

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