A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide

Bettina Bölter[1], Timo May, Jürgen Soll*

Botanisches Institut, Universität Kiel, Am Botanischen Garten 1–5, D-24118 Kiel, Germany

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Abstract The protein import complex of the chloroplastic outer envelope (Toc-complex) contains a prominent subunit of 86 kDa molecular weight (Toc86). Toc86 was identified as a putative precursor receptor. The *Arabidopsis* genome sequencing project indicates that Toc86 represents only a proteolytic fragment of a larger polypeptide of 160 kDa. The 160-kDa protein, which we name Toc160, is only present in significant amounts in pea chloroplasts isolated under stringent conditions. The capacity of chloroplasts to import an in vitro translated precursor protein correlates well with the integrity of Toc160. We conclude that Toc160 is still a bonafide subunit of the protein import machinery of chloroplasts.

Key words: Chloroplast; Protein import; Precursor protein; Receptor; *Pisum sativum*

1. Introduction

Posttranslational protein import into chloroplasts is accomplished by the joint action of two hetero-oligomeric protein complexes in the outer and inner envelope membranes. Key subunits of the translocon at the outer envelope of chloroplasts (Toc-complex) have been described and functionally characterized ([1–3], for a review see [4]). Toc75 forms the preprotein translocation channel with an aqueous pore >2 nm [5,6]. Toc34 is a GTPase in close association with Toc75 and is attributed a role in preprotein translocation regulation [7,8]. Two HSP70 homologues associated with either the *cis* or the *trans* site of the outer envelope, respectively, are also involved in a productive translocation cycle [1,9,10].

Toc86 was identified as a putative import receptor by different experimental approaches. (i) Toc86 forms a major cross-link product with precursor proteins in the absence of ATP [3]; (ii) Fab fragments generated from antibodies against Toc86 inhibit binding and subsequent translocation of a precursor protein [11]. Toc86 is a major protein in the chloroplastic outer envelope from pea and was used as a marker protein during the separation and isolation of outer and inner envelope membranes [12]. A large N-terminal domain is exposed to the cytosol and is easily degraded by proteases yielding a 52-kDa C-terminal fragment [11,13]. Indeed, during the standard isolation procedures of chloroplasts and subfractionation into membranes the 52-kDa fragment was generated to a very variable degree. The deduced protein sequence of the cDNA clones isolated [11,14] contained the N-terminus of Toc86, as deduced from protein micro-sequence data, and a putative presequence. However, no stop codon was found in the 5′-ends of the cDNAs isolated. Recently, a genomic sequence from *Arabidopsis thaliana* was published (Acc. Nos. AC002330 and AF069298) which showed high identity to Toc86 on the DNA as well as on the deduced amino acid sequence level. The open reading frame, however, coded for a protein of 160 kDa molecular mass. These data indicated that Toc86 represented a C-proximal portion of a much larger protein. We therefore reinvestigated the presence of Toc86 and the 160-kDa protein in pea chloroplasts. Our results indicate that protein translocation efficiencies correlate with the presence of the 160-kDa protein. The protein should therefore be renamed Toc160.

2. Material and methods

2.1. Isolation of intact chloroplasts

Chloroplasts were isolated from 8–10 days grown pea plants in a kitchen blender equipped with razor blades [1,15] in 330 mM sorbitol, 20 mM MOPS, 13 mM Tris, 3 mM $MgCl_2$ and 0.1% (w/v) BSA, pH 7.6. After filtration through 4 layers of cheese cloth and a nylon gauze (30 μm mesh) organelles were recovered by centrifugation at $2000\times g$ for 45 s. The crude chloroplast pellet was resuspended and the slurry layered directly on a 40/80% Percoll step gradient containing 330 mM sorbitol and 50 mM HEPES/KOH, pH 7.6. The Percoll gradient was centrifuged in a swinging bucket rotor at $7000\times g$ for 4 min. Intact purified chloroplasts were recovered from the 80% Percoll interface and washed twice in excess medium containing 330 mM sorbitol, 50 mM HEPES/KOH, pH 7.6 and 3 mM $MgCl_2$. Organelles were recovered by centrifugation at $1800\times g$ for 45 s. Chlorophyll was determined as described [16].

2.2. Isolation of envelope membranes

Outer envelope membranes were isolated from chloroplasts equivalent to 5 mg chlorophyll as described [17] with the following modifications. All media for the isolation of chloroplasts and membranes contained a mixture of protease inhibitors (Sigma Product No. P8340, Boehringer Mannheim Complete Product No. 1697 498, at conditions recommended by the manufacturers), in addition 100 μg/ml buffer α-macroglobulin were added. Chloroplasts were incubated in hypertonic sucrose solution (0.65 M sucrose in 50 mM HEPES/KOH, pH 7.6) and lysed by 50 passages in a Dounce homogenizer. The slurry was diluted to a chlorophyll concentration of 0.5 mg/ml and centrifuged for 45 s at $1800\times g$ to remove most of the thylakoid membranes and intact chloroplasts. The supernatant was layered on top of a sucrose step gradient of 0.465/0.8/1 M sucrose in 50 mM HEPES/KOH, pH 7.6, and centrifuged in a swinging bucket rotor (SW55, Beckman Instruments) at $300\,000\times g$ for 1 h. Outer envelope membranes were recovered from the 0.8 M sucrose interface, diluted five-fold in 50 mM HEPES/KOH, pH 7.6, and concentrated by centrifugation at $165\,000\times g$ for 10 min. Purified membranes were frozen in liquid nitrogen or used immediately for further analysis. All of the above purification steps were carried out at a temperature between 0 and 2°C.

*Corresponding author. Fax: (49) (431) 880-4222.
E-mail: jsoll@bot.uni-kiel.de

[1]These authors contributed equally to this paper.

Abbreviations: preSSU, precursor form of ribulose-1,5-bisphosphate carboxylase; mSSU, mature form of preSSU

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2.3. Miscellaneous methods

Protein import assays were carried out essentially as described before [1] using chloroplasts equivalent to 7.5 μg chlorophyll in a 100-μl import assay for the times indicated. Organelles were recovered by centrifugation prior to analysis by SDS-PAGE [18] and fluorography [19].

Immunoblots [20] were done using an antiserum against Toc34 [7] or Toc86. The Toc86 antiserum was raised in a rabbit against the authentic outer envelope protein which was purified by SDS-PAGE and electroeluted prior to injection.

3. Results and discussion

During a comparison of the protein sequences of known Toc components with sequences deduced from the *Arabidopsis* genome project we and others (D. Schnell, personal communication, [6]) observed that a gene coding for a polypeptide of 160.8 kDa molecular mass (Acc. Nos. AC002330 and AF069298) indicated strong similarities to the gene coding for Toc86 from pea. Comparison of the amino acid sequences deduced from the genomic clone from *Arabidopsis* and the cDNA clone from pea shows 66% identical amino acids and in addition 9% homologous exchanges (Fig. 1). Pea Toc86 might therefore represent only a C-terminal fragment of a much larger protein present also in pea. The calculated isoelectric point of 4.6 indicates a prevalence of negative charged residues in *Arabidopsis* Toc160 which are mostly located in the first 700 amino acids (Fig. 1). Clustering of negative charges in the cytosolic domains of this protein might be involved in the electrostatic binding process of positively charged transit sequences in plastidic precursor proteins. The preferences of the Toc75 import channel for positively charged ions and peptides [5,21] supports the functional properties of charged residues in the binding process. However, further independent protein-protein recognition and interaction must occur to con-

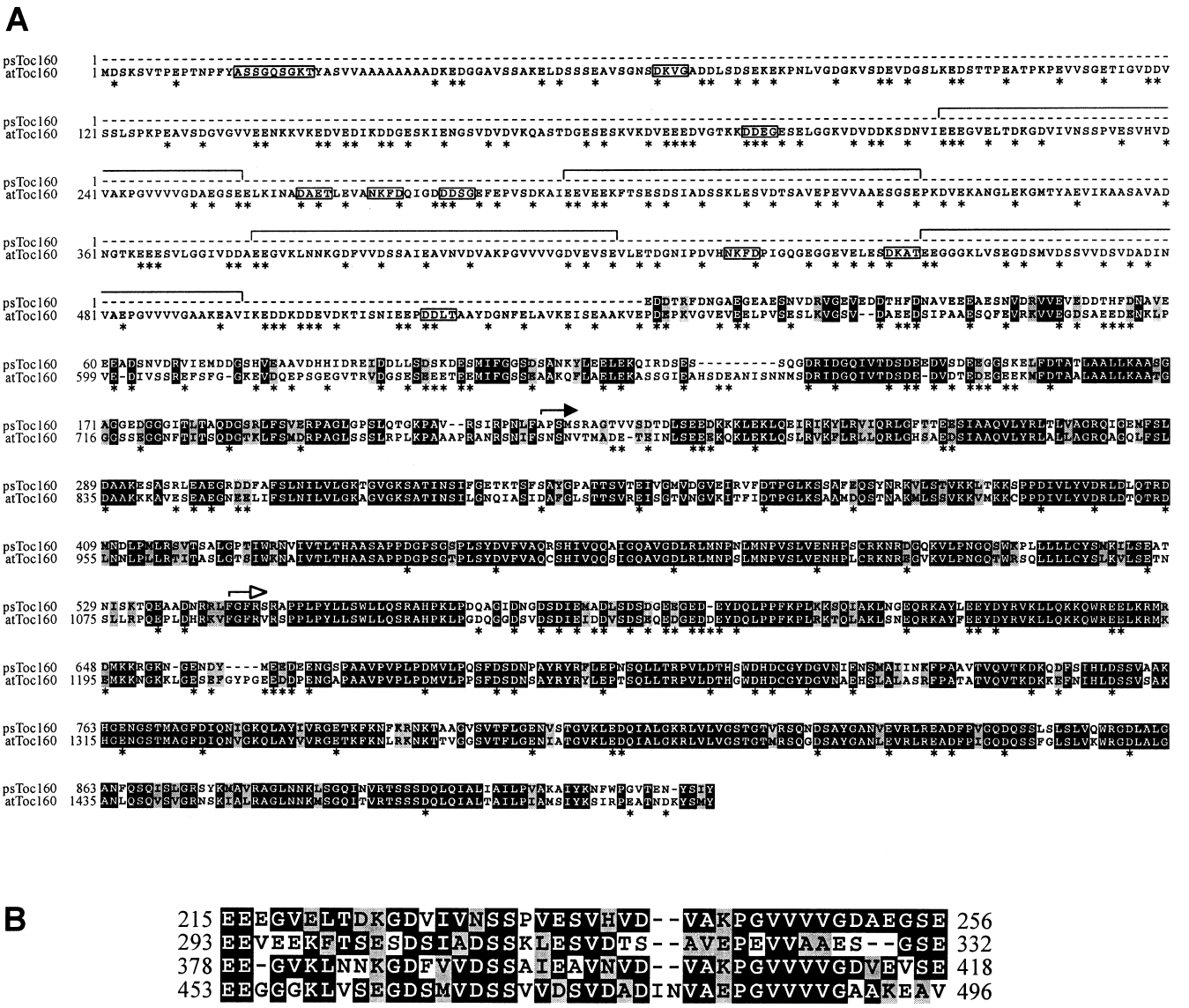

Fig. 1. A: Comparison of the deduced amino acid sequences of a pea (ps) cDNA (Acc. No. 1076524) and an *Arabidopsis* (at) genomic DNA sequence (Acc. No. AF069298). Identical and homologous amino acids are boxed in black and grey, respectively. The putative nucleotide binding site in the N-terminus is boxed. A repeated amino acid motif is indicated by brackets. The beginning of the 86-kDa and the 52-kDa fragments as determined by protein sequencing [11] are indicated by a closed and an open arrow, respectively. B: Alignment of the amino acid repeat. Numbers indicate amino acid position in the deduced sequence of atToc160.

vey specificity and selectivity of the binding event and to avoid an overlap with mitochondrial binding and translocation processes, which also seem to be supported by electrostatic interactions via acid-bristle domains present in different subunits of the TOM-complex [22,23]. Besides the previously described nucleotide binding domain [11,14] a further putative one could be detected (Fig. 1). The putative nucleotide binding domain is found in connection with a 4 times repeated stretch of 40–44 amino acids (Fig. 1B). This motif is highly conserved in the ATP binding region of protein kinases and shows weak homology to the SH3 domain as determined by the Blocks Search program [24]. The mechanistic consequences of this observation for Toc160 function remain to be established.

Next we tried to establish experimental conditions which allowed the isolation of silica-sol purified chloroplasts, still containing non-proteolyzed Toc160. Various commercial protease inhibitors were tested in our standard chloroplast isolation protocol ([1], see also Section 2) but this did not result in a significant increase in the appearance of Toc160. Only when we optimized our chloroplast and envelope membrane isolation procedures, $>70\%$ of Toc160 were actually detectable as full-length protein. Crucial experimental details were the quickness of the isolation of organelles and the temperature. Firstly, the time required from grinding the pea leaves to the start of an import experiment or to the solubilization of organelles in Laemmli buffer was optimized to 15 min instead of 40 min as done previously. Secondly, special care was taken that at no time during the isolation the temperature of the isolation media increased to more than 2°C. Under these optimized conditions more than 70% were not yet proteolyzed, however, the 86-kDa form of Toc160 was already detectable (Fig. 2, lane 1). Further proteolytic fragments were observed which are most likely intermediates in the degradation of Toc160 and were previously not detected (Fig. 2, lane 1). A prolonged incubation for 60 min on ice or ambient temperature resulted in a much more pronounced or complete degradation of Toc160, respectively (Fig. 2, lanes 2, 3).

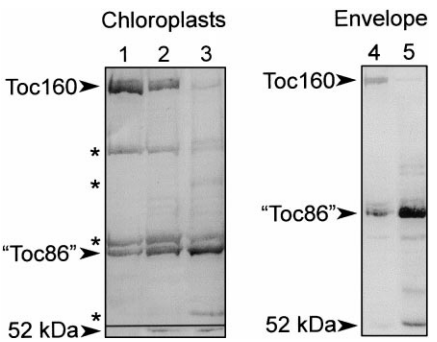

Fig. 2. Immunoblot analysis of pea chloroplasts and purified outer envelope membranes by an antiserum against Toc86. Intact pea chloroplasts were purified as described in Section 2 and prepared for SDS-PAGE by boiling in sample buffer either immediately (lane 1), after 45 min on ice (lane 2) or after 60 min at ambient temperature. Outer envelope membranes from pea chloroplasts were isolated by an improved fast isolation procedure in the presence of protease inhibitors or by standard procedures (as outlined above). The position of Toc160, 'Toc86' and the 52-kDa fragment is indicated. The asterisks (*) indicate further proteolytic fragments. The immunoblot (lanes 1–3) demonstrating the presence of the 52-kDa fragment was left for 10 min in staining solution instead of 5 min for the upper part of the blot.

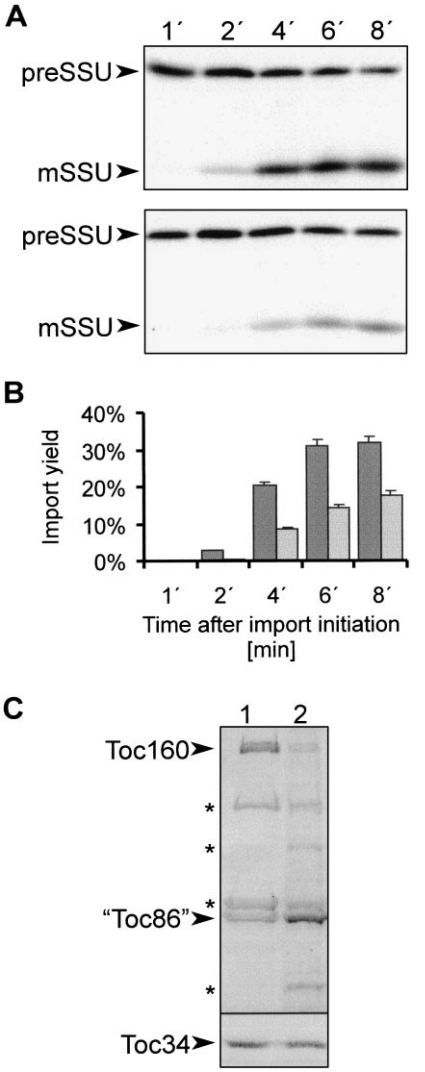

Fig. 3. The protein translocation capacity correlates with the presence of Toc160. A: ^{35}S-labeled preSSU translation product was incubated with intact chloroplasts for the various times indicated (1–8 min). Samples were analyzed by SDS-PAGE and fluorography. Upper panel: freshly isolated plastids; lower panel: import into organelles left on ice for 45 min. B: Quantification of the import yield of three independent experiments as presented in A. Dark bars: freshly isolated chloroplasts; light grey: organelles left on ice for 45 min. Error bars indicate the standard deviation. Import efficiency (appearance of mSSU) is given in percentage of total precursor protein added (100%). C: Immunoblot analysis of pea chloroplasts used for the import reaction in A. Lane 1: fresh chloroplasts; lane 2: chloroplasts left on ice for 45 min. The asterisks indicate intermediate breakdown products (see Fig. 2). The lower part of the immunoblot was incubated with an antiserum to Toc34.

When we analyzed purified outer envelope membranes isolated by standard procedures ([17], see also Section 2) for the presence of Toc160, only traces could be observed in an immunoblot (Fig. 2, lane 5). In contrast, rapidly isolated outer membranes still contained about 50% unproteolyzed Toc160 (Fig. 2, lane 4). In spite of the calculated molecular weight of 160 kDa Toc160 migrates at an apparent molecular mass of 200 kDa in Laemmli SDS-PAGE. This could be another reason why residual Toc160 escaped detection since in the most widely used polyacrylamide gels containing 12% acrylamide and 0.8% bisacrylamide polypeptides of this size migrate at

the border to the stacking gel. From these data we conclude that the 160-kDa protein, i.e. Toc160, represents the full-length polypeptide also in pea and that the previously identified Toc86 is only a C-terminal proteolytic fragment.

Does the protein import efficiency correlate with the presence of intact Toc160? To address this question freshly isolated chloroplasts or chloroplasts left on ice for additional 45 min were incubated with a ^{35}S-labeled precursor of ribulose-1,5-bisphosphate carboxylase small subunit (preSSU) for various times (Fig. 3A). Both chloroplast populations showed binding of preSSU and translocation as indicated by the appearance of the processed mature form of preSSU (mSSU). Quantification of the import process by measuring the appearance of mSSU demonstrated that the yield of import was two-fold higher for fresh chloroplasts in comparison to organelles which where left on ice for 45 min before initiating the import reaction (Fig. 3B).

Immunoblot analysis of the two chloroplast populations used for import by antisera against Toc86 and Toc34 demonstrated that the cytosolically exposed protease sensitive Toc34 [7] was not proteolytically degraded to a detectable extent. On the other hand the amount of intact Toc160 in chloroplasts left on ice for additional 45 min had decreased significantly, while the 86-kDa peptide became the prevalent form (Fig. 3C).

The preprotein translocation channel Toc75 is not protease sensitive under these conditions ([2,25], and data not shown). The drop in import efficiency might therefore be due to the drop in intact Toc160. The results from crosslinking studies and antibody inhibition [3,11] indicate that the 86-kDa form of Toc160 still contains polypeptide domains that can partially support the import reaction albeit at a lower rate. Complete removal of Toc160 and its 86-kDa fragment as well as Toc34 by an external protease such as thermolysin decrease the translocation yield to about 10% [2,11,25].

4. Conclusions

A previously identified precursor protein receptor in pea, Toc86, represents only a C-terminal proteolytic fragment of a much larger protein with a calculated molecular mass of 160 kDa. The correlation between the intactness of the 160-kDa protein and the import yield as well as previous data on 'Toc86' make it most likely that this protein, which we now name Toc160, continues to serve as an import receptor. The extreme protease sensitivity together with the unidentified nature of the protease will hamper further analysis of the full-length Toc160 in situ. Heterologous expression of the entire protein or parts of it might circumvent this problem and help to identify and characterize peptide domains within Toc160 involved in precursor protein recognition and binding. Conformational changes of Toc160 provoked by nucleotide binding and phosphorylation will influence this event.

Targeting and insertion of Toc160 into chloroplastic outer envelope is most likely accomplished by a hydrophobic carboxy-terminal domain which is also essential for the 86-kDa form to insert into the membrane [26]. The region N-proximal of the 86-kDa form of Toc160 was proposed to stimulate a step in the productive folding process of the polypeptide, which was proteolytically removed after completion of the reaction [26]. The observed cleavage of the putative precursor of the 86-kDa form of Toc160 could be due to the intrinsic sensitivity to proteases of Toc160 described above. Toc160 therefore seems to follow the general insertion pathway of outer envelope proteins without a cleavable presequence but aided by a hydrophobic membrane anchor region [27].

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