Tic40, a New “Old” Subunit of the Chloroplast Protein Import Translocon*

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The protein import translocon at the inner envelope of chloroplasts (Tic complex) is a heterologeric multisubunit complex. Here, we describe Tic40 from pea as a new component of this complex. Tic40 from pea is a homologue of a protein described earlier from *Brassica napus* as Cim/Com44 or the Toc36 subunit of the translocon at the outer envelope of chloroplasts, respectively (Wu, C., Seibert, F. S., and Ko, K. (1994) *J. Biol. Chem.* 269, 32264–32271; Ko, K., Budd, D., Wu, C., Seibert, F., Kourtz, L., and Ko, Z. W. (1995) *J. Biol. Chem.* 270, 28601–28608; Pang, P., Meathrel, K., and Ko, K. (1997) *J. Biol. Chem.* 272, 25623–25627). Tic40 can be covalently connected to Tic110 by the formation of a disulfide bridge under oxidizing conditions, indicating its close physical proximity to an established translocon component. The Tic40 protein is synthesized in the cytosol as a precursor with an N-terminal cleavable chloroplast targeting signal and imported into the organelle via the general import pathway. Immunoblotting and immunogold-labeling studies exclusively confine Tic40 to the chloroplast inner envelope, in which it is anchored by a single putative transmembrane span.

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‡ The abbreviations used are: Tic, translocon at the outer envelope membrane of chloroplasts; α, antiseraum; Cim, chloroplast inner envelope membrane protein; Com, chloroplast outer envelope membrane protein; DSP, dithiobisuccinimidyl propionate; Hsp, heat shock protein; PAGE, polyacrylamide gel electrophoresis; proSSU, precursor of ribulose-bisphosphate carboxylase/oxygenase small subunit; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; Tic, translocon at the inner envelope membrane of chloroplasts.



during endocytobiosis, chloroplasts and mitochondria have relinquished most of their genes to the host nucleus (1). Hence today, these organelles import the vast majority of their constituent proteins in a post-translational event from the cytoplasm. Nuclear-encoded chloroplast precursor proteins are imported to chloroplastic subcompartments by preprotein translocases, which are located both in the outer and inner envelope membranes of the organelle. The translocon at the outer membrane of chloroplasts (Toc complex) and the translocon inner membrane of chloroplasts (Tic complex) act cooperatively during the import process. According to the unified nomenclature (2), single subunits are named according to their calculated molecular size and their affiliation with either the chloroplastic outer or inner envelope membrane. The core subunits of the Toc complex from pea are Toc160, Toc75, and Toc34 (for review see Refs. 3–6). Toc160 is a putative precursor receptor and succeeds Toc86, which represents only a proteolytic C-terminal fragment of Toc160 (7). Toc75 forms the aqueous translocation pore, which is used by precursor proteins (8, 9). The function of Toc34 is less clear, but in the light of its GTP-binding properties, it could regulate precursor recognition (10–12) or translocation. *Brassica napus* bnToc36, formerly named chloroplast inner membrane, chloroplast outer membrane protein of 44 kDa (Cim/Com44) or *Bce*44B (13, 14), might be another import relevant protein. Conflicting results do not allow the clear assignment of the protein to either the inner or the outer envelope membrane, reflected by its name Cim/Com44 (13, 14). Finally the protein was named Toc36 (2, 15) without additional experimentation or elucidation of its role in import. Two Hsp70 homologues associated either with the cytosolic site, i.e. Com70 (16) or the intermembrane face of the outer envelope membrane (17, 18), seem also to be involved in the translocation process.

Subunits identified in the Tic complex include Tic110, Tic55, Tic22, and Tic20, however, their role in translocation is less well defined than for the Toc components. Tic110 might be involved in recruiting stromal Hsp100 or chaperonine 60 to the import site (10, 20, 21), whereas Tic110 domains exposed to the envelope intermembrane space could form translocation contact sites (22, 23). Tic55, containing a Rieske-type iron-sulfur motif, might act as a redox sensor to regulate import capacity of chloroplasts (24). Tic22 is peripherally associated with the intermembrane face of the inner envelope. Tic20 is an integral subunit of the Tic complex and is suggested to form part of the Tic translocation pore (5, 25).

Protein import into chloroplasts occurs jointly through the co-operation of both the Toc and the Tic complex (26, 27). At least, a certain population of joint Toc and Tic translocation sites is present even in the absence of precursor proteins. It is not clear, if contact sites between the outer and the inner envelope membranes, which can be seen in electron microscopic pictures (5, 26–28), are identical with or related to joint protein translocation sites. The isolation of either the outer or inner envelope membranes devoid of cross-contamination by each other is therefore not possible (29, 30). Consequently, isolated Tic complexes from purified chloroplastic outer envelopes still contain the prominent Tic components and vice versa (20, 21, 23, 31). It is therefore, important to establish the exact localization of a new translocon component.

In this paper we report that a 44-kDa polypeptide from pea is a bona fide component of the Tic complex. The 44-kDa polypeptide, which is a functional homologue of bnToc36, is exclusively localized in the inner envelope membrane. We propose to name the protein Tic40 but neither Cim/Com44 nor Toc36.
EXPERIMENTAL PROCEDURES

Isolation of a cDNA Clone for Tic40—The sequence of Bce44B (14) was used for a data base search. An expressed sequence tag from Arabidopsis thaliana (cDNA clone ATTS5476, accession number F15182) similar to Bce44B was identified and used as a template to synthesize a random-primed digoxigenin-labeled probe by polymerase chain reaction. A pea cDNA expression library (UniZAP XR, Stratagene, La Jolla, CA) was screened with this probe, resulting in the isolation of a partial-length cDNA. This clone was used again as a template for synthesizing a homologous digoxigenin-labeled probe. Three full-length cDNA clones were isolated in a second screen. DNA cloning and sequencing of both DNA strands were performed according to standard procedures (32).

Genomic Southern Blot Analysis—Genomic pea DNA was isolated by the cetyltrimethylammonium bromide method (33). 15 μg of DNA were digested in 100 μl with different restriction enzymes for 4 h at 37 °C. After separating the DNA fragments on an 0.7% agarose gel, the DNA tech) and hybridized with a 32P-labeled DNA probe.

Isolation of Chloroplast Subcompartments—Pea plants (Pisum sativum L. var. Golf) were grown for about 14 days. Intact chloroplasts were isolated from leaves and further purified by silica-acetone precipitation (17). After hypertonic treatment of intact chloroplasts in 0.65 M sucrose and 50 mM HCl, pH 7.5. Envelope membranes were solubilized with 2% SDS for 2 min at 95 °C. After diluting SDS to 0.2% with IP buffer (see above) 5 μl of aTic40 were added for immunoprecipitation. IgGs were bound to protein A-Sepharose and the associated proteins were analyzed under denaturing conditions on SDS-PAGE followed by immunostaining with aTic110.

RESULTS

We are currently trying to reconstitute the process of preprotein recognition and translocation as carried out by the Toc complex from heterologously expressed and purified subunits in vitro (9). As bntoc36 (13–15) is a putative subunit of this Toc complex, we wanted to clone and express its homologue from pea.

A pea cDNA library was screened using a heterologous DNA probe of an expressed sequence tag from Arabidopsis, which showed strong similarities to bntoc36. Several but identical cDNA clones were isolated and sequenced. The open reading frame of this cDNA clone, peace48 (Fig. 1), (Accession number AJ243758) coded for a protein of 436 amino acids with a calculated molecular mass of 48 kDa (Fig. 1A). The deduced protein sequence shows clear sequence homology to bntoc36 (54% identity, 8% similarity) (Fig. 1A) not only within the published sequence (14) but also in the sequence stretch, which was indicated as 5′-untranslated region in the B. napus clone (see Fig. 1), indicating that the native protein is indeed larger than previously thought. To resolve the question of where the N-terminus of bntoc36 started relative of that of its pea homologue, an antisense was raised against a heterologously expressed partial sequence, representing amino acids 128–436 of the pea protein (see “Experimental Procedures”). The authentic pea protein was then purified from detergent-solubilized envelope membranes by immunofinity chromatography containing the IgGs covalently cross-linked to protein A-Sepharose (see “Experimental Procedures”). Two proteins of an apparent molecular size of 44 and 42 kDa eluted from the affinity matrix (Fig. 1B). Both proteins were sequenced. The amino acid sequence determined from the 44-kDa protein was XISSNNGQETTSVG, which is identical to amino acids 74–88 of the deduced sequence of the 48-kDa protein. The amino acid sequence of the 42-kDa protein form was determined as VX-PQLSSPPPFST and represents amino acids 88–97 of the 48-kDa protein. From these data we conclude that (i) the 42-kDa form represents only a proteolytic fragment of the 44-kDa protein (see also below); (ii) the 44-kDa protein represents the mature protein, whereas the cDNA codes for a precursor form of 48 kDa. This notion is supported by the amino acid composition of the N-terminal 73 amino acids, which are typical for a chloroplast targeting signal, namely rich in hydroxylated amino acids and an overall positive charge (37) (see also below). Finally, we conclude that (iii) the cDNA clone coding for bntoc36 (14, 15) does not represent a full-length clone. Because of the following results we will name the 44-kDa protein Tic40. The molecular mass number represents the calculated molecular weight of the mature protein deduced from peace48 and not its apparent molecular weight. The precursor form is called preTic40.

Secondary structure prediction algorithms indicate that Tic40 contains a single transmembrane span (Fig. 1C) within the extreme N-terminal region of the protein, consistent with its putative localization in the envelope membranes. This hydrophobic region is missing in the deduced sequence of the partial bntoc36 polypeptide.

Earlier data had indicated that bntoc36 is coded for by a small multigene family (14), because two isoforms of slightly different size were detected in immunoblots (13, 14, see also below). We, therefore, addressed this question by digesting pea genomic DNA with various restriction enzymes, and in a subsequent Southern blot analysis transfer restriction fragments were hybridized with 32P-labeled probe of peace48. The labeling
pattern (Fig. 2) is indicative for a single gene. Furthermore, we isolated four different cDNA clones coding for Tic40, partial and full-length, all of which contained an identical coding region.

To verify that the cDNA clone peace48 indeed represents a chloroplast precursor protein, the polypeptide was synthesized in vitro in a reticulocyte lysate system. The 35S-labeled protein was incubated with isolated chloroplasts and either bound to the chloroplast surface or imported into the organelle in an ATP-dependent manner, as indicated by the appearance of a processed lower molecular weight mature form (Fig. 3A). The processed mature protein was protease-protected inside the organelle, whereas the chloroplast surface-bound precursor remained protease accessible (not shown). The ATP dependence of import is indicative for translocation via the general import pathway into the organelle, whereas all nuclear-coded outer envelope proteins use an ATP and presequence independent insertion route (for review see Ref. 3). The only known exception to the above rule is the translocation pathway of preToc75 (38).

Preproteins, translocating via the general import pathway, compete with each other for translocation sites. Therefore, we examined if import of labeled preTic40 could be competed for by the preSSU, a stromal protein, which was heterologously expressed and purified from E. coli and added to a chloroplast import assay after its denaturation in 8 M urea. In the presence
of excess preSSU, the import of 35S-labeled preSSU and 35S-labeled preTic40 translation product was completely abolished as indicated by the absence of the processed mature forms of both proteins (Fig. 3B). Simultaneous association of the 35S-labeled precursor forms of both polypeptides with the organelar surface increased. If this is a general effect remains to be established.

Together our data indicate that Tic40 is indeed synthesized as a precursor protein, which is imported into chloroplasts by the general translocation pathway. Most consistent with these results would be a location in the chloroplastic inner envelope but not in the outer envelope, as proposed for bnToc36, which bound to the chloroplast surface in a largely protease accessible manner (14), most likely because of the absence of the chloroplastic targeting signal and the transmembrane region. This notion is supported by our own observation, that an N-terminal truncated Tic40, which started at amino acid position 129, was neither imported nor bound to chloroplasts (not shown).

To determine the subchloroplast localization of Tic40, we used three different experimental approaches. First, simultaneous protein translocation assays of preTic40 were carried out using labeled reference proteins of established chloroplast localization, i.e., the outer envelope protein Toc34 and the inner envelope Tic110 precursor protein, or the hybrid protein, Rubisco small subunit transit peptide-110N. Rubisco small subunit transit peptide-110N, which contains the Rubisco small subunit transit peptide fused to the membrane anchor region of Tic110 (110N), is translated, imported, and localized to the chloroplastic inner envelope with much higher efficiency than authentic preTic110 (23) and, therefore, was used here. Chloroplasts were fractionated after import into thylakoids, stroma, and inner and outer envelope membranes. Processed mature Tic40 was clearly enriched in the inner envelope membrane fraction (Fig. 4A, lane 5) concomitantly with Tic110N. In contrast, labeled Tic34 was found predominantly in the puri-
Chloroplast Tic40

Is Tic40 involved in protein translocation? To address this question, cross-linking experiments were performed using either intact chloroplasts with bound 35S-labeled preSSU blocked from complete translocation (14, 20) or with inner envelope membrane vesicles using either DSP or the thiol oxidant CuCl2 (11, 13). After cross-linking of intact chloroplasts and complete solubilization of the membranes in SDS, cross-linked products were immunoprecipitated by an antiserum against Tic40 and analyzed by nonreducing SDS-PAGE and fluorography. A 35S-labeled high molecular complex was detected, which remained in the stacking gel of polyacrylamide gels (not shown). This result is consistent with the observation of Nielsen et al. (20) who describe the formation of a 600-kDa Tic complex upon cross-linking. Therefore, the nature of this complex was not further investigated.

Next, purified inner envelope membranes were incubated with the thiol oxidant CuCl2 (Fig. 6, lower panel), which yields the reversible formation of covalent disulfide bridges between polypeptides that are in close physical proximity to each other or the thiol cleavable chemical cross-linking reagent DSP (Fig. 6, upper panel). After cross-linking, inner envelope membranes were solubilized in SDS, and proteins were subjected to immunoprecipitation using an antiserum against Tic40. Immunoprecipitated cross-linked proteins were cleaved under reducing conditions using trypsin, electrophoresed in SDS-PAGE, and transferred onto nitrocellulose, and immunodecorated with a Tic110 antibody. The immunoblots show two immunoreactive bands in the absence or presence of either DSP or CuCl2, as indicated on top of the figure. Membranes were dissolved in 2% SDS and subsequently subjected to immunoprecipitation in the absence or presence of a Tic40 or preim- mune serum. Precipitated proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and immunodecorated with a Tic110 antibody.

**Fig. 6. Tic40 is present in a complex together with Tic 110.** Purified inner envelope membranes were incubated in the absence or presence of either DSP or CuCl2, as indicated on top of the figure. Membranes were dissolved in 2% SDS and subsequently subjected to immunoprecipitation in the absence or presence of a Tic40 or preimmune serum. Precipitated proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and immunodecorated with a Tic110 antibody.

Indicating that it is an integral transmembrane protein (not shown). This experimental finding is corroborated by the secondary structural analysis, which predicts a hydrophobic transmembrane span in the N-terminal region of the 44-kDa protein (Fig. 1C). The immunoblots show two immunoreactive proteins at 44 and 42 kDa apparent molecular mass in isolated envelope membranes. The lower molecular weight form is absent in rapidly isolated chloroplasts (Fig. 5A, lane 1) (7) or in a protein extract obtained from pea leaves homogenized in liquid N2 and followed by immediate denaturation in boiling SDS-PAGE sample buffer (not shown).

Third, ultra-thin sections of pea leaves were immunodecorated using an antiserum against Tic40 and immunogold-labeled secondary antibodies. Gold grains were confined almost exclusively to the chloroplasts (Fig. 5B). Within the chloroplasts, gold grains were confined only to the inner envelope membranes. The experimental evidence from three different experimental approaches point into a single direction. Accordingly, we conclude that Tic40 is a bona fide constituent of the chloroplast inner envelope membrane.

Fied chloroplast outer envelope membranes (Fig. 4A, lane 4). No or very little labeled proteins were recovered either in the stroma or in the thylakoid membranes, respectively. Quantification of the import experiment, shown in Fig. 4B, demonstrates, that more than 80% of mature Tic40 as well as Tic110N copurify with inner envelope membranes, whereas Toc34 is confined 70% to the outer envelope membranes (Fig. 4B). This nonexclusive distribution between both membranes is consistent with the presence of translocon components in stationary joint translocation sites and the reported purity of enriched outer and inner envelopes from chloroplasts (29, 30).

Second, protein blots representing the protein complement of thylakoid membranes, soluble stroma, and inner and outer envelopes were decorated with an antiserum against Tic40, Tic110, and Toc34. The results show that Tic40 is almost exclusively detected in the inner envelope membrane fractions as is Tic110, whereas Toc34 is largely confined to the outer envelope membrane fraction (Fig. 5A). The immunolocalization of Tic40 as well as Tic110 and Toc34 is consistent with the results from the protein import studies and strongly suggest that Tic40 is an inner envelope protein. When inner envelope membranes were treated either with 1 M NaCl or extracted at pH 11 (0.1 M Na2CO3), Tic40 was recovered with the insoluble fraction, indicating that it is an integral transmembrane protein (not shown). This experimental finding is corroborated by the secondary structural analysis, which predicts a hydrophobic transmembrane span in the N-terminal region of the 44-kDa protein (Fig. 1C). The immunoblots show two immunoreactive proteins at 44 and 42 kDa apparent molecular mass in isolated envelope membranes. The lower molecular weight form is absent in rapidly isolated chloroplasts (Fig. 5A, lane 1) (7) or in a protein extract obtained from pea leaves homogenized in liquid N2 and followed by immediate denaturation in boiling SDS-PAGE sample buffer (not shown).

**Fig. 5. Immunolocalization of Tic40 in chloroplasts.** A, an immunoblot is shown indicating the presence and distribution within chloroplasts of the inner envelope protein Tic110, the outer envelope protein Toc34, and Tic40. Chloroplasts (Chl) equivalent to 4 μg of chlorophyll were used, and 2 μg from each chloroplast subcompartment were used. B, immunogold labeling of ultra thin sections from pea mesophyll cells. An overview and three independent enlargements are shown.
conditions, separated by SDS-PAGE, and analyzed by immuno-
blotting. α-Tic40 was able to co-immunoprecipitate Tic110
when cross-linked by either DSP or CuCl₂ (Fig. 6), whereas in
the absence of cross-linking reagent or precipitation, using
preimmune serum did not result in any significant co-immuno-
precipitation (Fig. 6). Because oxidation by CuCl₂ results in a
reversible disulfide bond formation only when the thiol groups
are situated close to each other in inner envelope membranes.

DISCUSSION

In this present work Tic40 was identified as an inner enve-
loped-localized protein and a constituent of the protein import
machinery. Tic40 from pea is homologous to a protein previ-
ously named Cim/Com44 from B. napus. Cim/Com44 was identi-
fied by its ability to form a chemical cross-link with a radio-
labeled precursor protein trapped in the import machinery
under conditions that allow only partial translocation but not
complete import (13). We were able to confirm and extend this
observation in this study by demonstrating that the pea homo-
logue of Cim/Com44, namely Tic40, is in close physical prox-
imity to Tic110, a well established subunit of the protein translo-
con. After isolation of a cDNA clone coding for Tic40 from pea we
noticed that the open reading frame was larger than that which
previously reported for the Brassica homologue (14). The N-
terminal region of the deduced amino acid sequence from the
pea cDNA exhibited the typical features of a chloroplast-tar-
geting signal. Import studies confirmed that the putative pre-
cursor form of pea Tic40 was actually imported and processed
in chloroplasts by the general import pathway (3, 4). Therefore
the failure of the B. napus Cim/Com44 translation product to
import into chloroplasts is because of the missing targeting signal
and probably led to its fortuitous adhesion or aggrega-
tion at the chloroplast surface in vitro (14).

We have used two independent experimental techniques to
establish the cellular and subchloroplastic localization of Tic40.
First, biochemical fractionation of purified chloroplasts into
the different subcompartments either with or without the import
of labeled and differentially labeled markers, e.g. Tic110 and
Toc34, indicated that Tic40 is predominantly present in the
inner envelope. The currently available techniques to separate
outer and inner chloroplastic envelopes only yield enriched
populations of a single membrane (29, 30). Therefore, the
membrane “purity” might vary considerably (29, 30) but is around
80% (Figs. 4 and 5) for the preparations presented here. The
second approach was immunogold labeling of ultra-thin sections
from pea leaves. This technique excludes cellular frac-
tionation and presents the antibody to a large variety of poten-
tial antigens. This method is less prone to false positive
results than if only a purified organelle is used for labeling
or especially if labeling is done prior to fixation and embedding
(14, 39). Again our data show that Tic40 is confined to chloro-
plasts within the plant cell and to the inner envelope of the
organelle.

Tic 40 seems sensitive to proteolytic degradation. In isolated
inner envelope membranes as well as in chloroplasts after
prolonged incubation a smaller breakdown product was detect-
able in immunoblots (14). Protein sequencing of this smaller
polyepitope demonstrated that cleavage had occurred 15 amino
acids C-terminal from the N terminus. Further proteolytic
damage is most likely not occurring, because the single puta-
tive membrane anchor region of Tic40 starts shortly after the
cleavage site (compare Fig. 1) and renders the rest of the
polyepitope chain inaccessible to further degradation. A second
smaller Tic40-like protein, as suggested earlier (14), is, there-
fore, not likely to exist. This notion is corroborated by the

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