

In vitro targeting of the Toc36 component of the chloroplast envelope protein import apparatus involves a complex set of information

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Abstract

Toc36 is a family of 44-kDa envelope polypeptides previously identified as components of the chloroplast protein import apparatus. Toc36 exists as multiple outer and inner envelope membrane forms. One member, Toc36B (formerly Bce44B), is targeted to the envelope without the typical maturation event. Targeting and assembly into the envelope is thus likely to involve a complex interplay of indigenous signals. These signals were examined by testing the effects of truncations and chimeric fusions on the targeting of Toc36B. The targeting ability of Toc36B appeared unaffected by carboxyl truncations of up to 80% of the protein, but was abolished by N-terminal deletions. The N-terminal 39 residues of Toc36B conferred the same targeting profile to mouse dihydrofolate reductase as that displayed by unaltered Toc36B. However, removal of 18 residues from the carboxyl end of the N-terminal 39-amino acid segment abolished targeting to the chloroplast. Additional information in the remaining Toc36B segment was also apparent based on the import results of chimeric fusions between the transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase and Toc36B. The targeting of Toc36B to various destinations in the chloroplast envelope appears to be influenced by information from at least two segments of the protein. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Chloroplast; Targeting signal; Envelope membrane

1. Introduction

Nuclear-encoded chloroplast proteins synthesized outside the organelle are imported into the compartment via a complex process. This process requires the

participation of energy, transit signals, proteinaceous envelope membrane factors, processing peptidases and chaperones [1–3]. These factors facilitate various steps of the translocation process such as unfolding, binding to surface receptors, translocation across envelope membranes and maturation [1–3]. The multi-subunit protein translocation machinery of the chloroplast envelope membrane is central to the process [2]. The translocation of a protein into the interior of the compartment requires the close collaboration of outer and inner envelope membrane components of the machinery. Most of the components of the machinery identified to date appear to function exclu-

Abbreviations: IgG, immunoglobulin G; Dhfr, mouse cytosolic dihydrofolate reductase; Rbcs, precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase; Toc, translocon of the outer envelope of chloroplasts

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sively in either the outer envelope membrane or the inner envelope membrane. The immunologically related Toc36 components present in the outer and inner envelope membranes may play a role in both locations [4,5]. These immunologically related Toc36 proteins appear to be tightly associated components of the envelope, even though the hydropathy profile of one deduced amino acid sequence, Toc36B, predicts a hydrophilic non-membranous polypeptide [5]. The existence of Toc36 in both the outer and the inner chloroplast envelope membrane raises an intriguing question concerning the information governing the targeting and assembly of Toc36 proteins. It is not known whether the same protein is present in all locations or whether different but related proteins are present in each locale of the chloroplast envelope. The expression of Toc36B in bacteria provided evidence that the proteins were capable of associating with both the outer and inner membranes of bacterial cells [6]. A higher amount of Toc36 is found associated with the inner chloroplast envelope membrane relative to the outer envelope membrane [5], however, the pattern is reversed in bacteria with more in the outer membrane and less in the inner membrane [6]. Although these differences are consistent with the direction of protein translocation, the significance of the asymmetric distribution patterns in relation to protein transport remains unknown. The data arising from the bacterial experiments indicate that Toc36B contains information for targeting to both bacterial membranes. Therefore, the possibility that the various native Toc36 forms in the chloroplast envelope arose from a similar 'multi-site targeting' mechanism warrants further investigation. Initial examination of the N-terminus of the deduced Toc36B protein sequence did not reveal any obvious insights into the targeting and assembly of the component. The availability of a cDNA clone for one member of these immunologically related Toc36 envelope polypeptides allows us to investigate the targeting behavior of one specific Toc36 envelope protein. The *in vitro* targeting results of various Toc36B deletions and fusion constructs revealed a complex interplay of information for the targeting and assembly of the protein in the envelope. The main set of information for directing the protein is located in the N-terminus of Toc36B.

2. Materials and methods

2.1. Construction of plasmids and bacterial strains

DNA fragments encoding Toc36B were manipulated using established protocols [5,7]. The plasmids used were pGEM4 and pGEM11Z (Promega). C-terminal deletions of Toc36B were created either by linearizing pToc36B at unique restriction endonuclease sites within the cDNA insert or by exonuclease III/S1 digestion [8]. The resulting translation products of these deletion constructs (designated C1–C5 in Fig. 1) lacked 10, 24, 197, 234 and 284 amino acids from the C-terminus. These deletions represent 3, 7, 60, 72 and 80% of the Toc36B protein, respectively.

N-terminal deletions were generated by exonuclease III/S1 digestion and joining the digested DNA fragments to the DNA sequence for the first four amino acids of the pea precursor to the small subunit of the pea Rbcs transit peptide (MASM) [9,10]. The MASM sequence used to construct these deletions does not possess any chloroplast targeting properties [10]. The resulting translation products (designated N1–N4 in Fig. 2) lacked 42, 82, 140 and 181 amino acids from the N-terminus. The amino acid sequences of the fusion sites of N1–N4 are MASMISSLSVPPQ-, MASMISSLSVPPSV-, MASMISSLSRLF- and MASMMYPKMI-, respectively. The N6 construct was made by joining the *Bst*EII-*Not*I fragment of *toc36B* to the same MASM sequence. The sequence of the N6 fusion site is MASMISSVTSNAKKYAM-.

The N5 and Toc36B2-Dhfr fusion constructs were made by joining the DNA sequence for the first 23 amino acids of Toc36B to N1 and mouse Dhfr, respectively. The DNA fragment encoding the N-terminal 23 amino acids and the 5' untranslated region of *toc36B* was retrieved from C5 using *Eco*RI and *Hin*FI. The *Eco*RI-*Hin*FI DNA fragment was inserted into pGEM4 via *Eco*RI and *Sma*I sites, after conversion of the *Hin*FI site to a blunt end. The resulting vector was then used for the construction of N5 and Toc36B2-Dhfr. The N5 fusion was completed by inserting the Asp-718-*Hind*III DNA fragment retrieved from N1 into the *Bam*HI and *Hind*III sites of the above vector. The Asp-718 and *Bam*HI sites were made blunt. The Toc36B2-Dhfr construct was fin-

ished by inserting the 900 bp *EcoRI* DNA fragment encoding Dhfr into the *Bam*HI site of the above vector. Both sites were converted into blunt ends. The amino acid sequence of the fusion points are -GLGIVPP- and -GLGIIP-, respectively.

The Toc36B1-Dhfr fusion construct was made by joining the DNA sequences for the first 39 amino acids of Toc36B and mouse Dhfr. The DNA fragment encoding the first 39 amino acids and the 5' untranslated region of *toc36B* was available in the C5 truncation construct. A 900-bp *EcoRI* DNA fragment encoding Dhfr was inserted into the C5 construct. The *EcoRI* sites were converted into blunt ends. The amino acid sequence of the fusion point is -SSGGDRSSRIPALM-.

The Rbcs-Toc36B and RbcS-N1 fusion constructs were made by joining the DNA sequences for the pea Rbcs transit peptide and Toc36B. The Rbcs-Toc36B construct was made by inserting a *Bst*EII-*Hind*III DNA fragment encoding Toc36B into a vector containing the DNA sequence for the Rbcs transit peptide, pSSTP [10]. The amino acid sequence of the fusion point is -MDRSSRVVTS-. The Rbcs-N1 fusion was made by inserting into pSSTP the *EcoRI*-*Hind*III DNA fragment encoding the N-terminal truncated N1 protein. The protein sequence at the fusion point is -MDRSSRIRARYVPP-.

2.2. *In vitro* targeting assays

Radiolabeled protein precursors were prepared and used in *in vitro* import or binding assays as described [4,10,11]. Post-import treatment and subfractionation schemes were as described [12,13]. Import experiments involving antibody impairment were performed as described [9]. All samples were analyzed by standard protein gel electrophoresis and fluorography procedures.

3. Results

3.1. Targeting with C-terminal truncated Toc36B proteins

Various truncated Toc36B proteins were tested for targeting competence. The first set of deletions (ranging from 10 to 284 residues) were generated at the

C-terminus. The N-termini were left unaltered (Fig. 1A,B). All of the C-terminal truncated Toc36B proteins retained their targeting competence. The truncated proteins were associated with the envelope in the presence of nigericin or ATP (Fig. 1B). Truncated proteins C1–C4 displayed the same thermolysin sensitivity profile as unaltered Toc36B. The majority of the associated proteins were sensitive to thermolysin with approximately 5% showing resistance. Estimations were made relative to the amount of bound precursors per chloroplast and further adjusted to the size of the truncated protein. The low levels of proteins targeted to the chloroplast in a protease-resistant manner relative to precursors destined for the interior (e.g. stroma and thylakoids) is most likely reflective of the limited capacity of the envelope membrane for incorporating additional new polypeptides, especially envelope membranes of mature chloroplasts. Further perturbations to the low import levels are evident in the following experiments. Unlike C1–C4, all of the associated C5 proteins were degraded by thermolysin. The 5% thermolysin resistant level displayed by Toc36B was retained in truncated proteins C1–C4 but abolished in C5. This behavior indicates that the 5% level of thermolysin resistance observed was related to the protein precursors and was not attributed to incomplete protease degradation. The truncated precursors (C1–C5) were completely degraded by thermolysin in the absence of chloroplasts (data not shown). Unlike thermolysin, trypsin completely degraded all of the associated C3, C4 and C5 proteins. Approximately 5% of C1 and C2 remained resistant to trypsin, a level similar to Toc36B. These results indicate that the truncations represented by C3, C4 and C5 affected the way in which Toc36B associates with the envelope membrane.

We further tested and compared the import profiles of C3 and Toc36B. The C3 protein was the smallest deletion that retained its targeting competence and displayed a change in sensitivity to trypsin (Fig. 1C). Like unaltered Toc36B, the majority of the chloroplast-associated C3 proteins were thermolysin-sensitive. Approximately 5% of the associated C3 proteins were resistant to thermolysin. The C3 protein fractionated with both the outer and inner envelope membranes in the same manner as Toc36B. These results suggest that the efficacy of the envelope

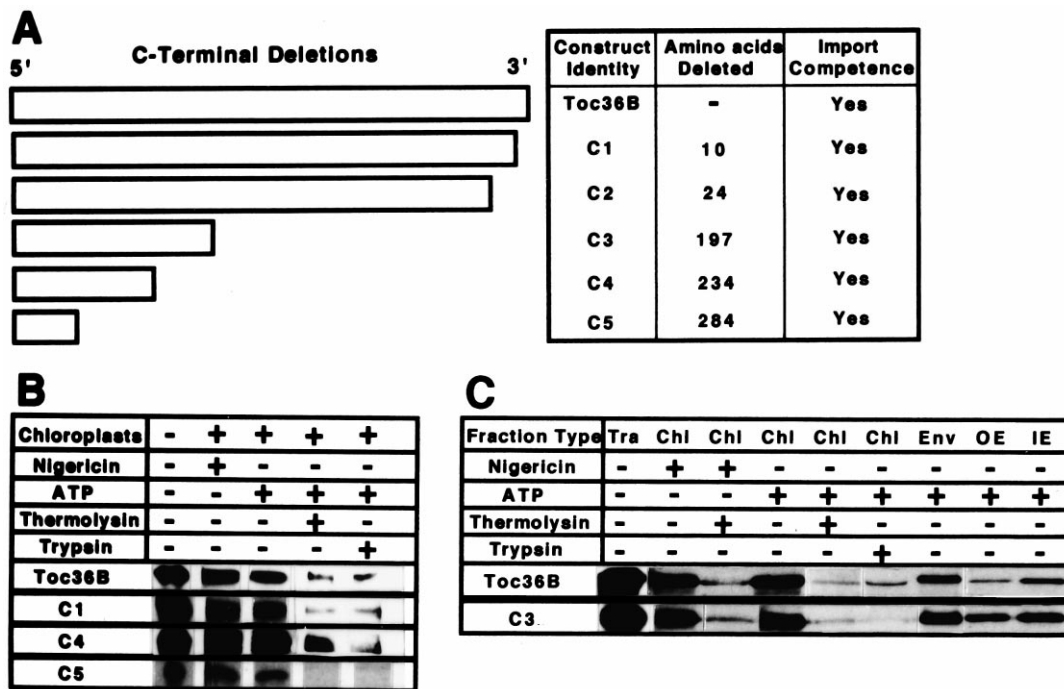


Fig. 1. The effects of carboxy-terminal truncations on the targeting of Toc36B. (A) The construction details are described in Section 2. Construct identities, the number of residues affected and the resulting import competence of the corresponding constructs are summarized in the table. (B) Fluorograms of the import assays representing constructs C1, C4 and C5 are shown. The import profile of Toc36B is provided for comparison. The first lane represents translation products only. (C) Import and subfractionation studies for Toc36B and deletion construct C3. The fraction types designated are as follows: translation products, Tra; chloroplasts, Chl; total envelopes, Env; outer envelopes, OE; inner envelopes, IE. Experimental parameters and post-import treatments used are indicated in each of the panels.

targeting signal was unaffected by the carboxyl deletions. Since none of the C-terminal deletions abolished the targeting ability of Toc36B, the primary plastid targeting information is unlikely to be located at the C-terminus. These results suggest that the main information for targeting to the envelope is located in the N-terminus, but additional information present in the C-terminus may be required for structural aspects of the protein.

3.2. Targeting studies with N-terminal deletions of Toc36B

Various N-terminal deletions (N1–N4) were tested and compared to unaltered Toc36B to confirm the location of the targeting signal (Fig. 2). The chloroplast-targeting ability of Toc36B was completely abolished in all cases (Fig. 2A,B). Since none of the C-terminal deletions abolished the targeting ability of Toc36B and the N-terminal deletions did, the

primary chloroplast-targeting information is most likely located within the extreme N-terminal 39 residues.

The addition of extra nine amino acids to the N-terminus (construct N6) also abolished the targeting competence of Toc36B (Fig. 2A,B). The extra residues were generated from the joining of the 5' untranslated *toc36B* region to the pea *rbcs* 5' untranslated sequence (see Section 2 for details) (Fig. 2A). The results suggest that the targeting signal was affected by the addition of extra amino acids to the N-terminus.

The removal of 18 amino acids from the carboxyl end of the 39-residue N-terminal segment (from position 25 to 42) also abolished the targeting competence of Toc36B (construct N5) (Fig. 2B). Neither binding nor import of the N5 protein was detected even though the N-terminal 21 amino acids were left intact. The series of 18 amino acids removed consisted of two unusual segments of alternating amino

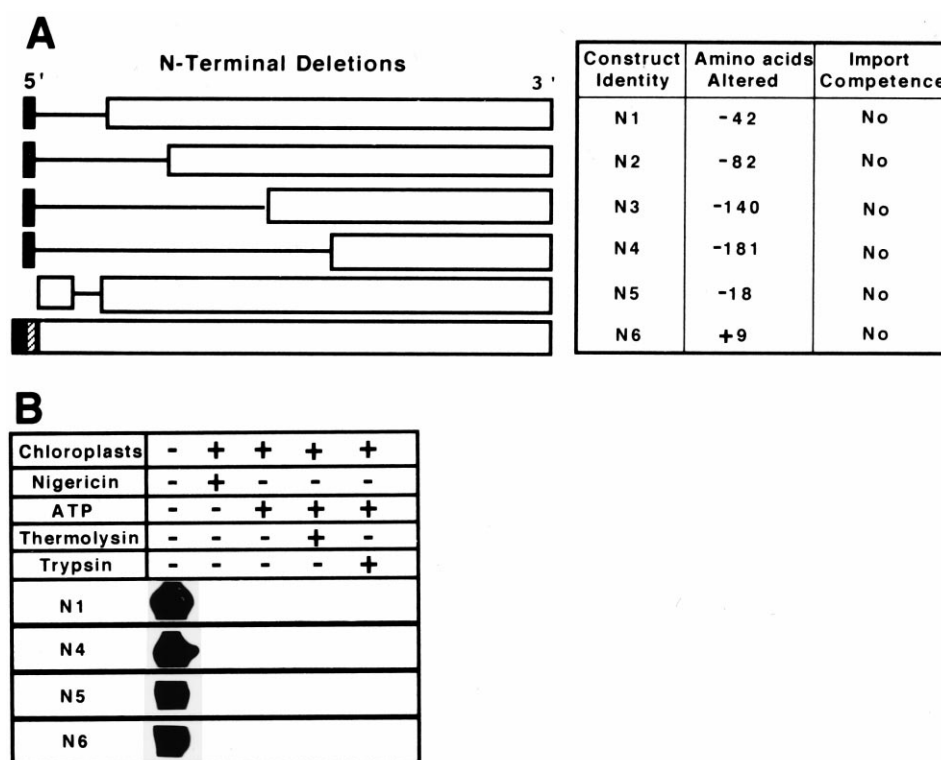


Fig. 2. The effects of amino-terminal manipulations on the targeting of Toc36B. (A) The construction details are described in Section 2. Construct identities, the number of residues affected and the resulting import competencies are summarized in the table. (B) Fluorograms of the import assays representing constructs N1, N4, N5 and N6 are shown. Experimental parameters and post-import treatments used are indicated.

acids, glycine-alanine and proline-phenylalanine. The two sequences are -GAGAGAG- and -PFPFPPF-, respectively. The two segments of alternating amino acids are in tandem and are separated by a single serine.

3.3. Targeting studies with Toc36B-Dhfr fusion proteins

The N-terminal 39-amino acid sequence was further assessed for its ability to target a foreign protein, mouse Dhfr, to the chloroplast envelope (designated Toc36B1-Dhfr). The targeting characteristics of Toc36B1-Dhfr were similar to unaltered Toc36B. The Toc36B1-Dhfr fusion protein was targeted to the chloroplast in the presence of nigericin or ATP (Fig. 3A), whereas Dhfr alone failed to do so (data not shown). Toc36B1-Dhfr remained unprocessed after targeting to the chloroplast. The majority of the chloroplast-associated proteins were thermolysin-sensitive. Approximately 5% of the associated proteins

were resistant to thermolysin. Toc36B1-Dhfr fractionated with the outer and inner envelopes in equal amounts which differs from the distribution pattern displayed by unaltered Toc36B (Fig. 1C). However, unlike Toc36B, Toc36B1-Dhfr was completely degraded by trypsin, similar to the behavior observed for C3, C4 and C5. These results suggest that the envelope targeting signal was unaltered by the passenger protein Dhfr. The sensitivity of Toc36B1-Dhfr to trypsin mimics the behavior displayed by the proteins with C-terminal deletions. The loss of C-terminal Toc36 sequences changes the protein's sensitivity to trypsin.

In contrast to Toc36B1-Dhfr, the Toc36B2-Dhfr fusion protein with the same 18 residues removed as N5, behaved like N5, with neither binding nor import being observed (Fig. 3A). The extreme N-terminal 23 residues alone do not appear to possess the capability to target Toc36B to the chloroplast envelope. There are at least two possibilities for this behavior: (1) the targeting information of Toc36B is

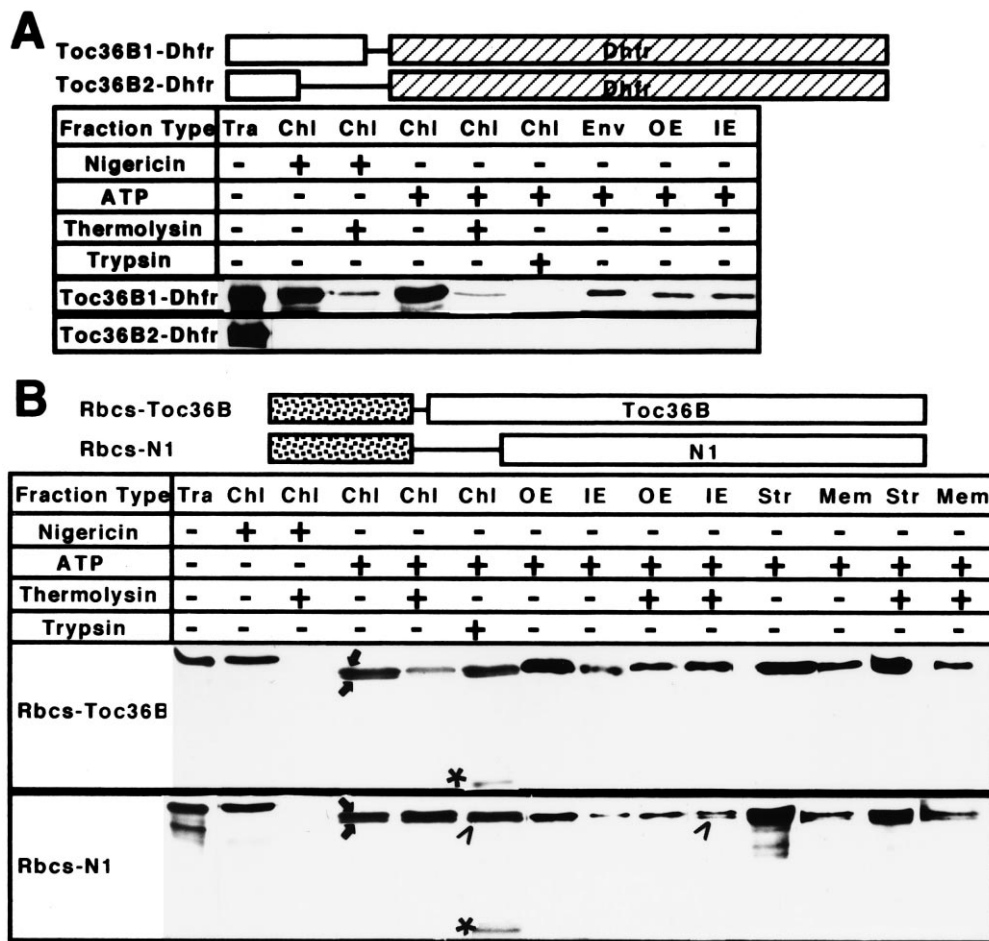


Fig. 3. Import studies for chimeric fusion proteins between Toc36B, Dhfr and Rbcs. (A) The construction details for Toc36B and Dhfr fusions are described in Section 2 and are represented as bar diagrams above the import assays. (B) The construction details for Rbcs and Toc36B fusions are described in Section 2 and are represented as bar diagrams above the import assays. Experimental parameters and post-import treatments used are indicated as in Fig. 1. The fraction type designated by Mem represents total membrane fractions. The positions of the two imported Rbcs-Toc36B and Rbcs-N1 products are marked by arrows in the first representative sample in each experiment. The positions of the trypsin-generated 20- and 38-kDa products are marked by asterisks and arrowheads, respectively.

located in the region containing the alternating amino acids or (2) the alternating amino acid segment functions in combination with the extreme N-terminal 23 residues to provide the needed targeting signal.

3.4. Targeting studies of Rbcs-Toc36B fusion proteins

Two chimeric fusions involving the Rbcs transit peptide and Toc36B were constructed and tested to gain a further insight into the targeting information. Both Rbcs-Toc36B and Rbcs-N1 bound to chloroplasts at high levels in the presence of nigericin and

were thermolysin-sensitive (Fig. 3B). High levels of import into the stroma were observed in the presence of ATP (3.3 mM and in the presence of light). Interestingly, the imported proteins were processed into two smaller-sized products (44 and 42 kDa for Rbcs-Toc36B, 40 and 38 kDa for Rbcs-N1) and were resistant to thermolysin. The two imported forms were not present in equal amounts and were not strictly in the stromal fraction. A small amount of the imported forms co-fractionated with the membranes, a mixture of thylakoids and envelopes. Some of the membrane-associated forms displayed differences in sensitivity to the proteases thermolysin and trypsin. Trypsin



Fig. 4. Impairment of protein targeting by anti-Com70 antibodies. Assays were conducted with anti-Com70 IgGs (designated Com70 Abs) and compared to control IgGs (antibodies against the 37-kDa inner envelope protein) (designated Control Abs). The presence or absence of IgGs is indicated by +/- . Increasing amounts of IgGs used in the assays are indicated by the number of indicated pluses (-, +, ++ and +++), representing 0, 100, 200 and 400 μ g of IgGs, respectively. The impairment assays were not post-treated with proteases.

treatment generated low amounts of a distinct, smaller-sized product of approximately 20 kDa in the Rbcs-Toc36B and Rbcs-N1 experiments (indicated by the asterisks in Fig. 3B). Unlike Rbcs-Toc36B, both thermolysin and trypsin gave rise to distinct lower sized Rbcs-N1 products. Thermolysin generated a small amount of a distinct 38-kDa product (indicated by the arrowheads in Fig. 3B). Trypsin produced additional 38-kDa products and a small amount of a 20-kDa product. All of the envelope-associated forms observed had already traversed the inner membrane, since they were all present as processed products. The protease-generated products likely represent a collection of forms that span both outer and inner envelope membranes as well as forms in the intermembrane space and/or traversing the inner envelope. Collectively, these results indicate that the proteins can exist in distinct states or locales in the outer and inner envelope membranes. The C-terminal segment of Toc36B contains information affecting the manner in which the proteins associate with the outer and inner envelopes. These results are consistent with the data obtained in the above experiments (the C-terminal deletions and the Toc36B-Dhfr fusions) and the multi-site observations reported in earlier studies [5,6].

3.5. *Toc36B1-Dhfr* and *Rbcs-Toc36B* are imported differently

Com70 has been shown to be in close physical proximity to a partially translocated precursor and plays a role in the early stage of the typical import pathway [4,14]. Antibodies against Com70 can therefore be used in import impairment experiments to provide an indication of differences in the transport

pathways of various precursor proteins. This approach was used to assess whether Toc36B1-Dhfr was recognized in a manner different from Rbcs-Toc36B at an early stage of the import process. Impairment studies were conducted without the use of protease post-treatments since Com70 works at an early stage of the import process. Increasing concentrations of anti-Com70 immunoglobulin G (IgG) reduced the level of imported Rbcs-Toc36B (Fig. 4), but had no apparent effect on Toc36B1-Dhfr. Antibodies against another envelope protein Cim37 (designated Control Abs), an inner envelope polypeptide, had no observable impact on the import of either fusion precursors (Fig. 4). The results indicate that the Toc36B targeting signal, unlike typical stroma- or thylakoid-destined transit peptides, may be routed/advanced in a different manner early in the protein import process.

4. Discussion

The import behavior exhibited by the various carboxyl and amino deletion constructs of Toc36B clearly establishes the N-terminal 39 amino acids as the main envelope targeting information of the protein, even though the N-terminal targeting signal is not processed like a typical chloroplast protein transit peptide. The lack of impairment of Toc36B import by anti-Com70 IgGs, which impairs early in the import of typical chloroplast protein precursors, provides more evidence that the targeting pathway/mechanism is different from the route typically utilized by most proteins. The Toc36B targeting signal, unlike typical stromal or thylakoidal transit peptides, may be advanced in a different manner early in the

protein import process. Removal of the N-terminal 39 amino acids from Toc36B abolishes targeting to the chloroplast, as is the case with the deletion of 18 residues from the C-terminal end of the N-terminal region. The remaining extreme N-terminal 21-amino acid segment itself is not sufficient to direct proteins (Toc36B or Dhfr) to the envelope. The 39-residue N-terminal region itself contained the necessary information to target Dhfr to the chloroplast. It is noteworthy to point out that the 18 residues removed were not the usual mixture of amino acids, but consisted of two conspicuous segments of alternating amino acids, glycine-alanine and proline-phenylalanine. The two alternating segments are in tandem and are separated by a single serine. This amino acid arrangement may suggest a role in the formation of a competent targeting signal or structure. Disturbance of the targeting signal can be observed in the import data of N6, where the addition of nine extra amino acids to the Toc36B N-terminus abolished its import competence. This type of effect indicates that the amino acid context surrounding the targeting signal may also contribute to the import behavior of Toc36B. It is thus important to note here that many of the constructions studied contain exogenously-added amino acids of various lengths that may also exert possible structural/conformational effects on the targeting signal and/or the whole protein to give rise to our observations. The import data should therefore be viewed as effects caused by a combination of the deletions and the amino acids added in the specifically manipulated region of the Toc36B protein. In addition to these features, the N-terminal 39 amino acids also contain a high concentration of methionine residues resembling methionine-rich regions or bristles involved in the binding of signal sequences by the 54-kDa protein of the signal recognition particle [15–17]. The methionine bristle-like feature of the N-terminus may also be a contributing factor to the targeting of the protein and/or an indication of its potential role in protein translocation and the mechanism underlying this role. In the context of the features and possibilities discussed above, the N-terminal 39 amino acids are likely involved in the formation of a complex targeting signal. These possibilities are currently being investigated at all levels.

The remaining carboxyl segment of Toc36B can

also affect targeting, as suggested by the truncation and the chimeric fusion import data. Although the attachment of a stromal-destined transit peptide onto Toc36B redirected the majority of the proteins into the stromal compartment as processed products, a portion of the products was associated with membranes and was present in different locations of the envelope. Different membrane-associated forms were revealed by post-import protease treatments (e.g. the 38- and 20-kDa protease-generated products). All of these forms were partially translocated to a stage of the import pathway allowing processing by the stromal peptidases. These forms may span both outer and inner envelope membranes, or the inner membrane only. Further experimentation is required to determine the topology of the various forms. This behavior indicates the presence of information in the carboxyl segment of Toc36B that may influence aspects such as membrane-association and conformation. The change in sensitivity to thermolysin displayed between C3 and C4 appears to point to such a possibility. Removal of the sequences represented by C3, C4 and Bce44B1-Dhfr perturbed or abolished the signal for proper assembly in the membranes. Although the precise role of the C-terminal information in the targeting and assembly of Toc36B is unknown at present, it does indicate that the mechanism underlying the process is complex and involves the interplay of signals throughout the protein. Unlike translocation intermediates generated by sub-optimal energy conditions, the distinct Toc36B-containing forms were present in conditions supporting normal import of proteins into the chloroplast. These complex signals appear to play a role in targeting and assembly of Toc36 in more than one site in the outer and inner plastid envelope. For example, the N-terminal information may govern the extent of importation into the chloroplast, whereas the C-terminal information may act in an integrative/stop-transfer capacity and/or in a structural capacity. The resulting interplay would in turn determine the final locale of the protein. The possibility that the various native Toc36 forms in the chloroplast envelope arise from a multi-site targeting mechanism appears to be in line with the previous observations reported for chloroplasts and bacteria [4–6]. The *in vitro* import results presented in this study provide further indication that at least some of the Toc36

proteins may be derived from a multi-site distribution mechanism. We are currently characterizing and studying other members of the Toc36 protein family to elucidate the import/assembly signals involved in the targeting of the various natural forms.

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