Positive Charges Determine the Topology and Functionality of the Transmembrane Domain in the Chloroplastic Outer Envelope Protein Toc34

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Abstract. The chloroplastic outer envelope protein Toc34 is inserted into the membrane by a COOH-terminal membrane anchor domain in the orientation N_{cyto}-C_{in}. The insertion is independent of ATP and a cleavable transit sequence. The cytosolic domain of Toc34 does not influence the insertion process and can be replaced by a different hydrophilic reporter peptide. Inversion of the COOH-terminal, 45-residue segment, including the membrane anchor domain (Toc34Cinv), resulted in an inverted topology of the protein, i.e., N_{in}-C_{cyto}. A mutual exchange of the charged amino acid residues NH_2- and COOH-proximal of the hydrophobic α-helix indicates that a double-positive charge at the cytosolic side of the transmembrane α-helix is the sole determinant for its topology. When the inverted COOH-terminal segment was fused to the chloroplastic precursor of the ribulose-1,5-bisphosphate carboxylase small subunit (pS34Cinv), it engaged the transit sequence–dependent import pathway. The inverted peptide domain of Toc34 functions as a stop transfer signal and is released out of the outer envelope protein translocation machinery into the lipid phase. Simultaneously, the NH_2-terminal part of the hybrid precursor remained engaged in the inner envelope protein translocon, which could be reversed by the removal of ATP, demonstrating that only an energy-dependent force but no further ionic interactions kept the precursor in the import machinery.

THE majority of chloroplast proteins are encoded for in the nucleus, synthesized in the cytosol, and post-translationally imported into the organelle. The standard import route is facilitated by two protein translocation machineries located in the chloroplastic outer and inner envelope membranes, which act jointly during translocation (Schnell and Blobel, 1993; Alefsen et al., 1994). Proteins destined to be translocated into chloroplasts carry NH_2-terminal transit sequences, which are both necessary and sufficient for targeting and import. The transit sequence recognition is mediated by a protease-sensitive receptor polypeptide located in the outer envelope membrane (Cline et al., 1985). A low ATP concentration, between 5–50 μM, is required for binding of the precursor protein to the outer envelope translocon in a step not well characterized yet, while high concentrations, between 50–1,000 μM, are necessary for complete translocation into the chloroplasts (Flügge and Hinz, 1986; Schindler et al., 1987; Olsen et al., 1989).

In contrast, proteins destined for the chloroplastic outer envelope are generally synthesized without an NH_2-terminal cleavable transit sequence but are targeted by internal information (Salomon et al., 1990; Li et al., 1991; Ko et al., 1992; Fischer et al., 1994; Kessler et al., 1994; Seedorf et al., 1995; Li and Chen, 1996; Chen and Schnell, 1997), except for the 86- and 75-kD subunits of the translocon at the chloroplastic outer envelope (Toc86,[1] Hirsch et al., 1994; Kessler et al., 1994; Toc75, Tranel et al., 1995; Tranel and Keegstra, 1996). The Toc75 precursor uses the general import machinery during the initial phase of the import. It is processed in the stroma to an intermediate-size form while the majority of the mature protein remains in the chloroplastic outer membrane by an unknown stop transfer mechanism. In a second phase of translocation, the protein is inserted into the membrane and terminally processed in a poorly understood way. The transit sequence–independent targeting and insertion seem to require neither protease-sensitive polypeptide components of the outer envelope membrane nor the hydrolysis of ATP. Current

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1. *Abbreviations used in this paper*: chl, chlorophyll; inv, inverse; mS, small subunit of ribulose-1,5-bisphosphate carboxylase; pS, precursor form; Tic, translocon at the inner envelope membrane of chloroplasts; TIM, translocation intermediates; Toc, translocon at the outer envelope membrane of chloroplasts.

evidence suggests that none of the protein translocon components of the chloroplastic outer envelope is involved in the presequence-independent insertion pathway (Salomon et al., 1990; Li et al., 1991; Ko et al., 1992; Fischer et al., 1994; Seedorf et al., 1995; Li and Chen, 1996; Chen and Schnell, 1997; for review see Soll et al., 1992). This insertion mechanism is different from the presequence-independent integration of proteins into the mitochondrial outer membrane, which uses subunits of the TOM machinery (for review see Lill and Neupert, 1996).

An issue that remains to be solved is how these membrane proteins achieve their correct topology. (*a*) Does the specific lipid composition influence targeting and topology of the chloroplastic outer envelope proteins, and (*b*) what is the role of the membrane spanning domains of these envelope polypeptides? Or, (*c*) do soluble segments of the protein influence its insertion and topology? Toc34 has a well-established topology, N_{cyto}-C_{in} with only one transmembrane segment. The α-helical hydrophobic membrane anchor is located close to the COOH terminus of the protein, which leaves only 3 kD of the polypeptide exposed to the intermembrane space. The major portion of the protein, including its GTP-binding domain, is cytosolic. Insertion of Toc34 translation product into the chloroplastic outer envelope requires the presence of the hydrophobic transmembrane segment (Seedorf et al., 1995; Chen and Schnell, 1997; Li and Chen, 1997). This insertion process renders Toc34 resistant to extraction at pH 11.5. Productive folding or assembly, which can be followed by the appearance of an 8-kD protease-protected fragment, seems to be stimulated by the presence of nucleoside-triphosphates and a protease-sensitive envelope membrane component (Seedorf et al., 1995; Chen and Schnell, 1997). Resistance to alkaline extraction together with the appearance of the 8-kD proteolytic fragment can be used to follow the productive insertion process of Toc34.

In this study, we demonstrate that a double-positive charge at the cytosolic side of the α-helicial membrane anchor dictates the topology of Toc34. Furthermore, this domain acts as a functional stop transfer signal for the outer envelope protein translocon. The signal anchor sequence is released laterally from the translocon into the lipid phase of the membrane.

Materials and Methods

Chloroplast Protein Import Assays

Chloroplasts were isolated from pea leaves of 10–12-d-old plants by standard procedures and purified further on silica-sol gradients (Waegemann and Soll, 1991). Chlorophyll (chl) concentration was determined as described (Arnon, 1949). Standard import assays contained chloroplasts equivalent to 30 μg chl in 100 μl import buffer (330 mM sorbitol, 50 mM Hepes-KOH, pH 7.6, 3 mM $MgCl_2$, 10 mM methionine, 10 mM cysteine, 20 mM potassium gluconate, 10 mM $NaHCO_3$, 2% [wt/vol] BSA) and 1–10% of reticulocyte lysate in vitro–synthesized ^{35}S-labeled proteins. Translocation reactions were initiated by the addition of organelles and allowed to continue for 10 min at ambient temperature. Chloroplasts were recovered from the import reaction by centrifugation (5,000 *g*, 5 min, 4°C) through a Percoll cushion (40% [vol/vol] Percoll in 330 mM sorbitol, 50 mM Hepes-KOH, pH 7.6), washed once in Hepes-sorbitol (Waegemann and Soll, 1991), and used for further treatments.

Chloroplasts were treated with thermolysin before a translocation experiment with 750 μg protease/mg chl for 30 min on ice in 330 mM sorbitol, 50 mM Hepes-KOH, pH 7.6, 3 mM $MgCl_2$, and 0.5 mM $CaCl_2$. The reaction was terminated by the addition of 10 mM EDTA. Intact chloroplasts were recovered on silica-sol gradients and washed twice as described above. When chloroplasts were treated with thermolysin after an import experiment, organelles were incubated at a final concentration of 100 μg protease/mg chl for 15 min at 4°C. The reaction was stopped by the addition of 10 mM EDTA, and the chloroplasts were recovered by centrifugation and washed once (Joyard et al., 1983; Cline et al., 1984). When indicated, chloroplasts were treated with trypsin (Marshall et al., 1990; Lübeck et al., 1996). Chloroplasts equivalent to 200 μg chl were incubated with 200 μg trypsin (10,700 Na-benzoyl-L-arginine ethyl ester U/mg from bovine pancreas) for 60 min at 25°C in 50 mM Hepes-KOH, pH 8.0, 0.1 mM $CaCl_2$, and various sorbitol concentrations (200 mM = hypotonic; 330 mM = isotonic; 600 mM = hypertonic conditions). The reaction was stopped by the addition of 1 mM PMSF and a fivefold molar excess of soybean trypsin inhibitor. Intact chloroplasts were recovered by centrifugation through a Percoll cushion and washed twice under isotonic conditions as described above. PMSF was present at all washing steps. Chlorophyll concentration was determined, and equal amounts of organelles were loaded onto the SDS-PAGE on a chl basis.

After hypotonic lysis in 10 mM Hepes-KOH, pH 7.6, chloroplasts were separated into a soluble and a total membrane fraction by centrifugation for 10 min at 165,000 *g*. Soluble proteins were precipitated by 10% TCA. In some cases, lysed organelles were treated with 0.1 M Na_2CO_3, pH 11.5, for 10 min on ice. Insoluble material was recovered by centrifugation as described before, and soluble proteins were precipitated by 10% TCA. Import products were analyzed by SDS-PAGE (Laemmli, 1970) followed by fluorography (Bonner and Laskey, 1974).

Construction of cDNAs Coding for Toc34 Hybrid Proteins and Synthesis of Labeled Proteins

cDNAs coding for the different hybrid proteins, including single amino acid exchanges, were constructed by recombinant PCR (Higuchi, 1990). Toc34Cinv was constructed using two extra-long (105-bp) primers, which coded for the inverted sequence of the Toc34 COOH terminus (see Fig. 1). Products were cloned into a vector suitable for in vitro transcription and controlled by DNA sequencing (Sanger et al., 1977). The original cDNAs coding for Toc34 and the precursor form (pS) have been described (Klein and Salvucci, 1992; Seedorf et al., 1995). In vitro transcription was done using either T7 or SP6 RNA-polymerase, as outlined before (Salomon et al., 1990). Proteins were synthesized in a reticulocyte lysate system in the presence of [^{35}S]methionine and [^{35}S]cysteine (1,175 Ci/mmol) for 1.5 h at 30°C. Overexpression of unlabeled or radioactively labeled proteins was done in *Escherichia coli* BL21 (DE3) cells using the pET vector system (Novagen Corp., Madison, WI) (Waegemann and Soll, 1995). Proteins were isolated from inclusion bodies and solubilized in 8 M urea. The final urea concentration in the import assays never exceeded 80 mM.

Chloroplastic ATP Levels

Intact chloroplasts were incubated in import buffer in the presence of 2 mM ATP and 2 μCi [γ-^{32}P]ATP for 10 min at 25°C. An aliquot was removed, and chloroplasts were separated from the aqueous medium by centrifugation (15 s, 9,000 rpm) through a silicon oil layer (Wirtz et al., 1980). Residual chloroplasts were recovered by centrifugation and resuspended in import buffer in the absence of ATP. Aliquots were removed from the organelle suspension at different time intervals and recovered by centrifugation through silicon oil. Chloroplasts were extracted with 80% dimethylketon. Nucleotides were separated by thin layer chromatography on glass plates coated with polyethyleneimine cellulose using 0.5 M KH_2PO_4 as solvent. ATP-containing spots were localized in UV light and scraped off the plates, and radioactivity was determined after addition of scintillation cocktail in a scintillation counter.

Results

Toc34 has an established topology, i.e., N_{cyto}-C_{in} with a single hydrophobic α-helical region at the COOH terminus acting as a membrane anchor domain. Our aim was to study the role of the membrane anchor region in the topology of Toc34 and its influence on the protein import pathway. Therefore, the hybrid protein Toc34Cinv was constructed containing 45 amino acids of the COOH terminus,

including the membrane anchor domain, but in an inverted sequence (for details see Fig. 1). Accurate targeting and insertion of Toc34 into the chloroplastic outer envelope can be proven by a combination of two experimental approaches: (*a*) Toc34 is largely resistant to extraction at pH 11.5 in situ (Seedorf et al., 1995); and (*b*) Toc34 is sensitive to thermolysin in situ, except for an 8-kD COOH-terminal fragment (Seedorf et al., 1995). When Toc34Cinv translation product was incubated with intact chloroplasts, the protein integrated into the outer envelope membrane in a largely protease-resistant form (Fig. 2 *A*, *bottom*, lane *2*), in contrast to the wild-type Toc34 (Fig. 2 *A*, *top*, lane *2*). Protease resistance of inserted hybrid protein is compatible with the sequence of Toc34Cinv because the protein contains only three amino acids COOH-terminal of the inverted membrane anchor (see Fig. 1). The appearance of proteolytic fragments, which are formed from a subpopulation of Toc34Cinv, was most likely due to an incomplete transfer of the hydrophilic domain across the outer envelope membrane. Toc34Cinv inserted into the membrane in a way that renders it largely resistant to extraction at pH 11.5, i.e., it behaves as an integral membrane protein like the wild-type Toc34 (Fig. 2 *A*, lanes *3* and *4*). The proteolytic fragments generated from inserted Toc34Cinv were also resistant to alkaline extraction, demonstrating that the membrane anchor region had integrated into the lipid bilayer even in those cases where translocation was not complete. Insertion of Toc34 translation products into chloroplasts yielded the expected results (Seedorf et al., 1995; Chen and Schnell, 1997), i.e., the integrated protein was sensitive to thermolysin but resistant to alkaline extraction (Fig. 2 *A*, *top*, lanes *3–6*).

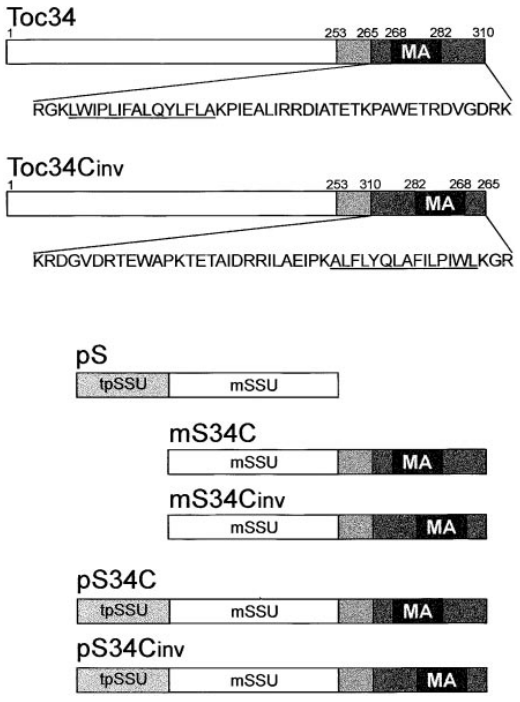

Figure 1. Peptide domain combination of various hybrid proteins used in this study and their nomenclature. The membrane anchor (*MA*) is underlined. Numbers indicate amino acid positions of the Toc34 sequence from pea (Seedorf et al., 1995).

Next we wanted to know if the hydrophilic NH_2-terminal part of Toc34 influences the insertion reaction. It was replaced by the mature form of the Rubisco small subunit (mS), which is a soluble stroma-localized protein. Both hybrid proteins, mS34C and mS34Cinv, inserted indistinguishably in the chloroplastic outer envelope to the analogue "wild-type" Toc34's (Fig. 2, *A* and *B*), i.e., inserted mS34C and mS34Cinv were resistant to extraction at pH 11.5 (Fig. 2 *A*, lanes *9* and *10*), and mS34C was susceptible to a thermolysin treatment while mS34Cinv proved to be largely resistant (Fig. 2 *A*, lanes *7* and *8*). The proteolytic fragments of inserted mS34Cinv are most likely due to incomplete translocation of the NH_2-terminal polypeptide chain across the outer envelope membranes. Solubilization of chloroplast membranes by detergent before protease treatment resulted in complete proteolysis (not shown).

To obtain further experimental evidence for the putative topology of the different Toc34 proteins, they were synthesized in a reticulocyte lysate system in the presence of [^{3}H]leucine. The 8-kD proteolysis-resistant fragment (see above) can only be detected in experiments using [^{3}H]leucine-labeled Toc34 because the COOH terminus does not contain any methionine or cysteine residues. The appearance of the 8-kD fragment either from [^{3}H]Toc34

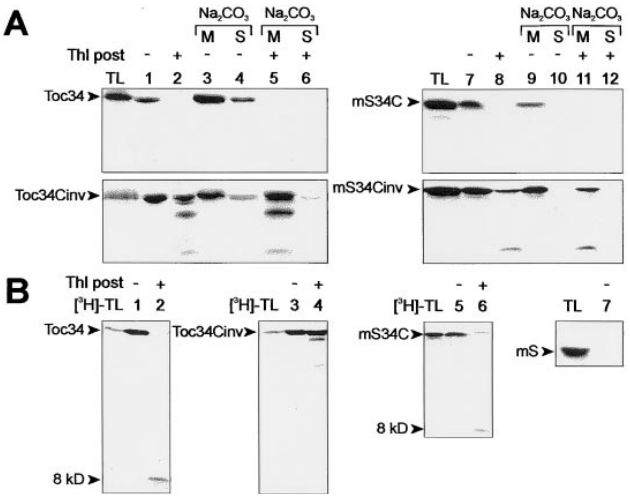

Figure 2. The COOH-terminal segment of Toc34 is the sole determinant for the protein topology. (*A*) ^{35}S-labeled Toc34, Toc34Cinv, mS34C, and mS34Cinv translation product (*TL*) was used in standard translocation experiments (2 mM ATP, 25°C, 10 min) with purified pea chloroplasts (equivalent to 30 μg chl). After completion of the translocation experiment, organelles were either not treated or treated with the protease thermolysin as indicated. Chloroplasts were lysed in hypertonic buffer (lanes *3–6*) and separated into a soluble (*S*) and insoluble (*M*) fraction by centrifugation after extraction at pH 11.5 with 0.1 M Na_2CO_3. (*B*) Toc34, Toc34Cinv, mS34C, and mS were synthesized in a reticulocyte lysate system in the presence of [^{3}H]leucine and used in a parallel translocation experiment under identical conditions as described for *A*. SDS-PAGE gels were loaded with equal amounts of organelles on a chl basis. A 12.5% (wt/vol) acrylamide-containing separation gel was used in *A*, while a 15% gel was used in *B*. Translocation product (*TL*) is shown as internal standard, 10% of which was added to a standard translocation reaction. Fluorograms are shown.

or [^{3}H]mS34C translation product shows that both polypeptides obtained a topology N_{cyto}-C_{in}, i.e., indistinguishable to Toc34 in situ (Fig. 2 *B*, lanes *1* and *2*, and *5* and *6*). Thermolysin treatment of inserted [^{3}H]Toc34Cinv yielded no 8-kD fragment, but did yield a fragmentation pattern similar to the ^{35}S-labeled Toc34Cinv (Fig. 2 *B*, lanes *3* and *4*). mS does not interact with chloroplasts to a detectable amount (Fig. 2 *B*, lane *7*). From these data, we conclude that the Toc34 COOH-terminal 45 amino acids seem to be the only determinant for chloroplastic targeting, and they simultaneously dictate the topology of the inserted protein. The inverted COOH terminus of Toc34 can function as a bona fide membrane anchor in the chloroplastic outer membrane but simultaneously inverts the topology of the protein into N_{in}-C_{cyto}.

As demonstrated above, the COOH-terminal region of Toc34 contains an outer envelope targeting domain regardless of its orientation. In light of this, we asked if the stroma-targeting envelope transfer signal present in the transit sequence of pS is able to override the membrane anchoring information present in the COOH terminus of Toc34. We therefore compared the import and binding of preSSU with its derived hybrid proteins pS34C and pS34Cinv. Under conditions that allow import to occur at optimal rates (25°C and 2 mM ATP), pS is efficiently translocated into the chloroplasts and processed to the mature form (Fig. 3 *A*, lanes *1* and *2*). The mature form mS is recovered as a protease-protected soluble protein (Fig. 3 *A*, lanes *3–6*). The ratio of radioactivity recovered in pS and processed mature mS was 1:19, as determined by laser densitometry of exposed x-ray films ($n = 5$). pS34C is imported into intact chloroplasts with a yield similar to preSSU. It is processed like the wild-type protein to yield mS34C (Fig. 3 *B*, lanes *1* and *2*). The ratio of chloroplast-bound precursor and mature mS34C was 2:8 ($n = 5$).

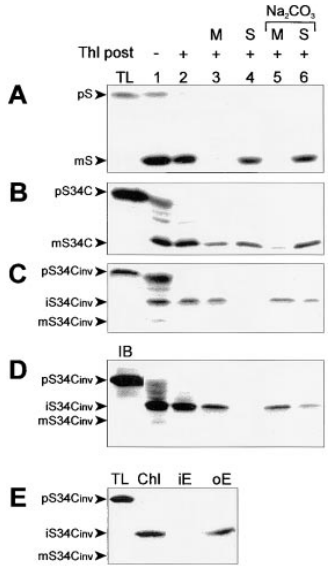

Figure 3. The inverted COOH-terminal segment of Toc34 functions as a stop transfer domain at the chloroplastic outer envelope. (*A–D*) Indicated ^{35}S-labeled, in vitro–translated (*A–C*) or overexpressed (*D*) proteins were incubated with intact pea chloroplasts under standard import conditions. After completion of the import reaction, chloroplasts were either not treated (lane *1*) or treated (lane *2–6*) with the protease thermolysin. Organelles were fractionated into a total membrane (*M*, lane *3*) or soluble protein (*S*, lane *4*) fraction. Chloroplasts were treated with 0.1 M Na_2CO_3 before separation into an alkaline-insoluble (*M*, lane *5*) or -soluble (*S*, lane *6*) fraction. (*E*) Chloroplastic outer and inner envelope membranes were fractionated after a standard import reaction as described by Keegstra and Youssif (1986). Chloroplasts were treated with thermolysin after import. Fluorograms are shown.

Upon subfractionation of the chloroplasts, an equal distribution of mS34C is observed between the total membrane and the soluble protein fraction (Fig. 3 *B*, lanes *3* and *4*). However, mS34C only adheres to the membranes since it is completely extractable at pH 11.5 (Fig. 3 *B*, lanes *5* and *6*). In comparison to preSSU, the binding of pS34C is only slightly increased, indicating that most but not all of the hybrid precursor might enter the standard import route. Translocation still takes place when pS34Cinv translation product was incubated with intact chloroplasts under standard import conditions. However, the processing resulted primarily in a product of intermediate size (iS34Cinv) and only in a very small amount of mature mS34Cinv (Fig. 3 *C*, lanes *1* and *2*). The ratio between pS34Cinv, iS34Cinv, and mS34Cinv was 6:3:1 ($n = 5$). iS34Cinv was completely recovered in the total membrane fraction (Fig. 3 *C*, lanes *3* and *4*). Upon alkaline extraction, 80% of iS34Cinv was recovered in the pellet fraction while 20% could be extracted (Fig. 3 *C*, lanes *5* and *6*). These latter results indicated that most of iS34Cinv had inserted into the lipid bilayer of the outer or inner envelope while a smaller portion of iS34Cinv remained in a proteinaceous environment, e.g., the protein translocation pore. When the outer and inner envelope membranes were separated from chloroplasts after pS34Cinv translocation, iS34Cinv was recovered in the outer envelope (Fig. 3 *E*). The translocation efficiency of pS34Cinv is less than for pS and pS34C because more pS34Cinv remained bound to the chloroplast surface in a protease-accessible manner (Fig. 3 *B*, lanes *1* and *2*). On the other hand, insertion of mS34Cinv translation product into the outer envelope attains a largely protease-protected topology (Fig. 2, lanes *7* and *8*). We do not think that these results are contradictory because pS34Cinv can enter two chloroplastic translocation pathways: a receptor-dependent pathway as well as a receptor-independent pathway, depending on the experimental conditions. This might result in a protein being fixed with both the NH_2 terminus and the COOH terminus at the surface of the organelle. In contrast, mS34Cinv has only one opportunity, namely to enter the receptor-independent pathway. This is also corroborated by further findings presented below.

Furthermore, when we used urea-denatured, overexpressed pS34Cinv in a standard import reaction, iS34Cinv was again the major reaction product (Fig. 3 *D*, lanes *1* and *2*). Processed, mature mS34Cinv was below 10% of the total. Only very little binding of urea-denatured pS34Cinv was detected. This might be due to the missing secondary structure of the unfolded precursor, which could prevent (*a*) the interaction with the membrane due to the absence of the α-helical conformation of the COOH terminus and (*b*) the accumulation of the precursor at receptor sites on the organellar surface.

The general import pathway into chloroplasts by the Toc and Tic machinery requires protease-sensitive receptor components at the chloroplastic surface, which recognize the NH_2-terminal transit sequences. In contrast, the insertion of chloroplastic outer envelope proteins, which do not contain a presequence, is independent of a protease-sensitive surface component. To investigate further if and to what extent pS34C and pS34Cinv used the standard import pathway, chloroplast surface–exposed precursor receptors were removed by treatment with the pro-

tease thermolysin. The binding of pS to protease-shaved chloroplasts was reduced to 10% in comparison to untreated organelles (Fig. 4, *C*, lanes *1* and *3*; and *D*). In contrast, the amount of binding of pS34C or pS34Cinv was not significantly influenced by a protease pretreatment, indicating that both proteins inserted largely via the COOH-terminal membrane anchor region under conditions that discourage the Toc pathway. Under import conditions, (2 mM ATP and 25°C), thermolysin-shaved chloroplasts were also blocked in complete translocation of pS (Fig. 4 *C*, lanes *7–10*). The residual import yield varied between 10 and 15% ($n = 6$; Fig. 4 *D*) for protease-pretreated chloroplasts. In parallel experiments, the efficiencies of pS34C import into thermolysin-shaved chloroplasts as measured by the appearance of processed mS34C dropped to 15–20% of control imports (Fig. 4, *A*, lanes *7–10*; and *D*). The import yield was even less for the translocation of pS34-Cinv into protease-pretreated chloroplasts (Fig. 4 *B*, lanes *7–10*). Only 5% iS34Cinv was still detected (Fig. 4 *D*). The precursor proteins pS34C and pS34Cinv, which were bound to protease-shaved chloroplasts, were resistant to alkaline extraction, indicating that they inserted by the COOH terminus into the outer envelope membrane.

To distinguish in vitro between presequence-dependent and -independent interactions with the organellar surface, i.e., via the COOH-terminal α-helix, chloroplasts were incubated with pS, pS34C, and pS34Cinv, respectively, under binding conditions (50 μM ATP and 4°C) for 10 min, followed by a chase period under translocation conditions (2 mM ATP and 25°C). pS bound to the chloroplastic surface and translocation intermediates (TIM 3/4), which are well characterized for the pS import pathway (Waegemann and Soll, 1991, 1996), were detected upon thermolysin treatment (Fig. 4 *C*, lanes *1* and *2*, and *13* and *14*). The bound pS could be chased into the chloroplasts upon the addition of ATP and raising the temperature to 25°C, demonstrating that the binding state was productive (Fig. 4 *C*, lane *15*). pS34C and pS34Cinv also bound to the chloroplast surface with a yield similar to pS (Fig. 4, *A* and *B*, lanes *1* and *13*). Upon thermolysin treatment, the translocation intermediates (TIM 3/4) could be detected (Fig. 4, *B* and *C*, lanes *2* and *14*). However, chloroplast-bound hybrid proteins did not translocate into chloroplasts to any significant extent upon establishing import conditions (Fig. 4, *A* and *B*, lane *15*), probably because they had also inserted during the course of the incubation period into the chloroplastic outer envelope with the COOH-terminal α-helical region. This is supported by the observation that the surface-bound pS34C and pSCinv were resistant to alkaline extraction (not shown). These results indicate that conditions that do not allow import, i.e., low ATP and low temperature, favor the insertion of Toc34 via the α-helical membrane anchor region.

From the data presented in Fig. 4, we conclude that pS34C and pS34Cinv use preferentially the Toc and Tic complex-dependent pathway under experimental conditions that favor this pathway (2 mM ATP, 25°C) and functional receptor polypeptides. Whenever we manipulate the optimal requirements by lowering the temperature or the ATP concentration or by removing precursor recognition sites, the membrane insertion route via the COOH-terminal membrane anchor becomes more prominent.

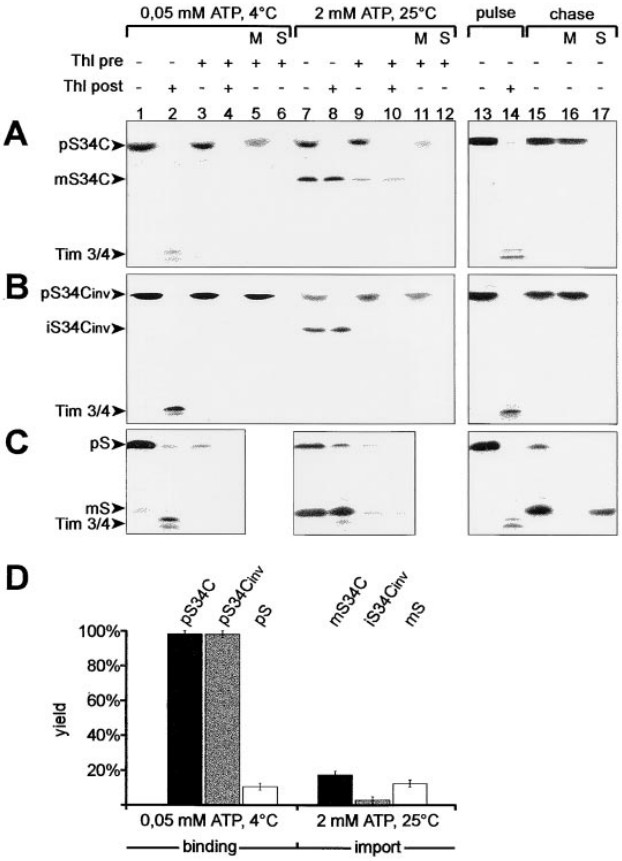

Figure 4. The hybrid proteins pS34C and pS34Cinv can engage different translocation pathways. (*A–C*) Chloroplasts were either not treated (lanes *1* and *2*, and *7* and *8*) or treated (lanes *3–6* and *9–12*) with the protease thermolysin before either binding or import reactions. ^{35}S-labeled pS34C (*A*), pS34Cinv (*B*), and pS (*C*) were incubated for 10 min with organelles under binding (50 μM ATP, 4°C) or import (2 mM ATP, 25°C) conditions. After completion of the reaction, chloroplasts were again either treated or not treated with the protease thermolysin as indicated on top of the figure (*Thl post*). Fractionation into membranes and soluble protein was done as outlined before. A typical result out of five repeats is shown. Chloroplasts were incubated with pS, pS34C, and pS34Cinv translation product, respectively, at 4°C for 10 min in the presence of 50 μM ATP (*pulse*, lanes *13* and *14*). Chloroplasts were reisolated by centrifugation and washed once, and import conditions were established, i.e., 2 mM ATP, 25°C, 10 min (*chase*, lanes *15–17*). The position of translocation intermediates TIM 3 and TIM 4 is indicated. All other manipulations were as indicated on top of the figure and carried out as described in Fig. 2. (*D*) Quantification of the binding and import efficiency from *A–C*. The yield of binding in lane *3* is compared with lane *1* and given as percentage thereof. The yield of translocation in lane *10* is compared with lane *8* and given as percentage thereof. Quantification was done by laser densitometry of the exposed x-ray film.

pS34Cinv is imported into chloroplasts at 2 mM ATP and processed to an intermediate-size form (see Fig. 4 *B*), most likely by the stromal-processing protease. Therefore, it should also engage the Tic complex during this process. Furthermore, iS34Cinv behaves as an integral membrane protein that cofractionates with the chloroplastic outer envelope (Fig. 3 *E*) when the inner and outer membrane are separated by shearing forces (Keegstra and Youssif, 1986).

To test if iS34Cinv is in contact with the Tic complex, we compared the sensitivity of the imported and processed forms of pS, pS34C and pS34Cinv, to thermolysin and trypsin, respectively. Under strictly controlled conditions, thermolysin only shaves proteins off the chloroplastic surface, while trypsin penetrates the outer envelope membrane and gains access to the intermembrane space and to proteins exposed on the surface of the inner membrane (Marshall et al., 1990; Lübeck et al., 1996; see also Fig. 5 *C*). mS and mS34C were resistant to both thermolysin and trypsin (Fig. 5 *A*, lanes *1–6*), indicating that they had reached the stroma. In contrast, iS34Cinv was resistant to thermolysin but sensitive to trypsin (Fig. 5 *A*, lanes *7–9*). A proteolytic fragment was generated by trypsin (iSΔC; Fig. 5 *A*, lane *9*), which was recovered in the total soluble protein fraction of chloroplasts (Fig. 5 *B*, lane *4*). These data indicate that trypsin removed the membrane anchor of iS34Cinv, so that translocation into the stroma could proceed through the inner envelope translocon. The predicted localization of iS34Cinv, i.e., anchored in the outer envelope membrane while simultaneously engaging the Tic complex, was further established by carrying out the trypsin treatment under different osmotic conditions. The rationale behind this approach is that at hypertonic conditions (0.6 M sorbitol), a retraction of the inner envelope membranes from the outer and a widening of the intermembrane space occurs because H_2O is extruded from the stroma into the surrounding medium (Block et al., 1983; see Fig. 5 *C*). Under these conditions, iS34Cinv should become trypsin sensitive. On the other hand, isotonic or slightly hypotonic conditions do not result in a membrane rearrangement, and trypsin treatment should result in the appearance of iSΔC. This was found to be the case (Fig. 5 *B*). Chloroplast-inserted iS34Cinv was completely degraded by trypsin treatment under hypertonic conditions (Fig. 5 *B*, lanes *9* and *10*). At isotonic and hypotonic conditions, iSΔC remained protease protected (Fig. 5 *B*, lanes *3* and *4*, and *7* and *8*). iSΔC is not protease resistant per se, as demonstrated by treating the soluble iSΔC with trypsin (Fig. 5 *B*, lane *6*), which results in complete proteolysis. We conclude that the inverted COOH-terminal segment of Toc34 can serve as a stop transfer signal for the Toc machinery. The stop transfer signal is recognized by the outer envelope translocon and laterally released into the plane of the membrane. Simultaneously, the iS part of the protein continuously interacts with the inner envelope translocon.

The latter prediction was analyzed using the following experimental procedures. Intact chloroplasts were preincubated either in the absence or presence of *E. coli* expressed pS (Fig. 6 *A*), pS34Cinv (Fig. 6 *B*), or Toc34 (Fig. 6 *C*) under import conditions for 5 min. Organelles were recovered by centrifugation and washed once in import buffer in the presence or absence of 2 mM ATP. A second round of protein translocation was started by the addition of ^{35}S-labeled pS reticulocyte lysate translation product. Import of ^{35}S-labeled pS was only slightly diminished when chloroplasts were preincubated in the presence of overexpressed pS, in comparison to a mock preincubation in the absence of overexpressed pS (Fig. 6 *A*, lanes *1* and *2*, and *5* and *6*). When ^{35}S-labeled pS reticulocyte translocation was added simultaneously with *E. coli* expressed unlabeled pS, binding and translocation of ^{35}S-labeled pS was almost completely abolished (Fig. 6 *A*, lanes *3* and *4*). This demonstrates that the heterologously expressed precursor could compete with the reticulocyte lysate–synthesized precursor. In contrast, ^{35}S-labeled pS import into chloroplasts that were preincubated with pS34Cinv was very strongly inhibited (Fig. 6 *B*, lanes *1* and *6*), independent of the presence of pS34Cinv during the second round of translocation. Binding of pS to the chloroplast surface was not influenced significantly (Fig. 6 *B*, lane *5*), indicating that inhibition occurred in a later step of the translocation

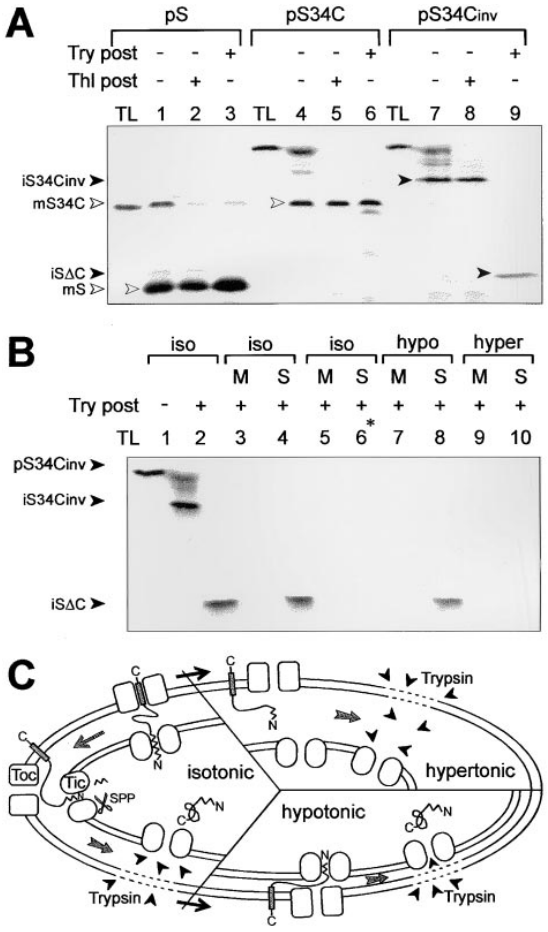

Figure 5. The stop transfer signal present in pS34Cinv is recognized by the Toc complex. pS, pS34C, and pS34Cinv were imported into pea chloroplasts under standard conditions. (*A*) After completion of the import reaction, organelles were treated with the noninvasive protease thermolysin or with trypsin as indicated on top of the figure. The positions of the processed proteins or the proteolysis products are indicated by arrowheads. (*B*) Chloroplasts were treated after import with trypsin in isotonic (330 mM sorbitol), hypotonic (150 mM sorbitol), or hypertonic (600 mM sorbitol) conditions. Chloroplasts were lysed after the trypsin treatment (lanes *3–10*) and separated into a total membrane (*M*) and a soluble (*S*) protein fraction by centrifugation. In lane *6*, the soluble fraction was treated a second time with trypsin to demonstrate its protease sensitivity. (*C*) Schematic representation of the different effects of the various sorbitol concentrations on the arrangement of the outer and inner chloroplastic envelopes and for the trypsin accessibility of iS34Cinv. Not drawn to scale.

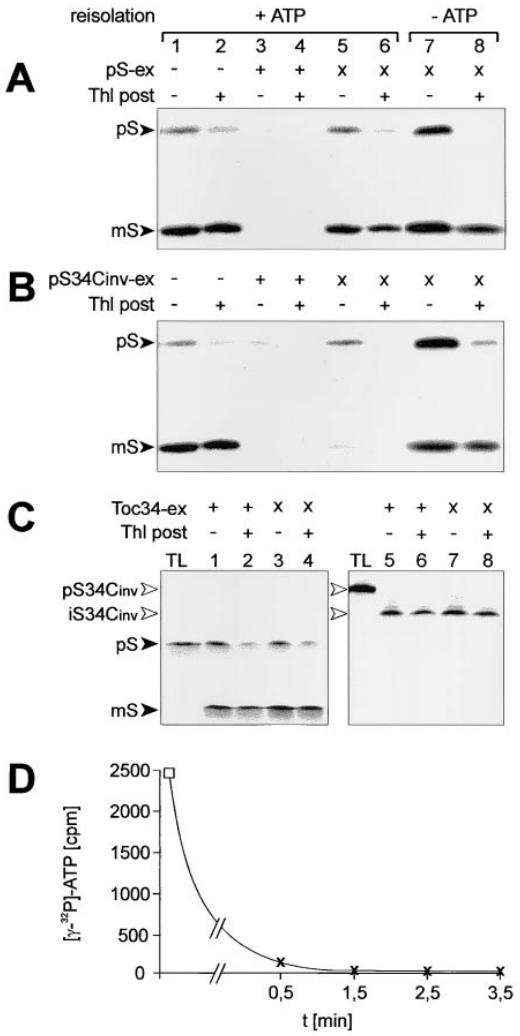

Figure 6. pS34Cinv competes with pS for the standard import pathway but not with Toc34 for insertion. (*A* and *B*) Chloroplasts were incubated either in the absence (lanes *1* and *2*) or presence (lanes *3–8*) of unlabeled urea-denatured pS-ex (*A*) or pS34Cinv-ex (*B*), 2 μM each, for 10 min under import conditions. The final urea concentration was 80 mM in all assays (lanes *1–8*). Chloroplasts were reisolated through a 40% Percoll cushion and washed once either in the presence (lanes *1–6*) or absence (lanes *7* and *8*) of 2 mM ATP. Chloroplasts were resuspended in import buffer (see Materials and Methods) in the presence of 2 mM ATP, and a second round of import was initiated by the addition of radiolabeled pS reticulocyte lysate translation product. Import experiments in lanes *3* and *4* (+) received unlabeled pS-ex (2 μM) or pS34Cinv-ex (2 μM), respectively, together with ^{35}S-labeled pS. Experiments in lanes *1*, *2*, and *5–8* (*x*) did not receive additional overexpressed preprotein during the second round of import. (*C*) Chloroplasts were incubated with heterologously expressed Toc34-ex (2 μM) during a first round of translocation as outlined above. The second round of translocation was started by the addition either of ^{35}S-labeled pS (lanes *1–4*) or pS34Cinv (lanes *5* and *6*). Experiments in lanes *1* and *2*, and *4* and *5* received simultaneously 2 μM unlabeled Toc34-ex (+) during the second round of translocation. (*D*) Changes of ATP levels in chloroplasts during reisolation. Chloroplasts were preincubated in the presence of 2 mM ATP and 2 μCi [γ^{32}P]ATP for 10 min at 25°C, and an aliquot was taken (*white box*) and centrifuged through silicon oil. Residual chloroplasts were recovered by centrifugation and resuspended in import buffer in the absence of ATP. Aliquots were drawn at different time intervals (*x*), and chloroplasts were separated from the surrounding aqueous mediums by centrifugation through a silicon oil layer. Organelles were extracted with 80% (vol/vol) dimethyl-ketone, and nucleotides were separated by thin layer chromatography on polyethylene-imine cellulose. Plates were developed in 0.5 M KH_2PO_4. Radioactivity recovered in ATP was quantified by a liquid scintillation counter.

reaction, e.g., at the level of the Tic complex. This is corroborated by the observation (Fig. 6 *B*, lanes *7* and *8*) that the inhibitory effect of pS34Cinv preincubation was completely reverted when chloroplasts were reisolated and washed in the absence of ATP before the second round of translocation. The ATP concentration in chloroplasts drops to below 10 μM ATP (Fig. 6 *D*) during the reisolation and washing procedure. This low level of ATP is not sufficient to support translocation (Olsen et al., 1989). We conclude that an ATP-dependent pulling force in the stroma, e.g., exerted by hsp70 or hsp100, looses its grip on the partly translocated protein, upon which the precursor retracts from the inner envelope translocon. The Tic complex then becomes available for new rounds of translocation.

Heterologously expressed pS34Cinv competes effectively with ^{35}S-labeled pS reticulocyte lysate translation product when added simultaneously to chloroplasts (Fig. 6 *B*, lanes *3* and *4*). This further shows that pS34Cinv enters the standard import pathway. Chemical amounts of Toc34 can neither compete for pS nor for pS34Cinv import (Fig. 6 *C*), corroborating our earlier notion that under optimal import conditions (2 mM ATP, 25°C), the presequence-dependent standard import route is preferred to the membrane insertion pathway.

Our data so far indicated two findings: (*a*) Inversion of the COOH-terminal 45 amino acids of Toc34 causes a reorientation of the membrane topology; and (*b*) the inverted COOH-terminal segment functions as a stop transfer signal for the Toc machinery. However, it remained to be established if the orientation determinants reside in the transmembrane or in the flanking sequences. As can be seen in Fig. 7, two positively charged amino acids are placed in direct vicinity (position-1 and -3) of the cytosolic side of the Toc34 transmembrane segment, while in the case that two positively charged amino acids are closely spaced in the lumenal segment of Toc34, they are directly accompanied by a negatively charged residue. This double-positive charge might represent a determinant for Toc34 orientation. Therefore, a mutual charge exchange was performed (see Fig. 7 *A*), which resulted in a $Toc34_{+\leftrightarrow-}$ and $Toc34Cinv_{+\leftrightarrow-}$. $Toc34_{+\leftrightarrow-}$ contains the membrane segment in a wild-type orientation, while the two positive charges at the NH_2-proximal site of the transmembrane segment were replaced by two negatively charged amino acids. At the COOH-proximal end, one negatively charged amino acid was replaced by a positively charged amino acid resulting in a double-positive charge close to the COOH-proximal site of the transmembrane segment. The similar amino acid exchanges were also placed in Toc34Cinv, resulting in $Toc34Cinv_{+\leftrightarrow-}$.

In contrast to wild-type Toc34, $Toc34_{+\leftrightarrow-}$ now inserts into the chloroplastic outer envelope in a way that renders

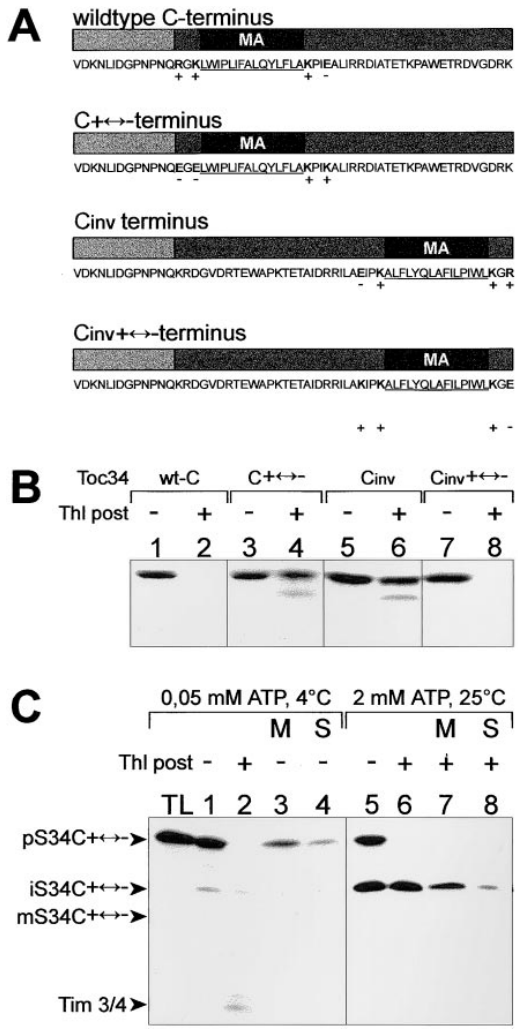

Figure 7. Positive charge distribution determines the functionality of the COOH-terminal segment of Toc34. (*A*) Charged amino acids (*bold*) directly proximal to the α-helical membrane anchor (*MA*, *underlined*) were mutually exchanged. (*B*) Intact chloroplasts were incubated with Toc34 (lanes *1* and *2*), $Toc34_{+\leftrightarrow-}$ (lanes *3* and *4*), Toc34Cinv (lanes *5* and *6*), and $Toc34Cinv_{+\leftrightarrow-}$ (lanes *7* and *8*) translation product for 10 min under standard conditions. Chloroplasts were either not treated or treated with the protease thermolysin after completion of the insertion reaction (*Thl post*, as indicated). (*C*) $pS34C_{+\leftrightarrow-}$ translation product was incubated with intact chloroplasts under binding (lanes *1–4*) or import (lanes *5–8*) conditions for 10 min as outlined on top of the figure. Chloroplasts were treated subsequently with thermolysin as outlined before. Reaction products were separated by extraction at pH 11.5 into a membrane (*M*, lanes *3* and *4*) and a soluble (*S*, lanes *7* and *8*) protein fraction. The position of the translocation intermediates (*Tim 3/4*) is indicated. Fluorograms are shown in *B* and *C*.

it mostly protease resistant (Fig. 7 *B,* lanes *3* and *4*), reminiscent of the insertion behavior of Toc34Cinv (compare Fig. 7 *B,* lanes *5* and *6*). $Toc34Cinv_{+\leftrightarrow-}$ instead now inserts in a protease-sensitive manner (Fig. 7 *B*, lanes *7* and *8*), reminiscent of wild-type Toc34 (compare Fig. 7 *B,* lanes *1* and *2*). These results indicate that the charge distribution flanking the transmembrane segment is the prime determinant for the orientation of Toc34.

As shown above (Fig. 3), pS34C can be imported into chloroplasts, where it is processed and recovered as soluble protein in the stroma. When we tested the binding and import properties of $pS34C_{+\leftrightarrow-}$, we found that it was no longer able to translocate fully into chloroplasts (Fig. 7 *C*, lanes *5* and *6)*. Instead, under import condition it was processed to $iS34C_{+\leftrightarrow-}$ and recovered in the membrane fraction, resistant to extraction at pH 11.5 (Fig. 7 *C*, lanes *7* and *8*). Under binding conditions, the translocation intermediates TIM 3/4 indicated that $pS34C_{+\leftrightarrow-}$ had entered the standard import pathway (Fig. 7 *C*, lanes *1* and *2*). We conclude that charge distribution around the transmembrane segment represents a stronger sorting signal than orientation and hydrophobicity of the membrane domain.

Discussion

Toc34 is anchored in the chloroplastic outer envelope by a single transmembrane hydrophobic membrane anchor in an orientation N_{cyto}-C_{in}. If we compare the region of 28 amino acids NH_2-terminal preceding and COOH-terminal following the membrane anchor, a charge difference of 4:7 positive charges can be observed. Hence, it is not obvious that posttranslational insertion of Toc34 obeys the positive inside rule (von Heijne and Gavel, 1988; von Heijne, 1989). Charge differences as sole determinant for the orientation of a membrane anchor region is difficult to reconcile because it would most likely require an intact electrochemical potential across the chloroplastic outer envelope (Andersson and von Heijne, 1994; Rapoport et al., 1996). At present, the evidence suggests that the outer envelope lacks a significant electrochemical potential (Joyard et al., 1991). However, the specific lipid composition of the chloroplastic outer envelope results in a strongly negatively charged membrane surface (Joyard et al., 1991). Positively charged amino acids selectively positioned to a transmembrane segment might be prominent determinants for the membrane orientation of a polypeptide. Our results establish that two positive charges in close proximity to the transmembrane segment dictate the topology of Toc34. The interconversion of the positive charge "motif" (Fig. 7) NH_2- and COOH-proximal to the transmembrane segment resulted in the interconversion of the Toc34 topology. From these observations we conclude that although the hydrophobic α-helix of Toc34 is necessary for its insertion (Seedorf et al., 1995), positively charged amino acids flanking this region are the major determinants for its orientation. Characterization of the insertion behavior of other single membrane spanning outer envelope proteins will be necessary to validate and generalize this conclusion.

The general protein import machinery of the chloroplastic outer envelope can translocate proteins containing integral membrane components of either the inner envelope or the thylakoid membrane. Consequently, amino acid sequences with the potential to form hydrophobic α-helices pass freely through the outer envelope translocon. The 2-oxoglutarate-malate translocator, a 12-helix-motif transporter of the inner envelope, uses the general import machinery as well as the three α-helices containing thylakoid localized light harvesting chlorophyll a/b binding protein (Hobe et al., 1994; Weber et al., 1995). The presence of a

stop transfer signal functional at the outer envelope protein translocon would obviously be deleterious to organelle biogenesis. Chloroplast preproteins localized in the thylakoid lumen contain a bipartite transit sequence. The NH_2-proximal part functions as an envelope transfer domain, while the COOH-proximal part functions as thylakoid transfer domain and dictates which translocation route it follows, i.e., a Sec-dependent or ΔpH-dependent route (for review see Robinson and Mant, 1997). Positive charges placed around a hydrophobic domain determines the translocation pathway to be used. The hydrophobic domain in thylakoid lumen presequences is much shorter, 10–13 amino acids, than in Toc34. Positively charged amino acids in combination with a critical length of a hydrophobic transmembrane segment might represent a necessary combination of signals that is recognized by the Toc machinery. The unique import pathway of preToc75 into the outer envelope membrane seems to involve a stop transfer signal that is present in the COOH-proximal part of the cleavable transit sequence (Tranel and Keegstra, 1996). However, the nature of this signal is not clear, nor is it clear whether it is deciphered by the Toc or the Tic machinery. Hydrophobic stop transfer regions from mouse immunoglobulin M or vesicular stomatitis virus glycoprotein, which are both necessary and sufficient to halt translocation of proteins across the endoplasmatic reticulum, do not halt translocation of proteins into chloroplasts (Lubben et al., 1987).

Our results (Figs. 3–6) clearly demonstrate that pS34Cinv is jointly imported by the Toc and the Tic complexes and not via a Toc-independent pathway. The recognition of the stop transfer signal must therefore occur within the Toc complex. A fraction of processed iS34Cinv is still in a proteinaceous environment, maybe in the pore component Toc75. Most of the protein is resistant to extraction at pH 11.5, which is indicative for the insertion and interaction with the lipid moiety of the membrane. Our results and those obtained for preToc75 (Tranel and Keegstra, 1996) indicate that the Tic complex harboring the precursor polypeptide translocation intermediate does not influence whether or not the Toc machinery can release a transmembrane segment into the lipid bilayer. Translocation arrest leaves the hydrophobic transmembrane segment in the pore of the Toc complex, where it might have sufficient time to "sense" the lipid environment, which induces the release into the bilayer. The mechanism of release of the protein into the plane of the membrane is not known but could be analogous to that proposed by Martoglio et al. (1995) and Liao et al. (1997) (for review see Siegel, 1997).

The partially processed polypeptide iS34Cinv remains locked in the Tic complex in the presence of ATP while the membrane anchor has already inserted into the lipid bilayer of the outer membrane. Simultaneously, the Toc complex becomes available for a new round of precursor recognition and translocation initiation (Fig. 6). This indicates that in intact chloroplasts, the Toc complex can function independently of the Tic machinery, corroborating earlier observations (Waegemann and Soll, 1991) that isolated purified outer envelope vesicles are also functional in specific precursor recognition and partial translocation.

The ATP-dependent lock that keeps iS34Cinv in the Tic complex could be represented by the action of hsp100, which interacts on the stromal site with the Tic complex (Akita et al., 1997; Nielsen et al., 1997). The engagement of the Tic complex by iS34Cinv leads to a block of these translocation sites and to the inhibition of further import. By the removal of ATP, the inhibition is released because iS34Cinv retracts from the Tic complex and becomes exposed to the intermembrane space, indicating that no further ionic interactions keep the protein in the Tic complex. The chloroplastic inner envelope contains an active ATP/ADP carrier that might be responsible for the rapid drop in chloroplastic ATP levels (Neuhaus et al., 1997). iS34Cinv can probably not reengage the Tic machinery because it possesses only an incomplete import signal.

Intermediate processing of the presequence of SSU can occur if the proper site is not available to the stromal-processing peptidase (Archer and Keegstra, 1993). About 120 amino acids are spaced between the authentic processing site and the beginning of the membrane anchor domain. This should suffice to guarantee faithful processing if both the outer and inner envelope translocons were in close proximity to each other during the entire import event. Our data indicate that upon the release of the transmembrane segment into the plane of the membrane, the joint translocation sites dissociate and clear away from each other. This scenario would require a much longer spacing between the outer envelope and the Tic complex, which could explain our results (see also Fig. 5 *C*). It seems also unlikely that iS34Cinv is inserted into the outer membrane in a loop structure for two reasons: (*a*) iSSU34Cinv is resistant to the protease thermolysin, and (*b*) a loop structure would require a topology N_{cyto}-C_{in}, but we have demonstrated (Fig. 2) that mS34Cinv inserts in a topology N_{in}-C_{cyto}.

In the constructs pS34C and pS34Cinv, competition exists between two targeting signals, which could lead the polypeptides on alternative translocation pathways. Under experimental conditions that are optimized for import (2 mM ATP and 25°C), pS34C and pS34Cinv use preferentially the standard import pathway via the Toc and Tic complex. Removal of the presequence receptors from the chloroplast surface shifts pS34C and pS34Cinv to the outer envelope insertion pathway via the COOH-terminal hydrophobic region. Similarly, preincubation of chloroplasts under binding conditions (50 μM ATP at 4°C) for 10 min also results in a shift to the Toc-independent insertion pathway. While both pathways may operate simultaneously, our data establish that transport conditions can be selected that favor one over the other.

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