Membrane insertion of the chloroplast outer envelope protein, Toc34: constrains for insertion and topology

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Summary

The insertion of the outer envelope protein Toc34 from chloroplasts was studied. Toc34 was chosen as a model protein because it contains one predicted transmembrane helix at the C-terminus and a large hydrophilic N-terminal located GTPase domain, which is exposed to the cytosol. Unlike proteins located in internal chloroplast compartments, Toc34 neither contains a cleavable presequence nor uses the general import pathway. The protein can insert into the outer envelope of chloroplasts but not into the outer membrane of mitochondria. Using protein-free liposomes we showed that Toc34 is able to insert directly into the lipid bilayer. This insertion is stimulated by GTP and the presence of nonbilayer lipids, but is independent of the presence or absence of charged lipids. The topology of the protein inserted into protein-free liposomes was not exclusively directed by the positive-inside rule but by the size of the hydrophilic domain.

Key words: Direct insertion, Protein-free liposomes, Chloroplast import

Introduction

Many chloroplast proteins are encoded by the nuclear genome and must be post-translationally imported into the organelle. In general, these proteins contain a cleavable N-terminal transit sequence, which facilitates the interaction with the Toc and Tic (translocon at the outer and inner chloroplastic envelope membrane, respectively) machinery (Keegstra and Cline, 1999; Schleiff and Soll, 2000; Schnell et al., 1997). This translocation pathway is also called the general import pathway. By contrast, most proteins of the chloroplast outer envelope do not contain such an N-terminal transit sequence. Furthermore, these proteins were shown to insert independently of the general import pathway in vitro (Schleiff and Klösgen, 2001; Soll and Tien, 1998). The insertion process of the outer envelope proteins (OEPs) has been investigated in some detail so far for OEP14 (Li et al., 1991; Tu and Li, 2000) and the import receptor Toc34 (Kessler et al., 1994; Seedorf et al., 1995; Tsai et al., 1999; Sveshnikova et al., 2000) from *Pisum sativum* and *Arabidopsis thaliana*, and OEP7 (Lee et al., 2001; Salomon et al., 1990; Schleiff et al., 2001) from spinach. For all proteins, insertion was not strictly dependent on ATP (Li and Chen, 1996; May and Soll, 1998; Salomon et al., 1990) or thermolysin-sensitive factors on the outer envelope (Chen and Schnell, 1997; Li and Chen, 1996; Salomon et al., 1990; Tsai et al., 1999). Furthermore, OEP14 and OEP7 were found to insert specifically into the chloroplast outer envelope but not into microsomal (Li and Chen, 1996) or mitochondrial membranes (Li et al., 1991; Soll et al., 1992).

The OEP7 has a single transmembrane domain and an N_{in}-C_{out} orientation (Salomon et al., 1990; Waegemann et al., 1992). The insertion of OEP7 is dependent on temperature, but independent of light and a membrane potential (Salomon et al., 1990). The protein is able to bind to and insert into a protein-free membrane. The topology of this protein is defined by positively charged amino acids of the C-terminus flanking the transmembrane domain (Lee et al., 2001; Schleiff et al., 2001). Additionally, it was shown that the topology of OEP7 is sensitive to the lipid asymmetry of the outer envelope (Schleiff et al., 2001).

Toc34 also contains a single transmembrane domain but with a C_{in}-N_{out} orientation (Seedorf et al., 1995). Insertion of Toc34 was found to be stimulated by ATP (Li and Chen, 1997; Seedorf et al., 1995; Tsai et al., 1999) and GTP (Chen and Schnell, 1997; Tsai et al., 1999). The cytosolic region was suggested to influence the insertion given that partial deletion resulted in the reduction of the insertion efficiency (Li and Chen, 1997). Two positive charges flanking the transmembrane domain at the cytosolic site seem to influence the orientation of Toc34 (May and Soll, 1998).

The outer envelope is a membrane with several unique and important features. The outer envelope of chloroplasts contains a lower concentration of phosphatidylcholine (PC) and a higher concentration of phosphatidylglycerol (PG) in the inner than in the outer leaflet of the bilayer (Dorne et al., 1985). Charged lipids like PG were found to be important for association and insertion of proteins into bilayers (van't Hof et al., 1991; van't Hof et al., 1993) because of electrostatic interaction with positively charged amino acids or rejection of negatively charged amino acids. The outer envelope is the only membrane facing the cytosol to contain the nonbilayer lipid monogalactosyldiacylglyceride (MGDG) (Bruce, 1998). Nonbilayer lipids are thought to play an important role in protein membrane interaction and insertion. For example, MGDG stimulates the association of the transit sequence of preferredoxin and pre-SSU (small subunit of rubisco) with lipid surfaces (Chupin et al., 1994; Pilon et al., 1995; van't Hof et al., 1991; van't Hof et al., 1993).

Fig. 1. Toc34 and mutants. Toc34, Δ(2-230)Toc34, Toc34C++ and Δ(2-230)Toc34C++ are shown, indicating the deletions and the charge distribution of the 15 amino acids flanking the transmembrane domain. The amino acid sequence of the short constructs is given and the exchanged amino acids are presented in bold. The position of the [^{35}S]-labelled methionines and [^{3}H]-labelled leucines is also shown.

Phosphatidylethanolamine (PE), another nonbilayer lipid, was found to assist protein folding of membrane proteins (Bogdanov and Dowhan, 1998; Bogdanov et al., 1999) and is required for efficient protein transport across the plasma membrane of *Escherichia coli* (Rietveld et al., 1995).

Toc34 is a subunit of a larger hetero-oligomeric translocation complex; therefore, binding and insertion into the membrane, as well as integration into the complex, probably represent distinct steps in the translocation pathway. To dissect this process we investigated insertional and topological constraints of Toc34 for translocation in chloroplasts and in a reconstituted protein-free liposome system. Our results show that Toc34 inserts into chloroplast outer envelopes even after inhibition of the translocation pore Toc75. Consistent with this observation is the ability of Toc34 to insert into liposomes. Interestingly, GTP also stimulates Toc34 insertion into protein-free liposomes. We suggest that the topology of Toc34 is partly determined and maintained by the size of the cytosolic domain. The positive-inside rule can be restored by deletion of the hydrophilic GTPase domain.

Materials and Methods

Constructs

Construction of Toc34, Toc34Cinv and Toc34C++ (Fig. 1) was previously described (May and Soll, 1998). Δ(2-230)Toc34 and Δ(2-230)Toc34C++ (Fig. 1) were constructed as follows: Δ(2-230)Toc34Cinv was obtained by recombinant PCR using the cDNA of Toc34Cinv as template (May and Soll, 1998). The PCR product was cloned into pet21d using the engineered *Nco*I and *Xho*I restriction side. The cDNA was confirmed by sequencing. Then pet21d containing the cDNA coding for Toc34 and Toc34C++ was digested using HincII, and the larger fragment (~3500 bp) containing 173 bp of Toc34 or Toc34C++ was purified. Pet21d containing the cDNA encoding for Δ(2-230)Toc34Cinv was also digested with HincII but the smaller DNA fragment (1394 bp) containing 74 bp of Δ(2-230)Toc34Cinv was isolated. Both fragments were ligated and the constructs were controlled by digestion with *Nco*I and *Xho*I, in vitro translation, in vitro transcription and overexpression. The cDNA encoding for Tic40 (Stahl et al., 1999) was amplified by PCR and inserted into pBSC or pet21d.

Transcription and translation

Both coupled and uncoupled transcription and translation was used. The uncoupled transcription translation is described elsewhere (Schleiff et al., 2001). For coupled transcription and translation the T7-TNT-Kit from Promega (Madison, WI) was used. Proteins were synthesised in 50 μl containing 100 units T7-polymerase, 25 μl reticulocyte-lysate, 2 μg DNA, 1 μl RNase-inhibitor, 2 μl TNT-buffer and 2 μl amino acid mix without methionine or leucine. The reaction mixture was supplemented with [^{35}S]-methionine (1000 Ci/mmol) or [^{3}H]-leucine (148 Ci/mmol), respectively, and the reaction was carried out for 1 hour at 30°C. The translation mixture was centrifuged for 1 hour at 250,000 ***g*** at 4°C and the post ribosomal supernatant was used for import.

Protein import into chloroplasts and mitochondria

Chloroplasts and mitochondria from garden pea were isolated by standard procedures and further purified on Percoll gradients (Schleiff et al., 2001; Day et al., 1985). Import into mitochondria was carried out as described in (Rudhe et al., 2002). For chloroplast use, chlorophyll concentration was determined to standardise import results (Arnon, 1949; Mourioux and Douce, 1981; Schindler et al., 1987). Standard import into chloroplasts equivalent to 40 μg chlorophyll was performed in 100 μl import buffer (10 mM methionine (or leucine), 20 mM potassium gluconate, 10 mM $NaHCO_3$, 3 mM $MgSO_4$, 330 mM sorbitol, 50 mM Hepes/KOH pH 7.6) containing 1-10% of in vitro translated [^{35}S]- or [^{3}H]-labelled proteins. Import was initiated by addition of organelles to import mixture and stopped after the times indicated. Intact chloroplast were reisolated through a Percoll cushion (40% Percoll in 330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6), washed once in 330 mM sorbitol, 50 mM Hepes/KOH, pH 7.6, 3 mM $MgCl_2$, and used for further treatments as described previously (Schleiff et al., 2001).

Liposome preparation and insertion experiments

Purified plant lipids were provided by Nutfield Nurseries (Surrey, UK). Outer envelopes of chloroplasts from pea were purified as described (Schleiff et al., 2001). Liposomes with various lipid content (Table 1) were prepared as follows. The lipids were mixed in a glass tube to yield a final concentration of 5 μmol total lipid content and dried under N_2-flow. Lipids were dissolved in 1 ml trichlormethane followed by N_2-drying and complete removal of the organic solvent under vacuum for at least 3 hours. The created lipid film was either stored at –80°C under argon or directly dissolved in buffer S (50 mM Hepes-KOH, pH 7.6, 0.2 M sucrose, degassed using N_2) for synthesis of liposomes S or in buffer N (50 mM Hepes-KOH, 125 mM NaCl, degassed) for the synthesis of liposomes N. The solution was vortexed and freeze-thawed five times. The multilamellar vesicles were extruded 21 times through a 100 nm pore polycarbonate filter mounted in the mini-extruder (Liposofast, Armatis, Mannheim, Germany) to give unilamellar liposomes (MacDonald et al., 1991). The insertion of Toc34 and mutants into the liposomes was carried out as described (Schleiff et al., 1999; Schleiff et al., 2001).

Quantification and data presentation

The amount of imported or inserted protein was quantified by two different methods. First, the SDS-page gel slice was dissolved in 30% H_2O_2 and 60% $HClO_4$ for 16 hours at 60°C followed by cooling and 1:10 dilution into Rotiszint 22 eco scintillation cocktail (Roth, Germany) and scintillation counting. Second, the radioactivity was quantified using the Phospho-Image Reader FLA 5000 (Fuji-Film, Tokyo, Japan) and quantified using Aida-Image Analyser (Raytest Isotopenmessgeräte GmbH, Staubenhard, Germany). The radioactivity of the proteins was normalised to the amount of labelled amino acids present in each construct and in the 8 kDa fragment in order to normalise for the amount of protein seen. Binding (B) and insertion (I) efficiency was quantified using the results of one experiment as follows:

$$B_{protein} = (C_{protein}/C_{10\%\ control})/(C_{Toc34wt}/C_{10\%\ control\ Toc34wt}) \times 100\%$$

$$I_{protein} = ([C_{8kDa\ frag.}/NL_{8kDa\ frag.}]/[C_{10\%\ control}/NL_{protein}])/(C_{Toc34wt}/C_{10\%\ control\ Toc34wt}) \times 100\%,$$

where $C_{protein}$ stands for the counts detected for the investigated protein, $C_{Toc34wt}$ for the counts detected for Toc34wt, $C_{8kDa\ frag}$ for the counts detected for the 8 kDa fragment after protease treatment, $C_{10\%\ control}$ for the counts detected for the 10% translation product loaded on the SDS-PAGE and $NL_{8kDa\ frag.}$ or $NL_{protein}$ for the number of labels present in the 8 kDa fragment or in the construct, respectively. When both quantification methods were used the results for binding and insertion were averaged for both techniques and the averaged values were used for further calculations. The data are presented using Adobe Photoshop 4.0 (Adobe Systems Inc.), Corel Draw 8.0 (Eastman Kodak Company) and Sigma Plot 5.0 (SPSS Inc., Chicago, IL).

Results

Toc34 insertion into the chloroplast outer envelope

As mentioned before, Toc34 is an outer envelope protein without a classical transit sequence (Kessler et al., 1994; Seedorf et al., 1995). To investigate whether outer envelope proteins are involved in the insertion process of Toc34, [^{3}H]-labelled Toc34 was imported into chloroplasts (Fig. 2A, lane 2). Chloroplasts were separated from unbound precursor by centrifugation through a Percoll cushion (see Materials and Methods). Insertion of Toc34 into the outer envelope was tested by thermolysin treatment. The proteolytic digestion of [^{3}H]-labelled Toc34 after membrane integration resulted in the expected 8 kDa fragment (Fig. 2A, lane 3) (Seedorf et al., 1995). The 8 kDa fragment became protease sensitive after membranes were solubilised by detergent (see below, (May and Soll, 1998)). To determine whether this 8 kDa fragment was the result of insertion of a nonspecific product of similar size also present in the translation mixture (Fig. 2A, lane 1), translation was initiated in the absence of Toc34 mRNA (Fig. 2B, lane 1). However, it was found not to be the case, because after incubation of this translation product with chloroplasts, no inserted or protease-resistant product could be detected (Fig. 2B, lanes 2 and 3). Also, the 8 kDa fragment is not a proteolytical fragment of the Toc34 translation product as shown by treatment with thermolysin, which resulted in complete degradation of labelled protein (Fig. 3A, lane 2). Both results confirm, with protease treatment, that the 8 kDa band is a specific fragment of membrane-inserted [^{3}H]-labelled Toc34. The observed 28 kDa fragment (Fig. 2A, lane 3) was only partly resistant against proteolysis (not shown). This fragment might be the result of the interaction between the cytosolic domain of Toc34 and/or Toc159 (Hiltbrunner et al., 2001; Sun et al., 2002). To control the import competence of the chloroplasts used, the precursor form of the small subunit of Rubisco, preSSU, was imported into chloroplasts. As judged by maturation (Fig. 2A, lane 2, lower panel) and protease resistance of the mature form (Fig. 2A, lane 3), the chloroplasts used were highly import-competent.

Heterologously expressed proteins containing a typical transit sequence, like preSSU, normally compete for translocation with other precursor proteins that use the general translocation pathway (Schleiff et al., 2001). However, no sensitivity of Toc34 insertion in the presence of excess preSSU could be observed as judged by the appearance of the 8 kDa fragment after proteolysis, whereas translocation of preSSU was abolished (Fig. 2A, lanes 4 and 5). We also used spermine, a known inhibitor of the import channel Toc75 (Hinnah et al., 1997). As before, spermine inhibited import of preSSU (Fig. 2A, lanes 2 and 6) but had no influence on the insertion of Toc34 into the outer envelope of chloroplasts, as judged by the appearance of the 8 kDa fragment after thermolysin treatment

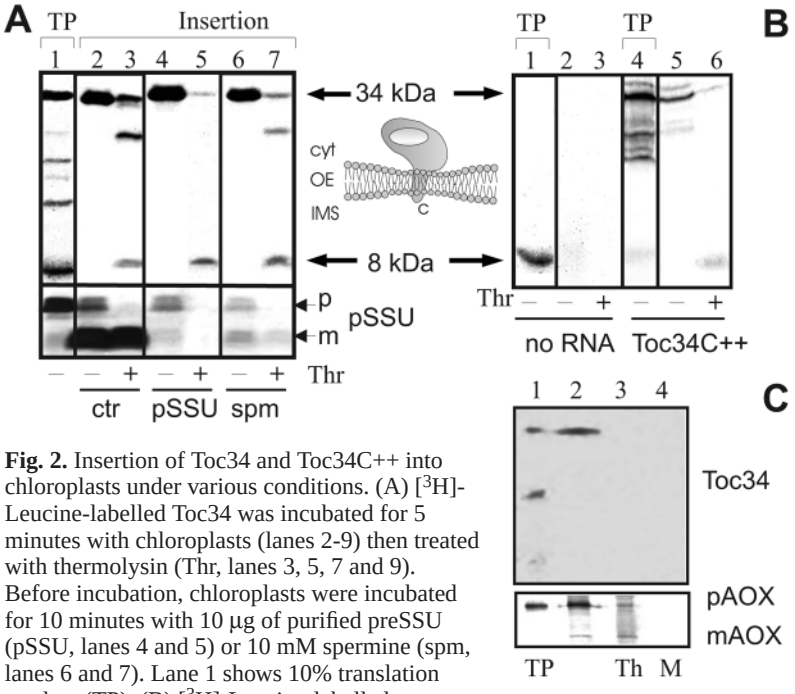

Fig. 2. Insertion of Toc34 and Toc34C++ into chloroplasts under various conditions. (A) [^{3}H]-Leucine-labelled Toc34 was incubated for 5 minutes with chloroplasts (lanes 2-9) then treated with thermolysin (Thr, lanes 3, 5, 7 and 9). Before incubation, chloroplasts were incubated for 10 minutes with 10 μg of purified preSSU (pSSU, lanes 4 and 5) or 10 mM spermine (spm, lanes 6 and 7). Lane 1 shows 10% translation product (TP). (B) [^{3}H]-Leucine-labelled translation product minus RNA (lanes 2 and 3), or Toc34C++ (lanes 5 and 6), was used for insertion into chloroplasts, which was followed by thermolysin treatment (Thr, lanes 3 and 6). Lanes 1 and 4 show 10% translation product. A model of the orientation of the wt protein and the charge mutant C++ is presented between A and B. (C) [^{3}H]-Leucine-labelled Toc34 or [^{35}S]-methionine-labelled precursor of AOX (10% translation product in lane 1) were incubated with mitochondria for 15 minutes (lanes 2-4); mitochondria were then treated with 120 μg/ml thermolysin (lane 3, Th) or 100 mM Na_2CO_3 (lane 4, M) for 30 minutes at 4°C. Thermolysin activity was stopped by the addition of EDTA (lane 3) and membranes were isolated by centrifugation at 200,000 ***g*** (lane 3).

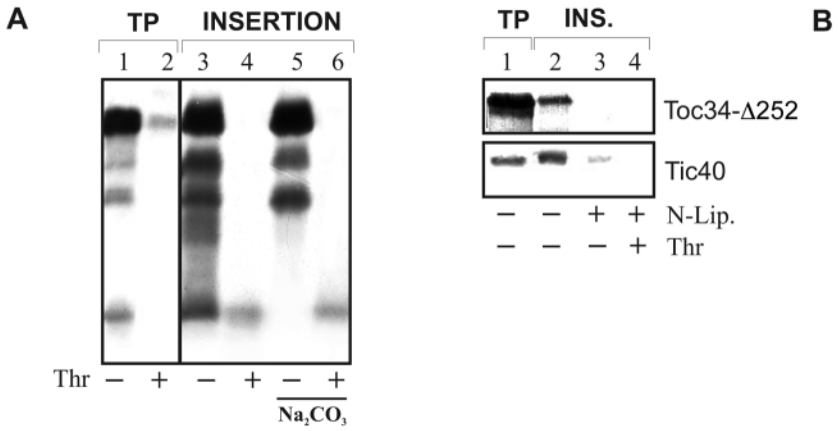

Fig. 3. Insertion of Toc34 into protein-free membranes depends on the transmembrane region. (A) [^{3}H]-Leucine-labelled Toc34 (lane 1) was treated with thermolysin (Thr, lanes 2, 4 and 6) before (lane 2) and after insertion into free liposomes (1 mM final lipid concentration, lanes 3-6), followed by incubation in 100 mM Na_2CO_3 (lanes 5 and 6) for 30 minutes at 4°C and pelleting of the membrane fraction. (B) [^{3}H]-Leucine-labelled Toc34-Δ252 and [^{35}S]-methionine-labelled Tic40 (10% TP, lane 1) were incubated with liposomes (lanes 2-4) without (lane 2) and with (lanes 3, 4) competition with N-liposomes, followed by thermolysin treatment (lane 4).

(Fig. 2A, lanes 6 and 7). A similar result was observed after treatment of the chloroplasts with $CuCl_2$ or spermidine (not shown), which both inhibit the function of the Toc complex (Hinnah et al., 1997; Seedorf and Soll, 1995). Together, the results suggest that pore forming and/or protease-sensitive proteins (Seedorf et al., 1995) are not involved in the insertion process of Toc34. However, from the current data it cannot be excluded that a protease-protected or -resistant receptor for Toc34 might exist.

Toc34 does not insert into the outer membrane of mitochondria

When Toc34 was expressed in *E. coli*, insertion of the protein into the inner membrane of the bacterium was observed (not shown). This raised the question of whether Toc34 can insert into any available membrane or if insertion is initiated by similarities of the lipid bilayers – that is, the existence of the nonbilayer lipid PE in the *E. coli* membrane. To answer this question we used pea mitochondria, which do not contain non-bilayer lipids, to study the insertion of Toc34. The import competence of the purified mitochondria was supported by import and maturation of alternative oxidase (AOX) (Fig. 2C, lane 2, lower panel) and the protease resistance of the mature form (Fig. 2C, lane3). When Toc34 was incubated with mitochondria, binding was observed (Fig. 2C, lane 2, upper panel). However, we did not observe insertion of Toc34 deduced from the absence of the proteolytical 8 kDa fragment after thermolysin treatment of mitochondria (Fig. 2C, lane 3). To confirm this conclusion the mitochondria were incubated with sodium carbonate and membranes were recovered. Toc34 was not observed in the pellet fraction (Fig. 2C, lane 4), further supporting our idea that Toc34 was not inserted into the membrane. Therefore, we conclude that Toc34 does specifically insert into the outer envelope of chloroplasts, but not into the outer membrane of mitochondria.

Toc34 inserts into protein-free membrane bilayer

Toc34 insertion seemed to be independent of the outer envelope translocation machinery. Therefore, we wanted to determine whether Toc34 could be inserted into a lipid bilayer directly. Liposomes with a lipid composition comparable to the average composition of the outer envelope were incubated with [^{3}H]-labelled Toc34. Toc34 was inserted into protein-free liposomes, as judged from the appearance of the 8 kDa fragment (Fig. 3A, lanes 3 and 4). To confirm that Toc34 was inserted into the bilayer, liposomes were extracted with sodium carbonate before (Fig. 3A, lane 5) and after (Fig. 3, lane 6) thermolysin treatment. Both Toc34 and the 8 kDa fragment were detectable in the membrane fraction, whereas the nonspecific 8 kDa product observed in the translation product was not. This is in line with the notion that the 8 kDa product observed in the translation product did not account for the 8 kDa observed after proteolysis. To prove that insertion of Toc34 is specific and dependent on the transmembrane domain, liposomes were incubated with Toc34 lacking the transmembrane region (Fig. 3B, upper panel, Toc34-Δ252) and with Tic40 (Fig. 3B, lower panel). Both proteins were found to associate with the membrane (Fig. 3B, lane 2); however, after competition for nonspecific binding using N-liposomes (Schleiff et al., 1999; Schleiff et al., 2001), only a small amount of Tic40 remained bound to the liposomes (Fig. 3B, lane 3), and this was rapidly degraded by the addition of thermolysin (Fig. 3B, lane 4). We conclude that neither Toc34 lacking the transmembrane domain nor Tic40 were inserted into the membrane under the conditions used.

Quantification of the insertion (Materials and Methods) revealed that about 20% of the bound Toc34 was inserted (Fig. 3A, lanes 2 and 3; Fig. 4A, lanes 6 and 7; C3), as judged by the appearance of the 8 kDa fragment after protease treatment. This result was also achieved by using lipids purified from chloroplast outer envelopes (Fig. 4A, lanes 12 and 13). This 8 kDa transmembrane segment became protease accessible after membrane solubilisation (Fig. 4A, lane 14). The association or insertion was not altered when the concentration of the zwitterionic lipid PC was increased to 50 mol% (Fig. 4A, C4, lanes 8 and 9; Table 1). However, a decrease in the PC concentration to 16 mol% (Fig. 4A, C2, lanes 4 and 5) resulted in an increase of the association by about 25% and an increase of the insertion efficiency by twofold when compared with the association and insertion into liposomes of average lipid composition (Fig. 4A, C3, lanes 6 and 7). To test which of the other lipids most strongly influenced the association and insertion, liposomes containing a second nonbilayer lipid, namely PE, were used for insertion experiments (C1). Addition of 2 mol% (final concentration) of PE resulted in an increase of insertion of Toc34 (Fig. 4A, C1, lanes 2 and 3) comparable to the increase found using the lipid mixture C2. However, not only the nonbilayer lipid concentration was increased in C2, but

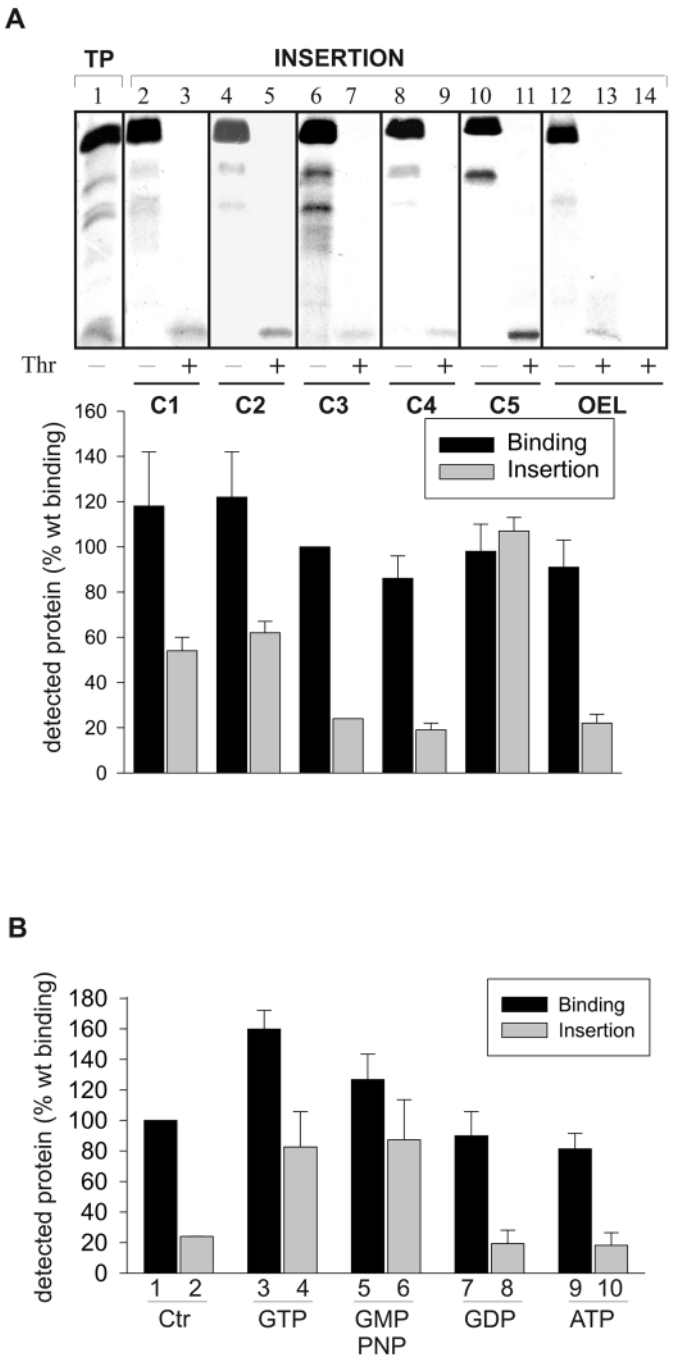

Fig. 4. Insertion of Toc34 into protein-free liposomes under different conditions. (A) [^{3}H]-Leucine-labelled Toc34 was incubated with protein-free liposomes (1 mM final lipid concentration, lanes 2-11) of different lipid composition (for nomenclature, see Table 1) or of outer envelope lipids (OEL, lanes 12-14), followed by thermolysin treatment (Thr, lanes 3, 5, 7, 9, 11, 13 and 14). In lane 14 the membrane was solubilised by TX-100 before thermolysin treatment. Binding (before thermolysin treatment, black bar) and insertion (8 kDa fragment after thermolysin treatment corrected for the number of leucine residues, grey bar) was quantified as described in Materials and Methods and is shown as a histogram. The binding of Toc34 to liposomes of composition C3 was set to 100%. The results represent an average of at least three independent experiments. (B) [^{3}H]-Leucine-labelled Toc34 was incubated with protein-free liposomes (1 mM final lipid concentration, composition C3, lanes 1 and 2) in the presence of 1 mM $MgCl_2$ (lanes 1-10) and 1 mM GTP (lane 3 and 4), 1 mM GMP-PNP (lanes 5 and 6), 1 mM GDP (lanes 7 and 8) or 1 mM ATP (lanes 9 and 10) followed by quantification of binding and insertion as for Fig. 3. Binding of Toc34 to liposomes in the absence of nucleotides was set to 100%. The results represent an average of at least three independent experiments.

Table 1. Lipid content of the used liposomes

Lipid*	OEL†	C1‡	C2	C3	C4	C5
DGDG	29	28	37	29	22	29
MGDG	17	17	21	17	13	17
PC	32	31	16	32	50	42
PG	10	10	12	10	7	0
PI	6	6	7	6	4	6
SL	6	6	7	6	4	6
PE	0	2	0	0	0	0

*Lipid concentration in mol%.
†Average lipid content of the outer envelope of chloroplasts (Bruce, 1998).
‡Used composition 1-5.
DGDG, digalactosyldiacylglyceride; MGDG, monogalactosyldiacylglyceride; OEL, outer envelope lipids; SL, sulfoquinovosyl diacylglycerol.

also the content of anionic lipids. To verify that the insertion was dependent on the nonbilayer lipids, liposomes lacking PG were used to study Toc34 insertion (C5). Toc34 associated with PG-free liposomes with similar efficiency as with liposomes of average lipid composition (Fig. 4A, compare lanes 6 and 10), but the insertion efficiency increased by a factor of four (Fig. 4A, compare lanes 7 and 11) when compared with liposomes with the average lipid composition of the outer envelope.

From the results presented in Figs 2 and 4 we conclude that the insertion of Toc34 into the membrane occurs independently of channel proteins in vitro. Using synthetic protein-free liposomes we can clearly show that the insertion efficiency, but not the association of Toc34, is dependent on the presence of nonbilayer lipids (MGDG and PE) and on the concentration of anionic lipids (PG).

Insertion of Toc34 into protein-free liposomes is stimulated by GTP

Insertion of Toc34 into the outer envelope of chloroplasts is stimulated by ATP and GTP (Chen and Schnell, 1997; Tsai et al., 1999). This result was taken as indication for the existence of a membrane-localised ATPase and GTPase involved in Toc34 insertion (Tsai et al., 1999). Because our results show that Toc34 inserted into protein-free liposomes, we investigated whether such a nucleotide effect also influences the insertion of Toc34 into liposomes. Therefore, the insertion of Toc34 into protein-free liposomes was carried out in the presence of different nucleotides. When GTP was added before the addition of liposomes, the association of Toc34 with the lipid surface increased, on average, by 60% compared with the association in the absence of GTP (Fig. 4B, lanes 1 and 3). The insertion efficiency in the presence of GTP increased by a factor of four in comparison to the absence of GTP as determined by the appearance of the 8 kDa fragment (Fig. 4B, lanes 2 and 4). The increase of insertion is dependent on GTP binding but not on GTP hydrolysis, because addition of the

non-hydrolysable GMP-PNP (guanosine 5′[imido]triphosphate) also increased the insertion of Toc34 (Fig. 4B, lanes 5 and 6). By contrast, the addition of GDP or ATP did not alter the association or insertion of Toc34 significantly (Fig. 4B, lanes 7-10). We conclude that the stimulation by GTP on the insertion of Toc34 is an intrinsic effect, e.g. accessibility of the hydrophobic membrane anchor due to a GTP-dependent conformational change rather than due to the presence of additional proteinaceous components in the envelope membrane.

The hydrophilic domain imposes a second constraint on membrane topology of Toc34

After establishing that Toc34 insertion does not require proteinaceous components, we wanted to investigate the constraints on the topology of Toc34. Previous work had established that charges flanking the transmembrane regions form one constraint for the topology of outer envelope proteins (Schleiff et al., 2001). We therefore incubated [^{3}H]-labelled Toc34 with an inverted charge distribution flanking the transmembrane domain (Toc34C++) with chloroplasts. We observed an 8 kDa fragment after thermolysin treatment, suggesting that Toc34C++ is inserted with C_{in}-N_{out} orientation (Fig. 2B, lanes 5 and 6). This result seems to contradict our notion of the charge dependency for Toc34 topology. However, the topology could be dependent on the size of the hydrophilic domain. Therefore, we created deletion mutants as shown in Fig. 1. One polypeptide, Δ(2-230)Toc34, contained a large deletion of the N-terminal hydrophilic domain. The second polypeptide, Δ(2-230)Toc34C++, contained the same deletion and, in addition, a membrane domain with inverted charges (see Fig. 1). Both proteins labelled either with [^{3}H] or [^{35}S] were imported into chloroplasts. Both mutants contain five leucines in the transmembrane domain and a further four leucines in the adjacent parts (see Fig. 1), but they contain only an N-terminal methionine. This asymmetric labelling allows the orientation of the inserted polypeptides to be determined (Schleiff et al., 2001). The quantification of association and insertion of the [^{3}H]-labelled proteins revealed that the association efficiency of Toc34 and Toc34C++ (Fig. 5A, upper panel, binding) was comparable. In addition, the insertion efficiency did not differ significantly (Fig. 5A, upper panel, insertion). When the truncated forms of Toc34 were used, we observed an increased association of Δ(2-230)Toc34 to the chloroplast surface (Fig. 5A, lower panel, binding wt; Fig. 5B, upper panel, lane 2); however, the insertion yield remained similar (Fig. 5A, lower panel, insertion wt; Fig. 5B, upper panel, lane 3) when compared with full-length Toc34. Although Δ(2-230)Toc34C++ revealed similar association with the chloroplast surface as the full-length protein (Fig. 5A, lower panel, binding C++; Fig. 5B, lower panel, lane 2), the insertion efficiency was drastically reduced, as judged by the appearance of the 8 kDa fragment (Fig. 5A, lower panel, insertion C++; Fig. 5B, lower panel, lane 3). Analysis of the [^{35}S]-labelled protein revealed that Δ(2-230)Toc34 was sensitive to thermolysin treatment and therefore inserted into chloroplasts with an N_{out}-C_{in} orientation (Fig. 5B, lane 6). We did not observe a significantly smaller proteolytic fragment for the [^{3}H]-labelled proteins, which might be due to the resolution capacity of the gel system used. Therefore, we conclude that the size of the hydrophilic region is one of the main constraining influences on the topology of Toc34.

The topology of Toc34 and mutants after insertion into protein free liposomes

To test whether the size of the hydrophilic region also influences the topology of Toc34 within protein-free liposomes, the mutants described (Fig. 1) were used for

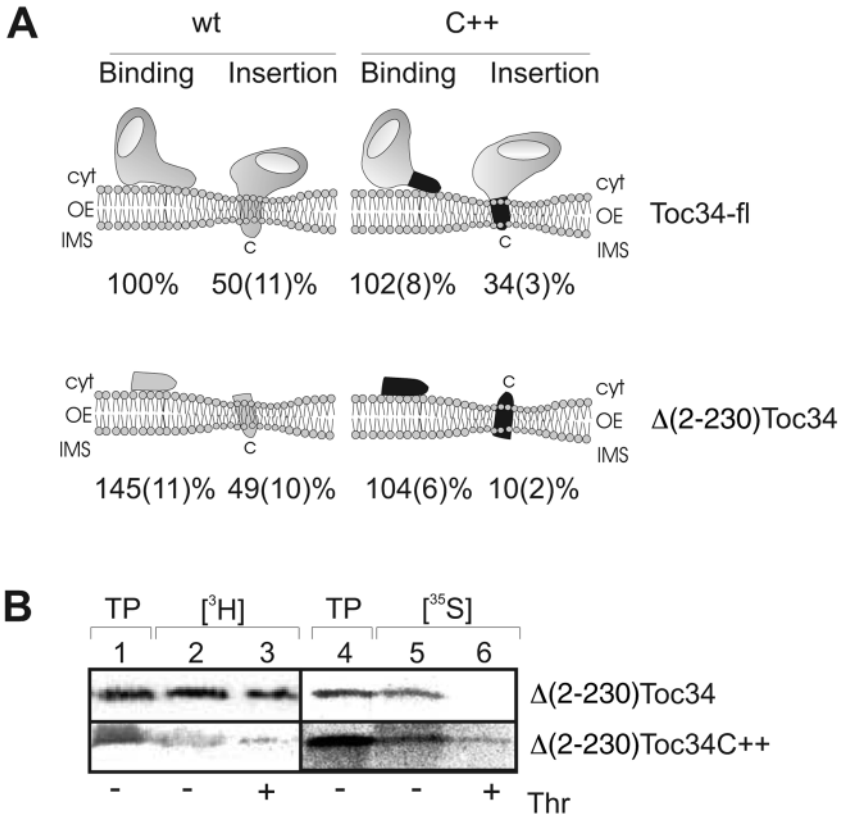

Fig. 5. Insertion of Toc34 and variants into chloroplasts outer envelope membrane. (A) [^{3}H]-Leucine-labelled Toc34 (upper, left), Δ(2-230)Toc34 (lower, left), Toc34C++ (upper, right) and Δ(2-230)Toc34C++ (lower, right) were incubated with chloroplasts as described and shown in the legend for Fig. 2. Binding and insertion was quantified as for Fig. 3. The binding of Toc34 was set to 100% and the numbers in brackets indicate the s.e.m. The models indicate the orientation of the proteins and the location of the C-terminus. The results represent an average of at least three independent experiments. (B) Insertion of [^{3}H]-leucine-labelled (lane 1-3) and [^{35}S]-methionine-labelled (lanes 4-6) Δ(2-230)Toc34 (upper panel) and Δ(2-230)Toc34C++ (lower panel) into chloroplasts (lanes 2, 3, 5 and 6) was performed as described in Fig. 2 and followed by thermolysin treatment (Thr, lanes 3 and 6). In lanes 1 and 4, 10% of the translation product is shown.

insertion experiments. Toc34C++ had a reduced association compared with wild-type Toc34 when liposomes with an average lipid composition of the outer envelope were used (Fig. 6A, upper panel, binding). However, all of the bound Toc34C++ was inserted as determined by the appearance of the 8 kDa fragment after protease treatment (Fig. 6A, upper panel, insertion C++). By contrast, only one quarter of the associated Toc34 was inserted into the bilayer (Fig. 6A, upper panel, insertion wt). The length deletion mutant with the original charge distribution showed a slightly reduced association (Fig. 6A, binding wt) but a higher insertion efficiency (Fig. 6, insertion wt) when compared with the full-length protein. The association of the length deletion with inverted charges was not reduced compared with Toc34wt (Fig. 6A, lower part, binding C++; Fig. 6B, lower panel, lane 2), but almost no insertion could be observed (Fig. 6A, lower part, insertion C++; Fig. 6B, lower panel, lane 3). In addition, using [^{35}S]-labelled mutant polypeptides, we could not detect any proteolytically resistant fragment when the truncated form of Toc34C++ was used (Fig. 6B, lane 6). This suggests that the insertion of this protein into liposomes of average lipid composition occurs with N_{out}-C_{in} topology, unlike the results observed using chloroplasts (Fig. 5B, lane 6).

The asymmetric distribution of PG between both leaflets of the outer envelope (Dorne et al., 1985) seems to be one of the most important determinants for the topology of OEP7 (Schleiff et al., 2001). Therefore, we tested whether the concentration of PG has an influence on the insertion efficiency of Toc34 into protein-free membranes. The association and insertion of Toc34C++ with liposomes lacking PG (Fig. 7A, upper panel, binding C++) was reduced compared with the association with liposomes containing PG (Fig. 6A, upper panel, binding C++). Toc34 inserted with higher efficiency than Toc34C++ into the liposomes not containing PG (Fig. 7A, upper panel, binding), which was comparable to the results seen for chloroplasts (Fig. 5A). Both truncated forms of Toc34, Δ(2-230)Toc34 and Δ(2-230)Toc34C++, showed a reduced association and insertion efficiency compared with Toc34 when liposomes lacking PG were used (Fig. 7A, lower panel; Fig. 7B, upper panel, lanes 2 and 3). However, the insertion of the length deletion of Toc34C++ into liposomes without PG (Fig. 7A, lower panel, insertion C++; Fig. 7B, lower panel, lane 2) increased compared with the insertion into liposomes of average composition (Fig. 6A, lower panel, insertion C++). Analysis of the translocation of the [^{35}S]-labelled proteins into liposomes not containing PG revealed the same result as seen using chloroplasts. Only for the truncated version of Toc34C++ was a protease-resistant form observed (Fig. 7B, lane 6), indicating that at least some of the protein had inserted in an N_{in}-C_{out} orientation.

Together, our results indicate that three different factors influence the membrane topology of Toc34: first, the lipid asymmetry present between outer and inner leaflet of the outer envelope; second, the size of the cytosolic domain of Toc34; and third, the charge distribution flanking the transmembrane domain.

Discussion

Insertion of Toc34 into the outer envelope membrane

Proteins required for the translocation of stromal-targeted proteins are identified in the outer and inner envelope (Keegstra and Cline, 1999; Schleiff and Soll, 2000). So far, no proteins present at the outer envelope have been shown to be required for insertion of outer envelope proteins (Schleiff and Klösgen, 2001), with the exception of the import of Toc75 (Tranel and Keegstra, 1996). Recently, evidence was presented for the existence of proteinaceous components influencing the insertion of proteins into the outer envelope (Tsai et al., 1999; Tu and Li, 2000). However, none of the putative accessory components could be identified. Furthermore, trypsin treatment of chloroplast resulted in a decrease, but not in a loss, of

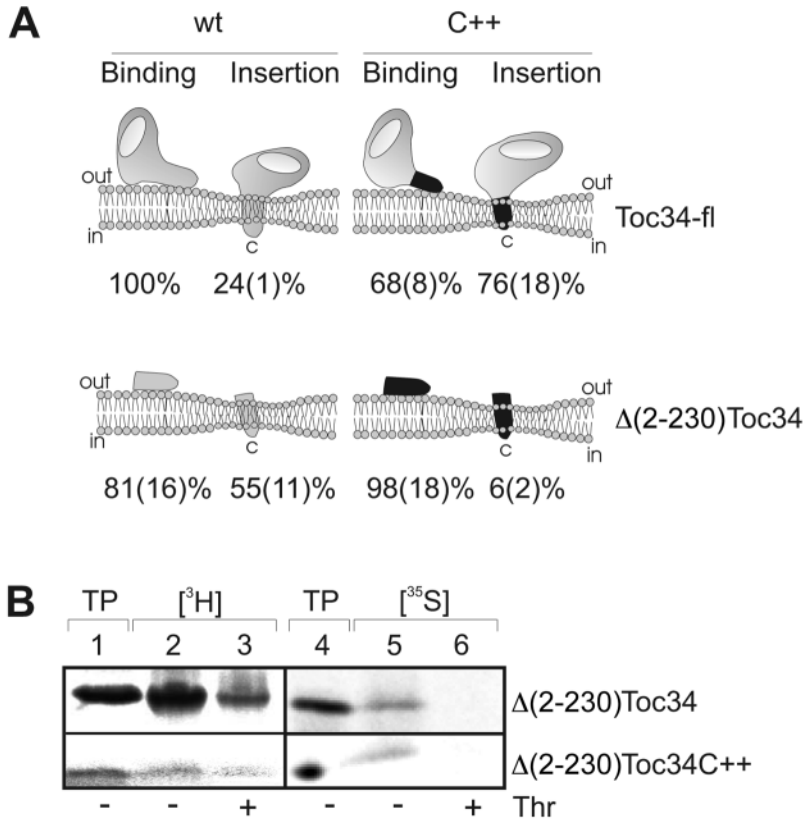

Fig. 6. Insertion of Toc34 and variants into protein-free liposomes. [^{3}H]-Leucine-labelled Toc34 (upper, left), Δ(2-230)Toc34 (lower, left), Toc34C++ (upper, right) and Δ(2-230)Toc34C++ (lower, right) were incubated with liposomes (1 mM final lipid concentration, composition C3), and binding and insertion was quantified as described in the legend for Fig. 5. The binding of Toc34 to protein-free liposomes was set to 100%. The results represent an average of at least three independent experiments and the numbers in brackets indicate the s.e.m. The models indicate the orientation of the proteins, and C the location of the C-terminus. (B) Insertion of [^{3}H]-leucine-labelled (lanes 1-3) and [^{35}S]-methionine-labelled (lanes 4-6) Δ(2-230)Toc34 (upper panel) and Δ(2-230)Toc34C++ (lower panel) into liposomes (lanes 2, 3, 5 and 6) was performed as described in Fig. 2 and followed by thermolysin treatment (Thr, lanes 3 and 6). In lanes 1 and 4, 10% of the translation product is shown.

insertion of outer envelope proteins. Here, chloroplasts were pre-treated with known inhibitors of channel activity like spermine and copper chloride, which did not result in a loss of Toc34 insertion (Fig. 2). Therefore, it seems unlikely that one as-yet-unidentified channel protein is involved in the translocation process. In addition, Toc34 inserts into the inner membrane of *E. coli*, which does not contain components of chloroplast translocation machinery (data not shown) and into protein-free liposomes (Fig. 3), but not into mitochondria (Fig. 2). This is consistent with the results found for the insertion of OEP7 (Schleiff et al., 2001).

In line with earlier observations for isolated chloroplasts (Chen and Schnell, 1997; Tsai et al., 1999), GTP binding was also found to stimulate insertion into protein-free liposomes (Fig. 4). This result supports the hypothesis (Chen and Schnell, 1997) that binding of GTP by Toc34 evokes a conformational change rendering Toc34 more capable of insertion – for example, by exposing the hydrophobic transmembrane domain. ATP had no effect on the insertion of Toc34 into protein-free liposomes when the post-ribosomal supernatant was used (Fig. 4). Therefore, we suggest that the previously observed ATP effect is partly due to chaperones present in the translation mixture and in the chloroplast preparation or to a conversion of ATP to GTP by nucleoside diphosphate kinase present in chloroplasts (Lübeck and Soll, 1995).

In summary, our data suggest that proteinaceous components are not essential for the insertion of the transmembrane domain into the lipid bilayer of the outer envelope.

Lipid dependence of association and insertion of Toc34

The effect of lipids on the association and insertion of outer envelope proteins has only recently received attention (Schleiff et al., 2001; Tu and Li, 2000). Investigation of insertion of OEP14 suggested that MGDG is not essential for the insertion of outer envelope proteins (Tu and Li, 2000). However, the result was obtained indirectly by treatment of chloroplasts with duramycin. Duramycin induces aggregation of membrane vesicles containing PE or MGDG (Navarro et al., 1985) and also induces artificial pore formation (Sheth et al., 1992). The mode of interaction between duramycin and nonbilayer lipids is not clear yet and the results are therefore rather difficult to interpret (Tu and Li, 2000). A more direct analysis of OEP7 insertion, using a protein-free liposome system, showed that the interaction is driven by the hydrophobicity of the transmembrane domain and possibly by galactosyldiacylglycerides like MGDG or/and sulfoquinovosyl-diacylgycerol (Schleiff et al., 2001). Here, we show that the nonbilayer lipids MGDG and PE stimulate the association of Toc34 to protein-free liposomes (Fig. 4). The insertion efficiency was enhanced when the nonbilayer lipid content was increased (Fig. 4) or 2 mol% of PE was added to the lipid mixture. From that we conclude that Toc34, like OEP7, associates with the surface of the organelle by hydrophobic interaction. This is also consistent with the observation that the free energy resulting from an association of the transmembrane segment with the lipid surface is in the range of the energy found for OEP7 (Schleiff and Klösgen, 2001). Furthermore, the insertion of Toc34, as well as of OEP7, into protein-free liposomes was largely stimulated after depletion of PG (Fig. 3). This supports our hypothesis that the insertion of outer envelope proteins is dependent on the lipid asymmetry present in the outer envelope (Dorne et al., 1985; Schleiff et al., 2001).

Constrains for the insertion efficiency and the topology of the outer envelope protein Toc34

The charge distribution flanking the transmembrane domain has been shown, using OEP7 as a model protein, to be one determinant of the topology of proteins in the outer envelope of chloroplasts (Schleiff et al., 2001). By contrast, Toc34 inserted into chloroplasts and liposomes with an N_{out}-C_{in} orientation, even after the reversal of the charges flanking the transmembrane domain (Figs 2, 6 and 7). Only the complete deletion of the cytosolic region resulted in a charge-sensitive

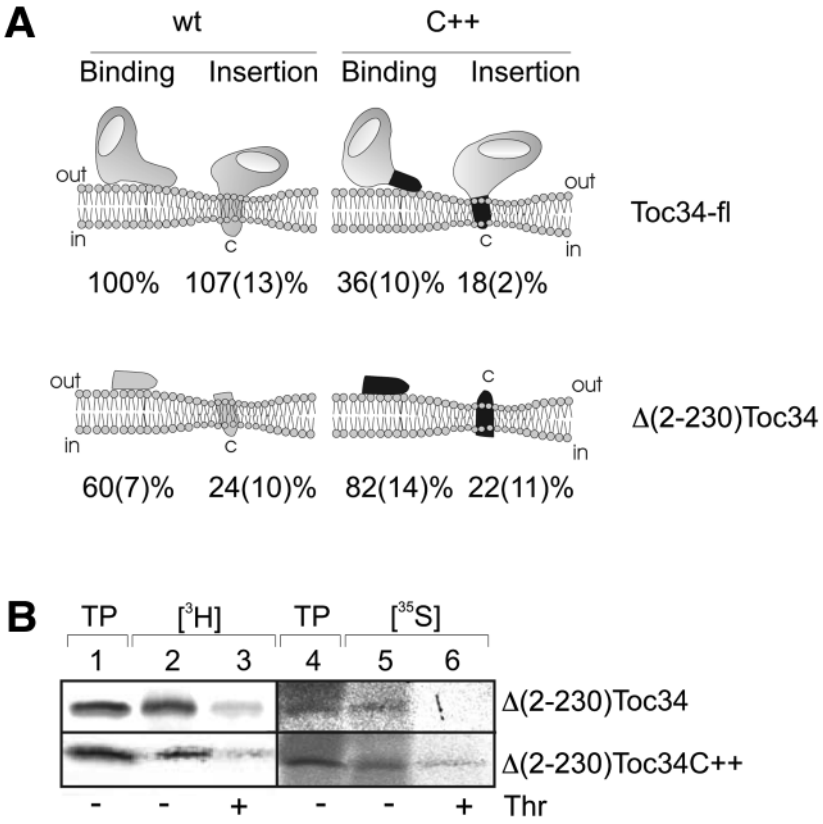

Fig. 7. Insertion of Toc34 and variants into protein-free liposomes lacking PG. (A) Insertion of [^{3}H]-leucine-labelled Toc34 (upper, left), Δ(2-230)Toc34 (lower, left), Toc34C++ (upper, right) and Δ(2-230)Toc34C++ (lower, right) into liposomes of the composition C5 (Table 1) was performed, quantified and presented as described in the legend for Fig. 6. (B) Insertion of [^{3}H]-leucine-labelled (lanes 1-3) and [^{35}S]-methionine-labelled (lanes 4-6) Δ(2-230)Toc34 (upper panel) and Δ(2-230)Toc34C++ (lower panel) into protein-free liposomes not containing PG (lanes 2, 3, 5 and 6) was performed as described in Fig. 2 and followed by thermolysin treatment (Thr, lanes 3 and 6). In lanes 1 and 4, 10% of the translation product is shown.

topology (see Figs 5 and 7), which, in combination with the lipid composition of the liposomes or the lipid asymmetry of the outer envelope, results in the predicted topology. But the large hydrophilic domain of Toc34 seems to represent an obstacle for the orientation of the transmembrane anchor in such a way that it is energetically unfavourable to translocate it across the lipid membrane. This suggests that the size of the hydrophilic region represents a retention force, which overrules the positive-inside rule.

Comparing the insertion of Toc34C++ into chloroplasts or liposomes without PG with its insertion into liposomes with PG clearly shows this. In all cases, the hydrophobic transmembrane domain inserts into the membrane with a N_{out}-C_{in} orientation. In the case of vesicles containing PG, the hydrophilic domain is exclusively retained on the membrane surface – most probably because of electrostatic interaction with the charged polar lipids. In the case of chloroplasts or liposomes without PG, this electrostatic interaction is less strong and allows the translocation of the hydrophilic domain across the membrane, although only to a small extent.

In summary, we conclude that early steps in the targeting and insertion process of the chloroplast outer envelope protein Toc34 can be faithfully studied in a reconstituted system. The liposome system shows that all the determinants for targeting, insertion and topology are present in the primary sequence and tertiary structure of Toc34, as well as in the lipid composition of the target membrane. Whether any of these steps are facilitated or accelerated by outer envelope proteins remains to be seen.

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