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Preprotein recognition by the Toc complex

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The Toc core complex consists of the pore-forming Toc75 and the GTPases Toc159 and Toc34. We confirm that the receptor form of Toc159 is integrated into the membrane. The association of Toc34 to Toc75/Toc159 is GTP dependent and enhanced by preprotein interaction. The N-terminal half of the pSSU transit peptide interacts with high affinity with Toc159, whereas the C-terminal part stimulates its GTP hydrolysis. The phosphorylated C-terminal peptide of pSSU interacts strongly with Toc34 and therefore inhibits binding and translocation of pSSU into Toc proteoliposomes. In contrast, Toc159 recognises only the dephosphorylated forms. The N-terminal part of the pSSU presequence does not influence binding to the Toc complex, but is able to block import into proteoliposomes through its interaction with Toc159. We developed a model of differential presequence recognition by Toc34 and Toc159.

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Introduction

In eukaryotic cells, two-thirds of all proteins have to traverse cellular membranes (Schatz and Dobberstein, 1996). The majority of proteins imported into chloroplasts contains an N-terminal transit peptide, which is cleaved off upon translocation. Phosphorylation of pSSU (preprotein of the small subunit of Rubisco) transit peptide enhances the import rate presumably through interaction with 14-3-3 proteins, which together with Hsp70 form a guidance complex for targeting to the chloroplast surface (May and Soll, 2000). Dephosphorylation of the preprotein has to occur before passage through the import channel of the outer envelope membrane (Waegemann and Soll, 1996). The translocation process across the outer envelope membrane is catalysed by the Toc complex (translocon of the outer envelope of chloroplasts; Soll, 2002), which contains Toc75, Toc159, Toc34 and the less tightly associated Toc64.

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The isolated Toc complex consisting of Toc159, Toc75 and Toc34 in a stoichiometry of 1:4:4–5 is able to transport pSSU in a GTP-dependent manner across a proteoliposome membrane (Schleiff *et al*, 2003a, b). The channel-forming Toc75 associates with Toc34 and Toc159 (Kouranov and Schnell, 1997; Nielsen *et al*, 1997). Both GTPases expose large domains to the cytosol, including the conserved GTP-binding domain, and interact with preproteins during the early stages of import (Hirsch *et al*, 1994; Kessler *et al*, 1994; Seedorf *et al*, 1995; Kouranov and Schnell, 1997). Toc34 in its GTP-bound state binds the phosphorylated C-terminus of preproteins with high affinity (Jelic *et al*, 2002; Schleiff *et al*, 2002). Preprotein association strongly enhances GTP hydrolysis and subsequently preprotein release. Toc34 is active for preprotein and GTP binding in a nonphosphorylated state, and can be inactivated by phosphorylation (Jelic *et al*, 2002). *Arabidopsis thaliana* encodes two isoforms of Toc34 with similar function, namely atToc33 and atToc34 (Soll, 2002). Toc159 consists of three domains. The N-terminal A-domain is highly acidic and of unknown function (Lee *et al*, 2003). The G-domain contains the GTP-binding region and the M-domain the membrane-anchoring region (Muckel and Soll, 1996; Chen *et al*, 2000). Due to its high protease sensitivity, Toc159 was first described as Toc86 comprising the G- and M-domains ($Toc159_f$). In the *A. thaliana* genome, four Toc159 isoforms were identified: atToc159, atToc132, atToc120 and atToc90 (Hiltbrunner *et al*, 2001a). A T-DNA insertion in *atToc159* gene leads to defects in chloroplast biogenesis, which cannot be compensated for by other members of the gene family (Bauer *et al*, 2000). This is in line with the observed central function of psToc159 as a GTP-driven motor during protein translocation (Schleiff *et al*, 2003a). The functional differences among members of the Toc159 family remain elusive. The GTP-binding domain might be involved in interaction with Toc34 (Smith *et al*, 2002) and is crucial for the import process, since the Toc159 knockout plant cannot be complemented by Toc159 constructs with a nonfunctional G-domain (Bauer *et al*, 2000). Toc159 was also found in a soluble cell extract (Hiltbrunner *et al*, 2001b).

Having two GTPases with proposed receptor function raises several questions, for example: Do both receptors interact directly with the preproteins? What are the conditions that favour binding and what are the conditions that favour release of the preproteins? And, in what order do the GTPases operate during translocation? In this ;article, we demonstrate that the soluble population Toc159 derives from partial membrane disruption due to the experimental set-up. This indicates that all preprotein interaction with the Toc complex occurs on the membrane. The GTP-dependent dynamics of the Toc complex are described. Furthermore, analysis of Toc159 interaction to pSSU reveals that the N-terminal part of pSSU is strongly bound by Toc159, whereas the nonphosphorylated C-terminal part induces GTP hydrolysis of the receptor.

Results

Localisation of the functional Toc159

Toc159 has been reported to exist in the cytosol as well as in the outer envelope membrane. A model was proposed in which Toc 159 binds to precursor proteins in the cytosol and targets them to the membrane. Owing to the implication that Toc159 functions before Toc34 in the import pathway, we thought it is important to reinvestigate this finding by following the procedure described (Hiltbrunner *et al*, 2001b). Intact chloroplasts and cell debris were removed from the homogenate by centrifugation (500 *g*). The presence of chloroplast proteins within the pellet was verified by immunoblotting (Figure 1A, lane 1). The supernatant was then subjected to ultracentrifugation (10^5 *g*, 2 h). Toc159 was found in the pellet as well as in the supernatant fraction. Consistent with previously reported results (Hiltbrunner *et al*, 2001b), Toc34 was restricted to the membrane pellet (lanes 2, 4). Toc64 and LHCPII were also restricted to the pellet fractions (lanes 2, 4, data not shown). Surprisingly, the outer envelope proteins Oep37 and Toc75 and the inner envelope proteins Tic110 and Tic40 were present in the soluble fractions as well (lanes 2, 4). Even after centrifugation at 3×10^5 *g* for 2 h, the integral membrane proteins Toc159, Toc75, Oep37, Tic110 and Tic40 partly remained in the supernatant (lane 5). Subsequently, we asked whether these envelope proteins are truly soluble forms or a result of partly disrupted chloroplasts leading to low-density membrane shreds. First, we analysed the lipid content of the supernatants. We detected monogalactosyldiglyceride (MGDG) and digalactosyldiglyceride (DGDG) in both supernatant fractions (Figure 1B, lanes 4, 6). The synthesis and presence of galactolipids within the plant cell is restricted to chloroplasts (Joyard *et al*, 1991), indicating the existence of chloroplast membrane shreds. Furthermore, we asked whether the 'soluble' form of Toc159 is present in membrane shreds by using Toc159 antiserum for coimmunoprecipitation. Translocon components as well as Oep37 were coimmunoprecipitated by Toc159 antiserum (Figure 1C, lane 2). Moreover, chloroplast lipids like MGDG and DGDG were also identified in the precipitate (Figure 1D, lane 6). In contrast, preimmunserum neither chloroplast envelope proteins nor lipids were precipitated (Figure 1C, lane 1; Figure 1D, lane 4). When the soluble nucleoside diphosphate kinase (NDPK) was immunoprecipitated (Figure 1F, lane 2), neither lipids (Figure 1E, lane 6) nor other proteins (not shown) could be detected. To confirm the lipid association of Toc159, we used antibodies against MGDG or DGDG (Radunz, 1976). Using these antibodies, we could precipitate Toc159 (Figure 1G, lane 2), confirming its lipid association.

We now asked whether membrane fragments with low protein content are not pelleted under the described conditions (Hiltbrunner *et al*, 2001b). For this, proteoliposomes containing Toc75 were centrifuged (10^5 *g* or 3×10^5 *g*, 2 h). Indeed, Toc75 proteoliposomes remained in the supernatant (Figure 1H, lanes 1, 2). Only after centrifugation at 6×10^5 *g* for 2 h, Toc75 proteoliposomes were recovered in the pellet fraction (Figure 1H, lane 6). We therefore investigated whether Toc159 can be pelleted out of the leaf extract at 6×10^5 *g*. After centrifugation for 2 h, Toc159 could only be detected in the pellet fraction together with Tic110 and Tic40 (Figure 1I, lanes 1, 4). In contrast, the soluble glycerinaldehyde-P-dehydrogenase (GAPDH) remained in the supernatant

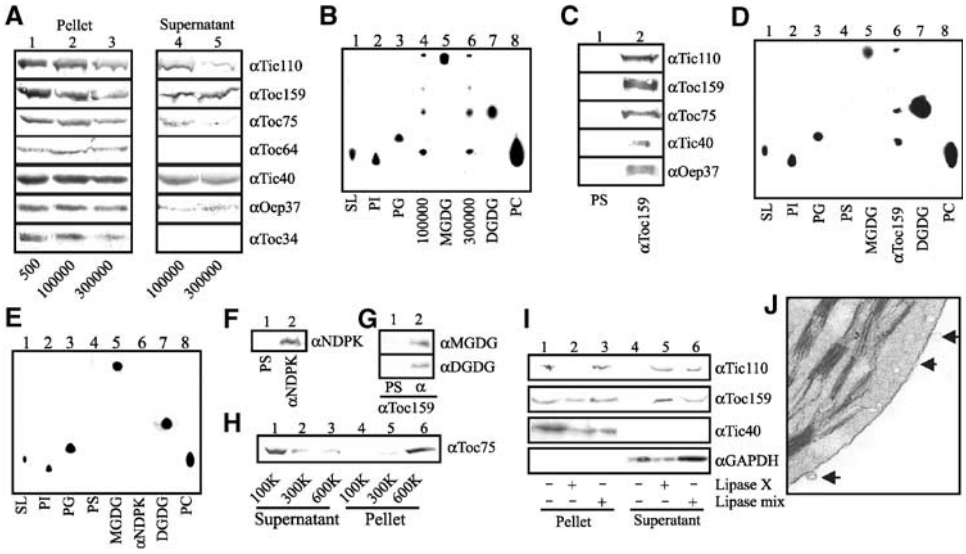

Figure 1 Isolation of Toc159 out of the soluble cell extract. (**A**) Leaf extract was prepared according to the materials and methods. The pellet (lanes 1–3) or supernatant (lanes 4, 5) of the 500 *g* (lane 1), 10^5 *g* (lanes 2, 4) and 3×10^5 *g* (lanes 3, 5) centrifugation step were loaded in equivalent amounts onto the chloroplast pellet on SDS–PAGE and immunodecorated with indicated antibodies. (**B**) The lipid content of the supernatant fractions (see (A)) was analysed by separation over TLC plate. Standard lipids were applied: sulphochinovosyldiglyceride (SL), phosphatidylinositol (PI), phosphatidylglyceride (PG), MGDG, DGDG and phosphatidylcholine (PC). The 10^5 *g* supernatant was used for coimmunoprecipitation with Toc159 (**C**, lane 2), NDPK (**F**, lane 2), MGDG (**G** up, lane 2) or DGDG-antiserum (G down, lane 2) and their corresponding preimmunsera (C, F, G lane 1). The precipitate was analysed for the presence of chloroplast envelope proteins (C) or Toc159 (G) by immunostaining and lipids by extraction (**D, E**). (**H**) Proteoliposomes containing Toc75 were centrifuged at 10^5 *g*, 3×10^5 *g* and 6×10^5 *g*. Supernatants (lanes 1–3) and pellets (lanes 4–6) were immunodecorated with Toc75 antiserum. (**I**) The soluble extract from 10^5 *g* spin (see (A)) was incubated in the absence (lanes 1, 4) or presence of lipase X (lanes 2, 5) or lipase mixture (lanes 3, 6) and subsequently subjected to a 6×10^5 *g* spin centrifugation. Pellets (lanes 1–3) and supernatants (lanes 4–6) were immunodecorated with Toc75 antiserum. (**J**) An electron micrograph shows an enlargement of the chloroplast periphery of an isolated chloroplast. Arrows mark membrane disturbances of the envelope membranes.

(lane 6). Additional evidence that the $6 \times 10^5 g$ pelleted envelope proteins are embedded in membrane fragments was obtained by treating the cell extract with phospholipases to disrupt bilayer structure and integrity. After phospholipase treatment, some of the Toc159 and Tic110 appeared in the supernatant of the $6 \times 10^5 g$ spin (Figure 1I, lanes 5, 6). Only Tic40 remained in the pellet (lanes 2, 3), which might be a result of aggregation. Again, the distribution of GAPDH was not affected by the addition of lipases (lanes 5, 6).

Toc159 was previously described as an integral membrane protein (Kessler *et al*, 1994; Hiltbrunner *et al*, 2001b). Performing immunogold labelling with antibodies against a C-terminal portion of Toc159, we found that 80% of the gold particles are located in the interior of the vesicles. This indicates that sections of the C-terminal part of Toc159 face towards the intermembrane space (Figure 2A, left side). In line with published results (Seedorf *et al*, 1995), 80% of the gold particles while using Toc34 antibodies were detected on the outside of the outer envelope vesicles (OEVs). The integral membrane nature of Toc159 is further consistent with its resistance against extraction by a chaotropic reagent (Figure 2B, lane 1) or sodium carbonate (Hiltbrunner *et al*, 2001b). For further topology studies of Toc159, OEVs were first treated with trypsin leading to the 52 kDa fragment of Toc159 (Figure 2C, lanes 2, 7), which is not extractable by high pH (lane 8). When the proteolysed OEVs were Na_2CO_3 extracted in the presence of trypsin to digest intermembrane space-facing regions, the 52 kDa fragment was degraded to smaller fragments. This and the presence of a fairly stable peptide of 20 kDa (lane 9–11, triangle) suggest the existence of intermembrane space-facing regions of Toc159. Trypsin treatment of Triton X-100-solubilised OEVs revealed the same result (data not shown). In contrast, the 52 kDa domain remained protease insensitive (lanes 4–6) when noncarbonate-treated but washed OEVs were treated with trypsin. Previously, it was shown that protease treatment of OEVs results in the appearance of an 8 kDa fragment of Toc34 (Seedorf *et al*, 1995) and a 55 kDa fragment of Toc75 (Sveshnikova *et al*, 2000). Therefore, the proteolysis of these peptides was used as an indicator of the accessibility of intermembrane space-facing regions after membrane disruption by carbonate treatment (lanes 12–16, square and arrow).

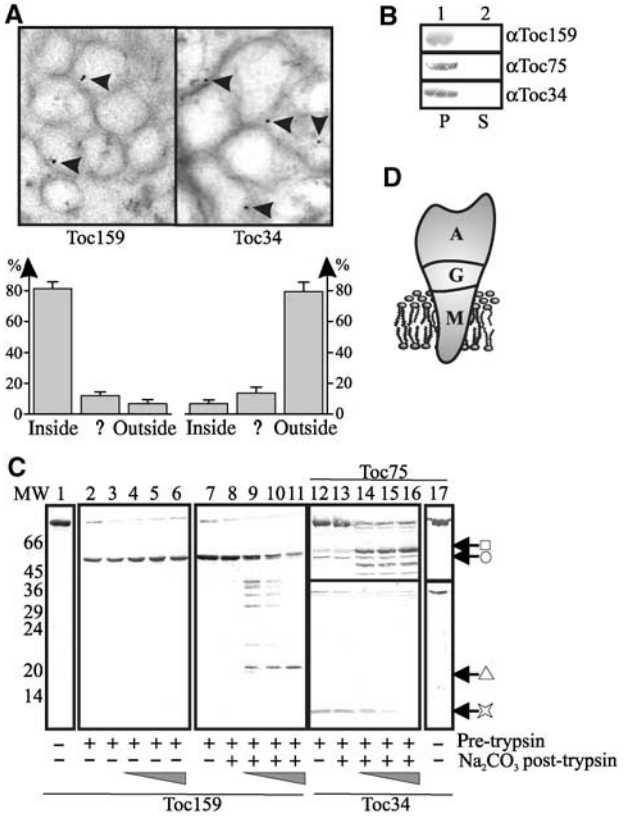

Figure 2 Membrane-bound Toc159. (**A**) Ultra thin sections of OEVs were incubated with gold-labelled antiserum against Toc159 (left) or Toc34 (right). The staining is visualised by electron microscopy and marked by arrows. The quantification of the distribution of the signals of three independent experiments is depicted as percentages of total signals. (**B**) Purified OEVs (30 μg protein) were incubated with 4 M urea for 30 min on ice. After separation of pellet (P) and supernatant (S), both fractions were analysed by immunoblotting. (**C**) Purified OEVs (120 μg protein) were treated with trypsin (lanes 2, 7, 12). After the digestion, OEVs were extracted by 100 mM Na_2CO_3 (lanes 8, 13) followed by centrifugation (lanes 3, 8, 13), and subjected to post trypsin incubation (lanes 4–6, 9–11, 14–16). The samples were analysed by immunoblotting with the indicated antisera. For comparison, nontreated OEVs were loaded (lanes 1, 17). Typical proteolytic products of Toc159 (circle and triangle), Toc75 (square) and Toc34 (asterisk) are marked. (**D**) A model of the topology of membrane-bound Toc159 is shown.

Taken together, we conclude that the identified soluble Toc159 indeed represents a membrane-bound population present in lipid shreds, which do not pellet by a simple $10^5 g$ centrifugation step. The results further establish that Toc159 is not a cytosolic protein and that the 52 kDa M-domain comprises an intermembrane space-facing part, which might contain multiple membrane-spanning regions. Thus, all interactions among precursor, Toc159 and Toc34 must occur on the membrane.

Toc159 binds to the nonphosphorylated form of pSSU

Previous studies established that Toc34 binds to precursors via their transit peptide, but the question of Toc159 binding to the precursor has not been adequately addressed. Outer envelope proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane, followed by renaturation. Subsequently, the binding of a ^{35}S-radiolabelled nonphosphorylated form of pSSU, as well as of ^{32}P-radiolabelled phosphorylated pSSU, to outer envelope proteins was assayed (Figure 3A). The binding was analysed in the absence or presence of GMP-PNP. GMP-PNP was previously found to enhance the binding of pSSU to Toc34 (Schleiff *et al*, 2002), to Toc159 (Kouranov and Schnell, 1997) and to the Toc complex, respectively (Schleiff *et al*, 2003b). GMP-PNP strongly stimulated the binding of both pSSU forms to Toc34 (Figure 3A, lanes 1–4). Similarly, the interaction of the nonphosphorylated form of pSSU with Toc159 was stimulated by GMP-PNP (lanes 1, 2). However, no interaction of Toc159 with the phosphorylated pSSU was detected (lanes 3, 4). Furthermore, neither the phosphorylated nor the nonphosphorylated pSSU interacts with the outer envelope protein Oep37, which served as a negative control (upper part). The identity of the protein bands was confirmed by immunoblotting (lower part). The same was observed when proteoliposomes containing $Toc159_f$, Toc34 or Oep16 were incubated with expressed and *in vitro* phosphorylated pSSU (Figure 3B, upper part), wheat germ *in vitro* translated and

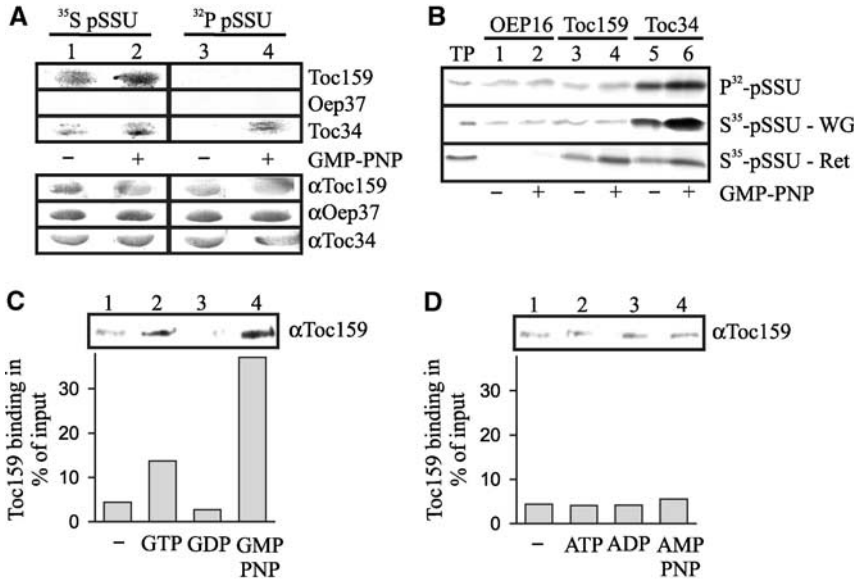

Figure 3 Recognition of nonphosphorylated pSSU by Toc159 is GTP dependent. (**A**) OEVs (50 μg protein) were separated by SDS–PAGE and transferred to the nitrocellulose membrane. Nonphosphorylated (lanes 1, 2) and phosphorylated (lanes 3, 4) pSSU in the absence (lanes 1, 3) or presence (lanes 2, 4) of GMP-PNP was bound and visualised by autoradiography. The same blot was immunodecorated with the indicated antisera (below). (**B**) Proteoliposomes containing Oep16 (lanes 1,2), Toc159 (lanes 3,4) or Toc34 (lanes 5,6) were incubated with *in vitro* phosphorylated pSSU (P^{32}-pSSU, up), wheat germ lysate *in vitro* translated and phosphorylated pSSU (S^{35}-pSSU-WG, middle), or reticulocyte lysate *in vitro* translated and nonphosphorylated pSSU (S^{35}-pSSU-Ret, down) in the absence (lanes 1, 3, 5) or presence of GMP-PNP (lanes 2, 4, 6). TP indicates 10% of the protein used. (**C**, **D**) pSSU (20 μg) was coupled to 20 μl Toyopearl AF-tresyl 650M column material and subsequently incubated with 250 ng $Toc159_f$. The incubation was performed in the absence or presence of guanine (C) or adenine nucleotides (D). The amount of bound Toc159 in percentage of input was quantified after immunostaining using AIDA software. One representative result of three experiments is depicted.

phosphorylated pSSU (middle part), or in reticulocyte lysate *in vitro* translated nonphosphorylated pSSU (lower part) in the absence (lanes 1, 3, 5) or presence of GMP-PNP (lanes 2, 4, 6). As before, Toc34 (lanes 5, 6) recognises pSSU with higher affinity in its GMP-PNP-bound stage. Furthermore, phosphorylation of the pSSU drastically enhances this interaction. In contrast, Oep16 and $Toc159_f$ only revealed background binding to the phosphorylated preprotein (lanes 1–4). As shown before, $Toc159_f$ interacts with the nonphosphorylated form of pSSU in a GMP-PNP-dependent manner (lanes 3, 4, lower part), whereas, as expected, Oep16 shows no binding at all (lanes 1, 2).

The observed nucleotide dependence of the interaction between Toc159 and the preprotein could also be assembled while incubation of immobilised pSSU with $Toc159_f$ in the absence or presence of different nucleotides. The strongest binding of $Toc159_f$ to pSSU was observed in the presence of GTP and GMP-PNP (Figure 3D, lanes 1, 3). The less pronounced binding of Toc159 to pSSU in the presence of GTP in comparison to GMP-PNP is probably due to a partial hydrolysis of GTP, which converts Toc159 to its GDP-bound state with a reduced affinity to the precursor protein (lane 3). No effect on the binding efficiency of Toc159 to pSSU by adenine nucleotides was observed (Figure 3D). We therefore conclude that the interaction between Toc159 and the nonphosphorylated pSSU is GTP-specific.

Toc159 and Toc34 have different affinities for different domains of the transit peptide

To define the part of the presequence that interacts with Toc159, three peptides of the presequence of pSSU, E2, A1 or B2 (Figure 4A), were used. The peptides were coupled via a C-terminal cysteine to thiol-activated sepharose. $Toc159_f$ (Figure 4B, lanes 1–3) was preincubated with GTP (lanes 4–6), GMP-PNP (lanes 7–9) or GDP (lanes 10–12), and then incubated with the affinity matrices. A strong interaction of $Toc159_f$ with the N-terminal peptide E2 was observed under all conditions tested (lanes 3, 6, 12), except in the presence of GDP (lane 9). In contrast, the binding of $Toc159_f$ to the nonphosphorylated peptide A1 increased in the presence of GMP-PNP in comparison to GTP or the absence of nucleotides (lanes 1, 4, 10). As before, the increment of $Toc159_f$ binding to A1 in the presence of GMP-PNP in comparison to GTP might be caused by GTP hydrolysis of $Toc159_f$ (lanes 6–9). In contrast, $Toc159_f$ recognises the phosphorylated C-terminal peptide (B2) to a lower extent than A1 and E2 (Figure 4A). The last result is consistent with the lack of $Toc159_f$ binding to phosphorylated pSSU (Figure 3A and B).

As a complementary approach, immobilised pSSU was incubated with $Toc159_f$ or $Toc159_{f\text{-GMP-PNP}}$ in the presence of peptides (A1, B2, E2) as competitors of binding (Figure 4C, lanes 2, 7) and the remaining interaction analysed by immunostaining. E2 preincubation reduces $Toc159_f$ binding to pSSU in the absence and presence of GMP-PNP, suggesting a strong nucleotide-independent interaction of $Toc159_f$ to this peptide (lanes 5, 10). A1 and B2 show no competition to pSSU binding by $Toc159_f$ in the absence and presence of GMP-PNP (lanes 3, 8). Comparison between the competition of pSSU binding by A1 (lane 8) or E2 (lane 10) and the direct binding of Toc159 to A1 (Figure 4B, lane 10) and E2 (lane 12) in the presence of GMP-PNP suggests that Toc159 can recognise the N-terminal portion of the presequence even though the binding pocket for the C-terminal portion is occupied but not *vice versa*. In contrast, $Toc159_f$ does not bind to the empty

column material (Figure 4C, lanes 1, 6). As a control for the experiment, we performed a competition assay using isolated Toc34, which revealed the strongest inhibition of pSSU binding by the phosphorylated peptide B2 in the presence of GMP-PNP (Figure 4D, lane 9). We conclude that a strong GTP-independent interaction of Toc159 occurs at the N-terminal part of the pSSU transit peptide.

Toc159 GTPase activity is stimulated by the nonphosphorylated transit peptide

The results from Figures 4C and 5A suggested a conversion of GTP into GDP by Toc159 in the presence of a preprotein. In order to test for a potential GTPase activity of Toc159, $Toc159_f$ was incubated with α^{32}P-GTP. As has been found for Toc34 (Jelic *et al*, 2002), $Toc159_f$ showed a slow endogenous GTP

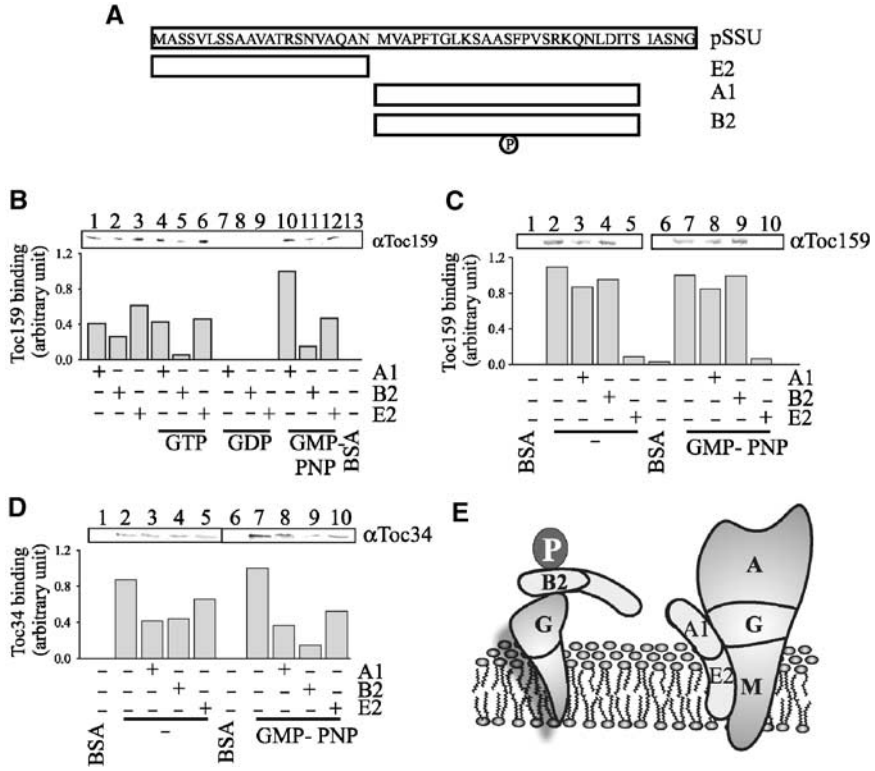

Figure 4 Toc159 recognises the N-terminal part of pSSU transit peptide with high affinity. (**A**) An outline of pSSU presequence and its peptides A1, B2 and E2 used in assays is presented. (**B**) Thiol-activated sepharose (20 µl) with bound peptides (0.6 mg/ml) was incubated with 250 ng $Toc159_f$ without (lanes 1–3) or with GTP (lanes 4–6), GDP (lanes 7–9) or GMP-PNP (lanes 10–12). A BSA-coated column was used as control (lane 13). The quantification of one representative example of three independent experiments is shown. The Toc159 binding to A1 in the presence of GMP-PNP was set to 1. (**C**) Toyopearl material (20 µl) coated with pSSU (1 mg/ml) was incubated with 250 ng Toc159 in the absence (lanes 2–5) or presence of 0.5 mM GMP-PNP (lanes 7–10). The binding was competed by addition of peptides to a final concentration of 5 µM. A BSA-coated column was used as control (lanes 1, 6). The quantification of one representative example of three independent experiments is shown. The Toc159 binding to pSSU in the presence of GMP-PNP was set to 1. (**D**) Same experiment as in (C), but with expressed Toc34. (**E**) A model of interaction of the presequence of pSSU is depicted.

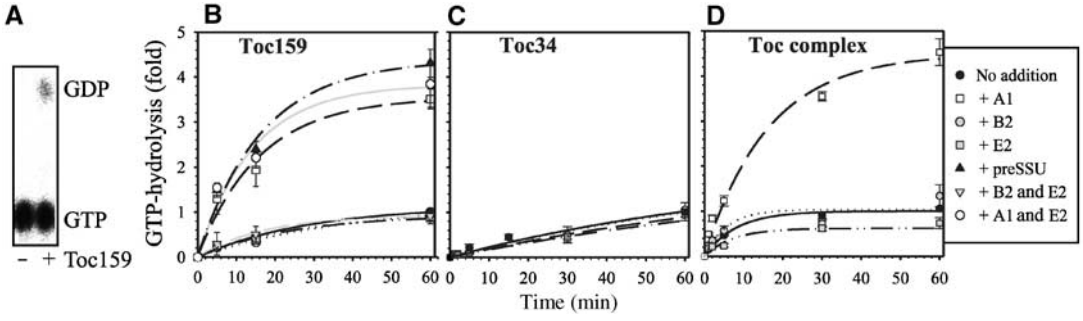

Figure 5 The nonphosphorylated C-terminal part of the pSSU presequence stimulates GTP hydrolysis by Toc159. (**A**) Toc159 was incubated with α^{32}P-GTP for 1 h at 20°C (lane 2) followed by spotting on a PEI cellulose plate. GTP alone without any protein was loaded as a control (lane 1). For measurements of GTP hydrolysis of α^{32}P-GTP by Toc159 (**B**, solid line), Toc34 (**C**, solid line) or Toc complex (**D**, solid line) was determined in the presence of pSSU (dashed-single dotted line), A1 (dashed line), B2 (dotted line), E2 (dashed-double dotted line), A1 and E2 (grey solid line), and B2 and E2 (dashed grey line). At indicated time points, GTP and GDP were separated on a PEI cellulose plate and hydrolysis was quantified. Data are shown as depicted on the right side and represent the average of at least three independent measurements. Lines represent the least-square analysis using an exponential equation.

hydrolysis activity (Figure 5A, lane 2). Therefore, the effect of pSSU on Toc159$_f$ GTPase activity was investigated. Under the conditions used, pSSU was found to stimulate GTPase activity of Toc159$_f$ by about four-fold (Figure 5B; for line and symbol description, see figure legend) compared to intrinsic hydrolysis. Furthermore, utilising the peptides A1, B2, E2 and the combinations A1 and E2, and B2 and E2, the stimulating activity could be localised to the peptide A1, while E2 or B2 had no influence on the GTPase activity. Interestingly, this peptide is recognised by Toc159$_f$ with lower affinity than the N-terminal peptide E2 (Figure 4B and C). In contrast, GTP hydrolysis by Toc34 was not affected by addition of the peptides (Figure 5C). We further asked how the peptides A1, B2 and E2 affect the GTPase activity of the isolated complex. It had been shown previously that pSSU stimulates GTP hydrolysis of the isolated complex (Schleiff *et al*, 2003a). Herein, we could demonstrate a stimulating effect of the A1 peptide on the GTPase activity of the Toc complex (Figure 5D). We conclude that the increased GTPase activity of the Toc complex by A1 is due to Toc159.

Guanine nucleotides affect the stability of the Toc complex

Toc34 and Toc159 function as receptor proteins. One central question still remained: how is the association of the two receptor proteins regulated? As both proteins are GTPases, we asked whether GTP or GDP affects the stability of the Toc complex. Therefore, solubilised OEVs were incubated with GDP or GMP-PNP. Subsequently, antisera against Toc34 or Toc159 were used for immunoprecipitation (Figure 6A). All the three Toc core components were coimmunoprecipitated with the same efficiency in the absence of nucleotides or in the presence of GMP-PNP by either Toc34 (Figure 6A, lanes 1, 3) or Toc159 antiserum (lanes 5, 7). In contrast, incubation with GDP leads to a reduced coimmunoprecipitation of Toc159 by Toc34 antiserum and *vice versa* (lanes 2, 6). Interestingly, the amount of coimmunoprecipitated Toc75 by Toc34 antiserum in the presence of GDP is reduced as well (lane 2), whereas Toc159 antiserum coimmunoprecipitates Toc75 in a similar manner regardless of added nucleotides (lanes 5–7), suggesting a nucleotide-independent

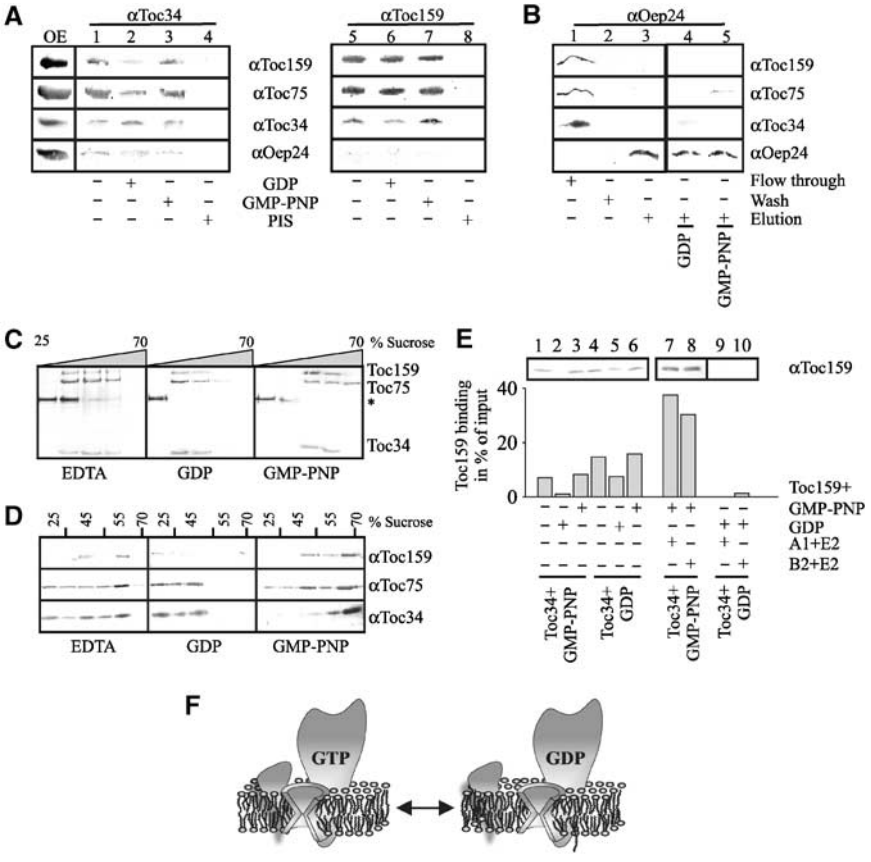

Figure 6 The stability of the Toc complex is guanine nucleotide dependent. Coimmunoprecipitation of purified OEVs (150 μg protein) with antisera against Toc34 (**A**, left), Toc159 (A, right), preimmunsera (A, lanes 4, 8) or Oep24 (**B**) was performed in the absence (A, lanes 1, 4, 5, 8; B, lanes 1–3) and presence (A, lanes 2–3, 6–7; B, lanes 4, 5) of guanine nucleotides. In (B), the flow-through (lane 1) and wash (lane 2) of the immunoprecipitation using Oep24 antibodies is shown. The bound proteins were identified by immunoblotting. (**C**) OEVs (150 μg protein) were incubated with EDTA (left), GDP (middle) or GMP-PNP (right), solubilised with 1.5% *n*-decylmaltoside and lipase treatment and separated by linear sucrose gradient centrifugation. Fractions of the gradient were collected and the protein content was analysed by silver staining. The identified Toc components and the lipase (*) are marked. (**D**) The Toc complex from a linear gradient in the absence of nucleotides was incubated with EDTA (left), GDP (middle) or GMP-PNP (right), and subjected to a sucrose step-gradient centrifugation. The protein content of the fractions was analysed by immunoblotting with the indicated antisera. (**E**) Toc34 (2 μg) was preincubated with GMP-PNP (lanes 1–3, 7, 8) or GDP (lanes 4–6, 9, 10) followed by incubation with 250 ng Toc159$_f$ (lanes 1, 4) preincubated with GMP-PNP (lanes 3, 6–8) or GDP (lanes 4–6, 9, 10) and pSSU presequence peptides (lanes 7–10). The amount of bound Toc159 is depicted as the percentage of total input. (**F**) A model of GTP-dependent association of Toc34 to Toc159/Toc75 is presented.

association of Toc159 and Toc75. The specificity of the reaction was confirmed by using preimmunsera (lanes 4, 8) and by investigating the presence of the outer envelope protein Oep24 as control protein. Although small amounts of Oep24 were coimmunoprecipitated by the used Toc34 antiserum (lanes 1–3), it presents only a small portion of total Oep24 content in OEVs (lane OE). Furthermore, Toc75, Toc159 and Toc34 were not coimmunoprecipitated by Oep24 antibodies regardless of the added nucleotides (Figure 6B, lanes 3–5). Therefore, we consider the precipitation of Oep24 as an unspecific reaction.

To confirm our observation, the stability of the Toc complex was analysed during isolation by sucrose gradient centrifugation. OEVs were incubated with EDTA, GDP or GMP-PNP before solubilisation and subjected to linear sucrose gradient centrifugation. In the presence of GMP-PNP, the Toc components are enriched in higher density fractions as compared to the absence of nucleotide (Figure 6C, right and left parts). In the presence of GDP, Toc components migrate only to lower-density fractions (middle part). Subsequently, Toc complex-containing fractions in the absence of nucleotides (left part) were pooled and incubated in the presence and absence of GMP-PNP or GDP. The samples were subjected to a sucrose step-gradient centrifugation. In the presence of GMP-PNP, the amount of Toc components, especially Toc34, is enriched in fractions of higher density in comparison to the amount in the absence of nucleotides (Figure 6D, left and middle parts). In the presence of GDP, no Toc components were found in a fraction of high sucrose concentrations (Figure 6D, middle part). The results from linear and step-gradient analysis of the Toc complex suggest that GMP-PNP leads to a stabilisation of the Toc complex, whereas GDP weakens the interaction between the Toc components (Figure 6C and D).

To further determine the role of the two GTPases in Toc stability, we analysed the interaction between Toc159 and Toc34 *in vitro*. We used $Toc159_f$ eluted from SDS–PAGE, which contains the G- and M-domain. We assume that the A-domain might not affect this interaction as drastically, since Toc159 lacking the A-domain is capable of promoting protein translocation in protoplasts (Lee *et al*, 2003). Toc34 was bound to a Ni-NTA column via a His-tag. Subsequently, the immobilised Toc34 was incubated with GDP (Figure 3E, lanes 4–6, 9, 10) or GMP-PNP (lanes 1–3, 7, 8). The Toc34 affinity matrix was incubated with Toc159 (lanes 1, 4) preloaded with GMP-PNP (lanes 3, 6–8) or GDP (lanes 2, 5, 9, 10). This approach allowed a different nucleotide-loaded stage of both GTPases. The amount of bound Toc159 was determined. The binding of Toc159 to $Toc34_{GDP}$ (lanes 4–6) was slightly increased in comparison to $Toc34_{GMP\text{-}PNP}$ (lanes 1–3). $Toc159_{GMP\text{-}PNP}$ binds to Toc34 to the same extent as nucleotide-free Toc159 (lanes 1, 3, 4, 6). In contrast, $Toc159_{GDP}$ shows reduced binding to Toc34 (lanes 2, 5). As $Toc34_{GMP\text{-}PNP}$ recognises pSSU with higher affinity compared to the nucleotide-free receptor (Schleiff *et al*, 2002), we investigated the effect of the pSSU transit peptide on the interaction between Toc159 and Toc34. Therefore, we incubated both receptors in the presence of GMP-PNP with peptides covering parts of the pSSU presequence (Figure 4A). The N-terminal peptide (E2) was combined either with peptides covering the nonphosphorylated (A1) or the phosphorylated C-terminal part (B2). Both peptide mixtures showed a strong stimulation of the interaction between the Toc GTPases in the GMP-PNP stage in comparison to peptide-free conditions (Figure 6E, lanes 3, 7, 8). In contrast, the interaction between both receptors loaded with GDP was drastically reduced (lanes 5, 9, 10). Taken together, the Toc complex is stabilised by GMP-PNP, whereas $Toc159_{GDP}$ favours Toc34 dissociation (Figure 6F). The binding of transit peptide further strengthened the interaction between the two GTPases in the GMP-PNP state.

Toc34 functions before Toc159 in the binding and translocation reaction

Having established that Toc159 recognises the N-terminal peptide with highest affinity, whereas the C-terminal peptide induces the GTPase function of the receptor, we asked whether the different peptides could influence protein import. Isolated chloroplasts were preincubated with the different peptides and import initiated by the addition of radiolabelled pSSU (Figure 7A). Preincubation with B2 and E2 led to a decrease of import yield of about 50%, whereas A1 did not reveal any effect (lanes 2–4), supporting our earlier result that B2 and E2 in contrast to A1 bind strongly to Toc34 and Toc159, respectively (Figure 4C, D). As expected, preincubation with expressed full-length pSSU almost completely abolishes the import of radiolabelled pSSU (Figure 7A, lane 5).

To confirm that this observation shows an effect on the core translocon, we reconstituted the isolated Toc complex in liposomes (Schleiff *et al*, 2003a). Proteoliposomes were incubated with radiolabelled pSSU in the presence of GMP-PNP to stimulate binding (Figure 7B, lanes 15, 16) or GTP to promote import (lanes 23, 24). To differentiate between the bound and the imported pSSU, proteoliposomes were treated with trypsin in order to digest surface-bound preproteins, whereas imported pSSU remains unaffected (Schleiff *et al*, 2003a). In the presence of GMP-PNP, the precursor remains protease sensitive, indicating its presence on the surface of the liposomes (lane 16), while in the presence of GTP pSSU was protease resistant, indicating translocation into the proteoliposomes (lane 24). Proteoliposomes were then preincubated with the peptides A1, B2 or E2 before addition of precursor protein (lanes 1–6, 9–14, 17–22). A1 slightly reduces the binding of pSSU in the absence or presence of GMP-PNP compared to the amount of bound pSSU in the absence of peptides (lanes 3, 11). However, A1 does not impair the import of pSSU into proteoliposomes because the protease-protected form of pSSU was found (lane 20). In contrast, E2 reduces pSSU binding in the absence of nucleotides (lane 5) and inhibits the import of pSSU like B2 (lanes 18, 22). Under binding conditions in the presence of GMP-PNP, B2 is the only peptide reducing pSSU interaction to the proteoliposomes (lane 9). This observation can be explained by a preceded binding of B2 to Toc34, while the interaction of Toc159 with E2 takes place at a later stage and inhibits import.

Next, proteoliposomes were loaded with a stromal extract containing the precursor-processing peptidase (Schleiff *et al*, 2003b). The processing event of pSSU to mSSU (mature form of pSSU) indicates a successful import reaction (Figure 7C, lane 1). To reveal the specificity of the peptides, the assays were conducted with proteoliposomes containing the Toc complex (Toc159, Toc75 and Toc34; upper part) or Toc159

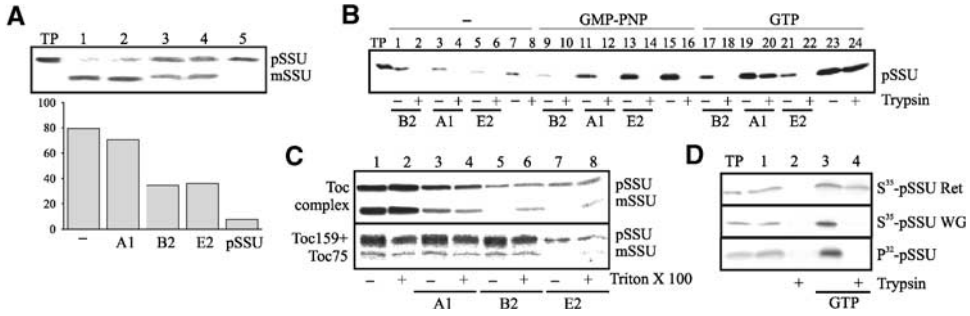

Figure 7 Import of pSSU is reduced by the N-terminal and phosphorylated C-terminal part of pSSU. (**A**) Isolated chloroplasts were incubated with ^{35}S-labelled pSSU translated in reticulocyte lysate for 10 min at 20°C in the absence (lane 1) or presence of 5 μM peptides of pSSU presequence (lanes 2–4) or pSSU (lane 5). Samples were separated via SDS–PAGE and visualised by a phospho-imager. The import rate into isolated chloroplasts is given as a percentage of the input. (**B**) Proteoliposomes with reconstituted Toc complex were incubated with ^{35}S-labelled pSSU translated in reticulocyte lysate in the absence (lanes 1–8) or presence of GMP-PNP (lanes 9–16) or GTP (lanes 17–24). The binding and import reaction was performed in the absence (lanes 7–8, 15–16, 23–24) or presence of peptides of the pSSU presequence (lanes 1–6, 9–14, 17–22). Surface-bound preproteins were digested by trypsin treatment (even lanes). (**C**) Import of ^{35}S-labelled pSSU translated in reticulocyte lysate into proteolipsomes with enclosed stromal fraction containing reconstituted Toc complex (upper part) or co-reconstituted Toc159 and Toc75 (lower part) was performed in the absence (lanes 1–2) or presence of peptides of the pSSU presequence (lanes 3–8). (**D**) Toc complex proteoliposomes were incubated with *in vitro* translated pSSU using reticulocyte lysate (S^{35}-pSSU-Ret, up), wheat germ lysate (S^{35}-pSSU-WG, middle) or *in vitro* phosphorylated pSSU (P^{32}-pSSU, down), in the absence (lanes 1,2) or presence of GTP (lanes 3, 4). Surface-bound preproteins were digested by trypsin treatment (lanes 2, 4).

and Toc75 as a minimal import-competent unit (lower part; Schleiff *et al*, 2003a). The proteoliposomes were then incubated with A1, B2 or E2, before addition of the full-length precursor. The B2 and E2 peptides are able to inhibit translocation of pSSU in proteoliposomes containing the Toc complex (lanes 5, 7, upper part), whereas only E2 prevents pSSU import into proteoliposomes containing Toc159 and Toc75 (lanes 7–8, lower part). This result supports the observation that B2 is strongly bound by Toc34 and not by Toc159, whereas E2 is only recognised by Toc159 with high affinity (Figure 4C, D). In contrast, the import of pSSU into proteoliposomes with the reconstituted Toc complex is only slightly reduced by the presence of A1 (lane 4, upper part). This can be explained by the low-affinity binding of A1 by Toc34 (Schleiff *et al*, 2002). Preincubation with A1 does not affect import into proteoliposomes with reconstituted Toc159 and Toc75. This result indicates a transient interaction of Toc159 to A1, because it is capable of stimulating GTP-hydrolysis of Toc159 but it does not block the import event. To confirm the activity of the signal peptidase in all cases, membranes were solubilised to release the signal peptidase, resulting in processing of pSSU (lanes 2, 4, 6, 8).

To confirm that the preprotein has to be dephosphorylated in order to be recognised by Toc159 and to be transported across the membrane, the translocation of pSSU *in vitro* translated in reticulocyte lysate (Figure 7D, upper part), in wheat germ lysate (resulting in phosphorylation, middle part) or pSSU purified and *in vitro* phosphorylated into Toc-proteoliposomes was studied. In line with previous results, translocation of pSSU-Ret was only achieved in the presence of GTP (lane 4; Schleiff *et al*, 2003a), resulting in a protease-resistant form. When phosphorylated pSSU was used (middle, lower part), no protease-resistant pSSU could be observed, indicating that phosphorylated pSSU cannot be translocated across the membrane.

Discussion

The Toc core complex consists of two G-proteins, Toc159 and Toc34, and the pore-forming Toc75 (Schleiff *et al*, 2003a). In addition, a soluble Toc159 pool was detected (Hiltbrunner *et al*, 2001b), which led the authors to propose a shuttling mechanism of Toc159 between a soluble cytosolic and a membrane-bound form. However, the function of this soluble intermediate remained elusive as no interaction of this soluble form with preproteins has been demonstrated so far. Utilising the published procedure (Hiltbrunner *et al*, 2001b), we found that the presence of Toc159 in the soluble extract is due to the presence of chloroplast membrane shreds in the soluble extract as a result of partial envelope membrane disruption (Figure 1). In electron micrographs of isolated chloroplasts, partial disruption of the envelope membrane can often be seen (Figure 1J). It might be that these membrane sections are shared off while isolating organelles. Moreover, our results demonstrate that the M-domain of Toc159 might comprise multiple membrane-spanning regions and an intermembrane space-facing region (Figure 2, Muckel and Soll, 1996). Toc159 is resistant to extraction by urea or carbonate. Therefore, the proposed shuttling model between a soluble and membrane-bound form of Toc159 seems unlikely. Therefore, we focused our analysis of the Toc complex dynamics and mechanism during preprotein recognition on the membrane-bound Toc159 and not on the function of the soluble form.

Based on the existing results and those presented here, we propose that Toc34 acts as an initial receptor. This hypothesis is based on multiple observations. First, Toc34 interacts with phosphorylated preprotein whereas Toc159 recognises only a nonphosphorylated one (Figure 3). Prior to complementation of translocation across the outer envelope, membrane dephosphorylation has to occur (Waegemann and Soll, 1996). It is therefore reasonable to think that Toc34 acts as an initial receptor. Second, addition of E2 peptide to the reconstituted Toc complex does not affect binding of pSSU in the presence of GMP-PNP, whereas B2 reduces the amount of bound pSSU drastically (Figure 7), indicating preceding recognition of the phosphorylated presequence. Third, Toc34 was predominantly found in association with cross-linked preprotein at early import stages, whereas Toc159 interaction was more pronounced in later stages (Kouranov and Schnell, 1997).

This result implies binding of phosphorylated pSSU by Toc34 before recognition by Toc159. Fourth, Toc159 was found to provide the driving force for translocation by GTP hydrolysis (Schleiff *et al*, 2003a). Fifth, the stoichiometry of Toc159:Toc75:Toc34 in the Toc complex is 1:4:4–5 (Schleiff *et al*, 2003b), suggesting Toc34 to function as a receptor and Toc159 as a central catalytic motor for translocation. Further, Toc34 is not essential for the translocation process (Figure 7C) and probably serves as a gate to regulate advancement to the channel. It can be speculated that Toc34 is a part of a timing mechanism and defines the targeting fidelity in that the precursor has to stay bound to Toc34 long enough for the phosphate to be removed, in order for it to be passed to the motor and the channel.

The dynamic Toc complex structure is influenced by GTP-binding and hydrolysis. GMP-PNP enhances the complex stability, whereas GDP induces Toc34 dissociation from Toc75 and Toc159 (Figure 6A–D). Even further, disassembly of Toc34 does not require GDP loading of both receptors but of Toc159 (Figure 6A, B). This supports the notion of a direct interaction between Toc34 and Toc159 (Smith *et al*, 2002), that Toc159 is a docking partner for recruiting Toc34 and that Toc159 acts as a GTP-dependent motor during translocation across a membrane (Schleiff *et al*, 2003a). A conformational change upon GTP hydrolysis is proposed in order to fulfil the pushing mechanism. Therefore, GDP-loaded Toc159 might provide an unfavourable conformation for Toc34 interaction. An enhanced interaction of two GTPases in their GTP-loaded stage is already described for SRP54 and SRα of the co-translational import pathway in the endoplasmatic reticulum (Keenan *et al*, 2001). Moreover, the nucleoside triphosphate-induced association of the receptors is further strengthened by the presence of the transit peptide of pSSU, suggesting that a GMP-PNP-loaded Toc complex is open for preprotein uptake. However, the observed association of Toc159 and Toc34 in the presence of GMP-PNP and the dissociation in the presence of GDP is not consistent with the previously proposed GDP-enhanced interaction between the G-domains of atToc33 and atToc159 (Smith *et al*, 2002). There, *in vitro* translated atToc33 was used, not excluding the presence of soluble factors influencing the interaction between the two receptors. In our experiments, the presence of chaperones can be precluded because the purity of expressed Toc34ΔTM and of $Toc159_f$ was controlled (data not shown). In addition, the interaction of the G-domains only of both proteins was investigated (Smith *et al*, 2002), whereas here $Toc159_f$ was used, comprising the G- and M-domain. An effect of the M-domain on the G-domain activity cannot be excluded.

Based on our results, we propose a model of pSSU recognition and translocation across the Toc complex (Figure 8). The phosphorylated C-terminal part of the pSSU presequence is first recognised by $Toc34_{GTP}$ (Schleiff *et al*, 2002; Figure 8, stage 2). Subsequently, by recognition of the N-terminal part of the pSSU presequence by Toc159, a trimeric complex is formed, consisting of Toc159, Toc34 and the preprotein (Figure 8, stages 3 and 4). The interaction of the preprotein with Toc34 stimulates GTPase activity of the receptor, leading to a dissociation of Toc34 from the preprotein (Jelic *et al*, 2002; Figure 8, stages 3 and 4). After dephosphorylation by an unidentified phosphatase, the C-terminal part induces GTP hydrolysis of Toc159 (Figure 8, stage 5), by which the receptor pushes the preprotein through the translocation channel (Figure 8, stage 6; Schleiff *et al*, 2003a). Upon GDP to GTP exchange, the Toc complex is transferred to its GTP-loaded state and it is prepared for the next round of preprotein uptake (Figure 8, stage 1). According to the presented data, the pSSU transit peptide provides both information for binding and for activation of Toc159 and Toc34.

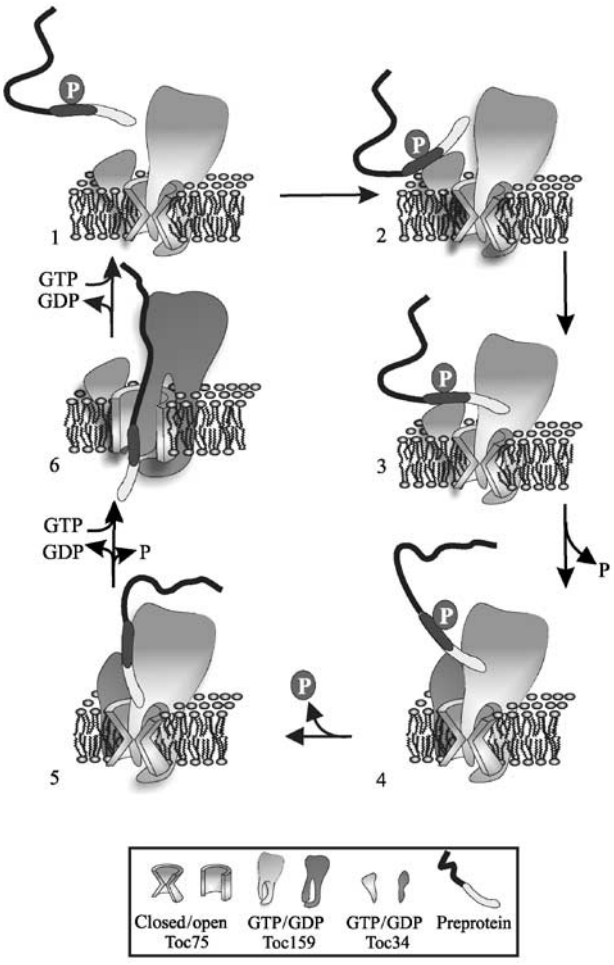

Figure 8 Model of preprotein recognition and translocation by the Toc complex.

Materials and methods

General procedures

The peptides A1, B2 and E2 were synthesised at the Department of Peptide and Protein Chemistry, Charite (Berlin, Germany). The used antisera were raised as described (Schleiff *et al*, 2003b). Lipids for reconstitution were supplied by Nutfield Nurseries (Surrey, UK), Ni-NTA^{2+} column material by Quiagen (Hilden, Germany), AfTresyl Toyopearl 650M by TosoHaas (Tokyo, Japan), *n*-decyl-maltoside by Glycon GmbH (Luckenwalde, Germany) and nucleotides by Roche Molecular Biochemicals (Mannheim, Germany). Protein concentrations were determined using Bio-Rad Laboratories protein assay (Hercules, USA). The expression and purification of Toc34 and Toc75 were conducted as described (Sveshnikova *et al*, 2000; Jelic *et al*, 2002). The Toc complex and Toc159 were isolated as in Schleiff *et al* (2003b). Soluble leaf extract was isolated following the published protocol (Hiltbrunner *et al*, 2001b), with the exception that a 3×10^5 or 6×10^5 *g* centrifugation was performed alternatively as the final step. Lipid extraction of the soluble fractions was earlier described (Schleiff *et al*, 2003b). For electron microscopy, OEVs were incubated with primary and secondary 15 nm gold-labelled

antibodies as described (Stahl *et al*, 1999). For analysis, cold particles were counted and data processed using Sigma Plot.

Trypsin digestion and extraction of isolated outer envelope vesicles

Isolated OEVs (120 μg protein) were pelleted (256 000 *g*, 10 min, 4°C) and resuspended in 20 mM Hepes/KOH pH 7.6, 120 mM NaCl, 0.5 mM $CaCl_2$, 1 mM β-mercaptoethanol. After incubation of the mixture with trypsin (0.05 μg/μl final) for 15 min at 20°C, the reaction was stopped by addition of 0.1 μg/μl trypsin inhibitor and PMSF (2 mM final). Subsequently, membranes were extracted by incubation with Na_2CO_3 (100 mM final) for 20 min on ice, followed by recovery of the membranes (256 000 *g*, 10 min, 4°C). After resuspension in 200 μl buffer, the trypsin treatment was performed as described above. At indicated time points, 50 μl aliquots were removed and the proteolytic reaction was stopped by protease inhibitors.

Coimmunoprecipitation

OEVs were solubilised by incubation with 1.5% *n*-decylmaltoside, 25 mM Hepes/KOH pH 7.6, 150 mM NaCl for 5 min at 20°C. Unsolved particles were removed (10^5 *g*, 10 min, 4°C) and the supernatant was diluted 10 times in IP-buffer (25 mM Hepes/KOH pH 7.6, 150 mM NaCl, 0.2% *n*-decylmaltoside) including 0.05% egg albumin. After the addition of 15 μl antiserum to the mixture, the sample was incubated for 1 h at 20°C, followed by incubation with 50 μl in IP-buffer pre-equilibrated protein-A sepharose (Amersham Bioscience, Freiburg, Germany) for 1 h at 20°C. After washing with IP buffer, the bound protein was eluted by cooking in SDS–PAGE loading buffer. The eluted fractions were separated by SDS–PAGE and subsequently transferred to a nitrocellulose membrane and immunodecorated with the indicated antisera. The coimmunoprecipitation of soluble cell extract fractions was conducted as outlined above.

Affinity chromatography

For binding analysis, recombinant Toc34 was coupled via a C-terminal His-tag on Ni^{2+}-NTA material to a final concentration of 0.1 mg/ml column resin. The concentration of bound pSSU on Toyopearl material was 1 mg/ml. The peptides of pSSU presequence were coupled to thiol-activated sepharose to 0.6 mg/ml resin. Each assay was performed with 20 μl of the coated material, which was pre-equilibrated with binding buffer (20 mM Tricine (pH 7.6); 50 mM NaCl, 0.5% Triton X-100, 0.1% BSA, 0.1 mM DTT, 1 mM $MgCl_2$) before incubation with 250 ng $Toc159_f$ for 30 min at RT. Peptides of the pSSU presequence and nucleotides were added to 5 μM and 0.5 mM final concentrations, respectively, and incubated with Toc159 before addition to the affinity matrix. After washing the column with binding buffer, the bound protein was eluted either by 250 mM imidazol in binding buffer (for Ni^{2+}-NTA) or with 8 M urea (for toyopearl or thiol-activated sepharose material). The eluted samples were subjected to SDS–PAGE analysis and immunoblotting.

Renaturation assay

The renaturation assay was performed as described (Fulgosi and Soll, 2002). Nonphosphorylated ^{35}S-labelled pSSU was synthesised by *in vitro* transcription and translation (Schleiff *et al*, 2003a). ^{32}P-labelled pSSU was yielded by incubation of 10 μg preprotein with wheat germ kinase fraction and ^{32}P-ATP (Jelic *et al*, 2002). Binding of the phosphorylated and nonphosphorylated pSSU was performed in coupling buffer (20 mM Tricine/KOH (pH 7.6), 15 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT) in the presence or absence of 0.5 mM GMP-PNP at 20°C for 2 h. After washing with the coupling buffer, bound preprotein was visualised by autoradiography.

GTP hydrolysis and import of pSSU in chloroplasts and into proteoliposomes

GTP hydrolysis of isolated Toc34, Toc159 and Toc complex was measured as described (Jelic *et al*, 2002). Import into isolated chloroplasts was performed as in Schleiff *et al* (2002). The synthesis of the liposomes, the reconstitution of the Toc components and the binding/import assay were performed as described (Schleiff *et al*, 2003a). For import competition analysis, 5 μM of pSSU presequence peptides were added to the reaction mix as indicated.

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