The function of

Toc34

and its regulation

Inaugural-Dissertation der Fakultät für Biologie

der Ludwig-Maximilians-Univerität München

vorgelegt von

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München

2003

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Tag der Mündliche Prüfung: 16. Oktober, 2003.

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ABBREVIATIONS:

APC…γ subunit of the chloroplast ATP synthetase;

CAO…chlorophyll a oxygenase;

FNR…Ferredoxin:$NADP^+$ Oxidoreductase;

GAP…GTPase activating protein;

GEF…GTP/GDP exchange factor;

HCF…high chlorophyll fluorescence phenotype protein 136;

mSSU… mature form of SSU;

p(re)APC/CAO/FNR/HCF/SSU…preprotein form of APC/CAO/FNR/HCF/SSU;

p(re)OE23/33… preprotein form of 23/33kDa subunit of the oxygen evolving complex;

SSU…small subunit of ribulose 1,5 biphosphate carboxylase-oxygenase (rubisco);

Tic/Toc…Translocon of the outer/inner envelope of chloroplast;

Toc33/34ΔTM…cytosolic domain of the 33/34kDa subunit of the Toc complex.

GMP-PNP... 5'-guanylylimidodiphosphate

KWGF... protein kinase containing fraction from wheat germ

BN-PAGE... blue native-polyacrylamide gel electrophoresis

TFA...trifluoroacetic acid

TLC-Plate...thin layer chromatography plates

PEI-cellulose plates...Poly(ethyleneimine)-cellulose plates

NTA agarose...Nitrilotriacetic acid agarose

1. ABSTRACT

Toc34 is a small receptor and a GTPase localised on the outer envelope of chloroplast. It is a constitutional protein of the Toc complex. This work describes some regulatory mechanisms of Toc34.

Pea Toc34 can be phosphorylated on the Ser113. The phosphorylation of the receptor switches off its ability to bind GTP, which further then excludes the binding of the preproteins to the Toc34. Based on the structural and functional features represents Toc34 a member of the Ras/Rho super family of the small GTPases, which consist of five GTP binding domains. As typical for these class of proteins, the point mutations in the G5 region change the specificity of Toc34 for GTP to XTP hydrolysis.

Furthermore, Toc34 has a low intrinsic hydrolysis rate which is typical for GAP stimulated GTPases. For the first time evidence is presented that preproteins cover the function of a GAP for the Toc34.

Further, Toc34 is a component of the large Toc translocase, which exhibits GTP hydrolysis. The GTP hydrolysis of the Toc complex was also found to be stimulated by preproteins. Furthermore, a detailed analysis of the interaction of the components of the Toc machinery with precursor proteins revealed that Toc34 is the initial receptor prior to translocation, since Toc34 recognises phosphorylated and non-phosphorylated preproteins, while the Toc159 does not recognise phosphorylated preproteins but clearly interact with non-phosphorylated preproteins.

In *A. thaliana*, two isoforms of this receptor were identified. In here, both isoforms were compared in order to understand their presence.

Firstly, both proteins are differentially regulated receptors in the manner of a phosphorylation, since atToc33 is phosphorylated where as atToc34 is not. The phosphorylation site of the atToc33 is on Ser181 a position, which influences the flexibility of the receptor. The amino acids composition of the phosphorylation site shares high similarity with the psToc34 phosphorylation site. As find for pea isoform, phosphorylation inhibits binding to preSSU or GTP.

Secondly, both *A.thaliana* homologues are GTPases with similar features for binding and hydrolysis of a GTP. However, the GTP hydrolysis of the two receptors is stimulated by different preproteins. This result is supported by the identification of differential affinities of

both receptors for different preproteins. *In silico* analysis of preprotein presequences exhibit differences in the amino acid content and length between preproteins better recognised by Toc33 or Toc34.

2. ZUSAMMENFASSUNG

Toc34 ist eine GTPase der äußeren Hüllmembran der Chloroplasten. Es ist als kleiner Rezeptor konstitutiver Bestandteil des Toc Komplexes. Diese Arbeit beschreibt regulatorische Mechanismen von Toc34.

Serin 113 von Toc34 aus Erbse kann phosphoryliert werden. Die Phosphorylierung führt zu einer Inhibierung der GTP Bindung, was wiederum die Anbindung von Vorstufenprotein verhindert. Basierend auf strukturellen und funktionellen Merkmalen läßt sich Toc34 zu der Ras/Rho Superfamilie kleiner GTPasen zurechnen, die durch den Besitz von fünf GTP bindenden Domänen gekennzeichnet sind. Typisch für diese Klasse von Proteinen ist, dass auch für Toc34 eine Punktmutation in der G5-Region zu einer Änderung der Spezifität von GTP- zu XTP-Hydrolyse führt.

Des weiteren weist Toc34 eine geringe intrinsische Hydrolyserate auf, die charakteristisch für GAP-stimulierte GTPasen ist. In dieser Arbeit wurde zum ersten Mal gezeigt, daß die GAP-Funktion von Toc34 durch das Vorstufenprotein übernommen wird.

Toc34 ist eine Komponente der GTP hydrolysierenden Toc-Translokase. Die GTP-Hydrolyserate des Toc-Komplexes wird ebenfalls durch das Vorstufenprotein stimuliert. Weiter zeigt eine detaillierte Analyse der Wechselwirkungen der Komponenten der Toc-Maschinerie, dass Toc 34 als initialer Rezeptor fungiert. So erkennt Toc34 phosphoryliertes Vorstufenprotein, während Toc159 nur mit nicht phosphoryliertem Vorstufenprotein interagiert.

In *A. thaliana* wurde zwei Isoformen des Rezeptors gefunden. Um die Existenz zweier Isoformen zu verstehen, wurden beide Proteine verglichen.

Zunächst werden beide Rezeptoren unterschiedlich reguliert. So wird atToc33 phosphoryliert, während für atToc34 keine Phosphorylierung gefunden werden konnte. Die Phosphorylierung von atToc33 erfolgt an Serin 181, was die Flexibilität des Rezeptors beeinflußt. Das Phosphorylierungsmotif von atToc33 weist große Ähnlichkeit zu dem von Toc34 aus Erbse auf. Wie für Toc34 aus Erbse demonstriert, inhibiert eine Phosphorylierung von atToc33 GTP- und preSSU-Anbindung.

Weiter zeigen beide Arabidopsis-Homologe ähnliche Merkmale in Bezug auf GTP-Bindung und –Hydrolyse auf. Davon abgesehen, wird die jeweiligen GTP-Hydrolyse von verschiedenen Vorstufenproteinen stimuliert. Diese Beobachtung wird weiter durch die Identifizierung verschiedener Affinitäten der beiden Rezeptoren für unterschiedliche

Vorstufenproteine unterstützt. So zeigen *in silico*-Analysen Unterschiede in der Länge und der Aminosäurenzusammensetzung zwischen den Vorstufenproteinen, die von Toc33 ode Toc34 erkannt werden.

3. INTRODUCTION

Chloroplasts are organelles developed from the photosythetic bacteria, which underwent an endosymbiosis with eukaryotic cell. During evolution the majority of the chloroplast genome, approx. 90%, was transferred to the nucleus. That event brought to the cell a new challenge: How can nucleus encoded and cytosolic synthesized proteins be efficiently transported to and into the chloroplasts?

The primary signal for the chloroplast localization is a N-terminal targeting presequence (Blobel 1979). The N-terminal sequence can be phosphorylated by a cytosolic kinase (Waegemann and Soll 1996) (Figure 1A). This phosphorylation is a signal for recruiting a cytosolic 14-3-3, which together with a cytosolic Hsp70 and probably some other so far not identified proteins, forms the guidance complex (May and Soll 2000). Preproteins with a non-phosphorylated presequence are expected to interact only with chaperones protecting them from aggregation. However, this pathway for chloroplast targeting is not yet understood. In general, the process of the cytosolic targeting remains still to be investigated in order to draw a clear picture of this event (Figure 1A).

Import of preproteins across the double envelope membranes of chloroplasts proceeds through the Toc complex of the outer membrane and prolongs through Tic complex (**t**ranslocon of **o**uter/**i**nner membrane of **c**hloroplasts) of the inner membrane. The suffix to the names of the translocon components stand for the molecular weight of the corresponding proteins in Kilo dalton (Schnell *et al.*, 1997). The central Toc complex consists of the two GTP receptors, Toc159 and Toc34, and the putative protein conducting channel forming Toc75 (Kessler *et al.*, 1994; Schnell *et al.*, 1994, Schleiff *et al.*, 2003a) (Figure 1A, B). Toc64 is the latest identified component of the Toc complex and is proposed to function as a docking site for preproteins accompanied by the guidance complex (Sohrt and Soll 2000). Two chaperons are involved in the translocation of precursor across the outer envelope membrane, Com70 on the cytosolic

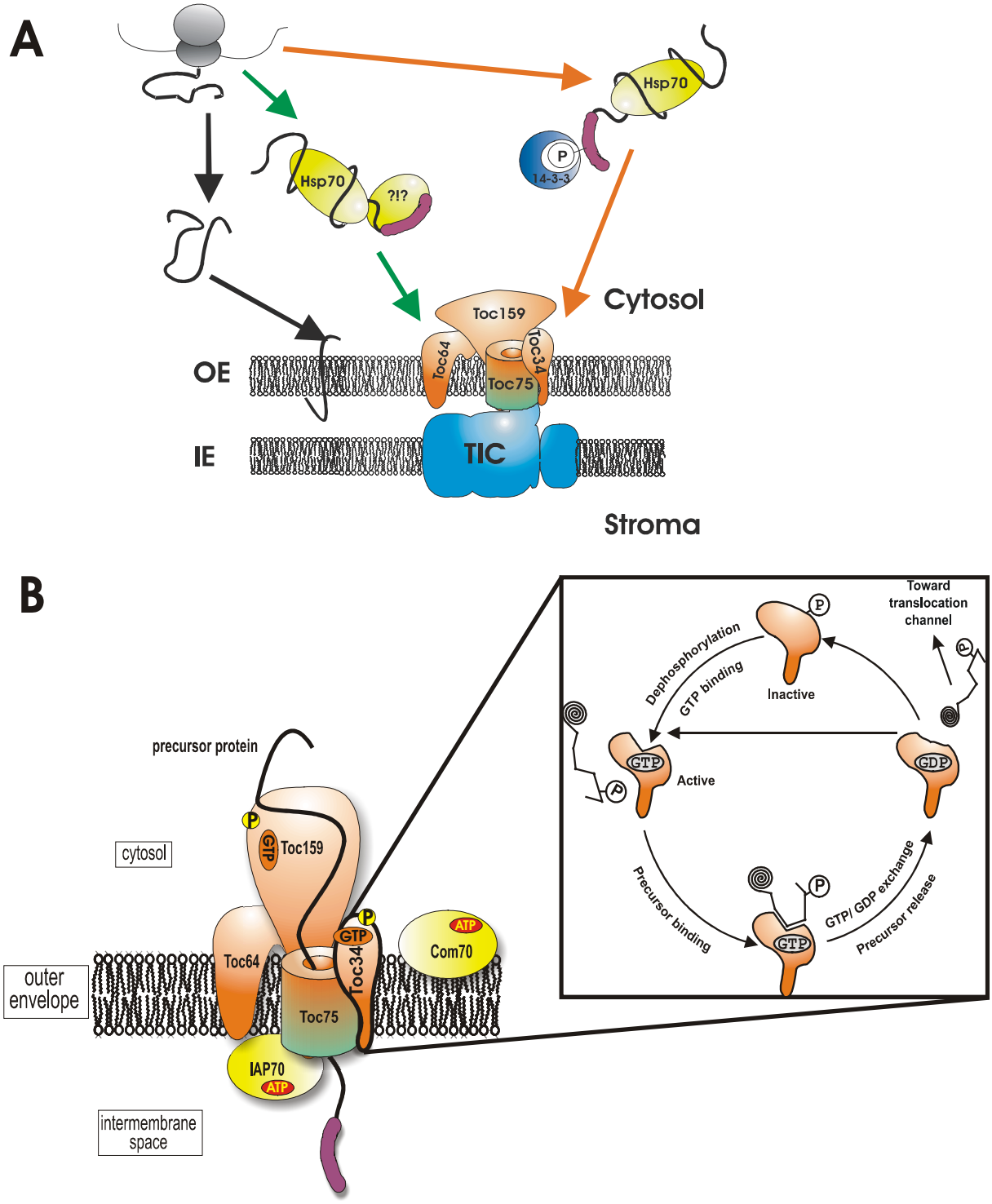

Figure 1. Cytosolic targeting and Toc complex regulation.
A) Nuclear encoded chloroplastic targeted preproteins are synthesized on the cytoplamic ribosomes. Freshly synthesized preprotein can go to the chloroplast via three distinct paths. Proteins which are self inserted don't possesses targeting signal (black arrows), proteins which contains a targeting signal but are not phosphorylated forming complexes with cytosolic Hsp70 and still not known components (green arrows) and proteins with a phosphorylated presequence forming a guidance complex with cytoplasmic Hsp70 and 14-3-3 proteins (orange arrows). The last two types of preproteins are imported into the chloroplasts over Toc complex (high lighted orange). B) Toc complex is regulated by phosphorylation and GTP binding. The Toc34 (bolded and framed) regulation is in the four steps. Interplay between GTP binding and hydrolysis and phosphorylation is the switch between the active and inactive state of receptor (Sveshnikova *et al.* 2000a).

side (Kourtz and Ko 1997) and a Hsp70 facing the intermembrane space (Marshall *et al.*, 1990; Waegemann and Soll, 1991; Schnell *et al.*, 1994) (Figure 1B). The translocation catalyzed by the Toc complex is a highly regulated and fine tuned occasion, as will be explained later on using the example of Toc34, which is the best understood component of Toc machinery (Figure 1B).

The translocation process proceeds further using the Tic complex(es), which is composed of six known components: Tic110, Tic20, Tic22, Tic55, Tic62 and Tic40. However, different cross-linking results, together with BN-PAGE suggest that two species of the Tic complex might exist (Kuranov *et al.* 1997, Caliebe *et al.* 1997, Kuchler *et al.* 2002). Both contain the identified translocation channel Tic110. One complex is formed by Tic110, Tic22 and Tic20, and the other by Tic110, Tic40, Tic55 and Tic62. The latter Tic complex is redox regulated by the action of Tic55 and Tic62 (Kuchler *et al.* 2002), but most characteristics of the Tic proteins are unknown.

The two molecular chaperones of the outer envelope of chloroplasts Com70 (Kourtz and Ko 1997) on the cytosolic side and Hsp70 facing the intermembrane space (Marshall *et al.* 1990, Waegemann and Soll 1991.; Schnell *et al.*, 1994) are engaged in translocation of preproteins across the outer envelope (Figure 1B).

The Com70 is sensitive to thermolysin treatment indicating exposure to the cytosol (Ko K and Ko ZW. 1992; Kourtz and Ko 1997). Hints for an involvement in protein import come from cross-links to preprotein at the early stage of import (Wu *et al.*, 1994; Kourtz and Ko 1997).

Another Hsp70 was found to be insensitive to high salt and alkali extraction and thermolysin treatment in isolated chloroplasts. However, after solubilization of the membranes by Triton X-100 the protein became susceptible to thermolysin indicating an orientation to the intermembrane space (Marshall *et al.*, 1990) (Figure 1B).

Toc64 is the newest described component of the Toc complex (Sohrt and Soll 2000) (Figure 1B). This protein is associated with the central components Toc34, Toc75 and Toc159. The

protein sequence is functionally divided into two modules. The N-terminal part exhibits homology to amidases. The C-terminal part has three tetra-trico peptide repeats (TPR). TPR repeats have been described in different protein translocation machineries. The mitochondrial import receptor Tom70 has seven TPR motifs interacting with precursor proteins that requires the mitochondrial import stimulating factor (MSF) a protein of the 14-3-3 family (Komiya *et al.*, 1997, Alam *et al.*, 1994). As discussed above the cytosolic guidance complex for chloroplast targeting is also thought to contain a 14-3-3. This leads to the proposal that Toc64 may present a docking site for the cytosolic guidance complex (Short and Soll 2000). In *Arabidopsis* three genes coding for atToc64 were found (Jackson-Constan and Keegstra 2001.).

The integral membrane protein Toc159 was found to be crosslinked to pSSU at the stage of energy independent binding of the preprotein (Hirsch *et al.*, 1994; Kessler *et al.*, 1994)(Figure 1B). Furthermore, fab fragments of Toc159 antiserum specifically inhibit the import event supporting the involvement in protein translocation (Hirsch *et al.*, 1994). Cross links of the 52kDa C-terminal domain to pSSU in all three import stages lead to the proposal that Toc159 is not only involved in protein recognition, but also in further translocation events (Kouranov and Schnell., 1997). However, only a C-terminal degradation product of Toc159 was analysed in these reports called Toc86 due to the high protease sensitivity of this protein (Bölter *et al.*, 1998).

In general, Toc159 can be divided in the three functional parts. First the N-terminal 600 amino acids long A-domain, rich in acidic amino acid with a calculated pI ~3.6, second the middle G-domain including 450 amino acids with the GTP binding motif and third the M-domain which forms a C- terminal membrane anchor of 450 amino acids (Chen *et al.*, 2000) (Figure 2). Bölter *et al.* (1998) had shown that chloroplasts with proteolytically degraded Toc159 lacking the N-terminal A domain, had a two times reduced import efficiency.

Toc159 provides two other so far unsolved properties. Firstly, the role of the GTP binding first shown by Kessler *et al.* (1994) remains to be elusive. GTP binding and hydrolysis are known to regulate many other biochemical processes involved in the complex and fine regulation of the cell metabolism and development, for example in the targeting and translocation process of proteins of the endoplasmic reticulum, in vesicular transport and cell division. GTP hydrolysis is also often a regulatory trigger initiating events. Therefore, the GTPases of the Toc complex are believed to represent such trigger for protein translocation across the chloroplast membrane.

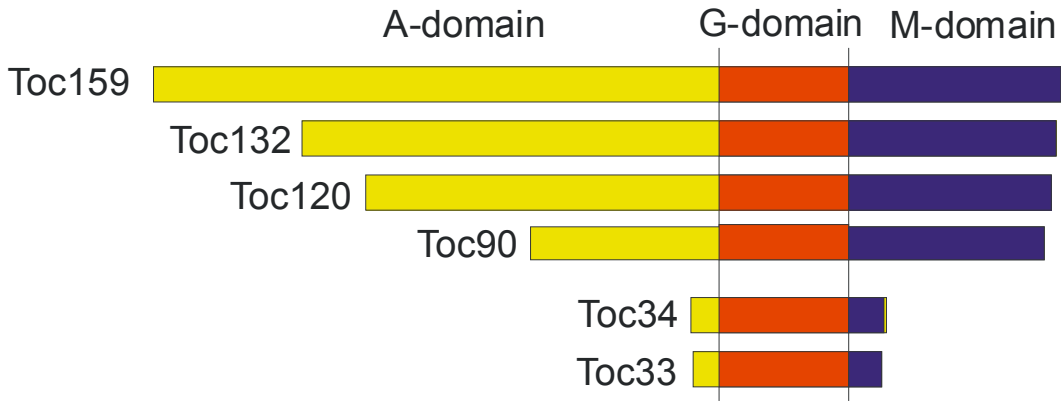

Figure 2. Domain structure of the Toc-GTPases.
All Toc-GTPases contain three different regions, a so-called A-domain, which is highly negatively charged, a G-domain containing the GTPase region and a M-domain containing the membrane anchor.

Import studies performed with thermolysin treated chloroplasts harbouring the 52 kDa membrane fragment of Toc159 without the A- and G-domain and the intact Toc34 and Toc75 show reduced efficiencies leading to the conclusion that GTP binding is necessary for the recognition of preproteins as is the case for Toc34 (Chen *et al.*, 2000; Svesnikova *et al.*, 2000a). Secondly, Toc159 was found to be phosphorylated by an outer envelope kinase (Fulgosi and Soll, 2001) (Figure 1B). The function of the phosphorylation remains to be established. Similarly to GTP binding and hydrolysis, phosphorylation of proteins is a way of regulation of a number of biochemical processes in the cell. It can be an effector or inhibitor of enzymes and might therefore be involved in fine tuning the translocation event.

The importance of Toc159 for import was presented *in vivo* studies in an *Arabidopsis thaliana* knock-out of Toc159 (ppi2, Bauer *et al.*, 2000). The plants show malformations, they develop with pale phenotype and are lethal at seedling stage. At the organelle level, ppi2 chloroplasts have prolamelar bodies similar to etioplast lacking the thylakoids and starch granules of mature chloroplasts. However, ppi2 plants show retardation of plastid growth and development in the non-photosynthetic roots and guard cells (Yu and Li 2001). These findings implicate a possible role of Toc159 in biogenesis of chloroplasts. *Arabidopsis* has further three homologues of Toc159: Toc120, Toc132 (Bauer *et al.*, 2000) and Toc90 (Hiltbrunner *et al.*, 2001). The roles of Toc120 and Toc132 are probably similar to Toc159 but remain to be investigated (Bauer *et al.*, 2000).

Recent studies claimed that a soluble form of Toc159 interacts with preproteins and Toc33. Subsequently, a model of partition of Toc159 between a soluble cytosolic fraction and the chloroplast outer envelope membrane was suggested (Hiltbrunner *et al.* 2001). This idea has to be confirmed by further investigations.

Toc75 was identified as a Toc component by the stable interaction with precursor proteins and by a close association with Toc159 and Toc34 in the presence or absence of preproteins (Waegemann and Soll 1991; Schnell *et al.* 1994; Perry and Keegstra *et al.* 1994) (Figure 1A, B). Furthermore, Toc75 antiserum inhibits protein import (Tranel and Keegstra, 1995). Toc75 is deeply embedded into the membrane with a barrel structure (Svesnikova *et al.* 2000b). Electrophysiological measurements with over-expressed Toc75 reconstituted into proteoliposomes supports this hypothesis. Toc75 was described as a voltage-gated, cation selective channel (Hinnah *et al.*, 1997, Hinnah *et al.*, 2002), which is in the interaction with preproteins (Hinnah *et al.*, 1997). In line with these observations a less strong association with precursor in the early binding step was demonstrated in comparison to the more pronounced interaction in the early import intermediate stage (Kouranov and Schnell 1997). Toc75 is the only known Toc component expressed with a N-terminal transit peptide of bipartite structure

using the general import pathway indicated by the competition of the pSSU translocation (Tranel and Keegstra, 1996; Inoue *et al.*, 2001).

Another receptor of the Toc complex is Toc34, a 310 amino acid long GTPase with the N-terminal 266 amino acids forming the GTP binding domain, which projects into the cytosol. Thermolysin treatment of chloroplasts leads to a degradation product of 8 kDa including the transmembrane alpha-helix and a small portion facing the intermembrane space (Seedorf *et al.*, 1995). Toc34 was co-purified with trapped precursor protein by a linear sucrose gradient and was found in close association with Toc75 (Seedorf *et al.*, 1995). Further, stable complexes with Toc159 and Toc75 in the presence or absence of precursor protein were identified by cross-linking studies and co-sedimentation (Waegemann and Soll 1991; Schnell *et al.*, 1994; Kouranov and Schnell 1997.; Nielsen *et al.*, 1997) (Figure 1A, B).

Svesnikova and co-workers (2000a) presented a mechanistic model, which states that GTP binding increases the affinity of Toc34 to precursors (Figure 1B(framed)). Thereby phosphorylated precursor proteins are bound with a five to ten fold higher affinity than non phosphorylated ones (Schleiff *et al.*, 2002a). Phosphorylation inhibits GTP binding to Toc34 and therefore interferes with transfer of precursor proteins to the translocation pore (Svesnikova *et al.*, 2000b) (Figure 1B(framed)). Such non-translocated precursor proteins might be bound by a cytosolic factor similar to that present in wheat germ lysate, which was found to inhibit protein translocation *in vitro* (Schleiff *et al.*, 2002b).

In *Arabidopsis thaliana* two isoforms of Toc34 were found called atToc33 and atToc34 (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000). Jarvis and co-workers (1998) isolated and characterized the first null mutant of a Toc component ppi1, which possesses a T-DNA insertion into the Toc33 gene. Ppi1 plants show a pale phenotype during the first two weeks due to the reduced chlorophyll content and a lower import rate of tested photosynthetic precursor proteins. However, older leaves of mature plants regain the appearance of wild type plants. A possible explanation for the accommodation of mutant plants in later stages of development is that

Toc34 may compensate for the Toc33 loss of function. Gutensohn *et al.*, (2000) supported the described phenotype by analysis of plants with an antisense repression of Toc33. Furthermore, the authors analyzed an antisense repression plant of Toc34 showing a less strong phenotype. Additionally, atToc33 was found to be an abundant protein in the tissues at developmental stages and of photosynthetically active leaves. In contrast, Toc34 has a constant but low expression during the whole lifetime, especially in peripheral tissues (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000).

Aim of this work was to show the mechanisms of Toc34 regulation in more detail, to explain an interplay between Toc34 phosphorylation and GTP binding as well as the consequences of the Toc34-preprotein interactions and GTP hydrolysis. In addition the features of two Toc34 homologues in *A. thaliana,* atToc33 and atToc34 were investigated and compared. Finally, an experimental approach was performed to address the question of which receptor is the initial receptor of the Toc complex.

4. MATERIALS AND METHODS

4.1 General

[γ^{32}P]-ATP (3000 Ci/mmol) and [α^{32}P]-GTP (3000 Ci/mmol) were purchased from Amersham Pharmacia Biotec, (Freiburg, Germany). All other chemical used were obtained from Roth (Karlsruhe, Germany) or Sigma (Munich, Germany). Standard procedures like the purification of outer envelopes of chloroplasts or expression are described elsewhere (Schleiff *et al.*, 2003a; Sveshnikova *et al.*, 2000a). A protein kinase containing fraction (KWGF) was partially purified from wheat germ as described earlier (Waegemann and Soll, 1996).

The bacterial strains BL21-DE3 and NovaBlue (DE3) were obtained from Stratagene Madison, USA), the vectors pET21d and pET23d from Novagen (La Jolla, USA). Standard procedures like the purification of outer envelopes of chloroplasts or SDS-Page are described elsewhere (Seedorf *et al.* 1995).

4.2 Generation and over expression of the preproteins and atToc33ΔTM, atToc34ΔTM and site-directed mutants.

Point mutations were introduced by standard polymerase chain reaction using the cDNA encoding for atToc34 and atToc33 as template. The DNA was then cloned into pET21d. Point mutations were confirmed by sequencing and the constructs were expressed and purified as described previously (Sveshnikova *et al.* 2000a). The cDNA encoding preHCF, preAPC, preFNR and preCAO were amplified by PCR and cloned into pET21d. Cloning and point mutations were confirmed by sequencing. Proteins were over expressed and purified as described previously using Talon chelated chromatography (Sveshnikova *et al.* 2000a). Inclusion bodies were solubilized in 25mM Hepes pH7.6, 8 M urea, 50 mM DTT. After

purification Toc33/34ΔTM and mutants were dialyzed into the buffer used for the experiments.

4.3 Introduction of point mutations into Toc34 and protein expression

Point mutations were introduced by standard polymerase chain reaction using the previously described Toc34ΔTM construct (Sveshnikova *et al.*, 2000a) as template. Point mutations were confirmed by sequencing and constructs expressed and purified as previously described (Sveshnikova *et al.*, 2000a). After purification Toc34ΔTM and mutants were dialysed into the buffer used for the experiments.

4.4 Immobilisation of Toc33 onto GTP or preSSU affinity matrix

0.1µM of purified and phosphorylated Toc33ΔTM in buffer A (20 mM Tricine, pH7.6, 50 mM NaCl, 1 mM $MgCl_2$, 1mM DTT) were incubated with GTP or GDP agarose (1mM immobilized nucleotide final) for 10 min at room temperature. After incubation the agarose was washed three times with 50 volumes of buffer A and remaining Toc33ΔTM was eluted by adding SDS-sample buffer and boiling at 95ºC for 3 min. The specificity of the binding was controlled by addition of equal amounts of albumin, which did not bind to the column under the conditions used.

PreSSU and mSSU were coupled to Toyopearl (TosoHaas Biosep, Germany) by a method described by Schleiff *et al.* 2002b. The phosphorylated Toc33 protein was incubated for 5 min in buffer A, with the prepared affinity matrix in the presence of 1 mM GMP-PNP. The affinity matrix was washed three times with 50 volumes of buffer A. The remaining sample was eluted by addition of SDS-sample buffer. All fractions were precipitated and subjected to SDS-PAGE analysis and immunoblotting.

4.5 GTP hydrolysis assay

Indicated amounts of atToc34ΔTM or atToc33ΔTM were incubated in 10µl containing 20 mM Tricine/KOH, pH7.6, 1 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT with [α^{32}P]-GTP or 1 mM XTP. Nucleotides were separated on PEI-cellulose plates (Merck, Darmstadt, Germany) using 600 mM NaH_2PO_4 at pH 3.4 as running buffer. The hydrolysis of GTP or XTP were analysed by the Michaelis-Menten equation:

$v_o = V_{max}[GTP]/ ([GTP] + K_m)$ (equation 1)

where v_o is the initial hydrolysis rate at a given GTP concentration, V_{max} the maximal rate of hydrolysis and K_m the Michaelis-Menten constant. The catalytic constant k_{cat} was calculated by:

$k_{cat} = V_{max} / [Enzyme]_T$. (equation 2)

4.6 Treatment of outer envelopes with non-hydrolysable analogue of GTP

Outer envelopes were incubated for 10 min with 1 mM of GTP-γ-S in 100 µl of buffer A followed by centrifugation. The envelope was resuspended in buffer A without NTP, again centrifuged and resuspended in phosphorylation buffer.

4.7 Phosphorylation of Toc34

Toc34ΔTM was incubated for 10 min with KWGF and 10 nM or 50 nM [γ^{32}P]-ATP (3000 Ci/mmol) or 10 µM ATP in 20 mM Tricine / KOH, pH7.6, 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$ at room temperature. Purified outer envelopes in 20 mM Tricine / KOH, pH7.6, 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$ were incubated for 10 min at room temperature in 50 µl final volume with 10 nM [γ^{32}P]-ATP. Proteins were subjected to SDS-PAGE without further treatment and phosphorylation was visualized by autoradiography.

4.8 Phospho-amino acid analysis of Toc34

Phospho-amino acid analysis was performed as earlier described (Soll *et al.*, 1988). In brief: phosphorylated Toc34ΔTM was hydrolyzed by 6N HCl in presence of unlabeled phosphor-amino acids (phosphor-tyrosine, phosphor-threonine, phosphor-serine, 20 µg each). The solvent was evaporated and the residual resuspended in water. The solution was spotted onto a precoated Silica thin-layer plate (Merck, Kieselgel 60) and electrophoresed using glacial acetic acid/formic acid/H_2O (78/25/897). The phospho-amino acids were located by ninhydrin staining and radioactivity was detected by autoradiography.

4.9 Isolation of phospho-peptides of Toc34

Phosphorylated Toc34 (over expressed or wtToc34 from outer envelopes) was cut out from the gel by electro-elution using an Electro-eluter (C.B.S., Scientific company, U.S.A) following the guidelines of the manufacturer. The eluted solution was precipitated by trichloracetic acid and the pellet resuspended in 50mM NH_4KCO_3. Then 20 µg of resuspended Toc34 was digested using 10 µg trypsin or chymotrypsin at 37ºC. After 180 min a further 10 µg of enzyme was added and digestion continued under the same conditions for 180 min. The protein was then lyophilised and resuspended in 0.1% TFA. Digestion products were separated on a reverse-phase column ultrasphere ODS 5µ (Beckmann) using a 60ml gradient from 15% acetonitril/ 0.1% TFA to 55% acetonitril/ 0.1% TFA, a flow rate of 1ml/min and a fraction size of 0.5 ml. Fractions were then lyophilised for further treatment.

4.10 Visualisation and quantification of the hydrolysis or phosphorylation

Phosphorylation or GTP binding and hydrolysis of Toc34ΔTM or outer envelope Toc34 by radioactive labelled nucleotides was visualised or quantified by different methods. For visualisation of radioactive probes, the gel or the TLC-plate was exposed to a X-OMAT-LS

film (Kodak, Rochester, USA) or to a Phospho-Image plate (Fuji-Film, Tokyo, Japan). XTP hydrolysis was visualised by ultra violet light exposure (254 nm) and picturing using a digital camera Coolpix 880 (Nikon, Tokyo, Japan). Data were transferred to Adobe Photoshop 5.0 LE. For quantification, the dried SDS-PAGE gel-slices were dissolved in 30% H_2O_2 and 60% $HClO_4$ for 16 h at 60ºC followed by scintillation counting. Alternatively, the Phospho-Image plate was scanned using a Phospho-Image Reader FLA 5000 (Fuji-Film, Tokyo, Japan) and quantified using Aida-Image Analyser (Raytest Isotopenmessgeräte GmbH, Staubenhard, Germany). Data were presented by using Sigma Plot 5.0 (SPSS Inc., Chicago, USA).

4.11 Activation of IAsys CMD-cuvette, Toc34 and Toc33 coupling and binding experiments

The activation and coupling procedure was described in detail (Schleiff *et al.* 2002a). In brief: each chamber of the Carboxylmethyl-Dextrane coated cuvette was incubated with 70 µl HBST (10 mM Hepes/KOH pH 7.4, 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20) until a stable baseline was observed. Then 100 µl of a fresh mixture containing 100 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 29 mM N-hydroxysuccinimide (EDC/NHS) was added. After 15 min EDC/NHS was added a second time for 15 min. The buffer was then replaced by 33µl of 100 mM NTA (Qiagen, Hilden, Germany) and cuvettes were incubated for 10min. Both chambers were washed three times with HBST and then incubated for at least 1 min in the same buffer. Additional coupling sites were blocked for 2 min by addition of 100 µl of 1M ethanolamine, pH 8.5. Nickel was loaded by incubation with 50 mM Nickel sulfate in 10 mM Hepes/KOH pH 8.0 for 5 min. This step was repeated three times followed by three wash steps with HBST. Both chambers were washed twice with 90 µl PGIW buffer (50 mM sodium phosphate pH 6.8, 10% glycerol, 300mM imidazole, 300 mM NaCl) followed by a 3 min incubation in the same buffer.

Prior to use, His-tagged proteins were dialysed for 16 h against 10 mM sodium phosphate pH 8, 50 mM NaCl, 5% glycerol, 0.7 mM β-mercaptoethanol and 0.01% Triton. Proteins were diluted to a final concentration of 0.1 µg/µl with HBST, and 100 µl were incubated in each chamber until equilibrium was reached. Both chambers were washed with HBST and incubated until equilibrium was reached again. This procedure was repeated to yield between 0.5 and 15 nM (in 100 µl reaction volume) coupled protein. Finally both chambers were incubated with HBST until experiments were performed.

For binding experiments, both chambers of the Ni-NTA cuvette were filled with 90 µl of the binding buffer (20 mM Hepes/KOH, pH 8.0, 50 mM NaCl, 0.05% Triton X100, 2.5% glycerol and 0.01% fatty acid free BSA) and allowed to equilibrate. A new baseline was established and binding was initiated by injection of 1-10 µl of the indicated amounts of proteins into their respective chambers as described in the figure legends. When binding was performed in the presence of GTP 0.5 mM $MgCl_2$ was added. Dissociation was performed in 90 µl of the same buffer (except GTP). For surface regeneration between experiments both chambers were extensively washed with 20 mM Hepes/KOH, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 2.5% glycerol, 0.01% BSA.

In some cases (Toc34ΔTM and for some experiments preSSU and preOE33) his-tagged proteins were used as ligand. The use of His-tagged ligands was possible for two reasons: firstly the association of preSSU containing and not containing his-tag with immobilized Toc34 was found to be identical (not shown). Secondly, for all measurements a His-tagged ligand was used and self-immobilization could not be observed as controlled in the parallel cuvette (not shown).

4.12 Analysis and quantification of IAsys Biosensor binding curves.

Data files from IAsys plus were further analyzed using Sigma Plot 2000 (SPSS Inc.). Association was analyzed by non-linear regression as previously described in detail (Schleiff

et al. 2002a), except for the binding of preproteins in the presence of GTP. This association was at least three-phasic.

The process should be described as outlined in Figure 13A. However, this type of reaction can only be numerically processed. Furthermore, complexes between the Toc proteins and GTP or GDP cannot be distinguished. Therefore, the analytically describable system has to be limited by describing the first fast association reaction (Figure 13B, step 1), the initial dissociation (Figure 13B, step 2) and reaching the equilibrium (Figure 13B, step 3). The process can not be described by a simple equilibriums reaction as outlined in Figure 13A, since using this type of scheme the increase after the initial decrease cannot be described. The resolution of changes is limited by that technique used so that differences between Toc/GTP or Toc/GDP can not be observed. Therefore, the signal (S; here response in arcsec) is only raised by the initial reaction and by the equilibrium.

4.13 Materials for isolation of the Toc complex

Plant lipids were obtained from Nutfield Nurseries (Surrey, UK), n-decyl-β-maltoside from Glycon Bioch. GmbH (Germany), octylglucoside and nucleotides from Roche (Germany). Protein concentration was determined using the Biorad Protein assay (BioRad, Germany).

4.14 Isolation of the Toc components and of the Toc complex

Toc75 containing a hexa-histidine tag was expressed and purified as previously described (Sveshnikova *et al.* 2000b). *E. coli* harbouring the pET21d-full-length-Toc34-6xHis expression vector was grown at 37ºC in 2YT media containing 50mg/l ampicilin and expression induced by addtion with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 3 hours and resuspended to a final concentration of 0.2mg/ml (wet cell pellet) in 100 mM Na-phosphate, 300 mM NaCl, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 10%

glycerol, 20 mM dodecylmaltoside, pH 7.0. After cell lysis non-soluble cell fragments and inclusion bodies were pelleted at 10000xg at 4ºC for 20 min. The pellet was washed and finally resuspended in 100 mM Na-phosphate, 8 M urea, 5mM β-mercaptoethanol, pH 8 and incubated for at least 10 min at room temperature. The suspension was centrifuged at 15000xg at 4ºC for 5 min and supernatant passed over pre-equilibrated Ni-NTA material (Qiagen, Germany). The column was washed with 5 volumes of 100 mM Na-phosphate, 6 M urea, 5mM β-mercaptoethanol, 20mM imidazol, pH 7 and protein eluted using 100 mM Na-phosphate, 6 M urea, 5 mM β-mercaptoethanol, 250 mM imidazol, pH 7. Prior to use, protein was dialysed into the buffer system indicated. To gain the 86 kDa fragment of Toc159 ($Toc159_f$) 300µl purified outer envelope was subjected onto 8% SDS-PAGE and $Toc159_f$ was electro-eluted. SDS was removed by addition of Serdolit PAD I (Serva, Heidelberg, Germany) in the presence of 4 M Urea. Toc complex was isolated as described (Schleiff *et al.* 2003a)

4.15 Western blot renaturation assay

In vitro transcription and translation of ^{35}S labelled pSSU was performed as described previously (Schleiff *et al.* 2003b). 10 µg pSSU was phosphorylated by the wheat germ kinase fraction as described in section 4.7. For the renaturation assay outer envelope vesicles were separated by SDS-PAGE and transferred to the nitrocellulose membrane. Subsequently the washing, blocking and renaturation steps were preformed as described (Fulgosi and Soll 2002).

Preprotein binding was preformed in the binding buffer: 20 mM Tricine/KOH pH 7.6, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT with or without 0.5 mM GMP-PNP at room temperature for 2 h. Membrane was washed 3 times for 10 min each in the binding buffer without preprotein. Results were visualized by autoradiography.

5.16 Statistical analysis of the transit sequences

The expression analysis data using the ppi1 plant from Richly *et al*. (2003) were transferred to SigmaPlot 2001 (SPSS Inc.) and sorted according to the amount of regulation of gene expression. The hundred strongest up-regulated and the hundred strongest down-regulated genes in the ppi1 background in comparison to wild type were selected. To all non-regulated genes random generated numbers were assigned. These numbers were generated using the implemented function “randm” of SigmaPlot 2001. The genes were sorted according to the random number and the fifty genes with the highest and the fifty genes with the lowest number were selected into the pool of non-regulated genes. The transit sequence of all selected genes was determined using ChloroP (http://www.cbs.dtu.dk/services/ChloroP). The size distribution of the selected sequences was determined by least square analysis using a single Gaussian distribution:

$$N = A * e^{-0.5*\left(\frac{L-L_0}{B}\right)^2} \qquad \text{(equation 3)}$$

where N stands for the number of sequences with a specific length, L is the length of the sequence, A is the hightt of the peak, B stands for the broadness of the peak and L_0 is the statistical center of the distribution.

For analysis of the statistical distribution of amino acids within this sequence the amino acids 2 to 31 seen from the N-terminus and the amino acids 1 to 30 seen from the cleavage site were used. The statistical analysis and the visualization were performed using the program GENIO/logo(http://genio.informatik.uni-stuttgart.de/GENIO/logo/logo.cgi).

5. RESULTS

5.1 The GTPase Toc34 from *Pisum sativum* is regulated by phosphorylation and preprotein binding

5.1.1 The phosphorylation site of Toc34

Toc34 can be phosphorylated *in situ* using isolated envelope membranes and *in vivo* as demonstrated by immunoprecipitation of Toc34 from isolated chloroplasts with phospho-serine antibodies (Sveshnikova 2000). To clarify if Toc34 is phosphorylated solely on a serine residue, Toc34ΔTM was phosphorylated using outer envelope membranes in the presence of [γ^{32}P]ATP, re-purified by affinity chromatography, hydrolyzed and phospho-amino acids were determined. Phospho-serine was found as the only labeled amino acid (Figure 3A). To determine the phosphorylation site, Toc34ΔTM was phosphorylated by the kinase present in the outer envelope followed by trypsin or Glu-C peptidase treatment. In addition, Toc34 present in the outer envelope was phosphorylated and proteolytically digested as well. Fragments were subjected to a reverse phase chromatography and fractions were tested for radioactivity. We observed for both proteins an almost identical fragmentation and phosphorylation pattern (not shown). In order to identify the phosphorylation site we used two approaches. The protein was phosphorylated by unlabelled ATP followed by proteolytic cleavage. Peptide fractions were collected and pooled based on radioactive pattern obtained before and subjected to mass spectroscopy. Further, Toc34ΔTM phosphorylated by outer envelope membranes was treated with chymotrypsin and all peptides were subjected to mass spectroscopy without further purification in order to analyze the homogeneity of the phosphorylation. Both approaches resulted in a single phosphorylation site at serine 113.

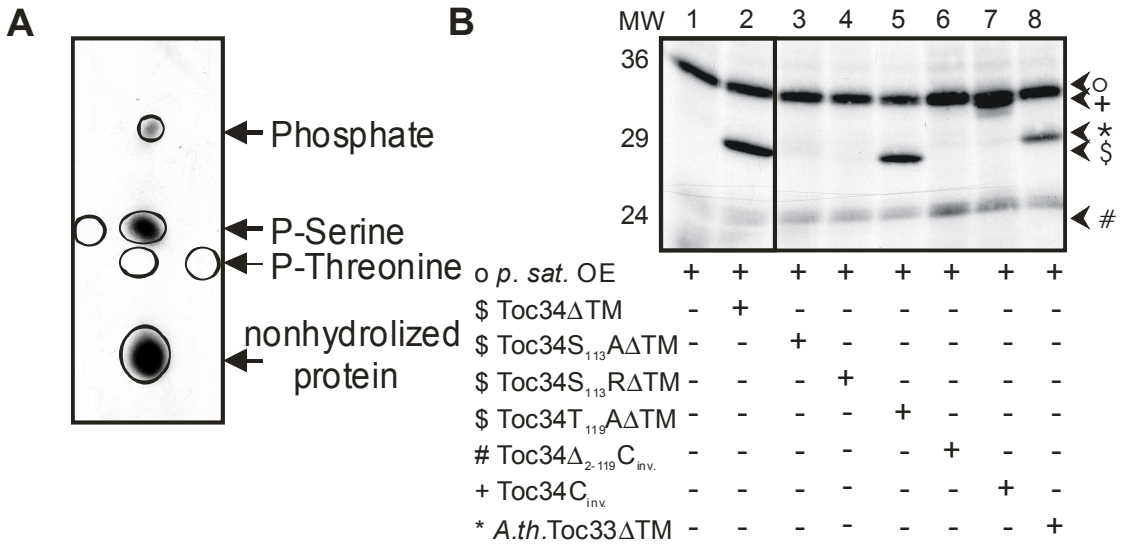

Figure. 3 Analysis of the phosphorylation side of Toc34

A) Toc34 was phosphorylated using [γ^{32}P]ATP by the kinase present in outer envelopes, hydrolyzed and amino acids separated. Ninhydrin stained phospho-amino acid marker phospho-serine (P-serine) and phospho-threonine (P-threonine) are indicated. B) Toc34 and mutants were phosphorylated by outer envelope of *Pisum sativum*. o indicates the endogenous Toc34, $ the position of Toc34ΔTM (lane 2), $Toc34S_{113}A\Delta TM$ (lane 3), $Toc3S_{113}R4\Delta TM$ (lane 4), $Toc34T_{119}A\Delta TM$ (lane 5), # the position of $Toc34\Delta_{2\text{-}119}C_{inv.}$ (lane 6), + the position of Toc34 $C_{inv.}$ (lane 7) and * the position of a.th Toc33ΔTM (lane 8).

To confirm this observation we created several point mutations of Toc34 replacing serine at position 113 by an alanine or by an arginine. In addition, threonine 119 was replaced by an alanine. The phosphorylation of Toc34 and the different mutants revealed that the replacement of serine 113 resulted in the loss of phosphorylation (Figure 3B, lane 2 vs. lane 3 and 4). The replacement of threonine did not result in a reduction of phosphorylation (Figure 3B, lane 2 vs. lane 5). Toc34 containing an inverted C-terminus (May and Soll, 1998) was phosphorylated (Figure 3B, lane 7, slightly smaller than endogenous Toc34). The same protein lacking the amino acids 2-119 was not phosphorylated, corroborating our finding that the phosphorylation site is in this N-proximal region. We always observed low but constant phosphorylation of a polypeptide of about 24 kDa. This polypeptide did not co-immunoprecipitate with Toc34 antibodies (data not shown) and thus does not represent a proteolytic fragment of Toc34. The exact nature of the 24 kDa protein remains to be

determined. However, we conclude that Toc34 from pea is phosphorylated at amino acid position 113.

In order to test if phosphorylation of Toc34 occurs also in other plants, the Toc34 homologue form *Arabidopsis thaliana*, Toc33, was used in the phosphorylation assay. It could be clearly demonstrated that this protein is also phosphorylated (Figure 3B, lane 8). Therefore, phosphorylation of the Toc34/33 receptor proteins seem to be a general regulatory step during chloroplast protein translocation.

5.1.2 Interplay between GTP binding and phosphorylation

Earlier work has established, that about 75% of Toc34 was phosphorylated (Sveshnikova *et al.*, 2000a). This suggests that phosphorylation could occur also in the GDP bound state of Toc34 and not only in the nucleotide free state of Toc34, since phosphorylated Toc34 still binds to GDP though with lower affinity than the non-phosphorylated one. To test this idea we investigated the influence of nucleotide binding of Toc34 on phosphorylation directly.

Toc34ΔTM was preincubated with ATP, GTP, GDP or XTP and subsequently phosphorylated by a protein kinase partially purified from wheat germ extract. A decrease of phosphorylation of Toc34ΔTM was observed in the presence of ATP (Figure 4A, lane 1 and 2) due to the competition between radioactivly labeled ATP and non-labeled ATP, and in the presence of GTP or XTP (Figure 4A, lane 1 and 3, lane 5 and 6), but much less in the presence of GDP (Figure 4A, lane 1 and 4). In comparison, phosphorylation of preSSU, a protein not interacting with nucleotides, was completely inhibited in the presence of ATP (Figure 4A, lane 8), whereas addition of GTP or GDP did not alter the phosphorylation efficiency of the kinase fraction (Figure 4A, lane 7, 9 and 10). We therefore conclude that the triphosphate binding of Toc34 inhibits phosphorylation of the receptor (discussed below). To support this finding we pre-incubated outer envelope membranes with different concentrations of GTP-γ-S or XTP and initiated phosphorylation by addition of radioactivly labeled ATP (Figure 4B). As

before, phosphorylation of Toc34 was significantly reduced when GTP was present. In the presence of 1mM GTP-γ-S only 28% and in the presence of 1mM XTP only 39% of Toc34 was phosphorylated in comparison to the control.

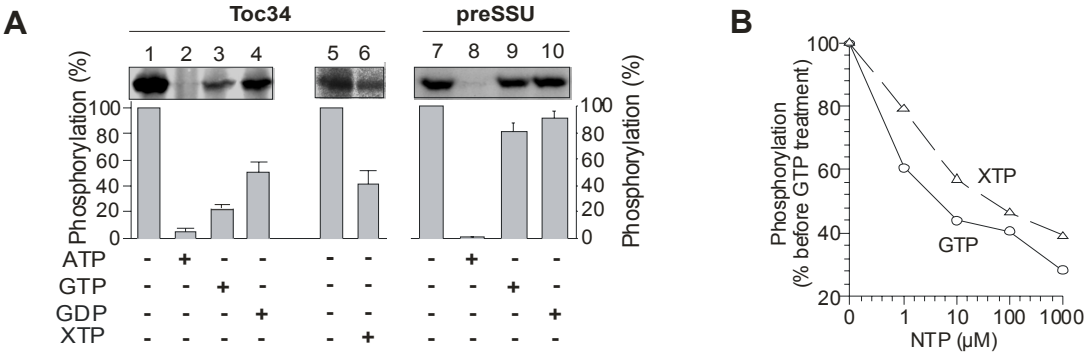

Figure. 4 Phosphorylation inhibits GTP binding and vice versa.
A) Toc34ΔTM (lane 1-6) or preSSU (lane 7-10) were phosphorylated by a kinase containing wheat germ fraction using [γ^{32}P]ATP before (lane 1, 5 and 7) and after preincubation with 1 mM ATP (lane 2 and 8), 1 mM GTP (lane 3 and 9), 1 mM GDP (lane 4 and 10) or 1 mM XTP (lane 6). The phosphorylation was quantified and compared to the phosphorylation in the absence of nucleotides (histogram). B) Outer envelope was incubated with increasing amounts of GTP-γ–S (open circle) or XTP (open triangle) followed by phosphorylation using [γ^{32}P]ATP. The envelope proteins were then separated on SDS-Page and phosphorylation of Toc34 was quantified as described in Materials and Methods. Values are presented as percent of the phosphorylation achieved without GTP or XTP treatment.

5.1.3 Intrinsic regulation of GTP hydrolysis by Toc34

In order to establish GTP hydrolysis by Toc34, an *in vitro* assay using purified Toc34ΔTM was developed. Purified Toc34ΔTM hydrolyses GTP at a slow rate (Figure 5A, Toc34ΔTM, lane 2-5). Even Toc34 binds to XTP (Sveshnikova 2000), we did not observe hydrolysis of XTP (Figure 5A, Toc34ΔTM, lane 7-10). Then we replaced aspartic acid at position 219 by asparagine, a mutation within the conserved nucleotide binding domain which was found to alter the GTP specificity to a XTP specificity (Hwang and Miller, 1987; Schmidt *et al.*, 1996).

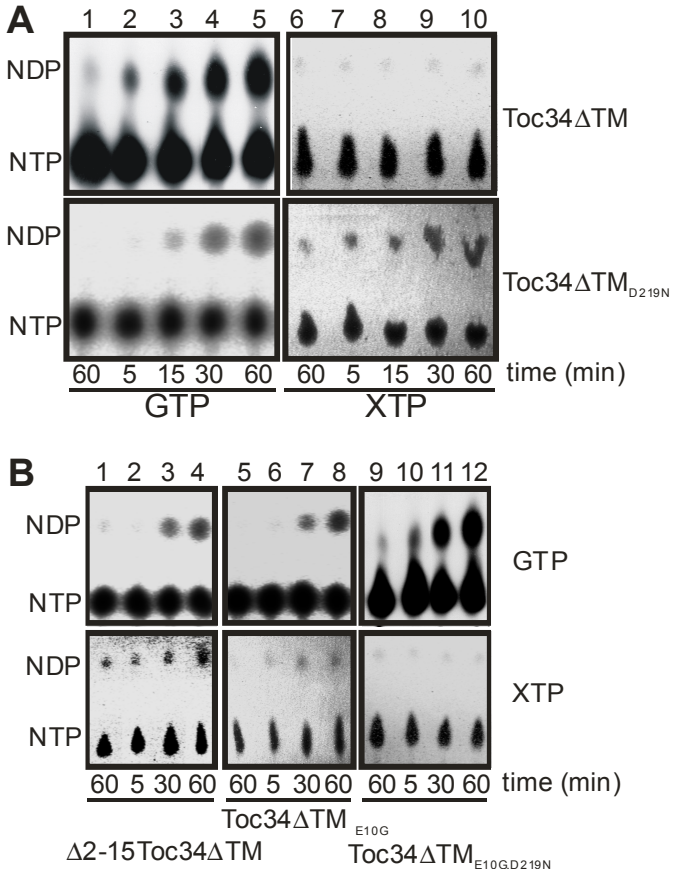

Figure 5. Toc34ΔTM hydrolysis GTP.
A) Toc34ΔTM or Toc34ΔTM$_{D219N}$ (100 ng) was incubated for 5, 15, 30 and 60 min with 1 µCi [α^{32}P]GTP (lane 2, 3, 4 and 5) or 2 mM XTP (lane 7, 8, 9 and 10). Self hydrolysis of GTP (lane 1) or XTP (lane 6) after 60 min under experimental conditions is shown. B) Δ2-15Toc34ΔTM (lane 2, 3 and 4), Toc34ΔTM$_{E10G}$ (lane 6, 7, and 8) or Toc34ΔTM$_{E10G, D219N}$ (lane 10, 11 and 12) (100 ng) were incubated for 5, 30 and 60 min with 1 µCi [α^{32}P]GTP (upper panel) or 2 mM XTP (lower panel). Self hydrolysis of GTP or XTP after 60 min under experimental conditions is shown in lane 1, 5 and 9.

Toc34 bearing this mutation was now able to hydrolyze XTP (Figure 5A, Toc34ΔTM$_{D219N}$, lane 7-10), while GTP hydrolysis was largely suppressed (Figure 5A, Toc34ΔTM$_{D219N}$, lane 2-5). We conclude that Toc34 is a GTPase and that the GTPase activity measured is not due to a protein that co-purifies with Toc34. Beside the classical aspartic acid point mutation, we identified a mutation in a region not present in other small GTPases, but influences the GTPase activity of Toc34. When the first fifteen amino acids of Toc34 were deleted, the specificity of Toc34 for GTP was lost and Δ2-15Toc34ΔTM was able to hydrolyze XTP (Figure 5B, lane 2–4). A similar but not as pronounced effect was observed when glutamic acid at position 10 was replaced by glycine, Toc34ΔTM$_{E10G}$ (Figure 5B, lane 6-8). The XTP

hydrolyzing activity of both mutants was lower than found for Toc34ΔTM$_{D219N}$. The double mutant Toc34ΔTM$_{E10G,D219N}$ was able to hydrolyze GTP but not XTP (Figure 5B, lane 10-12). Therefore, we conclude that Toc34 contains not only the known motifs conserved in small GTPases but also a novel region determining nucleotide selectivity at its extreme N-terminus.

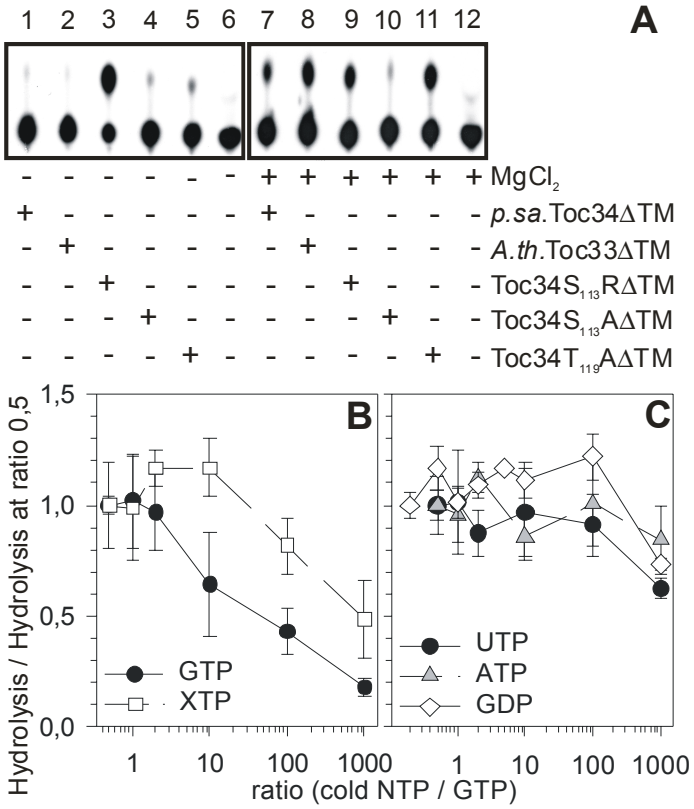

Figure 6. Properties of the GTP hydrolyses by Toc34ΔTM.
A) 100 ng of p.sa. Toc34ΔTM (lane 1 and 7), A.th. Toc33ΔTM (lane 2 and 8), Toc34S$_{113}$RΔTM (lane 3 and 9), Toc34S$_{113}$AΔTM (lane 4 and 10) and Toc34T$_{119}$AΔTM (lane 5 and 11) were incubated with 35 nM [α^{32}P]GTP in the absence (lane 1-6) or presence of $MgCl_2$ (lane 7-12) for 30 min and hydrolysis is shown. B,C) Hydrolysis of 35 nM [α^{32}P]GTP by 0,1 µg Toc34ΔTM was determined after 30 min in the presence of increasing amounts of XTP (B, open square), GTP (B, closed circle), GDP (C, open diamond), UTP (C, closed circle) and ATP (C, gray triangle). The amount of hydrolyzed NTP was compared to the hydrolysis in the presence of 0.1 fold competitor.

To further characterize the GTP hydrolysis, mutants of Toc34 were tested which contained mutations at the phosphorylation site. Interestingly, the serine to alanine replacement also resulted in a loss of hydrolysis (Figure 6A, lane 4 and 10), whereas the serine to arginine exchange resulted in a magnesium insensitivity (Figure 6A, lane 3 and 9). The threonine

replacement at position 119 had no influence on GTP hydrolysis (Figure 6A, lane 5 and 11). We conclude that the status of the phosphorylation site influences the hydrolysis activity of Toc34. Furthermore, the homologue from *Arabidopsis thaliana* atToc34 hydrolyzed GTP in a magnesium dependent manner (Figure 6A, lane 2 and 8) underlining that this polypeptide acts in a similar manner.

In order to determine the nucleotide specificity of Toc34 GTPase activity, hydrolysis assays were carried out in the absence or presence of XTP, UTP, ATP or GDP. In line with the observation made by Sveshnikova (2000), only XTP was able to compete for GTP binding and subsequently for hydrolysis by Toc34ΔTM (Figure 6B). Sveshnikova (2000) made the observation that ATP and GDP could replace GTP only at high concentrations, the hydrolysis of GTP by Toc34ΔTM could not be competed for by addition of ATP or GDP (Figure 6C). Only at a thousand fold excess of ATP or GDP a slight reduction of GTP hydrolysis was observed (Figure 5C). However, this reduction was also observed for UTP, which was not found to release Toc34ΔTM from the GTP or GDP affinity matrix. This is consistent with the hypothesis, that Toc34 recognizes the triphosphate with higher affinity than the diphosphate of guanosine, as suggested before. It further strengthens the idea, that Toc34 cannot only recognize the guanosine ring but also the xantosine ring to some extent.

5.1.4 Analysis of the GTPase activity of Toc34

A detailed analysis of the hydrolysis of GTP by Toc34ΔTM (Figure 7A) revealed a very slow maximal hydrolysis rate of 150 nM*min^{-1}. However, this rate is comparable to the intrinsic GTP hydrolysis rate of other small GTPases (for example see Downward *et al.*, 1990; Zhang *et al.*, 1997; Albert *et al.*, 1999). The catalytic constant of 0.02 min^{-1} is also comparable to the intrinsic catalytic constant found for other GTPases (for example see (Ruetthard *et al.*, 2001)). The analysis further revealed a Michaelis-Menten constant of 20 µM (Figure 7A, inset). We

conclude that Toc34 can act as a GTPase *in vivo*, but that its rate of hydrolysis is most likely stimulated by proteinaceous effectors.

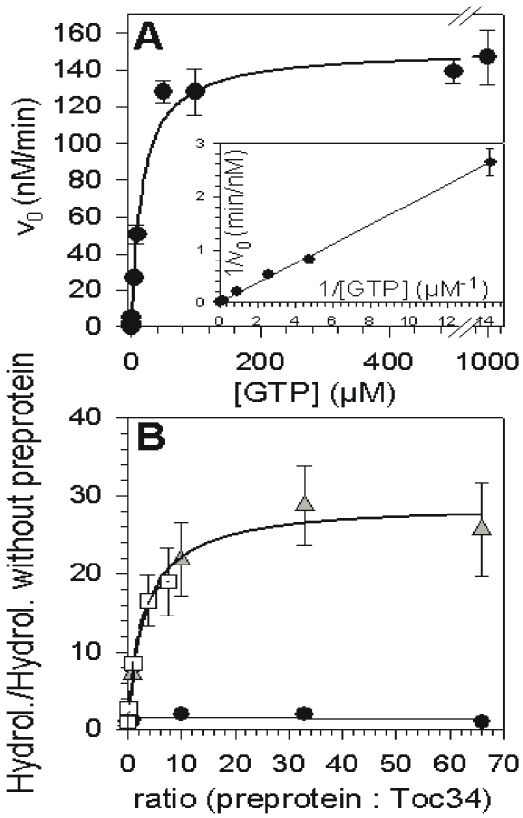

Figure 7. GTPase activity of Toc34ΔTM is stimulated by preproteins.
A) The Michaelis-Menten Kinetic of the hydrolysis was determined using 5 µg of protein. For that, the initial rate constant v_o of the hydrolysis of different amounts of GTP was determined. The insert shows the inverted plot of the Michaelis-Menten Kinetic for K_M determination. B) Toc34ΔTM (100 ng) was incubated for 30 min with [α^{32}P]-GTP in the presence of increasing amounts of mSSU (closed circle), preSSU (open square) and preOE33 ($_{gray}$ triangle). The GTP hydrolysis was compared to the initial hydrolysis of Toc34ΔTM in the absence of a preprotein. For all, each data point reflects the average of at least 4 independent experiments.

Therefore, we tested whether such stimulation can be observed in the presence of precursor protein. Indeed, preSSU and preOE33 but not the mature form of SSU clearly stimulated hydrolysis of GTP up to 28 fold (Figure 7B). The half maximum of stimulation was reached at a 2 fold molar excess of the preprotein over Toc34 again documenting the high affinity of Toc34ΔTM for the preprotein in the presence of GTP. Therefore, association of Toc34 with

the preprotein is one regulatory mechanism of the GTPase activity of Toc34 and its receptor function.

To investigate which part of the preprotein is responsible for the GTPase stimulation of GTP hydrolysis by Toc34, we used chemically synthesized peptides of the pSSU presequence: E1 contains N-terminal 20 amino acids, A1 assembles the C-terminal 20 amino acids and B1 is the A1 peptide which is phosphorylated on Ser34.

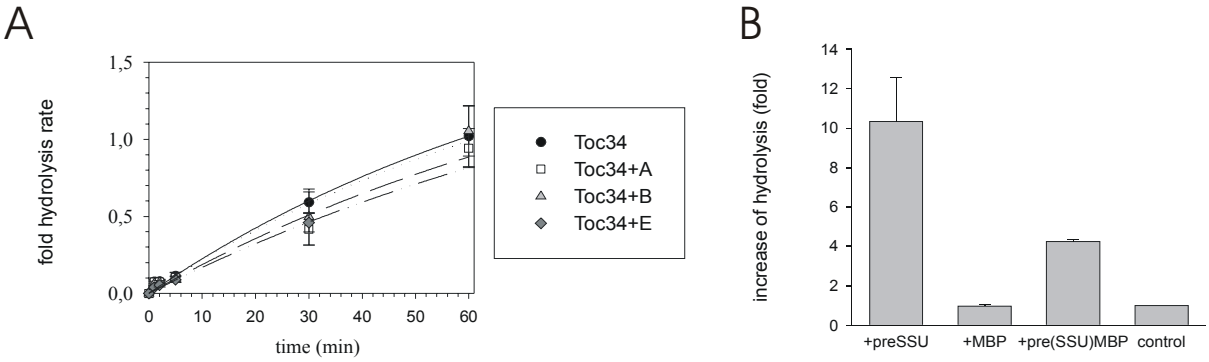

Figure 8. Stimulation of the Toc34 GTP hydrolysis by a prepeptides of the pSSU.
A) 0.1µg of Toc34 in the molar ratio 1:10 to the prepeptides are incubated for the indicated time. B) 0.1µg of Toc34 in the molar ratio 1:10 with pSSU, (pre)pSSU-maltose binding protein and MBP, are incubated with 1 µCi α^{32}PGTP for 60 minutes. Hydrolysis is visualised on PhosphoImager and quantfied by AIDA software. Control=1 (Toc34 alone).

However, all used prepeptides didn't show a stimulation of a GTP hydrolysis as shown in Figure 8A. This unexpected result leads to the question, whether the complete presequence or even further, complete precursor protein is required for the initiation of GTP hydrolysis by Toc34.

To answer this question we produced a chimeric clone where the presequence of preSSU is fused to the maltose binding protein (MBP). The MBP was used because it does not interact with Toc34 (not shown). Using this chimeric protein and MBP without presequence attached, stimulation of hydrolysis was only observed in the presence of presequence (Figure 8B). It clearly demonstrates that the presequence alone is sufficient to stimulate a GTPase activity of the Toc34.

5.2 The two Toc34 isoforms of *A.thaliana* are differentionally regulated and recognise different substrates

5.2.1 atToc33, but not atToc34 is regulated by phosphorylation

The Toc34 homologue proteins belong to a Ras/Rho like GTPase family (Section 5.1). However, in order to establish GTPase function of the homologous proteins of *A.thaliana* an *in vitro* assay using purified atToc34ΔTM and atToc33ΔTM was established. Both purified proteins hydrolyze GTP at a slow rate (Figure 9A, 4,5) in a magnesium dependent manner (Figure 9A, lane 2,3). Therefore, similar features as identified for the *P.sativum* homologue were expected for both proteins. One of these features of psToc34 is its regulation by phosphorylation (Sveshnikova *et al.* 2000a, Section 5.1). In order to reveal whether this regulation is common for all Toc34 homologues or only limited to a certain subclass, the phosphorylation of atToc34 and atToc33 was investigated. To our surprise, only Toc33ΔTM but not Toc34ΔTM (Figure 9B, lane 2, 3) was found to be phosphorylated by a kinase present in the outer envelope of chloroplast of *A.thaliana*. The same was observed using outer envelope membranes from *P.sativum* (Figure 9B, lane 5,6) or a kinase purified from wheat germ (data not shown). This result suggests, that Toc33 and Toc34 are differentially regulated. To further investigate whether phosphorylation causes the same regulation as found for the homologue in *P.sativum*, Toc33ΔTM from *A.thaliana* was phosphorylated using the outer envelope from chloroplasts of *A.thaliana* and incubated with GDP or GTP agarose affinity matrix (Figure 9C).

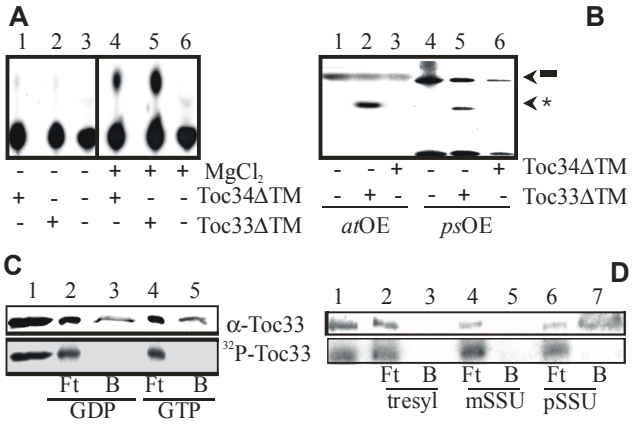

Figure 9. The receptor function of the GTPase atToc33 but not atToc34 is regulated by phosphorylation.

(A) Hydrolysis of [α-^{32}P] GTP by 1µg heterologously expressed atToc34ΔTM (lane 2, 4) and atToc33ΔTM (lane 3, 5) was determined in the absence (lane 1-3) or presence (lane 4-6) of 1mM $MgCl_2$. (B) Expressed atToc33ΔTM (lane 2, 5) and atToc34ΔTM (lane 3, 6) were incubated with outer envelope membranes from *A.thaliana* (lane 1-3) or *P.sativum* (lane 4-6) in the presence of [γ-^{32}P] ATP. The position of the endogenous psToc34 is indicated (-). (C) AtToc33ΔTM was incubated with outer envelope membranes *A.thaliana* in the presence of [γ-^{32}P]ATP. The phosphorylated protein (lane 1) was then incubated with GDP (lane 2,3) or GTP (lane 4,5) coated affinity matrix. The flow through (lane 2,4) and the bound protein (lane 3,5) were collected, subjected to SDS-Page and immunodecorated with Toc33 antibodies. The Western blot (upper panel) and the autoradiogram (lower panel) are shown. (D) Phosphorylated *at*Toc33ΔTM (lane 1) was incubated with tresyl affinity matrix (lane 2,3) coated with mSSU (lane 4,5) or preSSU (lane 6,7). The flow through (lane 2,4,6) and the bound protein (lane 3,5,7) were collected, subjected to SDS-Page and immunodecorated with Toc33 antibodies. The Western blot (upper panel) and the autoradiogram (lower panel).

Only the non-phosphorylated subpopulation of Toc33 was able to interact with both, GTP and GDP as determined by antiserum decoration (Figure 9C, lane 3, 5, upper panel) and autoradiography (Figure 9C, lane 3,5, lower panel). Therefore, phosphorylation seems to inhibit nucleoside phosphate recognition. Subsequently the interaction of Toc33 to the small subunit of ribulose 1,5 biphosphate carboxylase-oxygenase (rubisco; SSU) was investigated using the mature (mSSU, Figure 9D, lane 4,5) and precursor form (preSSU, Figure 9D, lane 6,7) coupled to Toyopearl material (Figure9D, lane2,3). We observed a specific interaction of Toc34 with the precursor form of the protein (Figure 9D, compare lane 5 and 7, upper panel), however, only the non-phosphorylated form of the receptor recognized the preprotein (Figure 9D, lower panel). We conclude that phosphorylation of Toc33 inhibits its GTP and preprotein binding capacity.

5.2.2 The phosphorylation site of atToc33

The phosphorylation site of psToc34 is located in the switch one region of the GTPase domain at serine 113 (Section 5.1). Interestingly, the alignment between psToc34 and atToc33 revealed that at the same position a glycine is present in the sequence of atToc33. However, we could confirm that atToc33 is phosphorylated at a serine residue like its homologue in *P.sativum* (Figure 10A). To identify the phospho-amino acid position, the phosphorylated protein was proteolytically digested using the protease V8. When the proteolytic fragments where subjected to SDS-PAGE, two small peptides of 8 kDa and 14 kDa could be visualised (Figure 10B, lane 1). The same sample was probed for the presence of the hexa-histidine tag present at the C-terminus of the expressed protein to identify the larger non-phosphorylated and the smaller phosphorylated fragment (Figure 10B, lane 2). To our surprise, the smallest fragment identified to contain the hexa-histidine tag (14 kDa) was the major phosphorylated fragment (Figure 10B, lane 3). We therefore concluded that phosphorylation must occur in the C-terminal half of the receptor. The first putative serine within this C-terminal 14 kDa was at position 164. Since this serine is conserved in Toc34 from *P.sativum*, we did not expect this serine to be phosphorylated in atToc33. In contrast, serine 170, 175, 181, 190 and 200 were found to be unique in Toc33. While analysing the sequence we identified a similar motif as in *P.sativum* containing the phosphorylated serine (Figure 10C). Even though serine 181 was therefore the most likely candidate, we exchanged all of the unique serines into alanines. The proteins harbouring the point mutations were phosphorylated (Figure 10D) using outer envelope of chloroplasts of *A.thaliana* (Figure 10D, lower panel) and *P.sativum* (Figure 10D upper panel). The only protein not phosphorylated was atToc33ΔTMS$_{181}$A (Figure 10D, lane 4) being in line with the identified phosphorylation motif (Figure 10C).

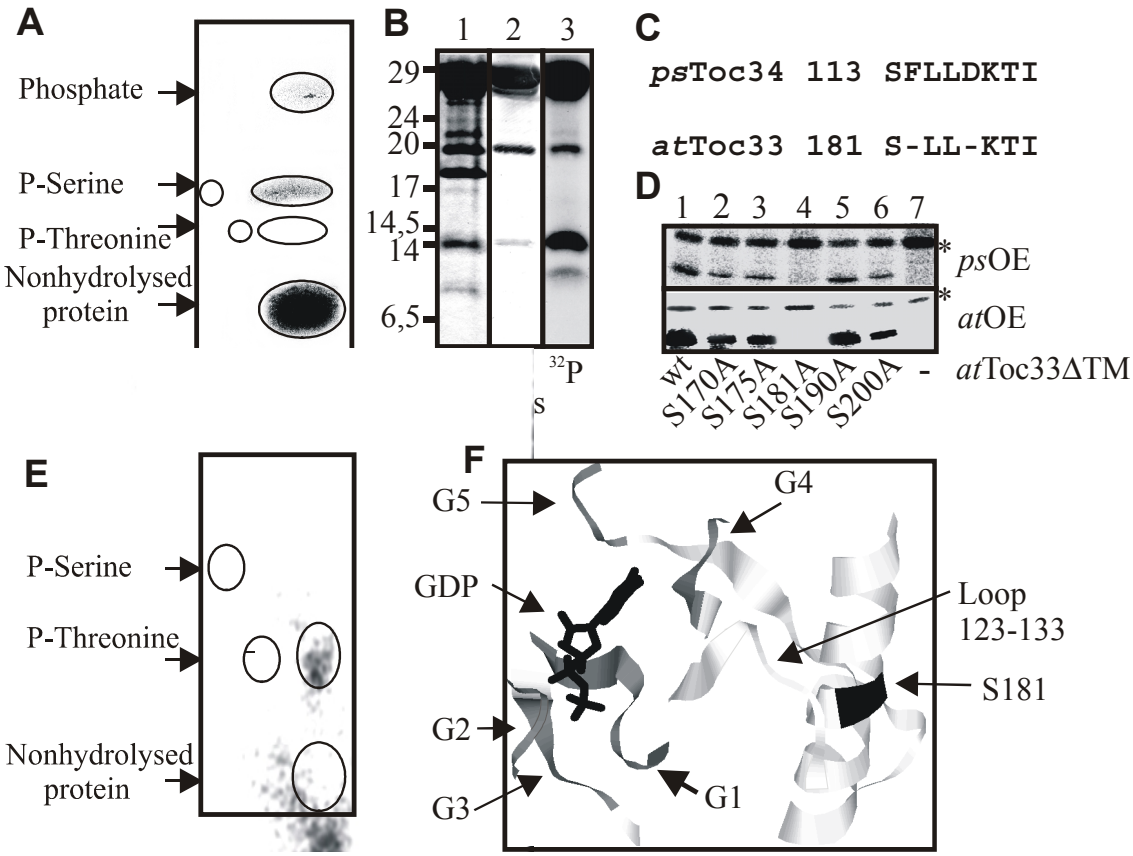

Figure 10. AtToc33 is phosphorylated on serine 181.
(A) Heterologously expressed atToc33ΔTM was phosphorylated by outer envelopes of *A.thaliana* in the presence of [γ-^{32}P]ATP. The protein was repurified, hydrolyzed and subjected to PEI cellulose. Positions of the non-labeled phosphoserine and phosphothreonine standards are indicated. (B) Phosphorylated atToc33ΔTM was digested using V8 and fragments were subjected to SDS-page followed by silver staining (lane 1), immunodecoration using anti-hexa-histidine antibodies (lane 2) or autoradiography (lane 3). (C) A conserved phosphorylation motif is found in *P.sativum* Toc34 and *A.thaliana* Toc33. (D) AtToc33ΔTM (lane 1) harboring a serine to alanine exchange on position 170 (lane 2), 175 (lane 3) 181 (lane 4) 190 (lane 5) and 200 (lane 6) was phosphorylated by outer envelopes of *P.sativum* (upper panel) or *A.thaliana* (lower panel) in the presence of [γ-^{32}P]ATP. Endogenous *ps*Toc34 and *at*Toc33 is indicated (*). (E) AtToc33ΔTM/S181T was phosphorylated by outer envelopes of *A.thaliana* in the presence of [γ-^{32}P]ATP. The protein was repurified, hydrolyzed and subjected to PEI cellulose. Positions of the non-labeled phosphoserine and phosphothreonine standards are indicated. (F) A model of the GTP binding domains and the location of the phosphorylation site is shown.

To further confirm that this is the only site phosphorylated in Toc33, we replaced serine 181 by threonine, since this amino acid should also be recognised by a serine/threonine kinase. Phosphorylation of Toc33ΔTMS181T was initiated by addition of outer envelope of chloroplasts of *A.thaliana*. We observed that this protein is now phosphorylated at a threonine residue (Figure 10E) confirming that atToc33 is phosphorylated at position 181.

5.2.3 GTP binding and hydrolysis properties of atToc34 and atToc33

Both Toc34 homologues of *A.thaliana* are able to hydrolyze GTP (Figure 9A). To further investigate the features of GTP binding, we utilized an IAsys Biosensor. This biosensor uses a dual chamber cuvette and a resonance mirror technique to monitor macromolecular interactions (Schleiff *et al.* 2002a). His-tagged atToc34ΔTM or atToc33ΔTM was immobilized to a NTA-Ni^{2+} modified carboxymethylated dextran coated cuvette as described in Materials and Methods. Then, 1mM of GTP or GDP was added to the cuvette in order to determine the kinetics of the nucleotide binding to the receptor proteins (Figure 11A). No drastic differences between Toc34 and Toc33 could be observed (Figure 11A, Table 1). For both proteins, GTP bound much more rapidly and to a higher extent than GDP (Figure 11A). The association rate (k_{on}) of GTP and GDP differs by a factor larger than three.

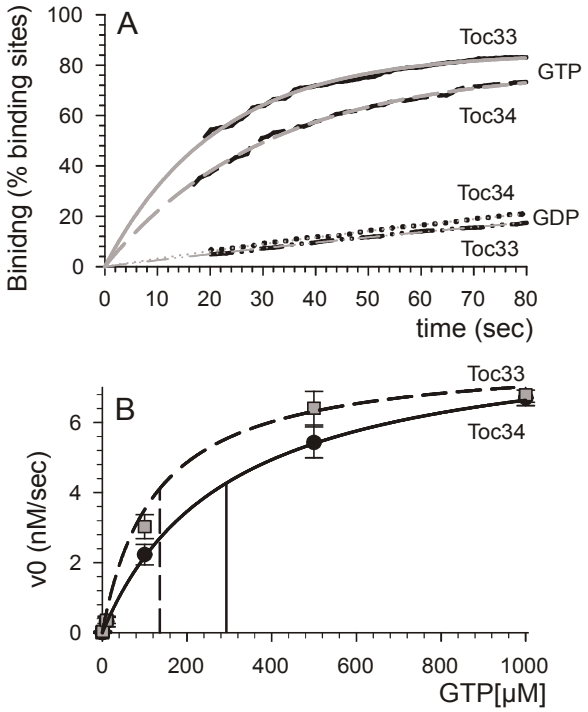

Figure 11. GTP binding and hydrolysis properties of *at*Toc33 and *at*Toc34.
(A) *at*Toc34ΔTM (dashed & dotted line) and *at*Toc33ΔTM (solid and dashed-dotted line) were coupled to an IAsys cuvette to a final concentration of 5nM and incubated with 1mM GTP (solid and dashed line) or 1 mM GDP (dotted and dashed-dotted line). The binding was normalized to possible binding by assumption of a single binding site. Grey lines represent the least square analysis using a hyperbolic function according to equation 2. (B) The initial rate constants for different amounts of GTP using 0.5 µg of each protein were determined. Drop lines show the half-maximum giving the K_m value listed in Table 1.

When the hydrolysis of GTP by atToc33 and atToc34 was analyzed in more detail, we observed an almost similar maximal hydrolysis rate (V_{max}) (Figure 11B). Both proteins revealed a very slow maximal hydrolysis rate of about 500 nM* min^{-1} (Table 1). This rate is comparable to the intrinsic GTP hydrolysis rate of other small GTPases (for example see Downhard *et al.* 1990, Zhang *et al.* 1997, Albert *et al.* 1999). The analysis further revealed a Michaelis-Menten constant of 290 µM and 190 µM for atToc33 and atToc34, respectively (Table 1).

TABLE 1. GTP binding rate constants and hydrolysis constants

Parameter		Toc33	Toc34
GTP binding[a]	k_{on} (sec^{-1})	0.045±0.003	0.041±0.002
GDP binding[a]	k_{on} (sec^{-1})	0.012±0.004	0.013±0.003
GTP hydrolysis[b]	V_{max} (nM/min)	520±30	470±20
	K_m (M)	(2.9±0.2) * 10-4	(1.3±0.3) * 10-4
	k_{cat}(min^{-1})	0.31±0.01	0.27±0.01

[a]IAsys measurement as described in Figure 11A, [b]Experiment described in Figure 11B

The catalytic constant (k_{cat}) of about 0.3 min^{-1} (Table 1) is comparable to the intrinsic catalytic constant found for other GTPases as well (for example see Albert *et al.* 1999). However, a turnover of one GTP molecule by one receptor every 3 minutes would not explain the rapid protein translocation into chloroplasts (Rutthard *et al.* 2001, Pilon *et al.* 1992)). We conclude that both Toc33 and Toc34 can act as a GTPase *in vivo*, but proteinaceous effectors most likely stimulate their rate of hydrolysis.

5.2.4 Dimerisation of atToc33 and atToc34

As a result of the three molecules of Toc34 in one crystallographic unit in the structure of the psToc34 a functional dimer between two receptor molecules was proposed (Sun *et al.* 2002).

Furthermore it was observed that Toc34 interacts with the highly homologous GTPase domain of Toc159 (Hiltbrunner *et al.* 2001). Here we wanted to investigate the nature of such dimerisation. The interaction of atToc33 and atToc34 was investigated in the absence (Figure 12A) or presence of nucleotides (Figure 12B,C). In the presence of GDP or without any nucleotides, highest binding was observed for the Toc34-Toc33 interaction (Figure 12A,B, solid line). In the presence of GTP, the association between Toc34 and Toc33 was in the range of the homo-dimerisation between Toc33 and Toc33 or Toc34 and Toc34, respectively. The homo-dimerisation seemed not to be as strongly dependent on the presence of nucleotides (Figure 12A-C). A more detailed analysis of the association revealed the highest affinity of any kind of interaction (homo or hetero dimerisation) in the absence of nucleotides reflected by the lowest dissociation constants (K_D, Table 2). Remarkably, the lowest dissociation constant (K_D) of about 200nM was observed for the heterodimerisation in the absence of nucleotides. The homodimerisation of Toc33, but not of Toc34, in the absence of nucleotides also revealed a low dissociation constant (K_D, Table 2). The homodimerisation of Toc34 revealed under all conditions the highest dissociation constant (K_D, Table 2). Therefore, these results suggest, that dimerisation is unfavourable in the presence of GTP and even further, heterodimerisation is preferred in comparison to homodimerisation. Taking into account that the G-domain of Toc159 is very homologous to the Toc34/33 G domain (Kessler *et al.* 1994, Hirsh *et al.* 1994) our result further supports the idea from Hiltbrunner and co-workers that the heterodimerisation of the G-domains of the Toc receptor components might be essential for the assembly and function of the Toc complex (Hilbrunner *et al.* 2001, Bauer *et al.* 2002). A more detailed analysis of the association between the receptor proteins revealed the highest dissociation rate constant (k_{diss}) for the hetero-dimers in the presence of GDP. In contrast, the dissociation rates (k_{diss}) in the presence of GTP or in the absence of nucleotides are comparable. This finding together with the observation that the dissociation constant (K_D) for the investigated interactions is highest in the presence of GTP (Table 2)

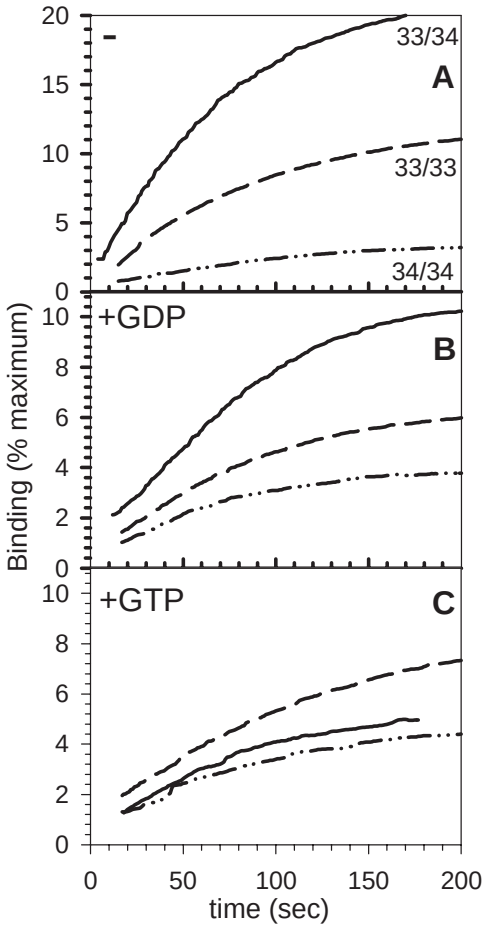

Figure 12. The homo- and hetero-dimerisation of atToc33 and atToc34.
The association between atToc33ΔTM and atToc34ΔTM was investigated facilitating the optical biosensor instrument (IAsys). The row data expressed in arcsec to reflect the increase of the density on the cuvette surface were calibrated for the maximal amount of possible binding assuming a single binding side (maximal binding, related to the amount receptor coupled) and data expressed as percent of the maximum. For that, 7ng of atToc33ΔTM (dashed line) and atToc34ΔTM (dashed - dotted and solid line) were coupled to the dextran surface as described in Materials and Methods followed by incubation with 175ng atToc33ΔTM (dashed and solid line) and atToc34ΔTM (dashed-dotted line) A) in the absence of nucleotides (upper panel), B) in the presence of 1mM GDP and 1mM $MgCl_2$. (middle panel) or C) in the presence of 1mM GTP and 1mM $MgCl_2$ (lower panel).

reveals that the association rate constant (k_{ass}) is lowest in the presence of GTP. This result directly contradicts the hypothesis that dimerisation acts as GTPase activating factor (GAP) (Reuber and Ausubel 1996). A GAP reveals the highest association rate constant and the lowest dissociation constant for its substrate in the presence of GTP (Kraemer *et al.*2002). In contrast, the high dissociation rate constant (k_{diss}) but the rather low dissociation constant (K_D, Table 2), which suggests a rather high association rate constant, found for the heterodimer in the presence of GDP leads to the speculation, whether this interaction might act as a GEF

(GDP/GTP exchange factor)(Hiltbrunner *et al.* 2001). This also could explain the results that abolishing of the interaction between the two GTPase domains leads to a reduction of the intrinsic GTPase activity (Reuber and Ausubel 1996).

TABLE 2. Binding constants for receptor dimerisation, for description see Figure 12.

nucleotide	constant	Toc33/Toc33	Toc34/Toc34	Toc33/Toc34
GTP	K_D (nM) [1]	720	1530	1020
	k_{diss} (sec^{-1})	0.0025±0.0006	0.007±0.003	0.002±0.001
GDP	K_D (nM)	830	2100	370
	k_{diss} (sec^{-1})	0.02±0.01	0.05±0.003	0.06±0.03
No	K_D (nM)	490	1670	200
	k_{diss} (sec^{-1})	0.006±0.004	0.004±0.001	0.004±0.002

1 … calculated using $K_D = 100\% * conc / R_{max} – conc$

5.2.5 Preprotein binding of atToc34 and atToc33

Toc33 and Toc34 are both GTPase type receptor proteins for chloroplast-targeted preproteins. We therefore wanted to investigate the interaction of different preproteins of *A.thaliana* with the two receptor proteins. When the interaction of Toc33 and Toc34 to the precursor of Ferredoxin:$NADP^+$ Oxidoreductase (FNR) in the absence of GTP was investigated, typical binding traces were observed (Figure 14A). In contrast, when GTP was present, we observed a rather new binding trace for the association between Toc34 and Toc33 and the precursor of the high chlorophyll fluorescence phenotype protein 136 (HCF). After initial rapid association, the signal decreased (Figure 14B,C). Finally, a second association could be observed (Figure 14B,C). In some of the cases the association was too rapid to observe the initial increase of the signal, since stirring and mixing effects do not allow the analysis for the first 5-10 sec. However, in order to analyze this data we had to develop a model for this process.

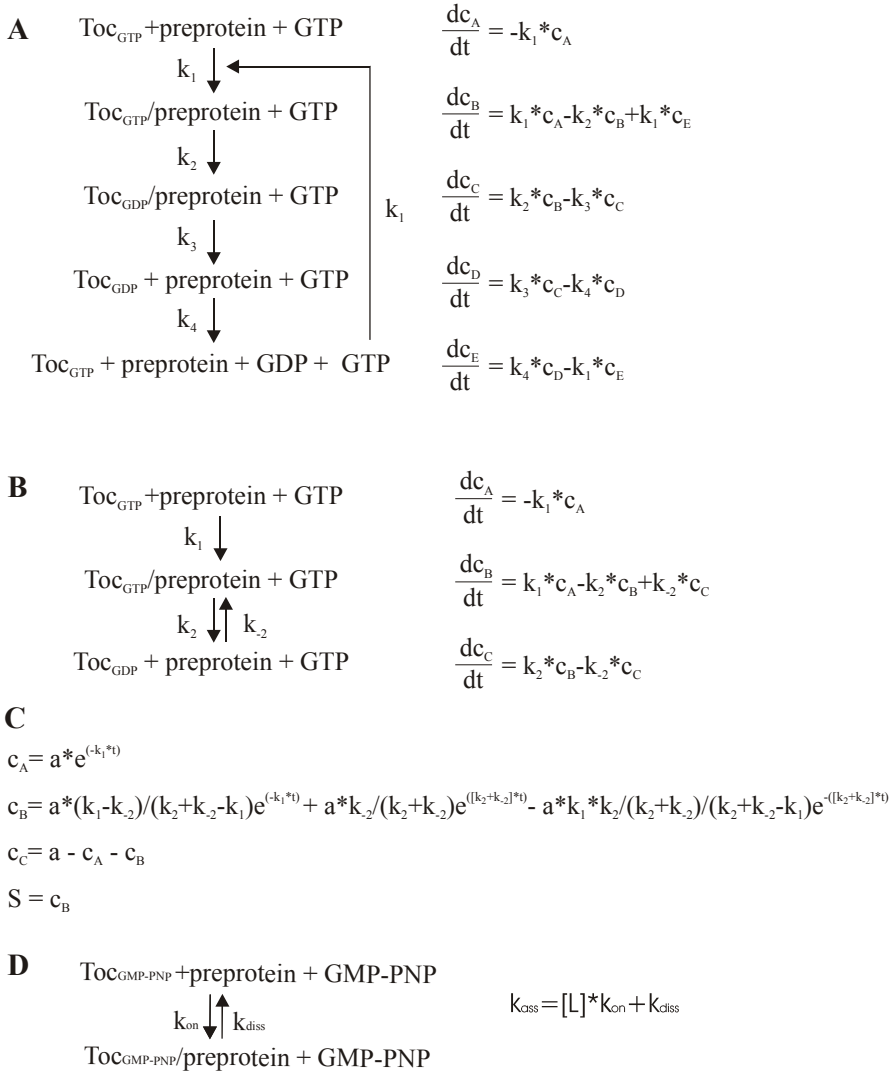

Figure 13. The reaction scheme for the preprotein recognition.
A) The exact reaction scheme for the Toc33/34 – preprotein recognition is outlined. B) The analytically describable reaction scheme is given. C) The solutions of the differential equations are given. The signal (S) observed is for preprotein/Toc complexes only. Therefore, the equation for the analysis is given. D) For reactions without hydrolysable GTP analogues the association can be described in a single step reaction. [L] is concentration of ligand.

Taking the existing knowledge about the behavior of Toc34 from *P.sativum* in account this curve can be explained as follows. The initial GTP preloaded Toc34 has a very high affinity for preproteins (Sveshnikova *et al.* 2000a, Schleiff *et al.* 2002a). This association stimulates GTP hydrolysis and dissociation of the preprotein (Section 5.1). Since GTP exists in excess, GDP to GTP exchange occurs since Toc34 binds GTP with higher affinity than GDP (Figure

11, Table 2). This association stimulates GTP hydrolysis and dissociation of the preprotein (Section 5.1, Sveshnikova *et al.* 2000a).

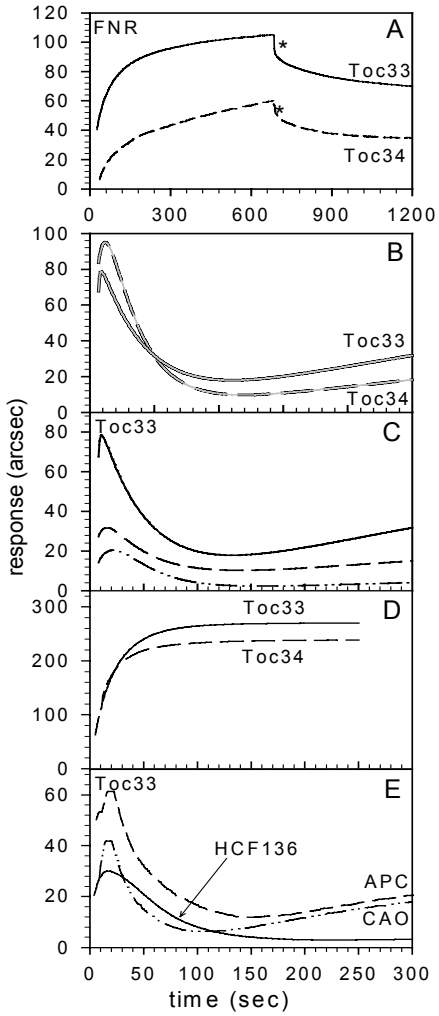

Figure 14. Differential preprotein binding ability of atToc33 and atToc34.
The association (A-D) between both receptor proteins and the indicated preproteins and the dissociation A) of the receptor preprotein complex was investigated using the optical biosensor instrument (IAsys). The increase in the density on the cuvette surface reflecting the increase of formed complexes is represented in arbitrary units and given as response in arcsec. In A) 5ng of atToc33ΔTM (solid line) and atToc34ΔTM (dashed line) were coupled to the dextran surface and incubated with 440nM FNR in the absence of GTP. After indicated time (*) dissociation was initiated by removal of the free FNR. In (B) 7ng of atToc33ΔTM (solid line) and atToc34ΔTM (dashed line) were coupled to the IAsys chamber and incubated with 23nM HCF in the presence of 1mM GTP and 1mM $MgCl_2$. In C) 7ng of atToc33ΔTM was incubated with 11.7nM (dotted line), 19.9nM, (dashed line) or 23nM HCF (solid line) in the presence of 1mM GTP and 1mM $MgCl_2$. D) 7ng of atToc33ΔTM (solid line) and atToc34ΔTM (dashed line) were coupled to the IAsys chamber and incubated with 23nM APC in the presence of 1mM GMP-PNP and 1mM $MgCl_2$. E) 7 ng of *at*Toc33ΔTM was coupled to the IAsys chamber and incubated with 23 nM HCF136 (solid line), 42.4 nM APC (dashed line), or 36.3 nM CAO (dashed-dotted line) in the presence of 1 mM GTP and 1 mM $MgCl_2$.

Finally, equilibrium between association and dissociation will be observed. In Figure 13A, the mathematical solution of the reaction is outlined, however, such model cannot be described analytically. Therefore we used a three-phase model as described in Figure 13B to extrapolate values from these binding trace. To confirm the assumption that we truly detect the GTPase activity of Toc34 and its stimulation by incoming preproteins, samples were taken out of the cuvette and spotted on PEI-cellulose plates. We observed increasing amounts of GDP at longer time points (not shown). Furthermore, for the precursor of the γ-subunit of the chloroplast ATP synthetase (preAPC, Figure 14D) and FNR (data not shown) the binding experiment was repeated in the presence of the non-hydrolysable GTP analogue GMP-PNP. Here, such three-phase behavior was not observed (Figure 14D).

When the association of preFNR was studied in more detail, we observed a high dissociation constant (K_D) in the absence of GTP for both receptor proteins Toc33 and Toc34 (Figure 15A, gray symbols, Table 3). In the presence of the non-hydrolysable homologue of GTP, GMP-PNP, the affinity between FNR and Toc33/34 was drastically increased (Figure 15A, open symbols, Table 3). The observed dissociation constants (K_D) are 30 fold lower than in the absence of the nucleotide (Table 3). We conclude that both receptor proteins recognize the incoming preprotein with highest affinity in the GTP bound state. The analysis of the amount of binding at equilibrium in the presence of GTP according to the three-phase binding model (Figure 13B) revealed a three fold higher dissociation constant (K_D) than in the presence of GMP-PNP. This is in line with the assumption, that this state reflects the equilibrium between bound and unbound precursor. Furthermore, the association of preFNR with Toc33 was found to be of higher affinity than the association with Toc34 (Figure 15A, Table 3). As expected, the difference of the dissociation constants in the presence or absence of the nucleotide is due to a drastic alteration of the association rate constant (k_{ass}) by a factor of ten (Figure 15B, Table 3).

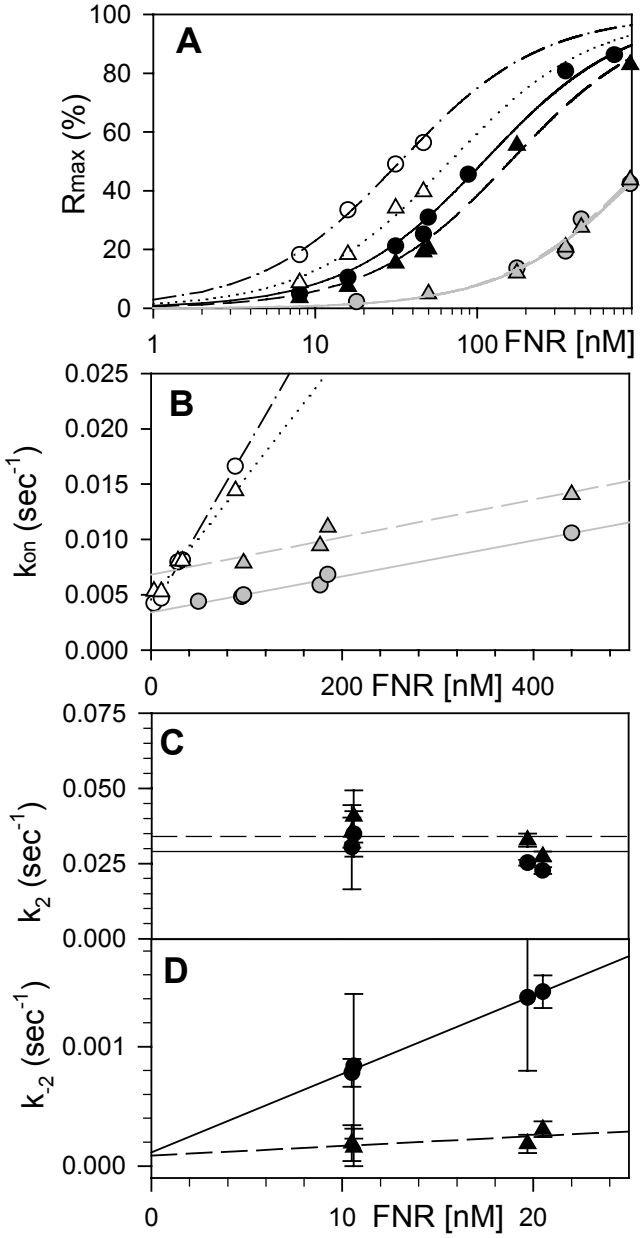

Figure 15. Binding properties of preFNR to atToc33 and atToc34.
As described in legend of Figure 14, the receptor proteins were coupled to the cuvette surface and association and dissociation between both proteins was determined. The binding traces were analyzed as described in Materials and Methods and the references therein. In (A) 7ng of atToc33ΔTM (solid and dashed-dotted line, circle) and atToc34ΔTM (dashed and dotted line, triangle) were coupled to the dextran surface and incubated with indicated amounts of preFNR in the absence of nucleotides (gray symbols and lines), in the presence of 1mM GTP and 1mM $MgCl_2$. (black symbols and solid or dashed line) or in the presence of 1mM GMP-PNP and 1mM $MgCl_2$ (open symbols and dashed-dotted or dotted line). Shown is the final amount of bound protein observed at equilibrium of the association (R_{max}) normalized for amount of binding sides assuming a single binding side on the receptor. Lines represent least square analysis of the data points to a hyperbolic curve for dissociation constant determination. In (B) the on-rates for the association in the absence or presence of GMP-PNP are plotted against the concentration precursor used to determine the association constant k_{ass}. Symbols and colors are according to (A). Lines represent the linear regression. (C,D) The rate constant 2 (C) and -2 (D) (according to Figure 13B) are plotted against the concentration of FNR used in the experiment. Symbols and colors are according to (A). Lines represent the linear regression. Constants are defined in Figure 13 B and D.

Analysis of the three-phase reaction in the presence of GTP revealed a second rate constant (k_2) of 0.028 or 0.034 sec^{-1} for Toc33 and Toc34, respectively (Table 3). This rate constant (k_2) is not dependent on the concentration of the ligand (Figure 15C), since the reaction described is a dissociation type process (Figure 13B). This further suggests, that the dissociation of the precursor protein FNR from the receptor is almost comparable between

TABLE 3. Binding constants for preprotein recognition

preprotein	nucleotide	Parameter	Toc33	Toc34
FNR[a]	-	K_D (nM)	1170±30 (460)	1140±30 (1140)
		k_{diss} (sec^{-1})	0.0068±0.0002	0.0182±0.0002
		k_{ass} ($M^{-1}sec^{-1}$)	(1.7±0.4)* 10^4	(1.6±0.4)* 10^4
	GTP	K_D (nM)	110±10	160±20
		k_2 (sec^{-1})	0.028±0.004	0.034±0.003
		k_{-2} ($M^{-1}sec^{-1}$)	(6.6 ± 0.3)*10^4	(8.1±0.7) *10^3
	GMP-PNP	K_D (nM)	33±1	68±2
		k_{ass} ($M^{-1}sec^{-1}$)	(1.5±0.4)* 10^5	(1.1±0.4)* 10^5
APC[a]	GTP	K_D (nM)	210±20	46±1
		k_2 (sec^{-1})	0.027±0.001	0.042±0.005
		k_{-2} ($M^{-1}sec^{-1}$)	(2 ± 1)*10^4	(6±2) *10^4
	GMP-PNP	K_D (nM)	199±10	22.2±0.5
		k_{ass} ($M^{-1}sec^{-1}$)	(9.0 ± 0.5)*10^4	(1.0±0.3) *10^6
HCF[a]	GTP	K_D (nM)	158±9	220±10
		k_2 (sec^{-1})	0.030±0.003	0.040±0.008
		k_{-2} ($M^{-1}sec^{-1}$)	(5.6 ± 0.8)*10^3	(2.8±0.6) *10^4
CAO[a]	GTP	K_D (nM)	270±30	102±8
		k_2 (sec^{-1})	0.024±0.004	0.035±0.006
		k_{-2} ($M^{-1}sec^{-1}$)	(2.9 ± 0.3)*10^4	(2.6±0.6) *10^5

[a]IAsys measurement as described in Figure 14&15

Toc33 and Toc34. The third rate constant (k_{-2}) is again concentration dependent (Figure 15D) since association is involved in the process covered by the last step (Figure 13B).

It is found that the association between Toc33 and FNR reaches the equilibrium faster than found for Toc34/FNR (Figure 15D) being in line with Toc33 having a slightly higher affinity for preFNR compared to Toc34.

Since we observed a different binding affinity of Toc33 and Toc34 for FNR (Table 3), we asked whether this could be seen for other preproteins as well. We subsequently investigated the association of three further preproteins to Toc33 and Toc34, namely preAPC (Figure 14D), the precursor of the HCF (Figure 14B,C) and precursor form of the chlorophyll a oxygenase (preCAO; not shown). The association of all four preproteins in the presence of GTP revealed a three-phase behavior (Figure 14B,C and data not shown). However, we observed that HCF has a slightly higher affinity and a lower dissociation constant (K_D), respectively, for Toc33 when compared to Toc34. In contrast, Toc34 has a significantly lower dissociation constant (K_D) compared to Toc33 when bound to APC or CAO (Table 3).

A closer look at the second rate constant (k_2) suggested that the dissociation in the presence of GDP is higher than in the absence of any nucleotide (for FNR), but comparable for all preprotein receptor combinations. This supports the hypothesis that the GDP bound state does not recognize any preproteins since otherwise strong variations of this rate constant would have been expected. The third rate (k_{-2}) is related to the association rate (k_{ass}) as seen for APC and FNR suggesting that the proposed model (Figure 13B) can be used to describe the observed binding behavior. However, the GTP binding seems not to be the rate-limiting step, since otherwise the same rate constants for all preproteins would have been expected.

In general we suggest that Toc33 has a higher affinity for FNR and HCF, whereas Toc34 revealed a higher affinity for APC and CAO.

5.2.6 Stimulation of GTP hydrolysis by atToc33 and atToc34 is substrate dependent

Toc34 and Toc33 are both GTPases (Figure 9), which differentially recognize different preproteins (Figure 14, 15; Table 3). Further, the intrinsic hydrolysis rate (Figure 11 and Table 1) suggests a stimulation of the hydrolysis by some kind of GAP component. The first hypothesis that homo-dimerisation might cause such stimulation (Sun *et al.* 2002) could not be confirmed for two reasons. First, homo- or heterodimerisation reveals the highest affinity in the absence of nucleotides (Figure 12, Table 2) and second, all hydrolysis experiments so far were performed in solution allowing dimerisation.

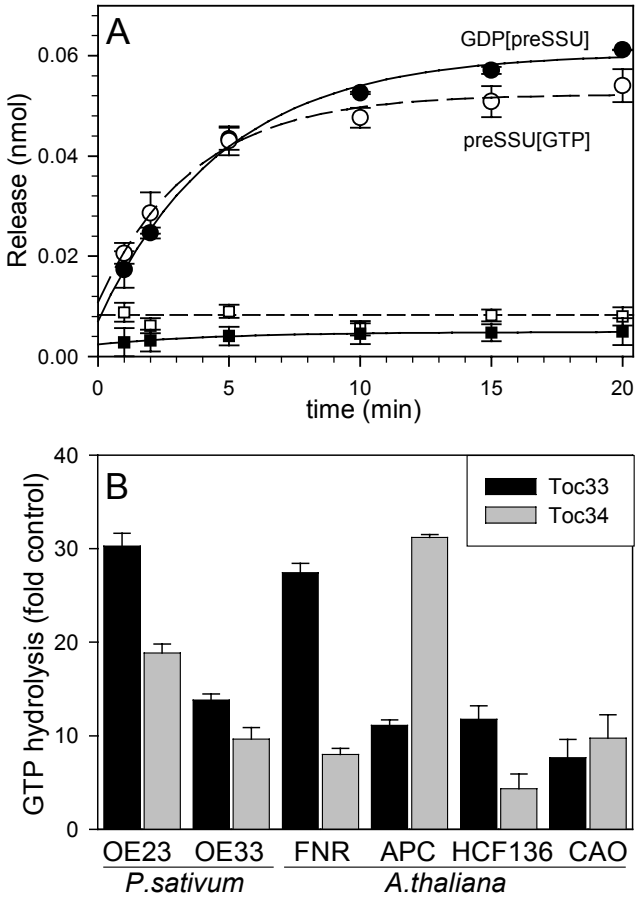

Figure 16. Differential stimulation of GTP hydrolysis of atToc33 and atToc34 by preproteins
A) 0.1 nmol of Toc33 was loaded with GTP and preSSU as described in Materials and Methods. The molar amount of released phosphate after hydrolysis of GTP in the presence (closed circles) or absence of preSSU (closed squares) and released preSSU in the presence of GTP (open circles) or GMP-PNP (open squares) at indicated time points are shown. Each data point is the average of at least two independent experiments. Lines represent least-squares analysis to a monophasic kinetic. B)The hydrolysis of the GTP by 0.1µg Toc34 and Toc33 proteins in the presence of 20 fold molar excess of the indicated preprotein was determined. The fold of hydrolysis increase compared to hydrolysis in the absence of preprotein is shown. Error bars indicated standard deviation of at least four independent experiments. Control=1 (Toc33/34 alone).

However, a second hypothesis suggested that incoming preproteins might function as GTPase stimulating factors (Fulgosi and Soll 2002). Since we had observed differential affinities for the two receptor proteins for preproteins we investigated the hydrolysis of Toc33 and Toc34 in the presence of 20 fold molar excess of the six preproteins FNR, APC, HCF, CAO (from A.thaliana) and the 23 and 33 kDa subunit of the oxygen evolving complex preOE23 and preOE33 (*P.sativum*). Strikingly, a stimulation of the hydrolysis was observed in all cases. However, as seen for the association, the increase was dependent on the receptor-preprotein combination. In line with the binding behavior, FNR stimulated Toc33 GTP hydrolysis 28 fold. Furthermore, OE23, OE33 and HCF were also found to stimulate the GTP hydrolysis of Toc33 more pronounced than the hydrolysis of Toc34. In contrast APC stimulated the GTP hydrolysis of Toc34 by a factor of 32 (Figure 16). However, in line with the observed dissociation constants, the stimulation by HCF and CAO was not as pronounced as found for the other precursor proteins. Therefore, preprotein recognition seems to initiate GTPase activation.

5.2.7 *In silico* analysis of prepeptide targeting sequences

To gain insights into the structural features of the presequences for the differential recognition by Toc33 and Toc34 we analysed the pools of nuclear encoded, chloroplast targeted proteins which are up, down or not regulated in the ppi1 mutant based on the transcription level. For the analysis we used sequences identified to be most drastically affected in their transcript presence either by up or down regulation (100 sequences each). Furthermore, from the genes of chloroplast targeted proteins not affected by the Toc33 knock out, 100 sequences where randomly chosen. The first observed difference was a significant difference in signal lengths. The down regulated preproteins contain statistically 10 amino acids shorter presequence than up and non regulated ones (Figure 17).

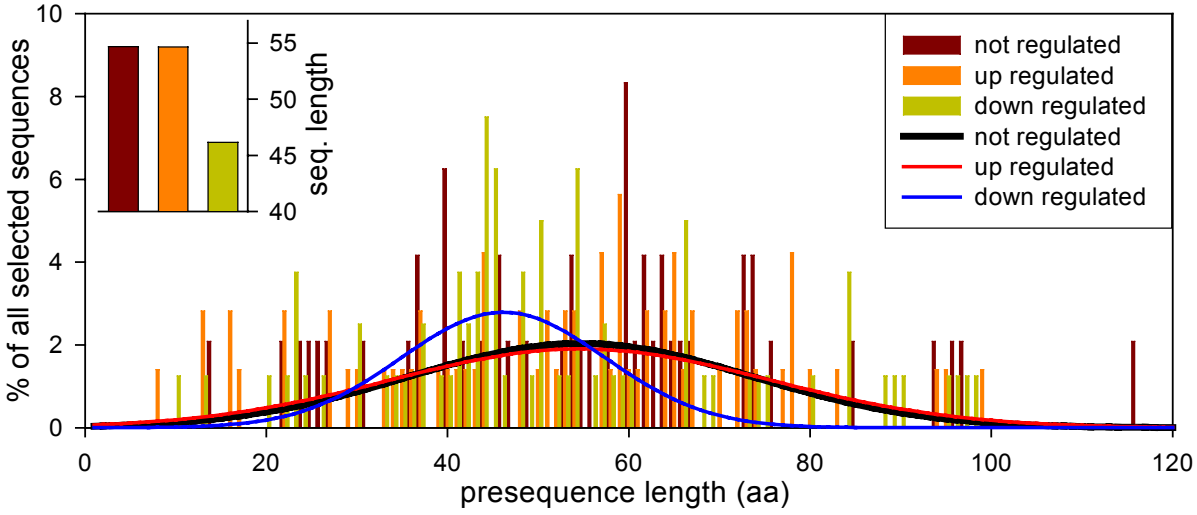

Figure 17. Comparison of the targeting sequence lengths for up, down and not regulated preproteins in ppi1mutant.
100 highest up regulated, down regulated and 100 computer randomly chosen not regulated presequences of the chloroplast targeted preproteins are statistically compared by lengths and presented by Gauss curve.

For further analysis of the amino acid distribution in the preprotein presequence, the first 30 N-terminal amino acids of the transit sequences were chosen. "Up regulated" preproteins exhibits hydroxylated and prolin rich region from amino acids 20 to 30 (Figure 18-up). "Not regulated" preproteins contain a positively charged and hydroxylated region from the amino acid 18 to 26 (Figure 18-non), while "down regulated" ones are enriched in hydroxylated amino acid just on the beginning of the sequence followed by hydrophobic residues in the length of ~10 amino acids (Figure18-down). These features are shared by the presequences of preFNR and preAPC, too.

A similar difference of the amino acid distribution was observed when the last 30 amino acids of the transit sequence were analysed. "Not regulated" preproteins possess two regions. The region from amino acid position 12 to 20 from end is strongly charged and enriched in prolines and the region from amino acid position 3 to 14 from end represents a serine cluster. "Down regulated" have on the very end charged and serine deputized region, while "up regulated" do not express any significant part (Figure 19). These results suggest that amino

acids distribution in the presequence is directly connected with differential recognition by Toc34 homologues, as discussed in Section 6.2.

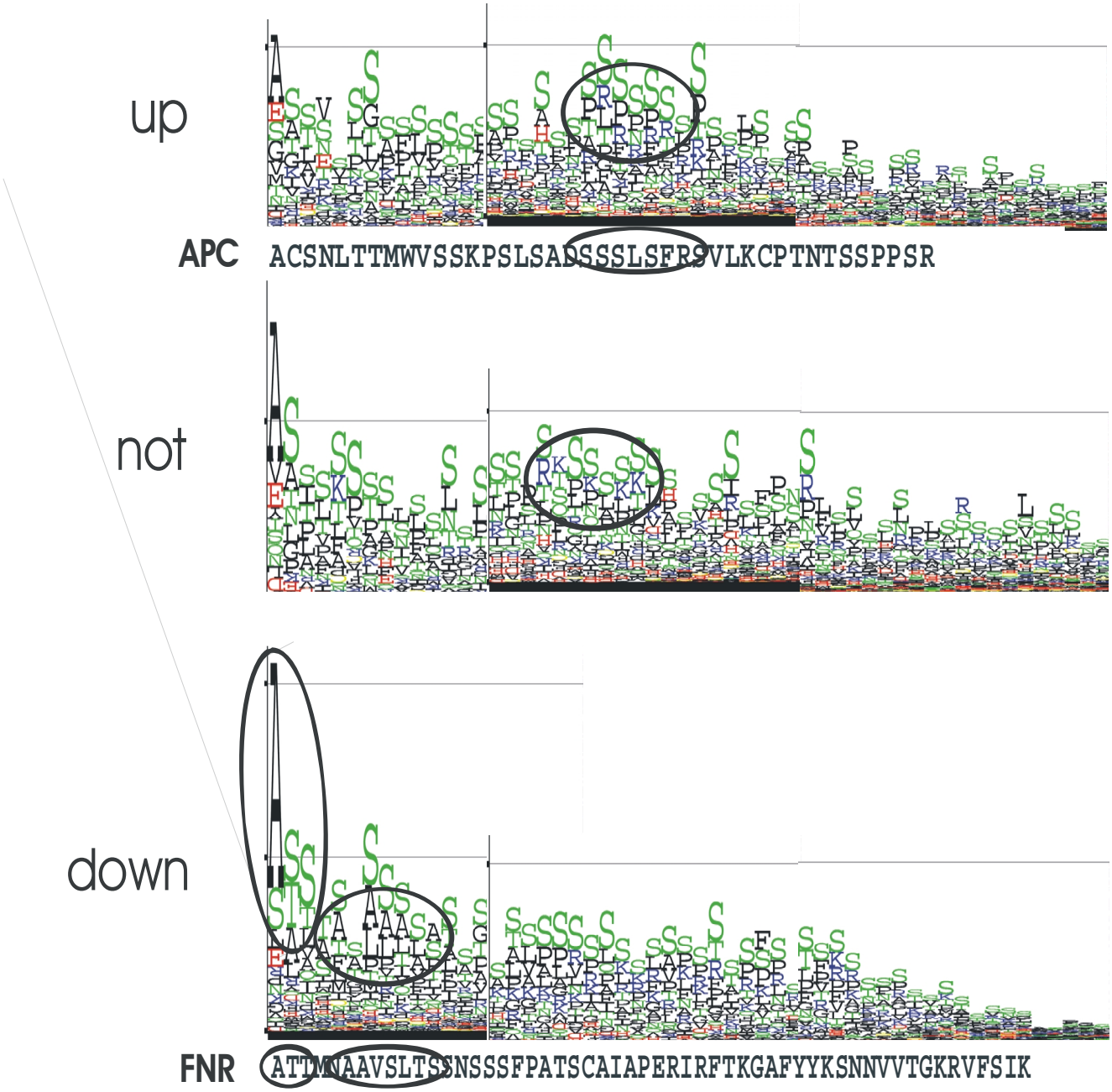

Figure 18. Content and distribution of the amino acids in the N-terminal part of a presequences.
100 presequences were analysed for each pool of preproteins. A domains of difference are marked with black ellipse. The presequence of preAPC is written below the graph of up regulated, while presequence of preFNR is written below the graph of down regulated preproteins.

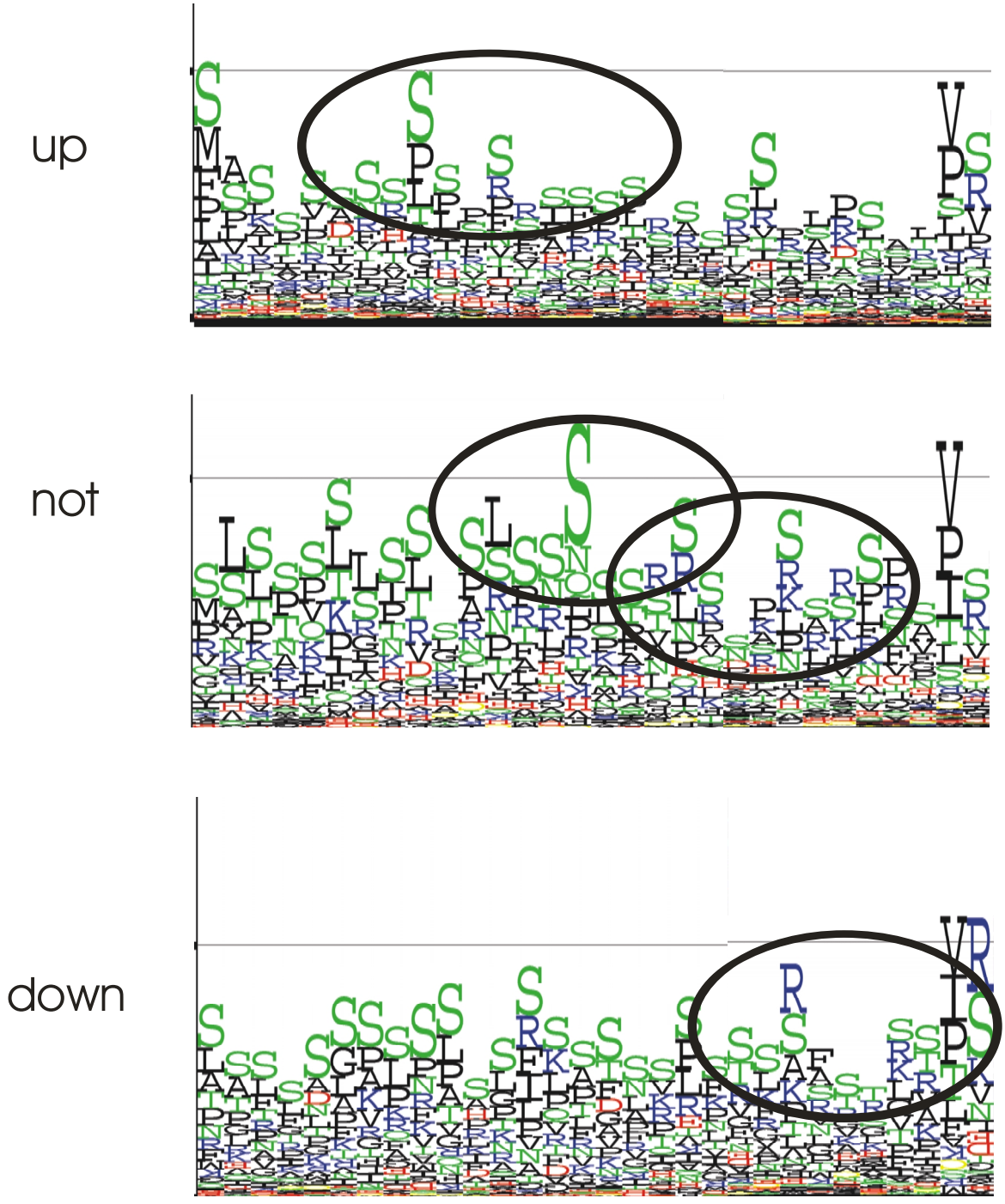

Figure 19. Content and distribution of the last 30 amino acids in the C-terminal part of a presequences.
Comparison of 100 up, down and not regulated preproteins presequences in ppi1 mutant from the last 30 amino acids of the C-terminus part of presequence. A domains of difference are marked with black ellipse.

5.3 The Toc complex GTP hydrolysis is regulated by preprotein recognition

5.3.1 The Toc complex GTP hydrolysis stimulation

Toc34 has endogenous GTPase activity, which is greatly stimulated by precursor proteins. Therefore we tested the capacity of the Toc complex to hydrolyze GTP (Figure 19A). In the absence of preprotein or in the presence of the mature form of the precursor protein, mSSU, a basal slow hydrolysis rate was observed (Figure 19B). In the presence of preSSU the hydrolysis rate was stimulated more than one hundred fold, demonstrating that preSSU functions as a GTPase activating protein.

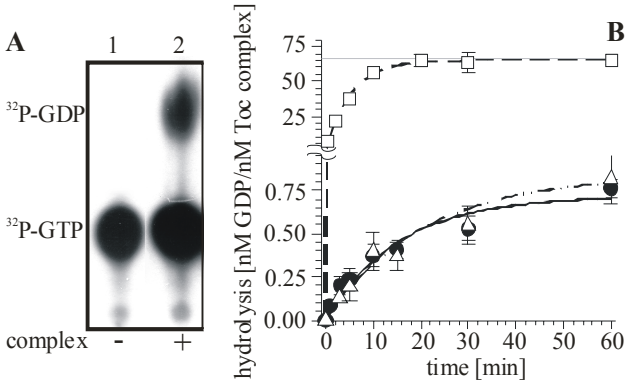

Figure 19. Stimulation of GTP hydrolysis of the Toc complex by pSSU.
(A) The hydrolysis of 330nM radiolabelled α-^{32}P-GTP in the absence (lane 1) or presence (lane 2) of the purified Toc complex (5 nM final concentration) was performed. (B) The amount of hydrolysed GTP at time points indicated are means of at least 3 independent experiments in the absence (B, ●) or presence of 100nM purified mSSU (B, △) or preSSU (B, □).

5.3.2 Interaction of Toc34 and Toc159 with the preproteins

One major question still remained: what is the mode of preprotein recognition?

Toc34 recognises a phosphorylated presequence with 4 to 5 times higher affinity than a nonphosphorylated one (Schleiff *et al.* 2002a). Therefore an experimental approach was designed where the recognition of phosphorylated preproteins by the Toc components was investigated.

Proteins of the outer envelope of chloroplasts were separated using SDS-PAGE and transferred to a nitrocellulose membrane, denatured and renaturated. This membrane was then

incubated with over expressed pSSU which was phosphorylated with ^{32}P-γATP (Figure 20B), or pSSU translated in the *E.coli* lysate containing ^{35}S-Met (Figure 20A), in the absence (Figure 20A,B) or presence of GMP-PNP (Figure 20A,B).

Both proteins Toc34 and Toc159 recognise more strongly nonphosphorylated ^{35}S labelled pSSU in the GMP-PNP bound state (Figure 20A,B +). In contrast, ^{32}P labelled pSSU is only recognised by the Toc34 also in the GMP-PNP loaded state. Control protein OEP37 did not recognise any of the pSSU variants.

Thereby, these results show that Toc34 is able to recognize phosphorylated and nonphosphorylated preprotein, while Toc159 interacts only with nonphosphorylated one.

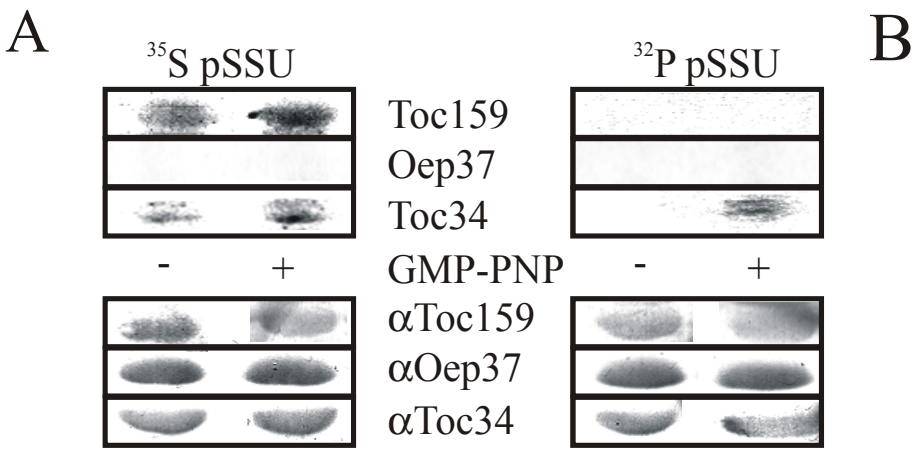

Figure 20. Interaction of Toc34 and Toc159 with pSSU.
20µg of total outer envelope proteins are after SDS-PAGE blotted on the nitrocellulose membrane, denaturated, renaturated and incubated with radioactively labelled pSSUs. A) Autoradiogramm of renaturated Toc34, Toc159 and OEP37 after incubation with the ^{35}S labelled pSSU in absence or presence of the GMP-PNP. Immunodecoration of the renatureted Western blot with antisera αToc34, αToc159 and αOEP37. B) Same as in A) after incubation with ^{32}P labelled pSSU.

6.0 DISCUSSION

6.1 Features of Toc34 from *Pisum sativum*

6.1.1 Phosphorylation of Toc34

Phosphorylation/dephosphorylation cycles are powerful tools to regulate diverse processes, such as biochemical pathways or cell and organelle cycle (Hardie, 1999). Specifically protein phosphorylation has been demonstrated to influence solute transport as well as protein translocation (Gruss *et al.* 1999; Deltrot *et al.*, 2000). Toc34, a GTP dependent protein import receptor represents a prominent phosphoprotein of the chloroplast outer envelope (Figure 3). The activity of Toc34 can be regulated by phosphorylation at serine 113 (Figure 3) causing the inactivation of its GTP binding capacity (Figure 4) and therefore resulting in a desensitization. Similar effects have been demonstrated for the eukaryotic initiation factor (Pain, 1996) or dynamine (Robinson *et al.*, 1993). Our data demonstrate that the phosphorylation shuts off the preprotein recognition (Sveshnikova *et al.*, 2000a) and therefore regulates translocation via the Toc34 receptor (Figure 1B(squared), Figure 21). The cell might require such a regulatory mechanism to adapt the protein repertoire of the chloroplast to the actual biochemical needs e.g. under high light conditions which require a response of the photosynthetic machinery but also of proteins involved e.g. in photorespiration. Recently it could be demonstrated that the import rates of preSSU at different stages of the plastid development are drastically altered (Su *et al.*, 2001). It was further demonstrated that this is due to a reduction of the preprotein recognition sites, which can also be the result of an increase of phosphorylated receptor proteins. These finding could also indicate a developmental dependent phosphorylation of Toc34 as a general regulatory mechanism (Figure 1B(squared), Figure 21). Alterations of illumination conditions result in changes of the redox state of the chloroplast. The Tic-complex contains Tic55, a Rieske type iron-sulphur

protein, which could be a component of a redox sensing circuit at the inner envelope (Caliebe *et al.*, 1997). Simultaneously, the Toc complex component Toc34 is regulated by phosphorylation and nucleotide binding (Figure 21). In combination such a system might build a regulatory network between the organelle and the parent cell (nucleus) to control protein targeting and organelle function.

6.1.2 Toc34, a GTPase with unique properties

Typical small GTPases have five conserved regions G1 – G5 (Bourne *et al.*, 1990). They specifically interact with GTP but their intrinsic hydrolysis rate is rather low (Downward *et al.*, 1990; Zhang *et al.*, 1997; Albert *et al.*, 1999). Toc34 recognizes all nucleoside triphosphates which contain a purine ring, but GTP with highest affinity (Figure 5, Sveshnikova 2000). This is consistent with earlier observations, that ATP cannot compete for GTP binding (Kessler *et al.*, 1994; Sveshnikova *et al.*, 2000a). Hydrolysis was only observed for GTP (Figure 5). GTP was hydrolyzed by the Toc34 with maximal hydrolysis rate (150 $nM*min^{-1}$), which is comparable to the intrinsic hydrolysis rate of other small GTPases (Downward *et al.*, 1990; Zhang *et al.*, 1997; Albert *et al.*, 1999) and already higher than an earlier reported hydrolysis rate for the dimerised form of Toc34 (Sun *et al.*, 2002). The increase of the hydrolysis rate compared to the earlier rate can be explained by the different experimental conditions. Furthermore, here determined catalytic constant of 0.02 min^{-1} was found to be similar to the intrinsic activity of other GTPases (Bourne *et al.*, 1991). From this we conclude that Toc34 acts as GTPase and hydrolysis can only be inhibited by XTP, a nucleotide that does not exist as a free substrate *in vivo* (Figure 5 and 6).

Mutations within or close to the G5 domain result in conversion of the GTPase to a XTPase (Hwang and Miller, 1987; Schmidt *et al.*, 1996). Therefore we changed aspartic acid 219 to asparagine (Figure 5). However, analysis of the structure of Toc34 (Sun *et al.*, 2002) revealed that D219 is not part of the G5 domain explaining the basal GTP hydrolysis of the D219N

mutant. However, Toc34 has properties, which are not common for GTPases: the extreme N-terminal region regulates the GTPase specificity and activity and the state of the phosphorylable serine 113 influences the hydrolysis by regulating GTP binding. The first observation can be explained by a stabilization of the G2 and G5 structure by the N-terminus. Analysis of the structure of Toc34 (Sun *et al.*, 2002) revealed that glycine 13 is in close proximity to isoleucine 56 and alanine 18 to serine 220. Destabilization of this region by deletion of the N-terminus or alteration of the amino acid composition would therefore result in loss of the conformational stability and in the observed loss of specificity. Furthermore, the phosphorylation site is located in the switch II domain (Sun *et al.*, 2002) explaining the loss of hydrolysis. In addition, the close proximity of serine 113 to arginine 76 located within the same loop as the magnesium bound glutamic acid 73 (Sun *et al.*, 2002) explains the observed desensibilization of hydrolysis to magnesium when serine was replaced by arginine (Figure 6). Further the close proximity of the large phosphate group after phosphorylation to the negatively charged arginine will cause a deformation of the magnesium binding loop by electrostatic interaction explaining the influence of phosphorylation on GTP binding and subsequent hydrolysis.

Most GTPases are regulated by very specific guanine nucleotide exchange factors (GEF (Cherfils and Chardin, 1999)) and GTPase activating proteins (GAP (Scheffzek *et al.*, 1998)). Recently it was suggested that dimerisation of Toc34 might cause a GTPase activation and therefore it may function as a GAP (Sun *et al.*, 2002). However, the hydrolysis rate of the dimerised form of Toc34 (Sun *et al.*, 2002) was even lower than the rate for Toc34 in the absence of a precursor reported here. In the presence of a transit peptide containing protein the hydrolysis of GTP was stimulated about 30 fold (Figure 7). Furthermore, the extent of stimulation by preSSU and preOE33 is similar to GAP proteins, which was found to be in the order of two to five orders of magnitudes (Albert *et al.*, 1999). We conclude that precursor proteins have a very pronounced effect as GTPase activating proteins in Toc34 function or co-

operate in an unknown manner with typical GTPase activating proteins (Figure 21). However, the association of Toc34 with the preprotein gives an additional stimulus to the hydrolysis of GTP by the GTPase. The resulting GDP-Toc34 precursor complex has a lower affinity for the preprotein than the GTP bound form (Sveshnikova *et al.* 2000a) and allows the preprotein to dissociate from the receptor and continue its passage through the translocon (Figure 21).

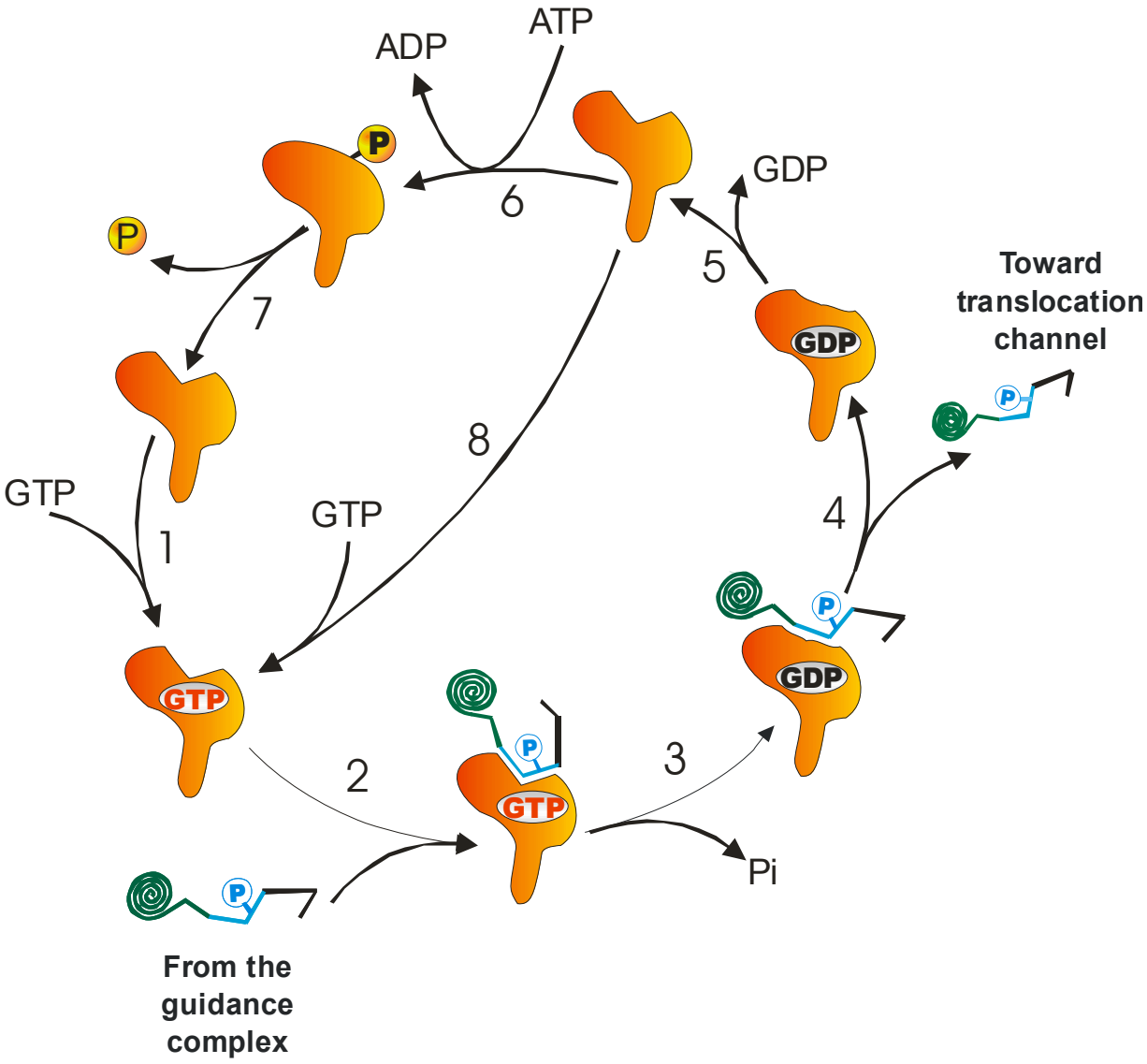

Figure 21. Mechanism of the Toc34 regulation.
Toc34 binding GTP (1), in the GTP loaded state Toc34 has a high affinity to bind a preprotein (2), preprotein acts like a GAP and stimulates GTP hydrolysis by the Toc34 (3). In the GDP loaded state Toc34 has low affinity for preproteins and the preprotein is released in the further steps of the translocation (4). Toc34 releases GDP (5) and in this nucleoside free state can be inactivated by phosphorylation by a outer envelope protein kinase which specifically phosphorylates Toc34 on Ser113(6), or can go in the next cycle of binding GTP (8). After inactivation by the phosphorylation, Toc34 can be activated by dephosphorylation (7), bind a GTP (1) and begin in the next cycle of preprotein recognition.

6.2 The Toc complex is regulated by expression of different isoforms

6.2.1 Differential regulation and preprotein recognition of Toc33 and Toc34

The Toc34 homologues were identified to belong to a P-loop type GTPase subfamily of Ras/Rho like proteins (Section 5.1). We confirmed that both, Toc33 and Toc34 from *A.thaliana* are able to bind (Figure 11A) and hydrolyse (Figure 8A, 10B) GTP. Interestingly, only Toc33 is phosphorylated. Phosphorylation occurs at position serine 181 (Figure 10) at a conserved motif (Figure 10C), which also can be found in the homologue of *Oryza sativa* (Vetter and Wittinghofer 2001). This phosphorylation inhibits GTP binding and hence precursor recognition (Figure 9C,D) (Figure 21). In the case of Toc34 from *P.sativum* the influence on GTP binding was rather obvious since phosphorylation occurs within the essential switch I domain (Section 5.2). Phosphorylation in atToc33 occurs further downstream. However, as indicated in Figure 10F, phosphorylation of serine 181 would disturb the general organization of the GTP-binding domain. Firstly, a structural deformation of the long helix might result in deformation of G5. Another explanation might be that the incorporation of the phosphate strongly interferes with the highly charged loop between amino acid 123 and 133. This loop, however, interacts with the G4 and G1 region. Therefore, three of the five important domains recognizing the GTP might be affected by phosphorylation of serine 181, explaining the interference with the GTP binding ability.

6.2.2 The receptor function of the two GTPases atToc33 and atToc34

GTP binding of a single receptor molecule was found to be rather slow (Figure 11A, Table 1). In line with this observation we found a slow turnover rate of the hydrolysis of GTP (Figure 11B, Table 1). The turnover rate of both receptors differs only slightly and is in the range found for other GTPases (Downward *et al.* 1990, Zhang *et al.* 1997, Albert *et al.* 1999) which are activated by associated proteins. The catalytic constant of about 0.3 min^{-1} (Table 1),

representing a turnover of one GTP molecule by one receptor every three minutes, does not explain the rapid protein translocation into chloroplasts. For example it was observed that about 20000 ferredoxin molecules per minute per chloroplast can be imported (Reutthard *et al.* 2001, Pilon *et al.* 1992). Taking into account that only 10% of about 20000 protein translocation complexes (Hirohashi and Nakai 2000) are active (Morin and Soll 1997), we must expect a stimulation of the GTP hydrolysis of the receptor by other components. For example it was proposed that homo-dimerisation might result in such stimulation. To our surprise, the highest affinity of dimerisation was found in the absence of nucleotides (Figure 12, Table 2). Even further, the presence of nucleotides drastically reduces the affinity for homodimerisation (Figure 12, Table 2). This result does not support the hypothesis that homodimerisation might function as a GAP protein, since GAPs have the highest affinity for the GTPase in the presence of GTP (Kraemer *et al.* 2002, Vetter and Wittinghofer 2001). Interestingly, the affinity of the heterodimeric complex was not significantly different in the presence of GDP or in the absence of nucleotides. This supports the idea of the hetero-oligomerisation of Toc34 or Toc33 with other G-domain containing Toc components like Toc159, Toc132, Toc120 or Toc90 (Hiltbrunner *et al.* 2001, Bauer *et al.* 2002). Since the affinity of Toc33 for Toc34 in the presence of GDP was found to be comparable with the affinity in the absence of nucleotides, this hetero-dimerisation might function like a GEF activation of GTPases, which recognize their substrate in the GDP bound form (Cherifils and Chardin 1999). Further, transit peptides are enriched in arginines, the amino acid supposed to reveal the catalytic amino acid of the GAPs (Friedman and Keegstra 1989). Therefore preproteins might function as GTPase activating proteins (Figure 21). In support of this hypothesis we observed an increase of GTP hydrolysis by the receptors up to 30 fold after preprotein recognition (Figure 16), hence giving a clearer picture of preprotein stimulated hydrolysis (Figure 21). This is further in line with the observation that 20000 preprotein molecules can be inserted into one chloroplast (Rutthard *et al.* 2001, Pilon *et al.*1992)

representing 1500-3000 active complexes (Morin and Soll 1997) since the turnover would be increased to one GTP molecule per six seconds revealing 15000-30000 translocation events per minute. The hypothesis of a GAP function of the preprotein is further in line with the observation that preproteins reveal the highest affinity for Toc33/34 in the presence of GTP (Figure 12, Table 3). The observed dissociation constants in the presence of GMP-PNP of Toc34/33 and FNR are 30 fold lower than in the absence of the nucleotide (Table 3) and comparable to the dissociation constant found for the interaction between preSSU and psToc34 in the presence of GTP (Schleiff *et al.* 2002a). Comparison between the off rate k_2 in the presence of GTP and the dissociation rate constant k_{diss} in the absence of nucleotides reveal a three fold increase in the presence of GDP (taking into account that dissociation occurs after hydrolysis). This is in line with the theory that hydrolysis moves the protein further in the translocation event. Taking together, the Toc34 acts as a GTPase type receptor for preproteins and hydrolysis is at least regulated by the recognition of the preprotein. The preprotein will be released in the presence of GTP and subsequently translocated. However, one question remained: Why do two isoforms exist if the mode of action is comparable?

Summarizing, both proteins were found to be differentially expressed (Gutensohn at al. 2000) and differentially regulated (Figure 9). We further identified that both receptors reveal differential affinities for different preproteins. Whereas Toc33 recognized preOE23, preOE33 (data not shown), FNR and HCF (Table 3) with higher affinity then Toc34, the latter revealed a higher affinity for APC and CAO. The same tendency was subsequently found for the stimulation of the GTP hydrolysis of the receptor proteins by the different preproteins (Figure 16). This result is in line with the observation that endogenous Toc34 cannot recover *A.thaliana* plants with a deletion of Toc33 (Jarvis *et al.* 1998). However, the ability of both receptor proteins to recognise all preproteins, even though with low affinity and drastic reduced GTPase activity stimulation (Table 3, Figure 16) together with the observed dominant localisation of Toc34 in roots (Gutensohn *et al.* 2000) might explain, why root plastids of the

Toc33 knock out line are able to differentiate into chloroplasts after prolonged light treatment (Yu and Li 2001).

The identification of different regulation of the two receptors as well as the preferential recognition of different preproteins explains the existence of two different isoforms of a single receptor component. The dissection of the features of the preproteins in order to be recognised by either one receptor, however, remains elusive and will require the investigation of a larger pool of preproteins to clearly draw conclusions on the regulation. The simple dissection into photosynthetic and non-photosynthetic proteins (Bauer *et al.* 2000, Jarvis *et al.* 1998) seems oversimplified, because even photosynthetic proteins reveal different affinities and stimulation behaviors (Figure 16).

6.2.3 Features of preproteins with differential binding characteristics for Toc34 isoforms

The analysis of the transcription level of chloroplast targeted proteins within the ppi1 mutant (Jarvis *et al.* 1998, Richy *et al*. 2003, Dario Leister personal communication) provided a tool to search for specific features of the presequence to be recognized preferentionaly either by Toc33 or Toc34. However, to understand results of this analysis, we made the following, non proven assumption: The “up regulated” genes represent proteins, which are classically targeted to the Toc33 receptor, but they can be recognized by Toc34 as well but with lower affinity. This might explain why such genes are up regulated. The “not regulated” genes encode for proteins targeted to the Toc34, since the lack of the Toc33 does not affect its expression. “Down regulated” genes are exclusively targeted by the Toc33 pathway and are therefore mainly affected by the knock out. This assumption is supported by the finding, that these proteins are not found in the chloroplast proteome in the early stages of chloroplast development (Paul Jarvis, personal communication).

Even though chloroplast presequences do not share many similar features in general, the statistical analysis of the three "pools" reveal some interesting properties: First, we observed a difference in the presequence length, statistically up and not regulated proteins are similar, while down regulated are shorter by approximately ten amino acids. This can mean that the length somehow effects the recognition of the preproteins by Toc33 or Toc34. However, the distribution of the length (Figure 17) would not support a "length based signal difference hypothesis".

Analysis of amino acid distribution of the three pools revealed further remarkable differences. The presequences of the pools, which can be recognized by Toc34 (up and non regulated) contain a hydroxylated/prolin rich region between amino acids 20 and 30 (Figure 18), which is not as pronounced in presequences exclusively recognized by the Toc33 (down regulated). In these sequences, a region of hydroxylated amino acids is placed in the first 10 N-terminal amino acids (Figure 18). However, experimental results suggest, that Toc33/34 recognize the C-terminal portion of the presequence (Sveshnikova *et al.* 2000a, Schleiff *et al.* 2002b). The analysis of the 30 amino acids affront of the cleavage site revealed, that Toc34 recognized proteins again contain a highly hydroxylated region (Figure 19), which is not as pronounced in Toc33 specific presequences (down regulated). Remarkably, these presequences contain positive charges at the –1 and –8 position (Figure 19). Therefore the physical properties of the presequence is a good ground for a differential recognition by Toc33 or Toc34, which has to be further investigated in the future.

6.3 Regulation of the Toc Complex

Toc34 is only one of two GTPases forming the Toc core complex (Schleiff *et al.* 2003b). However, in line with the observation with the isolated receptor, the GTPase activity of the Toc complex is strongly stimulated by preSSU (Figure 19). The excitation of the Toc complex GTPase activity by the pSSU is 3 to 4 folds higher than a stimulation of the Toc34 alone by

the same preprotein. Therefore, this effect can only be explained either by a cooperative action of other Toc components and the preprotein on Toc34 GTPase activity, or alternatively, by an increased stimulation of the GTPase activity of Toc159 by the preprotein as much as by Toc34. Indeed, using isolated Toc159 such stimulation was observed (Jelic, not shown)

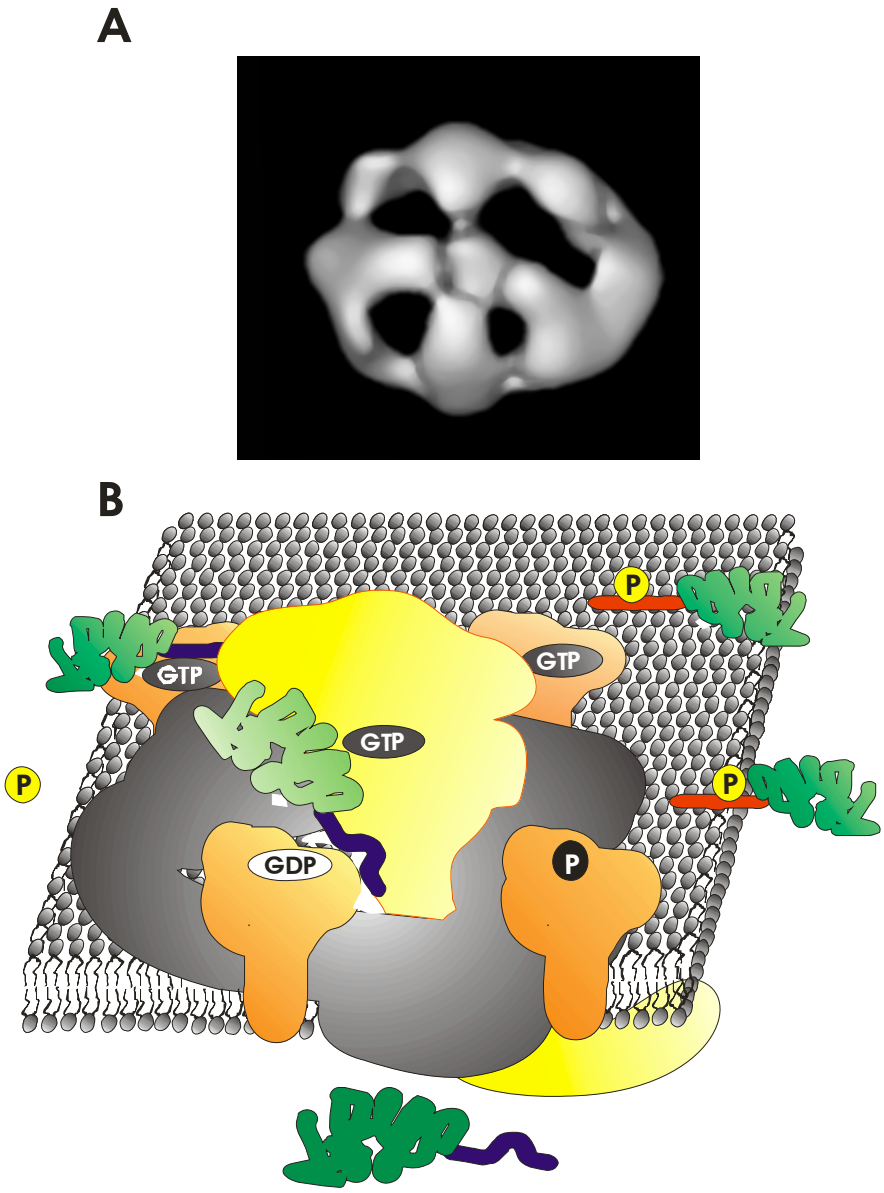

Figure 22. Proposed model of the Toc complex preprotein recognition.
A) Computer model of the electron microscopy data for the Toc complex (Schleiff *et al.* 2003) B) Proposed model of the Toc complex preprotein recognition. Four Toc34(orange), one Toc159(yellow) and four Toc75($_{gray}$) proteins assembling a Toc complex. Toc34 can exist in the three main functional species: nucleoside free state, phosphorylated state and preprotein competent GTP loaded state (Figure 21). Phosphorylated preprotein (red-green) binding on the Toc34-GTP, N-terminal part of the preprotein presequence interact with the Toc159, dephosphorylation of the presequence (blue-green) happened and preprotein stimulating GTP hydrolysis by the Toc34, Toc34-GDP has low affinity for preprotein and preprotein is transferred to the Toc159-GTP.

supporting the latter statement. Further, it was demonstrated that a minimal system of Toc159 and Toc75 reconstituted into liposomes are capable to import preproteins only when GTP is present. These results suggest that Toc159 is GTP driven motor of the Toc complex (Schleiff at al. 2003b). This finding would support, that Toc34 is the initial receptor of the Toc complex, contradicting the theory of a soluble active form of the Toc159. Further support for this hypothesis comes from the finding that Toc34, but not Toc159, is able to recognize a phosphorylated preprotein (Figure 20B). This leads to the proposal that the phosphatase must act during the transfer from the Toc34 to Toc159 and it can be suggested that A domain of the Toc159 might act as a docking site for this enzyme.

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Acknowledgments

I would like to express my gratitude to Herr Prof. Dr. J. Soll for giving me the opportunity to learn and work. Thank you Chef.

I thank Herr Prof. Dr. J. Ebel to be willing to serve as co-reviewer of the presented work.

I am especially grateful to PD. Dr. E. Schleiff, for his roles as a supervisor, leader and a friend. Enrico, my friend, thanks for everything.

Thanks to Prof. Dr. H. Fulgosi for all the advice and moral support. Hvala ti prijatelju, bez tebe bih vjerojatno odustao.

Many thanks to Thomas Becker for his critical attitude and for all the fruitful discussions about music, life and science.

My sincere thanks to all the people who helped me while writing this thesis, especially to Aleksandar Vojta for his essential support while suffering computer problems, and to Dr. Rowena Thompson and Rita Sharma for English language corrections.

To Catrin Claussen for friendship and understanding. Thanks Catrin.

I would like to thank all my colleagues, Ahmed, Cheol-Min, Claudia, Elena, Fatima, Friederike, Giesela, Iryna, Karl, Katharina, Katrin, Lanqin, Lars, Lea, Lisa, Masami, Michael K, Michael M, Sabine, Soumya, Sunčana, Theresa, Torsten, Ulrika, Ulrike, Ute, Walter and Wang Dong, for the friendly working atmosphere.

To my parents and my sisters for unselfish and unlimited support. Hvala vam za sve, ovaj uspjeh nije samo moj, već i vaš.

Thank you Nadana, Marija and Lucija Tereza. Darivate mi radost i život, volim vas neizmjerno.

Thank You God for your mercy, that You allowed me to discover some of Your secrets;

"Deduxit me super semitas iustitiae propter nomen suum."

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