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### ► **To cite this version:**

Mislav Oreb, Anja Höfle, Patrick Koenig, Maik S Sommer, Irmgard Sinning, et al.. Substrate binding disrupts dimerization and induces nucleotide exchange of the chloroplast GTPase Toc33. *Biochemical Journal*, 2011, 436 (2), pp.313-319. <10.1042/BJ20110246>. <hal-00592570>

**HAL Id: hal-00592570**

**<https://hal.science/hal-00592570v1>**

Submitted on 13 May 2011

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## SUBSTRATE BINDING DISRUPTS DIMERIZATION AND INDUCES NUCLEOTIDE EXCHANGE OF THE CHLOROPLAST GTPASE TOC33

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*Short (page heading) title: GTPase cycle of Toc33*

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GTPases act as molecular switches to control many cellular processes, including signaling, protein translation and targeting. Switch activity can be regulated by external effector proteins or intrinsic properties, such as dimerization. The recognition and translocation of pre-proteins into chloroplasts (via the Translocator of the outer/inner envelope membrane of chloroplasts; TOC/TIC) is controlled by two homologous receptor GTPases, Toc33 and Toc159, whose reversible dimerization is proposed to regulate translocation of incoming proteins in a GTP dependent manner. Toc33 is a homodimerizing GTPase. Functional analysis suggests that homodimerization is a key step in the translocation process, the molecular functions of which as well as the elements regulating this event are largely unknown. Here, we show that homodimerization reduces the rate of nucleotide exchange, which is consistent with the observed orientation of the monomers in the crystal structure. Pre-protein binding induces a dissociation of the Toc33 homodimer and results in the exchange of GDP for GTP. Thus, homodimerization does not serve to activate the GTPase activity as manifold discussed but to control the nucleotide loading state. We discuss this novel regulatory mode and its impact on the current models of chloroplasts protein import.

**Key words:** G proteins, dimeric GTPases, GDI function, protein translocation, substrate-based regulation

## INTRODUCTION

In eukaryotic cells, protein targeting and translocation are highly ordered and regulated processes. The targeting of proteins to the appropriate membrane is defined by specific signals within their amino acid sequence. Sequential transfer of an incoming protein across a membrane is achieved by transient protein-protein interactions within the multi-component translocation machineries (translocons). The initial steps of protein translocation across the membrane of the endoplasmic reticulum, the prokaryotic plasma membrane and the outer chloroplast membrane are regulated by dimeric GTPases [Fig. 1; e.g. 1, 2]. In the last decade, functional and structural analyses have significantly advanced our understanding of the GTPases controlling the targeting of proteins to the endoplasmic reticulum or the bacterial plasma membrane [3, 4]. However the transfer of precursor proteins across the chloroplasts membranes is not yet entirely understood [5]. It is widely accepted that the recognition of chloroplast precursor proteins with a typical N-terminal extension, called transit peptide, is performed by two GTP-dependent receptors, namely Toc159 and Toc33 [6, 7]. These are thought to cooperatively regulate the insertion of the precursor protein into Toc75, the translocation pore of the outer envelope [8, 9]. However, the individual role of both receptors as well as the sequence of events during the translocation process is not well understood.

The crystal structures of the GTPase (short G) domains of Toc33 from *Arabidopsis thaliana* (*atToc33*) and its orthologue Toc34 from *Pisum sativum* (*psToc34*) revealed a homodimeric interaction [10, 11]. Indeed, homodimerization of Toc33 at the chloroplast membrane was demonstrated by copper induced crosslinking [12]. Moreover, mutations reducing the affinity of homodimerization affect the import efficiency in *A. thaliana* plants [13]. This suggests that Toc33 is a GTPase regulated by dimerization [14]. The structural observations led to the proposal that GTP-regulated dimerization constitutes an element of the GTPase regulation within the translocon. Alternatively, it was proposed that the Toc33 homodimer mimics a heterodimeric GTPase complex between Toc33 and the highly homologous G domain of Toc159, a complex thought to be vital for translocation. Thus, the precise role of the Toc33 dimerization in the translocation reaction has not yet been defined.

One potential role of dimerization is to provide a reciprocal GAP function either in the homo- or the heterodimeric context. This hypothesis was based on the geometry of the active site, as a conserved arginine residue from one protomer was inserted into the active site of the other at a position near the  $\beta$ -phosphate of GDP. The positioning of this arginine residue is reminiscent of the interaction seen in GTPase/GAP complexes [e.g. Ras-like GTPases; 15]. However, experimental evidence indicates that the hydrolysis rate of GTP does not increase significantly at concentrations favoring the homodimeric state [16, 17]. It was speculated that a third molecule might be required as a "coGAP" or, alternatively, only the Toc33-Toc159 heterodimer might have the function of a mutual GAP complex (8). The larger G domain Toc159 receptor possesses an arginine residue at a similar position as Toc33 and additional insertions that may harbor the further needed catalytic amino acids (8). This concept would be consistent with the notion that both receptors preferably interact in the presence of GTP and precursor proteins [5].

Independent of a putative GAP function, Toc33 forms homodimers in the GDP loaded state *in vitro* [16] (see below). Furthermore, a stable interaction between Toc33 and a precursor protein in the context of the TOC complex could be detected by label-transfer experiments only in the presence of GDP, but not GTP [18]. In turn, reports have described an influence of the precursor protein on GTP hydrolysis of Toc33 [17,

19-22], an observation which cannot be reconciled directly with the GDP dependence of the label transfer. This prompted us to investigate a possible physiological function of Toc33 homodimers with respect to nucleotide exchange and precursor protein recognition.

For the biochemical analyses in this study, Toc33 was expressed in *E. coli* as a truncated version (*afToc33<sub>ΔC</sub>*), lacking the C-terminal membrane anchor. The truncated protein dimerizes in solution with low affinity [23], and thereby represents a suitable model to analyze dynamic interactions expected within the TOC complex. We demonstrate that the dissociation of GDP, and therefore nucleotide exchange is inhibited in the homodimeric state of *afToc33<sub>ΔC</sub>*. Dissociation of the homodimer is induced by the recognition of a precursor protein, which promotes binding of GTP. It was recently hypothesized that this might be required for a subsequent interaction of Toc33 with Toc159 [9]. Thus, the homodimeric conformation could reflect an inactive state of the translocon, preventing the initiation of GTP dependent downstream interactions that lead to pre-protein translocation across the outer membrane. By this we provide an example of GTPase regulation by dimerization which is distinct from the model presented for usual GADs (G proteins activated by nucleotide-dependent dimerization) [14].

## EXPERIMENTAL

### General

Protein expression and purification [19] was previously described. EDA-GTP-ATTO 550 (2'/3'-O-(2-Aminoethyl-carbamoyl)-Guanosine-5'-triphosphate, triethylammonium salt) and mantGDP (2'/3'-O-(N-Methyl-anthraniloyl)-guanosine-5'-diphosphate, triethylammonium salt) were obtained from Jena Bioscience (Jena, Germany). All calculations were performed with SigmaPlot (SPSS Inc.). Peptides were synthesized in the department of Peptide and Protein Chemistry at the Charité, Berlin. The sequences used are: B1 (derived from *Nicotiana tabacum* pSSU) - MVAPFTGLKSAA-S(PO<sub>3</sub>)-FPVSRKQNLDTSC [20]; control peptide: C - SKSMTEIEVTDVDMPC. Analytical ultracentrifugation of psToc34 was previously described [10].

### Nucleotide exchange on TOC GTPases

One hundred  $\mu$ M recombinant *atToc33*<sub>AC</sub> or *atToc33*<sub>AC</sub>-G1m, containing the triple mutation [G45R/K49N/S50R, see 24], were incubated on ice for 60 min in the presence of 1 mM GDP in HMK buffer (50 mM Hepes-KOH, pH 7.5, 2 mM MgCl<sub>2</sub>, 40 mM KAc). Free nucleotides were depleted by gel filtration [24]. The protein was then incubated at room temperature for 30 min to ensure complete hydrolysis of residual protein-bound GTP. The reactions were cooled to 0°C and incubated with 10  $\mu$ M [ $\alpha$ -<sup>32</sup>P]GTP (150 mCi/ $\mu$ mol) in the presence or absence of 5  $\mu$ M of urea-denatured (6M) recombinant pSSU. All samples contained a final urea concentration (<100 mM) to control for any effects of urea on intrinsic GTPase activity. A fraction of each reaction was blotted onto nitrocellulose membrane at the times indicated; the membrane was washed three times with 10 ml ice-cold HMK buffer containing 0.3% Tween-20 and subsequently air-dried. Bound [ $\alpha$ -<sup>32</sup>P]GTP was detected and quantified using a phosphor imager (Fuji Fla-5000) and Multi Gauge V2.02 software.

### Fluorescence GTP binding measurements

Binding of EDA-GTP-ATTO-550 to *atToc33*<sub>AC</sub> was determined in 20 mM Tris/HCl pH 8.0, 100 mM NaCl and 1 mM MgCl<sub>2</sub> with the indicated protein concentrations. Indicated amounts of GTP were produced by mixing GTP and EDA-GTP-ATTO-550 in a ratio of 1mM to 300  $\mu$ M. Fluorescence was excited by 554 nm and recorded at 576 nm. GTP binding by the monomeric species was analyzed by

$$k_{\text{apparent}} = k_{\text{diss}} + k_{\text{ass}} * [S] \quad \text{[E1]} \quad \text{and} \quad K_D = k_{\text{diss}} / k_{\text{ass}} \quad \text{[E2]}$$

with  $k_{\text{apparent}}$  as the apparent rate constant of GTP binding at a given GTP concentration,  $k_{\text{diss}}$  the dissociation rate of GTP,  $k_{\text{ass}}$  the association rate of GTP, [S] the GTP concentration and  $K_D$  the equilibrium dissociation constant.

### Analysis of dimerization by size exclusion chromatography

For molecular weight determination, 180  $\mu$ l of purified protein at indicated concentrations and in the presence of indicated concentrations of peptide was loaded onto a Superdex75 HR 10/300 gel filtration column (GE-Healthcare), equilibrated with 50 mM Tris/HCl at pH 8, containing 100 mM NaCl, 10 mM imidazole, 10 mM arginine, 5% glycerol and 1 mM MgCl<sub>2</sub>. Monomeric and dimeric species were analyzed by fitting two independent Gaussian distributions to the elution profile as described before [10, 23].

### Nucleotide dissociation measurements

*afToc33<sub>ΔC</sub>* was bound to Ni-NTA agarose and incubated over night with two column volumes of 1 mM GDP supplemented with 30 μM mantGDP in 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1 mM MgCl<sub>2</sub>, 10 mM imidazol, 5% (v/v) glycerol at 4 °C under constant agitation. The matrix was washed two times with the nucleotide free buffer before the protein was eluted with buffer supplemented with 500 mM imidazol. The dissociation rate of mantGDP from the protein was determined using Cary Eclipse fluorescence spectrometer (Varian) at 22 °C. Unlabeled nucleotides and peptides were first added to the cuvette and the baseline was recorded for 1 min. The measurement was initiated by the addition of mantGDP loaded protein. The fluorescence was excited at 355 nm and recorded at 448 nm for typically 10 minutes. Dissociation of GDP was analyzed by a single exponential equation. The dissociation rate ( $k_{\text{diss}}$ ) can be determined based on two mutually exclusive assumptions: (i) GDP can dissociate from both monomers and dimers with different rates or (ii) GDP dissociation occurs only in the monomeric state, implicating that the dissociation rate of the dimer determines the dissociation rate of GDP. Assuming that dissociation of the (homo-)dimer (*DIM*) is slower than dissociation of GDP one can write:



From this reaction scheme one can approximate

$$[F] = T * e^{-k_2 t} - D_0 * k_2 * (k_2 - k_1)^{-1} * (e^{-k_2 t} - e^{-k_1 t}) \quad [E3],$$

with  $F$  being the amount of *afToc33<sub>ΔC</sub>* without nucleotide,  $k_1$  being the dissociation rate of the dimer,  $k_2$  the dissociation rate of GDP and  $D_0$  the starting concentration of the dimer.

## RESULTS

### Pre-protein binding promotes nucleotide exchange on *atToc33*<sub>ΔC</sub>

The dimer interface of the TOC receptors is formed by several G-loops involved in nucleotide binding and a number of specific sequence elements [10, 11]. Thus upon Toc33 dimerization, GDP is bound within a cage, with no readily apparent route for nucleotide exchange [10, 11, 16]. Although it is unknown to date whether this structure represents only one example of a dynamic ensemble of possible modes of interactions occurring in the context of the TOC complex, this observation posed the question of whether nucleotide binding could be regulated by dimerization. To reconcile the pre-protein binding induced stimulation of GTPase activity of Toc33 [5, 19-21] with relevance to dimerization, we analyzed whether the pre-protein stimulates GTPase activity indirectly by dimer disruption, thereby promoting nucleotide exchange. As a first step to test this possibility, we examined the effects of pre-protein binding on GDP/GTP exchange of *atToc33*<sub>ΔC</sub>. We pre-incubated *atToc33*<sub>ΔC</sub> in buffer to promote hydrolysis of any residual bound GTP and subsequently with GDP to ensure that the maximum amount of protein was in its GDP-bound form. To assess nucleotide exchange, we incubated GDP-loaded *atToc33*<sub>ΔC</sub> with [ $\alpha$ -<sup>32</sup>P]GTP in the presence or absence of a urea-denatured precursor of the small subunit of Rubisco (pSSU), a well studied TOC model substrate. The reaction was performed at 0°C to minimize [ $\alpha$ -<sup>32</sup>P]GTP hydrolysis, and exchange was quantified by measuring protein-bound [ $\alpha$ -<sup>32</sup>P]GTP at various time points.

Low levels of [ $\alpha$ -<sup>32</sup>P]GTP binding to *atToc33*<sub>ΔC</sub> were detected in the absence of pSSU (Figure 1). In the presence of pSSU, the rate of nucleotide exchange increased significantly. The levels of bound [ $\alpha$ -<sup>32</sup>P]GTP in the presence of pSSU were 6-fold higher than in the absence of pSSU at the latest time point assayed (60 min). The same holds true for the rate of GTP binding, which is 10 fold lower in the absence of pSSU as determined by least square fit analysis using a single exponential equation (Figure 1). [ $\alpha$ -<sup>32</sup>P]GTP binding to pSSU alone or to *atToc33*<sub>ΔC</sub>-G1m, a receptor that lacks nucleotide binding, were undetectable, indicating that [ $\alpha$ -<sup>32</sup>P]GTP binding was specific to native *atToc33*<sub>ΔC</sub>. Thus, the previously reported stimulation of Toc33 GTP hydrolysis after pre-protein binding might be a result of an accelerated GDP/GTP exchange rates or an increased GTP affinity [5, 19-21, 25]. We therefore went on to dissect the influence of dimerization and precursor binding on the nucleotide binding of the Toc33 GTPase.

### GTPase dimerization reduces the rate of nucleotide exchange

First, we determined the apparent rate constants for association of purified *atToc33*<sub>ΔC</sub> (Figure 2a) with fluorescent EDA-GTP-ATTO at two different protein concentrations: 4 μM, containing predominantly monomeric receptor, and 200 μM with estimated 40% of the protein in the homodimeric conformation according to the established  $K_D$  [23].

The apparent rates ( $k_{apparent}$  in equation [E1,2]) were determined by a least square fit analysis to a single exponential function for the reaction with 4 μM and two independent exponential functions for the reaction with 200 μM protein (Figure 2b). For the latter we assumed that the dimeric and monomeric species bind GTP independently from each other, although co-operativity of the association of the two GTP molecules within the dimer cannot be excluded at this stage. The residuals of the least square fit analysis justify the usage of a double exponential (Figure 2b, inset). The apparent rates of GTP binding for the monomeric (Figure 2c, white circles for the 200 μM protein and black circles for the 4 μM protein) and dimeric *atToc33*<sub>ΔC</sub> (grey circles for 200 μM protein) are

linearly dependent on the concentration of the ligand. Analysis of the plots by equation [E1,E2] revealed an association rate constant ( $k_{ass}$ ) of  $4.9 \text{ M}^{-1}\text{s}^{-1}$  for the monomers and  $1.3 \text{ M}^{-1}\text{s}^{-1}$  for the dimeric species. Thus, the monomeric species has an approximately four-fold higher association rate constant for EDA-GTP-ATTO than the dimeric species (Figure 2c). Considering that recombinantly expressed *afToc33<sub>ΔC</sub>* is almost exclusively GDP loaded [10] and that GDP release from GTPases is thought to be the rate limiting step for nucleotide exchange [e.g. 26], the observed kinetics of EDA-GTP-ATTO binding is therefore most likely dependent on the velocity of GDP dissociation.

To quantify this effect, we investigated the influence of dimerization on GDP dissociation from purified *afToc33<sub>ΔC</sub>* (Figure 2a). The receptor was preloaded with mantGDP according to the protocol described in the experimental section. mantGDP dissociation was determined by the decay of fluorescence. The rates obtained in multiple measurements were plotted against the concentration of *afToc33<sub>ΔC</sub>* (Figure 3a, black circles). The highest mantGDP dissociation rate is found at the lowest receptor concentration and the rate is reduced at higher protein concentrations. This suggests a dimerization-dependent reduction of the GDP dissociation rate.

An opportunity to corroborate this observation was offered by the distinct physicochemical properties of *psToc34<sub>ΔC</sub>*, the functional orthologue of *afToc33<sub>ΔC</sub>* from pea (see above). Heterologously produced *psToc34<sub>ΔC</sub>* has an equilibrium dissociation constant for dimerization of about  $50 \text{ μM}$ , which is nearly 8 fold lower than observed for *afToc33<sub>ΔC</sub>* [10, 17, 23]. The reason for the discrepancy between the two proteins from different sources is, however, not yet understood. Nevertheless, here it is an advantage because we can use this information to confirm the relation between GDP release and dimerization. When mantGDP dissociation from purified *psToc34<sub>ΔC</sub>* (Figure 2a) was measured (Figure 3a, grey circles), the rates were reduced by increasing protein concentrations, consistently with the results obtained for *afToc33<sub>ΔC</sub>*. Comparison of this concentration-dependent effect between both proteins reveals a shift to lower concentrations for *psToc34<sub>ΔC</sub>*, which can be explained by the higher affinity of *psToc34<sub>ΔC</sub>* for homodimerization.

The suggested relation between GDP release and homodimerization was further substantiated by the analysis of dimerization inhibited variants of *afToc33* and *psToc34*, *afToc33R130A* and *psToc34R133A* [1, 17, 25, 27], which are able to bind nucleotides [16]. The GDP dissociation rate of these two proteins (Figure 2A) differs with respect to the source organism (Figure 3b), which parallels the observed distinct physicochemical properties of the native proteins (Figure 3a). However, the GDP dissociation rate of each of the mutants is independent of protein concentration in the range analyzed (Figure 3b). This observation supports the conclusion that dimerization reduces nucleotide release from the wild type protein. Following this notion, homodimer dissociation would be rate limiting for GDP dissociation.

To challenge this assumption by numerical analysis, a model representing a unidirectional reaction scheme was applied (equation [E3]). The model considers the transition of the nucleotide bound protein from its dimeric to its monomeric state and the subsequent dissociation of the nucleotide (excluding GDP dissociation from dimer).

This analysis reveals that both the rate constants for GDP dissociation from the monomeric receptor ( $k_2$ , [E3]) and dissociation of the homodimer ( $k_1$ , [E3]) are not altered by varying protein concentration (values listed in Table 1). Furthermore, the GDP dissociation rate constant determined by this approach resembles that of the monomeric species. In addition, the rate constant for *afToc33<sub>ΔC</sub>* homodimer dissociation is consistent with previous results [19]. By comparison of the rate constant for dimer and

nucleotide dissociation we can conclude that dissociation of the homodimer is the rate limiting step for GDP dissociation. In other words, the dimeric state prevents exchange of GDP by GTP, which is consistent with the occlusion of the nucleotide-binding pocket as observed in dimeric receptor structures [10, 11].

### **Precursor recognition disturbs dimerization and induces nucleotide release**

It has been shown that the transit peptide promotes an interaction between Toc33 and Toc159 when the receptors are in their GMP-PNP-bound forms [5]. In addition, our data indicate that pre-protein binding modulates receptor GTPase activity by stimulating nucleotide exchange [Fig. 1; e.g. 19]. This prompted us to analyze the influence of peptides representing the pSSU transit sequence on dimerization and nucleotide exchange by *at*Toc33<sub>ΔC</sub> or *ps*Toc34<sub>ΔC</sub>. For that purpose, a 26 amino acid peptide with a phosphoserine at position 13 exhibiting the highest affinity for *ps*Toc34<sub>ΔC</sub> in previous studies [B1; 20] was chosen. The same peptide was also used to study the interactions between Toc33 and Toc159 [5; therein referred to as B2].

Dimerization of 170 μM *ps*Toc34<sub>ΔC</sub> and 500 μM *at*Toc33<sub>ΔC</sub> was analyzed by size exclusion chromatography [16, 23] in the absence or presence of B1 peptide. The different concentrations were chosen to warrant a comparable amount of dimeric species for the two proteins with different dissociation constants for homodimerization (see above). We observed a peptide concentration dependent destabilization of the *at*Toc33<sub>ΔC</sub> and *ps*Toc34<sub>ΔC</sub> homodimer (Figure 4a, black circles, grey triangles). Remarkably, the concentration of the peptide at which 50% of the destabilization is observed (~200 μM) is comparable to the previously observed  $K_D$  for the interaction between B1 and *ps*Toc34<sub>ΔC</sub> [20]. In contrast to the influence observed for B1 on homodimerization, addition of a control (C, sequence given in experimental) peptide at the highest concentration used for B1 did not result in disturbance of the homodimer (Figure 4a, white circles).

Guided by this observation we next analyzed the influence of the peptide on GDP dissociation from the receptor. We observed a rather slow dissociation of mantGDP from the monomeric *at*Toc33<sub>ΔC</sub> (11 μM, according to the established  $K_D$ ) when no competing nucleotide was present (Figure 4b, black). The dissociation rate of the labeled nucleotide was increased in the presence of excessive unlabelled nucleotide due to the repressed rebinding of mantGDP (Figure 4b, black vs. grey). Addition of either control peptide (C) or B1 peptide to *at*Toc33<sub>ΔC</sub> in the presence of excessive unlabelled nucleotide did not alter the rate or amount of mantGDP released (Figure 4b, green & red). Therefore, we can conclude that the peptide does not influence the release of mantGDP from the monomeric receptor.

Next we determined the mantGDP release from *at*Toc33<sub>ΔC</sub> at a concentration of 330 μM containing about 45% of the receptor in a dimeric conformation (according to the established  $K_D$ ). Again, addition of unlabelled nucleotide (Figure 4c, black vs. grey) enhanced the dissociation rate due to the repressed rebinding of mantGDP. Addition of the control peptide did not further influence the release, neither in terms of the rate nor in terms of the amount of released mantGDP (Figure 4c, grey vs. green). In contrast, addition of the peptide B1 resulted in an increase of the total amount of released nucleotide in the course of the measurement (Figure 4c, grey vs. red). This increase of GDP release in the presence of B1 can be explained by its ability to enhance the dissociation rate of the homodimer (Figure 4a), thereby abrogating its rate-limiting role on nucleotide exchange.

To verify the influence of the peptide on nucleotide exchange we have added the peptide near the plateau phase of mantGDP release from *atToc33<sub>ΔC</sub>* after about 600 sec (Figure 4d). Addition of the control peptide (C) or buffer (Bu) did not alter the equilibrium, but once again B1 induced a significant release of mantGDP. This confirms that dimerization and, in consequence, nucleotide exchange are influenced by the transit peptide.

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## DISCUSSION

In the past several properties have been attributed to the homodimerization of Toc33, which is of functional relevance as documented *in vivo* and *in vitro* [12, 13]. In contrast to GADs described so far [14], homodimerization was not found to be GTP dependent and does not directly contribute to GTP hydrolysis [e.g. 10, 17] raising the question by which functional mechanism homodimerization contributes to the regulation of protein translocation. Our data indicate that dimerization decelerates the rate of GTP binding (Fig. 2) and GDP release (Fig. 3) by capturing the nucleotide in a cage at the dimer interface. In the case of monomeric Ras-like GTPases, an analogous function is fulfilled by GDP dissociation inhibitors [GDI, e.g. 26, 28, 29], which have to be replaced by a GDI-displacement factor [GDF; e.g. 30, 31] prior to nucleotide exchange. Thus, a function comparable to that of a GDF is accomplished by the receptor substrate, the transit peptide of the pre-protein. The peptide alters the kinetic properties of homodimerization and the affinity of this reaction results in an enhanced GDP release (Fig. 3). The transit peptide does not induce the release of the bound GDP from the monomeric receptor directly and thereby does not function as a classical nucleotide exchange factor (GEF). Thus, one has to conclude that the influence of the transit peptide on nucleotide exchange appears to result from an indirect effect on Toc33 dimerization as determined by size exclusion chromatography. However, this technique only senses the influence of the substrate on the dissociation rate ( $k_{diss}$  in equation E2) of the homodimer. Conclusions regarding the equilibrium dissociation constant ( $K_D$ , see [E2]) cannot be drawn because the monomers are immediately removed from the monomer/dimer equilibrium in the course of the chromatographic separation process. Indeed, addition of the transit peptide did not influence the dissociation constant determined by analytical ultracentrifugation (not shown). Thereby, the association rate constant appears to be altered to the same extent as the dissociation rate constant [E2]. A similar observation was made for the SRP-RNA, which kinetically controls the dimerization between Ffh and FtsY. Here the association rate ( $k_{ass}$ ) is accelerated to the same extent as the rate of dimer dissociation ( $k_{diss}$ ) resulting in the same equilibrium dissociation constant ( $K_D$ ) as in the absence of RNA [32]. Analogously, the transit peptide seems not to influence the affinity of heterodimerization but to lower the energetic barrier of dissociation. Expanding the current categorization of dimeric GTPases [14], we propose considering Toc33 as GID (G protein inhibited by nucleotide-dependent dimerization).

How can these observations be reconciled with current models of TOC receptor function? *In vitro* experiments suggest that Toc33 dimerization is not nucleotide dependent [9], although a slight preference for homodimerization in the GDP loaded state exists [10]. Initial recognition of precursor proteins by a GDP loaded Toc33 is concordant with chemical crosslinking experiments, where – in the context of the TOC complex – an interaction between Toc33 and the transit peptide was only observed in the presence of GDP, while GTP promotes its transfer to Toc159 [5, 18, 23]. Albeit speculative, precursor binding by the homodimeric receptor would enhance the association and dissociation rate of dimerization, resulting in nucleotide exchange driven by a high intracellular GTP to GDP ratio [33]. This in turn would promote heterodimerization with GTP loaded Toc159 [5], and subsequent GTP hydrolysis would ensure its dissociation again. The switch from homodimeric to the heterodimeric state and GTP hydrolysis are envisioned to initiate pre-protein translocation [34] and reformation of the Toc33<sub>GDP</sub> dimer.

The data presented herein, the structural interpretations given earlier [12, 15] and other experimental findings [e.g. 17 and references therein] all suggest that the Toc33

dimer exists in a GDP loaded ground state. However, a different scenario *in planta* cannot be ruled out, e.g. a mixed GTP/GDP loaded dimer. Our current hypothesis of the cooperative mechanism for Toc33 and Toc159 in precursor transfer proposes that the Toc33 homodimer is disrupted by the pre-protein to allow one Toc33 monomer to interact with Toc159. The role of the second Toc33 monomer is unclear, but this mechanism could account for the molar excess of Toc33 relative to Toc159 within TOC complexes [35-37]. Hence, an initial understanding of the molecular events of the TOC translocon emerges, and this model can now be tested by *in vivo* experiments.

#### **AUTHOR CONTRIBUTION**

Mislav Oreb, Anja Höfle, Patrick Koenig, Fei Wang and Maik S. Sommer conducted the experiments presented. Mislav Oreb, Irmgard Sinning, Ivo Tews, Danny J. Schnell and Enrico Schleiff were involved in the experimental design and data analysis. All authors were involved in manuscript preparation, which was drafted by Enrico Schleiff.

#### **ACKNOWLEDGEMENTS**

We thank Giesela English (Goethe University, Frankfurt) for constant technical assistance. Further, we thank Thorsten Hugel (Technical University, Munich) for constant support and critical discussions and Orla Slattery (Goethe University, Frankfurt) for helpful comments on the manuscript.

#### **FUNDING**

This work was supported by grants from the Deutsche Forschungsgemeinschaft in the frame of the SFB-807 (P17, to AH, MSS and ES), from the Center of Excellence "Macromolecular Complexes" Frankfurt (to PK and ES), from the Volkswagenstiftung (to MO, and ES) and from the interdisciplinary PhD program "Molecular Machines: Mechanisms and Functional Interconnections" of the Land Baden-Württemberg (to PK and IS).

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## TABLES

**Table 1. The dissociation rate constants for GDP release and dimer dissociation.**

Protein		Rate constant $k_{\text{diss}}$ (sec <sup>-1</sup> )*
<i>afToc33</i> <sub>ΔC</sub>	GDP <sup>a</sup>	$1.89 * 10^{-2}$
	GDP [E2] <sup>b</sup>	$(1.87 \pm 0.05) * 10^{-2}$
	dimer [E2] <sup>b</sup>	$(3.2 \pm 0.1) * 10^{-3}$
<i>afToc33</i> <sub>ΔC</sub> R130A	GDP <sup>a</sup>	$(1.90 \pm 0.04) * 10^{-2}$
	GDP <sup>a</sup>	$3.3 * 10^{-3}$
<i>psToc34</i> <sub>ΔC</sub>	GDP [E2] <sup>b</sup>	$(3.4 \pm 0.1) * 10^{-3}$
	dimer [E2] <sup>b</sup>	$(4.5 \pm 0.5) * 10^{-4}$
<i>psToc34</i> <sub>ΔC</sub> R133A	GDP <sup>a</sup>	$(4.1 \pm 0.1) * 10^{-3}$

\*Constants are calculated from results presented in Fig. 2A and C for wild type considering only the low receptor concentrations (a) and Fig. 2B and D according to the indicated equation (b).

## FIGURE LEGENDS

### Figure 1 The precursor pSSU stimulates GTP binding by Toc33.

GTP binding to 100 $\mu$ M *afToc33 $\Delta$ C* (circles), 100 $\mu$ M *afToc33 $\Delta$ C* in the presence of pSSU (triangles), 100 $\mu$ M *afToc33 $\Delta$ C*-G1m mutant (G45R/K49N/S50R; squares), 100 $\mu$ M *afToc33 $\Delta$ C*-G1m in the presence of pSSU (diamonds) or pSSU (hexagons) was determined. Recombinant protein was incubated at 25°C for 30 min to ensure complete hydrolysis of bound GTP. The reaction was cooled to 0°C and incubated with [ $\alpha$ -<sup>32</sup>P]GTP (150 mCi/ $\mu$ mol) for 0, 15, 30 or 60 minutes. Subsequently, a fraction of the incubation was blotted onto cellulose membrane, washed to remove unbound nucleotide, and bound [ $\alpha$ -<sup>32</sup>P]GTP was measured by phosphor imaging. The values are expressed in arbitrary units and the error bars reflect the standard deviation of three independent experiments. The lines represent the least square fit analysis to a single exponential function.

### Figure 2 GTP association depends on the protein concentration.

(a) Shown is an example of the isolated proteins (5 $\mu$ g) used in this study separated on SDS-PAGE and stained by Coomassie-Blue. The migration of the molecular weight standards is indicated on the left (in kDa). (b) Binding of 2 mM GTP supplemented with EDA-GTP-ATTO-550 to 4  $\mu$ M or 200  $\mu$ M *afToc33 $\Delta$ C* (concentration is indicated) was followed by fluorescence intensity increase (grey lines). The kinetics of GTP association was analyzed by a single exponential function (black line for 4 $\mu$ M) or two independent exponential functions (black line for 200 $\mu$ M, assuming two independent rate constants for the monomeric and dimeric receptor species) yielding P values < 0.0001. The inset shows the residuals for the analysis of the binding curve to *afToc33 $\Delta$ C* at 200 $\mu$ M using the single exponential (black line) or double exponential (grey line) equation. (c) Rate constants for GTP binding were determined from kinetic experiments at 4  $\mu$ M (white circles) or 200  $\mu$ M *afToc33 $\Delta$ C* (black circles for monomeric and grey circles for dimeric species) as shown in (b). Lines show the least square fit analysis to [E1,E2] for the monomeric (m) and dimeric (d) rate constants. The inset shows the logarithmic representation to visualize the distribution of the spots at low GTP concentrations. Dotted line in the inset indicates the region of extrapolation. For better readability the error bars are not shown which are smaller than 10% of the value shown.

### Figure 3 Dimerization reduces GDP dissociation.

(a) The rates of mantGDP dissociation determined by single exponential analysis are shown for *afToc33 $\Delta$ C* (black circles, left axis) and *psToc34 $\Delta$ C* (grey circles, right axis). The black and the grey arrow indicate the concentration corresponding to the  $K_D$  for dimerization of *afToc33 $\Delta$ C* and *psToc34 $\Delta$ C*, respectively. (b) The rates of mantGDP dissociation determined by single exponential analysis are shown for *afToc33 $\Delta$ C*(R130A) (black triangles, on y-axis 10<sup>2</sup>) and *psToc34 $\Delta$ C*(R133A) (grey triangles, on y-axis 10<sup>3</sup>). The average value is indicated as dashed line for *afToc33 $\Delta$ C*(R130A) and *psToc34 $\Delta$ C*(R133A), respectively. The dissociation rates of the *afToc33 $\Delta$ C* or *psToc34 $\Delta$ C* dimer and of GDP according to equation [E3] were calculated and are listed in Table 1.

**Figure 4 Influence of a transit peptide sequence on dimerization and nucleotide release.**

(a) *afToc33<sub>ΔC</sub>* (180 μl of 500 μM concentration, black circles) or *psToc34<sub>ΔC</sub>* (180 μl of 170 μM concentration, grey triangles) were subjected to size exclusion chromatography in the absence or presence of B1 peptide at indicated concentrations. For control, *afToc33<sub>ΔC</sub>* (180 μl of 500 μM concentration) was incubated with the control peptide C at indicated concentration (white circles). The absorption profiles were analyzed by two Gaussian distributions, the dimeric fraction was calculated from their area. The decrease of dimeric population was analyzed by a hyperbolic function. (b, c) The dissociation of mantGDP from 11 μM (a) or 330 μM *afToc33<sub>ΔC</sub>* (b) was determined by fluorescence (in arbitrary units) in absence (-, black) or presence of 1 mM GDP (Bu, GDP, grey). In addition to GDP, 1 mM control peptide (C, green) or B1 peptide (B1, red) were added. (d) The dissociation of mantGDP from 600 μM *afToc33<sub>ΔC</sub>* was determined by fluorescence and identical volumes of buffer (Bu, grey), control peptide (C, final concentration 1 mM) or B1 peptide (B1, final concentration 1 mM) were added at the time point indicated.

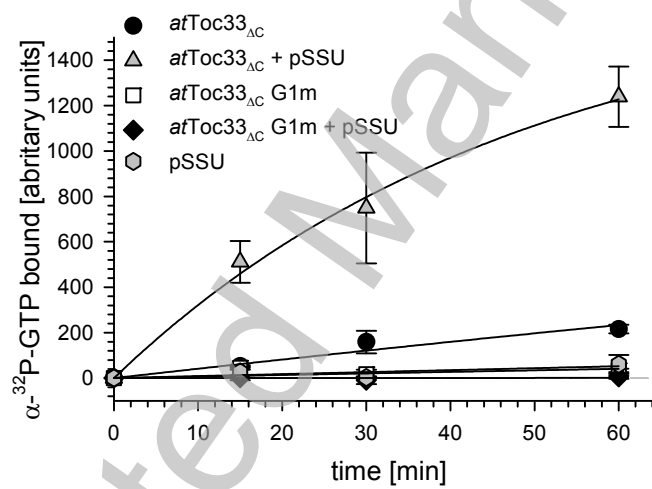


Figure 1, Oreb et al.

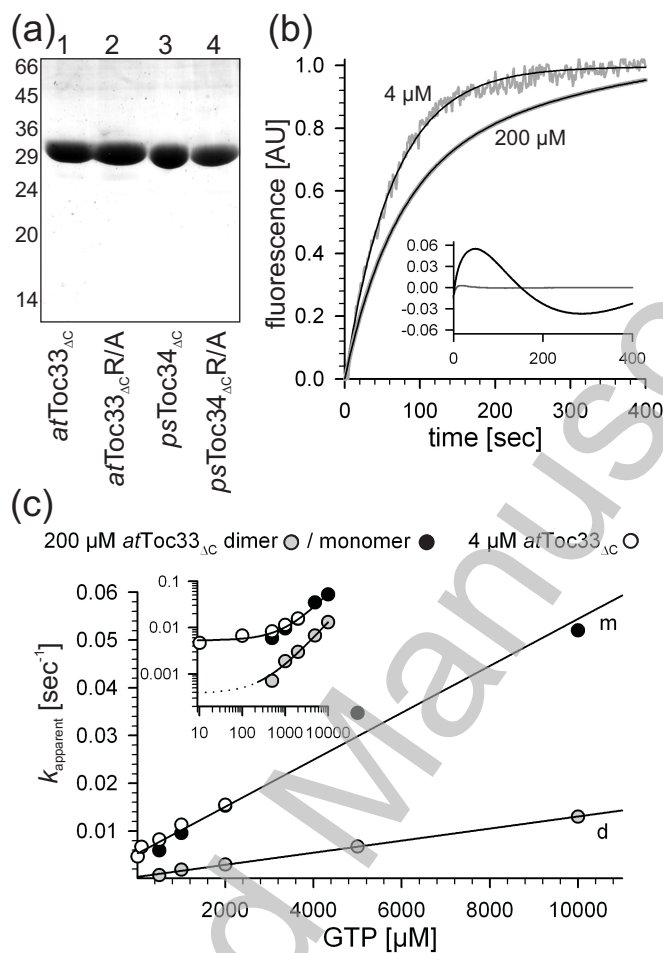


Figure 2, Oreb et al.

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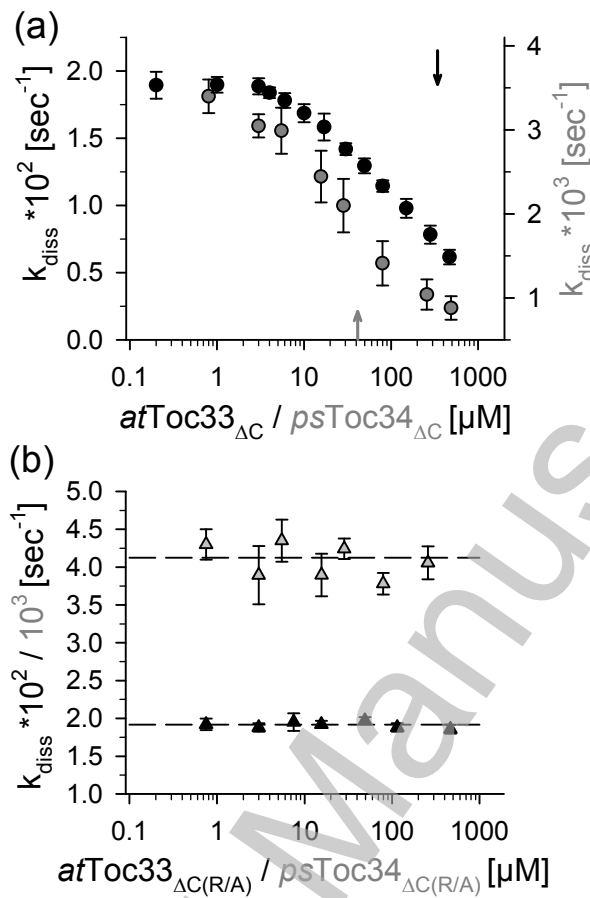


Figure 3, Oreb et al.

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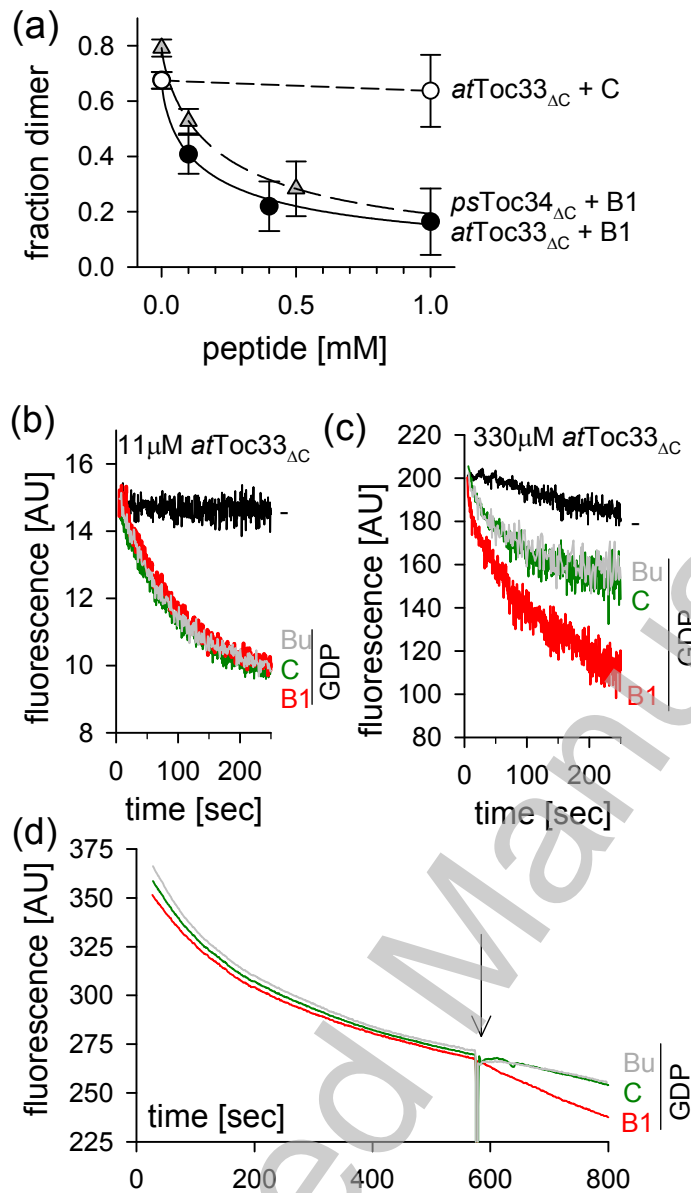


Figure 4, Oreb et al.

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