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online commentary

Substrate binding disrupts dimerization and induces nucleotide exchange of the chloroplast GTPase Toc33

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GTPases act as molecular switches to control many cellular processes, including signalling, 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 TOC/TIC (translocator at the outer envelope membrane of chloroplasts/inner envelope membrane of chloroplasts)] 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. In the present study, 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 discussed many times previously, but to control the nucleotide-loading state. We discuss this novel regulatory mode and its impact on the current models of protein import into the chloroplast.

Key words: dimeric GTPase, GDP-dissociation-inhibitor function (GDI function), G-protein, 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 (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 chloroplast 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 a transit peptide, is performed by two GTP-dependent receptors, namely Toc159 and Toc33 (translocator at the outer envelope membrane of chloroplasts of 159 or 33 kDa respectively) [6,7]. These are thought to co-operatively 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 are not well understood.

The crystal structures of the GTPase domains (abbreviated in the present paper as G domains) of *at*Toc33 (*Arabidopsis thaliana* Toc33) and its orthologue *ps*Toc34 (*Pisum sativum* Toc34) revealed a homodimeric interaction [10,11]. Indeed, homodimerization of Toc33 at the chloroplast membrane was demonstrated by copper-induced cross-linking [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 hetero-dimeric context. This hypothesis was based on the geometry of the active

Abbreviations used: EDA-GTP-ATTO-550, 2′/3′-*O*-(2-aminoethyl-carbamoyl)-GTP labelled with ATTO-550, triethylammonium salt; GAD, G-protein activated by nucleotide-dependent dimerization; GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GDF, GDI-displacement factor; mantGDP, 2′/3′-*O*-(*N*-methylanthraniloyl)-GDP, triethylammonium salt; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; pSSU, precursor of the small subunit of Rubisco; TOC/Toc, translocator at the outer envelope membrane of chloroplasts.

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site, as a conserved arginine residue from one protomer was inserted into the active site of the other at a position near the β-phosphate of GDP. The positioning of this arginine residue is reminiscent of the interaction seen in GTPase–GAP (GTPase-activating protein) complexes (e.g. Ras-like GTPases [15]). However, experimental evidence indicates that the hydrolysis rate of GTP does not increase significantly at concentrations favouring the homodimeric state [16,17]. It was speculated that a third molecule might be required as a 'co-GAP' or, alternatively, only the Toc33–Toc159 heterodimer might have the function of a mutual GAP complex [10]. The larger-G-domain Toc159 receptor possesses an arginine residue at a similar position to Toc33 and additional insertions that may harbour the further-needed catalytic amino acids [10]. 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 that 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 the present study, Toc33 was expressed in *Escherichia coli* as a truncated version (*at*Toc33$_{\Delta C}$), lacking the C-terminal membrane anchor. The truncated protein dimerizes in solution with low affinity [23] and thereby represents a suitable model to analyse 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 *at*Toc33$_{\Delta C}$. 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. In the present study, we provide an example of GTPase regulation by dimerization that is distinct from the model presented for usual GADs (G-proteins activated by nucleotide-dependent dimerization) [14].

EXPERIMENTAL

General

Protein expression and purification was performed as described previously [19]. EDA-GTP-ATTO-550 [2′/3′-*O*-(2-aminoethylcarbamoyl)-GTP, triethylammonium salt] and mantGDP [2′/3′-*O*-(*N*-methylanthraniloyl)-GDP, triethylammonium salt] were obtained from Jena Bioscience. All calculations were preformed with SigmaPlot (SPSS). Peptides were synthesized in the Department of Peptide and Protein Chemistry, Charité – Universitätsmedizin Berlin, Berlin, Germany. The sequences used were: B1 [derived from *Nicotiana tabacum* pSSU (precursor of the small subunit of Rubisco, where Rubisco is ribulose-1,5-bisphosphate carboxylase/oxygenase)], MVAPFTGLKSAA-S(PO_3)-FPVSRKQNLDITSC [20]; and C (control peptide), SKS-MTEIEVTDVDMPC. Analytical ultracentrifugation of *ps*Toc34 was as described previously [10]. The proteins analysed in the present study have the following accession numbers: *at*Toc33, GenBank® accession number NP_001117215.1 or TAIR (The *Arabidopsis* Information Resource; http://www.arabidopsis.org) accession number AT1G02280; *ps*Toc34, Swiss-Prot accession number Q41009.1.

Nucleotide exchange on TOC GTPases

Recombinant *at*Toc33$_{\Delta C}$ or *at*Toc33$_{\Delta C}$-G1m (containing the triple mutation G45R/K49N/S50R; see [24]) (100 μM) 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 $MgCl_2$ and 40 mM potassium acetate). Free nucleotides were depleted by gel filtration [24]. The protein was then incubated at room temperature (22 °C) for 30 min to ensure complete hydrolysis of residual protein-bound GTP. The reactions were cooled to 0 °C and incubated with 10 μM [α-^{32}P]GTP (150 mCi/μmol) in the presence or absence of 5 μM of urea-denatured (6 M) recombinant pSSU. For comparability, all samples contained a final urea concentration of 100 mM. A fraction of each reaction was blotted on to a nitrocellulose membrane at the times indicated; the membrane was washed three times with 10 ml of ice-cold HMK buffer containing 0.3 % Tween 20 and subsequently air-dried. Bound [α-^{32}P]GTP was detected and quantified using a FLA-5000 PhosphorImager (Fuji) and Multi Gauge V2.02 software.

Fluorescence GTP-binding measurements

Binding of EDA-GTP-ATTO-550 to *at*Toc33$_{\Delta C}$ was determined in 20 mM Tris/HCl, pH 8.0, 100 mM NaCl and 1 mM $MgCl_2$ with the indicated protein concentrations. Indicated amounts of GTP were produced by mixing GTP and EDA-GTP-ATTO-550 at a 1 mM:300 μM ratio. Fluorescence was induced by excitation at 554 nm and was recorded at 576 nm. GTP binding by the monomeric species was analysed by eqn (1) and eqn (2):

$$k_m = k_{diss} + (k_{ass} \times [S]) \quad (1)$$

and

$$K_d = \frac{k_{diss}}{k_{ass}} \quad (2)$$

where k_m is the measured rate constant of GTP binding at a given GTP concentration, k_{diss} is the dissociation rate of GTP, k_{ass} is the association rate of GTP, [S] is the GTP concentration and K_d is the equilibrium dissociation constant.

Analysis of dimerization by size-exclusion chromatography

For molecular-mass determination, 180 μl of purified protein at the indicated concentrations and in the presence of the indicated concentrations of peptide was loaded on to a Superdex 75 HR 10/300 gel-filtration column (GE Healthcare) equilibrated with 50 mM Tris/HCl, pH 8, containing 100 mM NaCl, 10 mM imidazole, 10 mM arginine, 5 % (v/v) glycerol and 1 mM $MgCl_2$. Monomeric and dimeric species were analysed by fitting two independent Gaussian distributions to the elution profile as described previously [10,23].

Nucleotide-dissociation measurements

*at*Toc33$_{\Delta C}$ was bound to Ni-NTA (Ni^{2+}-nitrilotriacetate)–agarose and incubated overnight with two column volumes of 1 mM

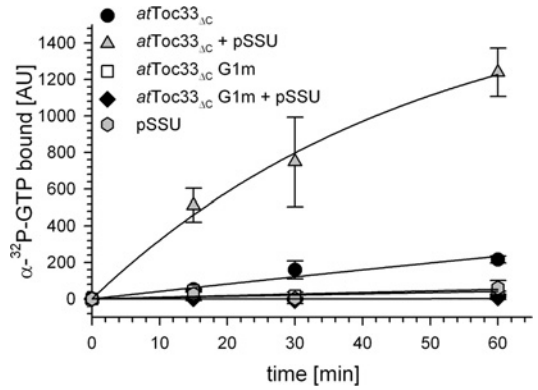

Figure 1 The precursor pSSU stimulates GTP binding by Toc33

GTP binding to 100 μM *at*Toc33$_{\Delta C}$ (circles), 100 μM *at*Toc33$_{\Delta C}$ in the presence of pSSU (triangles), 100 μM *at*Toc33$_{\Delta C}$-G1m mutant (G45R/K49N/S50R; squares), 100 μM *at*Toc33$_{\Delta C}$-G1m in the presence of pSSU (diamonds) or 5 μM 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 [α-^{32}P]GTP (150 mCi/μmol) for 0, 15, 30 or 60 min. Subsequently, a fraction of the incubation was blotted on to a cellulose membrane, washed to remove unbound nucleotide, and bound [α-^{32}P]GTP was measured by phosphorimaging. Values are means ± S.D. for three independent experiments. The lines represent the least-squares-fit analysis to a single exponential function. AU, arbitrary units.

GDP supplemented with 30 μM mantGDP in 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1 mM $MgCl_2$, 10 mM imidazole and 5 % (v/v) glycerol at 4 °C under constant agitation. The matrix was washed twice with 50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 1 mM $MgCl_2$, 10 mM imidazole and 5 % (v/v) glycerol before the protein was eluted with buffer supplemented with 500 mM imidazole. The dissociation rate of mantGDP from the protein was determined using a Cary Eclipse fluorescence spectrometer (Varian) at 22 °C. Unlabelled 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. Fluorescence was induced by excitation at 355 nm and was recorded at 448 nm for typically 10 min. Dissociation of GDP was analysed by a single exponential equation. The dissociation rate (k_{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, implying that the dissociation rate of the dimer determines the dissociation rate of GDP. Assuming that the dissociation of the (homo)dimer (DIM) is slower than the dissociation of GDP it follows that:

$$DIM_{GDP} \xrightarrow{k_1} MON_{GDP} \xrightarrow{k_2} MON + GDP$$

where DIM_{GDP} is dimer with bound GDP, MON_{GDP} is MON (monomer) with bound GDP, k_1 is the dissociation rate of the dimer, and k_2 is the dissociation rate of GDP from the monomer.

From this reaction scheme, eqn (3) can be approximated:

$$[F] = (T \times e^{-k_2 t}) - \left(D_0 \times k_2 \times \frac{1}{k_2 - k_1}\right) \times (e^{-k_2 t} - e^{-k_1 t}) \quad (3)$$

where F is the amount of *at*Toc33$_{\Delta C}$ without nucleotide and D_0 is the starting concentration of the dimer.

RESULTS

Pre-protein binding promotes nucleotide exchange on *at*Toc33$_{\Delta C}$

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 to date it is unknown 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 the relevance to dimerization, we analysed 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 *at*Toc33$_{\Delta C}$. We pre-incubated *at*Toc33$_{\Delta C}$ 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 *at*Toc33$_{\Delta C}$ with [α-^{32}P]GTP in the presence or absence of urea-denatured pSSU, a well-studied TOC model substrate. The reaction was performed at 0 °C to minimize [α-^{32}P]GTP hydrolysis, and exchange was quantified by measuring protein-bound [α-^{32}P]GTP at various time points.

Low levels of [α-^{32}P]GTP binding to *at*Toc33$_{\Delta C}$ 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 [α-^{32}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 was 10-fold lower in the absence of pSSU as determined by least-squares-fit analysis using a single exponential equation (Figure 1). [α-^{32}P]GTP binding to pSSU alone or to *at*Toc33$_{\Delta C}$-G1m, a receptor that lacks nucleotide binding, was undetectable, indicating that [α-^{32}P]GTP binding was specific to native *at*Toc33$_{\Delta C}$. Thus the previously reported stimulation of Toc33 GTP hydrolysis after pre-protein binding might be a result of an accelerated GDP/GTP exchange rate or an increased GTP affinity [5,19–21,25]. Therefore we proceeded 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 *at*Toc33$_{\Delta C}$ (Figure 2a) with fluorescent EDA-GTP-ATTO-550 at two different protein concentrations: 4 μM, containing predominantly monomeric receptor, and 200 μM, with an estimated 40 % of the protein in the homodimeric conformation according to the established K_d [23].

The apparent rates (k_m in eqns 1 and 2) were determined by a least-squares-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-squares-fit analysis justify the use 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 *at*Toc33$_{\Delta C}$ (Figure 2c,

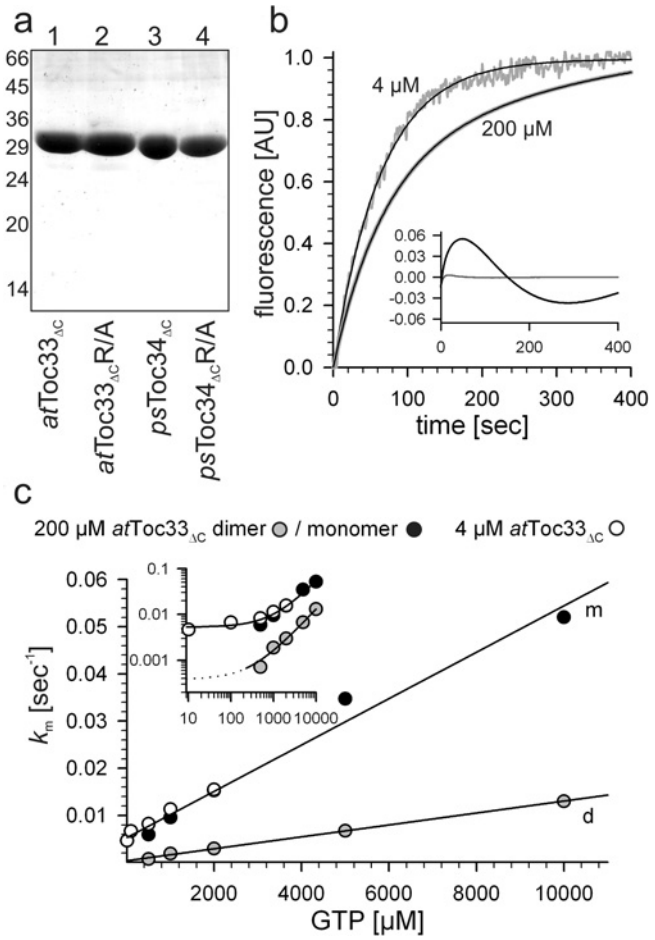

Figure 2 GTP association depends on the protein concentration

(**a**) Example of the isolated proteins (5 μg) used in the present study separated by SDS/PAGE and stained by Coomassie Blue. The migration of the molecular-mass standards is indicated on the left-hand side (in kDa). (**b**) Binding of 2 mM GTP supplemented with EDA-GTP-ATTO-550 to 4 μM or 200 μM *at*Toc33$_{\Delta C}$ was followed by the increase in fluorescence intensity (grey lines). The kinetics of GTP association was analysed by a single exponential function (black line for 4 μM) or two independent exponential functions (black line for 200 μM, 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 *at*Toc33$_{\Delta C}$ at 200 μM 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 μM (white circles) or 200 μM *at*Toc33$_{\Delta C}$ (black circles for monomeric species and grey circles for dimeric species) as shown in (**b**). Lines show the least-squares-fit analysis to eqns (1) and (2) for the monomeric (m) and dimeric (d) rate constants. The inset shows a logarithmic representation to visualize the distribution of the spots at low GTP concentrations. The dotted line in the inset indicates the region of extrapolation. For better readability, error bars are omitted (these are <10 % of the values shown).

grey circles for 200 μM protein) were linearly dependent on the concentration of the ligand. Analysis of the plots by eqns (1) and (2) revealed an association rate constant (k_{ass}) of 4.9 $M^{-1} \cdot s^{-1}$ for the monomers and 1.3 $M^{-1} \cdot s^{-1}$ for the dimeric species. Thus the monomeric species has an approximately 4-fold higher association rate constant for EDA-GTP-ATTO-550 than the dimeric species (Figure 2c). Considering that recombinantly expressed *at*Toc33$_{\Delta C}$ is almost exclusively GDP-loaded [10] and that GDP release from GTPases is thought to be the rate-limiting step for nucleotide exchange (for example, see [26]), the observed kinetics of EDA-GTP-ATTO-550 binding is therefore most probably dependent on the velocity of GDP dissociation.

To quantify this effect, we investigated the influence of dimerization on GDP dissociation from purified *at*Toc33$_{\Delta C}$

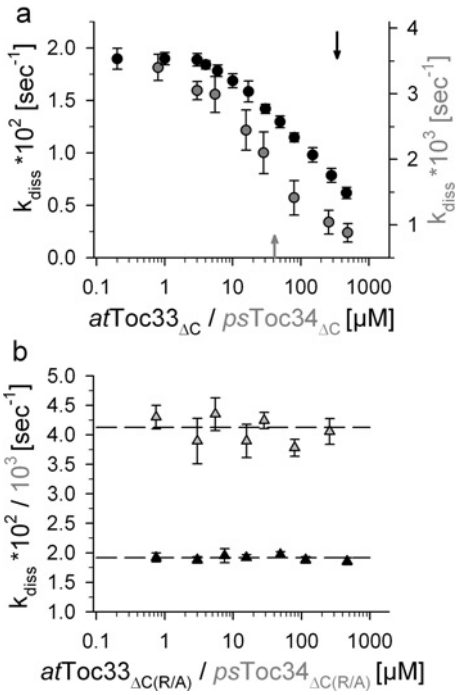

Figure 3 Dimerization reduces GDP dissociation

(**a**) The rates of mantGDP dissociation determined by single exponential analysis are shown for *at*Toc33$_{\Delta C}$ (black circles, left-hand axis) and *ps*Toc34$_{\Delta C}$ (grey circles, right-hand axis). The black and the grey arrows indicate the concentrations corresponding to the K_d for dimerization of *at*Toc33$_{\Delta C}$ and *ps*Toc34$_{\Delta C}$ respectively. (**b**) The rates of mantGDP dissociation determined by single exponential analysis are shown for *at*Toc33$_{\Delta C(R130A)}$ (black triangles, 10^2 on the *y*-axis) and *ps*Toc34$_{\Delta C(R133A)}$ (grey triangles, 10^3 on the *y*-axis). The average value is indicated as a broken line for *at*Toc33$_{\Delta C}$(R130A) and *ps*Toc34$_{\Delta C(R133A)}$ respectively. The dissociation rates of the *at*Toc33$_{\Delta C}$ or *ps*Toc34$_{\Delta C}$ dimer and of GDP according to eqn (3) were calculated and are listed in Table 1.

(Figure 2a). The receptor was pre-loaded with mantGDP as 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 *at*Toc33$_{\Delta C}$ (Figure 3a, black circles). The highest mantGDP-dissociation rate was found at the lowest receptor concentration and the rate was reduced at higher protein concentrations. This suggests a dimerization-dependent reduction in the GDP-dissociation rate.

An opportunity to corroborate this observation was offered by the distinct physicochemical properties of *ps*Toc34$_{\Delta C}$, the functional orthologue of *at*Toc33$_{\Delta C}$ from *P. sativum* (see above). Heterologously produced *ps*Toc34$_{\Delta C}$ has an equilibrium dissociation constant for dimerization of $\sim$50 μM, which is nearly 8-fold lower than observed for *at*Toc33$_{\Delta C}$ [10,17,23]. The reason for the discrepancy between the two proteins from different sources is, however, not yet understood. Nevertheless, it is an advantage in the present study because we can use this information to confirm the relationship between GDP release and dimerization. When mantGDP dissociation from purified *ps*Toc34$_{\Delta C}$ (Figure 2a) was measured (Figure 3a, grey circles), the rates were reduced by increasing protein concentrations, consistent with the results obtained for *at*Toc33$_{\Delta C}$. Comparison of this concentration-dependent effect between both proteins reveals a shift to lower concentrations for *ps*Toc34$_{\Delta C}$, which can be explained by the higher affinity of *ps*Toc34$_{\Delta C}$ for homodimerization.

The suggested relationship between GDP release and homodimerization was further substantiated by the analysis of dimerization-inhibited variants of *at*Toc33 and *ps*Toc34,

Table 1 Dissociation rate constants for GDP release and dimer dissociation

Protein	GDP release or dimer dissociation	Rate constant, k_{diss} (s^{-1})
*at*Toc33$_{\Delta C}$	GDP*	1.89×10^{-2}
	GDP (eqn 3)†	$(1.87 \pm 0.05) \times 10^{-2}$
	Dimer (eqn 3)†	$(3.2 \pm 0.1) \times 10^{-3}$
*at*Toc33$_{\Delta C}$R130A	GDP*	$(1.90 \pm 0.04) \times 10^{-2}$
*ps*Toc34$_{\Delta C}$	GDP*	3.3×10^{-3}
	GDP (eqn 3)†	$(3.4 \pm 0.1) \times 10^{-3}$
	Dimer (eqn 3)†	$(4.5 \pm 0.5) \times 10^{-4}$
*ps*Toc34$_{\Delta C}$R133A	GDP*	$(4.1 \pm 0.1) \times 10^{-3}$

*Rate constants are calculated from the results presented in Figures 2(a) and 2(c) for the wild-type considering only the low receptor concentrations.

†Rate constants are calculated from results presented in Figure 3(b) according to the indicated equation.

*at*Toc33$_{R130A}$ and *ps*Toc34$_{R133A}$ [1,17,25,27], which are able to bind nucleotides [16]. The GDP-dissociation rate of these two proteins (Figure 2a) differed with respect to the source organism (Figure 3b), which paralleled the observed distinct physicochemical properties of the native proteins (Figure 3a). However, the GDP-dissociation rate of each of the mutants was independent of protein concentration in the range analysed (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 (eqn 3). 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 the dimer). This analysis revealed that both the rate constants for GDP dissociation from the monomeric receptor (k_2, eqn 3) and dissociation of the homodimer (k_1, eqn 3) were not altered by varying protein concentration (values listed in Table 1). Furthermore, the GDP-dissociation rate constant determined by this approach resembled that of the monomeric species. In addition, the rate constant for *at*Toc33$_{\Delta C}$ homodimer dissociation was consistent with previous results [19]. By comparison of the rate constants 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 (guanosine 5′-[β,γ-imido] triphosphate, a non-hydrolysable GTP analogue)-bound forms [5]. In addition, our present (Figure 1) and previous [19] results indicate that pre-protein binding modulates receptor GTPase activity by stimulating nucleotide exchange. This prompted us to analyse the influence of peptides representing the pSSU transit sequence on dimerization and nucleotide exchange by *at*Toc33$_{\Delta C}$ or *ps*Toc34$_{\Delta C}$. For this purpose, a 26-amino-acid peptide with a phophoserine at position 13 exhibiting the highest affinity for *ps*Toc34$_{\Delta C}$ 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$_{\Delta C}$ and 500 μM *at*Toc33$_{\Delta C}$ was analysed 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$_{\Delta C}$ and *ps*Toc34$_{\Delta C}$ homodimers (Figure 4a, black circles and grey triangles respectively). Remarkably, the concentration of the peptide at which 50 % of the destabilization was observed ($\sim$200 μM) was comparable with the previously observed K_d for the interaction between B1 and *ps*Toc34$_{\Delta C}$ [20]. In contrast with the influence observed for B1 on homodimerization, addition of a control peptide (C; sequence given in the Experimental section) at the highest concentration used for B1 did not result in disturbance of the homodimer (Figure 4a, white circles).

Guided by this observation, next we analysed the influence of the peptide on GDP dissociation from the receptor. We observed a rather slow dissociation of mantGDP from the monomeric *at*Toc33$_{\Delta C}$ (11 μM, according to the established K_d) when no competing nucleotide was present (Figure 4b, black). The dissociation rate of the labelled nucleotide was increased in the presence of excessive unlabelled nucleotide due to the repressed rebinding of mantGDP (Figure 4b, compare black with grey). Addition of either control peptide (C) or B1 peptide to *at*Toc33$_{\Delta C}$ in the presence of excessive unlabelled nucleotide did not alter the rate or amount of mantGDP released (Figure 4b, green and 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$_{\Delta C}$ at a concentration of 330 μM containing $\sim$45 % of the receptor in a dimeric conformation (according to the established K_d). Again, addition of unlabelled nucleotide (Figure 4c, compare black with 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, compare grey with green). In contrast, addition of the peptide B1 resulted in an increase in the total amount of released nucleotide during the course of the measurement (Figure 4c, compare grey with red). This increase in 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 in nucleotide exchange.

To verify the influence of the peptide on nucleotide exchange, we added the peptide near the plateau phase of mantGDP release from *at*Toc33$_{\Delta C}$ after $\sim$600 s (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, as a consequence, nucleotide exchange are influenced by the transit peptide.

DISCUSSION

Previously, 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 with GADs described so far [14], homodimerization was not found to be GTP-dependent and does not directly contribute to GTP hydrolysis (for example, see [10,17]), raising the question by which functional mechanism homodimerization contributes to the regulation of protein translocation. Our present results indicate

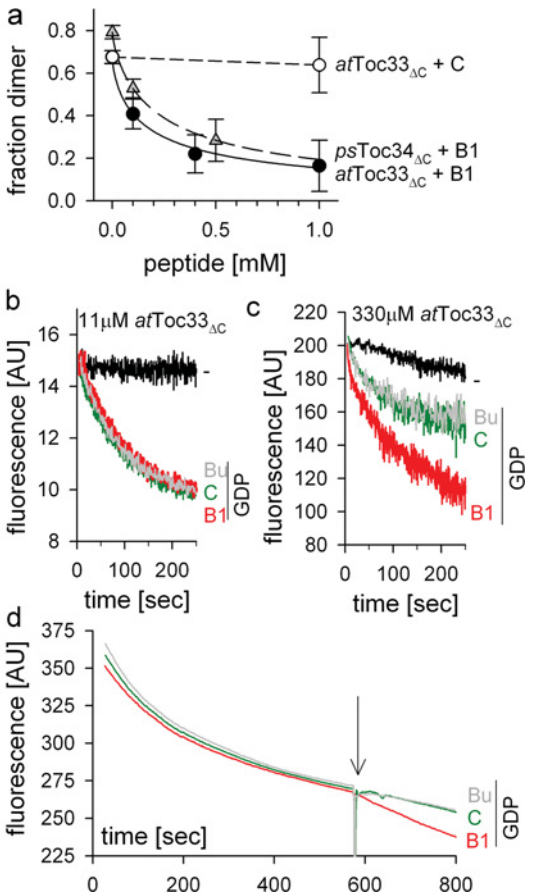

Figure 4 Influence of a transit peptide sequence on dimerization and nucleotide release

(**a**) *at*Toc33$_{\Delta C}$ (180 μl of 500 μM concentration, black circles) or *ps*Toc34$_{\Delta C}$ (180 μl of 170 μM concentration, grey triangles) were subjected to size-exclusion chromatography in the absence or presence of B1 peptide at the concentrations indicated. For a control, *at*Toc33$_{\Delta C}$ (180 μl of 500 μM concentration) was incubated with the control peptide C at the indicated concentrations (white circles). The absorption profiles were analysed by two Gaussian distributions, and the dimeric fraction was calculated from their areas. The decrease in the dimeric population was analysed by a hyperbolic function. (**b** and **c**) The dissociation of mantGDP from 11 μM (**a**) or 330 μM *at*Toc33$_{\Delta C}$ (**b**) was determined by fluorescence (arbitrary units, AU) in the absence (– , black) or presence (Bu, GDP, grey) of 1 mM GDP. 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 *at*Toc33$_{\Delta C}$ 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.

that dimerization decelerates the rate of GTP binding (Figure 2) and GDP release (Figure 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 GDIs (GDP-dissociation inhibitors; for example, see [26,28,29]), which have to be replaced by a GDF (GDI-displacement factor; for example, see [30,31]) prior to nucleotide exchange. Thus a function comparable with 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 (Figure 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 GEF (guanine-nucleotide-exchange factor). 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 eqn 2) of the homodimer. Conclusions regarding the K_d (see eqn 2) 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 (results not shown). Therefore the association rate constant appears to be altered to the same extent as the dissociation rate constant (eqn 2). A similar observation was made for the SRP (signal recognition particle) RNA, which kinetically controls the dimerization between Ffh and FtsY; here, the association rate (k_{ass}) was 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 does not seem 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 cross-linking 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, whereas GTP promoted its transfer to Toc159 [5,18,23]. Albeit speculatively, precursor binding by the homodimeric receptor would enhance the association and dissociation rates of dimerization, resulting in nucleotide exchange driven by a high intracellular GTP/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 the homodimeric to the heterodimeric state and GTP hydrolysis are envisioned to initiate pre-protein translocation [34] and reformation of the Toc33$_{GDP}$ dimer.

The results described in the present paper, the structural interpretations given previously [11,16] and other experimental findings (for example, see [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 co-operative 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 Sommer conducted the experiments. Mislav Oreb, Irmgard Sinning, Ivo Tews, Danny Schnell and Enrico Schleiff were involved in the experimental design and data analysis. All authors were involved in manuscript preparation, which was written by Enrico Schleiff.

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