A unique aspect of protein transport into plastids is the coordinate involvement of two GTPases in the Translocon of the outer chloroplast membrane (Toc). There are two subfamilies in Arabidopsis: the small GTPases (Toc33 & Toc34) and the large, acidic GTPases (Toc90, Toc120, Toc132, & Toc159). In chloroplasts, Toc34 and Toc159 are implicated in precursor binding, yet mechanistic details are poorly understood. How the GTPase cycle is modulated by precursor binding is complex and in need of careful dissection. To this end, we have developed novel *in vitro* assays to quantify nucleotide binding and hydrolysis of the Toc GTPases. Here we present the first systematic kinetic characterization of four Toc GTPases (cytosolic domains of atToc33, atToc34, psToc34, and the GTPase domain of atToc159) to permit their direct comparison. We report the $K_M$, $V_{max}$, and $E_a$ for GTP hydrolysis and the $K_d$ for nucleotide binding for each protein. We demonstrate that GTP hydrolysis by psToc34 is stimulated by chloroplast transit peptides; however, this activity is not stimulated by homodimerization and is abolished by the R133A mutation. Furthermore, we show peptide stimulation of hydrolytic rates are not due to accelerated nucleotide exchange, indicating that transit peptides function as GAPs and not GEFs in modulating the activity of psToc34. Finally, using the psToc34 structure, we have developed molecular models for atToc33, atToc34, and atToc159G. Combining these models with the measured enzymatic properties of the Toc GTPases, we provide new insights of how the chloroplast protein import cycle may be regulated.

Both mitochondria and chloroplasts arose through endosymbiotic internalization of either a free-living $\alpha$-proteobacteria or cyanobacteria. During the course of evolution, in both cases, the vast majority of the endosymbiont genes were relocated to the host nucleus (1). Moreover, in higher plants, most of both organelles’ proteome is derived from nuclear-encoded proteins that are translated on free, cytosolic ribosomes and then post-translationally translocated into the organelle via distinct protein complexes located in each of the two membranes that enclose these organelles (2,3). These mitochondrial and chloroplast translocators are denoted as Tim/Tom and Tic/Toc, respectively (Translocator of the inner/outer membrane of mitochondria/chloroplast). The Toc complex consists of three key proteins denoted by their apparent molecular masses: Toc34, Toc75 and Toc159 and most likely exist with a stoichiometry of 4:4:1, respectively (4). Proteins targeted from the cytosol to these mitochondrial and chloroplast translocators require additional ‘information’ in the form of an N-terminal targeting sequence known as a presequence and transit peptide, respectively (1). This additional targeting sequence has been added during evolution yielding a larger precursor protein, and is cleaved once the precursor is imported into the organelle (5).

In plant cells, despite colocalization of these two post-translational targeting processes, very little mis-sorting occurs *in vivo* or *in vitro* (6,7). The high fidelity of these two processes is maintained, in part, by the specific recognition of the targeting sequences by the import receptors associated with the translocator. In mitochondria, it has been shown that the outer membrane receptor, Tom20, recognizes and binds the presequence (8,9). In chloroplasts, both Toc34 and Toc159 contribute to import specificity through the coordinate or sequential recognition of the transit peptide (10-12). Although both the Tom and Toc receptors function
to promote preprotein binding specificity, only the Toc receptors perform as a molecular switch and/or molecular motor (13). These additional activities are derived from the intrinsic GTPase activity inherent in Toc34 and Toc159 (14). Further regulation of protein translocation has been suggested to occur via specific phosphorylation/dephosphorylation of the Toc GTPases (15,16) however the in vivo significance of this phosphorylation has not been established (17).

From sequence analysis of Arabidopsis, the Toc GTPases fall into two gene families: the small Toc GTPases (Toc33 and Toc34) (18) and the large, acidic Toc GTPases (Toc90, Toc120, Toc132, and Toc159) (19,20). However, in pea, only Toc34 and Toc159 isoforms have been detected. The small GTPases are inserted into the chloroplast outer membrane via their C-terminal transmembrane domain and contain a globular GTP-binding domain which contains the canonical G1, and G3-G5 domains involved in GTP/GDP binding similar to the Ras family of small GTPases (21). Arabidopsis Toc159 contains a central GTP-binding domain (residues 728-1092) with high sequence similarity (~63%) to the small Toc GTPases, however, Toc159 also contains two additional domains: a large, proteolytically-sensitive, N-terminal acidic domain (residues 1-727) and a C-terminal membrane anchoring domain (residues 1093-1503) (22). The other acidic Toc GTPases share this domain structure, yet the acidic regions are reduced in length (23). Very little is known about the structure and function of the acidic and membrane domains.

Although it has been shown in pea chloroplasts that an early preprotein binding intermediate is mediated in a GTP-dependent manner by the Toc complex (24), the details of how the two different GTPases, Toc34 and Toc159, participate in binding/translocation is debated. Several reports on the interaction of the Toc complex with preproteins have been recently published and result in two models: one in which a soluble form of Toc159 interacts with the preprotein in the cytoplasm and is subsequently targeted to Toc34 and Toc75 at the outer envelope to begin protein translocation (25), however these results have been recently challenged (26). The second model proposes that the preprotein first interacts with Toc34 at the chloroplast outer membrane surface and is then ‘handed over’ to translocon-associated Toc159 (15). After the recognition event involving transit peptide and/or preprotein interaction with the cytosol-exposed portions of Toc34 and Toc159 and concurrent rounds of GTP hydrolysis, the preprotein is inserted into the β-barrel protein Toc75 and translocated across the outer membrane of the chloroplast (27,28).

Despite multiple reports investigating the interaction of the Toc34 and Toc159 homologues with preproteins and transit peptides the role of GTP binding and hydrolysis is far from understood (1,4,5,10-12,15,16,18,21,26,29-33). In fact, the few reports on GTPase activity have been primarily focused the small GTPases and report enzymatic properties such as $V_{\text{max}}$ and $K_M$ that disagree by nearly two orders of magnitude (11,21). In Arabidopsis, the two related homologues, Toc33 and Toc34 have had their nucleotide-binding properties investigated, yet there have not been systematic investigations on their potentially different enzymatic properties (33). Finally, at-Toc159G has had only limited analysis performed on its nucleotide binding and/or hydrolysis properties (31,34). Mechanistically, from both the crystal structure (21) and the low resolution EM structure of the Toc translocon (4), it is clear that the Toc GTPases may be able to form either a functional homo- or heterodimer. The significance of this dimerization is still debated (35) yet it has been proposed that one possible explanation is that the two subunits can function as GTPase activating proteins that may reciprocally stimulate and/or coordinate the hydrolysis of GTP (21). This dimeric, self-stimulatory interaction was first reported in the crystal structure where the presence of Arg 133 from one monomer interacting deep within the GTP binding pocket of the other monomer can be observed. Reciprocal GAP activity is not a novel idea, for instance, reciprocal GAP activity has become an accepted model for the function of SRP54/Ffh and SRα/FtsY in protein secretion (36), the only other family of small GTPases associated with protein translocation (37).

Therefore, to enable a careful analysis of how preprotein binding, membrane association, and homo- and heterodimerization influence the GTPase activity of the various Toc proteins, we first present a systematic comparative kinetic analysis of the Toc34 sub-family and atToc159G. We report the $V_{\text{max}}$, $K_M$, $E_a$, and $k_{\text{cat}}$ for GTP hydrolysis, as well as the $K_d$ and $K_i$ of nucleotide
binding for each protein. Using psToc34 as a model, we also evaluate how concentration dependent homodimerization, an ‘arginine finger’ mutation and transit peptide interaction affect subunit dimerization and GTP hydrolysis for psToc34. Finally, using the crystal structure of psToc34, we have developed molecular models for atToc33, atToc34, and atToc159G. Comparing these models to each other and to p21Ras and other small GTPases may provide new insights on possible mechanisms of how the Toc GTPase cycle may be modulated during chloroplast protein import.

EXPERIMENTAL PROCEDURES

Expression & purification of Toc GTPases. The expression clones for the Toc proteins were kindly provided by the following people: psToc34, Prof. Jurgen Soll (Munich, DEU), atToc159, Prof. Danny Schnell (Amherst, MA), atToc33 and atToc34, Michael Gutensohn (Cologne, DE). The cDNA encoding the truncated, cytosolic domain of psToc34, atToc33, and atToc34 was inserted into pET21d (Novagen, Madison, WI), pQE60 (Qiagen, Valencia, CA), and pQE60 (Qiagen, Valencia, CA), respectively. The transmembrane spanning regions were deleted by insertion of His-tags C-terminal to residue D243, K257, and P259, of the respective Toc proteins. The cDNA for Toc159G, encoding the central GTPase domain, was inserted into pET21d (Novagen, Madison, WI) such that it expressed residues 727-1092 fused to a C-terminal His-tag. The plasmids containing atToc33 and atToc34 were each transformed into E. coli TG1 cells (Amer-sham/Pharmacia) and the plasmids containing psToc34 and atToc159G were transformed into E. coli BL21(DE3) cells. These cells were grown in LB media to an OD600 of 0.4 and induced with 1 mM IPTG for 4 hr at 37°C. The E. coli cells were lysed by three passages through a French Press (Thermo Electron Corporation, Waltham, MA) at 20,000 psi in the presence of lysis buffer (20 mM NaPO4, pH 8.0, 0.5 M NaCl, 1 mM MgCl2, 0.1% Triton X-100, 1 mM β-ME). The soluble protein was separated from inclusion bodies and cell debris by centrifugation at ~45,000 x g for 30 min. The His-tagged proteins were purified under non-denaturing conditions using Talon Co2+ IMAC (BD Biosciences, San Jose, CA). The bound proteins were washed with 15 mM imidazole and eluted with 250 mM imidazole. Eluent was immediately diluted with 2.5 X GBS and 50% glycerol yielding a storage condition of 1 X GBS (20 mM Tricine-KOH, pH 7.65, 1 mM MgCl2, 50 mM NaCl, 1 mM β-ME) (16) with 20% glycerol and frozen at -80°C until used. The concentrations of the purified recombinant proteins were determined using the Bradford assay (BioRad Laboratories, Hercules, CA). The purity of the proteins was estimated by scanning densitometry of a CBB stained SDS-PAGE gel to be greater than 95%.

Purification of the full-length transit peptides. The subcloning of the sequence encoding the transit peptide of the small subunit of Rubisco from N. tobacum (SStpNt) into PTYB2 vector (New England BioLabs, Beverly, MA, USA) was described previously (10). The growth and purification of SStpPs was performed as described previously for SStpNt (38). The eluted peptide was lyophilized to remove the β-ME and concentrate the protein. Upon dissolving the lyophilized peptide in water, the contaminating dnaK remained insoluble and could be removed by centrifugation (Fig. 1C). The purity of the peptide was initially determined by SDS-PAGE on a 19.2% Tris/Tricine gel and staining with Coomassie Brilliant Blue. The peptide was quantified using the BCA protein assay (Pierce Chemical Co., Rockland, IL) and then re-aliquotted and lyophilized in small, 0.5 mg aliquots. The transit peptide was then stored as a lyophilized powder at -20°C until used. The peptide was resuspended in ddH2O to the appropriate concentration for enzyme assays.

MALDI-TOF mass spectrometry. MALDI-TOF MS was performed on a Bruker Daltonics Microflex™ mass spectrometer, with Apomyoglobin used as an internal standard for calibration. Lyophilized standard protein and transit peptide were reconstituted into 50 pmol/µl in 10 µl of 50% ACN v/v with 0.1% TFA, v/v. The samples were mixed with 10 µl of CHCA matrix (10 mg/ml in 50% CAN v/v, 0.1% TFA v/v), spotted, and dried thoroughly. Mass spectra were acquired in positive ion mode.

Phosphate release assay for GTP hydrolysis. GTP hydrolysis of psToc34, atToc33, atToc34 and atToc159G were all performed in the same manner.
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using a modification of an activated charcoal, phosphate-release assay originally developed by our laboratory (39). In a flat-bottom 96-well microplate, reactions were carried out in GBS, 10 nM \[^{32}\text{P}\]GTP and varying amounts of cold GTP (and any additional substrate) in a final volume of 100 μl. The addition of Toc protein started the reaction and at various times, indicated in the figures, a 12.5 μl aliquot was removed and added to 200 μl 10% w/v activated charcoal in 50 mM HCl, 5 mM H₃PO₄. Vigorous pipetting with a multichannel pipette mixed the charcoal-aliquot mixture. At the completion of the reaction, the charcoal suspension was transferred to a 96-well 0.2 μm filter plate (Pall Corporation, Ann Arbor, MI) and vacuum filtered into a receiving 96-well plate. An 8 μl aliquot was taken of the filtrate and added to 150 μl MicroScint 40 scintillation fluid (Perkin Elmer, Downers Grove, IL). Again, vigorous pipetting was performed to ensure even distribution in the scintillation fluid. Counting was performed on a 96-well microplate scintillation counter (TopCount NXT Microplate Scintillation Counter, Perkin Elmer, Downers Grove, IL). In all cases, minimums of three independent experiments were performed, counted in duplicate for each data point, and cross-talk filters were applied. CPM data was graphed as CPM vs. Time using the Prism software (Prism 4c, GraphPad Software, San Diego, CA).

**Determination of Enzyme Velocity and Substrate Affinity.** The conditions were designed to ensure that the reaction rate was linear over the 30 min time course of the assay. By using multiple time points, A cpm/minute values were calculated from the slope of the line generated by CPM vs. Time. With the known specific activity of the isotope, the isotope purity, the enzyme concentration and the counting efficiency, the slope was then transformed into a velocity expressed as nmol GTP hydrolyzed/min/μmol Toc protein. To determine the \(V_{\text{max}}\) and \(K_M\) for GTP hydrolysis, the GTP concentration was varied from 1 to 100 μM. Once the rate values have been calculated for each GTP concentration, these rates (y-axis) are graphed against the substrate concentration (x-axis) and fit using the Michaelis-Menten equation (Equation 1) to determine the \(V_{\text{max}}\) and \(K_M\) using the GraphPad Prism program where \(v\) is the velocity, \(V_{\text{max}}\) is the maximum velocity, \(K_M\) is the Michaelis-Menten value and \([S]\) is the substrate concentration.

\[
\frac{v}{K_M + [S]} = \frac{V_{\text{max}}[S]}{K_M + [S]} 
\]  
(Eq. 1)

**Optimization of buffer composition, pH and storage conditions for Toc GTPases.** Buffers used to determine optimal storage conditions for the Toc proteins were the following: GBS, HMK (50 mM Hepes KOH, pH 7.5, 2 mM MgCl₂, 40 mM KOAc, 12.5 mM imidazole, 10% glycerol, 1 mM βMe) (34), KSS (50 mM Tris-HCl pH 8, 50 mM KCl, 5 mM MgCl₂, 5 μM GTP, 1 mM dithiothreitol) (25), and TTP (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 10 mM imidazole, 10% glycerol, 1 mM β-ME), a variation of the buffer used during the crystallization of psToc34 (21). In addition, psToc34 was subjected to the following freezing conditions: -80ºC slow, -80ºC fast, -20ºC, and storage in liquid nitrogen; ‘fast’ and ‘slow’ refer to the relative speed at which the samples reached -80ºC: ‘fast’ treated samples were snap frozen in liquid nitrogen for 30 seconds prior to being placed in a -80ºC freezer, while ‘slow’ treated samples were placed directly into a -80ºC freezer in a foam insulating container. Furthermore, psToc34 was incubated in GBS titrated through a pH range that extended from 6.1 to 11.9. GBS was made with the following buffering agents for a given pH range: MES (pH 6.1-6.7), HEPES (pH 6.8-7.5), Tricine (pH 7.6-8.4), Tris (pH 8.5-9.1) and CAPS (pH 9.2-11.9). At each pH titration step, psToc34 was assayed for maximal velocity as described earlier.

**Effect of cation replacement on psToc34 activity.** psToc34 was incubated in 1X GBS buffer in the presence of 5 mM (saturating conditions) selected metal salts: MgCl₂, CaCl₂, CdCl₂, CoCl₂, CuSO₄, MnCl₂, NiSO₄ and ZnCl₂ for 10 min at 25ºC to allow for cation replacement. A mixture of both \[^{32}\text{P}\]GTP (10 nM) and variable amounts of cold GTP were added to start the reactions. The hydrolysis activity of psToc34 was measured as described above and graphed as a percentage of psToc34 activity in GBS buffer.

**Effect of protein concentration on the hydrolysis rate of the Toc GTPases.** The four Toc proteins were assayed as described above in GBS at 1, 2, 3,
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5, 10, 15 and 20 μM protein concentration for hydrolysis activity. The resulting data is plotted as rate of hydrolysis as a function of protein concentration in μM.

Construction of psToc34(R133A) mutant. Mutation of Arg 133 was performed with the following mutagenesis primers from 5′ to 3′: GATGCCTACGCGGTGGACAACCTGGAC and GGTTGTCCACCGCGTAGGCATCCAGC. All mutagenesis was carried out with QuickChange PCR Mutagenesis (Stratagene).

Circular dichroism spectroscopic analysis of psToc34(R133A). Circular dichroism spectroscopy was performed on an Aviv Series 202 circular dichroism spectrometer (Aviv Biomedical, Lakewood, NJ, USA). After dialyzing extensively into 50 mM NaH 2PO 4, pH 7.8, 10 mM NaF; the final concentration of the psToc34(R133A) mutant protein was determined to be 7.58 μM using the Bradford assay. Circular dichroism spectra were collected at 25°C as 2 second averages at 1 nm intervals from 185 to 285 nm. Five spectra were averaged, corrected for buffer contributions, smoothed, and converted to molar ellipticity using the Aviv software, version 2.71. Deconvolution was performed as per (40) using the CDPRO software with IBASE3, a reference set of 37 soluble proteins, available at http://lamar.colostate.edu/~sreeram/CDPro. From this data, the secondary structural features of psToc34(R133A) were determined. The STRIDE program, available at http://webclu.bio.wzw.tum.de/stride/ (41), was employed to calculate the percent secondary structural elements of crystallized wild-type psToc34, chain B, pdb id 1H65 (21).

Sedimentation velocity analysis of psToc34. Sedimentation velocity analyses were performed using a Beckman Optima XL-1 analytical ultracentrifuge equipped with both absorbance and interference optical detection systems. After dialyzing samples extensively into GBS as described above, 400 μl was loaded into epon double sector centerpieces. For experiments at different concentrations, all dilutions were made using the dialysate and all experiments used dialysate as the reference buffer. Sample cells were loaded into the rotor and allowed to equilibrate to 25°C under vacuum for at least 1 hour. After fully equilibrated, samples were centrifuged at 50,000 rpm at 25°C using the An50Ti rotor and data was collected using interference optics. Analysis of the sedimentation velocity data was performed using the program SEDFIT, which uses Lamm equation modeling to carry out size distribution analyses of velocity profiles (42). Velocity profiles were analyzed by direct boundary modeling using distributions of Lamm equation solutions, c(s) (43,44). The solvent viscosity, η, and the density, ρ, were experimentally determined to be 0.00896 g cm 1 s -1 and 1.0003 g ml -1 respectively at 25°C using an Anton Parr densitometer by Prof. Jochen Weiss, UMass Amherst. The partial specific volume of the protein, v̅ was calculated to be 0.7441 ml g⁻¹ by inputting the protein sequences into the program SEDNTERP (45). The nonlinear regression using distributions of Lamm equation solutions, c(s), was combined with the determinations of the best fit weight average frictional ratio, the best fit meniscus position, and the algebraic calculation of systematic time-invariant and radial-invariant noise components and maximum entropy regularization using a confidence level of > 0.8 (42). The resulting sedimentation coefficient distributions, expressed in Svedberg units [S], were exported, normalized to respective protein concentrations and graphed using GraphPad Prism 4c (GraphPad Software, Inc.).

Activation Energies of the Toc GTPases. Reactions were performed ranging in temperature from 4°C (277 K) to 45°C (318 K) in 0.6 ml microfuge tubes. Prior to initiation of reaction by addition of Toc protein, the temperature of the reaction buffer and nucleotide was allowed to equilibrate for 10 minutes. Rates were determined as above in hydrolysis assays, and graphed as the natural log of the rate (ln k) versus the inverse of the absolute temperature, expressed in Kelvin (10 3/T, K⁻¹). E a was determined using the Arrhenius equation (Eq. 2):

\[
\ln k = \frac{-E_a}{RT}
\]

where k is rate in nmol GTP hydrolyzed per minute per μmol Toc protein, R is the universal gas constant, T is temperature in Kelvin and E a is the energy of activation expressed in kcal/mol.
Nucleotide Binding Studies using NTA-Magnetic Absorption. Using iron-incorporated agarose beads with Ni-NTA chemistry (Qiagen Inc., Valencia, CA), we are able to recapture the various Toc proteins via their C-terminal hexahistidinytags in a 200 μl reaction volume. This approach affords us the ability to selectively immobilize and extensively wash each protein in the presence of a 96-well hybrid plate magnet. Competition assays for nucleotide binding were performed in GBS buffer with each purified Toc GTPase. The assay contained 20 mM of the GTPase, [α-32P]GTP (MP Biomedicals, Irvine, CA) with a specific activity of 3000 Ci/mmol, an excess of Ni-NTA-agarose magnetic particles, and increasing concentrations of competing, unlabeled nucleotide as indicated in the Fig. 6 legend. The conditions were optimized to cover >3 orders of magnitude of the competitor with sufficient data points to permit an accurate non-linear fit. The reaction was carried out in a 96-well microplate in a total volume of 100 μl. First, a serial dilution of unlabeled competitor was carried out in a separate plate leading to a concentration 4-fold greater than indicated on the x-axis. Then, 25 μl of the resulting solution was transferred with a 12-well multichannel pipet into each row of wells in the reaction plate. The [α-32P]GTP was diluted and added to each well of the reaction plate, also as a 25 μl addition. Finally, the Ni-NTA-agarose magnetic particles and enzyme were added to start the reaction in a volume of 50 μl. The reaction proceeded for 30 min on ice before the plate was transferred to a novel, high-performance hybrid plate magnet. We used a new class of magnet plates recently developed at the D.O.E. Joint Genome Institute at Lawrence Berkeley National Laboratory (JGI/LBNL) for biological and industrial applications (46). They utilize hybrid technology that combines linear permanent magnetic and ferromagnetic material to produce significantly higher fields and gradients than currently available commercial magnetic plates. This hybrid structure exhibited maximum fields in excess of 9000 Gauss, which allowed for greater holding forces on magnetized targets that are being processed as well as permitting faster draw-down. Following immobilization in high field strength, the reaction mixture could be easily removed by aspiration and replaced with 300 μl GBS. Each well could then be washed continu-ously with GBS for 2-3 min with a microtiter plate washer (Skatron Microwash II, Norway) to remove all non-specific background. The wash buffer was then aspirated and replaced with 100 μl of elution buffer (GBS with 500 mM imidazole) and removed from the magnet for 5 min with periodic agitation. After replacement on the magnet for 5 min to ensure immobilization of the beads, 20 μl of the elution buffer was removed for scintillation counting. These 20 μl samples were removed from each well in triplicate and each competition reaction was performed in duplicate. In addition, to avoid the ambiguous results associated with homologous binding competitions performed at a single concentration of labeled nucleotide, at least two concentrations of [α-32P]GTP were used in each case. Thus, the entire competition experiment was repeated using a 3-fold higher concentration of the labeled nucleotide. The samples were counted in a 96-well format using the PerkinElmer Top Count scintillation counter with Microscint fluid, and crosstalk filters were applied before the data was analyzed.

Global-Fit Analysis of Nucleotide Binding Data. Using the experimental procedure described above, there were a total of 12 datasets per global fit analysis (i.e. 2 replicates, each counted in triplicate at two different nucleotide concentrations). These values were fit to Eq. 3 using Graphpad Prism 4.0c with the log Kd shared for global fitting and the amount of hot and cold nucleotide constrained according to the experimental design.

\[
\text{Total Binding} = \frac{B_{\text{max}} \times [\text{Hot}]}{[\text{Hot}] + [\text{Cold}] + K_d} + \NS \quad (\text{Eq. 3})
\]

The remaining variables were given best-fit values by non-linear regression. For heterologous binding competitions, the experiments were performed in the same manner, but the analysis was fit by Eq. 4:

\[
\text{Total Binding} = \frac{B_{\text{max}} \times [\text{Hot}]}{[\text{Hot}] + K_d \left(1 + \frac{[\text{Cold}]}{K_i}\right)} \quad (\text{Eq. 4})
\]

In this case the $K_i$ measured in the previous experiment was substituted into the equation while the log $K_i$ was shared for all data sets for global fitting. The amount of labeled and non-labeled GTP was held constant, and the remaining variables were given best-fit values by non-linear re-
gression. For data analysis, confidence of 95% was used.

**Peptide Stimulation of Hydrolysis Activity.** Hydrolysis experiments were performed with psToc34 and the addition of increasing concentrations of GTP, as described above, in the presence of a fixed concentration of SStpPs, SStpNt or the negative control RCMLA to generate linear CPM vs. time data. Linear regressions were applied generating a series of slopes for each concentration of GTP used. This was repeated for multiple concentrations of all peptides: 2.5, 4, 6.3, 10, 15.8, 25, 40, 63 and 100 fold molar concentrations to psToc34. The slopes from the CPM data were mathematically transformed to rate data and fit to Eq. 1 to determine V_{max} and K_{M}.

**Effect of transit peptide on nucleotide exchange.** psToc34 (0.5 mg) was incubated with [γ-32P]GTP (0.5 mCi of 800 Ci/mmol, MP Biomedicals) and EDTA in GBS for 1 hour at room temperature in a 500 μl reaction volume. The unbound nucleotide, EDTA and imidazole were removed by loading the mixture onto a PD-10 desalting column (Pharmacia) and eluted in GBS; approximately 2% of the psToc34 was routinely labeled. The eluent was collected in 60 μl fractions, psToc34 containing fractions began after ~3.2 ml of elution and the concentrations of these fractions were determined using the Bradford assay. The requisite amount of psToc34 was added to create a 200 nM solution and 50 μl was aliquotted into each well of a microtiter plate. A time-course of the dissociation of GTP label was started with a 50 μl addition of 100-fold GTP or 100-fold GTP and transit peptide at 0.3, 1.5, 7.5 or 37.5 fold molar excess over psToc34; GBS was used as a negative control. After each of the wells had incubated for the indicated time, the plate was placed on the hybrid magnet and then washed 12 cycles in a microtiter plate washer. The remaining GBS was aspirated away and replaced by GBS with 50 μl of 500 mM imidazole. Half of the elution was transferred into 100 μl of MicroScint scintillation fluid and counted in the TopCount (Packard). Data was fit using a single-phase exponential decay in Graphpad Prism 4.0c.

**Homology Modeling of Toc Proteins.** atToc33, atToc34, and atToc159G were modeled using the MODELLER modeling package (47) with the crystal structure of psToc34 as a template. The coordinates were obtained from the Brookhaven Protein Databank (pdb id 1H65). The sequence of each homologous Toc protein was aligned using the MODELLER algorithm SALIGN, which uses a variable gap penalty to avoid artificially introducing insertions into conserved areas of secondary structure. Since the percent identity was 62.9, 66.0, and 32.0 for atToc33, atToc34 and atToc159G respectively, the automodel scripts of MODELLER were used to generate 20 candidate models for each Toc protein ignoring out-gaps. These models were evaluated using the discrete optimized potential energy (DOPE) function from MODELLER and all amino acids aligned with psToc34 were compared summing the square of the differences relative to the DOPE function of the psToc34 crystal structure. All candidate models agreed closely with the DOPE function of psToc34; but for each set of candidate models, the lowest global score was chosen for further analysis. All structural models were rendered using VMD (48), and structural alignments were performed with STAMP (49). Rmsd values of the α-carbons were calculated relative to psToc34 using the rmsd plug-in of VMD, and per residue rmsd values were calculated by the rmsd calculator of MULTISEQ (50). All figures were ray traced in POV-Ray 3.6 (http://www.povray.org/download/).

**RESULTS**

**Expression and Purification of Toc Proteins and SStpPs/Nt.** We overexpressed atToc34ΔTM and atToc33ΔTM (*E. coli* TG1) and psToc34ΔTM and atToc159G (*E. coli* BL21[DE3]) as soluble protein and purified via IMAC using the C-terminal hexahistidinyl tag (Fig. 1A.) In order to verify purity, 100 ng of each protein was run on 15% acrylamide SDS-PAGE and concluded to be greater than 95% pure (Fig. 1B).

The transit peptide construct with a C-terminal Intein and chitin-binding domain was overexpressed in *E. coli* ER2566 cells as soluble protein. One-step purification was accomplished using a chitin affinity column with Intein-mediated self-cleavage and elution induced by addition of β-ME (Fig. 1C). Success of cleavage was assessed by removing chitin matrix, boiling with sample solubilizing buffer and running on SDS-PAGE (Fig. 1C, lane 7). The upper band in lane 7 is due to un-
cleaved transit peptide still bound to the chitin matrix via the chitin-binding domain. Cleaved protein was purified to homogeneity, as confirmed by gel electrophoresis (Fig. 1C, lane 9) and MALDI-TOF mass spectrometry (Fig. 1D). MALDI-TOF MS of transit peptides was performed with apomyoglobin used as an internal standard on a Bruker-Daltonics Microflex™ mass spectrometer shown in Fig. 1D. The +2 and +3 charged apomyoglobin species can be seen at 8477 m/z and 5651.7 m/z, respectively; the average mw of Apomyoglobin is 16952. The +1 and +2 charged SS-tpNt species are found at 6125.9 and 3062.6 m/z. The FindPept tool (http://ca.expasy.org/tools/findpept.html) was used to identify the peptides that correspond to the main peaks. The largest peak at 6125.9 m/z corresponds to the full-length transit peptide, while the minor peak at 4152.6 m/z represents either an alternative translation initiation at Met 22 or alternatively a degradation product where short N- and C-terminal regions were proteolytically removed during expression and purification (Fig. 1C). Similarly, MALDI-TOF analysis of the purified Pisum sativum SS-tp indicated that the full-length peptide has the expected mass of 6182.2 (data not shown).

Effects of Buffers, Storage Conditions, Enzyme Concentration and Metal Ions. One possible explanation for the variation in reported activities observed for the Toc GTPases is their sensitivity to various buffers, storage conditions, and metal ion contaminants. As such, it was important to test how different buffer systems affected the hydrolysis activity measurements. We tested four buffers for their effect on the GTPase activities of psToc34. A comparison of the enzymatic activity of psToc34 in these four buffers is shown in Supplemental Fig. 1A. Despite the differences in their composition, all of these buffers yielded relatively similar activities of psToc34. A comparison of the enzymatic activity of psToc34 in these four buffers is shown in Supplemental Fig. 1A. Despite the differences in their composition, all of these buffers yielded relatively similar activities of psToc34 however GBS appeared to have slightly higher levels of GTPase activity after background subtraction.

Upon determining the best buffer for enzymatic activity, an additional variable is how the protein is stored prior to use; therefore, we also tested several freezing and storage conditions: -80°C slow, -80°C fast, liquid nitrogen storage, and -20°C shown in Supplemental Fig. 1A. We found that -80°C slow freeze afforded the greatest maintenance of activity and that under optimal storage conditions, we were able to freeze and thaw the protein >3 times before activity became impaired (data not shown). Furthermore, GBS buffer was able to maintain the GTPase activity of the Toc proteins at -80°C in 20% glycerol for up to 4 weeks after which the activity began to diminish (data not shown).

The activity of psTo34 was assayed as a function of the pH in Supplemental Fig. 1B. We varied several buffers (each buffering agent at 20 mM) from pH 6.0 to 12.0 and tested the maximal velocity as described above. Consistent with previous publications reporting the activity of psTo34 in GBS buffer, pH 7.6 was chosen (16). According to Supplemental Fig. 1B, the pH optimum for psTo34 is between 7.5 and 8.2 and we therefore maintained the pH at 7.6 in accordance with the published GBS buffer.

Since it has been previously reported that Ras and other small GTPases are affected by the metal cofactor in the nucleotide binding site, we attempted to remove Mg²⁺ from the active site of the Toc proteins by dialysis with EDTA in Mg²⁺-free GBS. This treatment consistently resulted in protein precipitation (data not shown). Alternatively, we were able to add various metal salts at a large molar excess to determine if these metal ions could possibly exchange with the Mg²⁺ ion in the active site. The results of how MgCl₂, CaCl₂, CdCl₂, CoCl₂, CuSO₄, MnCl₂, NiSO₄ and ZnCl₂ affect activity is shown in Supplemental Fig. 1C. Suppression of activity was seen with all metals but two: CaCl₂ and ZnCl₂, of which, ZnCl₂ stimulated the hydrolysis activity nearly 2.7-fold. As a control, each metal salt was incubated with nucleotide (in the absence of enzyme) showing no effect on the spontaneous rates of GTP hydrolysis (data not shown).

Hydrolysis Activity of the Toc GTPases. The enzymatic parameters Kₘ and V_max have been reported in the past with a great deal of variation using a PEI-cellulose TLC technique and image analysis. To provide a more robust method, we used an activated charcoal, pull-down assay using radioactive [γ-³²P]GTP. This assay employs liquid scintillation counting to quantitate the amount of Pi released by GTP hydrolysis. In this assay, we incubated the ATM Toc proteins with [γ-³²P]GTP at 25°C. Aliquots of the reaction were added to 10% w/v activated charcoal where only the hydrolysis product ³²PO₃ along with H₂O, HCl and
\[ \text{H}_3\text{PO}_4 \text{ remain in the filtrate after a 0.2 \mu m vacuum filtration step. Aliquots of this filtrate were counted in a scintillation counter. A BSA control yielded counts analogous to spontaneous hydrolysis (data not shown). CPM data from at least three independent experiments were obtained and graphed as nmol GTP hydrolyzed per minute per \mu\text{mol Toc protein (Fig. 2A-D)}. This analysis indicates that atToc33, atToc34 and psToc34 share very similar K_M and V_max values, whereas atToc159G does not. Values from 3.5 to 6.3 \mu\text{M for the Michaelis-Menten constant, } K_M, \text{ and 35-37 nmol GTP hydrolyzed/min/\mu\text{mol Toc protein for the maximal velocity, or } V_max, \text{ were obtained for the smaller GTPases (Table I). The G domain of atToc159 had a } K_M \text{ and a } V_{\text{max}} \text{ values, whereas atToc159G did not, representative of its relatively low maximal velocity and decreased affinity for substrate.}

*Hydrolysis rates of the Toc GTPases are not influenced by concentration dependent dimerization.* The native state of psToc34 suggests that it may exist in a homo- or hetero-oligomeric complex (4,21,51). The recent crystallization paper indicated that psToc34 was able to form a stable dimer that had a 2-fold plane of symmetry during crystallization (21). Furthermore, several groups have shown using size exclusion chromatography, native gel electrophoresis and surface plasmon resonance that the Toc34 homologues can exist in equilibrium between monomeric and dimeric species (16,21,35). Direct boundary modeling of the SV data shows that psToc34 undergoes concentration-dependent dimerization, illustrated by the shift in sedimentation coefficient expressed as Svedberg units [S], towards larger S values as a function of increasing concentration of psToc34 analyzed at 3.4, 16.9 and 33.8 \mu\text{M (Fig. 3A, B & D)}. This relationship between monomer and dimer peaks and their relation to protein concentration is underscored in Fig. 3E. Notice that as concentration increases the line representing the increase in dimer peak height crosses the line showing the simultaneous decrease in monomer peak height at slightly less than ~12.5 \mu\text{M, suggesting that this is the equilibrium midpoint of psToc34. At concentrations below this threshold value, psToc34 is more likely to exist at a monomer; whereas, higher concentrations increase the population of psToc34 dimers. Interestingly, the GTP hydrolysis rate of the four Toc GTPases, when corrected for protein concentration, do not increase in rate as a function of protein concentration (Fig. 3C). The dashed and dotted lines in Fig. 3C represent 1.7 and 16.9 \mu\text{M, respectively, corresponding to concentrations where AUC analysis revealed psToc34 to be monomeric and mostly dimeric, respectively (Fig. 3D). At 33.8 \mu\text{M psToc34, the protein is predominantly dimeric, Fig. 3D. Furthermore, the monomeric species decreases concomitantly with the increase of the dimeric species as protein concentration is increased as is shown graphically in Fig. 3E. Therefore, the maximal rate of the psToc34 is not affected by protein dimerization in vitro, but this does not preclude the possibility that dimerization in vivo may affect the hydrolytic rate and thus protein import."

The mutant protein psToc34(R133A) is monomeric and lacks GTP activity. The crystal structure of the psToc34 homodimer reveals the side chain of arginine 133 of one monomer extending deeply into the GTP binding pocket of its dimeric partner suggestive of an arginine finger, a moiety common to many GTPase/GAP crystal structures. Although there are two closely positioned arginines in the dimeric interface of psToc34, Sun, et al. hypothesized that R133 functioned as the arginine finger. Equivalently positioned arginine fingers have been observed in the GAPs co-crystallized with other small GTPases such as H-Ras (52), CDC42 (53) and RhoA (54).

**Wild-type psToc34**, as shown in Fig. 3, has a defined profile in the analytical ultracentrifuge indicative of a concentration-dependent monomer/dimer equilibrium. However, AUC analysis of the mutant R133A reveals a single sedimenting species with a S value of 2.8. Moreover, this single species is similar in sedimentation behavior to the wild-type monomer (Fig. 3D). However, unlike wild-type psToc34, the R133A mutant remains monomeric at all three protein concentrations tested: 3.4, 3.53 and 170 \mu\text{M (Fig. 4A). Comparatively, dimerization of wild type was observed as low as 16.9 \mu\text{M (Fig. 3D). This AUC analysis in-**
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Indicates that the R133A mutation severely disrupts the psToc34 homodimeric interface.

In addition to eliminating concentration-dependent dimerization, the mutation of R133A also abolishes the in vitro GTP hydrolytic activity (Fig. 4B). The low level of hydrolysis is essentially indistinguishable from the rates of spontaneous GTP hydrolysis; whereas, wild-type psToc34 exhibits a sharp substrate-velocity curve, also shown for comparison in Fig. 4B. This very low activity precludes determination of the V_max or K_M with any statistical certainty. These two observations taken together indicate that the R133A mutation disrupts both GTP hydrolysis in the monomer and prevents homo-dimerization.

In order to rule out the possibility that the R133A mutant resulted in misfolding or some global structural change, we performed circular dichroism spectroscopy of purified psToc34(R133A) shown in Supplemental Fig. 2. Comparison of this data to the recently published CD spectra of wild-type psToc34 (10) reveals high spectral similarity. To provide more detailed insight into the structural properties of the mutant protein, we deconvoluted the CD spectra using the CDPRO program. The secondary structural content predicted by this program is shown in Supplemental Table I. Further, comparison of this data with the secondary structure content derived (using STRIDE, Supplemental Table I) from the crystal structure of wild-type psToc34 (21) is in close agreement with our measurements for R133A, indicative of a very similarly folded protein. Thus, the loss of activity of psToc34k(R133A) is not due to protein misfolding.

**Activation Energies of the Toc GTPases.** In order to determine the energy of activation for the various Toc proteins, we incubated Toc protein, in GBS buffer, at different temperatures ranging from 4°C to 45°C (the proteins were not stable above 55°C, data not shown). The hydrolysis assay was performed as described in experimental procedures, the rate of hydrolysis was calculated as before, and an Arrhenius plot was created from the natural log of the rate and the inverse of temperature expressed as 10^3/T (K^-1) shown in Fig. 5. The energies of activation, E_a, expressed in kcal/mol, for psToc34, atToc33, atToc34 and atToc159G are shown in Table I.

**Nucleotide Binding Activity of the Toc GTPases.** We used a novel approach to determine the equilibrium dissociation constants of the various Toc proteins for GTP, GDP and other nucleotides. The particular Toc protein is incubated with a known concentration of radiolabeled [α-^32P]GTP as well as increasing concentrations of unlabeled GTP in the case of homologous competition. Homologous and heterologous competition assays were carried out on the Toc GTPases using a variety of substrates in order to determine affinities and gain insight into the significance of the interacting moieties, shown in Fig. 6A-F. All of the Toc GTPases in this investigation bound GTP, but with varying degrees of affinity, ranging from 29 nM to 1.2 μM (Table II). Although these enzymes have already been implicated as GTPases, no systematic enzymatic comparison has been made. Unlike the hydrolysis data, psToc34 and atToc33 both bind GTP quite strongly with K_d values of 50 and 29 nM respectively, while atToc34 and atToc159G have considerably lower affinities. The difference in affinity of the psToc34 family for GTP and GDP was not great, showing only a 2-3 fold difference. In contrast, while atToc159G does not bind GTP as tightly as the small Tocs, it appears to be the most effective discriminator of GTP and GDP with nearly a 10-fold difference (Table II).

Heterologous competitions also demonstrated that these GTPases have nearly identical affinities for GTP and XTP; in the case of atToc33, the affinity for XTP is actually greater. Various other substrates were tested with psToc34 and strong affinity was also shown for GTP-γ-S and GMP-PNP (Fig. 6E). These results show that, although they fail to be hydrolyzable, the GTP-like properties of GTP-γ-S and GMP-PNP both compete for binding; however, GTP-γ-S with its lower K_i suggests it is the better non-hydrolyzable GTP analogue for psToc34. In this assay, psToc34 did not have any significant affinity for the cGMP analogues, the purine ATP, nor the pyrimidine, TTP (Fig. 6F). However, interestingly, it was able to slightly bind the pyrimidine UTP, which shares an analogous placement of a carbonyl oxygen with the purine GTP, suggesting that this oxygen is critical for recognition by psToc34. This may also explain the ability of psToc34 to bind XTP.

**Homology modeling of the Toc GTPases.** atToc33, atToc34, and atToc159G were aligned with the
protein sequence from the crystal structure of psToc34 (pdb id 1H65, chain B) using the SALIGN algorithm. Using this alignment, molecular models were made with the homology modeling software MODELLER. The high degree of identity/similarity and the small number of inserts between the proteins were well suited to this homology modeling technique and did not require loop-modeling refinement. The overall fold of each of the proteins was very similar according to the models, in fact, when superimposed they are indistinguishable over the majority of each structure as shown in Fig. 7A. Not including the inserts, the total average rmsd value for each of the modeled proteins was 0.128, 0.142, and 0.193 for atToc33, atToc34 and atToc159G, respectively. Fig. 7A is the structural alignment of atToc33, atToc34, and atToc159G models with the crystal structure of psToc34, the color scale denotes per residue rmsd values. There are a few small insertions, mostly in atToc159G. Fig. 7B displays the per residue rmsd as a function of amino acid position of psToc34; at each gap the inserted residues are denoted below. The residues with large rmsd values are for the most part near insertions since MODELLER satisfies spatial restraints based not only on the template but also creates bonds with reasonable chemical properties, therefore residues near gaps are moved to allow a feasible model of the insert.

In an attempt to explain the lower catalytic activity of atToc159G and higher K_M, we examined the details of the GTP binding pocket. The binding pockets of psToc34 and atToc159 are shown in stereo (Fig. 7C) with the backbones displayed in tan and gray transparent ribbons for psToc34 and atToc159G, respectively. Sun et al., suggest the possibility of R133 acting as an arginine finger in the psToc34 homodimer. The atToc33 and atToc34 models illustrate that the equivalent arginine from the alignment are likely performing the same function. The key residues identified by Sun et al., shown in pink, are Y132 and R133. Interestingly, the distance between Arg133 and the β-phosphate of GTP is nearly identical with the placement of the conserved Arg residues that have been implicated as arginine fingers found in several GAPs that have been co-crystallized in complex with their cognate GTPases (Fig. 7E and references within the Fig. 7 legend). Although the primary sequence alignment of atToc159G would suggest that that Arg953 may perform the equivalent function as an arginine finger upon hetero- or homodimerization, our model suggests that this is probably not the case. As shown in Fig. 7D, Arg953 from atToc159G is displayed in cyan and although still modeled to be within an α-helical segment with a similar backbone position, its side chain is clearly projecting away from the bound GDP, thus preventing the amino group from stabilizing the β-phosphate as has been postulated for other GAP arginine fingers.

Hydrolysis rate and affinity for GTP are stimulated by transit peptide. GTP is hydrolyzed at a linear rate by psToc34 (Fig. 2A) with a velocity of approximately 35 nmol GTP per minute per μmol Toc (Fig. 2B) Furthermore, when psToc34 is incubated with a 25:1 molar excess of reduced carboxy-methylated lactalbumin (RCMLA), an inherently unstructured peptide, the rate of 32PO3 release remains constant and is very similar to psToc34 alone (Fig. 8A) and the velocity reflects that of ‘unstimulated’ psToc34 (Fig. 8B). Furthermore, when psToc34 is incubated in the presence of a similar molar excess of either SStpPs or SStpNt (the transit peptide from the small subunit of rubisco from either pea or tobacco, respectively), the amount of 32PO3 release and the rate of GTP hydrolysis increases (Fig. 8A & B). Fig. 8A represents a single concentration of GTP (25 μM) with SStpPs, SStpNt and RCMLA all present at 25 molar fold excess to Toc protein. This was repeated with multiple concentrations of GTP, aliquots removed at 0, 10, 15 and 20 min and scintillation counted yielding CPM data that were fit to linear regression (example shown in Fig. 8A). Each linear regression yielded a slope that was mathematically transformed into a rate expressed in nmol GTP hydrolyzed per minute per μmol psToc34, as seen in Fig. 8B (data shown represents psToc34 with one concentration of peptide, 25 μM, and various GTP concentrations). A substrate-velocity plot was created for each of 11 peptide concentrations ranging from 0.58-fold to 100-fold molar excess (data not shown). No concentration of RCMLA stimulated the velocity of psToc34 at any of the GTP concentrations assayed (Fig. 8B & data not shown). SStpPs and SStpNt both stimulated the maximal velocity as well as reduced the K_M (Fig. 8B & D). Using the non-stimulated velocity curve in Fig. 8B (solid squares, solid line), we determined the fold increase in rate...
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at each of the 11 peptide concentrations of SSStpPs, SSStpNt and the negative control, RCMLA (Fig. 8C). Each data point for each treatment in Fig. 8C represents the $V_{\text{max}}$ obtained from non-linear regression for each treatment (cummulation of all data points for a given treatment) divided by the $V_{\text{max}}$ of psToc34 with no treatment (Fig. 8B). Both SSStpPs and SSStpNt increase the rate of GTP hydrolysis by psToc34 by $\sim$1.6-fold whereas RCMLA does not affect the rate (Fig. 8C). Furthermore, the molar excess of SSStpPs and SSStpNt required to give half the maximal rate increase is 3.5 and 3.8 molar fold, respectively. SSStpPs and SSStpNt also alter the $K_M$ or the affinity of psToc34 for the substrate, GTP (Fig. 8D). Each data point in Fig. 8D represents the $K_M$ calculated from the non-linear fit of each molar excess of SSStp to psToc34. The molar excess of SSStpPs and SSStpNt required to reduce the $K_M$ by half is 4.6 and 4.2, respectively.

**Nucleotide exchange of psToc34 is not stimulated by transit peptides.** In order to determine if transit peptides affect the rate of GDP/GTP exchange, psToc34 was preincubated with $[^{32}\text{P}]\text{GTP}$ and the dissociation of labeled nucleotide was monitored by scintillation counting as a loss of counts as a function of time under several conditions. The dashed line with closed squares of Fig. 9 represents the basal rate of GDP loss with psToc34 alone in GBS. The remaining data indicate that there is no significant difference between the rate of exchange with a 100-fold excess of GTP and no SSStpNt (open triangles) relative to a 100-fold excess of GTP at any amount of SSStpNt, as indicated in the Fig. 8 legend.

**DISCUSSION**

Key elements of the translocation complex of the outer membrane of the chloroplast have been identified. The functions of these individual components have been probed to some degree, but the mechanistic details of the Toc complex remain unclear. To better understand the function of the Toc complex, the behavior of the individual Toc components must be investigated *in vitro*, and more specifically, their basal enzymatic activities must be explored. Several groups have reported basal GTP hydrolysis rates for recombinant, ΔTM forms of psToc34, atToc33, atToc34, and at-Toc159G. Although it would not be surprising for these rates to vary between the different homologues, the substantial variation in the reported kinetic properties between different groups, and on occasion even within a given group for the same protein, is troubling. For example, with psToc34, careful analysis of the published data reveals hydrolysis rates within the same article that vary from 0.588 to 26.5, when expressed as nmol GTP/min/μmol Toc protein (21). Moreover, substantial variability is seen when comparing the hydrolytic rates (in nmol/min/μmol) of homologous proteins between pea and Arabidopsis, where values range from 0.588 for psToc34 (21) to $\sim$300 for atToc33 and atToc34 (16). Upon comparing reported values for rates of hydrolysis in the literature, a lack of standardization becomes apparent. Also, there were experimental design problems including reporting rates of GTP hydrolysis that are non-linear with time (14,26,33), kinetic analyses performed with sub-saturating levels of GTP (33,34), and application of non-linear fits to poor or limited data with no error analysis (35). To help reconcile these conflicting reports, we have concurrently determined the enzymatic properties of these four Toc GTPases in parallel, thus enabling a direct comparison of their GTPase properties.

One possible explanation of the observed variations in the literature is that different conditions were used by different labs to test their GTPase activities. By systematically testing several buffer systems and storage conditions it was clear that psToc34 was fairly robust and tolerant of different buffer and storage means. In light of our optimized buffer, pH and storage conditions, which are quite similar to those reported by others, the variations in reported rates can not be due to these conditions. All together, the buffers, storage conditions and pH could at most only account for $\sim$30% change in activity; those reported for the Toc GTPases vary in some cases by 100-fold (16).

It has been shown for other small GTPases that require Mg$^{2+}$ for their activity (30-34) that very few other cations can substitute for Mg$^{2+}$ in the catalytic site. We attempted to replace Mg$^{2+}$ in the active site of psToc34 with several different cations and found that only Zn$^{2+}$ could support hydrolysis activity of psToc34 at or above Mg$^{2+}$ level. Although zinc has a smaller radii than magnesium (1.60 vs. 1.34 Å) it may still be able to fit into the metal binding site of psToc34 thereby sta-
bilizing GTP. Indeed, other divalent metal cations have been shown to bind to other GTPases, for example, replacement of the Mg$^{2+}$ by Mn$^{2+}$ in p21Ras results in a higher GTPase activity (55). This stimulation has been suggested to involve an increase of the pK$_{a}$ of the $\gamma$-phosphate of GTP resulting in an increase of reaction rate at neutral pH. Schweins et al., determined that this relationship between pK$_{a}$ of nucleotide and $^1$H-abstraction potential indicated that the GTP itself acts as a general base in the reaction. This divalent metal cation mediated rate increase was also shown to be the case with two other GTPases Rap1A and Ran. The lack of elevated GTP hydrolysis by psToc34 with Mn$^{2+}$ is puzzling, however the non-Ras-like G2 motif and its interactions with the $\gamma$ phosphate of bound nucleotide may account for these observations.

As a result of our systematic comparison, we observed a number of differences between the enzymatic properties of atToc159G and the small Toc GTPases: atToc159G demonstrated a lower $V_{\text{max}}$, a higher $K_{M}$, a lower $E_{a}$, a higher $k_{\text{cat}}$, and a relatively lower affinity for GDP and GTP. The possibility that may account for these enzymatic differences are the residues coordinating the Mg$^{2+}$ ion. We took advantage of the molecular model that we built to explore this possibility. From the crystal structure of psToc34, the Mg$^{2+}$ is coordinated between residues E73 and S53, however from our homology-based model of atToc159G, this glutamate residue is replaced by a serine (Fig. 7D). The GDP and Mg$^{2+}$ are shown with residues from psToc34 in pink and equivalently aligned residues (S889 and S869) of atToc159G in cyan. Whether these two serines are capable of coordinating the Mg$^{2+}$ is not known, however analogous small GTPases (e.g. Rho) have been reported to contain a threonine-threonine Mg$^{2+}$ coordination site (56). This Ser-Mg$^{2+}$-Ser scenario is not likely since the participation of dual serines has not been observed in any GTPases to date nor have two serines been reported in the architecture of $>$200 other Mg$^{2+}$ coordination sites (57). Alternatively, our model suggests that another acidic residue, D884, may be positioned such that it could provide an alternative Mg$^{2+}$ coordination site for GTP/GDP binding to atToc159G (Fig. 7D). Altering the coordination site of the Mg$^{2+}$ ion may thus change the conformation of the bound nucleotide, leading to the enzymatic differences observed between atToc159G and the other small Toc GTPases. In the current work we have primarily utilized the homology models to provide some probable structural insight for our in vitro observations. As a future direction we intend to use these models to introduce mutations which modulate individual functions of Toc GTPases e.g. dimerization, hydrolysis, nucleotide specificity, and/or peptide affinity. These constructs will allow further examination of the interdependence of these functions as well as indicate the legitimacy of these models.

Although the enzymatic characterization of psToc34 clearly shows that the rate of GTP hydrolysis is insensitive to enzyme concentration, analysis by AUC reveals that the oligomeric properties of psToc34 over a similar concentration range leads to an increased population of the dimer. The behavior of psToc34 during centrifugation is suggestive of a fast equilibrium between the monomer and dimer, where the equilibrium is shifted towards the dimeric form as the protein concentration is increased. Whereas dimerization as a function of protein concentration is not a novel idea, (58,59), the dimeric form of psToc34 is no more active than its monomeric form. Interestingly, when we characterized the oligomeric behavior of a mutation, R133A, we observe a highly soluble, exclusively monomeric species at all protein concentrations tested, including those that were shown to be primarly dimeric for the wt protein. Furthermore, the R133A mutant was unable to hydrolyze GTP at any appreciable rate. In light of evidence that psToc34(R133A) is correctly folded, the structural basis for why this mutant is unable to undergo dimerization or perform GTP hydrolysis is unknown. Nonetheless, the causal relationship between loss of dimerization and GTP hydrolytic activity was not resolved by our current experimentation.

While the precise relationship between oligomerization and GTPase activity is complex, one possible explanation of these results is that when psToc34(R133A) is expressed in E. coli, it is loaded with Mg$^{2+}$GTP during expression and folding. However, since this mutation is catalytically inactive, there is no opportunity to hydrolyze this GTP to GDP + Pi, thus trapping this protein in the GTP-bound form. This is unlike the wt protein which when isolated and crystallized, was found to be exclusively in the GDP-bound form (21); there
is no GTP-bound crystal structure to date. Our data supports the proposal that Arg133 functions as the ‘arginine finger’ that contributes to the stabilization of the dimeric form of psToc34 (21). This is supported by our molecular models, which consistently place a conserved Arg at this corresponding residue, as spanning across the dimeric interface functioning to stabilize the β-phosphate associated with the GDP in the trans subunit (Fig. 7).

Since this work has been limited to the soluble forms of the Toc GTPases, how their transmembrane domains may influence either their oligomeric behavior or their catalytic activity remains unclear. However, using the soluble form of psToc34, we have clearly demonstrated the ability of this domain to form homodimers yet failed to see any evidence of its ability form homotetramers. This observation is consistent with the recent crystal structure, which also reported a dimeric structure with a two-fold axis of symmetry. The ability of psToc34 to oligomerize into only dimers is especially intriguing in light of the recent low-resolution structure of the pea Toc translocon that contains four Toc34 subunits with a single Toc159 subunit (4). This may suggest that the native Toc complex contains a pair of co-existing Toc34 dimers. Although speculative, the homodimerization that we observe in vitro may also exist in vivo and may become disrupted by either precursor binding, or alternatively, through interaction with Toc159, to yield a transient heterodimer. Our future work will be to dissect the interplay by which both nucleotides and transit peptides modulate the oligomeric properties of the Toc GTPases.

The comparison of activation energies for GTP hydrolysis between the Toc proteins show values of ~10-15 kcal/mol (Fig. 5). This is similar to the activation energy of another small GTPase, EF2, of ~20 kcal mol⁻¹ (60). It was, however, interesting to observe that although atToc159G has a lower Vₘₐₓ and a higher Kₘ, it appeared to have a significantly lower activation energy (10.1 vs. ~15). This would suggest that although atToc159G has a lower affinity for productive binding of GTP, upon binding, the orientation of the nucleotide was in some way more favorable for hydrolysis, thus a lower energy transition state. The lower nucleotide affinity reflected in the Kₘ could be the result of some steric constraints imparted by the additional N- and C-termini residues found in at-Toc159G. It is also possible that the addition of these flanking sequences may somehow contribute to its distinct kinetic and thermodynamic parameters. Unfortunately, we have no structural insight into these regions, since when building our molecular models, we necessarily truncated these sequences since they were not present in psToc34 and would have disrupted the structural alignment. How the acidic N-terminal and membrane-associated C-terminal residues influence the enzymatic properties as well as homo/heterodimerization of atToc159 will be difficult to ascertain since the full size protein is insoluble when expressed in a heterologous system.

Heterodimerization and reciprocal activation of GTP hydrolysis is not a novel idea, in fact, Sun et al. has proposed homodimerization between psToc34 monomers and possible heterodimerization between atToc33 and atToc34. This has been well studied in another protein transport system that involves GTPases, the SRP54/Ffh from the E. coli. In this system, it has been shown that heterodimerization elicits a reciprocal GAP activity from each monomer, thus coordinating GTP hydrolysis, completing the GTPase cycle, and thereby facilitating protein transport. Although the interactions between the Toc GTPases may mechanistically be different from the SRP54/Ffh GTPases, the possibility of homodimerization and/or heterodimerization (21,35) may still modulate the activities of the Toc GTPases and influence the functionalities of these proteins in vivo.

Furthermore, in the crystal structure, psToc34 appears to be a homodimer with an arginine finger extending from one monomer into the other (21). Fig. 7E is a stereo view of several small GTPase•GAP complexes showing the proximity of the arginine finger to the β-phosphate of GDP; R133 of psToc34 is colored according to element name and other proteins (Ras, Rho, Rab, CDC42 and psToc34) are in solid colors; see Fig. 7 legend for details. These complexes were aligned about the GDP and Mg²⁺ molecules; psToc34 as well as all of these canonical small GTPases appear to bind GDP and Mg²⁺ in a very similar conformation. Both atToc33 and atToc34 have equivalent arginine residues that appear to coincide with the position of the R133 of psToc34. The arginine finger of each GAP can be seen in the area near the β/γ-phosphate and psToc34 fits well with the terminal portion of the arginine side-chain. While
many GAPs do in fact have a tyrosine adjacent to the arginine finger, the tyrosine of psToc34 is likely not serving the same function as previously implicated (21).

Unfortunately, investigations into the ability of preproteins and/or transit peptide-derived synthetic peptides to stimulate the GTP hydrolysis rates of the Toc homologues is another area of some conflict and confusion in the literature. For instance, one group reported that there is a ~30-fold stimulation of hydrolysis by Toc34 when in the presence of preprotein (11), however this same group subsequently reported no effect upon the addition synthetic peptides comprising the N- and C-termini of the preSSU transit peptide (26). Furthermore, an N-terminal peptide from prSSU had the equivalent stimulatory activity as the full-length preprotein with atToc159G alone and with the reconstituted complex. However, in this more recent paper, they reported only ~4-fold stimulation and not the previously reported ~30-fold effect. Although the enzymatic properties of the native or reconstituted Toc complex may certainly be different than the individual components, our results (Fig. 3) clearly show that the oligomeric state of psToc34 and the other Toc homologues does not stimulate the hydrolysis rates in vitro. A further concern when comparing synthetic peptides to the full-length preproteins is that *E. coli* expressed preproteins are insoluble and require chemical denaturation for solubilization. This may interfere with the GTPase assay due to indirect effects of the denaturant. Additionally, the use of short synthetic peptides could have unintentionally split a continuous recognition element present in the full length transit peptide. Finally, short synthetic peptides would abrogate any structural contributions from the mature domain as has been clearly demonstrated for prSSU (61).

Nevertheless, it is clear from our results using a soluble, full-length transit peptide with psToc34, that the transit peptide alone does in fact stimulate the rate of GTP hydrolysis. We further show that this stimulation increases not only the $V_{\text{max}}$ of the enzyme, but also lowers the $K_M$ for GTP. The ~2-fold stimulation that we observe is very reproducible and is not the result of non-specific interactions. Although this is much more activity than the synthetic peptides were capable of stimulating psToc34 as reported by Becker *et al.* (26), it is still significantly less than the initial activity reported value by prSSU (11). Moreover, we have also tested the full-length prSSU and found that it does demonstrate a higher level of stimulation yet plateaus at ~3-fold (data not shown). Causes for the variability between laboratories in the level of peptide and preprotein stimulation of the hydrolysis of GTP by the Toc GTPases remain unclear. All the same, the ability of the full-length transit peptide to stimulate the rate of GTP hydrolysis suggests that this sequence may somehow regulate the activity/structure of the GTPase during Toc-mediated precursor recognition. The fact that this stimulation was not observed upon addition of the unstructured protein, RCMLA, suggests that the GTPase stimulation is a result of specific interaction(s) being mediated by one or more regions of the transit peptide and is not simply the interaction of the exposed peptide backbone as has been suggested for RCMLA stimulation of ATPase activity of the chaperone hsp70 (39). These observations suggest that some region or domain of the transit peptide somehow functions to regulate the activity/structure of the GTPase during Toc-mediated precursor recognition.

Transit peptide mediated stimulation of the GTPase activity of Toc34 and Toc159 has been reported (10,15,26,31). Although others have even used the term GTPase Activating Protein, we feel that this was premature. The regulatory mechanism of switch GTPases involves both activating proteins and exchange factors. If the only observable quantity is a hydrolysis product, then both exchange factors and activating proteins will lead to an apparent increase in rate. We have carefully examined the stimulatory effect on hydrolysis by titrating the transit peptide and saturating that effect as seen in Fig. 8. In a separate assay designed to follow the rate of exchange, no significant differences are observed with GTP alone and the addition of nearly a 40-fold excess of transit peptide (Fig. 9), which was well into the saturating concentration according to Fig. 8C. Therefore, taken together, these data suggest that the transit peptide is in fact functioning as a GAP and not a GEF. The ability of two different preproteins (prSSU and prOE33) has already been shown to stimulate the rate of GTPase hydrolysis of the purified translocation complex (11) suggesting that this may be a universal feature of chloroplast precursors and transit peptides.
The evolutionary process of how transit peptides acquired the ability to function as a GTPase-activating protein is unknown. Moreover, there are no structural details known on how transit peptides stimulate the GTPase activity. It has become clear that for many of the well-characterized, small GTPases (Rho, Rac1 and Cdc42), there are several divergent classes of proteins that are capable of stimulating the hydrolysis of GTP (62). The Rho family of small GTPases has been found to be the target of several bacterial cytotoxins that transiently down-regulate these GTPases. This can be done by both covalent modifications such as ADP ribosylation and glycosylation (63), as well as through cytotoxins or virulence factors, such as YopE, ExoS, and SptP, that mimic the activity of eukaryotic cytosolic GAPs (64). Interestingly, although these proteins have very little sequence similarity with each other, they have been shown to have very similar three-dimensional structures (65). Crystal structures of eukaryotic GAPs with their cognate GTPases show striking similarity in observed function with the bacterial cytotoxins, especially ExoS from Pseudomonas aeruginosa with Rac (66). Of significant note is the critical Arg residue (Arg144) that projects outward interacting with the bound GTP molecule of the GTPase. Comparison of this model with the structure of other eukaryotic GAPs is remarkable since outside of the conserved Arg residue, there are no other structural similarities of these bacterial cytotoxins to the cognate eukaryotic GAPs. This would suggest that the bacterial GAPs arose via convergent evolution with the functional analogs found in eukaryotes. This observation also suggests that multiple GAPs with highly divergent structures can perform the equivalent functional role. This common activity in the absence of a conserved structure may also be observed in how transit peptides function as GAPs.

Interestingly, the GAP activity associated with the bacterial cytotoxins is the disruption of the actin cytoskeleton in the host cell thereby avoiding engulfment and destruction by phagocytosis (67). To accomplish this activity they rely on a Type III secretory pathway to inject these virulence factors into the host cell. It has been proposed earlier that transit peptides could have evolved from cyanobacterial expressed virulence factors (1,68). Possibly these ancestral transit peptide progenitors were secreted into the host cytoplasm via the SynToc75 complex. Upon secretion, they function as virulence factors able to alter the actin cytoskeleton of their endocytotic host via the GAP mechanism, similar to YopE/ExoS/SptP. Although speculative, it is possibly through this GAP activity of the secreted peptides that these cyanobacteria were able to thwart the host defense response that would normally lead to their degradation. This GAP activity thus enables the transition from endocytosed bacteria into a semi-autonomous organelle. Finally, through unknown evolutionary changes, these ancestral host-encoded soluble GTPases were ultimately recruited and redeployed to the chloroplast envelope where they became receptor/gatekeeper GTPases. This ancestral, host origin of the Toc GTPases is consistent with the fact that to date, there is no clear cyanobacterial encoded Toc34 or Toc159 homologs.

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REFERENCES


FOOTNOTES

1 Contributed equally to this manuscript.

2 The online version of this article (available at [www.jbc.org](http://www.jbc.org)) contains supplemental Figs. 1 and 2 as well as supplemental Table I.

3 This work was supported by a grant from the Cell Biology Program (MCB-0344601) at the National Science Foundation to BDB and a Science Alliance Award from UTK to LER. IMC, a.k.a. Anion, is a UTK undergraduate and was supported in part by Hope, Volunteer and Merit Scholarships and an NSF Research Opportunity Award to BDB.

The abbreviations used are: TOC, translocator of the outer membrane of the chloroplast; TIC, translocator of the inner membrane of the chloroplast; TOM, translocator of the outer membrane of the mitochondria; TIM, translocator of the inner membrane of the mitochondria; MALDI-TOF, Matrix Assisted Laser Desorption Ionization-Time Of Flight; ACN, acetonitrile; TFA, trifluoro acetic acid; CHCA, α-cyano-4-hydroxycinnamic acid; AUC, analytical ultracentrifugation; SV, sedimentation velocity; CD, circular dichroism; XTP, xanthosine 5′-triphosphate; GTP-γ-S, guanosine 5-O-(3-thiotriphosphate); GMP-PNP, guanylyl-5′-[β,γ-imido]-triphosphate; 8-bromo-cGMP, 8-bromo guanosine-3′,5′-cyclic monophosphate.

FIGURE LEGENDS

Fig. 1. Overexpression and purification of Toc GTPases and transit peptides. *A*, atToc33 and atToc34 were overexpressed in *E. coli* strain TG1, atToc159G and psToc34 in *E. coli* strain BL21(DE3). Proteins were purified under nondenaturing conditions using cobalt IMAC chromatography and eluted with a step gradient from 15 mM (wash 3) to 250 mM imidazole (elution 1-3). Total protein, pellet, flow through (lanes 1-3), washes (lanes 4-6), elutions (lanes 7-9), and marker (lane 10) were separated by SDS-PAGE followed by Coomassie Blue staining. *B*, Lanes 1-5 are marker, psToc34, atToc33, atToc34 and atToc159G, respectively. Toc proteins were loaded under equal mass conditions (100 ng) separated by SDS-PAGE followed by Coomassie Blue staining. *C*, Transit peptide was purified using chitin affinity chromatography. Marker (lane 1), total protein, pellet, soluble fraction, flow through, wash (lanes 2-6), column matrix (lane 10) were separated by SDS-PAGE and visualized by Coomassie Blue staining. *D*, MALDI-TOF of SSStpNt was performed on a Bruker Daltonics MicroflexTM mass spectrometer, with
apomyoglobin used as an internal standard for calibration. The +2 and +3 charge states of apomyoglobin species can be seen at 8477 m/z (avg. MW of Apomyoglobin is 16952) and 5651.7 m/z, respectively. The +1 and +2 charged SStpNt species are found at 6125.9 and 3062.6 m/z. The species at 4152.6 m/z is a fragment resulting from proteolysis during isolation.

**Fig. 2.** Determination of $K_M$ and $V_{max}$ of Toc GTPases. Panels A-D represent CPM vs. Time (left) and Rate vs. Substrate concentration (right) of the four Toc proteins atToc33, atToc34, pStoc34 and atToc159G, respectively. In the left panel of A-D, the indicated concentration of total GTP (μM) is 1, ■; 5, ▲; 10, ▼; 25, □; 50, ●; 75, ○; and 100, X, respectively. The $[\gamma^{-32P}]$GTP in all experiments was held constant at 10 nM. Linear regression of CPM/min yielded a slope that was then mathematically transformed into a rate expressed as nmol GTP hydrolyzed per minute per μmol Toc protein, see experimental procedures. The rate at each GTP concentration is then graphed to yield the Michaelis-Menten substrate velocity plot. This data is fit to Equation 1 to determine the $V_{max}$ and $K_M$ of each protein.

**Fig. 3.** Hydrolysis rate of pStoc34 is not influenced by protein concentration but undergoes concentration-dependent dimerization. Panels A and B illustrate representative sedimentation velocity data from pStoc34 at low (3.4 μM) and high (33.8 μM) concentrations, respectively. Panel C is hydrolysis data of pStoc34, red line, ■; atToc33, green line, △; atToc34, blue line, ▼; and atToc159G, black line, ◊; were assayed at 1, 2, 3, 5, 15 and 20 μM Toc protein; two concentrations reported in panel D, 1.7 μM and 16.9 μM, are indicated by dashed and dotted vertical lines, respectively. Panel D shows the sedimentation coefficient distributions of pStoc34 at 1.7 μM, red line; 16.9 μM, green line; and 33.8 μM, blue line. Values on the x-axis in panel D below S=3 are representative of monomeric species whereas values above S=3 represent dimeric species. Panel E depicts the reduction of monomeric species, red line, ■; and increase of dimeric species, blue line, ◊ as the concentration of pStoc34 is increased.

**Fig. 4.** The mutant protein pStoc34(R133A) is monomeric and lacks GTP activity. Panel A shows the sedimentation coefficient distributions of pStoc34(R133A) at 3.4 μM, dotted line; 33.8 μM, dashed line; and 170 μM, solid line illustrating that this mutant form of pStoc34 can no longer undergo concentration-dependent dimerization. As shown in panel B, pStoc34(R133A) mutant protein, ○, dashed line; is severely reduced in its GTP hydrolytic activity as compared to wild-type pStoc34, ■, solid line.

**Fig. 5.** Energy of activation of the Toc GTPases. Panels A-D represent the dependence of the rate of pStoc34, atToc33, atToc34 and atToc159G on temperature, respectively. The maximal rate of GTP hydrolysis was determined for each protein at various temperatures. The natural log of the rates of GTP hydrolysis of the Toc GTPases are plotted as a function of the inverse of temperature, here, 10^{3/T} in Kelvin.

**Fig. 6.** Nucleotide binding activity of the Toc GTPases. Panels A-D represent the outcome of homologous and heterologous nucleotide binding competition assays for pStoc34, atToc33, atToc34 and atToc159G respectively. $K_d$ and $K_i$ values were determined for GTP, □; GDP, ◊; and XTP, △. Note that the atToc159G-XTP heterologous competition data was not of sufficient quality to allow a confident fitting of $K_i$, however a trend line is included for visual clarity. Panel E is the result of a heterologous competition of $[\alpha^{-32P}]$GTP with the non-hydrolysable analogs GTP-$\gamma$-S, △ and GMP-PNP, □. Various other nucleotides were used as competitors (Panel F), 8-bromo-cGMP, ■; 2’3’ cGMP, ▲; 3’5’ cGMP, ▼; ATP, ◆; CTP, ●; TTP, □; UTP, ▼; and CDP, △ most of which did not compete for binding. Both panels E and F were performed with pStoc34.

**Fig. 7.** Homology modeling of the Toc GTPases. A, The structural alignment of atToc33, atToc34, and atToc159G models with the crystal structure of pStoc34 illustrates the overall similarity in fold between the models and template. The color scale represents the per residue rmsd, which is displayed in B as a function of aligned amino acid position with pStoc34; inserted sequences are denoted below gaps. C
and $D$ represent details of the differences between the nucleotide binding pockets of psToc34 and at-Toc159G. The backbones of each superimposed structure are shown in brown and grey respectively, the GDP and Mg$^{2+}$ and water molecules from psToc34 are shown in elemental colors, side chains of residues from psToc34 are shown in pink while those of atToc159G are shown in cyan. The proximity of R133 to the position of other arginines in GTPase•GAP complexes is shown in panel $E$, residues of psToc34 are shown in elemental colors as well as the GDP and Mg$^{2+}$ molecules and the following color codes apply: yellow residues, H-Ras•P120 GAP (pdb id 1WQ1 (52)); blue residues, CDC42•CDC42 GAP (pdb id 1GRN (53)); orange residues, Rho•P50 Rho GAP (pdb id 1TX4 (56)); grey residues, RhoA•Rho GAP1 (pdb id 1OW3 (54)); and purple residues, Rab33B•GYP1 GAP (pdb id 2G77 (69)).

Fig. 8. Effect of transit peptide on hydrolysis rate of psToc34. Panel $A$ represents the increase in CPM as a function of time for psToc34 alone, ■; in the presence of 25 molar-fold SStpPs, △; SStpNt, ○; and RCMLA, ◇, respectively with a total of 25 μM GTP. The total GTP was titrated from 1 to 100 μM, as in Figure 3; only 25 μM is shown in panels $A$, $C$ and $D$ for clarity. $B$, Multiple concentrations of GTP were used to generate the substrate velocity plot for psToc34 alone or in the presence of a molar-fold excess of SStpPs, SStpNt or RCMLA at 2.5, 4, 6.3, 10, 15.8, 25, 40, 63 and 100 over psToc34; for clarity, only 25 molar-fold excess is shown. $C$, The fold increase, over unstimulated psToc34, in rate of GTP hydrolysis, is shown as a function of the peptide to psToc34 molar ratio at each titration of peptide; rate increase data was obtained from a substrate velocity plot as in panel $B$. $D$, $K_M$ values obtained from the substrate velocity plots in $B$ are plotted indicating an increase in affinity for substrate with the titration of SStpPs and SStpNt to a minimum $K_M$ at 25 molar-fold excess of peptide to psToc34. SStpPs, SStpNt and RCMLA are represented as open triangles, closed circles and open diamonds, respectively.

Fig. 9. Effect of transit peptide on GDP/GTP exchange rate. psToc34 was preloaded with [$\alpha$-32P]GTP and then incubated with the following: GBS, dashed line, ■; 100 fold GTP, solid line, △; and the following represented by dotted lines, 100 fold GTP with 0.3 fold SStpNt, ○; 100 fold GTP with 1.5 fold SStpNt, □; 100 fold GTP with 7.5 fold SStpNt, ▲; or 100 fold GTP with 37.5 fold SStpNt, ◇. No significant difference in rate of GDP loss was observed between 100 fold GTP and any amount of SStpNt in addition to 100 fold GTP although the hydrolytic stimulation had saturated in the presence of a lesser amount.

Supplemental Fig. 1. Buffer, storage condition, pH titration and metal ion exchange. $A$, Four buffers were chosen to assay the kinetic parameters of the Toc GTPases: GBS, KSS, HMK and TTP (see experimental procedures). psToc34 was assayed for maximal activity in each buffer and at four different storage conditions: -80°C slow, -80°C fast (liquid nitrogen first, then -80°C), -20°C and liquid nitrogen, depicted as white, diagonal lined, grey and horizontal lines, respectively. $B$, the pH of GBS was altered using different buffering agents to obtain buffer pH between 6.1 and 11.9. $C$, psToc34 was incubated with 50 mM of the following cations: MgCl$_2$ (small checker), CaCl$_2$ (large checker), CdCl$_2$ (horizontal lines), CoCl$_2$ (vertical lines), CuSO$_4$ (left-hand diagonal), MnCl$_2$ (right-hand diagonal), NiSO$_4$ (hatched) and ZnCl$_2$ (dotted). After sufficient time for metal ion exchange, GTP hydrolysis was measured in GBS supplemented with the appropriate metal cation. Rates are plotted as % control.

Supplemental Fig. 2. Far UV circular dichroism spectrum of psToc34(R133A). R133A was diluted to 7.5 μM in 50 mM NaH$_2$PO$_4$, pH 7.8. 10 mM NaF; data was collected at 25°C. [0] represents mean residue ellipticity with the units of millidegrees × cm$^2$ × dmol$^{-1}$. 

Nucleotide binding & hydrolysis of the Toc GTPases
## Table I

<table>
<thead>
<tr>
<th>Kinetic parameters of the Chloroplast Toc GTPases</th>
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<tbody>
<tr>
<td>$V_{\text{max}}$</td>
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<tr>
<td>nmol/min/µmol</td>
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<tr>
<td>psToc34</td>
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<tr>
<td>atToc33</td>
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<tr>
<td>atToc34</td>
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<tr>
<td>atToc159G</td>
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Table II

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<tr>
<th></th>
<th>GTP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GDP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>XTP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>GMP-PNP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>GTP-γ-S&lt;sup&gt;d&lt;/sup&gt;</th>
<th>UTP&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>psToc34</td>
<td>50 (42-61)</td>
<td>151 (135-170)</td>
<td>41 (36-47)</td>
<td>288</td>
<td>70</td>
<td>2.15x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>atToc33</td>
<td>29 (21-39)</td>
<td>78 (63-91)</td>
<td>63 (54-68)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>atToc34</td>
<td>1219 (807-1426)</td>
<td>2735 (1667-4375)</td>
<td>853 (710-1028)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>atToc159G</td>
<td>406 (270-611)</td>
<td>3365 (2535-4467)</td>
<td>N/A&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<sup>a</sup>values in nM, <sup>b</sup>values in parentheses represent 95% confidence, <sup>c</sup>K<sub>d</sub>, <sup>d</sup>K<sub>i</sub>, <sup>e</sup>data quality did not allow a confident non-linear fit, <sup>f</sup>not determined
Figure 1

A. 

B. 

C. 

D. 

Intensities (A.U.) x 10^-4

m/z

6125.9

8477

3062.6

4152.6
Figure 2

A.

B.

C.

D.
Figure 3

A. Fringes (a.u.) vs. Radius (cm)

B. Fringes (a.u.) vs. Radius (cm)

C. Enzyme (μM) vs. nmol GTP/min/μmol Toc

D. S vs. c(s)

E. [psToc34] μM vs. c(s)
Figure 4
Figure 5
Figure 6

A. 

% [$\alpha$-32P]GTP bound

log [unlabeled NTP]

B. 

% [$\alpha$-32P]GTP bound

log [unlabeled NTP]

C. 

% [$\alpha$-32P]GTP bound

log [unlabeled NTP]

D. 

% [$\alpha$-32P]GTP bound

log [unlabeled NTP]

E. 

% [$\alpha$-32P]GTP bound

log [GTP]

F. 

% [$\alpha$-32P]GTP bound

log [unlabeled NTP]
Figure 8
Figure 9
In vitro comparative kinetic analysis of the chloroplast TOC gtpases
L. Evan Reddick, Michael D. Vaughn, Sarah J. Wright, Ian M. Campbell and Barry D. Bruce

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