Dimerization is important for the GTPase activity of chloroplast translocon components atToc33 and psToc159

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Running Title: Structure and function of Toc GTPases

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Arabidopsis Toc33 (atToc33) is a GTPase and a member of the Toc (translocon at the outer-envelope membrane of chloroplasts) complex that associates with precursor proteins during protein import into chloroplasts. By inference from the crystal structure of psToc34, a homologue in pea, the arginine at residue 130 (Arg130) has been implicated in the formation of the atToc33 dimer and in inter-molecular GTPase activation within the dimer. Here we report the crystal structure at 3.2 Å resolution of an atToc33 mutant, atToc33(R130A), in which Arg130 was mutated to alanine. Both in solution and in crystals, atToc33(R130A) was present in its monomeric form. In contrast, both wild-type atToc33 and another pea Toc GTPase homologue, pea Toc159 (psToc159), were able to form dimers in solution. Dimeric atToc33 and psToc159 had significantly higher GTPase activity than monomeric atToc33, psToc159 and atToc33(R130A). Molecular modeling using the structures of psToc34 and atToc33(R130A) suggests that, in an architectural dimer of atToc33, Arg130 from one monomer interacts with the β-phosphate of GDP and several other amino acids of the other monomer. These results indicate that Arg130 is critical for dimer formation, which is itself important for GTPase activity. Activation of GTPase activity by dimer formation is likely to be a critical regulatory step in protein import into chloroplasts.

Chloroplast biogenesis relies on the import from the cytosol of the majority of chloroplast proteins, which are synthesized as precursors with N-terminal targeting signals. Import of precursor proteins into chloroplasts is mediated by a protein translocon complex, which is composed of the Toc (translocon at the outer envelope membrane of chloroplasts) and the Tic (translocon at the inner envelope membrane of chloroplasts) proteins (for reviews, see (1,2)). Three Toc proteins, Toc159, Toc75 and Toc34, form the core of the Toc complex (3). In Arabidopsis, Toc34 is encoded by two genes, atToc33 and atToc34, which share 59% and 64% amino-acid identity with pea Toc34 (psToc34), respectively (4,5). atToc33 is the major functional form in leaf chloroplasts (6,7).

Toc75 forms a protein-conducting channel across the outer membrane (8,9). Toc34 has a cytosolically-exposed GTPase domain followed by a C-terminal membrane anchor (10). Toc159 consists of an acidic N-terminal domain, followed by a GTPase domain homologous to that of Toc34, and a membrane-protected C-terminal domain (11). Toc159 and Toc34 function as the initial receptors for incoming precursor molecules. Non-hydrolyzable GTP analogues severely inhibit precursor binding to chloroplasts (12,13). It has also been shown in vitro that
the sequential transfer of precursors between these two receptors is regulated by GTP hydrolysis (14). Targeting of the two receptors themselves to chloroplasts is also dependent on GTP (15-19). Therefore activation and regulation of the GTPase activity of these two receptors are critical for protein import into chloroplasts.

Previously we have reported the crystal structure of the GTPase domain of psToc34 (20). We have shown that psToc34 forms a dimeric structure resembling a small GTPase complexed with a GTPase activating protein (GAP), with arginine 133 of one monomer contacting the β phosphate of the other monomer. We therefore proposed that arginine 133 functions as a GTPase-activating “arginine finger”. However, it has recently been shown that a mutant form of atToc33, atToc33(R130A), in which the arginine 130 residue (corresponding to psToc34 arginine 133) was mutated to alanine, had a reduced dimerization ability but the same GTPase activity as wild-type atToc33 (21). It was therefore concluded that the GTPase activity of atToc33 was not activated by dimerization and Arg130 did not function as an arginine finger.

Here we report the crystal structure of atToc33(R130A), and show that it indeed formed a monomeric structure. No significant amount of dimer could be detected in solution. In contrast, wild-type atToc33 and psToc159 GTPase domain were able to form dimers in solution whose GTPase activity was much higher than that of either their monomeric forms or atToc33(R130A). Therefore dimer formation is important for GTPase activity of atToc33 and psToc159. We also report the important role of magnesium ion in preserving the quaternary structure of these Toc GTPases.

**EXPERIMENTAL PROCEDURES**

Construction of plasmids encoding atToc33, psToc159, and atToc33(R130A) GTPase-domain - The coding regions for residues 1 to 256 of atToc33 and residues 700 to 1057 of psToc159, which correspond to their GTPase domain, were amplified from their cDNA clones (22,23) using primers with an NdeI site at the N terminus and a HindIII site at the C terminus. Amplified fragments were digested with NdeI and HindIII and subcloned into the NdeI/HindIII site of pET22b, resulting in translational fusion of the His6 tag to the C-terminus of atToc33(1-256) and psToc159(700-1057). The atToc33(1-256)R130A mutant was generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) on the plasmid encoding atToc33(1-256). The three proteins produced, wild-type atToc33(1-256)-His6, psToc159(700-1057)-His6, and atToc33(1-256)R130A-His6, are herein referred to as atToc33wt, psToc159G and atToc33(R130A) respectively.

Protein expression and purification - Plasmids for protein expression were transformed into *E. coli* strain BL21(DE3). Expression of proteins was induced with 1 mM IPTG after OD<sub>600</sub> of the culture reached ≈0.6. The cells were further cultured at 37°C for another 3 hours. Bacterial cells were harvested by centrifugation at 6000 rpm for 30 minutes at 4°C, resuspended in Ni-NTA column binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole), and lysed under high pressure using a microfluidizer. The clear cell lysate obtained after centrifugation at 30,000 rpm for 40 min at 4°C was purified using Ni-NTA affinity chromatography (Novagen). Proteins were eluted from the affinity resin by an elution buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 150 mM imidazole (for atToc33wt and atToc33(R130A)) or 100 mM imidazole (for psToc159G), at pH 7.9. A yield of approximately 20 mg/L culture was obtained.
for both atToc33wt and atToc33(R130A), and 50 mg/L culture for psToc159G.

**Gel filtration** - Gel filtration experiments were performed on HiLoad 16/60 Superdex™ 75 preparation-grade columns connected to an AKTA-Purifier FPLC system (Amersham Biosciences). Prior to protein loading, the column was equilibrated with a buffer containing 20 mM HEPES and 200 mM NaCl (pH 7.0) without MgCl₂ or with 2 mM, or 100 mM MgCl₂. Before loading, protein solutions were filtered through 0.22 μm filters. Small peaks are sometimes observed in the void volume and might be due to aggregation of small quantities of proteins. Molecular mass markers (Amersham Biosciences) previously run in the same column were used to determine molecular masses of sample proteins by elution comparison.

**GTPase assays** - GTPase assays were performed as described elsewhere (24). Briefly, assays were carried out in a 20 μl reaction mixture containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 1.0 μg of proteins, 1.7 nM [γ³²P] GTP (1μCi) (NEN, Boston, MA) and 200 μM GTP with 0, 2 or 100 mM MgCl₂ as specified for each figure. The reaction was incubated at 37°C for 30 minutes and stopped by addition of 5 μl of 500 mM EDTA. A fraction of the stopped reaction (0.35 μl) from each sample was spotted onto a polyethyleneimine cellulose thin layer chromatography plate (Sigma-Aldrich, St. Louis, MO) and developed in 1.0 M formic acid and 0.5 M LiCl. The plates were air-dried and spots corresponding to GTP and Pi were quantified using a phosphorimager (Fuji FLA-5000). For comparison of the GTPase activity of atToc33wt and psToc159G in different quaternary structures, peak fractions separated by gel filtration were used.

**Crystallization of the atToc33(R130A)-GDP complex** - Solubility of atToc33wt and atToc33(R130A) was very low and both proteins tended to precipitate at a concentration above 2 mg/ml. We found that adding 50 mM imidazole to the buffer could raise the solubility of atToc33wt and atToc33(R130A) to concentrations of 8 mg/ml and 6 mg/ml, respectively. Prior to crystallization trials, atToc33(R130A) protein was dialyzed against a buffer of 100 mM Na-acetate, pH 6.0, 50 mM imidazole, 100 mM NaCl, 5% glycerol, under slow equilibration without stirring and was concentrated using Amicon Ultra® (Millipore Corporation, Bedford, MA, USA) concentrators. Crystallization trials were setup with 6 mg/ml protein in the above buffer condition using the hanging-drop vapor-diffusion method. Initial screening was performed with sparse-matrix screen from Hampton Research. Preliminary crystals, which only diffracted to about 8.0 Å, appeared in the Hampton crystal screen condition containing 30% PEG 4000, 0.2 M ammonium acetate, 50 mM sodium acetate, pH 4.8. To improve crystal quality, various additives were used. Addition of nicotinamide nucleotide (NAD) at 100 μM resulted in greatly improved crystal quality. The crystal grown with this additive diffracted to 3.2 Å, and was used for collecting data for this report. Simultaneous attempts to crystallize atToc33wt were not successful.

**Data collection and refinement** - A complete data set was collected to a resolution of 3.2Å on a ADSC Quantum 4R CCD detector using a synchrotron radiation X-ray source at Beamline 17B2 of the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. The data was indexed and processed with HKL2000 (25). Initial phase estimates for tetragonal data were determined by molecular replacement (MR), using the previously-determined psToc34 GTPase
domain structure (PDB code: 1H65) as a search model. We removed Mg$^{2+}$, GDP and water molecules from the search model to calculate the phases. The program MolRep, from the CCP4 suite package, was used for molecular replacement (26). The sequence homology between the model and atToc33(R130A) was 60%. The final molecular replacement solution that was obtained included one subunit of the asymmetric unit with an R-value of 51.0% and a correlation factor of 34.0%.

The initial refinement of atToc33(R130A) was performed using CNS (27) with standard refinement procedures, including rigid body, minimization, simulated annealing, positional and individual temperature factor (B-factor) refinements. A total of 10% reflections were used to calculate R$_{free}$ and all remaining reflections were used in the refinement. The iterative model improvement was carried out using the graphics programs XtalView 4.1 (28), followed by further rounds of CNS refinement. Several rounds of refinement adding one GDP molecule, one Mg$^{2+}$ ion and 22 water molecules resulted in final R$_{free}$ of 29.1% and an R-value of 22.8%. The statistics of data collection and refinement are summarized in Table 1.

RESULTS

Quaternary structure evaluation - The GTPase domain of wild-type atToc33, atToc33(R130A) and psToc159 was over-expressed with a C-terminal His$_6$ tag and purified from E. coli. Hereafter, these three recombinant proteins are referred to as atToc33wt, atToc33(R130A) and psToc159G. Protein purity is shown in Fig. 1. Gel filtration was used for initial examination of the quaternary structure. In the presence of 100 mM MgCl$_2$, freshly prepared (see below) atToc33wt was eluted in two major peaks (Fig. 1B). By reference to molecular mass markers, peak I was determined to be the dimer form, peak II was the monomers (the shoulder of peak II might be caused by the high Mg$^{2+}$ concentration, see below). In contrast, under the same condition, atToc33(R130A) was present predominantly as monomers (Fig. 1B). These gel filtration results indicated that Arg130 indeed plays an important role in the dimerization of atToc33 (20, 21). The gel filtration profile of psToc159G was similar to that of atToc33wt except the dimer:monomer ratio of psToc159G was close to 1:1 (Fig. 1C), which was higher than that observed for atToc33.

**GTPase activities of atToc33, atToc33(R130A), and psToc159G** - GTP hydrolysis measurements of atToc33(R130A) and different forms of atToc33wt and psToc159G separated by gel filtration were carried out to determine the effect of dimerization on their GTPase activity. As shown in Fig. 2A, GTPase activity of atToc33wt dimers (peak I of Fig. 1B) was about 2- to 3-fold that of atToc33wt monomers (peak II of Fig. 1B). When assays were performed with unfractionated atToc33wt (i.e. atToc33wt without going through the gel filtration step), the GTPase activity was slightly higher than atToc33wt monomers. The GTPase activity of atToc33(R130A) was the same as that of atToc33wt monomers. The differences in GTPases activities were not due to difference in protein loading since equal amount of proteins were used as judged from protein concentration measurements and SDS-PAGE analyses (Fig. 2B). Similarly, as shown in Fig. 2C the GTPase activity of psToc159G dimers (peak I of Fig. 1C) was 2- to 3-fold that of psToc159G monomers (peak II of Fig. 1C). Unfractionated psToc159G had an intermediate GTPase activity. These results indicated that dimerization was important for the GTPase activity of atToc33 and psToc159G.
Role of magnesium in atToc33 dimerization and GTPase activity - During our GTPase assays, we noticed that atToc33wt prepared and assayed in the absence of Mg$^{2+}$ had very low GTPase activities. We therefore further explore the relationships among Mg$^{2+}$ concentrations, dimer formation and GTPase activity of atToc33. As shown in Fig. 3A, atToc33wt prepared and assayed in the absence of Mg$^{2+}$ had a very low GTPase activity. When 100 mM MgCl$_2$ was added to the assays, the GTPase activity increased. Interestingly, when only 2 mM MgCl$_2$ was added, which was the Mg$^{2+}$ concentration Weibel et al. (21) used for GTPase assays, the atToc33wt GTPase activity was even higher (Fig. 3A). This result suggested that Mg$^{2+}$ was important for atToc33wt GTPase activity but excess Mg$^{2+}$ might have an inhibitory effect. Inhibition by excess Mg$^{2+}$ has been observed for other GTPases (29,30).

When analyzed by gel filtration, while atToc33wt analyzed in the presence of 2 or 100 mM MgCl$_2$ eluted with similar dimer and monomer peaks, atToc33wt analyzed in the absence of Mg$^{2+}$ eluted entirely as monomers (Fig. 3B). This was also present only as monomers in the absence of Mg$^{2+}$ (Fig. 1C). When the dimer and monomer fractions atToc33wt were further separated and assayed individually, the GTPase activities of dimers were again much higher than those of monomers (Fig. 3C). In 2 mM MgCl$_2$, the dimer GTPase activity was almost 6-fold that of monomers.

Due to the possible inhibitory effect of 100 mM MgCl$_2$, we re-compared the GTPase activities of atToc33wt and atToc33(R130A) in the presence of 2 mM MgCl$_2$. Unlike psToc34, which belongs to the C2 space group with three molecules per asymmetric unit and forms a dimer each with adjacent monomer (20), the crystal of atToc33(R130A) contained single molecules packed as a monomer in an asymmetric unit.

atToc33wt lost the dimer conformation and GTPase activity upon storage - It needs to be pointed out that, for unknown reasons, atToc33wt dimers were observed only when the protein was fresh. To further analyze the influence of ageing on atToc33wt dimer formation and GTPase activity, assays were carried out with atToc33wt stored at 4°C or -20°C for one week. Upon storage at 4°C or -20°C, the GTPase activity of atToc33wt was reduced to a level similar to that of monomers (Fig. 4A). Gel filtration analyses indeed showed that storage at 4°C or -20°C resulted in complete loss of the dimer peak (Fig. 4B). These results provided further support for the importance of dimer formation for atToc33 GTPase activity.

Overall structure of atToc33(R130A) - To provide insight into the structure/function relationship of atToc33, we performed structural studies of atToc33wt and atToc33(R130A). However, crystals suitable for X-ray diffraction were only obtained from atToc33(R130A). Failure to obtain crystals of atToc33wt may be due to its presence in dual conformations in solution as shown in Fig. 1B, in which one conformation may act as a biological contaminant to the other, leading to failures during the crystallization trials. The atToc33(R130A) crystal belonged to the P4$_3$2$_1$2 space group, with cell dimensions a = b = 71.52 Å and c = 106.27 Å, and diffracted to 3.2 Å. Unlike psToc34, which belongs to the C2 space group with three molecules per asymmetric unit and forms a dimer each with adjacent monomer (20), the crystal of atToc33(R130A) contained single molecules packed as a monomer in an asymmetric unit.

Other than being a monomer, atToc33(R130A) shared major characteristics with psToc34 in terms of overall structure since the two proteins have a 59% amino-acid similarity.
sequence identity (Figs. 5 and 6A). The atToc33(R130A) structure we report here comprises amino acids 2 to 250. For comparison purpose, secondary structural elements of atToc33(R130A) were numbered according to the structure of psToc34 (20). The atToc33(R130A) structure is composed of eight $\beta$-strands flanked by nine $\alpha$-helices. The C-terminal helix $\alpha_7$ (residues 252-256), which is present in the psToc34 structure, is absent in atToc33(R130A).

Although the monomer structure of atToc33(R130A) highly resembles the monomer structure of psToc34, there are some structural variations between the two proteins. According to the structure of psToc34, the dimer interface is composed of four elements: switch I, switch II, the loop (residues 129-134) between the $\beta_4$ strand and the $\alpha_3$ helix, and the $\alpha_4'$ helix (residues 163-169). The superimposition of atToc33(R130A) and psToc34 shows significant differences in all of these regions (Fig. 6B). The $\alpha_4'$ helix of psToc34 was present in atToc33(R130A) as an extended coil rather than a helix. These differences in interface structure may force atToc33(R130A) to exist as a monomer, rather than a dimer. In addition, we did not observe the $\beta_3'$ strand that was present in the psToc34 structure. Instead this region is an extended coil in atToc33(R130A). Another small variation was observed in the region of residues 200 to 202, which forms the small $\beta_{10}$ helix ($\alpha_5'$) in psToc34, but forms an extended $\alpha$-helix ($\alpha_6$) in atToc33(R130A) (Figs. 5 and 6A). We also observed that switch I in atToc33(R130A) lies closer to the GDP moiety than it does in psToc34. However, without the atToc33wt structure, it is not clear whether these differences were caused by the overall sequence differences between psToc34 and atToc33, or caused by the R130A mutation.

**Structural comparison of atToc33(R130A) and psToc34 in the GDP-binding region** - In the psToc34 dimer structure, the guanine group of GDP is strengthened by the dimer interface. The sugar ring of GDP in one monomer is supported by residues Pro169, Asp170 and Tyr132 from the other monomer via van der Waals and hydrophobic forces. In the atToc33(R130A) monomer structure, significant variations were observed in the conformation of GDP. The guanosine group of GDP acquires a different orientation (Fig. 6C) to interact more with interior residues of the monomer by hydrogen bonding. For instance, the guanine group interacts with Asn209 by one hydrogen bond. However, the $\alpha$ and $\beta$ phosphates of GDP in atToc33(R130A) are organized in a similar way as that of GDP in psToc34. In psToc34, six residues (Gly49, Gly51, Lys52, Ser53, Ser54 and Ser68) are involved in stabilization of the phosphate groups, whereby five residues hydrogen bond with GDP, and Lys52 stabilizes the negative charge of the phosphates by forming salt bridges. Similarly, in atToc33(R130A) all the corresponding residues (Gly46, Gly48, Lys49, Ser50, Ser51 and Ser65 in atToc33) are engaged in stabilizing the phosphate groups.

Aronsson et al. (31) has shown that the atToc33(E208Q) mutant, in which the glutamic acid at residue 208 was mutated to glutamine, had the same nucleotide specificity and GTPase activity, but weaker nucleotide binding, compared to wild-type atToc33. In agreement with these results, in the crystal structure of atToc33(R130A) we observed that E208 forms a strong hydrogen bond with the O6 of the guanine group of GDP, suggesting that, while this residue may not influence the GTPase activity or nucleotide specificity, it may still play some role in the stabilization of GDP in the binding site.

**Molecular modeling of atToc33 dimers** - Since the structure of atToc33wt was not obtained, we performed molecular modeling
to generate an architectural dimer of atToc33wt using the psToc34 dimer structure as a probe. The monomer of atToc33(R130A) was superimposed onto each monomer in the psToc34 dimer, alanine at residue 130 was replaced with an arginine and energy minimization was performed using CNS (27). Around the GDP-binding site of the atToc33wt architectural dimer (Fig. 7), Arg130 from one monomer interacts with the β phosphate of GDP in the other monomer by a strong hydrogen bond. This interaction is the same as seen with Arg133 in psToc34. We also observed stabilization of the hydrophobic binding pocket by Tyr129 and hydrophobic stacking force by His158. In addition, the buried surface area of the psToc34 dimer and the atToc33 architectural dimer is ~2750 Å² and ~2570 Å², respectively. The similar buried surface area suggests that atToc33wt in its dimer conformation may resemble the psToc34 dimer. However, despite the similarity, psToc34 dimers have more hydrogen bonds between the two monomers than atToc33. For example, in the psToc34 dimer structure, Arg63 forms a salt bridge with Asp170 from the other monomer, and Arg214 interacts with Ser167 from the other monomer by forming a strong hydrogen bond. In contrast, we did not observe these interactions from the corresponding positions in the atToc33wt modeled dimer structure. It is possible that the sequence and local conformational differences result in less contact in the real atToc33wt dimer. This may be one of the reasons that atToc33wt seems less stable than psToc34 since psToc34 did not lose its dimer conformation upon storage and could remain soluble under a higher protein concentration (data not shown).

psToc159G was present with a higher proportion of dimers in solution than atToc33wt (Fig. 1C), and psToc159G also did not lose the dimer conformation upon storage (data not shown). To investigate the reason for the increased stability of psToc159, a psToc159 architectural dimer was also calculated based on the psToc34 structure. The buried surface area of psToc159 was calculated to be ~3066 Å² using a probe radius of 1.4 Å. The larger buried surface area of the psToc159G dimers might be one of the reasons for its enhanced stability in solution.

DISCUSSION

Weibel et al. (21) have shown that atToc33 and atToc33(R130A) hydrolyzed GTP with almost the same efficiency. Recombinant atToc33(R130A) had a reduced ability to bind in vitro-translated, [35S]-labeled wild-type atToc33, atToc159 and atToc33(R130A). They therefore concluded that Arg130 merely functioned in establishing monomer-monomer interactions but did not function as a GTPase activating “arginine finger”. Our gel filtration results (Fig. 1B) showed that Arg130, in the presence of exogenous Mg²⁺ and in freshly prepared samples, indeed played an important role in the dimerization of atToc33wt. However when the dimer population was separated from the monomer population, both atToc33wt and psToc159G had a significantly higher GTPase activity in their dimeric form (Fig. 2). Even in unfractionated samples, atToc33wt had a significantly higher GTPase activity than atToc33(R130A) in 2 mM MgCl₂ (Fig. 3D). It is possible that Weibel et al. did not assay freshly prepared samples so most of the dimers had dissociated into monomers.

Although direct evidence that Arg130 functions as an arginine finger is still lacking, our data show that dimer formation is important for the GTPase activity of atToc33wt and psToc159G. In the architectural dimer of atToc33, Arg130 from one monomer contacts the β phosphate of GDP in the other monomer. Therefore Arg130 as an arginine finger provides a
logical explanation for the higher GTPase activity of atToc33 dimers.

We observed that atToc33wt and psToc159G could not form dimers in the absence of exogenous Mg\(^2+\) (Figs. 1C and 3B). This is not due to depletion of Mg\(^2+\) from the GDP-binding pocket of each atToc33 molecule since Mg\(^2+\) was still present in the atToc33(R130A) monomer structure (Fig. 6A). In a recent study (32) with MnmE, a GTPase involved in the modification of tRNA, it has been found that G-domains of MnmE dimerise in a potassium-dependent manner and induce GTP hydrolysis. In our study although potassium ion was absent in the buffer we still observed the presence of atToc33wt and psToc159G dimers in the presence of MgCl\(_2\), indicating that Mg\(^2+\) alone can influence dimerization without the help of potassium ions. It is possible that there is a second Mg\(^2+\) binding site in the atToc33 dimer and this second Mg\(^2+\) may play a similar role as that of K\(^+\) in MnmE to enhance dimer formation.

It has been shown that GTP, when added as the sodium salt, could support precursor binding to chloroplasts at low GTP concentrations (50-100 \(\mu\)M), but binding decreased at higher GTP concentrations (33). It was then suspected that GTP was acting as a chelator for some required cation. Indeed when Mg\(^2+\) was added at the same concentration as GTP, precursor binding remained high even at 1 mM GTP (33). Our results indicate that, in vitro, Mg\(^2+\) promotes atToc33wt dimerization and psToc159G dimerization, which in turn is critical for their GTPase activities. It is likely that Mg\(^2+\) plays the same role in vivo during precursor import into chloroplasts. Therefore when Mg\(^2+\) is chelated away, dimerization and GTPase activity of atToc33 and Toc159 is inhibited, and precursors are then unable to bind to these receptor proteins. We propose that during protein import into chloroplasts, atToc33 and Toc159 may both form dimers. Their GTPase activity is then activated by this dimerization with the aid of Arg130 as the arginine finger. Although we have only shown homo-dimerization of atToc33wt, psToc159G and psToc34 in vitro, it has been shown that atToc33 is required for targeting of atToc159 to chloroplasts (17-19), and the Toc34 and Toc159 family proteins have homologous GTPase domains. It is therefore also possible that atToc33 and atToc159 could form heterodimers. How the GTPase activity of the Toc34 and Toc159 family proteins is regulated in vivo, and whether atToc33 and Toc159 form homodimers, heterodimers or even both, all require further investigations.

REFERENCES


**FOOTNOTES**

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The Abbreviations used are: Toc: translocon at the outer envelope membrane of chloroplasts; psToc: pea Toc; atToc: Arabidopsis Toc; atToc33wt: GTPase domain of wild-type atToc33; atToc33(R130A): GTPase domain of atToc33 R130A mutant; psToc159G: GTPase domain of psToc159; GAP: GTPase activating protein.

The atomic coordinates and structure factors (code 2j3e) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

FIGURE LEGENDS

Figure 1. SDS-PAGE and size exclusion chromatography of atToc33wt, atToc33(R130A) and psToc159G. A. Purified proteins were analyzed by SDS-PAGE and stained by Coomassie blue. Lane 1: atToc33wt (4.5 µg, ~1.5 mg/ml); Lane 2: atToc33(R130A) (4.5 µg, ~1.5 mg/ml); Lane 3: psToc159G (15 µg, ~5 mg/ml). B. atToc33(R130A) (brown line) and atToc33wt (blue line) were analyzed by gel filtration in the presence of 100 mM MgCl₂. C. Gel filtration pattern of psToc159G. Purified psToc159G was analyzed by gel filtration in the presence (green line) or absence (orange line) of 100 mM MgCl₂. Small peaks observed in the void volume of all gel filtration profiles shown here might be due to aggregation of small quantity of proteins.

Figure 2. Dimer formation is important for GTPase activity. A. Comparison of GTPase activities of different forms of atToc33wt and atToc33(R130A). atToc33wt dimers or monomer fractions separated by gel filtrations, or unfractionated atToc33wt or atToc33(R130A) was assayed for GTPase activity. B. SDS-PAGE analysis of protein samples used in A. Lane 1: separated dimers of atToc33wt; Lane 2: separated monomers of atToc33wt; Lane 3: unfractionated atToc33wt. Lane 4: unfractionated atToc33(R130A). 3 µg of each protein was analysed. C. Comparison of GTPase activities of different forms of psToc159G.

Figure 3. Magnesium affects the atToc33 dimerization and GTPase activity. A. GTPase activities of unfractionated atToc33wt in different concentrations of MgCl₂. B. Gel filtration patterns of unfractionated atToc33wt in different concentrations of MgCl₂. C. GTPase activities of atToc33wt monomers and dimers in different MgCl₂ concentrations. Monomer and dimer fractions were separated by gel filtration as shown in B and their GTPase activities were assayed in the respective MgCl₂ concentrations. D. GTPase activities of unfractionated atToc33wt and atToc33(R130A) in different MgCl₂ concentrations.

Figure 4. Effect of ageing on atToc33wt dimerization and GTPase activity. A. GTPase activities of unfractionated atToc33wt that was fresh prepared, or was further stored at 4°C or -20°C for 1 week. The GTPase assays were performed in 2 mM MgCl₂. B. Gel filtration patterns of atToc33wt under the three conditions shown in A.

Figure 5. Sequence alignment and secondary structure of psToc34, atToc33wt and psToc159G. Structural features on top of the alignment indicate those observed in the psToc34
and atToc33(R130A) crystal structures and are predicted for psToc159G based on sequence similarity. Numbering of residues on top of the alignment is based on atToc33. Helices and strands are colored in red and orange, respectively. The brown line shows the longest loop. Putative switch regions, switch I and switch II, are enclosed by red-colored boxes. Secondary structure elements that are observed only in psToc34 are shown in blue. Identical amino-acid residues are shown in orange when the sequences are identical in all three proteins, in green when the sequences are identical between two proteins, and in blue when similarity exists between any of the two proteins. The residues enclosed in the blue box represent the extended helix of $\alpha_5$ observed only in atToc33(R130A). Sequence alignment was performed using the Biology Work Bench from SDSC and displayed with the program BOXSHADE.

**Figure 6. Ribbon structure of the GDP-bound atToc33(R130A) monomer and stereo view of the superposition of the $C_\alpha$ position of atToc33(R130A) and psToc34 G domains.** A. GDP is denoted as a stick model, while Mg$^{2+}$ is shown in purple. The C-terminal and N-terminal regions of atToc33(R130A) are labeled. The eight $\beta$-sheets ($\beta_0$ to $\beta_7$) in the protein are shown in yellow, 9 $\alpha$ helices ($\alpha_0$, $\alpha_1$ to $\alpha_7$) are in red, and loops are in pale pink. The two potential switch regions (Sw I and Sw II) and the longest loop are also labeled. A GDP and magnesium ion omitted electron density (2Fo-Fc) at 1.0 $\sigma$ cutoff is displayed in green. Pymol graphic program (http://pymol.sourceforge.net) was used to generate Figs. 6 and 7. B. Stereo view of superposition of the G domain $C_\alpha$ atoms of atToc33(R130A) and psToc34. The atToc33(R130A) and psToc34 (PDB accession code 1H65) are colored in green and pink, the GDP of atToc33(R130A) and psToc34 are denoted as a stick model in blue and orange respectively, while the magnesium ion is shown as a sphere. The significant structure differences at switches I (Sw I), II (Sw II), residues 163-169 and residues 129-134 of atToc33(R130A) are labeled in red. C. A magnified view of the GDP-binding pocket region of B.

**Figure 7. Stereo view of the GDP-binding site of the architectural atToc33wt dimer.** The residues from one monomer are shown in gray. The arginine and tyrosine from the other monomer interacting with GDP are displayed in yellow. GDP is shown in green. Magnesium ion is illustrated as a pink sphere. All the residues are in stick model and are labeled.
### Table 1. Data collection and refinement statistics

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1\[ R_{merge} = \Sigma \Sigma |I_i - \langle I\rangle| / \Sigma \langle I\rangle. \]

2The number in parentheses is for outermost shell.
**Fig. 2**

- **A**: A bar chart showing CTP hydrolysis in pmol min⁻¹ μg⁻¹ for different samples: atToc33wt dimer, atToc33wt monomer, unfractionated atToc33wt, and unfractionated atToc33(K130A).

- **B**: A gel electrophoresis image with lanes 1 to 4, indicating molecular weights of 97, 66, 45, 30, and 13 kDa.

- **C**: A bar chart depicting CTP hydrolysis for psToc159G: dimer, monomer, and unfractionated.
Fig. 3
Fig. 4
Fig. 6
Fig. 7
Dimerization is important for the GTPase activity of chloroplast translocon
components atToc33 and psToc159
Yi-Hung Yeh, Muppuru M. Kesavulu, Hsou-min Li, Shu-Zon Wu, Yuh-Ju Sun,
Emadeldin H. E Konozy and Chwan-Deng Hsiao

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