Targeting of an abundant cytosolic form of the protein import receptor atToc159 to the outer chloroplast membrane

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Chloroplast biogenesis requires the large-scale import of cytosolically synthesized precursor proteins. A trimeric translocon (Toc complex) containing two homologous GTP-binding proteins (atToc33 and atToc159) and a channel protein (atToc75) facilitates protein translocation across the outer envelope membrane. The mechanisms governing function and assembly of the Toc complex are not yet understood. This study demonstrates that atToc159 and its pea orthologue exist in an abundant, previously unrecognized soluble form, and partition between cytosol-containing soluble fractions and the chloroplast outer membrane. We show that soluble atToc159 binds directly to the cytosolic domain of atToc33 in a homotypic interaction, contributing to the integration of atToc159 into the chloroplast outer membrane. The data suggest that the function of the Toc complex involves switching of atToc159 between a soluble and an integral membrane form.

Introduction

Approximately 2,000 different nuclear-encoded proteins must be imported to assemble the photosynthetic apparatus during chloroplast biogenesis (Cline, 2000; Bauer et al., 2001). These proteins are synthesized in the cytosol as precursors with an NH_2-terminal transit peptide, specifying chloroplast targeting (Chen and Schnell, 1999; Keegstra and Cline, 1999; Schleiff and Soll, 2000; Bauer et al., 2001). Both chloroplast envelope membranes contain translocon complexes to facilitate import of the precursor proteins. These are termed the translocon at the outer chloroplast membrane (Toc)* and translocon at the inner chloroplast membrane (Tic)-complexes (Schnell et al., 1997). The pea Toc complex consists of three major components forming a trimeric complex (Schnell et al., 1994; Ma et al., 1996), Toc75, Toc159, and Toc34. Toc75 (the number indicates the molecular mass in kD) forms at least part of a hydrophilic channel through which precursors are translocated across the outer membrane (Schnell et al., 1994; Hinnah et al., 1997). Toc159 and Toc34 are surface-exposed, integral membrane GTP-binding proteins (Hirsch et al., 1994; Kessler et al., 1994; Seedorf et al., 1995; Bölter et al., 1998; Chen et al., 2000). The two proteins share highly conserved GTP-binding domains (G domains) (Kessler et al., 1994), and substantial evidence indicates that the two proteins act concertedly in the recognition of the chloroplast-targeting signal (transit peptide) (Kouranov and Schnell, 1997; Chen et al., 2000; Sveshnikova et al., 2000). Based on direct cross-linking to transit sequences and inhibition of precursor binding by specific antibodies, Toc159 is thought to function as the primary receptor of the transit peptide at the chloroplast surface (Hirsch et al., 1994; Perry and Keegstra, 1994; Kouranov and Schnell, 1997).

Toc159 has a tripartite structure, consisting of the NH_2-terminal acidic domain (A domain), the G domain, and the COOH-terminal membrane domain (M domain) (Bauer et al., 2000). The G domain binds specifically to GTP (Kessler et al., 1994), but its function and that of the A domain are unknown. The M domain anchors Toc159 in the outer

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*Abbreviations used in this paper: A domain, acidic domain; G domain, GTP-binding domain; M domain, membrane domain; Ni-NTA, nickel-nitrilotriacetic acid–agarose; SRP, signal recognition particle; Tic, translocon at the inner chloroplast membrane; Toc, translocon at the outer chloroplast membrane.

Key words: chloroplasts; protein import; translocon; complex assembly; soluble receptor

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chloroplast membrane (Hirsch et al., 1994), but lacks conventional hydrophobic membrane-spanning sequences. In contrast, Toc34 is inserted into the outer membrane by a COOH-terminal hydrophobic transmembrane stub (Chen and Schnell, 1997; Li and Chen, 1997; May and Soll, 1998; Tsai et al., 1999).

In this study we have analyzed the *Arabidopsis* homologue of Toc159, atToc159. Analysis of the *ppi2* mutant lacking atToc159 has demonstrated an essential function of this protein in chloroplast biogenesis (Bauer et al., 2000), but it has not yet been characterized biochemically. We show that at the chloroplast surface, atToc159 is in a complex with atToc33 and atToc75, homologues of pea Toc34 and Toc75, respectively. Additionally, we demonstrate that in contrast to atToc34 and Toc75, a large soluble pool of atToc159 exists. In a pull-down assay, synthetically synthesized, soluble atToc159 was shown to bind to a hexahistidinyl-tagged, soluble form of atToc33, atToc33$_{1-265}$, lacking the COOH-terminal transmembrane stub. Furthermore, the addition of atToc33$_{1-265}$ inhibited outer membrane insertion of atToc159, likely in a competitive manner. Thus, the results indicate that atToc33 may function in the assembly of soluble atToc159 into the outer membrane and the Toc complex. Given its known function as an import receptor, these findings suggest that atToc159 may play a role in targeting cytosolic precursors to the chloroplast surface.

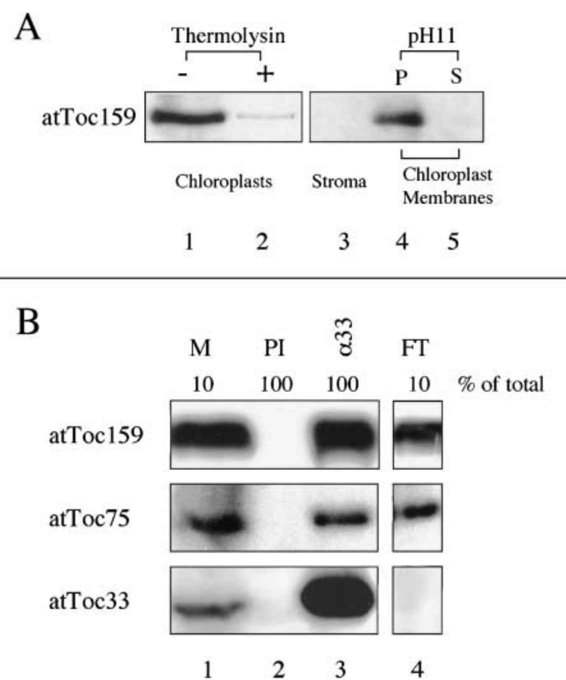

Figure 1. **Characterization of chloroplast atToc159.** A and B show Western blotting experiments. Antibodies used to probe the blots are indicated on the left side of the panels. (A) atToc159 is an integral protein of the outer chloroplast membrane remaining in the membrane pellet (P, lane 4) upon alkaline extraction (pH 11), and is sensitive to exogenously added protease (Thermolysin +, lane 2) in isolated *Arabidopsis* chloroplasts. AtToc159 is not present in the chloroplast stroma (Stroma, lane 3). (B) atToc159 is in a complex with atToc33 and atToc75. Anti-atToc33 antibodies (α33, lane 3) were used to immunoisolate a Toc complex from Triton X-100–solubilized chloroplast membranes (M, lane 1). atToc159 and atToc75 were specifically coprecipitated with atToc33 as they were absent from a preimmune control experiment (PI). Analysis of the flow-through fractions (FT, lane 4) indicates that atToc33 was quantitatively depleted from solubilized membranes, whereas only fractions of atToc159 and atToc75 were associated with atToc33. The percentage values indicate the fraction of the sample analyzed. S, supernatant.

Results

Association of atToc159 with chloroplasts and components of the Toc complex

In pea, Toc159 has been shown to be located at the chloroplast surface, exposed to the cytosol, and to associate with Toc34 and Toc75 to form a trimeric import complex (Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Ma et al., 1996). In *Arabidopsis*, the likely orthologue of pea Toc159, atToc159, has been described recently (Bauer et al., 2000), but the endogenous protein was not characterized in depth. The two proteins differ significantly at their respective NH_2 termini (Chen et al., 2000). Therefore, we further analyzed atToc159 to verify its function. atToc159, assayed by Western blotting using affinity-purified antibodies against the A domain of atToc159 (atToc159$_{1-740}$), was present in *Arabidopsis* chloroplast preparations and sensitive to treatment with the exogenously added protease (thermolysin+), confirming chloroplast surface exposure (Fig. 1 A, lane 2). The protein was not present in the stroma, the soluble content of the chloroplast (Fig. 1 A, lane 3), but behaved as an integral membrane protein as it remained in the pellet (P) upon alkaline extraction (pH 11) of isolated chloroplast membranes (Fig. 1 A, lanes 4 and 5).

To determine possible binding partners of atToc159, we did immunopurification experiments using Triton X-100–solubilized *Arabidopsis* chloroplast membranes as the starting material. Using affinity-purified atToc33 antibodies, we isolated a complex consisting of atToc33, atToc159, and atToc75 (Fig. 1 B, lane 3). None of the three proteins bound to preimmune antibodies (Fig. 1 B, lane 2), indicating that the three proteins interacted specifically among each other. Therefore, a Toc complex exists in *Arabidopsis* consisting of components homologous to those in pea. Interestingly, we observed that only a fraction of both atToc159 and atToc75 was associated with atToc33, whereas atToc33 itself was quantitatively removed from the solubilized membranes (Fig. 1 B, lane 4). Thus, the *Arabidopsis* Toc complex may be somewhat unstable, or its composition dynamically regulated. Alternatively, the Toc-proteins may also exist as monomers, or be able to form subcomplexes lacking one of the components.

Immunolocalization of atToc159

Immunofluorescence experiments using antibodies against atToc159$_{1-740}$ on isolated *Arabidopsis* protoplasts followed by incubation with FITC-coupled secondary antibodies were done to determine the localization of atToc159 at the cellular level (Fig. 2 A). By confocal laser scanning microscopy, the atToc159$_{1-740}$ antibodies gave a circular, rim-like fluorescence pattern consistent with a localization at the chloroplast periphery (Fig. 2 A, panel 3). A merge (Fig. 2 A, panel 2) between the transmission microscopic image (Fig. 2

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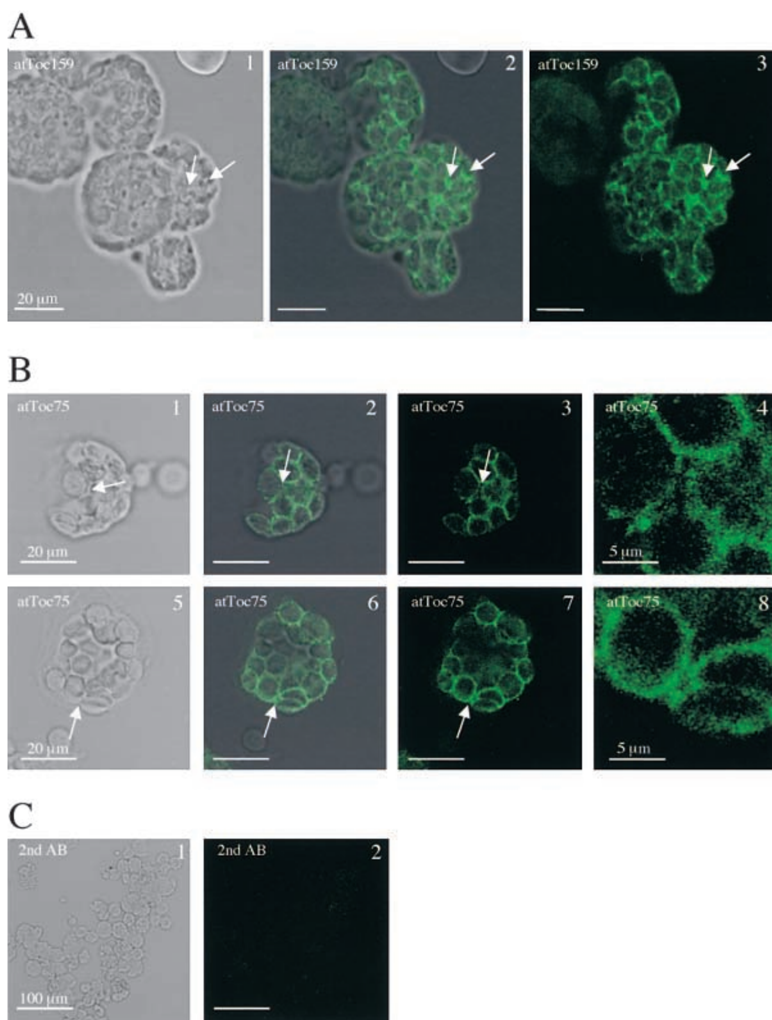

Figure 2. **Cellular localization of atToc159 and atToc75 using immunofluorescence.** (A) Anti-atToc159$_{1–740}$ antibodies give circular, rim-like fluorescence at the chloroplast surface in isolated *Arabidopsis* protoplasts (panels 2 and 3). Fluorescence is also present in spaces intervening between chloroplasts (arrows). Panel 1 shows a transmission microscopy image of isolated protoplasts. Panel 2 shows the merge of transmission (panel 1) and fluorescence microscopic images (panel 3). (B) Anti-atToc75$_{141–397}$ gives rim-like fluorescence at the chloroplast surface (panels 2–4 and 6–8) but not in the intervening spaces (arrows) enlarged in panels 4 and 8. Panels 1 and 5 show transmission microscopic images, 2 and 6 show merges, 3 and 4 as well as 7 and 8 show fluorescence microscopic images. (C) Control experiment using fluorescein-tagged secondary antibodies (2nd AB) in the absence of primary antibodies. Panel 1, transmission microscopic image of a field of protoplasts; Panel 2, fluorescence microscopic image of the same field.

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A, panel 1) and the fluorescence microscopic image (Fig. 2 A, panel 3) confirmed that the rim-like fluorescence indeed coincided with the chloroplast periphery. In addition, fluorescence occurred in areas between chloroplasts (Fig. 2, arrows), suggesting that atToc159 may also be present in the cytosol. In comparison, antibodies against atToc75$_{141–397}$ gave a rim-like fluorescence (Fig. 2 B, panels 1–8) comparable to that of the atToc159$_{1–740}$ antibodies, but fluorescence was absent from areas intervening between chloroplasts (Fig. 2 B, panels 4 and 8), consistent with the expected, exclusive localization of atToc75 at the chloroplast surface. Control experiments using only the FITC-coupled secondary antibodies gave only low levels of background fluorescence (Fig. 2 C, panel 2).

Toc159 is present in the soluble fractions of *Arabidopsis* and pea leaf proteins

To determine biochemically the subcellular distribution of Toc159 and compare it to that of other components of the chloroplast protein import machinery, equivalent amounts of soluble and chloroplast proteins (50 µg each, corresponding to 1.5 and 3 cellular equivalents, respectively) from *Arabidopsis* (Fig. 3 A) and pea (Fig. 3 B) were analyzed by Western blotting. Soluble fractions were prepared from *Arabidopsis* (Fig. 3 A, lane 2) and pea (Fig. 3 B, lane 3) leaf homogenates by low-speed centrifugation to remove chloroplasts, followed by a high-speed centrifugation step (100,000 *g*) to remove residual membranes. As expected, Toc75 and Toc34 were present in the chloroplast fraction of both *Arabidopsis* (Fig. 3 A, lane 3) and pea (Fig. 3 B, lane 2), but not detectable in the corresponding soluble fractions (Fig. 3 A, lane 2; Fig. 3 B, lane 3). In contrast, Toc159, the full-length protein migrating aberrantly at ~230 kD (Bölter et al., 1998; Chen et al., 2000), was present in both the soluble and chloroplast fraction of both species. It is improbable that the atToc159$_{1–740}$ antibodies reacted with proteins in the soluble fraction other than atToc159, as the antibodies showed no reactivity at all towards protein extracts from the *ppi2* mutant lacking atToc159 (Bauer et al., 2000) (Fig. 3 C). The additional, lower molecular mass bands appearing in the soluble fraction are probably due to NH_2-terminal proteolysis of Toc159 occurring despite the addition of protease inhibitors (Bölter et al., 1998; Chen et al., 2000). It is also unlikely that soluble atToc159 simply represents an assembly intermediate en route to its chloroplast destination, as such intermediates are generally not observed (unpublished data) and was not detected for the imported high turnover chlorophyll a/b binding protein (Fig. 3 A, CAB). Also, in contrast to the other components of the Toc complex not detectable among the soluble proteins, a large por-

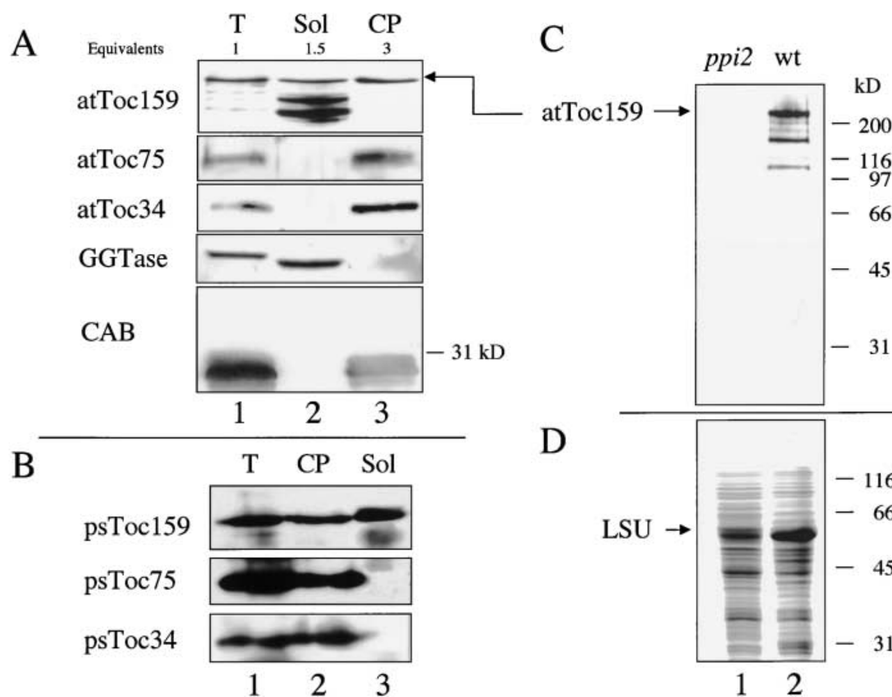

Figure 3. **Subcellular distribution of components of the chloroplast protein import machinery analyzed by Western blotting.** (A) *Arabidopsis* total leaf extracts (T, lane 1), soluble fraction (Sol, lane 2), and chloroplasts (CP, lane 3) (50 μg of protein each, cellular equivalents indicated at the top of the figure) were analyzed by Western blotting using antibodies specific to atToc159, atToc75, atToc34, cytosolic geranylgeranyl transferase (GGTase), and the chlorophyll a/b binding protein (CAB). (B) Pea total leaf extracts (T, lane 1), chloroplast (CP, lane 2) and soluble fractions (Sol, lane 3) (50 μg of protein each) were analyzed by Western blotting using antibodies specific to psToc159, psToc75, and psToc34. (C) Western blot analysis of *ppi2* (lane 1) and wild-type (wt, lane 2) *Arabidopsis* total protein extracts using specific antibodies against atToc159. (D) Coomassie blue–stained SDS-PAGE gel of *ppi2* and wild-type extracts indicating loading amounts in C. LSU, large subunit of Rubisco.

tion (∼50%) of intact Toc159 was present in the soluble fractions of *Arabidopsis* and pea. This observation, made in two different organisms using different antibodies, strongly suggests that Toc159 and its proteolytic products exist as bona fide soluble proteins in both *Arabidopsis* and pea.

Direct binding of atToc33$_{1–265}$ to atToc159 in a soluble phase assay

The presence of a soluble form, in addition to the membrane-bound form in the import machinery, suggests that the soluble form of atToc159 may be directly targeted to the Toc complex during its assembly. An analogous situation presents itself in targeting of the cytosolic signal recognition particle (SRP) to the ER, where homotypic interactions among three homologous GTP-binding proteins play an essential role (Walter and Johnson, 1994). In the process, the 54-kD subunit of SRP54 docks to the two SRP receptor subunits (SRα and SRβ). In analogy, membrane-bound atToc33 may be part of a docking site for soluble atToc159 in the Toc complex. This appears plausible, as atToc33 and atToc159 are components of the same complex (Fig. 1 B). Furthermore, atToc159 associates with Toc34, a close homologue of atToc33, upon import into pea chloroplasts (Bauer et al., 2000). It has been shown that chloroplast association of pea Toc159 is protease sensitive (Muckel and Soll, 1996), again consistent with a role of a surface-exposed protein such as atToc33. To test potential interactions between atToc159 and atToc33, we used a soluble phase-binding assay. For this purpose, a hexahistidinyl-tagged, soluble version of atToc33 (atToc33$_{1–265}$) (Gutensohn et al., 2000) containing the G domain but lacking the COOH-terminal transmembrane stub was produced in *Escherichia coli* and purified by nickel-nitrilotriacetic acid-agarose (Ni-NTA) chromatography (Fig. 4 A). To test whether atToc33$_{1–265}$ interacts directly with soluble atToc159, the purified recombinant protein was incubated with in vitro–synthesized ^{35}S-atToc159 under standard protein import conditions (containing 0.4 mM GTP to support a possible GTP dependency of the interaction), and reisolated by Ni-NTA chromatography. The eluates of the Ni-NTA resin were analyzed by SDS-PAGE, followed by autoradiography (Fig. 4 B) and quantitation using a PhosphorImager (Molecular Dynamics) (Fig. 4 C). In a control experiment, [^{35}S] atToc159 was incubated with Ni-NTA in the absence of atToc33$_{1–265}$ (Fig. 4 B, lane 2). [^{35}S] atToc159 bound to Ni-NTA resin only in the presence of atToc33$_{1–265}$ (Fig. 4 B, lanes 3–7). Furthermore, [^{35}S] atToc159 binding increased in parallel with increasing concentrations of atToc33$_{1–265}$ (Fig. 4 C). We conclude that in the soluble phase assay, [^{35}S] atToc159 and atToc33$_{1–265}$ interact directly.

atToc33$_{1–265}$ inhibits insertion of atToc159 into the outer chloroplast membrane

We have previously used an in vitro assay to study insertion of atToc159 into the outer membrane of isolated chloroplasts (Bauer et al., 2000). In the insertion assay, soluble [^{35}S] atToc159, synthesized in a reticulocyte lysate, assumed the properties of endogenous, membrane-bound atToc159 upon incubation with isolated pea chloroplasts, including its association with Toc34. To test whether atToc33 contributes to atToc159 membrane insertion, increasing concentrations of purified atToc33$_{1–265}$ were added to an in vitro experiment in which isolated *Arabidopsis* chloroplasts were incubated with [^{35}S] atToc159 (Fig. 5). Subsequently, total membrane fractions were prepared from reisolated chloroplasts and subjected to extraction with alkaline carbonate buffer. The extraction experiments were separated into supernatants and pellets. The membrane pellets were analyzed by SDS-PAGE, followed by autoradiography (Fig. 5, top) and quantitation using a PhosphorImager. Addition of atToc33$_{1–265}$ decreased

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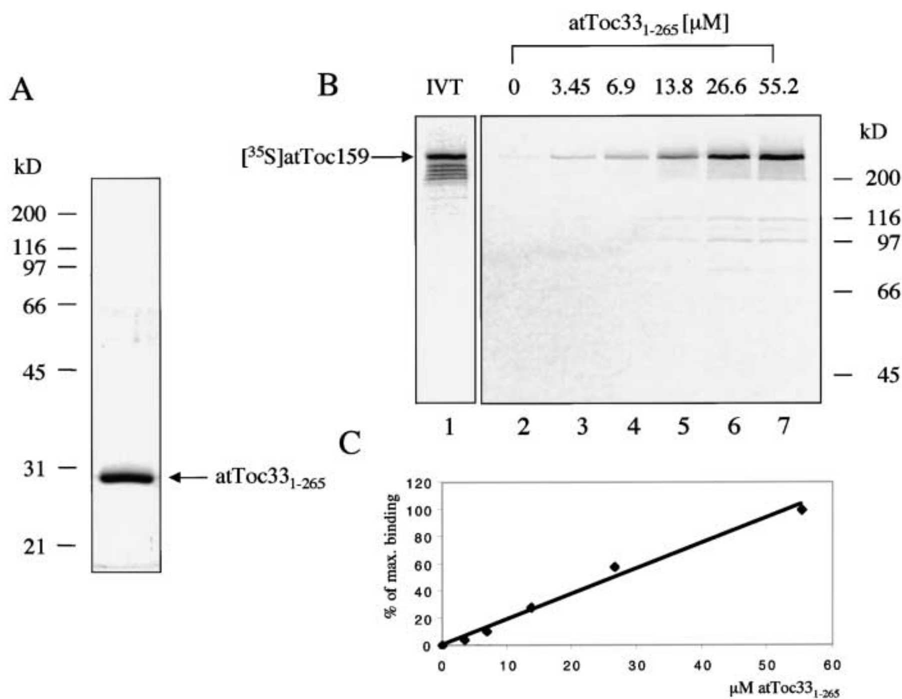

Figure 4. **Direct binding of ^{35}S-atToc159 to atToc33$_{1-265}$.** (A) Purification of atToc33$_{1-265}$ using Ni-NTA affinity chromatography. 1 μg of purified atToc33$_{1-265}$ separated by 10% SDS-PAGE followed by Coomassie blue staining. (B) Soluble phase binding assay. [^{35}S] atToc159 was synthesized in a reticulocyte lysate (IVT, lane 1). Increasing concentrations of purified, hexahistidinyl-tagged atToc33$_{1-265}$ (concentrations indicated in μM) were incubated with [^{35}S] atToc159 in a soluble phase assay and reisolated by Ni-NTA chromatography (lanes 3–7). In a control experiment, [^{35}S] atToc159 was incubated with Ni-NTA in the absence of atToc33$_{1-265}$ (lane 2). The experiments were analyzed by SDS-PAGE followed by autoradiography. (C) Binding of [^{35}S] atToc159 was quantitated using a PhosphorImager and is given in percentage of maximal binding (B, lane 7).

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the insertion of [^{35}S] atToc159 into the chloroplast outer membrane by up to ~60%. The result suggests that soluble atToc33$_{1-265}$ may compete with membrane-associated atToc33 for binding of [^{35}S] atToc159, thereby partially blocking its insertion into the outer membrane.

Discussion

Pea Toc159 was first identified in vitro, as a receptor component of the trimeric Toc complex (Hirsch et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994). Analysis of a knockout mutant revealed that the *Arabidopsis* orthologue of Toc159, atToc159, is essential for chloroplast biogenesis in vivo (Bauer et al., 2000). In this study we present novel data on the assembly of atToc159 into the Toc complex. We have identified a soluble, cytosolic form of atToc159. The soluble protein targets to the chloroplast and is integrated into the chloroplast outer membrane in a process involving atToc33. The findings reveal dynamics suggesting that atToc159 may be a mobile component of the Toc complex.

Toc159 has been biochemically characterized in pea and shown to be essential for chloroplast biogenesis in vivo, but questions regarding its subcellular distribution and association with the Toc complex in *Arabidopsis* remained open. Here we show that atToc159 exists in an integral membrane, as well as a soluble form. Protease sensitivity and resistance to alkaline extraction confirm that atToc159 is an integral, surface-exposed component of the chloroplast outer membrane (Fig. 1 A). An *Arabidopsis* Toc complex was identified and isolated in immunopurification experiments using solubilized chloroplast membranes. The complex contained atToc159, atToc33, and atToc75 (Fig. 1 B). Therefore, an *Arabidopsis* Toc complex exists, homologous in composition to that of pea (Schnell et al., 1994; Ma et al., 1996). However, it remains to be seen whether the *Arabidopsis* complex is trimeric, as has been demonstrated for the pea complex (Ma et al., 1996). Whereas in pea Toc75, Toc34, and Toc159 appear to be unique, the *Arabidopsis* homologues of Toc159 and Toc34 are encoded by a small gene family: three genes (atToc159, atToc132, and atToc120) encoding homologues of Toc159 (Bauer et al., 2000), and two genes (atToc34 and atToc33) encoding homologues of Toc34 (Jarvis et al., 1998). Future studies will reveal whether the members of this family act concertedly in a large supercomplex or whether they form a variety of trimeric Toc complexes. Taking into account that atToc75 is likely unique also in *Arabidopsis*, a combinatorial scope of six different Toc complexes may function in various tissues and plastid types (Cline, 2000).

Previous subcellular localization studies relied entirely on isolated pea chloroplasts (Hirsch et al., 1994; Kessler et al., 1994). In this study, we assessed the cellular distribution of atToc159 using specific antibodies in immunofluorescence (Fig. 2) and Western blotting experiments (Figs. 1 and 3). AtToc159 clearly located to the chloroplast surface by immunofluorescence (Fig. 2), but fluorescence also occasionally occurred in spaces intervening between chloroplasts, suggesting that atToc159 may be present in the cytosol (Fig. 2 A). Such cytosolic fluorescence was noticeably absent in immunofluorescence experiments using specific antibodies against atToc75 (Fig. 2 B). Western blot analysis of soluble and chloroplast proteins revealed partitioning of atToc159, but not of atToc75, between the two fractions (Fig. 3 A); thus, atToc159 exists in an unanticipated soluble form. Identical findings were made for pea Toc159 (Fig. 3 B). The identification of a soluble form of the well-characterized pea Toc159 underscores the significance of the finding.

Earlier targeting experiments indicated that targeting and insertion of atToc159 rely on protease-sensitive receptor components at the chloroplast surface (Muckel and Soll,

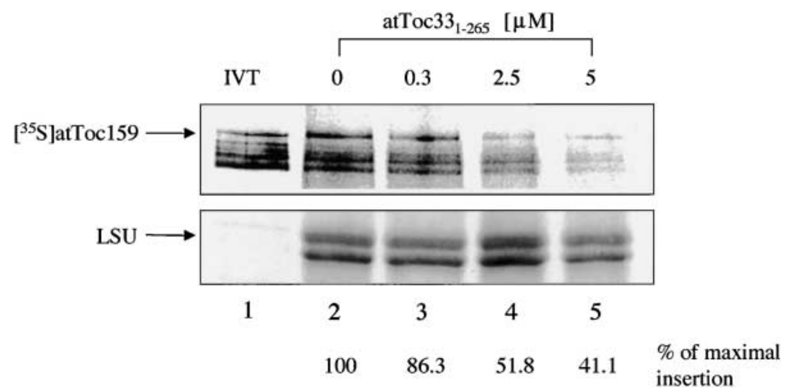

Figure 5. **Inhibition of [^{35}S]atToc159 membrane insertion by atToc33$_{1-265}$.** Soluble [^{35}S] atToc159 was synthesized in a reticulocyte lysate (IVT, lane 1). Reticulocyte lysate containing[^{35}S] atToc159 was incubated with isolated *Arabidopsis* chloroplasts (corresponding to 15 μg of chlorophyll) in the absence (lane 2) or presence of increasing concentrations of purified atToc33$_{1-265}$ (lanes 3–5). After the incubation, membranes were prepared from reisolated chloroplasts and subjected to extraction with alkaline carbonate buffer (lanes 2–5). The extracted membranes were analyzed by SDS-PAGE followed by autoradiography (upper panel, [^{35}S] atToc159). Insertion of [^{35}S] atToc159 was quantitated using a PhosphorImager and is given in percentage of maximal insertion (lane 2). The lower panel indicates that equivalent amounts of chloroplast membranes were loaded onto the gel based on the presence of the large subunit of Rubisco (LSU).

1996). This protease sensitivity, as well as the association of atToc159 with atToc33 in the *Arabidopsis* complex (Fig. 1 B) and with Toc34 upon insertion into isolated pea chloroplasts (Bauer et al., 2000), suggests that atToc33 may contribute to the proposed receptor. Therefore, we tested the ability of atToc159 to bind to atToc33 using a soluble phase binding assay (Fig. 4). In the assay, [^{35}S] atToc159 bound to atToc33$_{1-265}$, a soluble version of the protein lacking the COOH-terminal transmembrane stub (Fig. 4 A), in a concentration-dependent fashion. The result suggests that the soluble phase binding assay may have reconstituted a surface recognition step in the membrane assembly of atToc159. Therefore, we tested the ability of [^{35}S] atToc159 to insert into the outer chloroplast membrane in the presence of atToc33$_{1-265}$ using resistance to alkaline extraction as the criterion of insertion (Fig. 5). Increasing concentrations of atToc33$_{1-265}$ inhibited insertion of [^{35}S]atToc159 by up to 60%. It is likely that the added soluble atToc33$_{1-265}$ competed with endogenous atToc33 at the chloroplast surface for binding of [^{35}S] atToc159, thereby interfering with its subsequent insertion. Thus, binding to atToc33 at the cytosolic face of the chloroplast may represent a step in atToc159 recognition. It remains to be determined whether insertion of atToc159 requires the activity of additional outer membrane components. Nevertheless, we conclude that atToc33 may serve at least as part of a receptor complex in the recognition and insertion of atToc159. As both atToc159 and atToc33 are GTP-binding proteins, the possibility of GTP-dependent regulation of their interactions arises. GTP-regulated, homotypic interactions between GTP-binding proteins are not unprecedented. Notably, targeting of SRP to the SRP receptor involves such a mechanism (Walter and Johnson, 1994).

The data available on pea Toc159 and atToc159 summarily suggest that the proteins may function as mobile import receptors partitioning between the cytosol and the Toc complex. However, in vitro studies using isolated chloroplasts do not indicate a requirement for an added plant-specific soluble receptor, with the possible exception of a guidance complex (May and Soll, 2000) stimulating the import of unconventional phosphorylated precursor proteins (Waegemann and Soll, 1996). Moreover, isolated chloroplasts import a chemically denatured precursor in the complete absence of added soluble proteins (Pilon et al., 1992). Conceivably, atToc159 or other proteins present in isolated chloroplasts may serve as soluble import receptors upon release from the chloroplast surface. However, import of precursors into isolated chloroplasts still functions after proteolytic degradation of Toc159 to its 52-kD M domain (Chen et al., 2000), but precursor binding to the chloroplast surface was markedly reduced after degradation of Toc159 to the M domain (Chen et al., 2000). Although Toc159 can be bypassed in vitro, it appears unlikely that the M domain of atToc159 alone would be functional in vivo. Therefore, the strict requirement for atToc159 as a soluble and/or membrane-bound import receptor may be limited to the in vivo system.

This study has uncovered the existence of a soluble form of atToc159 and its targeting to the chloroplast surface. The occurrence of atToc159 in both a soluble and integral membrane form suggests that it may be able to switch. The switch would require that atToc159 be able not only to insert into, but also to dissociate from the outer membrane. Such a scenario implies conformational changes in atToc159, possibly induced by GTP-binding and hydrolysis. However, regulatory factors affecting membrane association of atToc159 are not presently known. AtToc159 may serve as a soluble import receptor, activated and targeted to the chloroplast surface upon precursor binding. Another possibility is that functional import machineries are assembled from pools of soluble components (i.e., atToc159, atToc120, and atToc132) in a regulated fashion. In certain aspects either scenario resembles the SRP system, but also the Sec pathway at the bacterial plasma membrane. SecA energizes protein translocation at the bacterial plasma membrane through ATP-dependent cycles of membrane insertion and deinsertion, thereby driving a precursor across the membrane (Driessen et al., 1998). The similarities between the SRP system and the Toc complex pertaining to the targeting mechanism have been discussed above. Assembly of the Toc complex may use a hybrid mechanism involving functional principles of both the SRP and Sec pathways.

In conclusion, the results of our study outline a possible function of atToc159 as a mobile transit sequence receptor in chloroplast protein import. In the future, precursor interaction and the role of GTP in the targeting of atToc159 to

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the outer membrane and the assembly of the Toc complex remain to be determined.

Materials and methods

DNA constructs used in protein synthesis

pET21d-atToc159 used for in vitro synthesis of atToc159 has been described previously (Bauer et al., 2000). To produce $atToc33_{1–265}$ lacking the COOH-terminal transmembrane stub, a DNA fragment corresponding to amino acids 1–265 was amplified from *Arabidopsis* EST clone 190I17T7 by PCR with primers that incorporated an NcoI site at the 5′ end and six histidine codons, followed by a HindIII site at the 3′ end and ligated into the NcoI-HindIII sites of pET21d (Novagen, Inc.). For bacterial overexpression, the plasmid pET21d-$atToc33_{1–265}$ was transformed into *E. coli* BL21(DE3) and the overexpressed protein was purified under nondenaturing conditions using Ni-NTA (QIAGEN). Bound $atToc33_{1–265}$ was eluted with an imidazole gradient from 40 to 250 mM, and fractions containing the protein were dialyzed against 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 μM GTP, and 1 mM DTT. The dialysate was centrifuged at 16,000 *g* to remove insoluble aggregates and glycerol was added to 5% wt/vol.

To produce antibodies against atToc75, a PCR product corresponding to amino acids 141–397 was amplified from *Arabidopsis* genomic DNA using primers incorporating an NcoI site at the 5′ end and six histidine codons, and a SacI site at the 3′ end. The resulting DNA fragment was ligated into the NcoI-SacI sites of pET21d, resulting in pET21d-$atToc75_{141–397}$. The plasmid pET21d-$atToc75_{141–397}$ was transformed into *E. coli* BL21(DE3) cells, and $atToc75_{141–397}$ was overexpressed and purified under denaturing conditions using Ni-NTA.

For in vitro synthesis of proteins in the presence of [^{35}S]methionine, a reticulocyte-based coupled transcription/translation system (Promega) was used.

Antibodies

Purified $atToc75_{141–397}$ was used to produce rabbit antibodies (Eurogentec). Antibodies were affinity purified using the antigen coupled to Affi-Gel 10 (Bio-Rad Laboratories) according to the supplier's recommendation. The antibodies against atToc159 have been described previously (Bauer et al., 2000). Antibodies against atToc33 and atToc34, as well as psToc159, psToc75, and psToc34 (Ma et al., 1996), were a gift from Dr. D.J. Schnell (University of Massachusetts, Amherst, MA). Antibodies against cytosolic *Arabidopsis* geranylgeranyl transferase were a gift from Dr. W. Gruissem (Institute of Plant Sciences, ETH, Zurich, Switzerland). Antibodies against chlorophyll a/b binding protein were a gift from the Dr. K. Apel (Institute of Plant Sciences, ETH).

Isolation of proteins from total plant extracts, soluble fractions, and chloroplasts

Seeds of plants (*A. thaliana* ecotype Columbia 2) used for extraction of soluble proteins or isolation of chloroplasts were vernalized at 4°C. Subsequently, plants were grown on 0.8% agar plates (0.5× Murashige-Skoog medium, 1% wt/vol sucrose) under long-day conditions (16 h light, 8 h dark) or on soil under short-day conditions (8 h light, 16 h dark). Total soluble protein and intact chloroplasts were isolated from leaves from 3–4-wk-old *Arabidopsis* plants. Leaves were harvested and floated on ice-cold tap water for 20–30 min before homogenization in HB buffer (450 mM sorbitol, 20 mM Tricine/KOH, pH 8.4, 10 mM EDTA, 10 mM $NaHCO_3$, 5 mM Na-ascorbate, 1 mM $MnCl_2$, 1 mM PMSF) using a Waring blender. The homogenized plant material was filtered through two layers of cheese cloth and one layer of Miracloth (Calbiochem) and centrifuged for 2 min at 500 *g* at 4°C. The supernatant was removed and used for isolation of total soluble protein as described below. The pellet was resuspended in 1 ml of RB buffer (300 mM sorbitol, 20 mM Tricine/KOH, pH 7.6, 5 mM $MgCl_2$, 2.5 mM EDTA), loaded on a Percoll step gradient (40% vol/vol and 85% vol/vol; Amersham Pharmacia Biotech) in RB buffer, and centrifuged for 10 min at 2,500 *g* at 4°C in a swing-out rotor. Intact chloroplasts were removed from the 40/85% Percoll interphase, washed with 10 vol RB buffer, and used for immunoblot analysis. The supernatant of the initial homogenate (see above) was supplemented with 0.1% vol/vol protease inhibitor cocktail (Sigma-Aldrich) and was cleared by centrifugation for 2 h at 100,000 *g* at 4°C. Protein was extracted from the supernatant by $CHCl_3$/MeOH precipitation (Wessel and Flügge, 1984). Cellular equivalents were calculated from the amounts of protein and/or chlorophyll contained in the total extracts, soluble and chloroplast fractions, respectively (unpublished data).

Immunopurification of the *Arabidopsis* Toc complex

To immunopurify the *Arabidopsis* Toc complex, chloroplasts were lysed under hypertonic conditions (0.6 M sucrose, TES [50 mM Tricine, pH 7.5, 10 mM EDTA containing 0.1% vol/vol protease inhibitor cocktail]) using a Dounce homogenizer. The lysate was separated into membrane and soluble fractions by centrifugation at 40,000 *g* for 20 min. The total membranes were solubilized in TES, 2% Triton X-100, and cleared by centrifugation at 100,000 *g* for 10 min. The cleared solubilisate was applied to a column containing an anti-atToc33 IgG fraction coupled to Sepharose. Subsequently, the column was washed with TES, 0.5% Triton X-100, and eluted with 0.2 M glycine, pH 2.2, 0.5% Triton X-100.

Western blot analysis was done according to standard protocols. Horseradish peroxidase–coupled secondary antibodies and enhanced chemiluminescence reaction were used for immunodetection as recommended by the supplier (Roche).

Immunolocalization in protoplasts and chloroplast protein insertion experiments

Immunolocalization using *Arabidopsis* protoplasts was done according to protocols at http://www.arabidopsis.org/cshl-course/7-gene_expression.html. Affinity-purified antibodies against $atToc159_{1–740}$ and $atToc75_{141–397}$ were used at 5 μg/ml. FITC-labeled secondary antibodies (Pierce Chemical Co.) were used at a dilution of 1:50. Protoplasts were then analyzed by confocal laser scanning microscopy (Leica DM IRBE microscope and Leica TCS SP laser) at excitation and emission wavelengths of 488 and 520 nm, respectively. Insertion experiments (using isolated chloroplasts corresponding to 15 μg of chlorophyll) and alkaline carbonate extractions were done as described previously (Chen and Schnell, 1997).

Soluble phase binding assay

Hexahistidinyl-tagged $atToc33_{1–265}$ was purified as described above and incubated, at the concentrations indicated, with 20 μl of reticulocyte lysate containing [^{35}S] atToc159 in import buffer (final concentrations: 50 mM Hepes, pH 7.7, 330 mM sorbitol, 2 mM $Mg[OAc]_2$, 40 mM KOAc, 0.4 mM GTP, 4 mM ATP, 0.1% Triton X-100 wt/vol) in a final volume of 50 μl and incubated on ice for 5 min. 10 μl of packed Ni-NTA was added, and the incubation continued for 30 min at 4°C under constant mixing to reisolate the hexahistidinyl-tagged $atToc33_{1–265}$. The resin was washed three times with 400 μl of import buffer, once with import buffer containing 40 mM imidazole, and then eluted with import buffer containing 200 mM imidazole. Eluates were analyzed by SDS-PAGE and Coomassie blue staining followed by autoradiography.

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