The Targeting of the atToc159 Preprotein Receptor to the Chloroplast Outer Membrane is Mediated by its GTPase Domain and is Regulated by GTP

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The targeting of the atToc159 preprotein receptor to the chloroplast outer membrane is mediated by its GTPase domain and is regulated by GTP

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The multimeric translocon at the outer envelope membrane of chloroplasts (Toc) initiates the recognition and import of nuclear-encoded preproteins into chloroplasts. Two Toc GTPases, Toc159 and Toc33/34, mediate preprotein recognition and regulate preprotein translocation. Although these two proteins account for the requirement of GTP hydrolysis for import, the functional significance of GTP binding and hydrolysis by either GTPase has not been defined. A recent study indicates that Toc159 is equally distributed between a soluble cytoplasmic form and a membrane-inserted form, raising the possibility that it might cycle between the cytoplasm and chloroplast as a soluble preprotein receptor. In the present study, we examined the mechanism of targeting and insertion of the Arabidopsis thaliana orthologue of Toc159, atToc159, to chloroplasts. Targeting of atToc159 to the outer envelope membrane is strictly dependent only on guanine nucleotides. Although GTP is not required for initial binding, the productive insertion and assembly of atToc159 into the Toc complex requires its intrinsic GTPase activity. Targeting is mediated by direct binding between the GTPase domain of atToc159 and the homologous GTPase domain of atToc33, the Arabidopsis Toc33/34 orthologue. Our findings demonstrate a role for the coordinate action of the Toc GTpases in assembly of the functional Toc complex at the chloroplast outer envelope membrane.

Introduction

The targeting and import of nuclear-encoded preproteins to chloroplasts is mediated by the coordinated action of preprotein translocons at the outer and inner envelope membranes (Keegstra and Cline, 1999; Schleiff and Soll, 2000; Bauer et al., 2001). The translocon of the outer envelope membrane of chloroplasts (Toc)* mediates the initial recognition of preproteins and initiates translocation across the membrane. Preproteins are recognized by interactions of their intrinsic NH2-terminal transit peptides with receptor components of the Toc complex, and translocation begins with the hydrolysis of ATP and GTP at the chloroplast surface (Olsen and Keegstra, 1992; Ma et al., 1996; Keegstra and Froehlich, 1999; Young et al., 1999). The Toc complex physically associates with the translocon at the inner envelope membrane of chloroplasts (Tic) to provide a direct conduit for preproteins from the cytoplasm to the internal stromal compartment (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998).

Three components of the Toc complex associate with preproteins during the early stages of import. Toc33/34 (Gutensohn et al., 2000; Sveshnikova et al., 2000; Schleiff et al., 2002) and Toc159 (Kessler et al., 1994; Perry and Keegstra, 1994; Kouranov and Schnell, 1997) are homologous, membrane-associated GTPases that directly interact with preproteins during preprotein binding. Direct crosslinking of transit sequences to isolated pea chloroplasts suggests that pea Toc159 (psToc159) functions as the primary preprotein receptor at the chloroplast surface (Perry and Keegstra, 1994; Ma et al., 1996). Furthermore, analysis of an Arabidopsis null mutant, termed ppi2, that lacks the psToc159 orthologue, atToc159, indicates that this protein is essential for chloroplast biogenesis (Bauer et al., 2000). The in vitro and in vivo
analysis of psToc34 and its Arabidopsis orthologue, atToc33, indicates that it interacts with preproteins at the early stages of import (Jarvis et al., 1998; Gutensohn et al., 2000; Sveshnikova et al., 2000; Schleiff et al., 2002) and that its GTPase activity is required for the initiation of membrane translocation (Kouranov and Schnell, 1997; Chen et al., 2000). The two GTPases associate with Toc75, an integral membrane protein that forms at least part of the channel through which preproteins pass during translocation across the outer membrane (Schnell et al., 1994; Ma et al., 1996; Hinnah et al., 1997).

psToc159 and atToc159 are 159 and 160 kD, respectively (Chen et al., 2000). Both have a distinct tripartite domain structure consisting of an NH2-terminal acidic domain (A-domain), a central GTPase domain (G-domain), and a COOH-terminal membrane anchor domain (M-domain; Bauer et al., 2000; Chen et al., 2000). Although the function of the A-domain is not known, the M-domain anchors the membrane-bound form of the protein to the outer membrane, exposing the A- and G-domains to the cytoplasm (Hirsch et al., 1994). psToc159 has been shown to bind GTP (Kessler et al., 1994); however, the role of the G-domain and the functional significance of GTP binding and hydrolysis have not been determined.

Originally, Toc159 was described as an exclusively integral membrane protein. Although the 52-kD M-domain clearly acts as a membrane anchor, the nature of the membrane association is unclear. A recent study indicates that atToc159 is equally distributed between a soluble cytoplasmic form and a membrane-bound form that is stably associated with atToc33 and atToc75 (Hiltbrunner et al., 2001). This observation led to the proposal that atToc159 functions as a mobile import receptor that cycles between the cytoplasm and outer membrane in alternating soluble and integral membrane states. Soluble atToc159 and the atToc33 GTPase domain (atToc33G, corresponding to amino acid residues 1–265) were shown to directly interact, and inclusion of atToc33G in an in vitro targeting assay inhibited association of atToc159 with isolated chloroplasts (Hiltbrunner et al., 2001). These data implicated atToc33 as a part of the receptor site for atToc159 at the chloroplast surface (Hiltbrunner et al., 2001).

The recently determined x-ray crystal structure of the GTPase domain of psToc34 demonstrated the capacity of this domain to dimerize and thereby potentially regulate its intrinsic GTPase activity. These data suggest that assembly of the functional Toc complex is regulated directly by GTP binding at the Toc GTPases.

Results

Energetics of atToc159 targeting to chloroplasts

As a first step in our analysis, we examined the energetics of atToc159 targeting to the outer envelope membrane to investigate a potential role for GTP. Targeting was assayed in an in vitro system using isolated Arabidopsis chloroplasts and in vitro–translated [35S]atToc159 in the presence of various nucleotides and their derivatives. Endogenous nucleotides were removed from the [35S]atToc159 in vitro translation mixture by EDTA treatment and gel filtration prior to the targeting assay. In addition, the isolated chloroplasts were preincubated in the dark to deplete endogenous nucleoside triphosphates (NTPs).

[35S]atToc159 that copurifies with chloroplasts after the in vitro targeting assay represents the sum of two populations of envelope-associated molecules: atToc159 that is specifically bound, but not inserted into the outer membrane, and atToc159 that is properly inserted in its native topology (Muckel and Soll, 1996). To discriminate these two forms, the total population of chloroplast-associated [35S]atToc159 was compared with the amount of 50–55 kD-[35S]atToc159 protease-protected fragments that were generated upon treatment of chloroplasts with thermolysin. Previous studies have shown that complete alkaline resistant insertion of Toc159 into the outer membrane protects the 52-kD M-domain from proteolytic digestion (Hirsch et al., 1994; Chen et al., 2000). Therefore, the 50–55-kD fragments represent properly inserted atToc159, whereas the population that is completely protease sensitive corresponds to the peripherally bound fraction.

Fig. 1 A shows that [35S]atToc159 binds to isolated chloroplasts in the absence of exogenous nucleotide (lanes 2 and 3). A portion of [35S]atToc159 is converted to an 86-kD fragment to varying degrees during the targeting assay (Fig. 1 A, lanes 2–6). This fragment has previously been shown to correspond to the G- and M-domains of atToc159 (atToc159GM) resulting from cleavage of the highly susceptible A-domain by an unknown endogenous protease (Bolter et al., 1998; Chen et al., 2000). A-domain cleavage does not affect binding or insertion of the receptor (see Fig. 5). We included both the full-length and 86-kD fragments in quantitating the levels of atToc159 binding to chloroplasts (Fig. 1 B). Treatment of the chloroplasts with thermolysin resulted in the complete degradation of bound atToc159 and conversion of inserted atToc159 to a set of proteolytic fragments at 50–55 kD (Fig. 1 A, lanes 8–12), demonstrating insertion of at least part of the full-length protein into isolated chloroplasts. The inclusion of apyrase in the samples lacking added nucleotides to insure complete depletion of NTPs had no additional effect on the levels of atToc159 binding and insertion (Fig. 1 B) although the enzyme treatment resulted in nearly complete degradation of atToc159 to its 86 and 55-kD fragments (Fig. 1 A, compare lanes 2 and 3). Therefore, EDTA treatment and gel filtration were sufficient to deplete free nucleotides from the assays. All
commercial preparations of apyrase and hexokinase tested resulted in significant degradation of the atToc159 translation product. For this reason, we opted to exclude an enzymatic NTP hydrolysis system from the targeting assays in further studies.

The addition of GTP does not have a significant effect on the binding of atToc159 to chloroplasts when compared with the controls lacking added nucleotide or containing ATP (Fig. 1 B). The efficiency of binding in all cases typically corresponds to 10–15% of added [35S]atToc159. In contrast, GTP specifically stimulates the productive insertion of atToc159 twofold over the levels observed in the absence of added nucleotide (Fig. 1 A, compare lanes 9 and 10, and B). Insertion corresponds to 5–8% of added [35S]atToc159 in the presence of GTP. These data suggest that binding and insertion are distinct reactions. Although binding does not appear to be strictly nucleotide-dependent, GTP specifically stimulates insertion. ATP has no measurable effect on insertion above the no nucleotide control (Fig. 1 A, compare lanes 9 and 11, and B). Furthermore, the addition of both ATP and GTP had no additive effect on either binding (Fig. 1 B) or insertion (Figs. 1 A, compare lanes 10 and 12, and 2 B) when compared to GTP alone. Therefore, ATP does not appear to play a direct role in atToc159 targeting.

To test whether GTP hydrolysis may be involved in the targeting reaction, we examined the effects of GDP and the nonhydrolyzable ATP analogue, GMP-PNP. Fig. 2 shows that GMP-PNP has little effect on binding (Fig. 2 B), but reduced insertion threefold when compared with GTP (Fig. 2, A, compare lanes 13 and 14, and B). Binding in the presence of GDP is ~60% of the level observed in the presence of GTP (Fig. 2 B), but GDP is equally as effective as GTP in promoting insertion of atToc159 into the outer membrane (Fig. 2, A, compare lanes 13 and 15, and B). To eliminate the possibility that GDP is converted to GTP by the plastid nucleotide diphosphate kinase during the targeting reaction, we tested the ability of the GDP analogue, GDPβS, to support targeting. GDPβS is equally as effective as GDP in supporting atToc159 binding (Fig. 2 B) and insertion (Fig. 2, A, compare lanes 15 and 16, and B). In addition, we included the nonhydrolyzable ATP analogue, AMP-PNP, in samples containing GDP to inhibit kinase activity. AMP-PNP had no effect on GDP-dependent insertion of atToc159 into the outer membrane (Fig. 2, A, compare lanes 15 and 19, and B). Therefore, the ability of GDP to support targeting is not due to its conversion to GTP in the in vitro assay. Taken together, the data in Fig. 2 indicate that GTP binding alone is not sufficient for proper insertion of atToc159. Hydrolysis of GTP to GDP is required for insertion. However, hydrolysis...
as such does not appear to drive insertion because atToc159 inserts equally well in the presence of GTP, GDP, or GDPBP. Rather, it appears that GDP, but not GTP, supports a conformation that is competent for insertion. In the presence of GDP, atToc159 may bypass the binding step or proceed from binding to insertion more efficiently resulting in lower levels of steady state binding.

A previous study indicated that insertion of a 96-kDa COOH-terminal fragment of psToc159 required ATP for insertion into the outer membrane. Our data indicate a requirement for ATP and suggest that ATP alone is insufficient to support insertion of the receptor (Fig. 1). Furthermore, AMP-PNP has no effect on atToc159 targeting (Fig. 2, A, compare lanes 10 and 11, and lanes 21 and 22, and B). Therefore, it appears that guanine nucleotides and not ATP are specifically required for atToc159 targeting. The previous report of an ATP requirement is likely the result of interconversion of the two nucleotides during the in vitro targeting assay.

**Effect of GTPase mutations on atToc159 targeting**

The analysis of the energetics of atToc159 targeting suggests that GTP/GDP play a role in the binding and insertion of the protein to the outer envelope membrane. To investigate whether the GTPase activity of atToc159 is directly involved in targeting, we generated two point mutations in the consensus GTP binding motif of the atToc159 G-domain. Fig. 3A shows an alignment of the motifs of atToc159 and atToc33 to human p21<sup>ras</sup> and examples of mammalian and prokaryotic signal recognition particle (SRP)54 and the SRP receptor. The Toc GTPases define a distinct subfamily of GTPases with limited but notable sequence similarity to other GTPases, and we used this conservation to select point mutations that could be predicted to affect either GTP binding or GTP hydrolysis specifically. The highest degree of sequence conservation among these GTPases is found within the G1 motif (P-loop). Therefore, we generated two independent point mutations in the G1 motif of atToc159, A<sup>864</sup> to R (atToc159-A864R) and K<sup>868</sup> to R (atToc159-K868R), that are predicted to affect GTP hydrolysis and binding, respectively (Althoff et al., 1994; Chen and Schnell, 1997).

The size and characteristics of atToc159 prevented expression of sufficient amounts of native protein to confirm the GTP-binding and hydrolytic activity of the full-length mutants. As an alternative, we took advantage of the *Escherichia coli* expression system to generate the corresponding G-domains of each mutant in soluble, native form. The wild-type G-domain (atToc159G, corresponding to residues 727–1092) and G-domains of the two GTPase mutants (atToc159G-A864R and atToc159G-K868R) each with COOH-terminal hexahistidine tags were purified by nickel-nitrilotriacetic acid agarose (Ni-NTA) chromatography (Fig. 3 B) and assayed for their GTP binding (Fig. 3 C) and hydrolysis activities (Fig. 3 D). The GTP binding and hydrolysis activities of the three G-domain variants are consistent with those predicted by the selected mutations. AtToc159G binds (Fig. 3 C) and hydrolyzes (Fig. 3 D) GTP as expected. The GTPase activity of the atToc159 G-domain under substrate saturation (unpublished data) is comparable to the intrinsic activity of isolated SRP receptor (Connolly and Gilmore, 1993). The atToc159G-A864R mutant binds ap-

![Figure 3](image-url)
Targeting of atToc159 to chloroplasts

Figure 4. The effects of GTPase mutations on the targeting of atToc159 to isolated chloroplasts. The targeting of in vitro-translated [35S]atToc159 (atToc159), [35S]atToc159-A864R (159-A864R), or [35S]atToc159-K868R (159-K868R) to isolated chloroplasts in the presence of GTP or GDP was performed as described in the legend of Fig. 1. (A) Phosphorimager analysis of SDS-PAGE-resolved chloroplast membranes from targeting reactions performed in the presence of GTP. Lanes 1, 4, and 7 contain 10% of the [35S]atToc159, [35S]atToc159-A864R, and [35S]atToc159-K868R in vitro translation products (IVT) added to each reaction, respectively. (B) Quantitative analysis of the data from triplicate experiments including those in A. [35S]atToc159 binding (Binding) and insertion (Insertion) were measured as described in the legend to Fig. 1. (C) GDP binding to wild-type and mutant atToc159-A864R G-domains. Binding of [3H]GDP (32 Ci/mmol) to purified atToc159G and atToc159G-A864R was measured in a filter binding assay in the presence of ATP. Bound [3H]GDP was quantitated by scintillation counting. (D) Phosphorimager analysis of SDS-PAGE-resolved chloroplast membranes from time courses of atToc159 and atToc159-A864R insertion performed in the presence of GTP or GDP. Lanes 1 and 7 contain 20% of the [35S]atToc159 (top) and [35S]atToc159-A864R (bottom) in vitro translation products (IVT) added to each reaction. The results from a typical experiment are shown. (E) Qualitative analysis of the data from duplicate experiments including those in D. Insertion of [35S]atToc159 was measured as described in the legend to Fig. 1. The positions of the atToc159 proteins, the 86-kD proteolytic fragments (159G) and the 50–55-kD thermolysin-protected fragments (159M) are indicated to the left of the figure. Error bars indicate standard deviation.

Deletion analysis of atToc159 targeting to chloroplasts

The direct role of atToc159 GTP/GDP binding activity in targeting suggests that the G-domain might participate directly in the targeting reaction. To examine the domains of atToc159 that are required for targeting, we performed the in vitro chloroplast targeting assay with a series of mutants corresponding to deletions of the A-, G-, and M-domains. Deletion of the 70-kD NH2-terminal A-domain of atToc159 to generate a construct corresponding to the G- and M-domains alone (atToc159GM) had no adverse effect on association or insertion into the outer membrane as compared to full-length atToc159 (Fig. 5 A, compare lanes 2 and 5, and lanes 3 and 6). Also, the A-domain itself (atToc159A) did not bind or insert into isolated chloroplasts (Fig. 5 A, lanes 2 and 5). Therefore, we conclude that the A-domain does not participate in targeting of atToc159 to the chloroplast outer membrane. The construct corresponding to the M-domain (atToc159M) bound to chloroplasts, but was not protected from thermolysin treatment (Fig. 5 A, lanes 11 and 12). This result is consistent with previous studies indicating that the M-domain is necessary but not sufficient for complete insertion of atToc159 into the outer membranes.
membrane (Muckel and Soll, 1996). The G-domain of atToc159 (atToc159G) bound to chloroplasts with efficiency equivalent to atToc159GM and full-length atToc159 (Fig. 5 B). As expected, bound atToc159G was completely sensitive to proteolysis indicating its inability to insert into the outer membrane alone (Fig. 5, A, lane 15, and B).

The efficient binding of atToc159G to isolated chloroplasts suggests that this domain participates directly in receptor targeting. To explore this possibility, we first examined the specificity of atToc159G binding with a competition assay. The inclusion of unlabeled atToc159G inhibited the binding of \[^{35}S\]atToc159 to isolated chloroplasts in a dose-dependent manner with \(\sim 80\%\) inhibition observed with 0.25 \(\mu\)M competitor (Fig. 6, A and B). Second, we examined whether atToc159G binding represents a productive step in the targeting of full-length atToc159 by testing the ability of atToc159G to compete with full-length atToc159 targeting. The binding and insertion of full-length \[^{35}S\]atToc159 is reduced by \(\sim 80\%\) in the presence of 0.25 \(\mu\)M atToc159G (Fig. 6, C and D), indicating that the atToc159G binding site represents a component of the receptor docking site at the chloroplast surface. These data also confirm that the fraction of \[^{35}S\]atToc159 that is bound but not completely inserted into chloroplasts represents a specific association with the outer membrane and not nonspecific binding to the chloroplast surface. On the basis of these data, we con-
clude that the G-domain directly participates in atToc159 targeting to chloroplasts.

To determine whether GTP binding/hydrolysis participates in the binding of atToc159G, we examined the binding of the mutant G-domains to chloroplasts. Both atToc159G-A864R and atToc159G-K868R associate with chloroplasts at levels ~50% that of control atToc159G (Fig. 7, A and B). Furthermore, their presence inhibits the targeting of full-length [35S]atToc159 to chloroplasts (Fig. 7, C and D). Specifically, atToc159G-A864R and atToc159G-K868R reduce atToc159 binding by 40–45%. Insertion is reduced by 70–80% in both cases (Fig. 7 D). Therefore, it appears that wild-type and mutant G-domains productively interact with the atToc159 docking site. The capacity of wild-type G-domain to bind at higher levels than the mutants (Fig. 7 B) could be explained by the ability of this construct to convert to a conformation with a higher relative affinity for the docking site upon hydrolysis of its bound GTP to GDP (see Fig. 9). These data are con-

Figure 7. Binding of wild-type and mutant atToc159 G-domains to chloroplasts in vitro. (A) In vitro–translated [35S]atToc159G (159G), [35S]atToc159G-A864R (159G-A864R), or [35S]atToc159G-K868R (159G-K868R) was incubated with isolated chloroplasts in a standard targeting assay (Fig. 1, legend). The total membrane fraction from each targeting reaction (lanes 2, 4, and 6) was resolved by SDS-PAGE and analyzed using a phosphorimager. 10% of the [35S]atToc159G, [35S]atToc159G-A864R, or [35S]atToc159G-K868R in vitro translation product (IVT) added to each reaction is shown in lanes 1, 3, and 5, respectively. (B) Quantitative analysis of the data from triplicate experiments including those in A. (C) Competition of purified atToc159G-A864R and atToc159G-K868R with atToc159 for targeting to chloroplasts. [35S]atToc159 was incubated with isolated, intact chloroplasts in a standard targeting assay (Fig. 1, legend) in the absence (−) or presence (+) of 0.25 μM purified 159G-A864R or 159G-K868R. Total membrane fractions were separated by SDS-PAGE and analyzed using a phosphorimager as described in the legend to Fig. 1. Lane 1 contains 20% of the [35S]atToc33G added to each reaction. (D) Quantitative analysis of the data from triplicate experiments including those in C. Error bars indicate standard deviation.

Figure 8. Direct binding of atToc33G to atToc159G. (A) [35S]atToc33G was incubated in the presence of GTP with the indicated amounts of hexahistidine-tagged atToc159G (159G) or cellular retinoic acid binding protein (CRABP) that had been immobilized on Ni-NTA resin. Bound proteins were eluted and separated by SDS-PAGE and analyzed using a phosphorimager. Lane 1 contains 20% of the [35S]atToc33G added to each reaction. (B) Quantitation of the data presented in A. Error bars represent standard deviation. (C) Competition of soluble atToc159G with immobilized atToc159G for binding to atToc33G. [35S]atToc33G was incubated with immobilized hexahistidine-tagged atToc159G in the absence or presence of increasing concentrations of soluble atToc159G. (D) Quantitation of the data presented in C.
To examine the nucleotide dependence of atToc159G–atToc33G binding, immobilized atToc159G was incubated with \(^{[35S]}\)atToc33G in the presence or absence of various nucleotides and nucleotide analogs. As shown in Fig. 9, the levels of binding in the presence of GTP (lane 4), ATP (lane 6), or their nonhydrolyzable analogues (lanes 5 and 7) was not considerably different than in the absence of nucleotides (lane 3). However, inclusion of GDP (lane 8) or GDP\(\beta\)S (lane 9) had a stimulatory effect on atToc33G–atToc159G binding, increasing binding 2.5–3-fold compared with other nucleotides (Fig. 9 B). Therefore, the interaction between the G-domains appears to be stabilized by GDP. This correlates with the effect of GDP on the insertion of full-length atToc159 into chloroplasts. On this basis, we propose that the GDP-stabilized atToc159G–atToc33G interaction observed in the solid phase binding assay corresponds to the conformational state resulting in stable insertion of atToc159 at the outer membrane and association of the receptor with the Toc complex.

**Discussion**

Toc159 is a major component of the Toc complex of chloroplasts and is proposed to function as the primary preprotein receptor of the complex. It has recently been demonstrated that this protein is equally distributed between the chloroplast surface and the cytoplasm, and may be a mobile component of the Toc complex, perhaps involved in targeting chloroplast preproteins to their site of import (Hiltbrunner et al., 2001). In this study, we focused on the mechanism by which soluble Toc159 is targeted to the Toc complex. An earlier study indicated that targeting and insertion of a 96-kD COOH-terminal fragment of psToc159 relied on proteinaceous components of the chloroplast (Muckel and Soll, 1996), and it has been suggested more recently that this putative receptor may include atToc33/psToc34 (Hiltbrunner et al., 2001). We have extended these studies, and present evidence that atToc159 is targeted to *Arabidopsis* chloroplasts in a guanine nucleotide-dependent manner via a direct interaction between the homologous G-domains of atToc159 and atToc33.

Analysis of the energetics of atToc159 targeting demonstrated that binding of the receptor to the outer membrane was not nucleotide-dependent. However, proper insertion of atToc159 into the outer membrane required GTP or GDP (Fig. 2). The nonhydrolyzable GTP analogue, GMP-PNP, inhibited insertion of atToc159 (Fig. 2), suggesting that the ability of GTP to support insertion required hydrolysis to GDP. These findings were confirmed by the analysis of two GTPase mutants of atToc159 with reduced GTP binding (atToc159-K868R) or hydrolysis (atToc159-A864R) properties (Fig. 3). The binding of both mutants to chloroplasts was similar to wild-type atToc159 (Fig. 4), whereas insertion was observed only with atToc159-A864R. These data support the hypothesis that the GDP-bound state promotes membrane insertion. GTP hydrolysis does not appear to provide the driving force for insertion because GDP binding alone is sufficient for stable insertion into the outer membrane (Fig. 2). Therefore, it is likely that the conformation of the GDP-bound state promotes insertion and association with the Toc complex.

**Diagram**

![Diagram of Energetics of atToc33G-atToc159G binding](image)

**Figure 9. Energetics of atToc33G-atToc159G binding.** Nucleotide-depleted in vitro–translated \(^{[35S]}\)atToc33G was incubated with immobilized hexahistidine-tagged atToc159G (159G) in the absence (−) or presence (+) of 0.1 mM GTP, GMP-PNP, ATP, AMP-PNP, GDP, or GDP\(\beta\)S. The bound proteins were eluted and separated by SDS-PAGE and analyzed using a phosphorimager. (A) Phosphorimager analysis of bound \(^{[35S]}\)atToc33G. 10% of the in vitro–translated \(^{[35S]}\)atToc33G (IVT) that was added to each reaction is shown in lane 1. Lane 2 contains the \(^{[35S]}\)atToc33G that bound to the Ni-NTA matrix in the absence of atToc159G. (B) Quantitation of the data presented in A.

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**Table**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Binding to atToc33G</th>
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<tr>
<td>GTP</td>
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<tr>
<td>ATP</td>
<td>80%</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>50%</td>
</tr>
<tr>
<td>GDP</td>
<td>30%</td>
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<tr>
<td>GDP(\beta)S</td>
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**Note:** The table above illustrates the percentage of binding of atToc33G to atToc159G under different nucleotide conditions.
A previous study demonstrated that ATP stimulated the targeting of a 96-kD COOH-terminal fragment (G- + M-domains) of psToc159 to isolated chloroplasts (Muckel and Soll, 1996). We did not observe a significant effect of ATP on targeting of atToc159 when compared with the control lacking added nucleotide or an NTP hydrolysis trap (Fig. 1). Likewise, ATP and GTP together had no additive effect on targeting, nor did the nonhydrolyzable ATP analogue AMP-PNP affect targeting in the presence or absence of GTP or GDP (Fig. 2). We conclude that ATP does not play a direct role in the atToc159 targeting reaction. The authors in the previous study used a hexokinase/glucose trap to eliminate ATP from the targeting reaction. We discovered that commercial preparations of hexokinase and another hydrolase, apyrase, degraded the full-length atToc159 translation product. This degradation could account for the apparent decrease in binding observed with the hexokinase trap in the previous study. Alternatively, generation of GTP from ATP and GDP by the plastid nucleoside diphosphate kinase (Lubeck and Soll, 1995) could account for the apparent ATP requirement.

Muckel and Soll (1996) used a series of deletion mutants and fusion proteins of the 96-kD COOH-terminal fragment of psToc159 to investigate targeting. Their studies indicated that portions of the M-domain bound to chloroplasts, but that only the 86-kD construct consisting of the G- + M-domains was capable of productively inserting into the outer membrane (Muckel and Soll, 1996). In the present study, we wished to more precisely determine which domains of the protein mediate the targeting of full-length atToc159, with the knowledge that the protein has a distinct tripartite domain structure and that GTP plays an important role (Fig. 5). Binding and insertion assays using isolated intact chloroplasts and a series of deletion mutants indicated that the G-domain on its own was able to efficiently and specifically bind chloroplasts (Fig. 6), but in the absence of the M-domain, wasn’t able to insert. The M-domain on its own bound to chloroplasts with very low efficiency but could not insert into the outer membrane, consistent with the previous findings. The A-domain apparently plays no role in binding or insertion of the protein. Together, these data suggest that the G-domain participates directly in the targeting of psToc159 to chloroplasts, and that the presence of the G-domain is required for the M-domain to productively insert into the outer membrane.

We examined the possibility that a direct interaction between the G-domains of atToc159 and atToc33 may be required for targeting and insertion. Using a solid phase pull-down assay, we demonstrated that atToc33G bound directly and specifically to purified atToc159G (Fig. 8). These results confirm that atToc33 acts as the cognate receptor for atToc159 at the chloroplast surface. In addition, binding was stimulated three-fold in the presence of GDP as compared to GTP (Fig. 9). We propose that the GDP-stabilized association of atToc159G and atToc33G corresponds to the nucleotide-bound state of the fully inserted form of atToc159. This hypothesis is consistent with a recent study showing that the soluble G-domain of atToc33 can compete for targeting and insertion of atToc159 to Arabidopsis chloroplasts in vitro (Hilbrunner et al., 2001). On the basis of these results, we propose a model depicting a multistep mechanism of atToc159 targeting to chloroplasts. The initial docking of atToc159 at the chloroplast surface is mediated by a cognate interaction between the G-domains of atToc159 and atToc33. The docking step does not require bound nucleotide, but does not result in efficient, stable association of atToc159 with the membrane. Our results and those of a previous study suggest that the M-domain also interacts with the outer membrane independent of the G-domain (Muckel and Soll, 1996). This interaction in conjunction with G-domain binding is likely to contribute to the initial binding of the full-length receptor. Subsequent to its initial docking, atToc159 inserts into the outer membrane and stably associates with the other Toc components. Insertion of the M-domain is likely to involve other envelope components (e.g., atToc75). This reaction requires nucleotide binding directly at the receptor. GTP or GDP function at this stage, but it is the GDP-bound state that is competent for insertion. Therefore, bound GTP must be hydrolyzed to GDP to allow insertion. In this scenario, the G-domain would function as a GTP-dependent switch that controls integration of atToc159 into the outer membrane. GDP also promotes tight binding of the G-domains of atToc159 and atToc33, thereby stabilizing the association of atToc159 with the Toc complex. The net result of the GTP-dependent insertion reaction is the unidirectional targeting of the receptor to the Toc complex, resulting in the assembly of the functional translocon.

The recently determined three-dimensional structure of the psToc34 G-domain demonstrates the capacity of the protein to form dimers (Sun et al., 2002). Interestingly, the subunits of each dimer appear to act as reciprocal GTP-ase activating proteins. The key residues involved in both GTP binding/hydrolysis and dimer formation are conserved in all atToc159 variants (Kessler and Schnell, 2002). This observation, in conjunction with the atToc159G-atToc33G binding data presented in this report, suggests that Toc159 and Toc34/33 might interact in a similar manner during Toc159 targeting and during their stable association in the Toc complex. It is interesting to speculate that the interaction between Toc34 and Toc159 may stimulate GTP binding or hydrolysis at one or both GTPases promoting the insertion of the receptor into the membrane. Furthermore, nucleotide binding and hydrolysis at Toc34/33 also may be involved in the targeting reaction. Although the dynamics of the Toc159-Toc34/33 association are not completely understood, it is clear that the G-domains of these proteins play a direct role in regulating their interactions.

The presence of a significant pool of soluble, cytoplasmic Toc159 raises the possibility that the GTP-dependent targeting reaction participates in the delivery of preproteins to the chloroplast surface. The membrane-bound form of Toc159 is known to interact with preproteins. Although it remains to be demonstrated directly that the soluble form of the receptor binds preproteins, it is intriguing to hypothesize that the GTP-dependent insertion and assembly of Toc159 with the Toc complex is coupled to the productive association of preproteins with the translocon and the initiation of membrane translocation.
Materials and methods

ToC159 mutations and deletion constructs

Point mutations were introduced into the G1 GTP binding motif of the atToC159 G-domain using the PCR-based overlap extension technique (Ling et al., 2001). To generate the atToC159-K868R mutant, PCR primers were used that changed codon CCC to CCG resulting in an A to G mutation at amino acid 864. For the atToC159-K868R mutant, PCR primers were used that changed codon AAA to AGA resulting in a Lys to Arg mutation at amino acid 864. Both mutant forms of atToC159 were inserted into the NcoI and Xhol sites of pET21d to generate pET21d-atToC159-A864R and pET21d-atToC159-K868R, respectively as templates. The atToC159M fragment was cloned into the NdeI/HindIII sites of pET21a (Novagen, Inc.) to generate pET21a-atToC159M. Construction of pET21d-atToC159A, encoding a fragment corresponding to 159 amino acids 1-740 was previously described (Bauer et al., 2000). All constructs resulted in in-frame fusions with a 6-histidine tag at their COOH-termini.

DNA fragments encoding atToC159G-A864R and atToC159G-K868R were generated by PCR using pET21d-atToC159G-A864R and pET21d-atToC159G-K868R, respectively as templates. The fragments were cloned into the NcoI/Xhol sites of pET21d to generate pET21d-atToC159G-A864R and pET21d-atToC159G-K868R, respectively. The atToC159M fragment was cloned into the NdeI/HindIII sites of pET21a (Novagen, Inc.) to generate pET21a-atToC159M. Construction of pET21d-atToC159A, encoding a fragment corresponding to 159 amino acids 1-740 was previously described (Bauer et al., 2000). All constructs resulted in in-frame fusions with a 6-histidine tag at their COOH-termini.

In vitro translation and expression in *E. coli*

All [35S]methionine-labeled in vitro translation products were generated in a coupled transcription-translation system containing reticulocyte lysate according to the manufacturer’s recommendations (Promega). Where indicated, the mixture was depleted of free nucleotides by gel filtration as previously described (Chen and Schnell, 1997). Where indicated, chloroplasts were depleted of exogenous energy by incubating chloroplasts containing 250 μM GDP, 250 μM GTP, GMP-PNP, GDP, or GDPβS was added to a final concentration of 2 mM and the targeting reactions were started with the addition of [35S]methionine-labeled in vitro translation products. Where indicated, the in vitro translation products were depleted of nucleotide triphosphates as described above. The reactions were incubated for 10 min at 21°C in the dark. After the targeting reaction, the chloroplasts were incubated in the presence or absence of 100 μg/ml thermolysin on ice for 30 min. The reactions were stopped by dilution with ice-cold HS buffer containing 5 mM EDTA, and the chloroplasts were reisolated by centrifugation at 2,500 g for 5 min through a 40% Percoll cushion. Chloroplasts were hypotonically lysed by resuspending the chloroplast membrane fraction was recovered by centrifugation at 50,000 g for 20 min. The membrane fractions were resolved by SDS-PAGE and radioactive signals in dried gels were detected and quantitated using a Storm 840 phosphorimager and ImageQuant v. 1.2 software. Counts from proteolytic fragments (atToC159GM and atToC159M) of atToC159 were normalized to the number of methionine residues lost due to proteolysis. Quantitative chloroplast targeting data are presented as the percent of maximal binding, the percent of maximal insertion or as the percent of added in vitro translation product.

Guanine nucleotide binding and hydrolysis assays

GTP binding to atToC159G, atToC159G-A864R, and atToC159G-K868R was measured using a solid phase GTP overlay assay. Purified atToC159G, atToC159G-A864R, and atToC159G-K868R were diluted to 25 μg/ml in 50 mM Hepes-KOH, pH 7.5, 40 mM KOAc (HK buffer) containing 2 mM EDTA and 200 μM of each sample was spotted onto nitrocellulose membrane. An equivalent sample corresponding to the eluate from Ni-NTA chromatography of an E. coli strain expressing preSSU (Ma et al., 1996) was used as a background reference. The nitrocellulose filter was incubated for 30 min at room temperature in 20 mM Tris-HCl, pH 7.5, 50 μM MgCl2, 0.3% Tween-20 (GTP binding buffer). The blot was transferred to GTP binding buffer containing 50 mM [α-32P]GTP (3,000 Ci/mmol; PE Life Sciences) and 1 μM ATP, and incubated for 1 h at 4°C. The blot was washed five times with 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.3% Tween-20, and binding of [α-32P]GTP was measured using a Storm 840 phosphorimager and ImageQuant v. 1.2 software. Data are presented as fmol of [α-32P]GTP bound per μg of protein.

GTP hydrolysis was measured using a method adapted from (Liang et al., 2000). Briefly, purified atToC159G, atToC159G-A864R, and atToC159G-K868R were diluted to 0.5 μM with HK buffer containing 2 mM EDTA and 1 μM [α-32P]GTP (150 μCi/mmol) in a final volume of 25 μl, and incubated for 5 min at 25°C. The hydrolysis reaction was initiated by the addition of MgCl2 to a final concentration of 5 mM. GTP hydrolysis was linear over a 60 min incubation period (unpublished data). Therefore, samples were removed after 0 min and 20 min of hydrolysis and the reactions immediately stopped by heating at 65°C for 5 min in 0.2% SDS, 10 mM EDTA, 4 mM GTP, 4 mM GDP. The samples were spotted onto PE-cellulose TLC plates (EM Science) and GTP and GDP were identified using a 1 M LiCl solvent. The plates were dried and radioabeled spots of GTP and GDP were quantified using a Storm 840 phosphorimagner. Data are presented as fmol of [α-32P]GTP hydrolyzed min⁻¹ pmol protein⁻¹. Hydrolysis of GTP by soluble E. coli proteins that nonspecifically bound to Ni-NTA resin was used as a background reference. GDP binding by purified atToC159G and atToC159G-A864R was measured using a filter-binding assay. The wild-type and mutant proteins (1 μM) were diluted with HK buffer containing 5 mM EDTA and incubated 5 min at 25°C. Binding was initiated with the addition of 2 μM [3H]GDP (32 Ci/mmol) and 10 mM MgCl2 in the presence of 2 μM ATP. The 50 μM reactions were incubated for 1 h at 25°C, rapidly diluted with 500 μl of ice-cold HK buffer and filtered through a nitrocellulose membrane using a vacuum manifold. After washing twice with 500 μl of ice-cold HK buffer, the radioactivity retained on the membrane was quantitated by liquid scintillation counting. Data are presented as fmol [3H]GDP bound per μg protein. GDP binding to cellular retinoic acid binding protein (CRABP; Clark et al., 1998) was used as a background reference.

Solid phase binding assays

Direct interaction between atToC159G and atToC33G was measured using a solid phase binding assay. Varying concentrations of purified hexahistidine-tagged atToC159G or CRABP were diluted in Hepes-KOH, pH 7.5, 2 mM MgCl2, 40 mM KOAc, 0.1% Triton X-100 (binding buffer) to give a final concentration of 40 mM imidazole. The samples were bound to 10 μl of packed Ni-NTA resin at 4°C for 30 min under constant mixing. The resin was washed twice with 400 μl of binding buffer containing 40 mM imidazole, 25 mM GTP, GMP-PNP, GDP, GDPβS, or GMP, as indicated. The resin was then incubated with 5–10 μl of [35S]atToC33G in vitro translation product in binding buffer containing 40 mM imidazole and the appropriate nucleotide in a final volume of 100 μl, for 1 h at 22°C, under constant mixing. The resin was washed three times with 400 μl of ice-cold
binding buffer containing 40 mMimidazole and the appropriate nucleotide. Proteins were eluted from the resin with SDS-PAGE sample buffer containing 0.9 M imidazole and resolved by SDS-PAGE. Gels were stained with coomassie blue to detect atToc159G (unpublished data), and [35S]atToc33G was detected in dried gels using a Storm 840 phosphorimager. Bound [35S]atToc33G is presented as the percent of maximal binding in each experiment.

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