The Roles of Toc34 and Toc75 in Targeting the Toc159 Preprotein Receptor to Chloroplasts*

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The Toc complex at the outer envelope of chloroplasts initiates the import of nuclear-encoded preproteins from the cytosol into the organelle. The core of the Toc complex is composed of two receptor GTPases, Toc159 and Toc34, as well as Toc75, a β-barrel membrane channel. Toc159 is equally distributed between a soluble cytoplasmic form and a membrane-inserted form, suggesting that assembly of the Toc complex is dynamic. In the present study, we used the Arabidopsis thaliana orthologs of Toc159 and Toc34, atToc159 and atToc33, respectively, to investigate the requirements for assembly of the trimeric Toc complex. In addition to its intrinsic GTPase activity, we demonstrate that integration of atToc159 into the Toc complex requires atToc33 GTPase activity. Additionally, we show that the interaction of the two GTPase domains stimulates association of the membrane anchor of atToc159 with the translocon. Finally, we employ reconstituted proteoliposomes to demonstrate that proper insertion of the receptor requires both Toc75 and Toc34. Collectively these data suggest that Toc34 and Toc75 act sequentially to mediate docking and insertion of Toc159 resulting in assembly of the functional translocon.

EXPERIMENTAL PROCEDURES

Toc33/34 Mutations and AtToc159 Deletion Constructs—Point mutations were introduced into the conserved G-domain of the Toc159 targeting domain (amino acids 1–265) of atToc33 (atToc33G) (17) using the overlap extension technique (19) and pET21d-atToc33G

Construction of pET21d-atToc159, pET21d-atToc159-A864R, pET21d-atToc159-K868R, and pET21a-atToc159MHis has been described previously (15). Constructs encoding atToc159G (pET21d-atToc159G) and atToc159M (pET21a-atToc159M) lacking hexahistidine tags were generated by modifying pET21d-atToc159G

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For the standard import reactions chloroplasts were incubated in the presence or absence of 100 μg/ml thermolysin as described (15, 22). Reisolated chloroplasts were hypotonically lysed in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and the total chloroplast membrane fraction was recovered by centrifugation at 100,000 × g for 20 min. The membrane fractions were resolved by SDS-PAGE, and [35S]GTP binding was measured using a filter binding assay as previously described (15).

Reconstitution of Proteoliposomes—Liposomes were generated using azolectin (type IV-S, Sigma) and a method adapted from Hinnah et al. (10). Azolectin was resuspended in 10 mM MOPS-KOH, pH 6.9, to give a final concentration of 50 mg/ml and pulse sonicated for 30 s to facilitate even dispersion. The liposomes were from egg yolk phosphatidylcholine, 3:1 (molar). Purified, urea-soluble psToc75, psToc34, or psToc34-G45R/K52N/S53R was diluted with an equal volume of 24 mM CHAPS, and incubated for 1 h on ice. The liposomes were thawed and added to the proteins at a ratio of 200 μl of liposomes/90 μg of protein. The mixture was pulse sonicated for 10 s, incubated for 1 h on ice, and dialyzed overnight into 50 mM Hepes-KOH, pH 7.5. Proteoliposomes were recovered by centrifugation at 90,000 × g for 40 min, and resuspended in HS buffer, containing 1 mM dithiothreitol.

Carbonate extraction of proteoliposomes was achieved by resuspending pelleted proteoliposomes containing psToc34 and psToc75 in 40 μl of 0.1 N Na2CO3, pH 11.5, and incubation for 20 min on ice. Proteoliposomes were recovered by centrifugation for 30 min at 18,000 × g, and the pellet and supernatant fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

Immunoprecipitation of Proteoliposome Proteins—Proteoliposomes were dissolved in 1% (v/v) Triton X-100 and applied to a column containing anti-psToc34 IgG coupled to Sepharose as previously described (9). Bound proteins were eluted, resolved by SDS-PAGE, and immunoblotted with anti-psToc34 and anti-psToc75 (9).

AtToc159 Proteoliposome Targeting Assays—Proteoliposome targeting assays were performed using 1–5 μl of freshly prepared proteoliposomes (corresponding to approximately 5 μg of protein) in import buffer containing 0.1 mg/ml bovine serum albumin, 2 mM ATP, and 2 mM GTP. Targeting measurements were initiated with the addition of [35S]methionine-labeled in vitro translation products and were incubated for 20 min at 26 °C in the dark. The reactions were stopped with the addition of ice-cold HS buffer and recovered by centrifugation for 30 min at 14,000 × g. The proteoliposomes were resuspended in 50 μl of HS buffer and incubated with or without thermolysin (10 μg/ml) for 30 min on ice. Protease treatments were terminated by adding EDTA to a final concentration of 10 mM in 1 ml of ice-cold HS buffer, and the liposomes were collected again by centrifugation. Finally, the proteoliposomes were resolved using SDS-PAGE and radioactive signals in dried gels were detected and quantitated using a Storm 840 PhosphorImager and ImageQuant version 5.2 software. Counts from proteolytic fragments of AtToc159 (i.e. atToc159M in thermolysin-treated proteoliposomes were normalized to reflect the number of methionine residues lost because of proteolysis.

RESULTS

The GTPase Activity of AtToc33 Is Required for AtToc159 Binding—Our previous studies demonstrated that chloroplast binding and membrane insertion of atToc159 were promoted by its intrinsic GTPase activity and involved a direct interaction with atToc33 (15, 18). To investigate whether GTP binding and the GTPase activity of atToc33 are also involved in atToc159 targeting, we studied the binding of atToc159 to a mutant of atToc33 with altered nucleotide binding and hydrolysis activities. The mutant, atToc33-G45R/K49N/S50R, contains three point mutations in the consensus G, motif (P-loop) of the conserved GTP-binding site (Fig. 1A). A comparable mutant of pea psToc34, psToc34-G45R/K52N/S53R, previously was shown to inhibit GTP binding and hydrolysis (20). To confirm the effects of the mutations, 29-kDa amino-terminal fragments of wild type atToc33 (atToc33G45R/K49N/S50R) and atToc33-G45R/K49N/S50R (atToc33G45R/K49N/S50R) containing hexahistidine tags and lacking the carboxyl-terminal transmembrane segment were expressed in E. coli, purified by Ni-NTA chromatography (Fig. 1B), and assayed for nucleotide binding and hydrolysis. As predicted, the GTP hydrolytic activity (Fig. 1C) and GTP bind-

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Fig. 1. Mutations incorporated into the GTPase domain of atToc33 and the effects on GTP binding and hydrolysis. A, the G1 GTP binding motif of wild type atToc33 and the corresponding motif of the atToc33-G45R/K49N/S50R mutant (G45R/K49N/S50R). Arrows indicate the sites of the mutations. B, Coomassie-stained SDS-PAGE profile of wild-type atToc33GHis and atToc33GHis-G45R/K49N/S50R purified from E. coli using Ni-NTA chromatography. Each lane contains 5 μg of protein. The positions of molecular size markers (kDa) are indicated to the right of the figure. C, GTP hydrolysis activities of wild type and mutant forms of atToc33GHis (α-32PGTP (2 μM) was incubated with 1.6 μM atToc33GHis or atToc33GHis-G45R/K49N/S50R (G45R/K49N/S50R) in a 20-μl reaction for 20 min at 25 °C. Radioiodinated GTP and GDP were resolved by thin-layer chromatography and radioactivity was quantitated using a PhosphorImager. D, GTP binding activities of wild type and mutant forms of atToc33GHis. Purified atToc33GHis or atToc33GHis-G45R/K49N/S50R (G45R/K49N/S50R) were incubated with 1 μM [α-35S]GTP at 25 °C for 30 min. The mixture was filtered through nitrocellulose, washed, and bound [α-35S]GTP was quantitated by scintillation counting.

The effects of atToc33 mutations on atToc159 binding, we incubated in vitro translated full-length atToc159 or a deletion mutant corresponding to the atToc159 GTPase domain, atToc159G, with immobilized atToc33GHis or atToc33GHis-G45R/K49N/S50R in a solid phase binding assay. The assays were performed either in the presence of GTP or the nonhydrolyzable GTP analog, GMP-P(NH)P. As previously shown, atToc33GHis binds both full-length [35S]atToc159 (17) and [35S]atToc159M (15) in the presence of GTP (Fig. 2, A, lane 2, and B, GTP). Binding is inhibited by GMP-P(NH)P (Fig. 2, A, compare lanes 2 and 5, and B) consistent with the proposal that GTP hydrolysis is required for high affinity binding (15). The binding of atToc159 or atToc159G to atToc33GHis-G45R/K49N/S50R was decreased by 50 and 65%, respectively, relative to binding to wild type atToc33G (Fig. 2, A, compare lanes 2 and 3, and B, GTP). Binding to the mutant in the presence of GMP-P(NH)P was similar to the levels of binding in the presence of GTP (Fig. 2A, compare lanes 3 and 6, and B) and to the levels of binding to wild type proteins observed in the presence of GMP-P(NH)P (Fig. 2, A, compare lanes 5 and 6, and B, GMP-PNP). These results provide additional evidence that the binding of the Toc GTPases is nucleotide regulated and confirm a role for the GTPase activity of atToc33 in the binding of atToc159 to the Toc complex.

The GTPase Domains of AtToc159 and AtToc33 Mediate Targeting of the Membrane Anchor of AtToc159—The results presented in Fig. 2 and those of previous studies (15, 18) provide convincing evidence that atToc159 targeting to the outer envelope membrane is mediated by a GTP-regulated cognate interaction between the atToc159 and atToc33 G-domains. We previously proposed that the binding of the G-domains and subsequent GTP hydrolysis results in the integration of the atToc159 M-domain into the outer envelope and assembly of functional Toc complexes (15). This proposal predicts that the activities of the G-domains are required for specific targeting and integration of the atToc159 M-domain at the chloroplast surface. The complementary roles of the M-domain and G-domains in receptor binding are supported by the observation that full-length atToc159 binds with greater efficiency to both isolated chloroplasts (15) and atToc33GHis (Fig. 2B) than either atToc159G or atToc159M alone. To test this hypothesis, we examined the role of the G-domains in M-domain targeting.

As a first step, we studied the influence of atToc159G on the binding of in vitro translated M-domain ([35S]atToc159M) to isolated chloroplasts in trans. Isolated chloroplasts were incubated with [35S]atToc159M in the presence or absence of E. coli expressed atToc159GHis, atToc159GHis-K868R, a mutant with reduced GTP binding and hydrolytic activity (15), or atToc33GHis. The amount of bound [35S]atToc159M was determined after reisolation of the chloroplasts through a Percoll cushion. Integration of [35S]atToc159M into the outer membrane was assessed by treating equivalent samples of chloroplasts in the presence or absence of thermolysin. The ~52-kDa M-domain is protected from proteolysis upon proper integration of atToc159 (14, 15, 24). In the absence of any additions, atToc159M binds with low efficiency to isolated chloroplasts and remains protease sensitive (Fig. 3, A, lanes 2 and 3, and B).

Previous studies have shown that this low level of binding represents a nonspecific interaction with the chloroplast surface (15). Addition of atToc159GHis increases [35S]atToc159M binding to ~7% of added translation product (Fig. 3, A, lane 4, and B), an increase of more than 2-fold, suggesting that the G-domain stimulates M-domain binding to chloroplasts. The atToc159GHis-K868R mutant is less effective at stimulating [35S]atToc159M binding (Fig. 3, A, lane 6, and B), consistent
Fig. 2. Nucleotide requirements for the interaction between atToc159 and atToc33G. Nucleotide-depleted in vitro translated [35S]atToc159 or [35S]atToc159G was incubated with nucleotide-depleted atToc33GHis or atToc33GHis–G45R/K49N/S50RHis (G45R/K49N/S50RHis) that had been immobilized on Ni-NTA resin, in the absence (−) or presence (+) of 0.1 mM GTP or GMP-PNP (GMPPNP). Bound proteins were eluted and resolved by SDS-PAGE, and analyzed using a PhosphorImager. A, PhosphorImager analysis of bound [35S]atToc159 (upper panels) or [35S]atToc159G (lower panels). Lanes 1 and 4 contain 10% of the [35S]atToc159 or [35S]atToc159G in vitro translation product (IVT) added to each reaction. B, quantification of the data from replicate experiments including those presented in A, with standard error bars.

Fig. 3. Binding of atToc159M to chloroplasts is stimulated by atToc33G. A, in vitro translated [35S]atToc159M was incubated with isolated, intact Arabidopsis chloroplasts in the presence of ATP and GTP for 15 min at 26 °C in the absence (−) or presence (+) of 5 μM atToc159GHis, atToc159GHis–K868R (159GHis–K868R), or atToc33GHis. After the incubation, reactions were divided equally and one-half was incubated in the absence (−) and the other half in the presence (+) of thermolysin (100 μg/ml) for 30 min on ice. The chloroplasts were isolated, lysed, and the total membrane fractions were analyzed by SDS-PAGE and phosphorimaging. Lane 1 contains 10% of the [35S]atToc159M in vitro translation product (IVT) added to each reaction. B, quantitative analysis of the data from replicate experiments including those in A with standard error bars.

with the reduced binding of this mutant to chloroplasts (15). Furthermore, the atToc33 G-domain (atToc33GHis) does not stimulate [35S]atToc159M binding (Fig. 3, A, lane 8, and B), indicating that the effect of atToc159GHis on M-domain binding is specific. Detectable integration of [35S]atToc159M into a protease-protected form is not observed with any addition (Fig. 3, A, lanes 5, 7, and 9, and B). Therefore, the association of the G-domain of atToc159 with the chloroplast surface may be a prerequisite for productive association of the M-domain, which may represent a step in a series of events that occur during receptor assembly into the Toc complex.

Given that the G-domain of atToc159 binds to atToc33 at the chloroplast, the ability of atToc159GHis to stimulate atToc159M binding to chloroplasts suggests that the M-domain might also bind to atToc33GHis. Therefore, we tested the effect of atToc159GHis on [35S]atToc159M binding to immobilized atToc33GHis in the solid phase binding assay. Indeed, Fig. 4, A and B, demonstrates that atToc159GHis stimulates the binding of [35S]atToc159M to atToc33GHis in a dose-dependent manner. Maximal binding corresponds to ~25% of added in vitro translated [35S]atToc159M. In contrast, the addition of excess unlabeled atToc159MHis inhibits [35S]atToc159M binding to atToc33GHis, demonstrating that the interaction between atToc159M and atToc33G is specific (Fig. 4, C, lanes 3 and 4, and D). On the basis of these data, we conclude that atToc159 binding at the chloroplast surface involves both the atToc159 G- and M-domains. The atToc159G-atToc33G interaction is a prerequisite for atToc159 M-domain binding, suggesting that the GTPases regulate the docking reaction.

Reconstitution of AtToc159 Targeting to Proteoliposomes—Although the atToc159-atToc33 interaction is required for the assembly of the Toc complex, additional membrane components are likely to play a role in the integration of the M-domain into the outer membrane. We proposed that Toc75 might play a role in Toc159 targeting by mediating insertion of the M-domain into the outer membrane (15). This hypothesis is supported indirectly by the observations that the membrane-integrated regions of psToc159 and psToc75 cooperate in prepolyprotein translocation across the outer membrane (9). To examine the role of Toc75 in Toc159 integration and define the minimal requirements for receptor targeting to the Toc complex, we attempted to reconstitute Toc159 targeting using chemically defined proteoliposomes. For this purpose, we took advantage of a modified protocol for the reconstitution of functional psToc34 and psToc75 into lipid vesicles. Previous studies using similar procedures have demonstrated that the refolding of psToc34 and psToc75 into proteoliposomes results in the reconstitution of native activities (10, 25). Both psToc75 and psToc34 proteins were expressed in E. coli and purified to near homogeneity under denaturing conditions in the presence of urea (data not shown). The proteins were refolded in the presence of CHAPS
**FIG. 4.** AtToc159M binds specifically to atToc33G, and binding is stimulated by atToc159G. A, in vitro translated [35S]atToc159M was incubated with Ni-NTA resin that had been saturated with atToc33G<sub>His</sub>, in the absence or presence of increasing concentrations of atToc159G<sub>His</sub>. Bound proteins were eluted and resolved by SDS-PAGE, and analyzed using a PhosphorImager. Lane 1 contains 10% of the [35S]atToc159M in vitro translation product (IVT) added to each reaction. B, quantitation of the data from replicate experiments including those in A, presented as % maximal binding with standard error bars. C, in vitro translated [35S]atToc159M was incubated with Ni-NTA resin that had been saturated with atToc33G<sub>His</sub> in the absence or presence of (+) 20 μM purified atToc159M<sub>His</sub>. Bound proteins were eluted, separated by SDS-PAGE, and analyzed using a PhosphorImager. Lane 1 contains 10% of the [35S]atToc159M added to each reaction. Lane 2 contains [35S]atToc159M that bound to the Ni-NTA matrix in the absence of atToc33G<sub>His</sub>. D, quantitation of [35S]atToc159M binding to atToc33G<sub>His</sub> from replicate experiments including those presented in C, expressed as % maximal binding with standard error bars.

**FIG. 5.** Reconstitution of psToc75 and psToc34 into proteoliposomes. A, titration of psToc75 and psToc34 reconstitution using varying concentrations of lipid. 100 μg of each protein was incorporated into liposomes as described under “Experimental Procedures.” The proteoliposomes were collected by centrifugation and the associated protein was quantitated. B, Coomassie-stained SDS-PAGE profile of proteoliposomes reconstituted with psToc34 or psToc75 at the optimal concentration of protein and lipid. Molecular size markers are indicated. C, proteoliposomes containing psToc75 or psToc34 were alkali extracted with 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.5, for 30 min on ice. The proteoliposome pellet (P) and supernatant (S) fractions were separated by differential centrifugation, resolved by SDS-PAGE, and stained with Coomassie Blue. The positions of psToc75 and psToc34 are indicated. D, proteoliposomes containing psToc75 and psToc34 (PL) were dissolved in buffer containing 1% Triton X-100 and loaded onto an anti-psToc34 IgG column. The unbound fraction (FT) was collected, and bound proteins were eluted with 0.2 M glycine, pH 2.2 (EF). The SDS-PAGE-resolved fractions were immunoblotted simultaneously with anti-psToc34 and anti-psToc75. Molecular size markers are indicated. E, GTP hydrolysis by proteoliposomes reconstituted with psToc34 or psToc34-G48R/K52N/S53R (G48R/K52N/S53R). [γ-<sup>32</sup>P]GTP (2 μM) was incubated with proteoliposomes at a final protein concentration of 2 μM in a 20-μl reaction for 20 min at 25 °C. Radiolabeled GTP and GDP were resolved by thin-layer chromatography and radioactivity was quantitated using a PhosphorImager.
Reconstitution of AtToc159 Targeting

Fig. 6. AtToc159 targeting to proteoliposomes containing reconstituted psToc34 and psToc75. A, [35S]AtToc159 was incubated with proteoliposomes prepared without (−) or with (+) psToc34 or psToc75 in the presence of ATP and GTP for 15 min at 25 °C. The proteoliposomes were recovered by centrifugation and incubated in the absence (−) or presence (+) of thermolysin (10 μg/ml) for 30 min on ice. The proteoliposomes were collected, resolved by SDS-PAGE, and analyzed using a PhosphorImager. Lanes 1 and 4 contain 10% of the [35S]AtToc159 in vitro translation product (IVT) added to the reactions. Molecular size markers (kDa) are indicated to the right of the figure, and the positions of atToc159 and the 50–55-kDa protease-protected fragments of atToc159 (159M) are indicated to the left. B, quantitative analysis of the data from replicate experiments, including those presented in A with standard error bars. [35S]AtToc159 binding (Binding) was measured as the amount of atToc159 that was associated with but not inserted into the proteoliposomes. The amount of inserted [35S]AtToc159 (Insertion) was determined directly from the amount of 50–55-kDa fragments (159M) present after the thermolysin treatment. Quantitations of binding and insertion were normalized based on the methionine content of full-length [35S]AtToc159 and the 50–55-kDa fragments. Lip, protein-free liposomes; Toc75, proteoliposomes containing psToc75; Toc34, proteoliposomes containing psToc34; Toc75/Toc34, proteoliposomes containing psToc75 and psToc34.

and incorporated into membrane vesicles by dialysis in the presence of plant lipids using a modification of the published procedure (10). The formation of unilamellar vesicles of ~200 nm average diameter was confirmed by electron microscopy (data not shown). The lipid concentration was optimized to give a maximum protein to lipid ratio of 90 μg of protein/10 mg of lipid (Fig. 5A). Under these conditions both psToc75 and psToc34 quantitatively associated with lipid vesicles when reconstituted alone or together (Fig. 5B). Furthermore, both proteins were resistant to alkaline extraction following reconstitution (Fig. 5C), indicating that their association with proteoliposomes represents true integration.

To confirm that the psToc34 and psToc75 complex was reconstituted upon refolding and membrane integration, we dissolved the proteoliposomes in buffer containing Triton X-100 and tested the ability of anti-psToc34 IgG to coprecipitate psToc75. As shown in Fig. 5D, the two Toc components coimmunoprecipitate in an approximate 1:1 molar ratio. These data confirm the assembly of the reconstituted components at a stoichiometry indistinguishable from that observed in Toc complexes isolated from chloroplasts (16, 17). As a final control, we tested whether the GTPase activity of psToc34 was recovered upon refolding and membrane integration. Fig. 5E shows that reconstituted psToc34 GTPase activity is greater than 2-fold higher than proteoliposomes reconstituted with a mutant form of psToc34, psToc34-G48R/K52N/S53R (20). The relative GTPase activities of the reconstituted proteins are nearly identical to those previously measured for the pea proteins in solution (20). These results provide additional evidence that the reconstitution protocol resulted in the proper folding and assembly of the psToc75 and psToc34 complex into the proteoliposomes.

Our next goal was to test whether the reconstituted proteoliposomes supported binding and integration of atToc159. For this purpose, in vitro translated [35S]AtToc159 was incubated with proteoliposomes under conditions used for a standard chloroplast targeting assay and the levels of targeting were measured after recovery of the proteoliposomes by differential centrifugation. Binding and insertion of atToc159 by proteoliposomes was calculated as it was for targeting of the receptor to chloroplasts (15). The total population of [35S]AtToc159 that co-purified with proteoliposomes was compared with the amount of 50–55-kDa protease-protected [35S]AtToc159 fragments (i.e., M-domain) that were generated by treatment of proteoliposomes with thermolysin. These fragments represent properly inserted atToc159, whereas the portion of the total population of proteoliposome-associated [35S]AtToc159 that is sensitive to thermolysin represents the peripherally bound protein. As shown in Fig. 6A, proteoliposomes containing either psToc75 or psToc34 alone bound low levels of [35S]AtToc159, whereas the binding of the receptor to proteoliposomes containing both proteins was ~2-fold higher (compare Fig. 6, A, lanes 5, 7, and 9; and B, Binding). However, only proteoliposomes containing the psToc34-psToc75 complex supported significant levels of [35S]AtToc159 integration as measured by the appearance of the protease-protected 50–55-kDa M-domain fragments.
of the receptor after thermolysin treatment of the targeting reaction (Fig. 6, A, compare lanes 6, 8, and 10; and B, Insertion). Protein-free liposomes did not exhibit significant levels of binding or integration of [35S]atToc159 (Fig. 6, A, lanes 2 and 3; and B). On the basis of these results, we conclude that both Toc34 and Toc75 are required for proper targeting and integration of Toc159. Furthermore, the results suggest that although Toc34 can support receptor binding, Toc75 is required for the productive integration of the M-domain into the membrane.

To confirm that the reconstituted targeting reaction reflects authentic atToc159 binding and assembly, we tested the targeting efficiency of two atToc159 GTPase mutants that were previously shown to exhibit reduced binding and integration in intact chloroplasts (15). The atToc159-A864R mutant has a similar GTP binding capacity compared with wild type atToc159, but possesses <10% of the GTP hydrolytic activity. The GTP binding capacity of the atToc159-K868R mutant is <20% of wild type and has no detectable hydrolytic activity. The binding of both mutants to psToc34-psToc75 proteoliposomes is reduced to ~50% of the levels of atToc159 (Fig. 7, A, compare lanes 2, 5, and 8, and B, Binding). Insertion of atToc159-K868R is less than 10% of wild type levels (Fig. 7, A, compare lanes 3 and 9, and B, Insertion). The insertion of atToc159-A864R is 60% of wild type (Fig. 7, A, compare lanes 3 and 6, and B, Insertion), which is consistent with its reduced hydrolytic activity and consequent slower conversion to the insertion-competent, GDP-bound state. Maximal binding and insertion of wild type atToc159 represent ~50% and ~10% of in vitro translated [35S]atToc159 added to the reaction, respectively. The effects of the mutations on atToc159 targeting closely parallel those observed in assays using isolated chloroplasts (15) providing additional evidence that the reconstituted system reproduces the in organello situation.

On the basis of binding studies with atToc159 and atToc33G_fr, we proposed that the cognate interaction between these two GTPases represents the initial step in the atToc159 chloroplast targeting reaction (15, 18). The data presented in Fig. 2 predict that the intrinsic GTPase activity of atToc33/psToc34 in addition to that of atToc159 plays a role in receptor targeting. To test this prediction directly, we compared the targeting efficiency of atToc159 to proteoliposomes containing psToc34 or psToc34-G48R/K52N/S53R. As shown in Fig. 8A, ~25% of added [35S]atToc159 binds to psToc34 proteoliposomes. In contrast, binding to psToc34-G48R/K52N/S53R proteoliposomes was reduced to 45% of the levels for psToc34 proteoliposomes (Fig. 8, A and B). This observation is consistent with the requirement for psToc34/atToc33 GTPase activity for binding of the receptor. Binding was also reduced 25% when the psToc34-G48R/K52N/S53R mutant was substituted for psToc34 in mixed proteoliposomes containing psToc75 (Fig. 8, C, compare lanes 2 and 4, and D, Bound). Furthermore, the insertion of the receptor was reduced by 70% in mixed proteoliposomes containing psToc34-G48R/K52N/S53R compared with wild type (Fig. 8, C, compare lanes 3 and 5, and D, Inserted). These data confirm that the intrinsic GTPase activity of psToc34/atToc33 is required for binding and insertion of Toc159 into the membrane. Therefore, the coordinate activities of the cognate G-domains of both Toc159 and Toc34 regulate Toc complex assembly.

**DISCUSSION**

Toc159 assembles with Toc34 and Toc75 to form the core of the preprotein translocon of the outer envelope membrane of chloroplasts. The equal distribution of Toc159 between soluble, cytoplasmic, and membrane-integrated forms indicates that the assembly of the Toc complex is dynamic, and has led to the proposal that Toc159 functions as a preprotein receptor that cycles between the cytoplasm and Toc complex (17). We have defined the minimal requirements for the assembly of atToc159 into Toc complexes by reconstituting targeting of the soluble receptor using purified atToc33/psToc34 and psToc75. Our studies demonstrate that both Toc components are necessary and sufficient to support binding and membrane integration of the receptor. The GTPase activity of atToc33/psToc34 is required for docking of the receptor at Toc complexes and appears to regulate the insertion of atToc159 into the Toc complex via Toc75.
Fig. 8. Targeting of atToc159 to proteoliposomes requires the GTPase activity of Toc34. A, [35S]atToc159 was incubated with proteoliposomes made with psToc34 or psToc34-G48R/K52N/S53R (G48R/K52N/S53R) as described in the legend to Fig. 6. The liposomes were collected by centrifugation and analyzed by SDS-PAGE and phosphorimaging. Lane 1 contains 10% of the [35S]atToc159 in vitro translation product (IVT) added to each reaction. B, quantitative analysis of the data presented in A with standard error bars. Bound [35S]atToc159 is expressed as % maximal binding. C, [35S]atToc159 was incubated with proteoliposomes containing psToc75 and psToc34 or psToc34-G48R/K52N/S53R (G48R/K52N/S53R) as described in the legend to Fig. 6. Lane 1 contains 10% of the [35S]atToc159 in vitro translation product added to each reaction. The positions of full-length atToc159 and the 50–55-kDa proteolytic fragments of atToc159 (159M) are indicated to the left of the figure and the positions of molecular weight standards (kDa) are indicated to the right. D, quantitative analysis of atToc159 binding (Bound) and insertion (Inserted) into proteoliposomes consisting of psToc34 and psToc75 (Toc34/Toc75) or psToc34-G48R/K52N/S53R and psToc75 (G48R/ K52N/S53R/Toc75) from replicate experiments, including those in C with standard error bars. [35S]AtToc159 binding and insertion was measured as described in the legend to Fig. 6, and is expressed as % maximal binding or insertion.

Previous studies implicated atToc33 as the initial docking site for atToc159 at the outer envelope and demonstrated the requirement for the intrinsic GTPase activity of atToc159 for targeting (15, 17, 18). Our analysis of atToc159 binding using solid phase assays and reconstituted proteoliposomes demonstrates that the GTPase activity of atToc33/psToc34 is also required for proper assembly of the receptor into Toc complexes (Figs. 2 and 8). Taken together, these studies suggest that the two GTPases work in concert to promote atToc159 association with the Toc complex.

Is there a role for the interaction of the G-domains in addition to promoting specific docking of the receptor at the Toc complex? In this study we show that the interaction of the G-domain of atToc159 with the cognate atToc33 GTPase promotes the specific association of the atToc159 M-domain with the chloroplast surface (Fig. 3). The observation that the M-domain binds specifically to atToc33G (Fig. 4) suggests that docking of the atToc159 G-domain might induce a conformation in atToc33 that reveals a high affinity M-domain binding site. The higher levels of atToc159 binding observed in the psToc34-psToc75 proteoliposomes compared with those containing psToc34 alone (Fig. 6) suggests that Toc75 might also contribute to initial binding of the receptor at the Toc complex. The predicted effect of these interactions is to position the M-domain for subsequent insertion into the membrane, an event that appears to require Toc75 (Figs. 6 and 8). The data presented here and in previous studies (15, 18) demonstrate that insertion of the atToc159 receptor into the outer membrane requires GTP hydrolysis at both GTPases. Although hydrolysis does not appear to provide the direct driving force for insertion, conversion of the GTPases to their GDP-bound forms increases their affinity for each other and is likely to trigger M-domain insertion (15). On the basis of these observations, we suggest that the interaction of the G-domains and subsequent GTP hydrolysis play a regulatory role in receptor targeting rather than provide a mechnochemical force for receptor insertion.

It has been shown previously that Toc75 and the M-domain of Toc159 cooperate during preprotein translocation across the outer membrane, each perhaps making contributions to the structure and function of the preprotein channel (9, 25). Interestingly, whereas the ~52-kDa M-domain of membrane-inserted Toc159 is protease-resistant and alkali-extractable, it is a largely hydrophilic domain, which suggests that it may not simply act as a passive membrane anchor, but rather a dynamic, functionally essential component of the receptor and the Toc complex in general. Indeed, proteolytic removal of the A- and G-domains of membrane-integrated Toc159 slows but does not abolish preprotein import into chloroplasts in vitro (14), an observation that is consistent with a vital role for the M-domain of Toc159 in preprotein translocation. Recent reconstitution studies led to the proposal that Toc159 might contribute to the translocation driving force (25). This raises the possibility that the GTP-regulated docking and insertion of the receptor at the Toc complex is coupled to preprotein docking and insertion at the outer membrane translocon. This idea is consistent with the observation that Toc159 is distributed between the cytoplasm and membrane and is found in substoichiometric amounts relative to Toc34 and Toc75 in Toc complexes (16–18). As such, the GTP-regulated insertion of the receptor could represent the GTP-dependent switch that is required for the
initiation of preprotein translocation.

The docking and insertion of Toc159 at the Toc complex has been proposed to represent not only a step in the assembly of the Toc complex, but also a step in the targeting of nucleus-encoded preproteins from the cytoplasm to the translocon (15, 17, 18). The fact that both preprotein binding and Toc159 targeting are dependent upon GTP hydrolysis provides a molecular basis for integrating Toc159 and preprotein targeting. We propose that Toc159 and Toc33/34 sequentially interact with transit peptides, as part of a GTP-regulated targeting cycle. There is evidence that both Toc33/34 and Toc159 bind preproteins and that their GTPase activities are required for protein import in vitro and in vivo (5–7, 9, 12–14, 24). Furthermore, in organello cross-linking data demonstrate that pSoc159 is the initial docking site for transit peptides at the chloroplast surface (5). Cross-linking to pSoc34 is detected later upon initiation of the translocation reaction (5). Our current data are consistent with a GTP-regulated targeting cycle in which soluble Toc159 would deliver the preprotein from the cytoplasm to the Toc complex by docking at Toc33/34. Docking could lead to transfer of the transit peptide to Toc33/34 or alternatively, Toc159 and Toc33/34 could interact with distinct regions of the transit peptide. Subsequent hydrolysis of GTP would trigger the insertion of Toc159 via Toc75 and initiate translocation of the preprotein through the formation of a translocation channel composed of Toc75 and the M-domain of Toc159. It is intriguing to speculate that transit peptide binding regulates the Toc159 targeting reaction by controlling the nucleotide state of the two Toc GTPases. Transit peptide binding at pSoc34 stimulates its GTPase activity (26, 27), and the GDP-bound forms of the Toc GTPases favor the insertion of Toc159 into the Toc complex (15, 18). This could provide a part of the molecular switch that triggers the insertion of Toc159 and the initiation of preprotein translocation. Others have proposed that Toc33/34 acts as a primary transit peptide receptor and that Toc159 functions only as a component of the translocation motor (7, 12, 25). However, all current data can be reconciled by the model in which the two Toc GTPases act coordinately to regulate transit peptide recognition and initiate membrane translocation by interacting directly with each other in a manner regulated by their intrinsic GTPase activities.

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