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The majority of nucleus-encoded chloroplast proteins are targeted to the organelle by direct binding to two membrane-bound GTPase receptors, Toc34 and Toc159. The GTPase activities of the receptors are implicated in two key import activities, preprotein binding and driving membrane translocation, but their precise functions have not been defined. We use a combination of in vivo and in vitro approaches to study the role of the Toc159 receptor in the import reaction. We show that atToc159-A864R, a receptor with reduced GTPase activity, can fully complement a lethal insertion mutation in the ATTOC159 gene. Surprisingly, the atToc159-A864R receptor increases the rate of protein import relative to wild-type receptor in isolated chloroplasts by stabilizing the formation of a GTP-dependent preprotein binding intermediate. These data favor a model in which the atToc159 receptor acts as part of a GTP-regulated switch for preprotein recognition at the TOC translocon.

Introduction

Plastids (e.g., chloroplasts) are a diverse group of interrelated organelles that have evolved to perform numerous and diverse metabolic processes within all plant cells, including essential roles in photosynthesis, amino acid and lipid synthesis, and cell signaling (Neuhaus and Emes, 2000). The biogenesis of these organelles and their differentiation into specialized forms in different tissues relies on the import of thousands of nuclear-encoded proteins from the cytoplasm (Jarvis, 2004; Richly and Leister, 2004). Protein import is mediated by several translocation systems, designated translocons, which reside within the envelope (Gutensohn et al., 2006). The majority of nucleus-encoded chloroplast proteins are targeted to the organelle by direct binding to two membrane-bound GTPase receptors, Toc34 and Toc159. The GTPase activities of the receptors are implicated in two key import activities, preprotein binding and driving membrane translocation, but their precise functions have not been defined. We use a combination of in vivo and in vitro approaches to study the role of the Toc159 receptor in the import reaction. We show that atToc159-A864R, a receptor with reduced GTPase activity, can fully complement a lethal insertion mutation in the ATTOC159 gene. Surprisingly, the atToc159-A864R receptor increases the rate of protein import relative to wild-type receptor in isolated chloroplasts by stabilizing the formation of a GTP-dependent preprotein binding intermediate. These data favor a model in which the atToc159 receptor acts as part of a GTP-regulated switch for preprotein recognition at the TOC translocon.

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Abbreviations used in this paper: A-domain, acidic domain; G-domain, GTPase domain; M-domain, membrane domain; PIC, protease inhibitor cocktail; preSSu, precursor to the small subunit of rubisco; SSu, mature small subunit of rubisco; TIC, translocon at the inner chloroplast membrane; TOC, translocon at the outer chloroplast membrane.

The online version of this article contains supplemental material.

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Multiple lines of evidence indicate that Toc34 and Toc159 play key roles in regulating protein targeting and plastid biogenesis. First, the two receptors mediate preprotein recognition at the chloroplast surface by binding directly to transit peptides (Perry and Keegstra, 1994; Ma et al., 1996; Kouranov and Schnell, 1997; Sveshnikova et al., 2000; Schleiff et al., 2002; Smith et al., 2004). The import of bound preproteins is inhibited by nonhydrolyzable GTP analogues, indicating that GTP hydrolysis at the TOC regulates transfer of the preprotein into the protein-conducting channel (Olsen and Keegstra, 1992; Ma et al., 1996; Young et al., 1999). The GTPase domains of the two receptors are involved in transit peptide binding, and binding stimulates the GTPase activities of the receptors, providing a link between preprotein recognition and GTPase activity (Jelic et al., 2002, 2003; Smith et al., 2004). As such, Toc34 and Toc159 are hypothesized to act as a coordinated GTPase switch that controls protein import into the organelle.

Second, members of the Toc159 and Toc34 gene families in arabidopsis have been shown to form structurally and functionally distinct translocon complexes that constitute distinct pathways for protein import into plastids. Genetic and biochemical analyses indicate that different members of the arabidopsis Toc159 (atToc159) and Toc34 (atToc33) families function in the import of different classes of preproteins that are required for specialized (e.g., photosynthesis) and housekeeping functions of the organelles (Jarvis et al., 1998; Bauer et al., 2000; Kubis et al., 2003, 2004; Ivanova et al., 2004). Mutations in genes encoding specific atToc159 and atToc33 family members interfere with plastid differentiation at different stages. These studies demonstrate an important role for the TOC GTPase receptors in coordinating protein trafficking with the changes in gene expression that accompany plant development.

Despite the central role for the TOC GTPases in regulating protein import, the molecular mechanism by which their GTPase activities control preprotein recognition and translocation remain undefined. Two major models have been proposed as working hypotheses for TOC receptor function. In one model, Toc159 and Toc34 form a GTP regulated gate that controls access of preproteins to the TOC channel, thereby maintaining the fidelity of import and regulating the specificity of translocons for distinct classes of preproteins (Seedorf et al., 1995; Kessler and Schnell, 2004). Although this model includes roles for both GTPases in preprotein binding at the translocon, Toc159 is proposed to function primarily in defining the selectivity of the translocon, whereas Toc34 is envisioned to function in conjunction with Toc159 to initiate transfer of the preprotein to the TOC channel. This proposal is supported by covalent cross-linking studies that demonstrate that Toc159 forms the primary contact with transit peptides during preprotein binding (Perry and Keegstra, 1994; Ma et al., 1996). Furthermore, the atToc159 family members appear to largely dictate the specificity of different TOC complexes for distinct classes of preproteins (Hiltbrunner et al., 2004; Ivanova et al., 2004; Kubis et al., 2004; Smith et al., 2004), consistent with a role for this receptor family in regulating initial preprotein recognition. In contrast, the two Toc34 receptors in arabidopsis appear to be largely functionally redundant in vivo (Jarvis et al., 1998).

A second model proposes that Toc159 GTPase activity provides the energetic driving force for preprotein translocation through the TOC channel (Schleiff et al., 2003; Becker et al., 2004). In this scenario, Toc34 is proposed to function primarily in preprotein recognition and Toc159 is envisioned to function downstream as a repetitive GTP-driven motor that forces the preprotein through the TOC channel. This model is based on the observation that reconstituted proteoliposomes containing Toc75 and a fragment of Toc159, corresponding to its G- and M-domains, can support a minimum level of GTP-dependent preprotein translocation (Schleiff et al., 2003).

The proposals for TOC GTPase function are largely in agreement on the critical role of the functional interactions between the two GTPases and their involvement in GTP-regulated preprotein recognition at the TOC complex. However, the models present very distinct hypotheses for the molecular mechanism of TOC GTPase function during import. The major differences between these models center on the role of Toc159 and its GTPase activity in preprotein recognition and translocation. Toc159 is alternatively proposed to function early in import as the primary preprotein receptor or late in import as the driving force for membrane translocation. One major issue in defining the specific role for Toc159 in TOC complex function is the lack of in vivo analysis of its GTPase function. The models based on in vitro biochemical analysis of Toc159 also are qualified by the fact that they rely on the use of purified or overexpressed fragments of the receptor that lack one or more of the domains found in the full-length native protein.

In this study, we aimed to more clearly define the role of Toc159 GTpase activity in TOC translocon function using in vivo genetic approaches combined with in vitro import assays using intact chloroplasts with full-length receptor. To this end, we took advantage of an arabidopsis mutant, ppi2, which lacks expression of atToc159, the major Toc159 family member expressed in chloroplasts (Bauer et al., 2000). We expressed atToc159-A864R, a mutated form of atToc159 that has significantly reduced GTPase activity, in ppi2 and examined the effects of the mutation on protein import and chloroplast biogenesis. Remarkably, the atToc159-A864R receptor was able to fully complement the ppi2 lethal phenotype. Furthermore, chloroplasts containing the atToc159-A864R mutant exhibited an increased rate of protein import relative to wild-type chloroplasts. Biochemical analysis indicated that the atToc159-A864R receptor increases the efficiency of preprotein targeting to chloroplasts. Our studies demonstrate that the nucleotide state of Toc159 regulates the formation of a stable preprotein binding intermediate, and thereby plays a regulatory role in preprotein recognition at the TOC translocon.

**Results**

**Expression of an atToc159 GTPase mutant can complement the ppi2 lethal phenotype**

Our strategy for assessing the role of atToc159 GTPase activity centered on expressing the atToc159-A864R mutant in stable transformants of the atToc159 null mutant, ppi2, and investigating the effects on chloroplast biogenesis and protein import.
AtToc159-A864R contains a single point mutation that converts a conserved alanine to an arginine in the G1 motif (P-loop) of atToc159. Previous studies showed that the *Escherichia coli*–expressed G-domain of atToc159-A864R binds GTP and GDP with equal or slightly higher affinities compared with wild-type receptor, but its GTPase activity is reduced to ~10% of that of atToc159 (Smith et al., 2002). To confirm that the activities of the full-length receptors are comparable to those measured with isolated G-domains, we purified full-length wild-type atToc159 and atToc159-A864R from a reticulocyte lysate in vitro translation system using immunoprecipitation and measured their activities using a standard GTPase assay. Fig. S1 A (available at http://www.jcb.org/cgi/content/full/jcb.200803034/DC1) shows that the hydrolytic activity of atToc159-A864R (K_M = 1.9 ± 0.30 μM; k_cat = 0.0042 ± 0.0001 min^{−1}) is ~30% of that measured for wild-type atToc159 (K_M = 3.2 ± 0.34 μM; k_cat = 0.014 ± 0.0022 min^{−1}). The hydrolytic activity of wild-type atToc159 is similar to that measured for purified, soluble atToc159 G-domain (K_M = 9.0 ± 1.35 μM; k_cat = 0.019 ± 0.0015 min^{−1}), indicating that the activities of the full-length and isolated G-domains are comparable. Furthermore, these data demonstrate that immunoprecipitation does not significantly affect the enzymatic activity of the receptor. As negative controls, we included two additional receptor G-domain constructs in the assay, atToc159-G1m and atToc33G-G1m. These receptors carry triple mutations within the G1 motif of their GTP-binding sites and previously were shown to lack GTP binding and hydrolytic activities (Bauer et al., 2002; Smith et al., 2002; Wallas et al., 2003). No measurable hydrolytic activity was detected in either case (Fig. S1 B). Together, the data are consistent with the results using the purified G-domains and indicate that the atToc159-A864R mutant has a significantly lower hydrolytic rate than wild-type receptor.

A transformation vector encoding atToc159-A864R under control of the cauliflower mosaic virus 35S promoter (CaMV35S) was transformed into heterozygous *ppi2* plants. Independent transformants were segregated to select for plants homozygous for both *ppi2* and CaMV35S::atTOC159-A864R (A864R::*ppi2*). The genotypes of the transformed plants were confirmed by PCR amplification of the wild-type authentic atTOC159 gene, the *ppi2* T-DNA marker, and the CaMV35S::atTOC159-A864R transgene (Fig. 1 A). To confirm that the transgene was expressed in the *ppi2* background, we performed immunoblots of plant extracts with anti-atToc159 serum. A *ppi2* line expressing CaMV35S::atTOC159 (WT::*ppi2*) that was previously generated was used as a control (Bauer et al., 2002). Full-length atToc159 was present in wild-type and WT::*ppi2*, whereas a full-length protein corresponding to atToc159-A864R was detected in A864R::*ppi2* plants (Fig. 1 B). Immunoblots of serial dilutions of extracts from these plants indicate that receptor expression in WT::*ppi2* and A864R::*ppi2* plants is similar, and both transgenes are expressed at levels only moderately higher than authentic atToc159 in wild-type (WT) plants. Alkaline extraction of membrane fractions from wild-type and A864R::*ppi2* plants indicated that atToc159-A864R was membrane integrated, consistent with correct targeting to the outer envelope membrane (Fig. 1 C). Moreover, atToc75 and atToc33 coimmuno-precipitated with atToc159 and atToc159-A864R from detergent-soluble extracts, demonstrating that the mutation did not detectably affect receptor targeting or TOC complex assembly (Fig. 1 D).

The homozygous *ppi2* mutant is albino and seedling lethal, resulting in plants that die shortly after germination on soil (Fig. 2 A) (Bauer et al., 2000). Remarkably, the atToc159-A864R gene complemented the *ppi2* mutation. A864R::*ppi2* plants were visibly indistinguishable from WT::*ppi2* or wild-type plants at all stages of development under normal growth conditions (Fig. 2 A). Furthermore, the levels of chlorophyll (Fig. 2 C) and representative chloroplast proteins, including other TOC components (Fig. 2 B), were indistinguishable from wild-type plants.

To investigate if the atToc159-A864R mutation might have a more subtle effect that was not apparent under normal growth conditions, we performed a de-etiolation experiment (Fankhauser and Casal, 2004) to more closely examine chloroplast biogenesis in the complemented plants (Fig. 2 D). The atToc159 receptor has previously been shown to be required for the development of chloroplasts from undifferentiated plastids in the majority of green tissues (Bauer et al., 2000). Therefore, we tested whether the atToc159-A864R mutation might delay this developmental process by partially disrupting the protein import process. In this experiment, the seeds were stratified in the dark at 4°C and exposed to a short pulse of light to initiate germination. The germinating seeds were equilibrated in the dark at 20°C, resulting in etiolated (nongreen) seedlings that lack fully developed chloroplasts. The seedlings were then transferred to light to trigger photomorphogenesis and chloroplast development. We monitored chlorophyll accumulation at 20 and 28 h after transferring the plants to light. As shown in Fig. 2 D, WT::*ppi2* and A864R::*ppi2* plants accumulated chlorophyll at similar rates. These data indicate that the atToc159-A864R mutation does not disrupt chloroplast biogenesis and can fully complement *ppi2*.

**AtToc159-A864R chloroplasts have an increased rate of import relative to wild-type chloroplasts**

To further understand the role of atToc159 GTPase activity, we examined the levels of preprotein import in chloroplasts isolated from wild-type, WT::*ppi2*, and A864R::*ppi2* plants. For these studies, we modified our chloroplast isolation procedure to minimize the degradation of atToc159 based on published procedures (Brock et al., 1993; Schulz et al., 2004). Our standard isolation procedures for pea and *arabidopsis* chloroplasts result in proteolytic cleavage of the amino-terminal A-domain of Toc159, yielding an 86-kD receptor fragment (Bolter et al., 1998; Chen et al., 2000). We found that modification of the standard isolation methods based on published procedures (Brock et al., 1993; Schulz et al., 2004) resulted in a dramatic decrease in atToc159 degradation (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200803034/DC1). Comparison of the import capabilities of these chloroplasts also demonstrates a ~2-fold increase in the efficiency of import (Fig. S2, B and C). All subsequent experiments were performed with chloroplasts isolated using this procedure.
but did not digest the processed form (Fig. 3 B), demonstrating that the mature SSu represented completely imported protein and not a partially imported substrate. Interestingly, import into atToc159-A864R chloroplasts remained sensitive to the GTP analogue, GTP\_H9253 (Fig. 3 C). Import into both wild-type and atToc159-A864R chloroplasts was reduced by 60% in the presence of the analogue. Therefore, GTP hydrolysis within the TOC translocon is still required for the import reaction, even when atToc159 hydrolytic activity is reduced by 70%. The inhibition by the analogue could be due to an effect on the residual activity of atToc159-A864R or on atToc33.

To examine the nature of the increased levels of import observed in atToc159-A864R chloroplasts, we examined import.

Isolated chloroplasts were incubated with [35S]-labeled chloroplast preprotein, preSSu, in the presence of 1 mM GTP and 2 mM ATP to promote import for 5 or 20 min at 25°C (Fig. 3 A). Import into chloroplasts from wild-type and WT:ppi2 plants were indistinguishable, demonstrating that expression of wild-type atToc159 under a non-native promoter does not measurably affect the import process. Surprisingly, the levels of preSSu import were significantly higher in atToc159-A864R chloroplasts compared with the two controls at each time point. The accumulation of SSu in atToc159-A864R chloroplasts was approximately double both wild-type or ppi2 chloroplasts expressing wild-type atToc159 (Fig. 3 A). Thermolysin treatment of the atToc159-A864R chloroplasts following the import reaction removed bound preSSu but did not digest the processed form (Fig. 3 B), demonstrating that the mature SSu represented completely imported protein and not a partially imported substrate. Interestingly, import into atToc159-A864R chloroplasts remained sensitive to the GTP analogue, GTP\_H9253 (Fig. 3 C). Import into both wild-type and atToc159-A864R chloroplasts was reduced by ~60% in the presence of the analogue. Therefore, GTP hydrolysis within the TOC translocon is still required for the import reaction, even when atToc159 hydrolytic activity is reduced by 70%. The inhibition by the analogue could be due to an effect on the residual activity of atToc159-A864R or on atToc33.

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were similar. Therefore, the increased rate of preSSu does not appear to result from an overall increase in turnover number. In contrast, the import rate constants (apparent $K_m$) for atToc159-A8684R (158 ± 18 nM) and atToc159 (224 ± 11 nM) chloroplasts were significantly different. The lower apparent $K_m$ for atToc159-A8684R chloroplasts suggests that the TOC translocons have a higher apparent affinity for preSSu relative to wild-type translocons, accounting for the increased rate of preSSu import under substrate-limiting conditions.

The atToc159-A864R receptor enhances preprotein binding to TOC translocons

GTPyS has previously been shown to inhibit the early stages of protein import by preventing the formation of an early import intermediate that has inserted into the TOC channel, but remains largely exposed at the chloroplast surface and therefore has not completed translocation across the membrane (Schnell and...
brane translocation is not disrupted by inhibiting atToc159 GTPase activity.

To determine whether subsequent translocation of the early import intermediate across the chloroplast envelope is affected by atToc159-A864R, we examined the complete translocation of the intermediate by chasing it into chloroplasts using high concentrations of ATP (5 mM). Although the levels of early import intermediate are higher in mutant chloroplasts (Fig. 5 B, lanes 3 and 4; Fig. 5 C), ~50% of the bound intermediate can be chased into chloroplasts in both atToc159-A864R and wild-type chloroplasts (Fig. 5 B, compare lanes 4 and 7; Fig. 5 D, black bars). In contrast to formation of the early import intermediate, complete translocation from the intermediate stage does not appear to require additional GTP hydrolysis at either TOC receptor because the inclusion of GTPγS in the chase experiment had no effect on import of the late intermediate.

Figure 3. The levels of protein import into atToc159-A864R chloroplasts are increased compared with wild-type chloroplasts. (A) Isolated chloroplasts from WT, WT:ppi2, and A864R:ppi2 plants were incubated with in vitro-translated [35S]preSSu under import conditions for the times indicated. The graph presents quantification of triplicate experiments from import of preSSu at the 5-min and 20-min time points. (B) Chloroplasts from preSSu 20-min import reactions in A were treated with 100 μg/ml thermolysin on ice for 30 min to discriminate between bound and fully imported protein. (C) Effect of GTPγS on protein import into WT:ppi2 and A864R:ppi2 chloroplasts. Chloroplasts were incubated under standard import conditions for 20 min in the presence of GTP or GTPγS. The graph presents quantification of preSSu import from triplicate experiments. The position of preSSu and imported mature SSu are indicated at the left of each panel. IVT, 10% of in vitro-translated [35S]preSSu added to each import reaction. Dashed lines indicate that the figures were generated from different regions of the same SDS-PAGE gel using samples from the same experiment.

Blobel, 1993; Kessler et al., 1994; Young et al., 1999; Inoue and Akita, 2008). We examined the formation of the early intermediate in wild-type and atToc159-A864R chloroplasts in more detail to determine if the mutation affected insertion into the outer membrane channel or subsequent translocation across the envelope. Isolated chloroplasts were incubated with [35S]preSSu in the presence of GTP and low levels of ATP (0.1 mM) to promote formation of the early import intermediate. The levels of early import intermediate in atToc159-A864R chloroplasts were nearly twofold higher than in chloroplasts from WT:ppi2 plants (Fig. 5 A, compare lanes 2 and 5 and graph). These results demonstrate that the reduction of atToc159 GTPase activity actually enhances, rather than reduces, the amount of early import intermediate. Consistent with its effects on import, GTPγS inhibits the formation of the early intermediate (Fig. 5 A). These results confirm that the initiation of outer membrane translocation is not disrupted by inhibiting atToc159 GTPase activity.

To determine whether subsequent translocation of the early import intermediate across the chloroplast envelope is affected by atToc159-A864R, we examined the complete translocation of the intermediate by chasing it into chloroplasts using high concentrations of ATP (5 mM). Although the levels of early intermediate are higher in mutant chloroplasts (Fig. 5 B, lanes 3 and 4; Fig. 5 C), ~50% of the bound intermediate can be chased into chloroplasts in both atToc159-A864R and wild-type chloroplasts (Fig. 5 B, compare lanes 4 and 7; Fig. 5 D, black bars). In contrast to formation of the early import intermediate, complete translocation from the intermediate stage does not appear to require additional GTP hydrolysis at either TOC receptor because the inclusion of GTPγS in the chase experiment had no effect on import of the late intermediate.
intermediate (Fig. 5 B, compare lanes 8–9 and 10–11; Fig. 5 D, gray bars). These results demonstrate that the intermediate in both wild-type and mutant chloroplasts represents a productive intermediate and that membrane translocation after initial insertion in the TOC channel is not disrupted by inhibiting TOC GTPase activity.

Low levels of preprotein binding to the TOC GTPase receptors have been observed in the absence of energy or in the presence of GTP alone (Perry and Keegstra, 1994; Ma et al., 1996). This prompted us to examine the initial binding step to determine its role in the increased levels of early intermediate formation and protein import observed in atToc159-A864R chloroplasts. Consistent with previous observations, wild-type chloroplasts exhibit low levels of preSSu binding in the absence of added nucleoside triphosphates (NTPs) or in the presence of the NTP hydrolytic enzyme, apyrase (Fig. 6 A). The addition of GTP or the nonhydrolyzable GDP analogue, GDPβS, do not measurably increase or decrease binding relative to the no-energy controls (Fig. 6 A and graph). The addition of the slowly hydrolyzable GTP analogue, GTPγS, reduces the basal levels of binding by nearly 50%, suggesting that forcing both TOC GTPases into their GTP bound forms inhibits the stable interaction of the preprotein with the receptors.

PreSSu binding to atToc159-A864R chloroplasts in the absence of energy or in the presence of GDPβS (Fig. 6 A, lanes 5 and 6 and graph) is indistinguishable from wild-type chloroplasts. GTPγS reduces binding to atToc159-A864R to levels similar to those observed with wild-type chloroplasts (Fig. 6 A, compare lanes 12 and 13). In contrast, GTP increases the level of binding to atToc159-A864R chloroplasts approximately
Figure 5. Preprotein translocation across the outer membrane is not affected by decreasing atToc159 GTPase activity. (A) In vitro–translated [35S]preSSu was incubated with isolated chloroplasts from WT:ppi2 or A864R:ppi2 plants in the presence of 0.1 mM ATP and 0.1 mM GTP to initiate translocation across the outer membrane (early import intermediate), but preclude complete translocation across the envelope. Where indicated, 1 mM GTPγS was substituted for GTP. The graph presents the quantification of results from triplicate experiments. (B) WT:ppi2 and A864R:ppi2 chloroplasts containing bound early import intermediates (Inter.; lanes 3 and 6) were reisolated and resuspended under protein import conditions in the presence of 5 mM ATP (Chase; lanes 4, 7, and 8–11). Where indicated, 1 mM GTPγS was added to the chase reactions (lanes 9 and 11). (C) Quantification of the levels of early import intermediate formed in B from triplicate experiments. (D) Quantification of the effects of GTPγS on the chase of the early intermediate (B) from triplicate experiments. Dashed lines indicate that the figures were generated from different regions of the same SDS-PAGE gel using samples from the same experiment.

threefold over the no-energy control (Fig. 6 A, compare lanes 4 and 7, and graph). To ensure that the GTP-dependent binding represents a productive intermediate, we tested whether it could be chased into chloroplasts by re-isolating the chloroplasts and adding sufficient ATP to support complete envelope translocation (Fig. 6 B, lane 2 and graph). Approximately 50% of the bound preprotein can be imported and processed to its mature form, indicating that the bound precursor represents an intermediate on the import pathway. These data indicate that the GTP-bound form of atToc159 functions as the active component in stable preprotein binding to the TOC translocon. The increased binding in the presence of GTP likely results from the stabilization of the initial binding of preprotein to the TOC translocon that previously was observed only when stabilized by chemical cross-linkers (Perry and Keegstra, 1994; Ma et al., 1996). The increased binding can account for the increase in the import rates observed in atToc159–A864R chloroplasts.

In contrast to complete translocation of preproteins from the early import intermediate stage, GTPγS inhibits translocation and processing of the GTP-dependent intermediate (Fig. 6 B, compare lanes 2 and 3 and graph). The reduction of binding by GTPγS in both the wild-type and atToc159–A864R chloroplasts suggests that stable preprotein binding is blocked when both atToc159 and atToc33 are locked in their GTP-bound forms. Furthermore, it is consistent with the hypothesis that both receptor GTPases participate in the initial binding of preproteins to the translocon.
As a final step in our analysis, we examined the components involved in the GTP-dependent binding observed with atToc159-A864R chloroplasts. To this end, we used the homobifunctional cysteine cross-linker, DTME (dithio-bismaleimidoethane), to cross-link bound preSSu to isolated chloroplasts in the presence of GTP. As previously observed, preSSu binding to atToc159-A864R chloroplasts was significantly higher than the wild-type controls (Fig. 7 A, top). Consistent with this observation, approximately three times the levels of preSSu coimmunoprecipitated with atToc159-A864R compared with wild-type atToc159 from detergent solubilized chloroplasts (Fig. 7 A and B). The levels of preSSu that coimmunoprecipitated with atToc33 were increased by ~50% in atToc159-A864R chloroplasts. We conclude that the stable, GTP-dependent binding to TOC translocons occurs due to increased binding to the GTP-bound form of atToc159 that is predominant in atToc159-A864R chloroplasts.

Discussion

In this paper, we report the characterization of a novel intermediate in the pathway of protein import into chloroplasts. Identification of the intermediate was made possible by increasing the GTP-bound form of the atToc159 receptor using the atToc159-A864R mutant that binds but does not efficiently hydrolyze GTP. In the presence of GTP, atToc159-A864R promotes stable binding of preprotein to the TOC translocon (Fig. 6). This results in an apparent increase in the affinity of the translocon for preSSu (Fig. 4). The bound preprotein is efficiently chased into chloroplasts under import conditions, indicating that it represents a productive step in the import process. The GTP-dependent binding observed in atToc159-A864R chloroplasts results in an increased rate of protein import (Figs. 3 and 4) and accounts for the ability of the mutant receptor to complement the atToc159 null mutant, ppi2 (Figs. 1 and 2). On the basis of these results, we conclude that GTP-dependent binding represents an early step in docking of the preprotein to the TOC translocon.

Previous studies have shown that GTP alone supports low levels of preprotein binding. However, the nature of this binding has been difficult to define because the levels were similar to, or only slightly higher than, binding observed in the absence of added NTP (Olsen and Keegstra, 1992; Young et al., 1999) and were captured only by covalent cross-linking (Perry and Keegstra, 1994; Ma et al., 1996). The atToc159-A864R receptor increases GTP-dependent preprotein binding, apparently by stabilizing the GTP-bound form of the receptor. In contrast to the early import

Figure 6. atToc159-A864R promotes GTP-dependent binding to the TOC translocon. (A) Energy-depleted, in vitro–translated [35S]preSSu was incubated with isolated chloroplasts from WT:ppi2 or A864R:ppi2 plants in the absence of added nucleotide (−), in the presence of apyrase or in the presence of 0.1 mM of the indicated nucleotide. Chloroplasts were isolated and bound preSSu was analyzed by SDS-PAGE and phosphorimaging. The graph presents the quantification of results from triplicate experiments. (B) atToc159-A864R chloroplasts were incubated with [35S]preSSu in the presence of 0.1 mM GTP to stimulate binding. The chloroplasts were isolated and resuspended in the absence of added nucleotide (−), in the presence of 5 mM ATP or 5 mM ATP and 1 mM GTP-S and incubated under standard import conditions (Chase). The graph presents quantification of imported preSSu from triplicate experiments. Dashed lines indicate that the figures were generated from different regions of the same SDS-PAGE gel using samples from the same experiment.
intermediate, the bound preprotein appears not to have initiated membrane translocation because import from the bound state requires GTP hydrolysis (Fig. 6), whereas complete translocation of the early import intermediate does not (Fig. 5) (Young et al., 1999). Furthermore, the early import intermediate requires low concentrations of ATP (Olsen et al., 1989; Olsen and Keegstra, 1992; Schnell and Blobel, 1993). Therefore, the GTP-dependent binding intermediate represents a stage in import that precedes preprotein insertion in the TOC channel and likely corresponds to the initial interaction of preproteins with the translocon.

This work and numerous previous studies clearly have established that GTP hydrolysis regulates the initiation of translocation across the outer membrane (Olsen and Keegstra, 1992; Kessler et al., 1994; Ma et al., 1996; Kouranov and Schnell, 1997; Young et al., 1999). Although atToc159-A864R promotes preprotein binding to the translocon, it does not appear to affect membrane translocation. Nevertheless, GTP analogues inhibit subsequent insertion of the preproteins into the TOC channel (i.e., formation of the early import intermediate) (Fig. 6). These observations are consistent with a role for GTP hydrolysis in initiating membrane translocation, but suggest that this occurs downstream of the GTP-dependent binding step and likely involves the atToc33 GTPase. The results favor models in which GTP-binding and hydrolysis at atToc159 are primarily involved in preprotein targeting to the translocon and are not strictly required for initiating membrane translocation (Li et al., 2007; Rounds et al., 2007). This hypothesis is consistent with several other observations. Pea chloroplasts that have been proteolyzed to selectively remove the amino-terminal A-domain and the G-domain of psToc159, but retain intact psToc34 and psToc75, exhibit reduced preprotein binding (Chen et al., 2000). Nonetheless, these chloroplasts remain import competent, albeit with reduced import rates. Importantly, import in these proteolyzed chloroplasts is still sensitive to nonhydrolyzable analogues, demonstrating a direct role for psToc34 GTPase activity in initiating membrane translocation (Chen et al., 2000). Moreover, an atToc159 deletion construct, consisting solely of the M-domain, can partially complement the arabidopsis ppi2 mutant when expressed in stable transgenic plants (Lee et al., 2003).

Although our data support a primary role for Toc159 in targeting preproteins to the translocon, the data also demonstrate that the initial binding of preproteins involves the concerted activities of both atToc159 and atToc33. AtToc33 and atToc159 both covalently cross-link to the preprotein at the GTP-dependent docking stage (Fig. 7). This is consistent with previous label-transfer cross-linking experiments in which both psToc159 and psToc34 were shown to interact with preproteins during initial binding (Kouranov et al., 1999). Together, these results indicate that both GTPases participate in this early binding step. Although decreasing GTP hydrolysis at atToc159 and therefore favoring its GTP-bound state appears to increase the stability of preprotein binding, the fact that GTPγS inhibits binding suggests that locking both atToc159 and atToc33 in their GTP-bound forms inhibits binding. These data suggest that atToc159 and atToc33 are not in the same nucleotide-bound state (i.e., GTP bound) during this initial interaction.

The accumulated evidence suggests that the Toc159 family of receptors constitute the key determinants of preprotein binding specificity at TOC translocons. Arabidopsis contains four atToc159 family members, and atToc159 and two other family members, atToc132 and atToc120, form separate translocons that have been shown to bind distinct preproteins in vitro and to mediate distinct import pathways in vivo (Hiltbrunner et al., 2004; Ivanova et al., 2004; Kubis et al., 2004; Smith et al., 2004). In contrast, the two Toc34 proteins in arabidopsis,
atToc33 and atToc34, exhibit modest effects in preprotein binding studies, and their functions are not redundant in vivo (Jarvis et al., 1998; Kubis et al., 2003; Constan et al., 2004). The different import pathways generated by the Toc159 receptors appear to be critical for plastid biogenesis (e.g., chloroplast formation) at various stages of plant development. The observation that the GTP-bound form of atToc159 promotes preprotein binding suggests that atToc159 GTPase activity regulates the initial docking of preproteins to the translocon and likely plays a key role in selective preprotein targeting. In this scenario, productive preprotein binding to the GTP-bound form of Toc159 would promote the formation of a stable preprotein–TOC complex that includes binding to Toc34. Subsequent GTP binding and hydrolysis at Toc34 would result in preprotein dissociation from the receptors and transfer to the TOC channel, thereby initiating the translocation reaction. We propose that GTP hydrolysis at Toc159 resets the translocon to a prebinding state following translocation. The prebinding state would prevent translocation in the absence of productive preprotein binding. This mechanism would allow the different Toc159 family members to selectively target preproteins to specific translocons and preclude the binding and translocation of preproteins from other import pathways. Preprotein binding and import in atToc159-A864R chloroplasts are more efficient because the GTP-bound form of the receptor would stabilize the import-competent state of the translocon, thereby bypassing the pre-binding state of the translocon.

A previous reconstitution study suggested that Toc159 GTPase activity was the major driving force for membrane translocation (Schleiff et al., 2003). In that work, translocation of a chloroplast preprotein was observed in artificial proteoliposomes that were reconstituted with renatured psToc75 and a fragment of psToc159 containing its G- and M-domains. Our studies demonstrate that inhibition of atToc159 GTPase activity does not inhibit membrane translocation in vivo. These data are inconsistent with a function for Toc159 as a translocation motor. However, Toc159 GTPase activity might contribute to dissociation of the preproteins from the receptor complex and transfer to the Toc channel, thereby facilitating insertion in the channel. In the reconstituted system, the activity might be sufficient to allow some transport across the membrane in the absence of Toc34. In this model, the functions of the Toc159 and Toc34 GTPase activities within native translocons are partially overlapping, with Toc159 GTPase activity primarily involved in preprotein recognition and Toc34 GTPase activity primarily involved in the initiation of translocation. Together, it is clear that cooperative activities of the Toc159 and Toc34 GTPases are required for translocon function in vivo. Future studies in which Toc34 GTPase activity can be manipulated independently and in combination with Toc159 should provide more detailed insights into the role of GTP in regulating the import process.

Materials and methods

Plant materials and intact chloroplast isolation

Arabidopsis thaliana seedlings were grown on agar plates containing 0.5x Murashige and Skoog growth medium (MS medium), 1% sucrose under long-day condition at 23°C for 14 d. To isolate intact chloroplasts, a grinding method was adapted as described previously (Brock et al., 1993; Schulz et al., 2004). In brief, plants were homogenized in ice-cold GB buffer (50 mM Hapes-KOH, pH 7.5, 2 mM EDTA, 1 mM MnCl2, 1 mM MgCl2, 330 mM sorbitol, 100 mM ascorbic acid, 0.05% protease inhibitor cocktail (PIC; Sigma-Aldrich), and 0.25% (w/v) BSA) filtered through layers of miracloth. The chloroplasts were collected by centrifugation for 8 min at 1,000 g at 4°C. The chloroplasts were resuspended in 5 ml of GB buffer and applied to a Percoll step gradient (35%/85% Percoll in GB buffer). After centrifugation at 7,000 g for 15 min at 4°C, the intact chloroplasts were collected from the 35%/85% Percoll interface and washed once by diluting into 50 ml ice-cold HS buffer (50 mM Hapes-KOH, pH 8.0, and 330 mM sorbitol). The chloroplasts were collected by centrifugation at 1,000 g for 6 min at 4°C and resuspended in 300 μl HS buffer and kept on ice.

Toc159 mutations and deletion constructs

The atToc159-A864R point mutation was previously introduced into pET21d-atToc159 using the PCR-based overlap extension technique (Smith et al., 2002). A C-terminal 6-histidine tag containing a TEV protease site was introduced into the construct for convenience. For expression of atToc159-A864R-TEVhis in arabidopsis, the coding region was cloned into the XbaI-EcoRI sites of the binary vector, pSMB (Mylne and Botella, 1998) to yield pSMB-CaMV35S:atToc159-A864R.

Arabidopsis transformation and the detection of transgene expression

The homozygous ppi2 plants expressing wild-type atToc159 were described previously (Bauer et al., 2002). The pSMB-CaMV35S:atToc159-A864R construct was introduced into Arabidopsis thaliana (ecotype Wassilewskija) ppi2 heterozygous plants via Agrobacterium tumefaciens-mediated transformation by the floral dip method (Clough and Bent, 1998; Chua et al., 2002). Transformed plants were selected by resistance to BASTA (glufosinate ammonium), which is encoded by the transgene-linked marker, and the presence of the transgene was confirmed by PCR of genomic DNA.

For detecting transgene expression, isolated chloroplasts were analyzed directly by SDS-PAGE and immunoblot analysis. For atToc159-A864R chloroplast membrane localization, wild-type or atToc159-A864R chloroplasts were isolated from 2-wk-old atToc33 plants, followed by alkaline carbonate treatment (Li and Schnell, 2006). The proteins from soluble and membrane fractions were dissolved in SDS-PAGE sample buffer, resolved by SDS-PAGE, and immunoblotted using atToc159 antibody.

De-etiolation experiment

The de-etiolation experiment was performed as previously described (Fankhauser and Casal, 2004). In brief, plate-sown seeds were kept in the dark for 3 d at 4°C (stratification) and treated with a 1-h exposure to white light. Plants were germinated in the dark at 20°C for 23 h. Cotyledon seedlings were then placed under long-day growth conditions to initiate photophoresis and chloroplast development. Chlorophyll accumulation was monitored by the method of Arnon (Arnon, 1949) at 20 and 28 h after transferring the plants to light.

Coimmunoprecipitation of TOC core complex proteins

Isolated intact chloroplasts corresponding to 200 μg chlorophyll from atToc159 or atToc159-A864R transformed plants were dissolved in 50 mM Hapes-KOH, pH 7.5, 150 mM NaCl, 1% Triton X-100 containing 1% protease inhibitor cocktail at 4°C for 30 min with constant gentle shaking. After a 30-min centrifugation at 18,000 g, the supernatant was removed and incubated with 2 μg/ml affinity-purified atToc159 antibody overnight. The sample was incubated with 20 μl packed protein A-Sepharose beads (CL-4B; GE Healthcare) for 2 h. The solution was removed and the beads were washed three times with 50 ml washing buffer (50 mM Hapes-KOH, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 0.1% PIC). The bound protein was eluted directly into SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting with atToc159 antisera and affinity-purified atToc75 and atToc33 antibodies.

For immunoprecipitation under denaturing conditions, chloroplasts (150 μg chlorophyll) were dissolved in 2% SDS containing 2% PIC by vigorous mixing. The samples were heated for 5 min at 97°C and cooled to room temperature. The samples were diluted with 720 μl dilution buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 167 mM NaCl, 5.6 mM EDTA, and 1% PIC) and allowed to incubate on ice for 5 min. After a 30-min centrifugation at 18,000 g at 4°C, the supernatant was divided into two equal volumes and incubated with 2 μg/ml affinity-purified atToc159 A-domain antibody or atToc33 antibody for 1.6 h. 20 μl of packed protein A-Sepharose was added to each sample and incubated for 2 h at
4°C with constant rotation. The bound samples were washed and analyzed by SDS-PAGE.

**GTP hydrolysis**

GTP hydrolysis was measured using a modified method (Liang et al., 2000). Atfoc159, atfoc159-A864R, atfoc159-G151, and atfoc33G1m were in vitro translated using the TNT coupled T7 reticulocyte system (Promega) and labeled by [35S]methionine. 50 μl of each translation mixture was diluted into 500 μl NaH2 buffer (50 mM Hepes-KOH, pH 7.5, and 150 mM NaCl) containing 0.5% Triton X-100 and 0.5% P1C. Atfoc159 antibody (for atfoc159 and atfoc159 mutant proteins) or His6-specific antibody (for His6-tagged soluble atfoc 33 G1 protein) was added to be a 2 μg/ml final concentration and the mixtures were incubated overnight at 4°C with constant rotation followed by the addition of 20 μl protein A-Sepharose beads for 2 h. The beads were recovered and washed five times with 500 μl NaH2 buffer containing 0.1% Triton X-100. The beads were incubated with 10 mM ATP in NaH2 buffer for 30 min at 4°C. The atfoc159G protein was expressed in E. coli and purified as described previously (Smith et al., 2002). GTP hydrolysis reactions were performed on Sepharose-bound protein as previously described (Liang et al., 2000). The immunoprecipitated proteins were analyzed by SDS-PAGE and phosphor-imaging to determine the amount of immunoprecipitated GTPase used in each assay. The amounts were calculated based on the specific radioactivity of the in vitro translation products.

**Chloroplast import, binding, and translocation**

Chloroplasts corresponding to 10 μg chlorophyll or 25 μg of chlorophyll were used for import or binding reactions, respectively, as described previously (Chen et al., 2002). For the chase experiments, chloroplasts (200 μg chlorophyll) containing bound preprotein or the early import intermediate reactions were generated as above. Chloroplasts recovered after isolation over the 35% Percoll cushion were washed once and resuspended in import buffer. Preprotein translocation was initiated with the addition of 5 mM ATP and samples were incubated at 26°C for the times indicated. Where indicated, chloroplasts were first incubated for 5 min in the dark with 0.1 mM GTP-γS. All samples were resolved by SDS-PAGE and analyzed by phosphor-imaging. Equivalent amounts of chloroplasts based on chlorophyll content were loaded in all lanes. Where necessary, radioactivity from in vitro translation products was normalized to reflect differing numbers of methionine residues.

The kinetics of preSSu import were measured using increasing concentrations of purified E. coli expressed [35S]preSSu. The [35S]preSSu was denatured with 6 M urea and denatured into import buffer before the addition of chloroplasts to initiate the reaction. The maximum import velocity and apparent Km were calculated from the y-axis intercept and slope of the plot, respectively.

**Cross-linking reactions**

Chloroplast binding reactions were performed using intact chloroplasts corresponding to 200 μg of chlorophyll as described above. After dilution in ice-cold HS buffer and re-isolation through a 35% Percoll cushion, chloroplasts containing bound precursors were washed twice with HS buffer. The chloroplasts were resuspended in 475 μl HS buffer and 25 μl of DMSO-dissolved DTME (dithio-bismaleimidoethane; Thermo Fisher Scientific) was added to a final concentration of 2.5 μM. Cross-linking reactions were conducted on ice in the dark for 45 min. Glycine (50 mM final concentration in HS buffer) was then added to quench the reactions. Chloroplasts were pelleted and washed once with HS buffer before further manipulation. Immunoprecipitation reactions were performed under denaturing conditions as described above, and the amounts of precipitated receptors and bound preSSu were determined by quantitative immunoblotting and phosphorimaging analysis, respectively.

**Online supplemental material**

Fig. S1 shows Michaelis-Menten (Fig. S1 A) and rate (Fig. S1 B) analysis for GTP hydrolysis by full-length atfoc159 and atfoc159-A864R compared with the isolated atfoc159 G-domain and atfoc159 mutants known to lack GTPase activity. Fig. S2 shows the reduced degradation of atfoc159 and increased protein import efficiency of chloroplasts isolated using the optimized purification procedure. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200803034/DC1.

The authors would like to thank Matthew Smith (Wilted Lauer University, Canada), and Ming Li and Caleb Rounds (University of Massachusetts, Amherst, MA) for their technical assistance.

This work was supported by National Institutes of Health (NIH) grant GM61893 to D.J. Schnell and by the Swiss National Science Foundation (SNSF) grant 3100AO-109667 to F. Kessler.

Submitted: 7 March 2008

Accepted: 4 September 2008

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