Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane

Jörg Bauer, Andreas Hiltbrunner, Petra Weibel, Pierre-Alexandre Vidi, Mayte Alvarez-Huerta, Matthew D. Smith, Danny J. Schnell, and Felix Kessler

1Plant Physiology and Biochemistry Group, Institute of Plant Sciences, ETH Zürich, CH-8092 Zürich, Switzerland
2Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003
3BASF Plant Science GmbH, 67056 Ludwigshafen, Germany

Two homologous GTP-binding proteins, atToc33 and atToc159, control access of cytosolic precursor proteins to the chloroplast. atToc33 is a constitutive outer chloroplast membrane protein, whereas the precursor receptor atToc159 also exists in a soluble, cytosolic form. This suggests that atToc159 may be able to switch between a soluble and an integral membrane form. By transient expression of GFP fusion proteins, mutant analysis, and biochemical experimentation, we demonstrate that the GTP-binding domain regulates the targeting of cytosolic atToc159 to the chloroplast and mediates the switch between cytosolic and integral membrane forms. Mutant atToc159, unable to bind GTP, does not reinstate a green phenotype in an albino mutant (ppi2) lacking endogenous atToc159, remaining trapped in the cytosol. Thus, the function of atToc159 in chloroplast biogenesis is dependent on an intrinsic GTP-regulated switch that controls localization of the receptor to the chloroplast envelope.

Introduction

Approximately 2,000 different nuclear-encoded proteins must be imported to acquire photosynthetic capacity during chloroplast biogenesis (Cline, 2000). These proteins are synthesized in the cytosol as precursors with an NH₂-terminal transit peptide, specifying targeting to the chloroplast. Both chloroplast envelope membranes contain translocon complexes to facilitate import of precursor proteins. These are termed the translocon at the outer chloroplast membrane (Toc)* and translocon at the inner chloroplast membrane complexes (Keegstra and Cline, 1999; Chen et al., 2000b; Schleiff and Soll, 2000; Bauer et al., 2001). The Arabidopsis–Toc complex consists of the three major components forming a trimeric complex (Hiltbrunner et al., 2001b). AtToc159 (the number indicates the molecular mass in kDa) and AtToc33 are surface-exposed, integral membrane GTP-binding proteins sharing highly conserved GTP-binding domains (G-domains; Hiltbrunner et al., 2001a; Jackson-Constan and Keegstra, 2001). The pea homologue of atToc75, the third component of the Toc complex, forms at least part of a hydrophilic channel through which precursors are translocated across the outer membrane (Hinnah et al., 1997).

Evidence, obtained with isolated chloroplasts, indicates that atToc33 and atToc159 act concertedly in the recognition of the chloroplast-targeting signal (transit peptide). Based on direct cross-linking to transit sequences and inhibition of precursor binding by specific antibodies, pea Toc159 is thought to function as a primary precursor receptor at the chloroplast surface (Perry and Keegstra, 1994; Ma et al., 1996). The pea homologue of atToc33 (psToc34) has also been shown to specifically interact with the transit peptide of precursor proteins, and may therefore contribute to precursor receptor function (Sveshnikova et al., 2000; Schleiff et al., 2002). Moreover, GTP binding and hydrolysis at psToc34 regulate insertion of precursor proteins across the outer membrane.
membrane (Chen et al., 2000a). In Arabidopsis, atToc159 is essential for viability. The Arabidopsis ppi2 mutant, disrupted in TOC159, has an albino phenotype coincident with the absence of differentiated chloroplasts. Furthermore, the failure of ppi2oplasts to accumulate photosynthetic proteins in vivo is consistent with a defect in protein import (Bauer et al., 2000). Disruption of atToc33 has a less pronounced effect, leading to a pale green phenotype. The phenotype is indicative of a role in chloroplast biogenesis, but persists only during early seedling development (Jarvis et al., 1998).

AtToc159 has a tripartite structure, consisting of an NH2-terminal acidic domain (A-domain), the G-domain, and the COOH-terminal membrane domain (M-domain; Bauer et al., 2000; Chen et al., 2000a). The function of the A-domain is unknown. The M-domain anchors atToc159 in the outer chloroplast membrane while lacking predicted membrane-spanning α-helices. The G-domain binds GTP specifically, but its functional significance is not yet understood. We have previously demonstrated that atToc159 exists in a complex with atToc33 and atToc75 (Hiltbrunner et al., 2001b). However, in contrast to atToc33 and atToc75, about half of the protein exists in a soluble, cytosolic form (Hiltbrunner et al., 2001b). Soluble atToc159 binds directly to atToc331–265, rendered soluble by deletion of the COOH-terminal transmembrane region. Furthermore, atToc331–265 competed for outer membrane insertion of atToc159 in isolated Arabidopsis chloroplasts. The results suggest that atToc33 functions as a receptor in the assembly of soluble atToc159 into the Toc complex (Hiltbrunner et al., 2001b). Support for this hypothesis stems from the crystal structure of pea Toc34 (Sun et al., 2002). Pea Toc34 forms homodimers when in the GDP-bound state. Conservation of the G-domains and the novel dimerization motifs in atToc159 as well as atToc33 suggests that the two proteins may form heterodimers via their G-domains, thus providing a structural basis for targeting of cytosolic atToc159 directly to the Toc complex (Kessler and Schnell, 2002; Sun et al., 2002). Here, we demonstrate that the functional G-domain is sufficient and necessary to target cytosolic atToc159 to the outer chloroplast membrane.

Results

Expression and targeting of atToc159 domain GFP fusions in living Arabidopsis cells

To determine the domains required for targeting of cytosolic atToc159 to the chloroplast, we engineered constructs encoding a series of NH2-terminal GFP fusions under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The constructs were transformed into living Arabidopsis protoplasts and analyzed by confocal laser scanning microscopy (Fig. 1). Due to the length and instability of the full-length atToc159 cDNA (Bölter et al., 1998; Chen et al., 2000a), we were unable to obtain a construct encoding the corresponding GFP fusion. However, a T-DNA construct encoding the combined G- and M-domains (atToc159GM) complemented the ppi2 mutant lacking atToc159, suggesting that atToc159GM is largely functional (unpublished data). Therefore, we fused GFP to the NH2 terminus of a construct corresponding to the G- and M-domains in which the A-domain is replaced by GFP (GFP-atToc159GM). To confirm that GFP-atToc159GM behaves like full-length endogenous atToc159, we performed a Western blotting experiment on fractions of transiently transformed protoplasts using mAbs against GFP (α-GFP), as well as antibodies against the A-domain to detect endogenous atToc159 (Fig. 1 A; α-atToc159). Both GFP-atToc159GM (detected by α-GFP) and endogenous atToc159 (detected by α-atToc159) were present in the carbonate-extracted total membrane fraction and to a lesser degree in the soluble fraction. In contrast, GFP was present almost exclusively in the soluble frac-
Role of the G-domain in atToc159 targeting | Bauer et al.

GTP-binding and -hydrolysis measurements of atToc159mGTP

Targeting of the fusion between GFP and the G-domain to the chloroplast (Fig. 1 B) suggests that the G-domain directs atToc159 to the organelle. Therefore, it also appears probable that the targeting and distribution of atToc159 is regulated by guanosine nucleotide binding and/or hydrolysis. To determine the role of GTP binding to atToc159, a mutant protein (atToc159mGTP) containing three-point mutations in the G1 motif (A864R, K868N, and S869R) was designed (Fig. 2 A). The mutations are predicted to strongly reduce both guanosine nucleotide binding and hydrolysis (Chen and Schnell, 1997). To determine GTP-binding and -hydrolysis activities, we expressed the G-domain of wild-type atToc159 (159G) as well as that of atToc159mGTP (159G-mGTP) as hexahistidinyl-tagged proteins in *Escherichia coli*. Isolated G-domains were used because functional expression of full-length atToc159 in *E. coli* was not feasible due to the instability of the protein and low levels of expression. However, we expect the mutations to have the same relative effects on the isolated G-domain as on the full-length protein. The recombinant G-domains were purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography. GTP binding to the isolated G-domains was measured using a solid-phase GTP overlay assay. In this assay, the purified proteins were spotted onto nitrocellulose and incubated in buffer containing α-[32P]GTP (3,000 Ci/mmol) in the presence of 1 μM ATP. Bound α-[32P]GTP was quantitated using a phosphorimager. Error bars indicate SD. (C) GTP hydrolysis by wild-type and mutant atToc159 G-domains. 1 μM α-[32P]GTP (150 mCi/μmol) was incubated with 0.5 μM 159G or 159G-mGTP for 60 min at 25°C. Radionabeled GTP and GDP were resolved by TLC on PEI-cellulose F plates using 1 M LiCl as the solvent, and radioactivity was quantitated using a phosphorimager. Error bars indicate SD. N.D., not detectable above background.

Functional dissection of atToc159 in vivo

AtToc159 is essential for the onset of photoautotrophic growth, and hence, plant viability (Bauer et al., 2000). To determine whether the G-domain contributes to the essential function, an in vivo complementation assay was designed. The assay relies on the restoration of the wild-type green phenotype in the albino *ppi2* mutant (lacking wild-type atToc159 due to T-DNA insertion) as the criteria for complementation. We engineered T-DNA constructs (conferring phosphinothricine resistance) encoding either full-length wild-type atToc159 (atToc159wt) or atToc159mGTP under the control of the CaMV 35S promoter. The constructs were introduced into plants heterozygous for the *ppi2* mutation (conferring kanamycine resistance) by agrobacterium-mediated transformation (Bechtold and Pelletier, 1998). *ppi2* plants transgenic for either atToc159wt or atToc159mGTP were identified by dual phosphinothricine and kanamycine selection. We determined the *ppi2* background of plants carrying either of the transgenic constructs by PCR. Three lines, each carrying either heterozygous (B0101, B0201, B0502, B1105, or...
B0505) or homozygous (B0205) ppi2 mutations as well as either the atToc159wt or the atToc159mGTP transgene, were chosen for segregation analysis (Fig. 3 A). Complementation was scored by determining the ability of transgenic atToc159wt or atToc159mGTP to restore a wild-type, green phenotype in plants expressing atToc159wt (Fig. 3 A). PCR analysis of the background using RT-PCR. (C) Control PCR on total RNA after DNase treatment, but without reverse transcription.

To substantiate the results of the segregation analysis, we molecularly characterized the complementation lines (Fig. 4). PCR analysis using transgene-specific primers demonstrated that the T-DNA constructs encoding atToc159wt or atToc159mGTP were indeed present in the lines analyzed (Fig. 4 A). PCR analysis of the ppi2 background showed that albino plants of line B0502 carrying the atToc159mGTP construct were homozygous for ppi2 (Fig. 4 A, middle), whereas green plants were heterozygous (Fig. 4 A, left). Therefore, the PCR analysis confirms the inability of the atToc159mGTP construct to complement ppi2. On the other hand, green plants of line B0205 carrying the atToc159wt construct were homozygous for ppi2 (Fig. 4 A, right), confirming complementation by the construct.

To rule out that the failure of the atToc159mGTP construct to complement ppi2 was due to a lack of gene expression, RT-PCR experiments were done on transgenic plants homozygous for ppi2 (Fig. 4 B and C). The RT-PCR analysis using transgene-specific primers indicate that in albino ppi2 plants carrying the atToc159mGTP construct, atToc159mGTP (Fig. 4 B, 159mGTP) was at least as strongly expressed as atToc159wt (Fig. 4 B, 159wt) in green ppi2 plants carrying the atToc159wt construct (Fig. 4 B). In a control experiment (Fig. 4 C) in which the RNA was pretreated with DNase and not reverse transcribed, PCR failed to yield any products, indicating that the RT-PCR products (Fig. 4 B) were not due to amplification of genomic DNA. The RT-PCR experiments demonstrate that the inability of atToc159mGTP construct to complement ppi2 is not due to a lack of transgene expression.

The presence of transgene-encoded proteins was determined by Western blotting using affinity-purified antibodies specific to the A-domain of atToc159 (Fig. 5). Albino ppi2 plants (line B0502) carrying the atToc159mGTP construct expressed the corresponding protein (Fig. 5 A, lane 1). However, atToc159mGTP was present at somewhat lower levels than atToc159wt in homozygous ppi2 plants (Fig. 5 A, lane 3) or than total atToc159 in heterozygous ppi2 plants expressing atToc159mGTP (Fig. 5 A, lane 2). Nevertheless, the data suggest that the inability of atToc159mGTP to complement the ppi2 phenotype is
probably not due to a lack of protein production or protein stability, but is rather due to the inability of the mutant G-domain to bind and hydrolyze guanosine nucleotides.

The functional significance of the atToc159–GTPase cycle is currently not known. However, the transient expression of GFP fusion proteins suggests that the G-domain is required for targeting of cytosolic atToc159 to the chloroplast outer membrane and may regulate partitioning between subcellular compartments (Fig. 1). To determine whether the subcellular distribution of atToc159mGTP is affected when compared with atToc159wt, we separated homozygous ppi2 plants expressing atToc159mGTP as well as homozygous ppi2/TOC159, lane 2) ppi2 plants expressing atToc159mGTP as well as homozygous ppi2/2ppl2) ppi2 plants expressing atToc159mGTP (lane 3). 50 µg of protein each were separated by SDS-PAGE, blotted onto nitrocellulose and stained with amido black (bottom). The blot was probed with affinity-purified antibodies raised against the A-domain of atToc159 (top). RbcL, large subunit of Rubisco. (B) Distribution of atToc159mGTP (159mGTP) and atToc159wt (159wt) between an alkaline-extracted membrane fraction (M, lanes 1 and 3) and a soluble fraction (S, lanes 2 and 4) prepared from homozygous ppi2 plants (ppi2/2ppl2) expressing atToc159mGTP (lanes 1 and 2) or atToc159wt (lanes 3 and 4), respectively. 20 µg of protein each were separated by SDS-PAGE, blotted onto nitrocellulose, and stained with amido black (bottom). The blot was probed with affinity-purified antibodies raised against the A-domain of atToc159 (top). RbcL, large subunit of Rubisco.

suggest that atToc159mGTP is not properly targeted to plastids, and is therefore unable to integrate into the outer chloroplast membrane in vivo.

**Insertion of atToc159 into the outer chloroplast membrane requires the intact GTP-binding site**

The presence of atToc159mGTP in the soluble fraction, as well as its absence from carbonate-extracted membranes, suggests that the GTP binding and hydrolysis at the G-domain may be required not only for chloroplast targeting, but also for membrane insertion. Therefore, in vitro chloroplast-targeting experiments were performed using either synthetic [35S]atToc159wt or [35S]atToc159mGTP as the substrate. Association of [35S]atToc159wt or [35S]atToc159mGTP with isolated Arabidopsis chloroplasts and formation of the 52-kD protease-resistant fragments on membrane insertion (Hirsch et al., 1994; Kessler et al., 1994; Bauer et al., 2000) were used as criteria for correct targeting and insertion of atToc159, respectively (Bauer et al., 2000). Here, association is defined as the sum of peripherally bound and inserted [35S]atToc159, whereas the 52-kD fragment reflects only the membrane-inserted protein. Both [35S]atToc159wt (Fig. 6 A, lane 1) and [35S]atToc159mGTP (Fig. 6 A, lane 5) were synthesized in a cell-free reticulocyte lysate and subsequently incubated with isolated Arabidopsis chloroplasts. Aliquots of reisolated chloroplasts that had been incubated with either [35S]atToc159wt (Fig. 6 A, lane 2) or [35S]atToc159mGTP (Fig. 6 B, lane 6) were either directly analyzed by SDS-PAGE and autoradiography (to determine chloroplast association) or treated with protease (thermolysin; Fig. 6 A, lanes 3, 4, 7, and 8) before analysis (to determine membrane integration). [35S]atToc159wt associated with isolated chloroplasts, and on treatment with the protease thermolysin yielded a 52-kD fragment, indicating the expected func-
tionality of the wild-type protein. In contrast, mutant \([^{35}\text{S}]\text{atToc159mGTP}\) associated with chloroplasts to a much lower degree (~30% of wild type; Fig. 6 B, Chloroplast Association), and formation of the 52-kD fragment was reduced to ~10% of the \([^{35}\text{S}]\text{atToc159wt}\) control (Fig. 6 B, 52-kD Fragment). Thus, both targeting to the chloroplast and insertion into the outer membrane may be affected in \([^{35}\text{S}]\text{atToc159mGTP}\).

It can likely be ruled out that the targeting and insertion defects of atToc159mGTP were due to other reasons than the guanosine nucleotide–binding deficiency. Smith et al. (2002) report additional experiments in the accompanying paper in which GTP was removed from \([^{35}\text{S}]\text{atToc159wt}\) by incubation in the presence of EDTA and subsequent gel filtration before the in vitro targeting experiment. In a control experiment, \([^{35}\text{S}]\text{atToc159wt}\) was incubated and subjected to gel filtration in the presence of Mg\(^{2+}\) and GTP. Both association and formation of the protease-protected M-domain fragments of EDTA-treated \([^{35}\text{S}]\text{atToc159wt}\) were strongly reduced when compared with the Mg\(^{2+}\) and GTP-treated sample. Therefore, the results obtained with EDTA-treated \([^{35}\text{S}]\text{atToc159wt}\) are in agreement with the data obtained for \([^{35}\text{S}]\text{atToc159mGTP}\), suggesting that its targeting and insertion defect correlates with the guanosine nucleotide–binding deficiency.

### Interaction between atToc159 and atToc33 may be regulated by GTP binding at atToc159

We have previously demonstrated that the soluble G-domain of atToc33 (atToc33\textsubscript{1–265-H6}) binds directly to atToc159 and prevents its association with the chloroplast (Hiltbrunner et al., 2001b). Thus, it appears that targeting of atToc159 to the chloroplast outer membrane involves its interaction with atToc33. The presence of dimerization motifs in the G-domains of both atToc159 and atToc33 (Sun et al., 2002) suggests that the interaction between the proteins involves the G-domains and may possibly be regulated by GTP binding and hydrolysis. To determine whether atToc159mGTP is affected in its ability to interact with atToc33, equivalent concentrations of either \([^{35}\text{S}]\text{atToc159mGTP}\) or \([^{35}\text{S}]\text{atToc159wt}\) were incubated with increasing concentrations of a recombinant soluble version of atToc33 (atToc33\textsubscript{1–265-H6}) lacking a short COOH-terminal transmembrane region (Fig. 7 A: Hiltbrunner et al., 2001b). As a control, we used the in vitro synthesized \(^{35}\text{S}\)-labeled A-domain (atToc159\textsubscript{1–731}). The transient expression experiments (Fig. 1 D) indicated that the fusion between the A-domain and GTP remained cytosolic, suggesting that the A-domain may not directly interact with atToc33, atToc33\textsubscript{1–265-H6}, together with any bound atToc159 or A-domain, was reisolated by Ni-NTA agarose chromatography. The eluates were analyzed by SDS-PAGE followed by autoradiography and phosphorimager quantitation (Fig. 7 B). The data show that dose-dependent binding of \([^{35}\text{S}]\text{atToc159mGTP}\) to atToc33 is reduced by up to threefold when compared with that of the wild type. Neither of the proteins bound to Ni-NTA resin significantly in the absence of atToc33\textsubscript{1–265-H6}, confirming that both mutant and wild-type atToc159 had directly bound to atToc33 (Fig. 7 A, lanes 1 and 5). Furthermore, \([^{35}\text{S}]\text{atToc159}_{1–731}\) gave no detectable binding to either the Ni-NTA resin alone or to atToc33\textsubscript{1–265-H6}, supporting the notion that the A-domain does not play a role in chloroplast targeting of atToc159. Moreover, these results suggest that the binding of \([^{35}\text{S}]\text{atToc159wt}\) and \([^{35}\text{S}]\text{atToc159mGTP}\) to atToc33\textsubscript{1–265-H6} is indeed specific. The reduced binding of atToc159mGTP to atToc33\textsubscript{1–265-H6} suggests that the interaction of the two proteins is indeed regulated by GTP binding and hydrolysis. The M-domain is also likely to participate in targeting of the receptor to the Toc complex. In addition, other Toc components that are not included in this in vitro binding assay, such as Toc75, may be involved in the targeting reaction.

### Discussion

The trimeric Toc complex contains two homologous GTPases (atToc33 and atToc159) in addition to atToc75, a protein-conducting channel component. In contrast to atToc33 and atToc75, which exclusively behave as integral outer membrane proteins, atToc159 also exists in a cytosolic form (Hiltbrunner et al., 2001b). Targeting of soluble, cytosolic atToc159 involves an interaction with atToc33 at the chloroplast surface. The presence of novel dimerization motifs in the G-domains of both atToc159 and atToc33 supports a role for atToc33 as an atToc159 receptor, and potentially provides a structural basis for targeting of cytosolic atToc159 to the outer chloroplast membrane via heterodimer formation between the G-domains (Sun et al., 2002). In this paper, we demonstrate that the G-domain of atToc159 mediates and likely regulates chloroplast targeting by GTP binding and hydrolysis. To determine which of the domains of atToc159 are required for targeting to the chloroplast, a series of GFP fu-
sions was engineered and transiently expressed in isolated *Arabidopsis* protoplasts. Technical difficulties prohibited the construction of a fusion between full-length atToc159 and GFP. However, a T-DNA construct encoding the combined G- and M-domains complements the *ppi2* mutant lacking atToc159, suggesting that the truncated protein without the A-domain is largely functional (unpublished data). Therefore, not surprisingly, GFP, fused NH2-terminally to the combined G- and M-domains (GFP-atToc159GM), was present both at the chloroplast periphery and in the cytosol (Fig. 1 B), recapitulating the partitioning of endogenous atToc159 observed in earlier fractionation experiments and immunofluorescence (Hilbrunner et al., 2001b). Therefore, these data provide additional evidence for the functionality of the truncated protein lacking the A-domain. Moreover, GFP-atToc159GM corresponds to 'Toc86', the proteolytic fragment atToc159 originally described in Hirsch et al. (1994), Kessler et al. (1994), and Schnell et al. (1994). The data shown here confirm that Toc86 may represent a targeting-competent form of atToc159 (Muckel and Soll, 1996). A fusion protein between the G-domain and GFP was also efficiently targeted to the chloroplast periphery of isolated *Arabidopsis* protoplasts (Fig. 1 C). This finding indicates that the G-domain is sufficient for targeting to the chloroplast. In contrast, GFP fusions to the A- and M-domains, respectively, were predominantly present in the cytosol on transient expression in protoplasts, suggesting that neither the A- nor the M-domain are sufficient for targeting. In the case of the A-domain, a role in targeting was not expected; pea Toc159 lacking the A-domain has been previously shown to be targeted and inserted into the chloroplast outer membrane in vitro (Muckel and Soll, 1996). The M-domain GFP fusion protein was evenly distributed in the cytosol, suggesting that it may behave as a soluble protein, although it was originally identified as the integral M-domain (Hirsch et al., 1994). This appears possible, as the primary sequence of the M-domain consists mostly of hydrophilic residues and does not contain predicted hydrophobic transmembrane helices. In the integral membrane form of atToc159, however, the M-domain is resistant both to alkaline extraction and protease treatment. Therefore, the M-domain may conformationally switch to expose a hydrophobic region. Conceivably, this switch may underlie membrane insertion, and may ultimately be coupled to a GTPase-regulated conformational switch taking place at the G-domain.

Transient expression of GFP fusion proteins in isolated *Arabidopsis* protoplasts suggest that the G-domain controls targeting of cytosolic atToc159 to the chloroplast. To analyze the role of GTP binding to atToc159, we engineered a mutant of atToc159 (Fig. 2 A, atToc159mGTP) that is almost completely unable to bind and hydrolyze GTP (Fig. 2 B) when compared with the wild type. AtToc159mGTP was inactive in an in vivo complementation assay in which the albino *ppi2* mutant lacking atToc159 was transformed with constructs encoding atToc159mGTP or wild-type atToc159 (atToc159wt), respectively (Fig. 3). Though homozygous *ppi2* plants expressing the atToc159mGTP protein were obtained, the mutant protein, in contrast to the wild type, failed to restore the normal green phenotype (Fig. 4 A). Thus, the functional G-domain of atToc159 is essential for chloroplast biogenesis.

To further characterize the defect of atToc159mGTP, *ppi2* plants expressing either atToc159mGTP or atToc159wt were fractionated into soluble and integral membrane fractions. Although atToc159wt partitioned between the two fractions, atToc159mGTP was present exclusively in the soluble fraction of *ppi2* plants. The result suggests that atToc159mGTP is neither properly targeted nor inserted into the outer membrane (Fig. 4 B), thereby providing further evidence for the role of the G-domain. Furthermore, the nature of the mutations in atToc159mGTP suggests that GTP binding and hydrolysis by the G-domain may regulate targeting and membrane insertion. In agreement with the in vivo fractionation experiment, wild-type atToc159, but not synthetic atToc159mGTP, was able to insert into the outer membrane of isolated chloroplasts (Fig. 6 A). A marked reduction of association with the chloroplast outer membrane was also observed (Fig. 6, A and B). Moreover, synthetic atToc159wt, from which GTP had been biochemically removed, also did not associate with isolated chloroplasts efficiently and did not insert into the outer membrane in vitro (Smith et al., 2002). The reduced association may reflect a decreased affinity for either atToc33 or other outer membrane components. This conclusion is supported by in vivo binding data, demonstrating that atToc159mGTP binds to the soluble G-domain of atToc33 less efficiently than the wild type (Fig. 7). In this experiment, the A-domain showed no detectable interaction with the soluble G-domain of atToc33, suggesting that the A-domain does not contribute to the interaction between atToc159 and atToc33. In summary, the results suggest that the G-domain of atToc159 controls targeting of the cytosolic form to the outer membrane in a two-step reaction in which binding to outer membrane receptors precedes membrane insertion.

Questions regarding the function of cytosolic atToc159 remain open. However, targeting of cytosolic atToc159 to the chloroplast outer membrane most likely does not reflect the maturation of an immature cytosolic precursor version of the protein, as is the case during the biogenesis of other known chloroplast outer membrane proteins (Schleiff and Klösgen, 2001). Our results suggest that atToc159 must be in an active form in the cytosol, as its targeting requires the correctly folded protein to be able to bind GTP. GTP-regulated targeting of atToc159 to the outer membrane represents a novel, possibly reversible targeting mechanism to the outer chloroplast membrane. This is in contrast to other studied proteins that spontaneously insert into the outer membrane (Schleiff and Klösgen, 2001) or are targeted to the outer membrane by a mechanism involving a bipartite transit sequence, as is the case for pea Toc75 (Tranel and Keegstra, 1996). As cytosolic atToc159 is likely a functional protein, it is tempting to speculate that it binds to newly synthesized precursors, escorting them to the Toc complex and promoting their subsequent import into the stroma. After precursor delivery, atToc159 may deinsert from the outer membrane and undergo further precursor targeting cycles. Current research is directed at the elucidation of the function of soluble atToc159.
Materials and methods

DNA constructs used in protein synthesis

pET21d-atToc159G and pET21d-atToc159Gm-GTP used for the production of recombinant 159G and 159Gm-GTP, respectively, were obtained as follows: using a forward primer (5'-CAGCCTGTCGAAATCTCATGACGGCGATCG-GCATCACTACCATCACATGATTCGTAGGTCGAGAGCCTACATGATGCAGTTGCAATAGGAA-3') including a SpeI site instead of the NcoI site in the PCR-Script® (Stratagene) vector, resulting in the coding sequence under GAGATGTTATG-3/H11032 in cloning vectors used in the complementation assay. In brief, the 3.9-kb NcoI fragments of pET21d-atToc159 or pET21d-atToc159mGTP were ligated into the Ncol site of pTD31 (Tinland et al., 1995), resulting in the constructs pTD31-atToc159Ncol and pTD31-atToc159mGTPNcol, respectively. By ligating a Stul-XbaI fragment of pET21d-atToc159 into the corresponding sites of pTD31-atToc159Ncol/pTD31-atToc159mGTPNcol, the complete sequences were obtained. The resulting constructs were named pTD31-atToc159 and pTD31-atToc159mGTP. From the two pTD31 constructs, the promoter (CaMV 35S) gene-terminating constructs were named pTD31-atToc159G and pTD31-atToc159mGTP. The promoter (CaMV 35S) gene-terminating constructs were named pTD31-atToc159G and pTD31-atToc159mGTP. For isolation of total soluble and membrane protein, plants were subjected to a 24-hr incubation period. Therefore, samples were removed after 0 min and 60 min of hydrolysis, and the reactions were stopped by adding 1 ml HS (330 mM sorbitol and 50 mM Hepes-KOH, pH 7.5) for 5 min in 0.2% wt/vol SDS, 10 mM EDTA, 4 mM GTP, and 4 mM GDP. The samples were spotted onto PEI cellulose-F TLC plates (EM Science), and GTP and GDP were resolved using 1 M LiCl solvent. The samples were dried and radiolabeled spots of GTP and GDP were quantified using a Storm 840 phosphorimager. Data are presented as fmol of α-[32P]GTP bound per protein pmol in min⁻¹. Hydrolysis of GTP by soluble E. coli proteins that nonspecifically bound to Ni-NTA resin was used as a background reference.

Preparation of plant protein extracts and subfractions

Isolation of total protein was done as described elsewhere (Rensing et al., 1998). For isolation of total soluble and membrane protein, plants were grown in liquid nitrogen. TE (50 mM Tris-HCl, pH 7.5, and 2 mM EDTA) was added and the homogenate was filtered and centrifuged at 100,000 g at 4°C for 30 min. The supernatant was considered total soluble protein. To obtain total membrane protein samples, the pellet was resuspended in 50 mM Tris-HCl, pH 7.5. For carbonate extraction, the pellet was resuspended in 0.2 M NaCO₃ and centrifuged at 100,000 g at 4°C for 30 min. The supernatant was removed and the pellet subjected to a second round of carbonate extraction. The final pellet was considered total integral membrane protein and resuspended in 50 mM Tris-HCl, pH 7.5. All samples were concentrated by CHCl₃/MeOH precipitation (Wessel and Flügge, 1988) and used for SDS-PAGE and immunoblotting according to standard protocols. HRP-coupled secondary antibody (Bio-Rad Laboratories) and enhanced chemiluminescence reaction (Roche) were used for immunodetection.

Chloroplast isolation and insertion assay

A. thaliana plants var. Columbia 2 were grown under long day conditions (16 h light, 8 h dark) on agarose plates (0.8% wt/vol) containing 0.5% Murashige-Skoog medium supplemented with 1% wt/vol sucrose, and were used for isolation of intact chloroplast as described previously (Fitpatrick and Keegstra, 2001), but with minor modifications. 1% wt/vol cellulase (Serva) was used instead of 4%, and 0.25% wt/vol macerozyme (Yakult Honsha Co., Ltd.) instead of 1%. For purification of intact chloroplasts, a 40%–85% wt/vol Percoll step gradient was used rather than a linear gradient.

Chloroplasts corresponding to 15 μg chlorophyll were used for insertion assays. Insertion assays contained import buffer, 2.5 mM ATP, 1 mM GTP, and 8 μl in vitro translate in a final volume of 100 μl. The insertion reaction was started by adding the in vitro translate, incubated for 15 min at 25°C, and stopped by adding 1 ml HS (330 mM sorbitol and 50 mM Hapes/KOH, pH 7.5). Chloroplasts for insertion assays in the absence of GTP were depleted of endogenous nucleotides for 20 min at KT in darkness. The insertion reaction was done in darkness, too. ATP and GTP were omitted from these reactions; instead, 5 mM EDTA was added.

Chloroplasts used for thermolysin treatment were washed in HS. Thermolysin digest was performed in HS supplemented with 30 mM CaCl₂ for 15 min on ice.

GFP constructs and transient transformation of A. thaliana protoplasts

Plants used for protoplast isolation were grown as described above. Transient transformation of A. thaliana protoplasts was done as described elsewhere (Liu et al., 2001) using the following modifications: 1 and 4%, respectively, we used only 1% wt/vol cellulase (Serva) and 0.25% wt/vol macerozyme (Yakult Honsha Co., Ltd.) for transient expression of ToC159G, ToC159M, and ToC159GM-NH₂-terminally fused to the COOH terminus of GFP, we used pSH11, a derivative of pC60 (Dr. C. Lupi, Institute of Plant Sciences, ETH Zürich). pC60 is a pBluescript-based construct consisting of the GFP under the control of the CaMV 35S promoter and the nos terminator. pSH11 was derived from pC60 by omitting the stop codon of GFP and inserting a NotI site at the 3' end of GFP. cDNA fragments coding for atToc159G, atToc159M, and atToc159GM were obtained by PCR us-
ing 5′ and 3′ primers including a NotI site (5′ primer for atToc159G and atToc159GM: 5′-AAGGAAAAAACGGCGCCACACTGGCTAAGCTTTACGAC-3′; 5′ primer for atToc159M, 5′-AAGGAAAAAACGGCGCCACACTGGCTAAGCTTTACGAC-3′; 3′ primer for atToc159G, 5′-AAGGAAAAAACGGCGCCACACTGGCTAAGCTTTACGAC-3′; 3′ primer for atToc159M and atToc159GM, 5′-AAGGAAAAAACGGCGCCACACTGGCTAAGCTTTACGAC-3′). The respective PCR products were then ligated into the NotI site of pCL60, resulting in pCL60-atToc159A-GFP. For transient expression with pSH11, the corresponding PCR fragment (−579 to +120 of the GUS gene) with a NotI site was added, resulting in pSH11-atToc159A-GUS. The respective PCR products were then ligated into the NotI site of pSH11, resulting in pSH11-atToc159A-GFP. For Western blot analysis, protoplasts transiently expressing GFP or the GFP-(CATGGACAAACCAGCAGGTCGATCCA-3′) fusion protein were collected by centrifugation at 100,000 g.

Role of the G-domain in atToc159 targeting | Bauer et al. 853

DNA extraction and diagnostic PCR
DNA PCR on Arabidopsis plants was isolated from single leaves according to the following procedure: one leaf was ground in 500 μl extraction buffer (0.2 M Tris-HCl, pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% wt/vol SDS). After 5′ centrifugation at 16,000 g at 4°C, the supernatant was transferred to an equal volume of isopropanol and centrifuged for 30 min at 16,000 g at RT. The supernatant was discarded and the pellet washed with 80% vol/vol ethanol, air dried, and resuspended in 50 μl water. The DNA was used for PCR reactions according to standard protocols. The following primers were used to identify individual Toc159 knockout plants: (pp22; 5′-GGATGTTTTAAAATCCGAGAAG-3′ and 5′-CATCCTGAGTTTACGATGACG-3′) and plants carrying the transgene (Toc159 and Toc159GTP, respectively) under the control of the 35S CaMV promoter and nos terminator (5′-CAATCCGACCTGAGTTTACG-3′ and 5′-CATGATGGACTCAAGTACG-3′). For detection of the transgene, the primers 5′-CCATGACTGGAATGATAACG-3′ and 5′-CTGGCAGGTCCGACCATAG-3′ were used as positive control.

RT-PCR experiments
Total RNA was isolated from Arabidopsis plants using the RNeasy Plant Mini Kit (QIAGEN). Preceding reverse transcription RNA was treated with 5 U RQ1 Dnase (Promega) in presence of 40 U Rnase Inhibitor (Promega). After inactivation of Dnase by heating, first-strand synthesis was performed as described elsewhere (Gubler and Hofmann, 1983). The cDNA was directly used for PCR according to standard protocols. The primers 5′-CATGATGGACTCAAGTACG-3′ and 5′-CAATCCGACCTGAGTTTACG-3′ were used for detection of the transgene, and under the control of the 35S CaMV promoter and nos terminator (5′-CAATCCGACCTGAGTTTACG-3′ and 5′-CATGATGGACTCAAGTACG-3′) were used in a control reaction to amplify part of ats1B (GenBank/EMBL/DDBJ accession no. X14564).

This work is dedicated to professor Nikolaus Anreisz on the occasion of his 60th birthday. The authors would like to thank Martha Stadler-Waibel for technical assistance and Nikolaus Anreisz for support.

This work was supported by Swiss National Science Foundation grant 3100-05602/7 and ETH grant TH-23/01-3 to F. Kessler, and National Institutes of Health grant GM61893 to D.J. Schnell.

Submitted: 2 August 2002
Revised: 21 October 2002
Accepted: 21 October 2002

References
Bauer, J., K. Chen, A. Hilbrunner, E. Wehli, M. Eugster, D. Schnell, and F. Kessler. 2000. The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature. 403:203–207.

Bauer, J., A. Hilbrunner, and F. Kessler. 2001. Molecular biology of chloroplast biogenesis: gene expression, protein import and intraorganellar sorting. Cell. Mol. Life Sci. 58:420–433.

Bechhold, N., and G. Pelletier. 1998. In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol. Biol. 82:259–266.

Böltler, B., T. May, and J. Soll. 1998. A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. FEBS Lett. 441:59–62.

Chen, D., and D.J. Schnell. 1997. Insertion of the 54-kDa chloroplast protein import component, IAP34, into the chloroplast outer membrane is dependent on its intrinsic GTP-binding capacity. J. Biol. Chem. 272:6614–6620.

Chen, K., X. Chen, and D.J. Schnell. 2000a. Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. Plant Physiol. 122:813–822.

Chen, K., X. Chen, and D.J. Schnell. 2000b. Mechanism of protein import across the chloroplast envelope. Biochem. Soc. Trans. 28:485–491.

Cline, K. 2000. Gateway to the chloroplast. Nature. 403:148–149.

Connolly, T., and R. Gilmore. 1993. GTP hydrolysis by complexes of the signal recognition particle and the signal recognition particles receptor. J. Cell Biol. 125:799–807.

Fitzpatrick, L.M., and K. Keegstra. 2001. A method for isolating a high yield of Arabidopsis chloroplasts capable of efficient import of precursor proteins. Plant J. 27:59–65.

Gubler, U., and B.J. Hoffmann. 1983. A simple and very efficient method for generating cDNA libraries. Gene. 25:263–269.

Hilbrunner, A., J. Bauer, M. Alvarez-Huerta, and F. Kessler. 2001a. Protein translocation at the arabidopsis outer chloroplast membrane. Biochem. Cell Biol. 79:1–7.

Hilbrunner, A., J. Bauer, P.-A. Vidi, S. Infanger, P. Weibel, M. Holdway, and F. Kessler. 2001b. Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. J. Cell Biol. 154:309–316.

Hinna, S.C., K. Hill, R. Wagner, T. Schlicher, and J. Soll. 1997. Reconstitution of a chloroplast protein import channel. EMBO J. 16:7351–7360.

Hirsch, S., E. Muckel, F. Heemeyer, G. von Heijne, and J. Soll. 1994. A receptor component of the chloroplast protein translocation machinery. Science. 260:1989–1992.

Jackson-Constan, D., and K. Keegstra. 2001. Arabidopsis genes encoding components of the chloroplastic protein import apparatus. Plant Physiol. 125:1567–1576.

Jarvis, P., L.J. Chen, H. Li, C.A. PetO, C. Funkhauser, and J. Chory. 1998. An Arabidopsis mutant defective in the plastid general protein import apparatus. Science. 282:100–103.

Jin, J.B., Y.A. Kim, S.J. Kim, S.H. Lee, D.H. Kim, G.W. Cheong, and I. Hwang. 2001. A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in Arabidopsis. Plant Cell. 13:1511–1526.

Keegstra, K., and C. Kline. 1999. Protein import and routing systems of chloroplasts. Plant Cell. 11:557–570.

Kessler, F., and D.J. Schnell. 2002. A GT-Pase gate for protein import into chloroplasts. Nat. Struct. Biol. 9:81–83.

Kessler, F., G. Blobel, H.A. Patel, and D.J. Schnell. 1994. Identification of two GTP-binding proteins in the chloroplast protein import machinery. Science. 260:1035–1039.

Liang, Z., T. Mathew, and G. Li. 2000. GTPase mechanism and function: new insights from systematic mutational analysis of the phosphate-binding loop residue Ala30 of Rab5. Biochem. J. 346:501–508.

Ma, Y., A. Kouranov, S.E. LaSala, and D.J. Schnell. 1996. Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. J. Cell Biol. 134:315–327.

Muckel, E., and J. Soll. 1996. A protein import receptor of chloroplasts is inserted into the outer envelope membrane by a novel pathway. J. Biol. Chem. 271:23846–23852.

Perry, S.E., and K. Keegstra. 1994. Envelope membrane proteins that interact with chloroplast precursur proteins. Plant Cell. 6:93–105.

Rensink, W.A., M. Pilon, and P. Weisbeek. 1998. Domains of a transit sequence during the recognition and translocation of precursor proteins at the outer envelope. J. Cell Biol. 141:441:59–62.

Schleiff, E., and R.B. Klose. 2001. Without a little help from ‘my’ friends: direct

Downloaded from jcb.rupress.org on July 6, 2017
insertion of proteins into chloroplast membranes? Biochim. Biophys. Acta. 1541:22–33.

Schleiff, E., J. Soll, N. Sveshnikova, R. Tien, S. Wright, C. Dabney-Smith, C. Subramanian, and B.D. Bruce. 2002. Structural and guanosine triphosphate/diphosphate requirements for transit peptide recognition by the cytosolic domain of the chloroplast outer envelope receptor, Toc34. Biochemistry. 41:1934–1946.

Schnell, D.J., F. Kessler, and G. Blobel. 1994. Isolation of components of the chloroplast protein import machinery. Science. 266:1007–1012.

Smith, M., A. Hiltbrunner, F. Kessler, and D.J. Schnell. 2002. The role of GTP in the targeting and insertion of atToc159, the major preprotein receptor of chloroplasts. J. Cell Biol. 159:833–843.

Sun, Y.J., F. Forouhar, H. Li, S. Tu, Y.H. Yeh, S. Kao, H.L. Shr, C.C. Chou, C. Chen, and C.D. Hsiao. 2002. Crystal structure of pea Toc34, a novel GTPase of the chloroplast protein translocon. Nat. Struct. Biol. 9:95–100.

Sveshnikova, N., J. Soll, and E. Schleiff. 2000. Toc34 is a preprotein receptor regulated by GTP and phosphorylation. Proc. Natl. Acad. Sci. USA. 97:4973–4978.

Tinland, B., F. Schoumacher, V. Gloeckler, A.M. Bravo-Angel, and B. Hohn. 1995. The Agrobacterium tumefaciens virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. EMBO J. 14:3585–3595.

Tranel, P.J., and K. Keegstra. 1996. A novel, bipartite transit peptide targets OEP75 to the outer membrane of the chloroplastic envelope. Plant Cell. 8:2093–2104.

Wessel, D., and U.-I. Flügge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138:141–143.