Import of chloroplast precursor proteins is controlled by the coordinate action of two homologous GTPases, Toc159 and Toc33, located at the cytosol-outer membrane interface. Recent studies in Arabidopsis showed that the cytosolic form of the precursor binding protein Toc159 is targeted to its receptor at the import machinery, Toc33, via heterodimerization of their GTP-binding domains. Toc33 may also form GDP-bound homodimers, as suggested by the crystal structure of its pea ortholog. Moreover, the structural data suggested that arginine 130 (Arg130) of Arabidopsis Toc33 may function as a GTPase-activating “arginine-finger” at the other monomer in the Toc33 dimer. Here, we demonstrate that Arg130 of Toc33 does not function as an Arginine-finger. A mutant, Toc33-R130A, binds and hydrolyzes GTP like the wild type. However, we demonstrate that Arg130 is involved in both homodimerization of Toc33 and in heterodimerization with the GTP-binding domain of Toc159. The dependence of Toc33 homodimerization on Arg130 is mutual, requiring the presence of Arg130 at both monomers. As the GTPase is not activated by dimerization, it may be activated independently at either monomer, possibly even before dimerization. Independent regulation of GTPase activity may serve to coordinate the interactions of the GTPases during the import of proteins into the chloroplast.

Chloroplast biogenesis requires the import of ~2,000 nuclear-encoded proteins from the cytosol (1). Most of these proteins are synthesized as cytosolic precursors with cleavable N-terminal transit sequences specifying targeting to the chloroplast. Translocation of precursors across the chloroplast envelope membranes requires the activity of translocon complexes located at the outer (Toc1 complex) and inner membrane of the chloroplast (Tic complex) (2-4). The Arabidopsis Tic complex consists of at least Toc159, Toc33, and Toc75 (5). In addition, the Arabidopsis Tic complex may also contain the homolog of Toc33 (Toc34) as well as those of Toc159 (Toc120, -132, and -90) (6, 7). Toc159 and Toc33 are GTPases sharing similarity in their GTP-binding domains (G-domains) (8). The GTP-binding domains of both proteins are exposed to the cytosol consistent with their known functions as precursor binding proteins. Toc75 is deeply embedded in the outer membrane of the chloroplast (9) and shares homology with channel proteins in the outer membrane of Gram-negative bacteria (10, 11). Indeed, Toc75 has channel properties upon reconstitution into lipid bilayers, consistent with a function as part of a protein-conducting channel (12).

A large body of experimental evidence supports the role of Toc-GTP-binding proteins in chloroplast protein import, but their precise mode of action remains largely unknown. Recent results have contributed to a more detailed understanding of the Toc-GTPase system: Both Toc33 and Toc159 interact directly with precursors (13, 14). Based on sequential chemical cross-linking to transit sequences (13, 14) and antibody inhibition of precursor binding to isolated chloroplasts (15), precursor recognition by Toc159 is likely to precede binding to Toc33 (16). It is therefore assumed that Toc159 functions as a primary import receptor. Moreover, Toc159 exists in an abundant soluble form (5), supporting an early role of Toc159 as a cytosolic precursor receptor. Targeting studies of cytosolic Toc159 to the outer chloroplast membrane indicate that Toc33 functions as part of a docking site for Toc159 (17, 18). In vivo analysis indicates that targeting requires the GTP-binding site of Toc159 to bind and hydrolyze GTP as a mutant of Toc159, unable to bind and hydrolyze GTP, remains trapped in the cytosol (17). A recent reconstitution study suggests that Toc159 may utilize a sewing machine mechanism to “stitch” precursor proteins across the outer membrane (19). The proposed mechanism is analogous to that of SecA, which reversibly inserts into the bacterial membrane during the transport process (20).

GT-dependent targeting of Toc159 to the outer chloroplast membrane constitutes an essential switch in chloroplast biogenesis (17). Detailed analysis of guanosine nucleotide requirements, demonstrated that docking of Toc159 at Toc33 and insertion of Toc159 into the outer membrane are stimulated by GDP and thus are likely to depend on the conversion of GTP into GDP in situ (18). A possible role of GDP in dimerization is supported by the recently solved crystal structure of psToc34, the pea ortholog of Toc33, which forms GDP-bound homodimers (21, 22). Dimer formation involves a dimerization motif, D1, conserved in the G-domains of Toc33 and Toc159. Two critical arginine residues in psToc34, Arg129 (corresponding to Arg125 of Arabidopsis Toc33) and Arg133 (corresponding to Arg130 of Arabidopsis Toc33), have been implied in self-dimerization of psToc34. R128A makes a number of interactions with amino acid residues of the other monomer. The mutant R128A had reduced GTPase activity and failed to
ARG130, which is mutated in Toc33-R130A is indicated above the sequences (*). The D1 motif is overlined with a black bar. The arrow indicates the position of the hexahistidinyl tag in Toc33-wt and Toc33-R130A. Residues identical in all three sequences are underlined in black, residues identical in two sequences in gray, B. Overexpression and purification of Toc33-wt and Toc33-R130A. Toc33-wt (Toc33w-H6) and Toc33-R130A (Toc33R-H6,R130A) were overexpressed in E. coli BL21(DE3) and purified under nondenaturing conditions using Ni-NTA affinity chromatography. Protein were eluted with an imidazole gradient. 25 μl equivalents of non-induced cultures (~IPTG, lanes 1 and 5), induced cultures (~IPTG, lanes 2 and 6), soluble protein fractions (s, lanes 3 and 7), as well as the purified proteins (1 μg each) (el, lanes 4 and 8) were separated by SDS-PAGE followed by Coomassie Blue staining.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs used for Bacterial Overexpression and in Vitro Transcription/Translation** — The cloning of pET21d-Toc33w-H6 (Toc33-wt) has previously been described (5). This construct was used to mutate Arg130 via site directed mutagenesis. Toc33w-H6,R130A (Toc33-R130A) was amplified in two pieces from pET21d-Toc33w-H using PCR with the following primer pairs: forward primer 5'-GAA ATT AAT-3' and reverse primer 5'-CCC AAG CTT-3', producing an approximately 350 bp insert. The insert was ligated into the BspHI site of pET21d resulting in pET21d-Toc33w-R130A. The ligation was transformed into E. coli BL21(DE3), and the overexpressed protein was purified under nondenaturing conditions using Ni-NTA (Qiagen) chromatography. Protein concentration was determined using the Bradford assay (24).

**GTP Binding Assays** — GTP binding to Toc33-wt and Toc33-R130A was determined using the Bradford assay (24).
ice-cold wash buffer (20 mM Tris-Cl, pH 8, 5 mM MgCl₂, 0.3% Tween 20) and air-dried. Bound [α-32P]GTP was detected and quantified using a phosphorimager.

To determine [α-32P]GTP (50 nM) binding to Toc33-wt and Toc33-R130A in the presence of non-labeled GTP or GDP, the binding buffer was supplemented with increasing concentrations of unlabeled GTP and GDP, respectively. Quantification was done as described above. From this the binding of [α-32P]GTP to Toc33-wt and Toc33-R130A (expressed as percentage of binding without competition) in presence of increasing concentrations of unlabeled GTP or GDP was plotted against the ratio of unlabeled nucleotide versus [α-32P]GTP.

**GTP Hydrolysis Assay**—GTP hydrolysis of Toc33-wt and Toc33-R130A was measured using a method adapted from a recently published protocol (25). 0.5 μl recombinant protein was incubated at 25 °C in 20 mM Tris-Cl, pH 8, 25 mM KOAc, 2 mM MgCl₂, 0.1 g/liter bovine serum albumin, and 50 nM [α-32P]GTP (3 Ci/μmol) in a final volume of 50 μl. After 0, 60, 120, and 240 min of incubation 10 μl of the reaction was removed and stopped by the addition of 10 μl of 0.4% SDS, 20 mM EDTA, 8 mM GTP, 8 mM GDP and heated to 65 °C for 5 min. 2 μl of the samples were spotted onto PEI-cellulose TLC plates (Macherey-Nagel). GTP and GDP were separated using 0.75 M KH₂PO₄, pH 3.5 as the solvent. The plates were air-dried and the spots corresponding to GTP and GDP were detected and quantified using a phosphorimager.

To determine kcat of Toc33-wt and Toc33-R130A the same approach was used. 10 μl of recombinant protein was incubated as described above, but additionally increasing concentrations of non-labeled GTP (0, 10, 20, 50, 100, 200, 500, 750, and 1000 μM) were added to the reaction. Samples were removed after 0, 30, 60, and 120 min and quantification was done as described above. From this, nanomoles of [α-32P]GTP hydrolyzed per minute were calculated and plotted against the concentration of unlabeled GDP. The catalytic constant kcat was calculated according to kcat = Vmax/[E].

**Blue Native PAGE**—5.5–16% polyacrylamide gradient gels, as well as the buffers used for electrophoresis, were prepared according to Schagger and von Jagow (1991) (26). Increasing amounts of Toc33-wt and Toc33-R130A (15–22.5 μg) were loaded. Bovine serum albumin (10 μg) was used as a standard. Electrophoresis, performed at 4 °C, was started at 80 V and increased to 200 V after the proteins had reached the separating gel. The gels were additionally stained with Coomassie Blue prior to analysis.

After blue native PAGE, the protein bands corresponding to the monomers and dimers of Toc33-wt and Toc33-R130A were excised from the gel. The gel pieces were destained and the protein eluted from the pieces with 200 μl of 2% SDS, 50 mM Tris-Cl, pH 7.5, and 1 mM dithiothreitol and precipitated using the chloroform/methanol method (27). Then it was used for SDS-PAGE gel electrophoresis and subsequent Western blotting. The blot was probed with antibodies recognizing Toc33 (5) and developed using enzyme-linked chemiluminescence.

**In Vitro Transcription/Translation**—[35S]Toc159 and [35S]Toc159G used in the pull-down assays were in vitro synthesized directly from the plasmid described above using a reticulocyte-based coupled transcript-
tion/translation system (Promega), following the instructions of the supplier.

Templates for in vitro synthesis of wild-type and mutant Toc33 lacking a His6 tag were obtained by PCR amplification from the respective constructs containing a His6 tag using the primers 5/-H11032-GAA ATT AAT ACG ACT CAC TAT AGG GG-3/-H11032/5/-H11032-CCC AAG CTT GAC GTC TTA CTT TCC TTT ATC ATC AGA G-3/.

Soluble Phase Binding Assay—His6-tagged proteins were purified as described above and incubated, at the concentrations indicated, with 10/-H9262 l of the respective [35S]Met-labeled protein in import buffer (final concentrations: 50 mM Hepes/KOH, pH 7.5, 330 mM sorbitol, 40 mM KOAc, 2 mM Mg(OAc)2, 5 mM dithiothreitol, 0.4 mM GTP, 4 mM ATP, 0.1% Triton X-100) for 10 min on ice. 10/-H9262 l of packed Ni-NTA agarose equilibrated in import buffer was added, and the incubation continued for 30 min at 4°C under constant mixing to reisolate the His6-tagged proteins. The resin was washed three times with 0.5 ml import buffer, once with 40 mM imidazole and then eluted with 200 mM imidazole. Eluates were analyzed by SDS-PAGE and Coomassie Blue staining followed by autoradiography and quantification on a Phosphorimager.

RESULTS

Expression and Purification of Toc33-wt and Toc33-R130A—We expressed wild-type Toc33 (Toc33-wt) and the mutant R130A (Toc33-R130A) as soluble proteins encompassing amino acids 1–265, but lacking the C-terminal hydrophobic transmembrane domain, which was replaced by a hexahistidinyl tag (His6) (Fig. 1A). Both Toc33-wt and Toc33-R130A were expressed at high levels (Fig. 1B, lanes 2 and 6). The proteins were largely soluble (Fig. 1B, lanes 3 and 7) suggesting correct folding and functionality. The proteins were purified to near homogeneity (Fig. 1B, lanes 4 and 8) from soluble bacterial protein fractions using Ni-NTA-agarose affinity chromatography.

Guanosine Nucleotide Binding to Toc33-wt and Toc33-R130A—Arg130 has been predicted to function as a GTPase-activating arginine-finger. To test this hypothesis, we determined both GTP binding and hydrolysis properties of Toc33-R130A and compared the results to those of Toc33-wt. In the GTP binding assay, we incubated the recombinant proteins with radioactive [32P]GTP. Aliquots of the reactions were applied to nitrocellulose and washed with buffer. Protein together with bound radioactive nucleotides remained attached to the nitrocellulose. Bound [32P]GTP was quantified using a phosphorimager (Fig. 2A). At 100 nM [32P]GTP both Toc33-wt and Toc33-R130A bound [32P]GTP with similar efficiency (Fig. 2A). The binding appeared to be specific as a bovine serum albumin control failed to retain [32P]GTP. Total GTP hydrolysis was plotted against GTP concentration and kcat was calculated (kcat = Vmax/[E]).

Dimerization of Toc-GTPases
To determine the affinity of Toc33-wt and Toc33-R130A for GDP and GTP, respectively, the recombinant proteins were incubated with 50 nM [α-32P]GTP in the presence of increasing concentrations of either unlabeled GDP or GTP. Aliquots of the reactions were applied to nitrocellulose and [α-32P]GTP remaining bound to the nitrocellulose after washing was quantified using a phosphorimager (Fig. 2B). Both GDP and GTP were considered competitive inhibitors of [α-32P]GTP binding. Competitive inhibition indicates that both proteins bind GDP (Toc33-wt: $K_d = 4.5 \mu M$; Toc33-R130A: $K_d = 8.8 \mu M$) with a slightly lower affinity than GTP (Toc33-wt: $K_d = 2.6 \mu M$; Toc33-R130A: $K_d = 3.8 \mu M$), Toc33-R130A having a slightly lower affinity than Toc33-wt for both nucleotides. The GTP dissociation constants obtained for Toc33-wt and Toc33-R130A are higher than those measured for Ras (Ras: $K_d = 0.0001–0.1 \mu M$), but in the range of those of SRP/SRα (SRP/SRα: $K_d = 1–10 \mu M$). At saturating concentrations of GTP ($30 \mu M$) close to 100% of Toc33-wt and Toc33-R130A had bound GTP indicating that most of the protein was functional with regard to GTP-binding (data not shown). Furthermore, the data suggest that GTP-binding to Toc33 may be saturated at cellular concentrations of GTP ($0.1 \mu M$) (28). As dimerization of the Toc-GTPases may depend on binding of guanosine nucleotides, we point out that binding affinities of both Toc33-wt and Toc33-R130A for GTP and GDP are comparable.

**GTP Hydrolysis by Toc33-wt and Toc33-R130A**—GTP hydrolysis measurements of Toc33-wt and Toc33-R130A were carried out to determine whether Arg130 functions as an arginine-finger. Toc-GTPases appear to dimerize preferentially in the GDP-bound state. It is therefore of interest whether Toc33-R130A is able to convert GTP to GDP. First, we analyzed the ability of Toc33-R130A to hydrolyze GTP at low concentration over time and compared it to the wild type, Toc33-wt (Fig. 3A). Toc33-wt and Toc33-R130A were incubated with 50 nM [α-32P]GTP. After the incubation, guanosine nucleotides contained in an aliquot of the reaction were separated by polyethyleneimine (PEI)-cellulose thin layer chromatography (Fig. 3A). Radioactive spots corresponding to either GDP or GTP were quantified using a phosphorimager. The results suggest that both Toc33-wt (Fig. 3A, lanes 1–4) and Toc33-R130A (Fig. 3A, lanes 5–8) hydrolyze GTP, at a low concentration, with similar efficiencies.

To determine catalytic rates of GTP hydrolysis by Toc33-wt and Toc33-R130A, the recombinant proteins were incubated with increasing concentrations of non-radioactive GTP in the presence of 50 nM [α-32P]GTP (Fig. 3, B and C). After incubation, guanosine nucleotides contained in an aliquot of the reaction were separated by PEI-cellulose thin layer chromatography. Radioactive spots corresponding to either GDP or GTP were quantified using a phosphorimager, and the $k_{cat}$ were calculated for both recombinant proteins. Toc33-wt (Toc33-wt: $k_{cat} = 0.013$ min$^{-1}$) and Toc33-R130A (Toc33-R130A: $k_{cat} = 0.019$ min$^{-1}$) had similar catalytic constants (Fig. 3, B and C). The results indicate that Arg130 in Toc33 probably does not function as the predicted, GTPase-activating arginine-finger. Furthermore, the measured hydrolysis rate of Toc33-wt is low, comparable to the basal activity of Ras (0.008–0.03 min$^{-1}$) (29), suggesting that Toc33 is probably not a self-activated GTPase. As Toc33-R130A converts GTP to GDP with kinetics similar to the wild type, it was of interest to determine whether...
homo- and heterodimerization properties of Toc33-R130A were affected by the point mutation.

Homodimerization of Toc33-wt and Toc33-R130A—Though R130A does not function as an arginine-finger, the residue has previously also been implied in dimerization by forming hydrostatic bonds with the GDP molecule bound to the other monomer. We analyzed dimerization of recombinant Toc33-wt and Toc33-R130A using blue native PAGE (Fig. 4) as well as a pull-down assay (Fig. 5). Blue native PAGE of preparations of purified Toc33-wt resulted in two bands, one migrating below (Fig. 4A, lanes 2–5, monomer) the bovine serum albumin standard (Fig. 4A, lane 1), the other above (Fig. 4A, lanes 3–5, dimer), suggesting that the lower band (±30 kDa) corresponds to the monomer and the upper band (±70 kDa) to the dimer. Higher molecular mass bands were not observed suggesting that other oligomers than the dimer are not present in detectable quantities. In contrast to Toc33-wt, the putative 70-kDa dimer band of Toc33-R130A was much weaker (Fig. 4A, lanes 6–9, dimer), suggesting reduced dimerization when compared with Toc33-wt. To confirm that the upper, putative dimer band, indeed contained Toc33-wt or Toc33-R130A, both the lower and upper bands were excised from the gel. Protein was eluted from the excised bands and analyzed by SDS-PAGE and Western blotting (Fig. 4B). SDS-PAGE of the lower bands of both Toc33-wt (Fig. 4B, lane 1, m) and Toc33-R130A (Fig. 4B, lane 3, m) resulted in ±31 kDa proteins as visualized by Amido Black staining after transfer of proteins to nitrocellulose. The upper bands of both Toc33-wt (Fig. 4B, lane 1, d) and Toc33-R130A (Fig. 4B, lane 3, d) resulted in much weaker bands of about ±31 kDa when stained with Amido Black. To confirm that the proteins stained by Amido Black were indeed Toc33-wt and Toc33-R130A, respectively, the blot was probed with antibodies recognizing Toc33 (aatToc33). The lower bands of both Toc33-wt (Fig. 4B, lane 1) and Toc33-R130A (Fig. 4B, lane 3) were recognized by aatToc33, giving strong signals, suggesting that the lower bands indeed contained Toc33-wt and Toc33-R130A, respectively, most likely as monomers. The upper bands of both Toc33-wt (Fig. 4B, lane 1) and Toc33-R130A (Fig. 4B, lane 3), analyzed by Western blotting, also reacted with aatToc33. The result suggests that the upper bands also largely consist of Toc33-wt and Toc33-R130A, respectively, most likely as dimers. However, the upper band of Toc33-R130A gave a much weaker signal than that of Toc33-wt, likely due to reduced dimer formation and/or streaking of the protein across the length of the gel (Fig. 4A, lanes 6–9).

The results of the native gel experiments were substantiated by pull down experiments (Fig. 5) in which the recombinant, hexahistidinyl-tagged Toc33-wt or Toc33-R130A were incu-
bated, in increasing concentrations, with synthetic, radioac-
tively labeled Toc33-wt ([35S]wt) or Toc33-R130A ([35S]R130A)
lacking the hexahistidinyl tag. Upon incubation of the hexahis-
tidinyl-tagged protein with its radioactive binding partner, Ni-
NTA agarose was added to reisolate the tagged protein to-
gether with any radioactive binding partner. The Ni-NTA
agarose was washed and bound proteins were eluted with
imidazole. The eluates were analyzed by SDS-PAGE followed
by autoradiography. Bound radioactive proteins were quan-
tified using a phosphorimager (Fig. 5).

Although the nature of the pull-down experiments does not allow exact determination of the binding constants, qualitatively significant effects of the R130A substitution on homo- and heterodimerization were ob-
served: Whereas recombinant Toc33-wt pulled down around
20% of the synthetic, radioactive wild-type protein ([35S]wt) (Fig. 5A, lanes 1–6), recombinant Toc33-R130A pulled down only trace amounts of its radioactive equivalent (Fig. 5A, lanes 7–9). Moreover, recombinant Toc33-wt pulled down similarly low amounts of radioactive Toc33-R130A (Fig. 5A, lanes 10–12) and vice versa (Fig. 5A, lanes 13–15). These results demon-
strate that Arg130 must be present in either monomer to allow for stable homodimerization. Thus, Toc33-R130A, while not dramatically affected in its GTP binding and hydrolysis prop-
erties, appears to be strongly compromised in dimerization, suggesting that GTP-hydrolysis may be uncoupled from
dimerization.

**Heterodimerization of Toc33 and Toc159 involves Arg130 of Toc33—Toc33 has been proposed to function as a receptor for Toc159 at the chloroplast surface. We therefore also tested the ability of recombinant Toc33-wt and Toc33-R130A to interact with soluble, radioactive Toc159 ([35S]Toc159) (Fig. 6) or its GTP-binding domain ([35S]Toc159G) (Fig. 7) lacking hexahis-
tidinyl tags. Hexahistidinyl-tagged Toc33-wt or Toc33-R130A were incubated with either [35S]Toc159 (Fig. 6) or [35S]Toc159G (Fig. 7). Ni-NTA agarose was added to reisolate hexa-
histidinyl-tagged protein and any radioactive protein bound to
it. The Ni-NTA agarose was washed and bound proteins were
eluted with imidazole. The eluates were analyzed by SDS-
PAGE followed by autoradiography (Figs. 6A and 7A).

In the pull-down assay, Toc33-wt (Fig. 6A, lanes 1–4) bound more efficiently to soluble [35S]Toc159 (~5% of the total radioactive protein) than Toc33-R130A (~2% of the total radioactive protein) (Fig. 6A, lanes 5–8) suggesting that heterodimerization of Toc33 and Toc159 may also require Arg130 of Toc33 (Fig. 6, A and B).

Heterodimerization of Toc33 and Toc159 has been reported to involve the GTP-binding domain of Toc159 (Toc159G). We therefore analyzed the ability of [35S]Toc159G to interact with both Toc33-wt and Toc33-R130A (Fig. 7, A and B). Toc33-wt pulled-down [35S]Toc159G far more efficiently (up to 5% of the total radioactive protein) than Toc33-R130A (up to 1% of the total radioactive protein). These results indicate that Arg130 is also critical for heterodimerization of Toc33 with the GTP-
bounding domain of Toc159. Furthermore, the results suggest that homo- and heterodimerization of the Toc-GTPases may rely on the same molecular mechanisms.
DISCUSSION

The GTP-regulated heterodimerization between atToc159 and atToc33 likely plays a key role in chloroplast protein import (5, 17, 18, 30). The physiological significance of Toc33/Toc159 heterodimerization is underscored by its essential role in chloroplast biogenesis in vivo (17). Our results shed light on the role of the amino acid Arg 130 in both homodimerization of Toc33 and its heterodimerization with Toc159 and allow conclusions regarding the Toc-GTPase dimerization mechanism. An earlier model suggests that dimerization may stimulate GTP hydrolysis by the insertion of the Arg130 arginine-finger into the GTP-binding pocket (21). Our results however suggest that stable dimerization could possibly occur after GTP hydrolysis at the monomers.

We compared the guanosine nucleotide binding and hydrolysis properties of wild-type Toc33 (Toc33-wt) with the alanine mutant of Arg130 (Toc33-R130A). Toc33-R130A binds to GTP and GDP with affinities comparable to those of Toc33-wt (Fig. 2, A and B). Moreover, the GTP hydrolysis catalytic constants of both the mutant and wild type are similar suggesting that GDP hydrolysis by Toc33-R130A is not compromised (Fig. 3). On the one hand, these results provide direct evidence against the role of Arg130 as an arginine-finger. On the other hand, the data show that Toc33-R130A is able to convert GTP to GDP. This is significant as heterodimerization of Toc33 and Toc159 appears to occur preferentially in the GDP-bound state. 1) The crystal structure of pea Toc34 revealed GDP-bound homodimers (21). 2) Heterodimerization of Toc33 with Toc159 was stimulated by GDP (18). 3) Self-dimerization of Toc33 was stimulated by GDP over GTP (30). The GDP dissociation constants of both Toc33-wt and Toc33-R130A are slightly higher than those for GTP but still suggest that GDP is tightly bound, possibly stabilizing Toc-GTPase dimers (21). Arg130 has not only been postulated to function as an arginine-finger but, due to its interactions with the GDP molecule and amino acids of the other monomer, to play a role in dimerization. Using blue native PAGE (Fig. 4) and pull-down assays (Figs. 5, 6, and 7), we found that Toc33-R130A has a strongly reduced ability to form stable dimers either with itself, with Toc33-wt, with Toc159 or the isolated GTP-binding domain of Toc159. Together with GTP binding and hydrolysis data, these findings indicate that Arg130, though not functioning as a GTPase-activating arginine-finger, plays a key role in both homo- and heterodimerization. Moreover, Arg130 must be present in both monomers as dimerization is reduced to near background levels if Arg130 is replaced by an alanine in just one of the two monomers (Fig. 5). Given that Toc33-R130A is compromised in dimerization but binds and hydrolyzes GTP with kinetics comparable to the wild type and that Toc-GTPase dimers preferentially form in the GDP-bound state, we propose that Toc-GTPases may be able to hydrolyze GTP prior to dimerization.

These experiments were done in the absence of precursor proteins or other potential interacting proteins. Thus, these results reflect the “idling” protein import machine. However, the findings suggest dynamics of the protein translocation process. Hydrolysis rates of both Toc33 and Toc159 are low and have potential for activation. Independent GTPase activating events occurring at Toc159 in the cytosol and Toc33 at the outer membrane may convert the GTPases into what may be their dimerization-competent GDP-bound forms. Preferential binding of Toc159 to Toc33 over homodimerization of Toc33 may be key to targeting of the soluble receptor to the outer membrane and this hypothesis will need to be tested. Likely, precursor proteins play a key role in GTPase activation as precursor proteins stimulate GTPase activity of psToc34 by up to 100-fold (31). Other components of the Toc complex, such as Toc75, may...
potentially exert effects on GTP hydrolysis or binding. We envisage that GTP-driven dynamic interactions may function to orchestrate transfer of cytosolic precursors from Toc159 to Toc33 and finally to the protein-conducting channel component, Toc75.

Acknowledgments—We thank colleagues in our laboratory and M. Stadler and Prof. N. Amrhein (ETH Zürich) for their continued support and encouragement.

REFERENCES

1. Cline, K. (2000) *Nature* 403, 148–149
2. Jarvis, P., and Soll, J. (2002) *Biochim. Biophys. Acta* 1590, 177–189
3. Chen, K., Chen, X., and Schnell, D. J. (2000) *Biochem. Soc. Trans.* 28, 485–491
4. Bauer, J., Hiltbrunner, A., and Kessler, F. (2001) *Cell. Mol. Life Sci.* 58, 420–433
5. Hiltbrunner, A., Bauer, J., Vidi, P.-A., Infanger, S., Weibel, P., Hoekwy, M., and Kessler, F. (2001) *J. Cell Biol.* 154, 309–316
6. Hiltbrunner, A., Bauer, J., Alvarez-Huerta, M., and Kessler, F. (2001) *Biochem. Cell Biol.* 79, 1–7
7. Jackson-Constan, D., and Keegstra, K. (2001) *Plant Physiol.* 125, 1007–1012
8. Kessler, F., Blobel, G., Patel, H. A., and Schnell, D. J. (1994) *Science* 266, 1035–1039
9. Schnell, D. J., Kessler, F., and Blobel, G. (1994) *Science* 266, 1007–1012
10. Bolter, B., Soll, J., Schulz, A., Hinnah, S., and Wagner, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 15831–15836
11. Reumann, S., Davila-Aponte, J., and Keegstra, K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 784–789
12. Hinnah, S. C., Hill, K., Wagner, R., Schlacher, T., and Soll, J. (1997) *EMBO J.* 16, 7351–7360
13. Perry, S. E., and Keegstra, K. (1994) *Plant Cell* 6, 93–105
14. Kouranov, A., and Schnell, D. J. (1997) *J. Cell Biol.* 139, 1677–1685
15. Hirsch, S., Muckel, E., Heemeyer, E., von Heijne, G., and Soll, J. (1994) *Science* 266, 1889–1992
16. Sveshnikova, N., Soll, J., and Schnell, D. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 4973–4978
17. Bauer, J., Hiltbrunner, A., Weibel, P., Vidi, P.-A., Alvarez-Huerta, M., Smith, M. D., Schnell, D. J., and Kessler, F. (2002) *J. Cell Biol.* 159, 845–854
18. Smith, M. D., Hiltbrunner, A., Kessler, F., and Schnell, D. J. (2002) *J. Cell Biol.* 159, 833–843
19. Schleiff, E., Jelic, M., and Soll, J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 4604–4609
20. Economou, A., and Wickner, W. (1994) *Cell* 78, 835–843
21. Sun, Y. J., Forouhar, F., Li, H., Tu, S., Yeh, Y. H., Kao, S., Shu, H. L., Chou, C. C., Chen, C., and Haseo, C. D. (2002) *Nat. Struct. Biol.* 9, 95–100
22. Kessler, F., and Schnell, D. J. (2002) *Nat. Struct. Biol.* 9, 81–83
23. Bauer, J., Chen, K., Hiltbrunner, A., Wehrli, E., Jelic, M., Schnell, D., and Kessler, F. (2000) *Nature* 403, 203–207
24. Bradford, M. M. (1976) *Anal. Biochem.* 255, 248–254
25. Liang, Z., Mather, T., and Li, G. (2000) *Biochem. J.* 346, 501–508
26. Schagger, H., and von Jagow, G. (1991) *Anal. Biochem.* 194, 254–260
27. Wessel, D., and Flugge, U.-I. (1985) *Annu. Rev. Biochem.* 54, 143–149
28. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) *Nature* 349, 117–127
29. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) *Nature* 348, 125–132
30. Jelic, M., Soll, J., and Schleiff, E. (2003) *Biochem. J.* 42, 5906–5916
31. Schleiff, E., Soll, J., Sveshnikova, N., Tien, W., Wright, S., Dahney-Smith, C., Subramanian, C., and Bruce, B. D. (2002) *Biochem. J.* 41, 1934–1946