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Dimerization of Toc-GTPases at the Chloroplast Protein Import Machinery*

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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 (Arg^{130}) of *Arabidopsis* Toc33 may function as a GTPase-activating "arginine-finger" at the other monomer in the Toc33 dimer. Here, we demonstrate that Arg^{130} 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 Arg^{130} is involved in both homodimerization of Toc33 and in heterodimerization with the GTP-binding domain of Toc159. The dependence of Toc33 homodimerization on Arg^{130} is mutual, requiring the presence of Arg^{130} 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 (Toc[1] complex) and inner membrane of the chloroplast (Tic complex) (2–4). The *Arabidopsis* Toc complex consists of at least Toc159, Toc33, and Toc75 (5). In addition, the *Arabidopsis* Toc 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).

GTP-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, Arg^{128} (corresponding to Arg^{125} of *Arabidopsis* Toc33) and Arg^{133} (corresponding to Arg^{130} 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

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[1] The abbreviations used are: Toc, translocon at the outer chloroplast membrane; Tic, translocon at the inner chloroplast membrane; wt, wild type; PEI, polyethyleneimine; Ni-NTA, Ni-nitrilotriacetic acid; G-domain, GTP-binding domain.

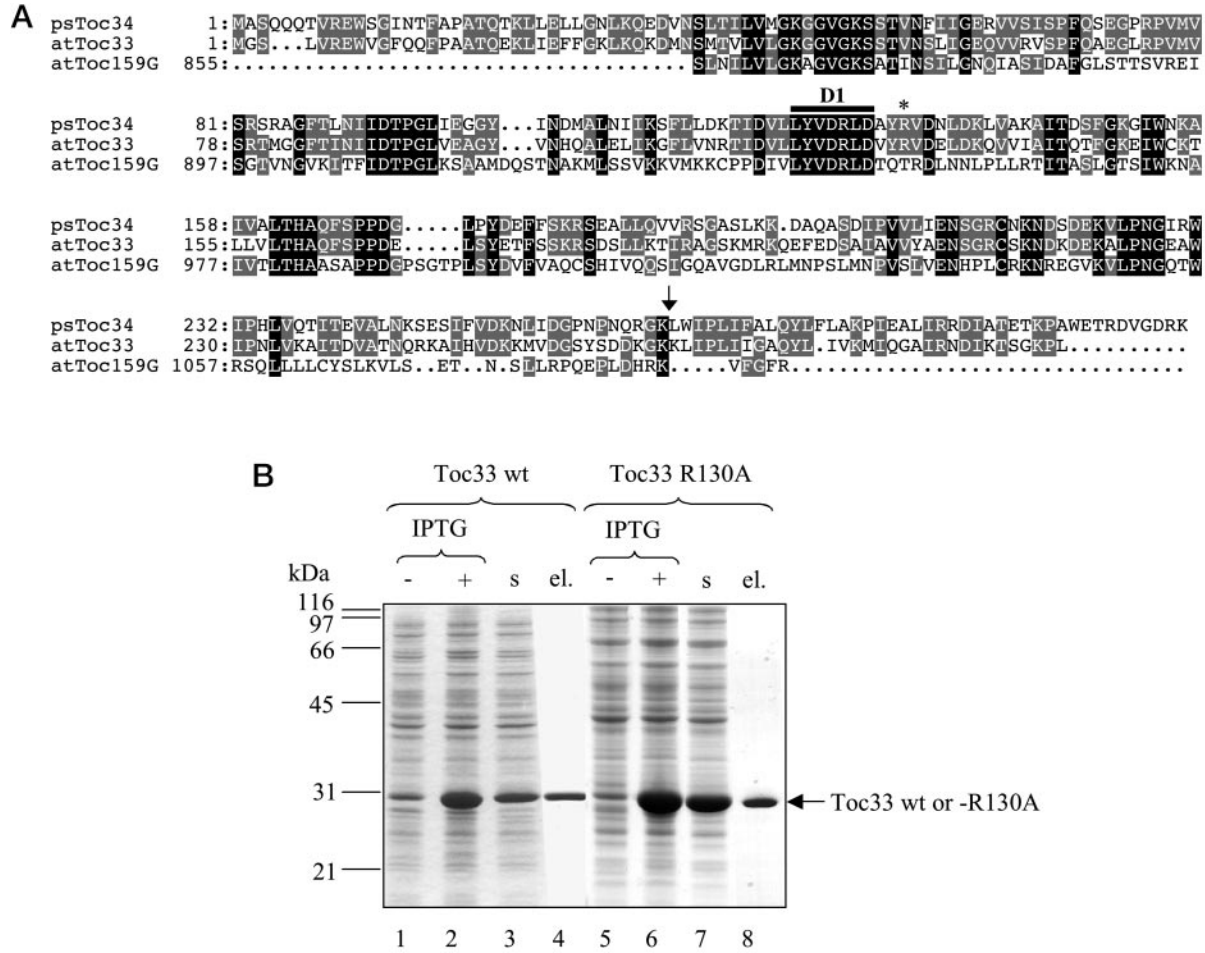

FIG. 1. **Sequence alignment and expression of hexahistidinyl-tagged versions of Toc33.** *A*, sequence alignment of pea Toc34, atToc33, and atToc159G ($Toc159_{727–1093}$). Arg^{130}, which is mutated in Toc33-R130A is indicated above the sequences (*). The D1 motif is *overlined* with a *black bar*. The *arrow* indicates the position of the hexahistidinyl tag in Toc33-wt and Toc33-R130A. Residues identical in all three sequences are *underlayed* in *black*, residues identical in two sequences in *gray*. *B*, overexpression and purification of Toc33-wt and Toc33-R130A. Toc33-wt ($Toc33_{1–265}H_6$) and Toc33-R130A ($Toc33_{1–265}H_6$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.

dimerize. Arg^{133}, on the other hand, makes contact with the GDP molecule as well as several amino acids at the other monomer. Its positioning resembles that of the arginine-finger of GTPase-activating proteins, leading to the suggestion that Arg^{133} may function as such (21). The structural data suggested that dimerization may activate the GTPase resulting in GDP-bound dimers (21). But a corresponding mutant has not been characterized so far.

In this study, we used the *Arabidopsis* ortholog of psToc34, Toc33, to address the interdependency of GTPase activity and dimerization. We analyzed the mutant Toc33-R130A (Arg^{133} in psToc34) and compared it to the wild type, Toc33-wt. The data indicate that Arg^{130} is not required for GTPase activity and therefore does not function as an arginine finger. Instead, Arg^{130} appears to be necessary for dimerization. The data also address heterodimerization of Toc33 with Toc159, demonstrating that Arg^{130} is required for heterodimerization of Toc33 with Toc159.

EXPERIMENTAL PROCEDURES

DNA Constructs used for Bacterial Overexpression and in Vitro Transcription / Translation—The cloning of pET21d-$Toc33_{1–265}H_6$ (Toc33-wt) has previously been described (5). This construct was used to mutate Arg^{130} via site directed mutagenesis. $Toc33_{1–265}H_6$R130A (Toc33-R130A) was amplified in two pieces from pET21d-$Toc33_{1–265}H_6$ using PCR with the following primer pairs: forward primer 5′-GAA ATT AAT ACG ACT CAC TAT AGG GG-3′ and reverse primer 5′-CCC AAG CTT GAG CTC ATC GAC TGC ATA CAC ATC CAA ACG ATC A-3′, including a *Sac*I site and forward primer 5′-CAT GGA GCT CGA TAA GCA AGT TGT TAT AG-3′, including a *Sac*I site, and reverse primer 5′-TTA TGC TAG TTA TTG CTC AG-3′. PCR fragments were then ligated into the *Eco*RI-*Bam*HI site of pET21d-$Toc33_{1–265}H_6$ in a triple ligation. pET21d-Toc159 has previously been described (23). $Toc159_{727–1093}$ (Toc159G) was amplified from pET21d-Toc159 using primers including a *Bsp*HI (5′-CAT GTC ATG ACT AGT CAG GAT GGT ACG-3′) and a *Not*I site (5′-ATA AGA ATG CGG CCG CTT AAA CTC GGA AAC CAA ATA CTT TAC G-3′), respectively. Ligation of the *Bsp*HI/*Not*I digested fragment into the *Nco*I/*Not*I site of pET21d resulted in pET21d-$Toc159_{727–1093}$.

For bacterial overexpression, the constructs were transformed into *E. coli* BL21(DE3), and the overexpressed protein was purified under nondenaturing conditions using Ni-NTA (Qiagen) chromatography. The bound protein was eluted with an imidazole step gradient ranging from 50 to 250 mM. Fractions containing the protein were dialyzed against 50 mM Tris-HCl, pH 8.0, 25 mM KOAc, 1 mM dithiothreitol, and 1 mM $MgCl_2$. The dialysate was centrifuged at 15000 × *g* to remove insoluble aggregates. The protein concentration of the purified recombinant protein was determined using the Bradford assay (24).

GTP Binding Assays—GTP binding to Toc33-wt and Toc33-R130A was determined as follows: 1 μM recombinant protein was incubated on ice for 60 min in the presence of binding buffer (20 mM Tris-Cl, pH 8, 50 μM $MgCl_2$, 0.3% Tween 20, 1 mM ATP) containing 0.1 μM [α-^{32}P]GTP (3 Ci/μmol) in a final volume of 10 μl. 2 μl of the reaction were spotted onto nitrocellulose membrane, which had been preincubated in binding buffer and air-dried. The membrane was washed three times with 10 ml

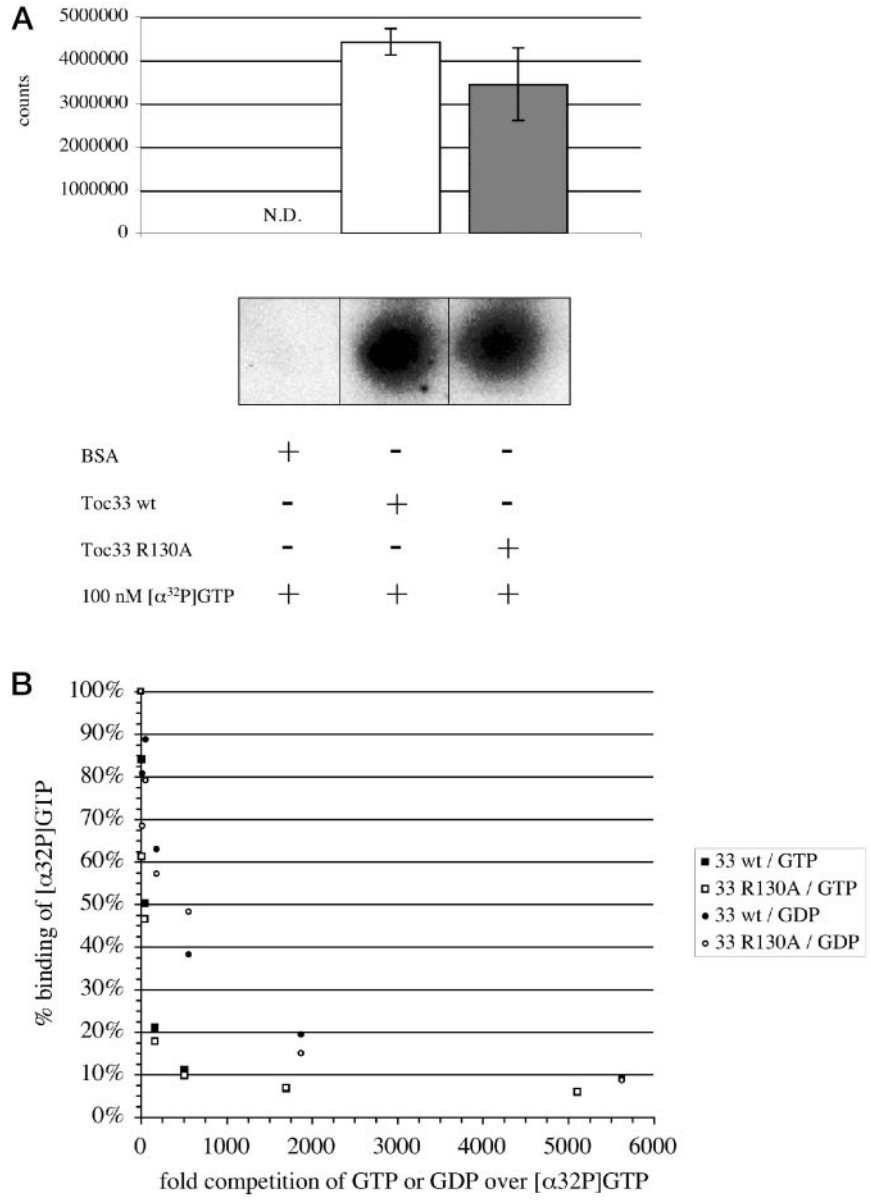

FIG. 2. **Guanosine nucleotide binding to Toc33-wt and Toc33-R130A.** *A*, purified Toc33-wt ($Toc33_{1-265}H_6$) and Toc33-R130A ($Toc33_{1-265}H_6R130A$) was incubated on ice with 0.1 μM [α-^{32}P]GTP (3 Ci/μmol). Bovine serum albumin was used as a negative control. Aliquots were spotted onto pretreated nitrocellulose and non-bound [α-^{32}P]GTP was washed off. The *middle panel* shows the autoradiography of a representative binding experiment. Bound [α-^{32}P]GTP was quantitated using a PhosphorImager (*top panel*). *Bars* represent mean values of three independent experiments. *Error bars* indicate the standard deviation. *B*, the competition of [α-^{32}P]GTP (50 nM) binding to Toc33-wt and Toc33-R130A by GTP and GDP was analyzed as described above by adding increasing concentrations of unlabeled GTP and GDP. From this the binding of [α-^{32}P]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-^{32}P]GTP.

ice-cold wash buffer (20 mM Tris-Cl, pH 8, 5 mM $MgCl_2$, 0.3% Tween 20) and air-dried. Bound [α-^{32}P]GTP was detected and quantified using a phosphorimager.

To determine [α-^{32}P]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-^{32}P]GTP to Toc33-wt and Toc33-R130A (expressed as the percentage of binding without competition) in presence of increasing concentrations of unlabeled GTP or GDP was calculated and plotted against the ratio of unlabeled nucleotide *versus* [α-^{32}P]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 μM recombinant protein was incubated at 25 °C in 20 mM Tris-Cl, pH 8, 25 mM KOAc, 2 mM $MgCl_2$, 0.1 g/liter bovine serum albumin, and 50 nM [α-^{32}P]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 KH_2PO_4, pH 3.5 as the solvent. The plates were air-dried and the spots corresponding to GTP and GDP, respectively, were quantified using a Phosphorimager.

To determine k_{cat} of Toc33-wt and Toc33-R130A the same approach was used. 10 μM 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-^{32}P]GTP hydrolyzed per minute were calculated and plotted against the concentration of unlabeled GTP. The catalytic constant k_{cat} was calculated according to $k_{cat} = V_{max}/[E]$.

Blue Native PAGE—5.5–16% polyacrylamide gradient gels, as well as the buffers used for electrophoresis, were prepared according to Schägger 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—[^{35}S]Toc159 and [^{35}S]Toc159G used in the pull-down assays were *in vitro* synthesized directly from the plasmid described above using a reticulocyte-based coupled transcrip-

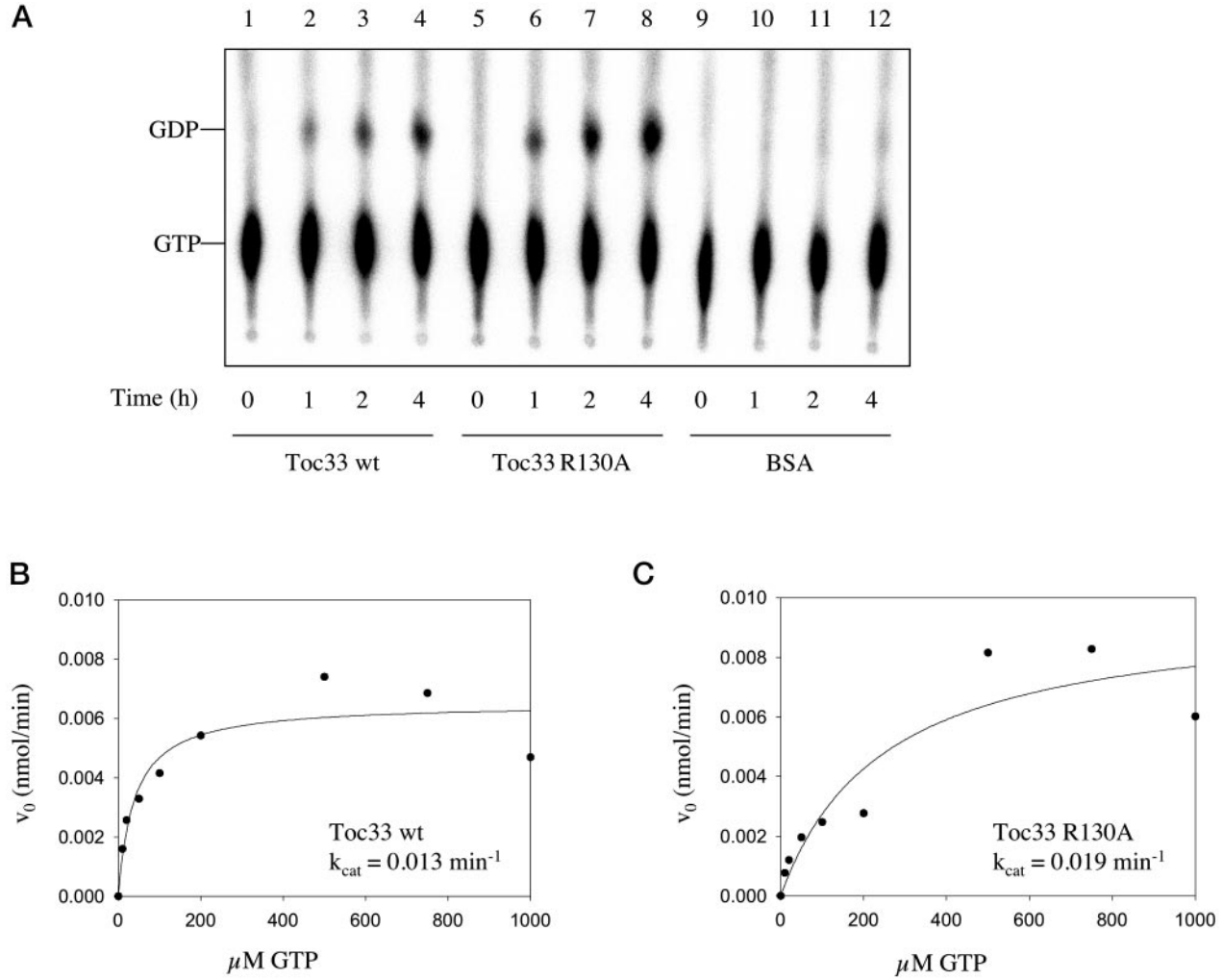

FIG. 3. **Measurements of the rates of GTP hydrolysis for Toc33-wt and Toc33-R130A.** *A*, time course of GTP hydrolysis. 0.5 μM recombinant protein was incubated at 25 °C in buffer containing 50 nM [α-^{32}P]GTP. After 0, 1, 2, and 4 h an aliquot of the reaction was stopped and 2 μl of the samples were spotted onto PEI-cellulose TLC plates to resolve GTP and GDP. *B*, determination of k_{cat} of Toc33-wt. 10 μM protein was incubated with 50 nM [α-^{32}P]GTP and increasing concentrations of non-labeled GTP. Samples were taken after 0, 30, 60, and 120 min and separated on PEI-cellulose plates. Spots corresponding to GTP and GDP were quantified using a PhosphorImager. Total GTP hydrolysis was plotted against GTP concentration and k_{cat} was calculated ($k_{cat} = V_{max}/[E]$). *C*, determination of k_{cat} of Toc33-R130A was done as described under *B*.

tion/translation system (Promega), following the instructions of the supplier.

Templates for *in vitro* synthesis of wild-type and mutant Toc33 lacking a His_6 tag were obtained by PCR amplification from the respective constructs containing a His_6 tag using the primers 5′-GAA ATT AAT ACG ACT CAC TAT AGG GG-3′/5′-CCC AAG CTT GAC GTC TTA CTT TCC TTT ATC ATC AGA G-3′.

Soluble Phase Binding Assay—His_6-tagged proteins were purified as described above and incubated, at the concentrations indicated, with 10 μl of the respective [^{35}S]Met-labeled protein in import buffer (final concentrations: 50 mM Hepes/KOH, pH 7.5, 330 mM sorbitol, 40 mM KOAc, 2 mM $Mg(OAc)_2$, 25 μM dithiothreitol, 0.4 mM GTP, 4 mM ATP, 0.1% Triton X-100) for 10 min on ice. 10 μl 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 His_6-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 (His_6) (Fig. 1*A*). Both Toc33-wt and Toc33-R130A were expressed at high levels (Fig. 1*B*, *lanes 2* and *6*). The proteins were largely soluble (Fig. 1*B*, *lanes 3* and *7*) suggesting correct folding and functionality. The proteins were purified to near homogeneity (Fig. 1*B*, *lanes 4* and *8*) from soluble bacterial protein fractions using Ni-NTA-agarose affinity chromatography.

Guanosine Nucleotide Binding to Toc33-wt and Toc33-R130A—Arg^{130} 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 [α-^{32}P]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 [α-^{32}P]GTP was quantified using a phosphorimager (Fig. 2*A*). At 100 nM [α-^{32}P]GTP both Toc33-wt and Toc33-R130A bound [α-^{32}P]GTP with similar efficiency (Fig. 2*A*). The binding appeared to be specific as a bovine serum albumin control failed to retain [α-^{32}P]GTP. These results suggest that both Toc33-wt and Toc33-R130A bind GTP with high affinity.

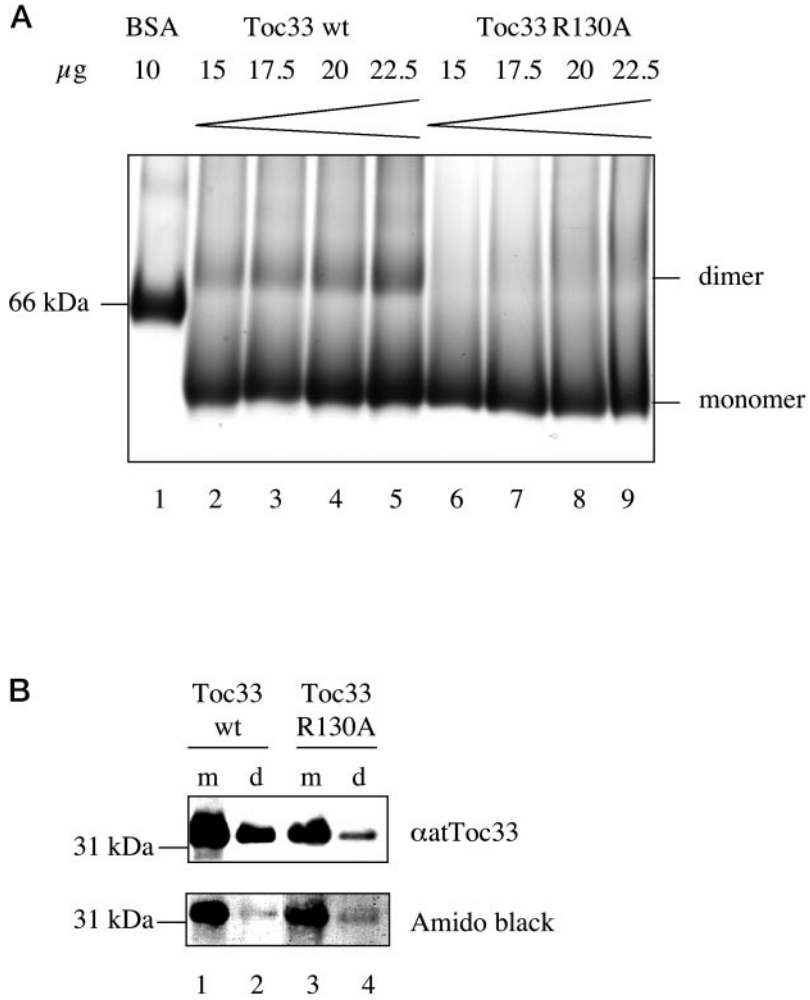

FIG. 4. **Homodimerization of Toc33-wt and Toc33-R130A.** *A*, increasing amounts of purified Toc33-wt and Toc33-R130A (15–22.5 μg) were separated by blue-native PAGE followed by additional Coomassie Blue staining. Bovine serum albumin was used as a standard (66 kDa). *B*, bands corresponding to monomers (*m*) and dimers (*d*) of Toc33-wt (*lanes 1* and *2*) and Toc33-R130A (*lanes 3* and *4*) were excised and eluted. The eluted protein was analyzed by SDS-PAGE and Western blotting. The blot was probed with antibodies recognizing Toc33.

To determine the affinity of Toc33-wt and Toc33-R130A for GDP and GTP, respectively, the recombinant proteins were incubated with 50 nM [α-^{32}P]GTP in the presence of increasing concentrations of either unlabeled GTP or GDP. Aliquots of the reactions were applied to nitrocellulose and [α-^{32}P]GTP remaining bound to the nitrocellulose after washing was quantified using a phosphorimager (Fig. 2*B*). Both GDP and GTP were considered competitive inhibitors of [α-^{32}P]GTP binding. Competitive inhibition indicates that both proteins bind GDP (Toc33-wt: K_d = ~4.5 μM; Toc33-R130A: K_d = ~8.8 μM) with a slightly lower affinity than GTP (Toc33-wt: K_d = ~2.6 μM; Toc33-R130A: K_d = ~3.8 μM), 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 μM), but in the range of those of SRP/SRα (SRP/SRα: K_d = ~1–10 μM). At saturating concentrations of GTP (~30 μM) 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 mM) (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 Arg^{130} 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. 3*A*). Toc33-wt and Toc33-R130A were incubated with 50 nM [α-^{32}P]GTP. After the incubation, guanosine nucleotides contained in an aliquot of the reaction were separated by polyethyleneimine (PEI)-cellulose thin layer chromatography (Fig. 3*A*). Radioactive spots corresponding to either GDP or GTP were quantified using a phosphorimager. The results suggest that both Toc33-wt (Fig. 3*A*, *lanes 1–4*) and Toc33-R130A (Fig. 3*A*, *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-^{32}P]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 Arg^{130} 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

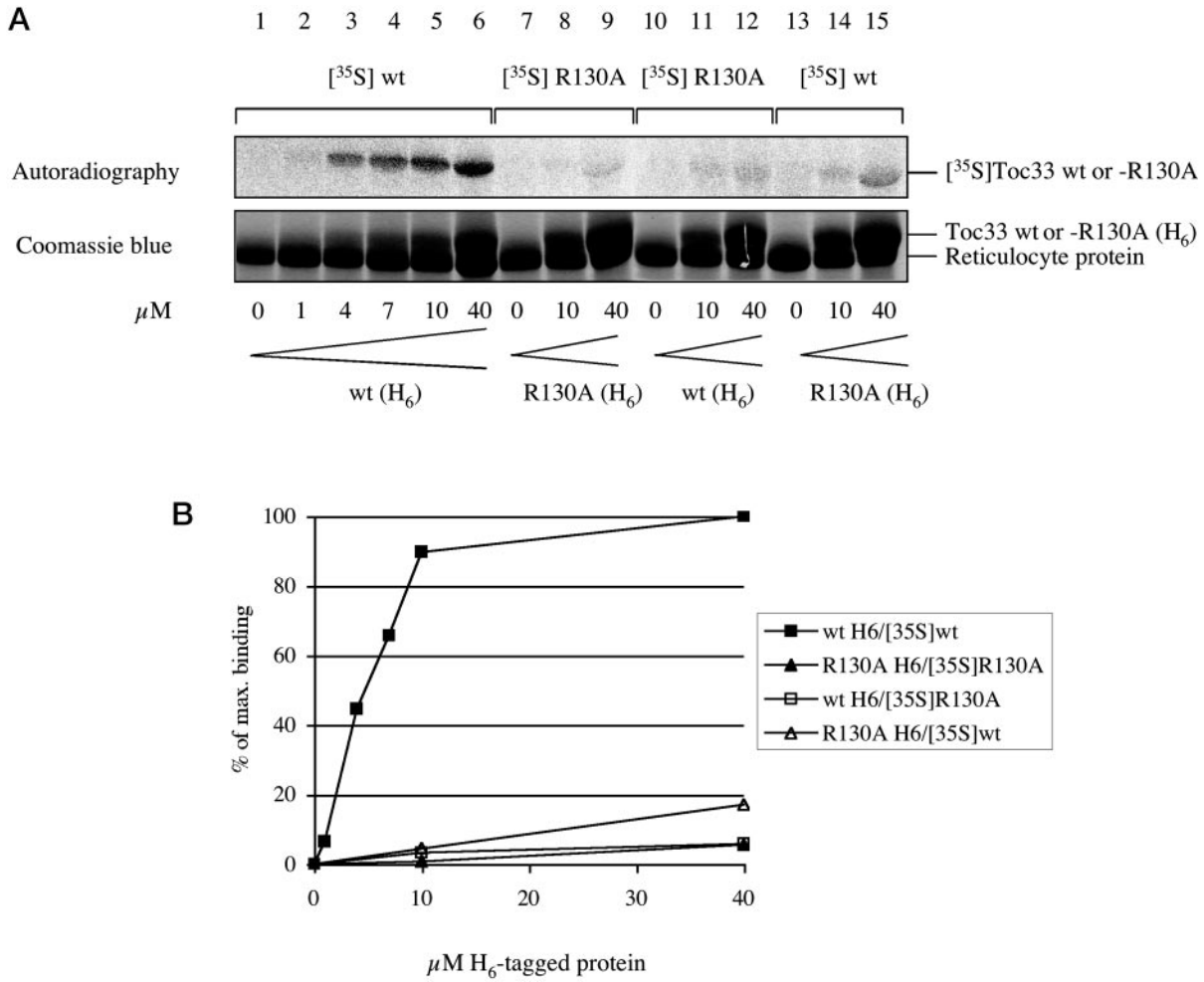

FIG. 5. **Homodimerization of Toc33-wt and Toc33-R130A.** *A*, increasing concentrations of hexahistidinyl-tagged Toc33-wt (wt H_6) or Toc33-R130A (R130A H_6) (0–40 μM) were incubated with ^{35}S-labeled Toc33-wt ([^{35}S]wt) or Toc33-R130A ([^{35}S]R130A) in the four possible permutations. Recombinant, hexahistidinyl-tagged protein together with any bound radioactive protein was captured using Ni-NTA chromatography, washed with buffer, and eluted with imidazole. Eluates were analyzed by SDS-PAGE and Coomassie Blue staining followed by autoradiography. *B*, PhosphorImager quantification. The amount of ^{35}S-labeled Toc33-wt bound to 40 μM recombinant, hexahistidinyl-tagged Toc33-wt was defined as 100% binding. Binding in absence of recombinant, hexahistidinyl-tagged protein was adjusted to zero.

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. 4*A*, *lanes 2–5*, monomer) the bovine serum albumin standard (Fig. 4*A*, *lane 1*), the other above (Fig. 4*A*, *lanes 2–5*, 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. 4*A*, *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. 4*B*). SDS-PAGE of the lower bands of both Toc33-wt (Fig. 4*B*, *lane 1*, *m*) and Toc33-R130A (Fig. 4*B*, *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. 4*B*, *lane 2*, *d*) and Toc33-R130A (Fig. 4*B*, *lane 4*, *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 (αatToc33). The lower bands of both Toc33-wt (Fig. 4*B*, *lane 1*) and Toc33-R130A (Fig. 4*B*, *lane 3*) were recognized by αatToc33, 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. 4*B*, *lane 2*) and Toc33-R130A (Fig. 4*B*, *lane 4*), analyzed by Western blotting, also reacted with αatToc33. 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. 4*A*, *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-

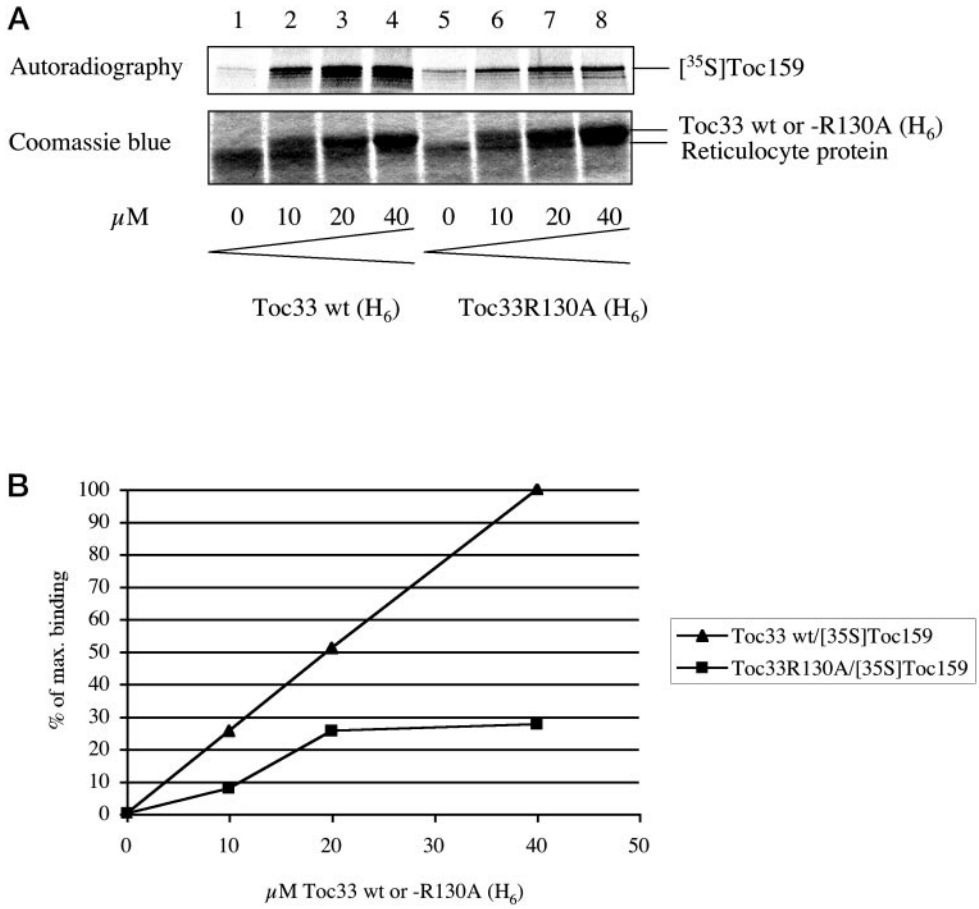

FIG. 6. **Binding of [^{35}S]Toc159 to Toc33-wt and Toc33-R130A.** *A*, increasing concentrations (0–40 μM) of hexahistidinyl-tagged Toc33-wt and Toc33-R130A were incubated with [^{35}S]Toc159 synthesized in a reticulocyte lysate. Recombinant, hexahistidinyl-tagged protein together with any bound radioactive protein was captured using Ni-NTA chromatography, washed with buffer, and eluted with imidazole. The eluates were subjected to SDS-PAGE and Coomassie Blue staining followed by autoradiography. *B*, PhosphorImager quantification. The amount of [^{35}S]Toc159 bound to 40 μM Toc33-wt was defined as 100% binding. Binding in absence of recombinant protein was adjusted to zero.

bated, in increasing concentrations, with synthetic, radioactively labeled Toc33-wt ([^{35}S]wt) or Toc33-R130A ([^{35}S]R130A) lacking the hexahistidinyl tag. Upon incubation of the hexahistidinyl-tagged protein with its radioactive binding partner, Ni-NTA agarose was added to reisolate the tagged protein together 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 quantified using a phosphorimager (Fig. 5*B*). 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 observed: Whereas recombinant Toc33-wt pulled down around 20% of the synthetic, radioactive wild-type protein ([^{35}S]wt) (Fig. 5*A*, *lanes 1–6*), recombinant Toc33-R130A pulled down only trace amounts of its radioactive equivalent (Fig. 5*A*, *lanes 7–9*). Moreover, recombinant Toc33-wt pulled down similarly low amounts of radioactive Toc33-R130A (Fig. 5*A*, *lanes 10–12*) and *vice versa* (Fig. 5*A*, *lanes 13–15*). These results demonstrate that Arg^{130} must be present in either monomer to allow for stable homodimerization. Thus, Toc33-R130A, while not dramatically affected in its GTP binding and hydrolysis properties, appears to be strongly compromised in dimerization, suggesting that GTP-hydrolysis may be uncoupled from dimerization.

Heterodimerization of Toc33 and Toc159 involves Arg^{130} 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 ([^{35}S]Toc159) (Fig. 6) or its GTP-binding domain ([^{35}S]Toc159G) (Fig. 7) lacking hexahistidinyl tags. Hexahistidinyl-tagged Toc33-wt or Toc33-R130A were incubated with either [^{35}S]Toc159 (Fig. 6) or [^{35}S]Toc159G (Fig. 7). Ni-NTA agarose was added to reisolate hexahistidinyl-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. 6*A* and 7*A*). Radioactive proteins bound to Toc33-wt or Toc33-R130A were quantified using a phosphorimager (Figs. 6*B* and 7*B*).

In the pull-down assay, Toc33-wt (Fig. 6*A*, *lanes 1–4*) bound more efficiently to soluble [^{35}S]Toc159 (~5% of the total radioactive protein) than Toc33-R130A (~2% of the total radioactive protein) (Fig. 6*A*, *lanes 5–8*) suggesting that heterodimerization of Toc33 and Toc159 may also require Arg^{130} 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 [^{35}S]Toc159G to interact with both Toc33-wt and Toc33-R130A (Fig. 7, *A* and *B*). Toc33-wt pulled-down [^{35}S]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 Arg^{130} is also critical for heterodimerization of Toc33 with the GTP-binding domain of Toc159. Furthermore, the results suggest that homo- and heterodimerization of the Toc-GTPases may rely on the same molecular mechanisms.

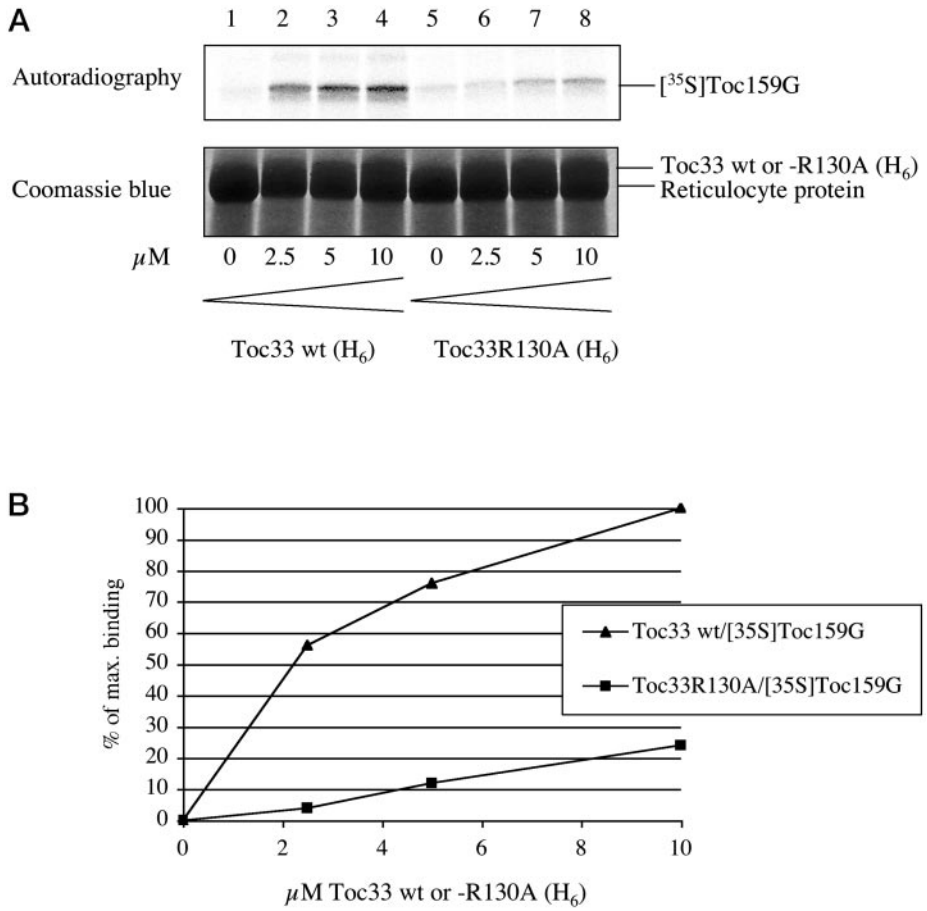

FIG. 7. **Binding of [^{35}S]Toc159G to Toc33-wt and Toc33-R130A.** *A*, increasing concentrations (0–10 μM) of Toc33-wt and Toc33-R130A were incubated with [^{35}S]Toc159G. Recombinant, hexahistidinyl-tagged protein together with any bound radioactive protein was captured using Ni-NTA chromatography, washed with buffer and eluted with imidazole. The eluates were subjected to SDS-PAGE and Coomassie Blue staining followed by autoradiography. *B*, PhosphorImager quantification. The amount of [^{35}S]-Toc159G bound to 10 μM Toc33-wt was defined as 100% binding. Binding in absence of recombinant protein was adjusted to zero.

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 Arg^{130} 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 Arg^{130} (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 GTP hydrolysis by Toc33-R130A is not compromised (Fig. 3). On the one hand, these results provide direct evidence against the role of Arg^{130} 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). Arg^{130} 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 Arg^{130}, though not functioning as a GTPase-activating arginine-finger, plays a key role in both homo- and heterodimerization. Moreover, Arg^{130} must be present in both monomers as dimerization is reduced to near background levels if Arg^{130} 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 occuring 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.

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