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Chloroplast protein import: solve the GTPase riddle for entry

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The fidelity of the numerous intracellular protein-trafficking pathways to different organelles is dictated by the interactions between the intrinsic targeting signals of substrate proteins and specific receptors that deliver the substrate to the proper organelle. Recent studies of protein targeting to chloroplasts suggest a novel mechanism in which GTP-dependent substrate recognition is coupled to a GTP-driven motor that initiates the translocation of proteins into the organelle.

Chloroplast biogenesis requires the import of ~2500 different nuclear-encoded preproteins. For the vast majority of these proteins, targeting to the organelle is determined by the interaction between the N-terminal targeting signals (transit peptides) of the protein and the receptor components of the Toc (translocon at the outer membrane of chloroplasts) complex [1]. Two GTPase subunits of the Toc translocon, Toc159 and Toc34, make the initial contacts with preproteins and are hypothesized to form the receptor system for the transit peptide at the chloroplast surface. Toc159 and Toc34 associate with Toc75, which is a component of the translocation pore, to constitute the core of the outer-envelope translocation machinery (Table 1). It has long been known that GTP hydrolysis is required for protein import [2], which implicates the nucleotide-binding and hydrolysis activities of Toc159 and Toc34 as being key regulators of the import reaction. Becker *et al*. [3] have now provided evidence to support a model for GTP-dependent preprotein recognition and translocation by the coordinated activities of Toc159 and Toc34.

The targeting and motor hypotheses

In recent years, the precise roles of Toc159 and Toc34 in transit-peptide recognition, and the function of their GTPase activities have become a major focus of investigation. Two key questions drive these studies. First, which of the two Toc GTPases forms the initial transit-peptide receptor and, thereby, constitutes the primary determinant of targeting specificity? Second, what are the individual contributions of the GTPase activities of Toc159 and Toc34 in regulating preprotein recognition and/or membrane translocation?

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Table 1. Components of the Toc translocon[a]

Core Toc components			Null phenotype		Characteristics	Function		Refs
						Motor hypothesis	Targeting hypothesis	
Toc159 family	Pea	*Arabidopsis*			N-terminal acidic, central GTPase and C-terminal membrane-anchor domains	Translocation motor	Primary receptor	
	psToc159	atToc159	Albino					[22]
		atToc132	None					[24]
		atToc120	None					[24]
				atToc132 + *atToc120* double mutant: lethal				[24]
		atToc90	None					[23]
Toc34 family	psToc34	atToc34	None		N-terminal GTPase domain (homologous to Toc159) and C-terminal transmembrane segment	Primary receptor	Secondary receptor	[25]
		atToc33	Pale					[26]
				atToc33 + *atToc34* double mutant: lethal				[25]
Toc75	psToc75	atToc75	nd		β-Barrel pore-like protein	Protein-conducting channel	Protein-conducting channel	

[a]Abbreviations: at, *Arabidopsis thaliana*; nd, not determined; ps, *Pisum sativum*; Toc, translocon at the outer membrane of chloroplasts.

An early hypothesis (known as the 'targeting' hypothesis) proposes that Toc159 is the primary preprotein receptor, subsequently associating with Toc34 and transferring the precursor for insertion into the Toc75 protein-conducting channel [1] (Figure 1). This model has been extended to include the possibility that preprotein recognition by a recently described, soluble form of Toc159 can occur not only at the membrane but also in the cytoplasm [4–7]. In this hypothesis, GTP serves primarily to coordinate the stepwise interactions between the Toc components, in addition to the transfer of preproteins into the membrane channel, thus ensuring unidirectional transport of the preprotein. This model is now being challenged by an alternative hypothesis (the 'motor' hypothesis) from the Soll and Schleiff laboratories [3]. These groups propose that Toc34 functions as the primary preprotein receptor that transfers the substrate to Toc159, which functions as a motor that threads the preprotein through the Toc75 channel via multiple rounds of GTP hydrolysis [8] (Figure 2). The authors also propose that a phosphorylation cycle by an unidentified kinase–phosphatase system occurs at the same time as the targeting reaction, to regulate interaction of the Toc complex with preproteins.

The motor hypothesis represents the culmination of three recent lines of research from the Soll and Schleiff groups. (i) Initial studies demonstrated that Toc34 binds to a variety of chloroplast preproteins, suggesting that it functions as a receptor for protein import [9]. Phosphorylation of the transit peptide, although not essential for preprotein recognition by Toc34, promotes high-affinity binding to Toc34 [10]. In a key observation, preprotein binding was shown to stimulate GTPase activity in Toc34 [11]. (ii) In a subsequent study, cryoelectron microscopy of purified Toc complexes [12] revealed a largely toroid complex with an electron-dense knob at its center. Based on the symmetry and stoichiometry of the complex, the central knob was proposed to correspond to one Toc159 molecule, and was surrounded by a ring composed of four Toc75 and four Toc34 molecules. (iii) The Soll and Schleiff groups demonstrated that the GTPase activity of a fragment of Toc159 (Toc159f) that corresponds to its GTPase and membrane-anchor domains is required for precursor translocation by the reconstituted Toc complex, and that Toc159f and Toc75 (in the absence of Toc34) are sufficient for translocation [13]. The results of (ii) and (iii) led Schleiff *et al*. [12] to propose that Toc159, positioned at the center of the Toc complex, functions as a revolving translocation motor that serves the four Toc75 channels in a motion similar to that of a Gatling gun. However, the role of Toc34 in this model remained uncertain.

In their latest work, Becker *et al*. [3] address the precise role of Toc34 in the functional hierarchy of the Toc components by examining its interactions with Toc159 and Toc75. First, they argue that Toc159 functions exclusively at the chloroplast membrane (because soluble Toc159 was shown to derive from partial membrane disruption). The authors then confirm that Toc34 binds preferentially to the phosphorylated form of the precursor of the Rubisco small subunit (pSSU) and provide evidence that Toc159f binds exclusively to the non-phosphorylated form. GTP promotes preprotein binding in both cases. Furthermore, Becker *et al*. used binding assays with synthetic peptides to demonstrate that Toc34 and Toc159f bind to different

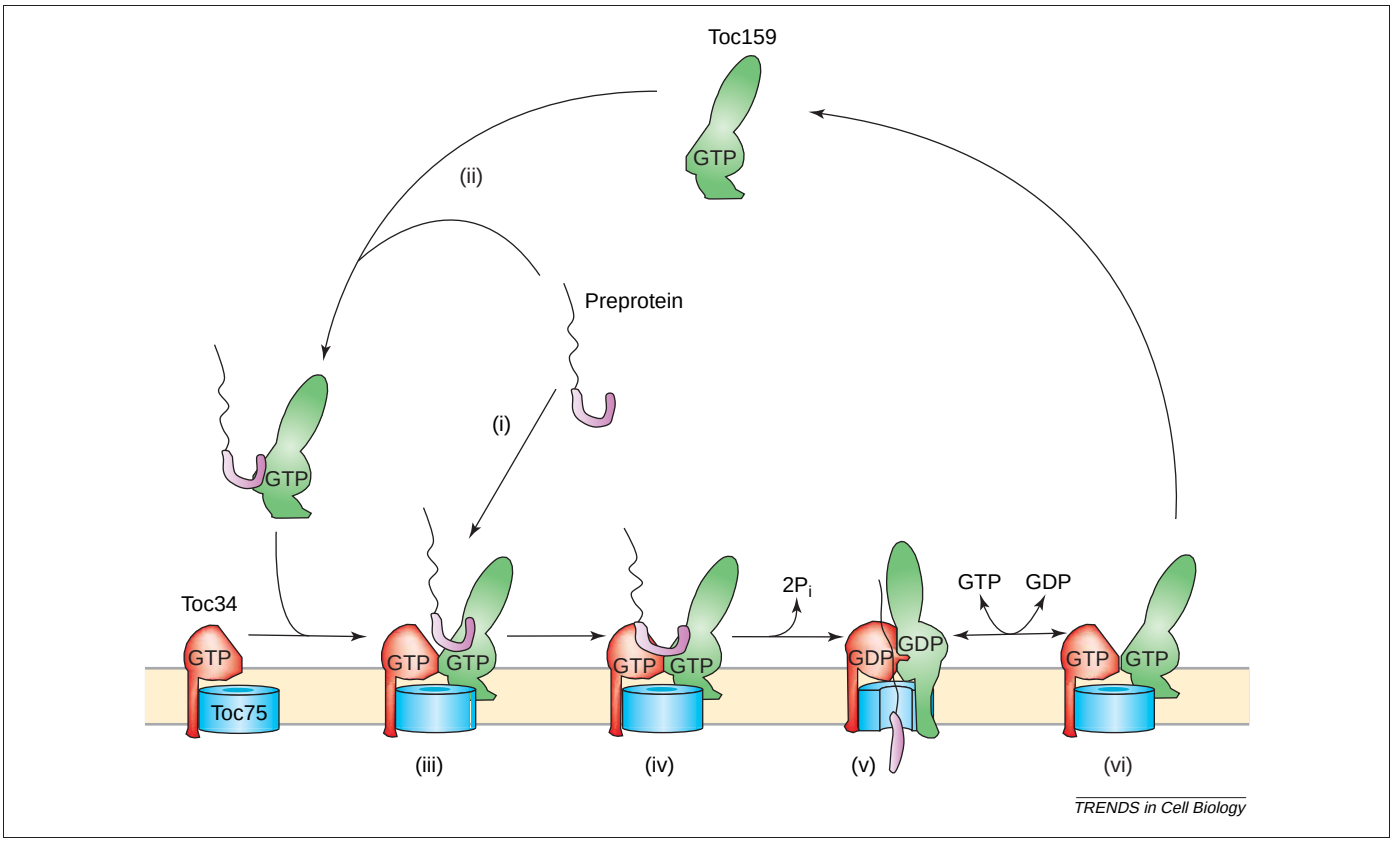

Figure 1. The 'targeting' hypothesis of preprotein binding and translocation by the Toc (translocon at the outer membrane of chloroplasts) complex. Preprotein recognition is mediated by **(i)** direct binding of the transit peptide to Toc159 at the outer membrane or **(ii)** binding of the transit peptide to cytoplasmic Toc159. **(iii)** Toc159–GTP (GTP) and Toc34–GTP (GTP) associate in a low-affinity interaction through a homotypic association between their GTPase domains. **(iv)** Formation of the quaternary complex (comprising Toc34, Toc75, Toc159 and preprotein) stimulates the activity of both Toc34 and Toc159 GTPases. **(v)** Toc34–GDP (GDP) and Toc159–GDP (GDP) form a high-affinity association between the two GTPases that triggers the insertion of Toc159 into the Toc complex, and the initiation of preprotein translocation across the membrane. **(vi)** GDP–GTP exchange resets the Toc complex and might lead to the dissociation of Toc159 to the cytoplasm for subsequent cycles of targeting.

regions of the pSSU transit peptide. Toc34 binds preferentially to the phosphorylated C-terminal region, whereas Toc159f exhibits a preference for the non-phosphorylated N-terminal peptide. In experiments that were crucial to the conclusions of the paper, the authors demonstrated that the C-terminal phosphopeptide and the N-terminal peptide inhibit the import of pSSU into isolated chloroplasts. The same effects were observed with the reconstituted Toc complex. Moreover, pSSU import by the minimal reconstituted translocon that consists of Toc159f and Toc75 is inhibited only by the N-terminal peptide. Finally, Becker *et al*. report that the pSSU precursor must be dephosphorylated before binding to Toc159. Assuming that the transit peptide is phosphorylated in the cytoplasm, the authors conclude that Toc34 interacts with the preprotein before Toc159 does.

What is the role of GTP in the import process? Using a combination of immunoprecipitation and sucrose density centrifugation, Becker *et al*. [3] present evidence that the Toc complex is stabilized by GTP. Complex formation between Toc34 and Toc159 is promoted further by the presence of the transit peptide. In the presence of GDP, a fraction of Toc34 seems to dissociate from the complex, which led the authors to propose that there is an intermediate ternary complex between the preprotein and the GTP-bound forms of the two Toc GTPases. Bound transit peptide leads to the stimulation of both GTPase proteins, resulting in the dissociation of Toc34 and the transfer of preprotein to Toc159. It seems that the low-affinity C-terminal region, rather than the high-affinity N-terminal region, of the pSSU transit peptide stimulates the GTPase activity of Toc159f. Thus, the authors propose that dephosphorylation of the C-terminal region of the transit peptide occurs during this reaction, causing its dissociation from Toc34 and its association with Toc159. Subsequent rounds of GTP hydrolysis at Toc159 drive the preprotein across the membrane.

The role of the GTPase cycle in regulating the interactions of Toc34 and Toc159, as presented by Becker *et al*., is unexpected. The crystal structure of Toc34 [14], in conjunction with solution- and solid-phase binding assays [7,15,16], demonstrates that dimerization of the GTPase proteins occurs in the GDP-bound state and is precluded by bound GTP. How can these different observations be explained? One possibility is that another component of the translocon (e.g. Toc75 or unidentified effectors) or a domain other than the GTPase domain of Toc159 (e.g. the membrane-anchor domain) plays additional roles in regulating the association of the GTPase proteins in the Toc complex.

Cycling receptor or translocation motor

The motor and targeting hypotheses agree on the essential aspects of the Toc complex, such as the precursor-binding

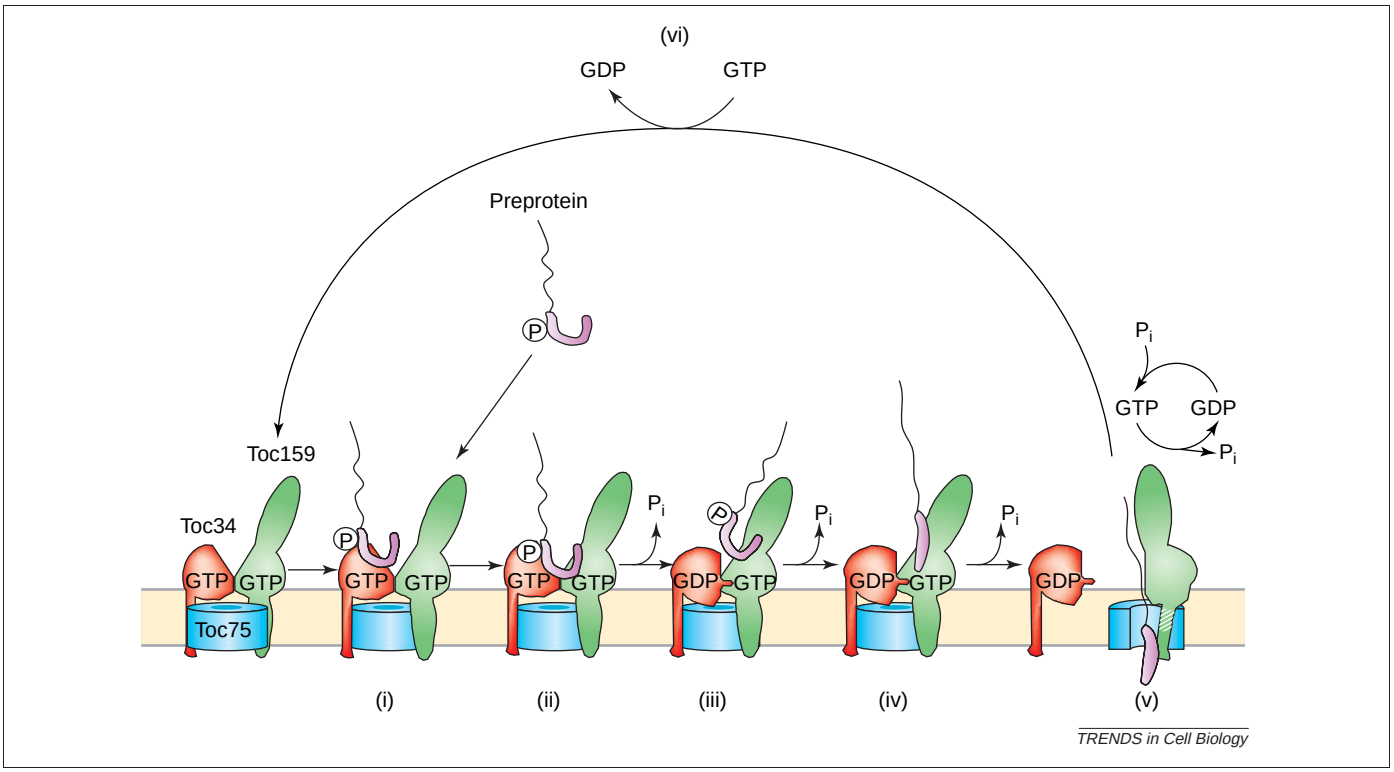

Figure 2. The 'motor' hypothesis of preprotein binding and translocation by the Toc (translocon at the outer membrane of chloroplasts) complex. **(i)** Preprotein recognition occurs by binding of Toc34–GTP (GTP) at the outer membrane to the C terminus of the phosphorylated transit peptide. **(ii)** Toc159–GTP (GTP) subsequently binds to the N-terminal region of the transit peptide. **(iii)** Transit-peptide binding stimulates the GTPase activity of Toc34, which triggers dissociation of the transit peptide. **(iv)** The transit peptide is dephosphorylated, leading to association of the C-terminal region with Toc159–GTP. **(v)** The C-terminal region of the transit peptide stimulates Toc159 GTPase activity, and Toc34–GDP (GDP) dissociates from the Toc complex, which enables Toc159 to thread the preprotein through the channel via repeated cycles of GTP hydrolysis. **(vi)** After translocation is finished, the trimeric Toc complex is regenerated by GDP–GTP exchange and can participate in additional rounds of targeting and translocation.

function of Toc34 and Toc159, and the channel function of Toc75. In two other aspects – receptor hierarchy and GTPase function – the hypotheses diverge. The studies by Becker *et al*. provide a compelling argument in favor of the motor hypothesis. Where does this leave the targeting hypothesis? The model was proposed on the basis of preprotein-binding [17] and crosslinking [18–20] studies with isolated chloroplasts that demonstrate that preproteins first interact with Toc159 during the initial stages of binding at the chloroplast surface. It has also been demonstrated that the soluble form of Toc159 binds selectively to a subset of preproteins that are required for biogenesis of the photosynthetic apparatus [21].

Genetic analyses of the Toc159 receptor family members in *Arabidopsis thaliana* – atToc159, atToc132, atToc120 and atToc90 – indicate that they define pathways for the targeting of specific subsets of plastid proteins [22–24]. By contrast, the two *A. thaliana* Toc34 homologs – atToc33 and atToc34 – do not seem to define the specificity of targeting because they have been shown to overlap functionally *in vivo* [25–27]. In addition, Nakrieko *et al*. [28] have shown that precursor phosphorylation is not essential for the fidelity of protein import in *A. thaliana*. These observations are consistent with Toc159 receptors being the primary determinants of the specificity of preprotein binding, which is an activity unlikely to occur downstream of an initial receptor.

With regard to the GTPase activity of Toc159, the two models differ in terms of whether hydrolysis is involved primarily in driving repeated rounds of translocation (motor hypothesis) or in insertion of the receptor into the Toc complex (targeting hypothesis). Both models agree that Toc159 plays a crucial role in membrane translocation, which is a concept supported by the *in vivo* and *in vitro* data. However, based on three observations, the targeting hypothesis stops short of proposing a motor activity. First, deletion constructs of Toc159 that completely lack the GTP-binding domain localize to the chloroplast surface and support the import of a precursor protein [6]. Second, isolated chloroplasts that are proteolyzed to remove the GTPase domain of Toc159 selectively, but leave Toc34 and Toc75 intact, retain the ability to import preproteins in a GTP-dependent manner [17]. Third, GTP-binding mutants remain soluble and do not associate with the Toc complex either *in vitro* [7,16] or *in vivo* [4,6].

Although considerable evidence has accumulated that supports the hypothesis that Toc159 is a primary receptor at the chloroplast surface, the existence of the soluble form of Toc159 [5] and its potential role in the targeting hypothesis remain unclear. Despite the soluble form of Toc159 binding specifically to a subset of preproteins *in vitro*, it remains to be seen whether this form targets newly synthesized cytoplasmic precursors to the Toc complex for translocation. Toc159 also needs to exit the Toc complex and re-enter the cytoplasm to complete the targeting cycle, but experimental evidence of this is still lacking.

Concluding remarks

In summary, two alternative hypotheses now exist for the roles of the Toc GTPases in preprotein recognition and translocation. The motor hypothesis proposes a primary role for Toc34 in transit-peptide recognition, with GTP hydrolysis serving roles in both passing the precursor from Toc34 to Toc159 and driving translocation across the membrane through a Toc159 motor activity. By contrast, the targeting hypothesis proposes Toc159 to be the primary receptor, with GTP hydrolysis at Toc34 and Toc159 regulating the formation of the functional translocon and the insertion of the preprotein into the translocon channel. Elements of both hypotheses are compelling and provide testable models to explain the molecular basis of the GTP-dependent control of preprotein targeting to chloroplasts.

The challenge now is to resolve the apparently contradictory results that are the bases for the two models, both of which have relied heavily on the biochemical analysis of *in vitro* systems. Although the reconstituted systems are powerful tools, the advent of *A. thaliana* mutants of the Toc GTPases with clear phenotypes provides a means of assessing the relevance of the biochemical data to the *in vivo* situation. For example, it is now feasible to test whether plastids from individual mutants in the Toc34 or Toc159 family members exhibit specific defects in preprotein binding and/or membrane translocation. This approach can be complemented by the generation of additional specific mutations within the GTPases and transit peptides that affect specific steps in the targeting and translocation reactions predicted by the two models. These experiments will be facilitated by the existing 3D structure of Toc34 and the determination of additional structures in the future. A closer examination of the molecular details of the two models using combined *in vitro* and *in vivo* analyses will lead to common ground in the form of modified hypotheses that can incorporate the valid features of both models.

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