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Protein Import Into Chloroplasts: An Ever-Evolving Story

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REVIEW / SYNTHÈSE

Protein import into chloroplasts: an ever-evolving story[1]

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Abstract: Chloroplasts are but one type of a diverse group of essential organelles that distinguish plant cells and house many critical biochemical pathways, including photosynthesis. The biogenesis of plastids is essential to plant growth and development and relies on the targeting and import of thousands of nuclear-encoded proteins from the cytoplasm. The import of the vast majority of these proteins is dependent on translocons located in the outer and inner envelope membranes of the chloroplast, termed the Toc and Tic complexes, respectively. The core components of the Toc and Tic complexes have been identified within the last 12 years; however, the precise functions of many components are still being elucidated, and new components are still being identified. In *Arabidopsis thaliana* (and other species), many of the components are encoded by more than one gene, and it appears that the isoforms differentially associate with structurally distinct import complexes. Furthermore, it appears that these complexes represent functionally distinct targeting pathways, and the regulation of import by these separate pathways may play a role in the differentiation and specific functions of distinct plastid types during plant growth and development. This review summarizes these recent discoveries and emphasizes the mechanisms of differential Toc complex assembly and substrate recognition.

Key words: chloroplast protein import, organelle biogenesis, Toc159, preprotein receptors, Toc complexes, Tic complexes.

Résumé : Les chloroplastes ne constituent qu'un seul des divers type d'organelles qui distinguent les cellules végétales, et contiennent plusieurs sentiers métaboliques critiques, incluant la photosynthèse. La biogenèse des plastes est essentielle à la croissance et au développement des plantes, et repose sur le ciblage et l'importation à partir du cytoplasme de milliers de protéines codées dans le noyau. L'importation de la majorité de ces protéines dépend de translocons, localisés dans les enveloppes membraneuses externes et internes du chloroplaste, nommés complexes Toc et Tic, respectivement. Les composantes centrales des complexes Toc et Tic ont été identifiées au cours des dernières 12 ans; cependant, les fonctions précises de plusieurs constituants, sont toujours en voie d'être élucidées, et de nouvelles composantes en voie d'être identifiées. Chez l'*Arabidopsis thaliana* (et autres espèces), plusieurs des nouvelles composantes sont codées par plus d'un gène, et il semble que les isoformes s'associent différentiellement avec des complexes d'importation distincts. De plus, il semble que ces complexes représentent des sentiers de ciblage fonctionnellement distincts, et que la régulation de l'importation par ces sentiers séparés pourrait jouer un rôle dans la différenciation et les fonctions spécifiques des divers types de plastes, au cours de la croissance des plantes et de leur développement. Cette revue résume ces découvertes récentes et met l'accent sur les mécanismes différentiels du complexe d'assemblage Toc et de la reconnaissance des substrats.

Mots clés : importation des protéines chloroplastiques, biogenèse des organelles, Toc159, récepteurs de protéines, complexes Toc, complexes Tic.

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Introduction

Plant cells, like those of all eukaryotes, contain numerous membrane-bound organelles that partition and organize many of the biochemical processes required for proper cellular development and function. The cells of photosynthetic eukaryotes such as higher plants and green algae, however, are distinguished from those of other eukaryotes by a diverse group of essential organelles called plastids. The most familiar plastids are chloroplasts, which house the photosynthetic apparatus in green tissues; but plastids are also the sites of crucial steps in numerous other biochemical processes, including amino acid and lipid biosynthetic pathways (Kirk and Tilney-Bassett 1978). Therefore, plastid biogenesis and maintenance of plastid identity and function are vital to the growth and development of the plant as a whole. The biogenesis of all organelles, including chloroplasts, relies on the import of thousands of nuclear-encoded proteins from the cytoplasm. Eukaryotic cells therefore possess elaborate intracellular protein trafficking systems to ensure that proteins are delivered to all subcellular compartments with

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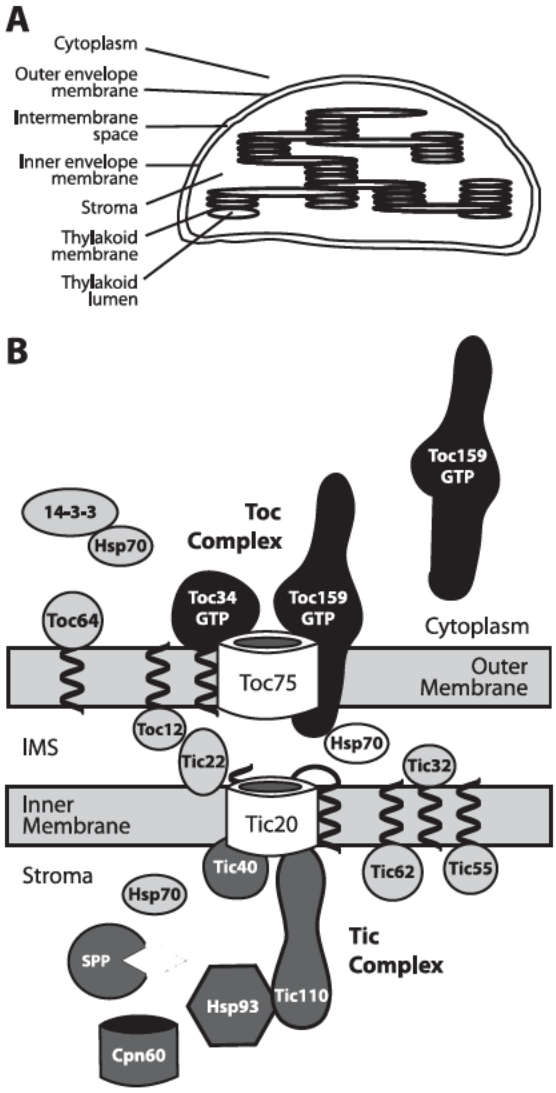
high fidelity. However, many of the mechanistic details of protein recognition and translocation remain unresolved, and these aspects of virtually all protein targeting systems are the focus of many research programs. The mechanism of protein targeting and import into chloroplasts is studied as a model for intracellular protein trafficking and organelle biogenesis in all eukaryotes and because of the central role that it plays in chloroplast function and plant growth and development.

It is well established that plastids originated from the endosymbiosis of a primitive photosynthetic cyanobacterium by a nucleated cell (Dyall et al. 2004; Reumann et al. 2005). As part of the evolutionary transition from free-living cyanobacterium to organelle, the vast majority of the endosymbiont's genes were transferred to the nuclear genome. A small plastid genome of about 120 kb remains, which contains approximately 120 genes, and whose expression is coregulated with the nuclear genome (Sugira 1992; Surpin et al. 2002). Approximately 95% of plastid proteins, representing 3000–4000 distinct proteins, are now encoded in the nucleus, translated in the cytoplasm, and delivered posttranslationally to the organelle (Leister 2003). Proteinaceous machinery located at the double membrane envelope that separates the internal stromal compartment of plastids from the cytoplasm (Fig. 1A) recognizes and imports these proteins to maintain organelle identity and function. This machinery appears to be unique to plastids and likely evolved in response to gene transfer to the nucleus following endosymbiosis to facilitate delivery of proteins back to the organelle (Reumann and Keegstra 1999). The components of the machinery appear to be of dual origin; some components (Toc75, Tic20, Tic22, and Tic55) were inherited from the cyanobacterial endosymbiont; the others (e.g., Toc159, Toc34, and Tic110) evolved from preexisting nuclear genes (Reumann et al. 2005).

The compositions and quantity of imported proteins vary considerably between plastid types and during plant development. For example, during the photomorphogenic transition of proplastids into chloroplasts, the expression of genes encoding photosynthetic proteins increases dramatically (Mache et al. 1997). As a result, the protein trafficking apparatus must adapt from a relatively low-capacity system for targeting constitutively expressed plastid proteins to one that can accommodate the massive influx of specific photosynthetic cargo. This requires remarkable flexibility in the capacity and selectivity of the protein import machinery to maintain organelle homeostasis.

For many years, it was believed that there was one "general import apparatus" responsible for the import of all plastid proteins, a belief that was based on biochemical evidence obtained using pea chloroplasts (for reviews, see Kouranov and Schnell 1996; Heins et al. 1998; Keegstra and Cline 1999). Recently, this notion has been challenged by genetic and biochemical data from *Arabidopsis thaliana* that suggest that there are multiple complexes capable of importing proteins into plastids and that these distinct complexes may play temporally and spatially differential roles in import. This review will focus on recent progress on the emerging idea that distinct import complexes exist and what is known about their differential functions.

Fig. 1. Organization of chloroplasts and the chloroplast protein import apparatus. (A) Diagrammatic representation illustrating the six distinct subcompartments of the chloroplast. (B) Schematic representation of components of the Toc and Tic complexes responsible for the recognition and import of preproteins from the cytoplasm into the stroma. Core translocon components involved in preprotein targeting are shown in black; components involved in membrane translocation are shown in white, and the components involved in translocation and maturation of preproteins in the stroma are shown in dark gray. Proteins with undefined functions or putative roles in facilitating or regulating import under specialized circumstances are shown in light gray. IMS, intermembrane space; Hsp, heat shock protein; SPP, stromal processing peptidase; Cpn, chaperonin; Tic, translocon in the inner envelope membrane of the chloroplast: Toc, translocon in the outer envelope membrane of the chloroplast.



General features of plastid protein import

With few exceptions (for a review, see Smith and Schnell 2004), nuclear-encoded plastid proteins are translated on free cytoplasmic ribosomes as preproteins, which include cleavable N-terminal extensions called transit peptides that mark them for delivery to plastids. Upon translocation into

the stroma, transit peptides are cleaved to yield proteins that can be folded, assembled into a functional complex, or targeted to one of the six subchloroplast compartments (Fig. 1A). The translocon at the outer envelope membrane of chloroplasts (Toc complex) is responsible for recognizing transit peptides and initiating preprotein import into plastids. It acts coordinately with the Tic (translocon at the inner envelope membrane of chloroplasts) complex to complete translocation of preproteins into the stroma (Fig. 1B).

Typically, transit peptides that mark preproteins as being destined for chloroplasts are enriched in both hydroxylated and hydrophobic amino acids and are devoid of acidic residues (Von Heijne et al. 1989). However, these targeting sequences are surprisingly variable in amino acid sequence and overall amino acid composition and range in length from 20 to 100 amino acids. Despite this variability, transit peptides can be predicted with reasonably high accuracy using programs such as ChloroP, iPSORT, and Predotar (Emanuelsson et al. 1999; Bannai et al. 2002; Small et al. 2004; Richly and Leister 2004). It has been suggested based on in vitro experiments that some transit peptides must be phosphorylated to be recognized by a 14-3-3 protein component of a cytoplasmic "guidance complex" that is postulated to target preproteins to chloroplasts (May and Soll 2000). However, this suggestion has been called into question by the finding that removal of the proposed phosphorylation sites from transit peptides does not affect the import efficiency of these proteins in vivo (Nakrieko et al. 2004). Therefore, it is not clear precisely what role transit peptide phosphorylation plays in preprotein import. While the putative 14-3-3-containing guidance complex may enhance targeting efficiency of proteins to chloroplasts under some conditions, it does not appear to be an essential component of the import machinery.

Translocation of preproteins from the cytoplasm across the double-membrane envelope and into the stroma is an energy-dependent process that requires the hydrolysis of both ATP and GTP but does not require a membrane potential, as is the case for mitochondrial protein import. By manipulating the nucleotides added to in vitro chloroplast import assays, three distinct stages of import have been identified. Preprotein recognition and binding by preprotein receptors at the chloroplast surface has been shown to be a reversible and energy-independent process (Perry and Keegstra 1994; Ma et al. 1996). More recently, it has been demonstrated that the nucleotide-bound form of the primary preprotein receptor of the Toc complex, which is a GTPase (see below), is the form of the receptor that is competent for preprotein binding (Smith et al. 2004). Interestingly, it does not appear to matter whether the bound nucleotide is GTP or GDP, which may help explain why preprotein binding is a nucleotide-independent process when assayed using in vitro chloroplast import assays. The second stage of import is characterized by the formation of an early import intermediate in which the preprotein has engaged the translocation machinery and has inserted across the outer envelope membrane (Olsen and Keegstra 1992). This step requires the presence of GTP and low levels (<100 μmol/L) of ATP, is thought to trigger the association of the outer and inner translocation machinery (i.e., the Toc–Tic supercomplex), and represents the committed, nonreversible step of import (Ma et al. 1996; Young et al. 1999). Finally, the preprotein inserts across the inner membrane, and translocation proceeds simultaneously across both envelope membranes and into the stroma. This step requires higher concentrations of ATP (>100 μmol/L) in the stroma (Theg et al. 1989), a requirement that has been attributed to molecular chaperones such as Hsp70, Hsp93, and perhaps Cpn60 (Pain and Blobel 1987; Jackson-Constan et al. 2001). Evidence for the involvement of stromal Hsp93 in protein import into chloroplasts has been accumulating, and it is now thought that this is the chaperone that cooperates with Tic110 and Tic40 (see below) to achieve inner membrane translocation (Akita et al. 1997; Nielsen et al. 1997; Inaba et al. 2003; Kovacheva et al. 2005). Hsp70/DnaK binding sites have been predicted and experimentally identified in a number of transit peptides suggesting a role for cytoplasmic chaperones (Ivey and Bruce 2000; Rial et al. 2000; Zhang and Glaser 2002); however, mutating such binding sites does not decrease the in vitro import efficiency of at least one preprotein (Rial et al. 2003). These data indicate that stromal Hsp70 may not be directly involved in import, making Hsp93 a more likely candidate to explain the stromal ATP requirement. Of note is that two Hsp93 isoforms are found in the stroma (Jackson-Constan and Keegstra 2001; Sjögren et al. 2004). These isoforms appear to have at least partially redundant functions, and while evidence for the involvement of one, or both, in import is accumulating, they are also thought to participate in processes such as protein degradation and photosystem biogenesis at the thylakoid membrane (Park and Rodermel 2004; Sjögren et al. 2004; Kovacheva et al. 2005).

After translocation across the double-membrane envelope, transit peptides are recognized and cleaved by the stromal processing peptidase, which recognizes the C-terminal portion of transit peptides and cleaves at the recognition site to yield the mature protein (Richter and Lamppa 2002). Until recently, it was believed that the loosely conserved stromal processing peptidase recognition and cleavage site was V/I-X-A/C_A (Zhang and Glaser 2002). However, a recent report suggests that it is the phyiscochemical properties rather than the precise amino acid sequence that identifies a transit peptide processing site (Rudhe et al. 2004). The mature proteins that arise following processing of the transit peptide are thought to be engaged by chaperones such as Cpn60, which may be involved in the folding and (or) assembly of larger stromal protein complexes (Lubben et al. 1989). Alternatively, these proteins may be targeted to the inner envelope membrane of chloroplasts or targeted to thylakoids via one of the multiple pathways involved in thylakoid protein targeting (for recent reviews, see Jarvis and Robinson 2004 and Smith and Schnell 2004).

The plastid protein import apparatus

The "signal hypothesis" was proposed in 1971 to explain how secretory proteins are targeted to the ER (Blobel and Sabatini 1971). The "envelope carrier hypothesis" was later proposed by Blair and Ellis (1973) to explain how and why chloroplasts contain so many proteins that are not produced by the organelle. Finally, in 1978, it was demonstrated that chloroplasts are able to take up proteins posttranslationally,

when the process is reconstituted in vitro (Chua and Schmidt 1978; Highfield and Ellis 1978). Both of these studies demonstrated that the nuclear-encoded small subunit of Rubisco is converted to a lower molecular weight form upon being taken up by chloroplasts, a result of the removal of the transit peptide. The small subunit of Rubisco continues to be the most commonly used and best-studied cargo protein substrate of the chloroplast protein import machinery.

It was not until the mid-1990s that components of the import apparatus from pea (*Pisum sativum*) were first identified using in vitro biochemical cross-linking approaches (Kessler et al. 1994; Perry and Keegstra 1994; Schnell et al. 1994). Initially, many components of the import apparatus were referred to by different names by different research groups. However, a consensus nomenclature was agreed upon that denotes the location and size of each protein (Schnell et al. 1997). The three-letter designation of Toc or Tic implies a direct involvement in preprotein import and is followed by a number that indicates the molecular mass of the protein in kilodaltons. Collectively, all components of the translocon at the outer membrane are said to comprise the Toc complex. Similarly, the Tic complex refers collectively to Tic proteins that are assembled into a functional unit in the inner membrane. Since the first Toc and Tic components were identified in pea, *A. thaliana* has emerged as a powerful model system, and in recent years, numerous Toc and Tic components have been identified in pea, *Arabidopsis*, and other species (Summer and Cline 1999; Hirohashi and Nakai 2000; Jackson-Constan and Keegstra 2001; Davila-Aponte et al. 2003; Hofmann and Theg 2004; Fulgosi et al. 2005; Voigt et al. 2005). Identification and characterization of all components have relied on a combination of genomic information and in vitro biochemical, molecular, and in vivo genetic approaches.

The Tic complex

Four polypeptides of the inner membrane, namely Tic20, Tic22, Tic40, and Tic110 (Fig. 1B), have been shown to be directly involved in import by covalent cross-linking to preproteins during inner membrane translocation and are therefore considered genuine components of the Tic complex. Tic22 is a resident of the intermembrane space (Kouranov et al. 1998), a location that suggests a function in the assembly of Toc–Tic supercomplexes, perhaps in cooperation with Toc12 (Becker et al. 2004*a*). Tic20 and Tic110 have both been implicated in inner membrane translocation (Chen et al. 2002; Heins et al. 2002), and Tic40 is believed to play a role in coordinating the association of stromal chaperones with preproteins at the late stages of translocation (Chou et al. 2003).

Tic20 is distantly related to bacterial branched-chain amino acid transporters and to the Tim17/23 components of the mitochondrial inner membrane translocase (Reumann and Keegstra 1999). It is a polytopic integral membrane protein that interacts with preproteins during translocation (Kouranov et al. 1998). Down-regulation of Tic20 in *Arabidopsis* using antisense technology results in a specific defect in transport across the inner membrane (Chen et al. 2002), which has led to the hypothesis that Tic20 forms at least part of the channel through the inner membrane.

Tic110 is a central component of the Tic complex and is essential to protein import into chloroplasts (Inaba et al. 2005; Kovacheva et al. 2005). It is an abundant inner membrane protein anchored by two transmembrane domains near the N-terminus and a large (~95 kDa) C-terminal globular domain that extends into the stroma (Kessler and Blobel 1996; Jackson et al. 1998; Inaba et al. 2003). A fraction of the protein is associated with Toc components under steady-state conditions (Kessler and Blobel 1996; Nielsen et al. 1997), which indicates a possible role for this component in the formation of functional Tic complexes as well as Toc–Tic supercomplexes. In vitro analysis of Tic110 led to the proposal that it coordinates the late events in preprotein import (Nielsen et al. 1997; Inaba et al. 2003). In this scenario, the stromal domain of the protein possesses two critical activities. First, it contains a transit peptide-binding site adjacent to its membrane anchor segments (Inaba et al. 2003). This site is proposed to form the initial binding site for the preprotein as it emerges from the Tic channel, thereby preventing it from slipping back into the intermembrane space. Tic110 also specifically associates with stromal heat-shock protein 93 (Hsp93), a chaperone believed to bind preproteins and provide the driving force for subsequent translocation (Akita et al. 1997; Kovacheva et al. 2005). In vitro evidence has been presented suggesting that Tic110 forms the preprotein translocation channel through the inner membrane (Heins et al. 2002). However, it is difficult to reconcile the experimentally determined structure and topology of Tic110 (Jackson et al. 1998; Inaba et al. 2003) with such a function, unless, as suggested by Inaba et al. (2003), Tic110 assembles with Tic20 (and perhaps other Tic proteins) in response to outer membrane translocation to form a functional Tic channel. In vivo studies reveal that Tic110 is an essential protein and support the notion that it cooperates with both Hsp93 and Tic40 during protein import (Inaba et al. 2005; Kovacheva et al. 2005).

Tic40 is a third integral membrane component of the Tic complex, which is anchored in the membrane by a single transmembrane domain (Chou et al. 2003). Although its role in import is not essential, as demonstrated by *Arabidopsis* T-DNA knockout mutants, there is good evidence that it may be involved in optimizing or modulating import, perhaps during times of stress (Chou et al. 2003; Ko et al. 2004; Kovacheva et al. 2005). Its sequence similarity to several cochaperones suggests that Tic40 plays a role in coordinating the association of chaperones with preproteins during the late stages of import (Chou et al. 2003), a proposal that is consistent with data presented by Kovacheva et al. (2005). In addition to assisting in translocation, molecular chaperones likely facilitate folding of newly imported proteins in the stroma. Cpn60, the plastid GroEL homologue, also associates with import complexes, suggesting coordination between preprotein translocation, processing, and folding (Kessler and Blobel 1996). Stromal Hsp70 has not been shown to directly participate in the import reaction, but it does associate with some nuclear-encoded thylakoid proteins to assist in their transit through the stroma from the translocons to the thylakoid membrane (Yalovsky et al. 1992).

At least three other proteins have been implicated as being involved in preprotein translocation across the inner membrane (Fig. 1B). Tic62, Tic55, and Tic32 have all been

proposed to potentially interact with Tic110 and to play regulatory roles in import (Caliebe et al. 1997; Kuchler et al. 2002; Hormann et al. 2004). While Tic32 appears to be an essential protein in *Arabidopsis* (Hormann et al. 2004), direct evidence for its role in import is still lacking, as it is for Tic62 and Tic55.

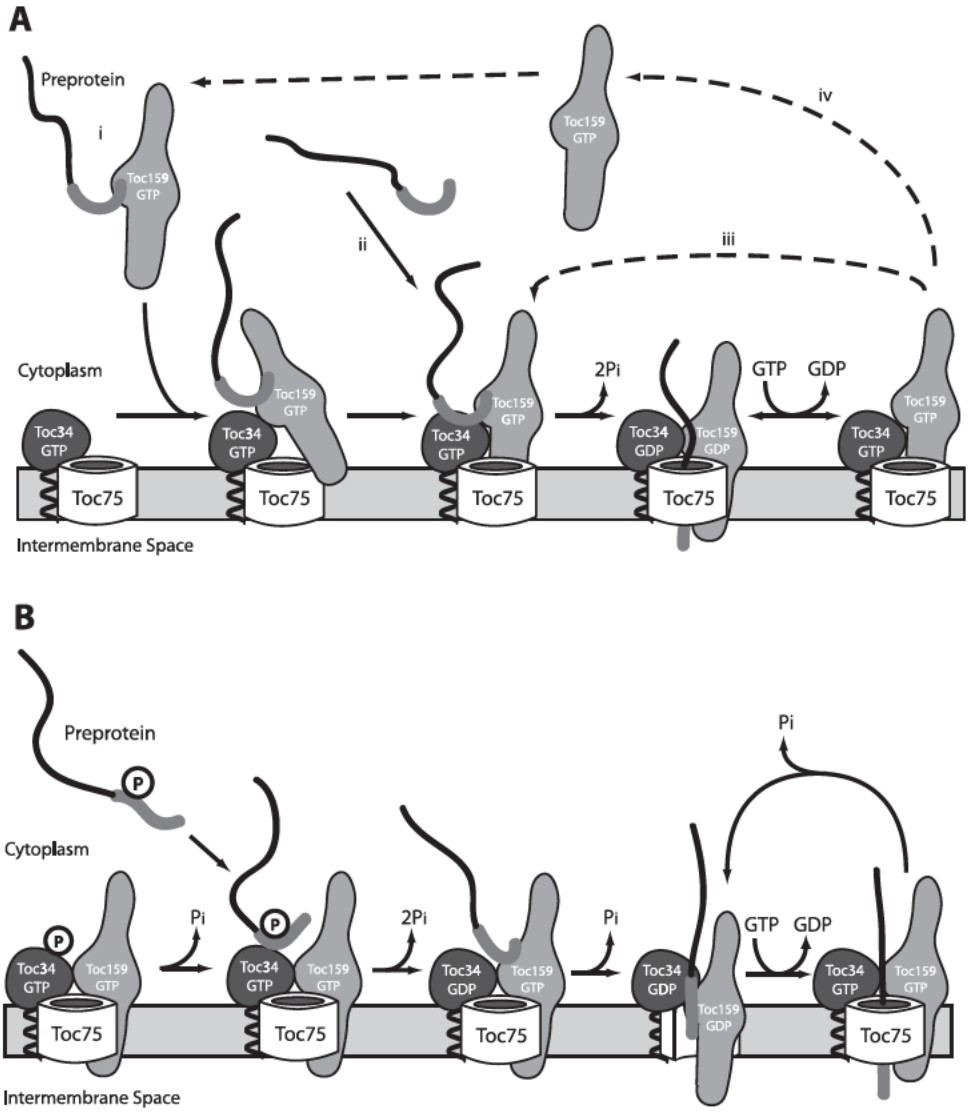
The Toc complex

The Toc complex is of critical importance to plants, as it ensures proper targeting of many essential proteins. The core of the Toc complex, which was first identified and characterized in pea, consists of Toc75, Toc159, and Toc34 (Fig. 1B). Toc75, a β-barrel membrane protein, forms at least part of the channel in the outer membrane through which preproteins are translocated. A number of homologues of pea Toc75 have been identified in *Arabidopsis* (Inoue and Potter 2004; Reumann et al. 2005). The names of the *Arabidopsis* homologues of Toc75 (and all other *Arabidopsis* Toc and Tic homologues) include the two-letter prefix "at", which indicates the species of origin, according to the nomenclature rules laid out by Schnell et al. (1997). Each Toc75 homologue is referred to as "atToc75", but is differentiated by the addition of a Roman numeral suffix that indicates which chromosome the corresponding gene is carried on. It appears that only one of the *Arabidopsis* homologues, designated atToc75-III, is the true functional orthologue of pea Toc75. This is partially evident by the fact that an *Arabidopsis* knockout mutant lacking atToc75-III is embryo lethal, whereas an atToc75-IV knockout is phenotypically normal (Baldwin et al. 2005). There is no evidence that one of the homologues, atToc75-I, is expressed at any time during development. A protein originally identified as atToc75-V (Eckart et al. 2002) has since been renamed AtOEP80, as it is not clear what, if any, involvement it might have in protein import (Inoue and Potter 2004). Interestingly, AtOEP80 (as well as the other Toc75 paralogues) is distantly related to Omp85, a bacterial protein involved in the biogenesis of other outer membrane β-barrel proteins (for a review, see Gentle et al. 2005), and it has been suggested that AtOEP80 might play a similar role in the outer envelope membrane of chloroplasts (Inoue and Potter 2004; Reumann et al. 2005). In addition to being the central component of the Toc core complex, Toc75 has also been shown to exist as a "free" protein in the outer membrane (Kouranov et al. 1998; Ivanova et al. 2004). Recently, it has been demonstrated that this pool of free Toc75 may mediate the insertion of some outer membrane resident proteins that are targeted to chloroplasts without the use of a cleavable transit peptide (Tu et al. 2004).

Toc159 and Toc34 are related GTPases that are involved in preprotein recognition and regulation of import. In *A. thaliana*, these GTPases are represented by small gene families. There are four *Arabidopsis* Toc159 family members (atToc159, atToc132, atToc120, and atToc90) and two Toc34 homologues (atToc34 and atToc33) (Jarvis et al. 1998; Bauer et al. 2000; Hiltbrunner et al. 2001*a*; Constan et al. 2004). Evidence has been presented that Toc159 exists in both a soluble cytoplasmic form and a membrane-bound form in both *Arabidopsis* and pea and that the soluble form may be involved in targeting preproteins to chloroplasts (Hiltbrunner et al. 2001*b*). In vitro biochemical studies involving wild-type and mutant forms of Toc159 containing single point mutations in their GTPase domains demonstate that GTP is required for targeting the soluble form of the protein to chloroplasts but that it is the GDP-bound form that is competent for insertion into the membrane (Bauer et al. 2002; Smith et al. 2002; Wallas et al. 2003). Studies in which Toc159 was transiently expressed in protoplasts independently demonstrated that Toc159 exists as a soluble protein in plants and requires GTP for targeting to chloroplasts (Lee et al. 2003). These data were used to extend an existing hypothesis that proposed Toc159 to be the primary preprotein receptor (Keegstra and Froehlich 1999). The modified model includes the possibility that preproteins could also be recognized and targeted to chloroplasts by the soluble form of the receptor (Fig. 2A). In this so-called "targeting" model, GTP coordinates the assembly of the functional translocon and the sequential promotion of preproteins through the Toc complex until they reach the channel, thus ensuring unidirectional translocation (Fig. 2A) (Kessler and Schnell 2004; Bédard and Jarvis 2005). This hypothesis has been recently challenged (Becker et al. 2004*b*), however, bringing the precise role of soluble Toc159 into question. More experimental evidence is required to demonstrate that the soluble form of Toc159 exists and is relevant to import. Furthermore, if a soluble form of the protein does exist, the question remains as to whether the protein cycles on and off the membrane or whether its targeting to chloroplasts is a one-way pathway. Answers to these questions may require the application of novel experimental approaches.

It has been proposed that Toc159 is also directly involved in outer membrane translocation of preproteins. In vitro biochemical data were used recently to argue that Toc159 acts in a "sewing machine mechanism" to thread preproteins across the outer membrane in a GTP-dependent fashion (Schleiff et al. 2003*a*), which led to the so-called "motor" model for membrane translocation (Fig. 2B) (Becker et al. 2004*b*; Kessler and Schnell 2004; Bédard and Jarvis 2005). In this alternative to the "targeting" model (Fig. 2A), Toc34 acts as the primary preprotein receptor, which passes the substrate on to Toc159, which in turn works as a motor to thread the preprotein across the outer membrane through Toc75 using multiple rounds of GTP hydrolysis (Fig. 2B). In contrast with this model, another study demonstrated that the membrane (M-) domain of Toc159 is the minimum domain required for preprotein import into plastids in vivo (Lee et al. 2003), suggesting that GTP is not required. These findings were consistent with an earlier study in which proteolytic removal of all domains of Toc159 except the M-domain did not completely abolish preprotein import in vitro (Chen et al. 2000). Furthermore, it is known that while GTP is required for import, GTP alone is not sufficient to support preprotein translocation (Young et al. 1999). A key aspect of the "motor" model is the phosphorylation state of both the transit peptide and Toc34; Toc34 is inactivated by phosphorylation, and dephosphoryated Toc34 has the highest affinity for phosphorylated transit peptides, which must be dephosphorylated before being transferred to Toc159 (Fig. 2B) (Sveshnikova et al. 2000; Jelic et al. 2002; Becker et al. 2004*b*). A recent study examined the functional significance of atToc33 phosphorylation in vivo by testing the ability of

Fig. 2. The so-called (A) "targeting" and (B) "motor" models for preprotein recognition and tanslocation by the Toc complex. In the "targeting" model for preprotein recognition and import, transit peptides (TPs) are recognized and bound by the central GTPase domain of cytosolic Toc159 (*i*); however, evidence also suggests that membrane-bound Toc159 is capable of acting as a preprotein receptor (*ii*). The soluble Toc159–preprotein complex docks at the outer envelope membrane of plastids via a low-affinity homotypic interaction between the GTP-bound GTPase domains of Toc159 and Toc34. Formation of the quaternary complex between Toc34, Toc75, Toc159, and the preprotein stimulates GTP hydrolysis by both GTPases, leading to the formation of a high-affinity complex between Toc159 and Toc34, insertion of Toc159 into the membrane, and initiation of preprotein translocation. Exchange of GDP for GTP at both Toc159 and Toc34 "resets" the complex, which might be available to receive another preprotein (*iii*) or might lead to the release of Toc159 back to the cytosol (*iv*), where it would be available for subsequent targeting cycles. In the "motor" model for preprotein targeting, the transit peptide (TP) must be phosphorylated by an unidentified kinase, and Toc34 must first be activated by dephosphorylation by an unidentified phosphatase. The preprotein then docks at the Toc complex via an interaction between the C-terminal end of the TP and Toc34. TP binding triggers GTP hydrolysis by Toc34, which together with TP dephosphorylation leads to the transfer of the preprotein to Toc159. This interaction triggers GTP hydrolysis by Toc159, which causes a conformational change that leads to the preprotein being inserted into the channel. Repeated cycles of GTP-for-GDP exchange and GTP hydrolysis are then used to complete preprotein translocation.



three phosphorylation mutants of the protein to complement an atToc33 knockout (Aronsson et al. 2006). All mutants examined in this study were able to fully complement the mutation in vivo, suggesting that phosphorylation of atToc33 does not play an important role in preprotein import. Therefore, while there is substantial evidence that Toc34 proteins can bind to transit peptides, and it seems possible that Toc159 is involved in preprotein translocation, the mechanism by which it does so remains unresolved, as do the precise roles of GTP and the soluble form of Toc159 in the earliest stages of preprotein recognition and translocation initiation. Both models offer compelling alternatives for the mechanism by which preproteins are recognized and translocated across the outer envelope membrane. Future studies designed to test both models will be of great interest; perhaps, as is so often the case, the data from these future stu-

dies will lead to the development of a new model that is a hybrid of the two existing hypotheses.

At least two other putative components of the Toc complex have been identified, namely Toc64 and Toc12. Toc64 has been reported to copurify with other Toc components under some conditions but not others, and direct evidence for the involvement of Toc64 in protein import into chloroplasts has not been presented (Sohrt and Soll 2000; Schleiff et al. 2003*b*). Interestingly, a second Toc64 homologue has been localized to mitochondria in *Arabidopsis* (Chew et al. 2004). A recent study using genetic and biochemical approaches with the moss *Physcomitrella patens* indicates that Toc64 is not required for preprotein import (Hofmann and Theg 2005). The authors conclude that Toc64 is therefore not a genuine component of the Toc complex and propose that the protein should be renamed OEP64 (Hofmann and Theg 2005). It is therefore unclear what role Toc64 might play in chloroplast protein import in higher plants, but the finding that it is associated with the Toc core complex under some conditions and not others might suggest that it plays a regulatory role. Toc12, a DnaJ domain containing protein, has recently been shown to interact with Toc64 and Tic22 and to recruit Hsp70 in an ATP-dependent manner in the intermembrane space (Becker et al. 2004*a*). These properties suggest that Toc12 many be involved in the formation of Toc–Tic supercomplexes during preprotein import. Evidence for its direct involvement in import is still lacking, and null mutants lacking this protein will be very instructive in determining its precise role in import.

Multiple Toc complexes and differential recognition of cargo proteins

Biochemical studies using pea chloroplasts led to the hypothesis that there is one "general import apparatus" responsible for the translocation of all preproteins into all plastids (e.g., see Jarvis and Soll 2002). The advent of the model plant *A. thaliana*, the use of molecular genetics, and the availability of genomic information and knockout mutants has led to this hypothesis being challenged; it is now widely accepted that complexes with distinct compositions are collectively responsible for the import of the diverse collection of preprotein substrates that are targeted to plastids (Ivanova et al. 2004; Kubis et al. 2004; Smith and Schnell 2004; Bédard and Jarvis 2005). Indeed, the discovery of distinct import pathways and structurally distinct Toc complexes with preferences for specific classes of preproteins in plastids of *Arabidopsis* has led to the suggestion that the import machinery plays an active, rather than passive, role in the biogenesis and differentiation of plastids. Therefore, a much more dynamic picture of the import apparatus has emerged.

As mentioned above, there are four Toc159 homologues in *Arabidopsis*, and it has been demonstrated that atToc159 and atToc132/atToc120 are components of structurally distinct Toc complexes, all of which appear to assemble around the central translocation channel protein, Toc75; complexes containing atToc132/atToc120 also possess atToc34, whereas those containing atToc159 preferentially contain atToc33 (Ivanova et al. 2004). AtToc90 appears to specifically associate with atToc33, although the precise role of atToc90 in import remains to be defined, as null mutants do not exhibit obvious phenotypes, even in combination with mutants lacking other Toc159 homologues (Hiltbrunner et al. 2004; Kubis et al. 2004). Of particular note is that the structurally distinct Toc complexes also appear to be functionally distinct.

Observations of an *Arabidopsis* null mutant lacking atToc159 (*ppi2*) were consistent with earlier biochemical cross-linking data that suggested Toc159 is the first Toc component to interact with preproteins and is therefore the primary preprotein receptor (Perry and Keegstra 1994; Ma et al. 1996; Kouranov and Schnell 1997; Bauer et al. 2000). These observations also led to the initial hypothesis that atToc159 is specifically required for the import of photosynthetic proteins (Bauer et al. 2000). The hypothesis is based, at least in part, on the inability of the *ppi2* mutant to accumulate chlorophyll or photosynthetic proteins and on the failure of leaf plastids to differentiate into chloroplasts. In addition, the mutants do not survive beyond the cotyledon stage of development if grown on soil but continue to develop if grown on sucrose-supplemented agar plates. Of particular note is that the undifferentiated proplastids of leaves accumulate normal levels of essential nonphotosynthetic proteins and that the plastids in nonphotosynthetic tissues such as roots develop normally (Bauer et al. 2000). It has proven difficult to isolate plastids from these mutants (M.D. Smith, unpublished observation), making in vitro import assays unfeasible. Fortunately, an in vivo approach to testing the hypothesis that atToc159 is specifically required for the import of photosynthetic proteins has been informative. GFP fusion proteins including the transit peptides of either a representative photosynthetic protein or a representative nonphotosynthetic protein, the small subunit of Rubisco and the E1α subunit of pyruvate dehydrogenase, respectively, were expressed in both wild-type and *ppi*2 genetic backgrounds, and subcellular localizations were determined using confocal microscopy and Western blotting (Smith et al. 2004). Whereas the nonphotosynthetic transit peptide directs GFP to the plastids of both wild-type and *ppi2* mutant plants, the photosynthetic transit peptide is unable to direct GFP to the plastids in which atToc159 is missing (Smith et al. 2004). These data are consistent with the hypothesis that atToc159 is specifically involved in the targeting and import of photosynthetic proteins, as are in vitro biochemical data on the preprotein binding characteristics of atToc159 (Smith et al. 2004). These assays demonstrate a direct and specific interaction between the GTPase domain of atToc159 and transit peptides and that the receptor preferentially interacts with the transit peptides of photosynthetic proteins.

The corollary to the hypothesis that atToc159 is specifically involved in the import of photosynthetic proteins is that atToc132 and atToc120 are required for the import of nonphotosynthetic, yet equally essential, cargo proteins of plastids. This is supported by in vitro biochemical data that indicate that the atToc132 and atToc120 receptors preferentially recognize and bind nonphotosynthetic cargo proteins that are not the preferred substrates of atToc159 (Ivanova et al. 2004). These findings are also consistent with the observation that *Arabidopsis* null mutants lacking one of atToc132 or atToc120 have no discernible phenotype,

whereas double knockouts lacking both atToc132 and atToc120 are lethal (Ivanova et al. 2004; Kubis et al. 2004). Collectively, these data lend support to the hypothesis that members of the Toc159 family of proteins are transit peptide receptors that represent distinct targeting routes for preproteins to plastids and that these separate pathways are required to ensure balanced import of proteins that are essential to the many biochemical pathways housed within plastids.

One aspect of the "motor" model hypothesis (Fig. 2B) (Schleiff et al. 2003*a*) is that Toc34 acts as the primary preprotein receptor. Interestingly, neither of the *Arabidopsis* Toc34 homologues appears to be essential individually, as single null mutations of either atToc33 (*ppi1*) or atToc34 (*ppi3*) do not display severe phenotypes, although atToc33/atToc34 double mutants are embryo lethal (Jarvis et al. 1998; Constan et al. 2004). Collectively, these data indicate that the Toc34 isoforms are redundant, yet essential, in vivo. The *Arabidopsis* Toc34 homologues do appear to exhibit selectivity in binding to different preproteins in vitro (Jelic et al. 2003; Kubis et al. 2003), and this selectivity appears to extend to the Toc34 homologues from other species (Voigt et al. 2005). The preprotein binding preferences of atToc33 and atToc34 are similar to those of atToc159 and atToc132/atToc120, respectively, which is also consistent with the composition of the structurally and functionally distinct Toc complexes. Taken together with the early biochemical cross-linking data (see above), it therefore seems unlikely that the Toc34 homologues represent the primary receptors providing the main element of substrate recognition and import fidelity. In light of the finding that atToc33 preferentially associates with atToc159 and that atToc34 preferentially associates with atToc132/atToc120, it seems possible that both of the Toc GTPases that comprise individual Toc complexes contribute to the preprotein binding capacity of these complexes. More work is required to determine the precise sequence of events during preprotein binding and translocation and therefore which, if any, of the Toc159 and Toc34 proteins represents the primary receptor.

There is evidence to suggest that import complexes distinct from and in addition to the atToc159/atToc33- and atToc132/atToc120/atToc34-containing Toc complexes exist in *Arabidopsis*. These complexes are proposed to have unique compositions and to be responsible for the import of specialized cargo proteins, such as NADPH-dependent protochlorophyllide oxidoreductase A (Kim and Apel 2004; Reinbothe et al. 2004; Kim et al. 2005). Of note is that even these specialized import complexes seem to be assembled around the translocation channel Toc75. A thorough explanation of these recently described complexes is beyond the scope of this review.

Perspectives

Components of the Toc and Tic complexes that are responsible for the import of nuclear-encoded plastid proteins were first identified in the early to mid-1990*s*. In the years since, many more components have been identified, and the molecular functions of many have been determined. However, owing to the emergence of *Arabidopsis* as a model system, the availability of its genomic sequence and T-DNA knockout mutants, and novel biochemical approaches, the field remains one of exciting and intense investigation. Indeed, new putative Toc and Tic components continue to be identified, as do new pathways for targeting proteins to many of the chloroplast subcompartments. Furthermore, the exact sequence of events that leads to preprotein translocation has not yet been agreed upon.

The presence of structurally distinct plastid protein targeting pathways with considerable but incomplete functional specialization likely reflects the need to maintain balanced import of a diverse array of preproteins and to accommodate the dramatic changes in substrate levels that occur during plastid differentiation. In addition, these distinct pathways may be specialized to provide a level of regulation for the import of specific subsets of preproteins, a function that may be critical for the maintenance of basic plastid function regardless of the developmental state of the organelle. Although good evidence for the existence of these distinct import complexes is rapidly accumulating, the molecular basis by which these receptor complexes assemble and differentiate between preprotein substrates remains unknown. It is possible that the most divergent domain among the Toc159 family members, the N-terminal A-domain, may impart the substrate specificity to the receptors; however, evidence for this has not yet been presented. There is good evidence that the Toc159 family of proteins represents the primary preprotein receptors of chloroplasts, and it seems likely that these proteins are primarily responsible for ensuring the fidelity of import. However, unique structural features that might only be formed upon assembly of the Toc GTPases could possibly be what provide the capability to accurately differentiate between substrate classes. In light of the findings that multiple Toc complexes exist, it is interesting to speculate that perhaps one reason why it has been difficult to identify transit peptide consensus sequences is that there are multiple Toc receptors that recognize different transit peptide sequences. This would be in contrast with cargo recognition by the Signal Recognition Particle, for example, which serves as the signal peptide receptor for virtually all cargo proteins targeted to the ER. It will be interesting to see if consensus sequences begin to emerge for transit peptides that are preferentially recognized by one Toc159 homologue over the other. To be sure, the discovery of distinct and specialized Toc complexes that are involved in the recognition and import of discrete sets of preproteins and may be involved in regulating the import of these proteins as part of the differentiation programs of different plastid types ensures that many more exciting discoveries in the field are still to come.

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