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New insights into the mechanism of chloroplast protein import and its integration with protein quality control, organelle biogenesis and development

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Abstract

The translocons at the outer (TOC) and inner (TIC) envelope membranes of chloroplasts mediate the targeting and import of several thousand nuclear encoded preproteins that are required for organelle biogenesis and homeostasis. The cytosolic events in preprotein targeting remain largely unknown, although cytoplasmic chaperones have been proposed to facilitate delivery to the TOC complex. Preprotein recognition is mediated by the TOC GTPase receptors, Toc159 and Toc34. The receptors constitute a GTP-regulated switch, which initiates membrane translocation via Toc75, a member of the OMP85 (Outer Membrane Protein 85)/TpsB (two partner secretion system B) family of bacterial, plastid and mitochondrial β -barrel outer membrane proteins. The TOC receptor systems have diversified to recognize distinct sets of preproteins, thereby maximizing the efficiency of targeting in response to changes in gene expression during developmental and physiological events that impact organelle function. The TOC complex interacts with the TIC translocon to allow simultaneous translocation of preproteins across the envelope. Two inner membrane complexes, the Tic110 and 1 MDa complexes, have both been implicated as constituents of the TIC translocon, and it remains to be determined how they interact to form the TIC channel and assemble the import-associated chaperone network in the stroma that drives import across the envelope membranes. This review will focus on recent developments in our understanding of the mechanisms and diversity of the TOC-TIC systems. Our goal is to incorporate these recent studies with previous work and present updated or revised models for the function of TOC-TIC in protein import.

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Keywords

TOC complex; TIC complex; chaperones; plastid biogenesis

Introduction

Plastids have evolved an array of protein import and suborganellar targeting systems to facilitate establishment of the structural complexity and functional diversity of this class of organelles [1-4]. The majority of information on protein targeting in plastids has been derived from chloroplasts, the predominant form of plastid found in algae and the green tissues of vascular plants. The biogenesis of chloroplasts during the early stages of plant growth and development involves the proliferation and near complete proteome remodeling of undifferentiated proplastids or non-photosynthetic etioplasts into photosynthetically competent mature chloroplasts [1,5]. These events give rise to mature chloroplasts comprised of three independent membrane systems (outer and inner envelope and thylakoid membranes) and three internal subcompartments (envelope intermembrane space, stroma, and thylakoid lumen). As such, the mechanisms and regulation of protein targeting in chloroplasts provide an excellent model for understanding the contributions of targeting pathways to the physiological and developmental changes that control organelle biogenesis and function.

The initial step in the targeting of nucleus-encoded chloroplast proteins is mediated by, at least, four known sorting and import systems at the double-membrane of the chloroplast envelope [2,3]. These include a cytosolic sorting system that recognizes chloroplast tail- or signal-anchored integral membrane proteins and facilitates their insertion into the outer envelope, thereby avoiding mistargeting of these proteins to the ER or mitochondrial outer membrane [6]. A second, largely uncharacterized system, mediates the targeting of a set of proteins that lack cleavable targeting signals to the chloroplast [7-9]. A third pathway, which is likely a vestige of early events in endosymbiosis, initially targets proteins to the Sec translocon at the ER and subsequently utilizes delivery of the proteins to the chloroplast via vesicle trafficking through the endomembrane system [10-13]. Analyses from chloroplast proteomics studies suggest that up to several hundred polypeptides could utilize these three targeting pathways [14].

In the fourth pathway, nucleus-encoded chloroplast preproteins are imported from the cytoplasm via sequential interactions between their cleavable, N-terminal transit peptides and the translocons at the outer (TOC) and inner (TIC) envelope membranes [2,3]. This pathway constitutes the major protein import system in plastids, mediating the import of ~3,500 polypeptides [15,16]. TOC-TIC serves as the initial gateway for the targeting of the majority of inner membrane, stromal and thylakoid proteins, in addition to a select number of outer membrane proteins. The inner envelope and thylakoid membranes contain their own sorting systems, which function downstream of TOC-TIC and appear to consist largely of pathways conserved and adapted from the cyanobacterial-like ancestor of chloroplasts [17]. The core components of the TOC and TIC systems are present across all plant lineages that evolved from primary endosymbiosis, demonstrating the central role of these import systems

in chloroplast biogenesis [3,18,19]. Analyses of the components and the mechanism of TOC-TIC function reveal an interesting hybrid of translocon functions adapted from the cyanobacterial-like endosymbiont and those imposed by the eukaryotic host cell. As a result, comparisons between our understanding of the chloroplast protein import system and bacterial export pathways provide novel insights into the workings of these translocons. Furthermore, the recent characterization of functionally diverse isoforms of TOC components and the developmental control of the import system shed light on how protein import has evolved in land plants to accommodate functional adaptation and specialization of plastids [20-22]. This review will focus on recent developments in our understanding of the composition, mechanism and diversity of TOC-TIC import systems. Our goal is not to be exhaustive, but focus on incorporating these recent findings into existing models of translocon function and explore new models that integrate protein import into the network of gene expression, protein targeting and quality control/turnover controlling plastid biogenesis and development.

Function of the TOC translocon

Cytosolic events

The majority of chloroplast preproteins appear to be targeted to the chloroplast surface in an unfolded state after they are synthesized on cytoplasmic ribosomes. Preproteins bind directly to the TOC complex via interactions between their intrinsic transit peptides and TOC receptors (Figure 1C). To date, no specific cytosolic targeting factors analogous to the PTS targeting receptors for peroxisomes (PEX) or the SRP targeting system for the ER have been identified in chloroplasts (for review see [6]). However, the analysis of preproteins synthesized in *in vitro* translation systems has identified the cytosolic Hsp70 and Hsp90 family members as potential molecular chaperones that facilitate delivery of the unfolded protein to the TOC complex (Figure 1) [23-26].

Cytosolic Hsp90 also has been shown to interact with both the transit peptide and mature regions of some preproteins and its presence stimulates import *in vitro* [26,27]. Hsp90-preprotein complexes generated in wheat germ extracts also contain the HSP70/HSP90 organizing protein (HOP) and the immunophilin, FKBP73 (Figure 1A)[27]. Docking of the chaperone complexes at the chloroplast surface involves Toc64, an integral outer membrane protein (Figure 1A) [28-30]. Toc64 contains a cytosolic tetratricopeptide repeat (TPR) domain typical of proteins that participate in the formation of Hsp70-Hsp90 chaperone complexes in the cytoplasm, and it is proposed to facilitate transfer of the preproteins from Hsp90 to the TOC complex to facilitate targeting (Figure 1) [30,31]. The model for Hsp90-Toc64 function is analogous to the role for Hsp90 and the mitochondrial outer membrane TPR protein, Tom70, in the targeting of nuclear encoded proteins to the TOM translocase of yeast mitochondria [32]. Plants lack a Tom70 homolog, suggesting that Tom70 in animals and fungi evolved after divergence of these organisms [33]. However, many plant species contain a mitochondrial homolog of Toc64, OM64, suggesting that plant mitochondria and plastids might have evolved related, but specific pathways from a common ancestral gene to assist cytosolic targeting to endosymbiotic organelles [29,33-36].

Hsp70 in association with a 14-3-3 protein of unknown identity increases the efficiency of import *in vitro* using isolated chloroplasts [25,37]. Together, the Hsp70 and 14-3-3 proteins have been designated the cytosolic guidance complex (Figure 1B). The transit peptides of several abundant preproteins contain consensus motifs for the binding of 14-3-3 proteins and binding is controlled via reversible phosphorylation of transit peptides *in vitro* [37]. This has led to a model in which reversible phosphorylation regulates the delivery of preproteins to TOC complexes by the guidance complex [37]. It remains to be determined if the guidance complex docks directly at the TOC complex or interacts with a separate outer membrane receptor system prior to delivery of preprotein cargo to the translocon. The latter possibility is consistent with the recent identification of OEP61, an outer envelope membrane protein that specifically associates with cytosolic Hsp70 (Figure 1B) [38,39]. However, OEP61 has not been directly implicated in protein import and the mechanism by which the guidance complex interacts with TOC remains to be investigated.

It is unclear whether cytosolic Hsp90 and the Hsp70-14-3-3 guidance complex act independently, in concert or as redundant chaperone systems [24]. Knockout of Toc64 in *Physcomitrella patens* resulted in slight disturbances in chloroplast shape, and knockout in *P. patens* and *Arabidopsis* resulted in no visible import defects [40,41]. Modest phenotypes were observed in the *Arabidopsis* double mutant lacking both Toc33 and Toc64 when plants were grown under low fluency light, in the absence of sucrose, or under salt stress [42]. Plants lacking Toc64 show modest, if any, protein import phenotypes in *Arabidopsis* under very specific growth conditions such as light, osmotic or cold stress, whereas Toc64 mutants in the moss, *Physcomitrella patens*, exhibit no observable phenotypes [40-42]. Likewise, mutations that eliminate the phosphorylation sites on several transit peptides implicated in 14-3-3 binding have no measurable effect on targeting *in vitro* or *in vivo* [43,44], and mutation of the putative phosphorylation site of the *Arabidopsis* Toc34 ortholog, atToc33, which is proposed to regulate GTP binding (and hence precursor binding) fully complemented the atToc33 null mutant phenotype [43]. Therefore the physiological role of the guidance complex model remains to be fully understood. Although Hsp90 is proposed to have a distinct substrate specificity from the guidance complex, it is possible that the lack of clear phenotypes in mutants that disrupt either the guidance complex or Hsp90-Toc64 chaperone systems could result from functional overlap between the pathways [24]. Alternatively, the chaperone systems might provide auxiliary functions that are not required for the majority of preproteins. In this scenario, the chaperone complexes could function as a “rescue system” for preproteins that have escaped the normal targeting pathway or have partially misfolded prior to recognition by the TOC system (Figure 1). This function could be particularly important for highly abundant chloroplast proteins, such as rubisco small subunit, or during early stages of chloroplast development when the flux of protein import could approach levels that saturate the capacity of the import apparatus.

Preprotein recognition by TOC receptors

The recognition of the transit peptide of cytosolic preproteins at the chloroplast surface is mediated by the coordinated activities of two integral membrane GTPases, Toc159 and Toc34, and a β -barrel membrane channel, Toc75 [20,45,46]. These three components form a stable complex in the outer membrane and are designated the TOC core complex (Figure

2A). The GTPase domains of the TOC receptors are classified into the translation factor (TRAFAC)-related superclass, which includes many major regulatory GTPases [47]. The Toc34 receptors consist of a cytosolic GTPase domain (G-domain) anchored to the outer membrane by a single C-terminal α -helical transmembrane segment [48-51]. The Toc159 receptors are structurally more complex, consisting of an N-terminal, variable acidic domain (A-domain), a central Toc34-related G-domain, and a large (~54 kDa) outer membrane-anchor domain (M-domain) [52,53]. Direct binding and *in organello* cross-linking studies clearly demonstrate that both Toc159 and Toc34 specifically bind preproteins during the early stages of protein import into chloroplasts, establishing their roles as the initial receptors for preproteins at the TOC translocon [54-59].

Early studies on protein import demonstrated that non-hydrolysable analogs of GTP inhibit preprotein binding and translocation in isolated chloroplasts [60], implicating the GTPase activities of the TOC receptors as key elements in controlling preprotein recognition. Interestingly, the GTPase activity of Toc159 or Toc34 individually is dispensable for protein import *in vivo* [61,62]. However, viable Arabidopsis double mutants lacking GTP binding and hydrolytic activities of both receptors have not been isolated. Taken together, these studies indicate that the receptors constitute an essential GTP-dependent receptor system for preprotein import and suggest that their GTPase activities work in concert to provide partially overlapping functions in controlling the transition from preprotein recognition to the initiation of membrane translocation (Figure 2).

Toc34 and Toc159 exhibit low intrinsic rates of GTP hydrolysis ($k_{cat} < 0.5 \text{ min}^{-1}$), consistent with their proposed roles as switch-activated receptors [63,64]. This feature is characteristic of the GAD (GTPases activated by dimerization) subclass of GTPases, which lack the external GTP/GDP exchange factors (GEFs) or GTPase activating proteins (GAPs) characteristic of small and heterotrimeric G-proteins involved in cellular signaling [65]. GAD GTPases include the signal recognition particle and its receptor [66], and are distinguished by internal regulation of GTPase activity by the reciprocal activities of each domain within the dimer [47,65,67]. Structural information on Toc34 and *in vitro* receptor binding studies revealed a key role for G-domain dimerization in controlling GTPase activity and receptor function. Crystal structures of the G-domains of Toc34 orthologs from pea and Arabidopsis reveal that the GTP/GDP-binding sites on the receptors lie at the dimer interface and form a cage around bound nucleotide with no obvious conduit for nucleotide exchange in the dimer [68,69]. Biochemical studies using full-length or specific domains of the receptors confirmed that the Toc34 G-domain can form homodimers as well as heterodimers with the Toc159 G-domain [69,70]. Both Toc34 homodimers and Toc34-Toc159 heterodimers also have been detected *in vivo* [71]. These studies provided the first clues that dimerization could play a central role in controlling nucleotide exchange and hydrolysis as a component of the preprotein recognition cycle.

Considerable information is available on the role of homodimerization at Toc34 receptors from biochemical and genetic analyses of the pea and Arabidopsis orthologs. Unlike other members of the GAD family, the G-domains of Toc34 do not appear to reciprocally activate GTP hydrolysis in the homodimer [64,68,72,73]. Although the crystal structures suggest the potential involvement of a classical “GAP arginine-finger” extending from one monomer

into the active site of the other monomer in the homodimer [69], mutation analysis of the key arginine residue or other residues involved in dimerization did not reveal measurable effects on nucleotide hydrolysis [64,73]. Furthermore, dimerization does not appear to be highly sensitive to the GTP or GDP bound state of the receptor [72]. Therefore, a classical model of reciprocal GTPase activation within the homodimer observed in other GAD GTPases does not appear to apply to the Toc34 GTPases. Nonetheless, mutations that disrupt dimerization reduce the rate of protein import *in vitro* and *in vivo* demonstrating a key role for G-domain interactions in controlling preprotein recognition at TOC complexes.

Much less is known about the biochemistry or roles of Toc34-Toc159 heterodimerization, and even less is known about the existence or activities of Toc159 homodimers, largely because of the technical challenges of studying the large membrane receptor. It has been shown that Toc34 dimerization mutants also reduce Toc34-Toc159 interactions [73]. This has led to the hypothesis that Toc34 switches between homo- and heterodimers as part of the preprotein recognition cycle (Figure 2) [74-76]. This hypothesis also includes the proposal that Toc34 and Toc159 could reciprocally activate their GTPase activities in a so-called pseudo-*trans*-homodimerization mechanism [65,72], although evidence for *trans*-activation is lacking.

While the relative contributions of homo- versus heterodimerization remain to be determined, a critical relationship between dimerization, GTPase activity and transit peptide binding at the receptors is beginning to emerge. The G-domains of each receptor contain transit peptide binding sites, and they appear to interact with overlapping, but distinct regions of transit peptides [54,57-59,77]. Transit peptide binding is known to stimulate the GTPase activity of Toc34 by promoting GDP/GTP exchange [54,57,63,78]. Interestingly, the stimulation of GTPase activity upon transit peptide binding correlates directly with the disruption of Toc34 dimers [78], and structural models suggest that the transit peptide binding site on Toc34 overlaps the dimer interface [72]. This observation is consistent with a model in which transit peptide binding disrupts the homodimers and allows exchange of bound GDP for GTP (Figure 2B and 2C) [76]. It is not clear if transit peptide recognition also acts as a classic GTP exchange factor (i.e. GEF) in addition to its indirect role of opening up the dimer into a more flexible conformation that allows free exchange of nucleotide. Independent observations suggest that transit peptide binding has the converse effect on Toc34-Toc159 binding by actually stabilizing heterodimer formation [74]. Taken together, these studies suggest that transit peptide recognition triggers dimer reorganization at the receptor GTPases and promotes the exchange and hydrolysis of nucleotide, which underlie the GTP-regulated switch controlling protein import (Figure 2).

The outer membrane preprotein translocation channel

Toc75 is the third essential component of the core TOC complex, and it plays a central role as the major component of the outer membrane preprotein translocation channel. It is encoded by a single gene in all available plant genomes, and null mutants of Arabidopsis Toc75 are embryo lethal [79-81], indicating the essentiality of Toc75 for plant viability. Toc75 is a member of the OMP85/TpsB superfamily of β -barrel proteins found exclusively in the outer membranes of Gram-negative bacteria, mitochondria and plastids (Figure 3)

[46,82-85]. Within this family, Toc75 appears to be most closely related to the BamA/Sam50 β -barrel family of proteins, which function in the biogenesis of β -barrel outer membrane proteins in bacteria and mitochondria [46,82,83,85-87].

The C-terminal region of Toc75 is predicted to form a β -barrel membrane channel consisting of 16-18 membrane-spanning β -strands [88]. The 3-D structure of the Toc75 β -barrel membrane domain containing 16 β -strands (Figure 3A) can be modeled with a high degree of confidence based on the recently determined crystal structures of BamA from *Neisseria gonorrhoeae* (Figure 3B) [86]. The N-terminal region of Toc75 contains three repeats of POTRA (POLypeptide-TRAnsport Associated) domains [89], which are characterized by a $\beta 1\alpha 1\alpha 2\beta 2\beta 3$ secondary structural motif organization [84] (Figure 3A). These domains can be modeled into the predicted 3-D structure based on the crystal structure of the three POTRA domains from the closely related OMP85 protein from the cyanobacterium, *Anabaena* sp. PCC 7120 [88] (Figure 3A). The POTRA domains of Toc75 have been proposed to function in TOC complex assembly, interactions with the TOC GTPases, preprotein recognition or to provide a chaperone-like activity for the preprotein during membrane translocation [89,90].

Toc75 forms stable complexes with Toc34 and Toc159 in stoichiometric ratios estimated at 4:4:1 or 3:3:1 (Toc75:Toc34:Toc159) [91-93]. Toc75 was first identified based on its association with preproteins and envelope-bound protein import intermediates [53,94], and antibodies to Toc75 inhibit protein import into isolated chloroplasts [95]. Toc75 crosslinks to the transit peptides of preproteins during initial binding as well as during later stages of membrane translocation, suggesting that it participates in preprotein recognition with the TOC receptors in addition to mediating membrane translocation [56]. A purified N-terminal segment of the Toc75 POTRA domain from pea Toc75 has been shown to interact with chloroplast preproteins and Toc34, suggesting that the domain could participate in preprotein recognition in concert with the GTPase receptors [90]. This activity would be consistent with the role of POTRA domains in other OMP85/TpsB family members, which serve as docking sites for protein substrates and/or factors that participate in substrate delivery to the β -barrel transporter [87,96].

Recent structures of BamA homologs from *Neisseria gonorrhoeae* and *Haemophilus ducreyi* suggest that POTRA domains also might act to control access to the cavity of the β -barrel membrane domain. These structures revealed two conformations, open and closed, based on the orientation of POTRA domains relative to the β -barrel channel [86]. These two states were proposed to represent a gating mechanism for regulating access to the interior of the β -barrel channel [86]. A crystal structure and molecular dynamics simulations of POTRA domains of OMP85 from *Anabaena* revealed a similar conserved feature in these proteins [88], and mutations in the unique loop region of the POTRA domains adjacent to the β -barrel in cyanobacteria influence the pore properties of the β -barrel [88,90]. It is tempting to speculate that a similar gating mechanism might function at Toc75, thereby providing a mechanism by which access of preproteins to the outer membrane channel could be controlled by transit peptide binding and reorientation of the POTRA domains.

Defining the roles of Toc75 POTRA domains in TOC function are complicated by conflicting reports on the topology of the protein. A number of biochemical studies, including immunoprecipitation using reconstituted Toc75, proteolytic treatment of intact chloroplasts, and N-terminal amino acid sequencing of proteolytic cleavage fragments are consistent with a topology in which the POTRA domains extend into the intermembrane space between the outer and inner envelope membranes, a topology consistent with mitochondrial and bacterial OMP85/TpsB family members [83,97,98]. However, cryo-EM imaging and studies of fluorescent protein fusions to Toc75 have suggested that the POTRA domains face the cytoplasm [99]. This latter study did not address the possibility that the chimeric fusions interfered with the proper insertion and assembly of the protein at the outer envelope, and therefore it is unclear whether the fusion protein represented a functional conformation and topology of Toc75. Figures 1 and 2 present the Toc75 topology with the POTRA domains in the intermembrane space; however, resolution to the question of Toc75 topology will be essential in refining models for the participation of the POTRA domains in the early stages of transit peptide binding at the organelle surface or at later stages by facilitating membrane translocation or interactions with TIC components in the intermembrane space.

It is clear that Toc75 plays a central role in the translocation of preproteins across the outer membrane; however, the exact nature of the membrane channel of the TOC complex remains to be fully defined. Electrophysiological studies of recombinant pea Toc75 reconstituted into planar lipid bilayers demonstrated that the protein forms a cation-selective β -barrel channel [100,101]. Furthermore, transit peptides alter the channel activities of reconstituted Toc75, consistent with the hypothesis that it contains a recognition site for preproteins that could function in conjunction with, or just downstream of the TOC receptors. Toc75 is predicted to form a pore size of approximately 14 to 26 Å (Figure 3A) [100,101], which is sufficient to transport a largely unfolded polypeptide chain, although it remains to be determined if the β -barrel pore of Toc75 alone forms the functional channel for protein import.

Although it is generally accepted that most preproteins are imported into plastids from the cytoplasm in a largely unfolded state, early studies on protein import demonstrated that chloroplasts could transport preproteins that were fused to tightly folded or covalently stabilized domains. These included the import of fusions to dihydrofolate reductase (DHFR) in the presence of methotrexate [102] or bovine pancreatic trypsin inhibitor (BPTI) containing intra-chain disulfide linkages [103]. Similar fusions to mitochondrial presequences block import into mitochondria [104], suggesting that the TOC channel might be more flexible or constitute a more complex assembly of TOC components than the translocase of the outer membrane of mitochondria (TOM) [46]. Interestingly, the crystal structures of the BamA homologs suggest relatively weak hydrogen bond interactions between the first and last β -strands of these proteins, implying a low energetic barrier to lateral opening of the β -barrel membrane domain to the membrane core, compared to the more stable structures observed in other OMP85/TpsB family members, such as the TpsB transporter FhaC (Figure 3B and 3C, compare the β -strands highlighted in green) [84,86]. Structural models of Toc75 predict an elastic conformation similar to that observed in BamA (Figure 3, compare A and B). Toc75 is proposed to exist in multiple copies within TOC complexes [91-93], and therefore it is possible that a Toc75 dimer or oligomer is the active

form of the channel. It is very tempting to speculate that a larger membrane pore could be formed to accommodate the translocation of small folded substrates if the interface of Toc75 monomers included the flexible region, and the β -barrels cooperated to form the channel during protein import.

There is also evidence that the β -barrel domain functions in coordination with the receptor GTPases (e.g. Toc159 M-domain) during preprotein translocation. The M-domain of Toc159 cross-links to preproteins during membrane translocation in isolated chloroplasts, indicating that it is closely associated with the translocating preprotein subsequent to its binding at the G-domain of the receptor [55,56]. The nature of membrane integration of the M-domain is unclear. The 54 kDa domain does not possess any predicted hydrophobic TMDs, but is resistant to protease treatment in intact chloroplasts [52,105], indicating that it is embedded in the outer membrane. *In vivo* studies demonstrate that expression of the M-domain is required for the formation of the minimal functional translocon [106]. These observations have led to proposals that Toc75 and the Toc159 M-domain both participate in membrane translocation [56,106]. It remains to be determined if the M-domain might mediate the assembly of TOC components to form the active translocon or participate in events within the intermembrane space that facilitate insertion of the preprotein or interactions between the TOC and TIC translocons.

Model for TOC function

Insights into the roles of the TOC receptor GTPases and their interactions with Toc75 are consistent with a model in which the receptors function as a GDP/GTP regulated switch to control the initiation of membrane translocation (Figure 2)[107-109]. In the first stage of import, Toc34 and Toc159 act coordinately to recognize the transit peptide of the cytosolic preprotein at the chloroplast surface (Figure 2B). The guidance complex and OEP61 or the Hsp90-Toc64 chaperone system could aid preprotein delivery to the TOC complex (Figure 1). Evidence suggests that Toc64 transiently interacts with Toc34 in a manner that could facilitate transfer of the preprotein from cytosolic chaperones to the TOC receptors [26,42]. There has been considerable debate regarding which TOC GTPase functions as the primary receptor [108], however the observation that Toc34 and Toc159 interact preferentially with distinct regions of the transit peptide suggests that they could bind simultaneously during preprotein recognition (Figure 2B) [74]. This is consistent with covalent cross-linking data demonstrating that both receptors interact with transit peptides at the early stages of preprotein binding [56], and molecular genetic studies in *Arabidopsis* that reveal important roles for both receptors in preprotein recognition [105,110-115].

In the second stage of import, binding of the transit peptide at Toc34, and perhaps Toc159, would dissociate GDP-bound homodimers (Figure 2C). This would lead to two key events in the switch model. First, dimer dissociation triggers exchange of GDP for GTP at the receptor(s) [78]. Second, dissociation of receptor homodimers would promote heterodimerization between Toc34 and Toc159 [74]. This step is envisioned to represent an 'activated' state in which the receptor-transit peptide complex is primed to initiate transfer of the preprotein into the TOC channel. The next set of molecular events leading to insertion of the preprotein into the TOC channel remains a black box. Most models predict that GTP

hydrolysis initiates preprotein insertion at the membrane channel (Figure 2D) and resets the receptor complexes to their GDP-bound homodimeric configurations for a subsequent round of translocation (Figure 2E). As such, GTP hydrolysis would provide the thermodynamic driving force to transition the preprotein from a moderate affinity, bound state at the receptors to an essentially irreversible commitment to membrane translocation. The irreversible state would represent insertion of the preprotein across both the outer and inner envelope membranes and binding of the preprotein to molecular chaperones in the intermembrane space (e.g. Tic22) and the stroma (e.g. cpHsp70, Hsp90C and Hsp93/ClpC) (Figure 2D; see below for details).

Toc159 and Toc34 are predicted to play complementary roles in the initiation of protein import. Mutants that stabilize the GTP-bound state of Toc159 promote protein import, implicating the GTP-bound state of Toc159 as the activated form that promotes the transition from preprotein binding to insertion [116]. Preprotein import in these mutants remains sensitive to non-hydrolyzable GTP analogs, implicating the GTPase activity of Toc34 as key to catalyzing the transition to translocation [116]. This hypothesis draws heavily on the model proposed for the function of SRP and SRP receptor in targeting ribosome-nascent chain complexes to the Sec61 translocon at the ER [117,118]. In the SRP model, GTP hydrolysis catalyzes transfer of the RNC complex from the SRP-SRP receptor complex to the translocon. Hydrolysis also serves to dissociate the SRP from its receptor and thereby provides the free energy to shift the equilibrium from a reversible targeting reaction to stable association of the RNC complexes with the translocon to initiate membrane translocation. An alternative model proposes that Toc159 functions as a GTP-driven motor for translocation across the outer membrane [119]. However, the analysis of the Toc159 GTPase mutants argues against such a motor function [61], and suggests that GTP hydrolysis at Toc159 or Toc34 initiates but does not drive translocation across the envelope membranes.

Nature and function of TIC complexes

Events in the intermembrane space

TOC and TIC components physically associate indicating that the outer and inner membrane translocons directly interact to facilitate import across the double membrane of the envelope [120,121]. To date, the nature of this interaction remains to be defined, but the analyses of stable early intermediates in the import reaction demonstrate that the preprotein engages TIC components in the intermembrane space and the inner envelope membrane upon insertion across the TOC complex (Figure 4)[120,122]. Tic22, a soluble protein, is the only component of the intermembrane space that has been shown to interact with preproteins during import [120]. It is bound to preproteins that span both the TOC and TIC complexes, and co-precipitates with other TIC components [120]. Double mutants lacking the two Tic22 isoforms in *Arabidopsis* show retarded growth, defects in chloroplast biogenesis, and reduced protein import [123] [124]. Structural and functional studies of the Tic22 homolog from apicoplasts of apicomplexan parasites are consistent with Tic22's proposed function as a molecular chaperone [125]. For example, apicoplast Tic22 was able to prevent aggregation of insulin in an *in vitro* aggregation assay, similar to other chaperones including

SecB and Hsp90 [125]. Tic22 chaperone activity was mediated by conserved hydrophobic grooves within the protein [125]. As such, Tic22 is envisioned to prevent misfolding or missorting of the preprotein to the intermembrane space and potentially serve as a component that links the TOC and TIC complexes [30]. Interestingly, the cyanobacterium *Anabaena* sp PCC 7120 also possesses a Tic22 homolog, which is localized to the periplasm and can be functionally replaced by Arabidopsis Tic22 [126]. *Anabaena* Tic22 is implicated in outer membrane biogenesis in concert with the cyanobacterial Omp85, a homolog of BamA [126]. *Anabaena* Tic22 interacts *in vitro* with the POTRA domains of Omp85, which are oriented toward the periplasm [126]. A role for Tic22 in plastid outer envelope biogenesis has not yet been established, although knockout of both Arabidopsis Tic22 homologs does not appear to have an effect on the *in vitro* targeting of the outer envelope proteins OEP24 and Toc34 [123].

The TIC channel

A number of key components of the TIC system have been identified and their specific functions proposed based on biochemical and genetic studies [2,3]. However, the precise nature of TIC complexes remains to be defined despite considerable investigation. Several components of the inner envelope membrane were shown to associate with preprotein intermediates in early studies of import and to co-purify with TOC complexes. These include Tic20/21, Tic22, Tic40 and Tic110 (Figure 4A) [120,127,128] and are referred to collectively as the Tic110 complex. Tic20 contains four α -helical transmembrane segments and reconstitution of the protein into proteoliposomes demonstrated that it functions as a cation-selective membrane channel [129]. Down-regulation of Tic20 expression results in reduced protein import and severe effects on chloroplast biogenesis [130-132]. These observations in conjunction with preprotein crosslinking studies led to the proposal that Tic20 functions as a component of the TIC membrane channel. Interestingly, the Tic20 homolog from the apicomplexan parasite, *Toxoplasma gondii*, has been shown to be essential for protein import into the apicoplast, an organelle evolutionarily related to plastids [133].

A second inner membrane protein of similar structural organization and topology, Tic21 (CIA5/PIC1), was identified in a forward genetic screen for protein import mutants [134]. Tic21 also associates with other TIC and TOC components and Tic21 null mutants in Arabidopsis exhibit specific defects in the accumulation of photosynthetic proteins [134]. It has therefore been proposed that Tic21 plays a complementary role to Tic20 as a part of the TIC channel or in assembling TIC complexes. Tic21 also has been implicated in iron transport, suggesting that it might perform dual functions in chloroplast physiology [135].

Tic110 associates with TOC components in TOC-TIC supercomplexes and therefore is proposed to function as a central component of the inner membrane translocon (Figure 4A) [128,136,137]. It contains two transmembrane helices at its N-terminus with a very short intervening loop region, and a ~95 kDa C-terminal region that extends into the stroma [138]. Tic110 also crosslinks to preproteins during import, and the region of the stromal domain adjacent to the second transmembrane helix has been shown to bind directly to transit peptides [122]. This has led to the proposal that it serves as the initial docking site for

preproteins as they emerge from the TIC channel into the stroma. Null mutants of Tic110 in *Arabidopsis* are embryo lethal, and the expression of deletion mutants in both the N- and C-terminal regions of the Tic110 stromal domain generate dominant negative effects that impact both the assembly of TIC complexes and protein import [139,140].

There has been considerable discussion as to the role of Tic110 as part of the translocation channel. Antibodies to Tic110 alter the activity of an anion channel in the inner membrane that is activated upon protein import [141], and reconstitution of Tic110 fragments in proteoliposomes exhibited cation-selective channel activity [142]. However, structural studies of the Tic110 stromal domain show that a C-terminal amphipathic helix that was proposed to form part of the channel exists in an extended and elongated form that is inconsistent with a channel function [143]. Furthermore, considerable evidence indicates that the stromal domain of Tic110 serves as a scaffold for the binding of several stromal chaperone complexes (see below). These observations are consistent with topology and expression studies in chloroplasts showing that the C-terminal domain of Tic110 resides in the stroma and is not membrane integrated [122,138]. Therefore, it remains to be seen if the channel activities correspond to a direct role for Tic110 in membrane translocation or reflect its close association with other channel components.

Recently, a second 1 MDa protein complex was identified in association with protein import intermediates at the inner envelope membrane, and this complex was proposed to constitute the TIC translocon instead of the previously identified Tic110 complex (Figure 4B) [144,145]. This complex lacks Tic110 and Tic40, but contains Tic20/Tic21 and three novel components, designated Tic214, Tic100 and Tic56. Tic214 contains six transmembrane helices and is the first putative import component encoded by the plastid genome [144]. Tic100 and Tic56 are nuclear encoded and are peripherally associated with Tic214 and Tic20 at the inner membrane [144]. Tic214, Tic100 and Tic56 co-purify with major TOC components, suggesting that they might engage the outer membrane translocon during protein import. *Arabidopsis* Tic100 and Tic56 null mutants exhibit severe growth and greening defects [144], and mutants lacking Tic214 expression in *Chlamydomonas reinhardtii* are lethal [146]. The 1 MDa complex exhibited preprotein responsive channel activity in reconstituted proteoliposomes, consistent with a role as a component of an inner membrane protein import complex [144]. In contrast to the TOC components and Tic110, Tic56, Tic214 and Tic100 are absent from some algal and plant lineages (glaucophyta, rhodophyta and the poaceae) [144], indicating that the function of the complex is not required in all plants.

The 1 MDa complex does not fractionate with Tic110, indicating that they are not stably associated in the inner membrane. This has raised the possibility that the 1 MDa complex constitutes the translocon at the inner membrane, with Tic110 and Tic40 serving roles downstream as organizers of the stromal chaperones that assist at the late stages in import [144]. However, previous studies have demonstrated an association of Tic110 with Tic20 [120], suggesting that there is a shared component and potential physical interaction between these two complexes of the TIC machinery.

At least two models can be proposed to account for the participation of both the 1 MDa and Tic110 complexes in protein import. In the first model, the two complexes could function independently and thereby constitute distinct translocons that mediate the import of different subsets of preproteins. In this model, Tic20 could form a common component of the translocation channel with the specificity of each translocon dictated by assembly with either Tic110-Tic40 (Figure 4A) or Tic214-Tic100-Tic56 (Figure 4B). The fact that both complexes were identified with protein import intermediates generated with the same or similar preproteins makes this hypothesis unlikely [128,145]. As an alternative, we propose a model in which the two complexes could function coordinately during import (Figure 4C) [145]. For example, the 1 MDa complex (Figure 4B) could engage the preprotein early as it emerges into the intermembrane space and serve to facilitate formation of the TIC translocon, which would include Tic110 and Tic40 (Figure 4A). The functional translocon could consist of a complex containing Tic110, Tic40 and the 1 MDa complex, or the 1MDa complex could pass Tic20 and its associated preprotein to Tic110 and Tic40 to facilitate membrane translocation with the assistance of the stromal chaperones (Figure 4C). In this model, the TIC translocon would be dynamic and would assemble in response to preprotein import. The dynamic nature of the interactions between the Tic110/Tic40 and 1 MDa complexes could account for the inability to detect a stable interaction in biochemical experiments. The scenario in which the 1MDa complex mediates an intermediate step in TIC assembly may not be required in all organisms, accounting for the absence of these components in some plant and algal species [144].

Stromal events and the energetics of protein import

Under physiological conditions, protein import proceeds simultaneously across the TOC and TIC translocons. Upon accessing the stroma, the transit peptide of the preprotein is removed by the activity of the stromal processing peptidase (SPP), a soluble metallopeptidase related to the mitochondrial processing peptidase [147-149]. Arabidopsis mutants lacking SPP are lethal, indicating that transit peptide processing is an essential process [148,150,151]. SPP does not stably associate with the TOC-TIC import apparatus, and preprotein processing can occur after completion of preprotein translocation or upon emergence of the transit peptide cleavage site into the stroma [121].

Although the TOC receptor GTPases control the initiation of protein import, their nucleotide binding and hydrolytic activities are consistent with a transit peptide regulated switch that initiates preprotein insertion into the translocon, rather than providing the catalytic driving force for transport of the polypeptide chain across the envelope membranes. ATP hydrolysis alone appears to be sufficient for translocation across both the outer and inner envelope membrane [60,152]. Import is an energetically costly process, requiring the hydrolysis of an estimated 650 ATP molecules per polypeptide transported or a ΔG of ~27,300 kJ/mol protein imported [153]. The analysis of import intermediates and the kinetics of import under ATP limiting conditions demonstrate that ATP hydrolysis by stromal chaperones likely provides the motor for membrane translocation.

Based on its association with a number of stromal chaperones, Tic110 also has been proposed to serve as a scaffold or docking site for the assembly of the import associated

chaperone network that participates in membrane translocation (Figure 5A) [128,136,137,154]. Consistent with this proposal, structural studies of Tic110 from the red algae, *Cyanidioschyzon merolae*, indicate that the stromal domain consists of a rod-shaped helix-repeat that is most similar to the HEAT-repeat motif that forms a platform for protein–protein interactions [143]. Tic40 is loosely associated with Tic110 and also crosslinks to preproteins at later stages in protein import (Figure 5A) [127,155,156]. It contains a single N-terminal transmembrane helix, and its stromal domain contains a central TPR domain and a C-terminal region that is functionally and structurally related to the Hip (Hsp70-interacting protein) and Sti1/Hop (Stress inducible/Hsp70-Hsp90-organizing protein) co-chaperones found in the cytoplasm of eukaryotes [155,157]. Preprotein binding promotes the interaction between the central TPR domain of Tic40 and Tic110, and the C-terminal Sti1/Hop domain has been shown to interact with stromal Hsp93/ClpC chaperone [158]. Arabidopsis Tic40 null mutants exhibit defects in growth and chloroplast biogenesis, and chloroplasts isolated from the mutant exhibit selective defects in import across the inner envelope membrane [127]. Arabidopsis double mutants affecting Tic40 and Tic110, or Tic40 and Hsp93/ClpC activities exhibit no additive phenotypes, consistent with the conclusion that Tic40 works in conjunction with these two components as a co-chaperone to assemble stromal chaperones at the Tic110 scaffold to facilitate protein import (Figure 5A) [140].

The energetics of protein import into plastids is reminiscent of the energetics of the Hsp70-driven motors that function in post-translational translocation into the ER [159] or mitochondria [160]. However, a significant difference between plastids and other organelles is the apparent complexity of the import-associated chaperone system [24,161]. To date, four molecular chaperones have been shown to be associated with Tic110 and the TIC machinery: cpHsp70, Hsp90C, Hsp93/ClpC, and Cpn60 (Figure 5) [128,136,154,162–164].

CpHsp70, the stromal Hsp70 family member, has been shown to associate with the TIC translocon and protein import intermediates in a variety of species examined [163,164]. In Arabidopsis, double null mutants of the two genes encoding cpHsp70 proteins are embryo lethal, and knockout mutants of either of the two genes individually results in defects in chloroplast biogenesis and in reduced levels of protein import [164]. However, the potential pleiotropic consequences resulting from manipulations of cpHsp70, a general chaperone involved in multiple organelle processes including photosystem assembly and maintenance [165,166], thylakoid biogenesis [167], and thermotolerance in germinating seeds [168], left open the possibility that the impact on protein import resulted from indirect effects. An elegant genetic strategy in the moss, *Physcomitrella patens*, addressed this issue [163]. In this study, the gene encoding an essential cpHsp70 isoform was replaced with temperature sensitive mutant varieties based on similar mutations from cytosolic Hsp70s. No differences in protein import were observed between control and mutant chloroplasts under permissive conditions, but treatment of isolated chloroplasts at the non-permissive temperature caused reduced protein import. Furthermore, the same study demonstrated that reduction in the levels of the chloroplast cpHsp70 co-chaperone, CGE (chloroplast GrpE homologue), also reduced protein import levels [163]. More recently, the same investigators generated mutations in moss cpHsp70 resulting in 2- to 3-fold higher K_m values for ATP hydrolysis [169]. Remarkably, measurements of the kinetics of protein import as a function of ATP

concentration in chloroplasts carrying these altered chaperones resulted in nearly identical 2- to 3-fold increases in the K_m for preprotein translocation. These studies provide compelling evidence that the stromal cpHsp70 functions as a rate limiting step in membrane translocation, and therefore is a central component of the protein import motor.

The chloroplast isoform of Hsp90, Hsp90C, also has been implicated in protein import [162], and is encoded by a single essential gene in Arabidopsis [162,170]. Hsp90C was identified in complexes containing import intermediates in the later stages of protein import and was shown to co-immunoprecipitate with membrane complexes containing Tic110, cpHsp70 and Hsp93/ClpC. The reversible Hsp90 ATPase inhibitor, radicicol, blocks protein import into isolated chloroplasts. Interestingly, radicicol does not inhibit transit peptide binding at TOC or the early stage of import when the preprotein has inserted across the TOC complex and engaged the TIC translocon, but it does block subsequent translocation of the polypeptide across the two membranes. This observation led to the proposal that Hsp90C participates with other stromal chaperones as part of the import motor. This hypothesis is supported by studies in cyanobacteria, Chlamydomonas and Arabidopsis demonstrating that the bacterial/organelle Hsp70 and Hsp90 family members interact in complexes containing additional co-chaperones [171-173].

Stromal Hsp93/ClpC is a member of the Hsp100 AAA+ (ATPases associated with various cellular activities) family, and it was shown to associate with Tic110 complexes and preproteins in an ATP-dependent manner [136,154]. Two functionally redundant isoforms of Hsp93/ClpC exist in chloroplasts, and knockout of the gene encoding the most abundant isoform results in defects in chloroplast biogenesis and protein import [140,174,175]. Hsp93/ClpC can form hexameric rings similar to related Hsp100 family members in bacteria and the cytoplasm of eukaryotes [176]. Early models predicted that Hsp93/ClpC docks at Tic110 and functions to drive membrane translocation by threading the polypeptide through the central channel of the hexameric ring. Subsequent biochemical reconstitution studies demonstrated that preprotein binding at Tic110 triggers recruitment of Tic40, which promotes dissociation of the preprotein from Tic110 and transfer of the polypeptide to Hsp93/ClpC [158]. The C-terminal Sti1 domain of Tic40 stimulates Hsp93/ClpC ATPase activity, leading to the hypothesis that Tic40 plays a critical function as a co-chaperone in assembling the translocation motor complex [122,158]. The central TPR repeat domain and C-terminal Sti1p/Hop/Hip domains of Tic40 are structurally related to the domains of co-chaperones that mediate the assembly of cytosolic Hsp70-Hsp90 chaperone complexes in the cytoplasm [127,155,157]. In yeast, cytosolic Hsp90 co-chaperones, including Sti1, also have been shown to interact directly with Hsp104, a member of the Hsp100 AAA+ family [177]. Therefore, Tic40 is an excellent candidate to coordinate the assembly and activities of the larger import associated chaperone network at the TIC complex in addition to its proposed role in mediating Hsp93/ClpC-Tic110 interactions.

The remarkable complexity of the import-associated chaperone network in chloroplasts compared to other organelle translocation systems raises interesting questions regarding the relative contributions of each chaperone to the import reaction. Double mutants affecting cpHsp70 and Hsp93/ClpC function result in more severe phenotypes relative to the individual mutants, suggesting that they function in independent or partially overlapping

pathways [164]. This has led to the proposal that the two chaperones exhibit selectivity toward distinct preprotein substrates, depending upon the physical properties of the preprotein, and thereby constitute distinct translocation motors that function in parallel pathways during import (Figure 5C). However, a number of observations disfavor a model in which the chaperones function independently. The pharmacological studies of Hsp90C and its physical interactions with cpHsp70 suggest the two proteins physically associate and participate in the same stages of membrane translocation [162,171-173,178]. Furthermore, studies on cpHsp70, Hsp90C and Hsp93/ClpC demonstrate that they each function in the import of diverse and overlapping sets of preproteins [158,162-164]. In addition, the energetics studies in moss strongly implicate cpHsp70 activity as the rate-limiting component for the import of multiple preproteins [169]. This favors a model in which the Hsp90C and Hsp93/ClpC chaperones function in concert with the cpHsp70 motor to facilitate membrane translocation of preproteins (Figure 5B).

Quality control

The flux of protein import into plastids varies widely, depending on diurnal cycles, developmental programs, and stress conditions, and both the levels and profiles of import substrates can change dramatically as part of the plant's response to physiological adaptations [179-183]. For example, expression levels of the most abundant photosynthetic proteins alone can increase from nearly undetectable amounts in etiolated tissues to greater than 25% of total cellular transcript levels during greening and early seedling development [5]. Considerable proliferation in the number of chloroplasts per cell in newly expanding green tissues accompanies this transition to photoautotrophic growth. Consequently, the capacity of the protein import apparatus must rapidly adapt to facilitate the changes in gene expression that alter the plastid proteome. Recent studies have provided evidence that quality control systems have evolved to monitor the capacity of the TOC-TIC system to import proteins efficiently and avoid the accumulation of chloroplast preproteins in the cytoplasm, and also potentially to eliminate newly imported proteins that have misfolded or have been mistargeted in the stroma.

Under normal circumstances, chloroplast preproteins are not detected in the cytosol prior to import, demonstrating the existence of efficient mechanisms to prevent the accumulation of preproteins in the cytosol if the capacity for import has been exceeded or preproteins misfold prior to engaging the TOC-TIC systems [6]. However, accumulation of cytosolic preproteins is detectable in several TOC/TIC mutants that reduce the efficiency of import [61,106,111,112]. In one such case, significant transcriptional upregulation of cytosolic chaperones and the cytosolic ubiquitin-proteasome system (UPS) was observed in response to defects induced by the absence of atToc159 in the *ppi2* mutant [184,185], and inhibition of the 26S proteasome in these plants resulted in the appearance of chloroplast preproteins in the cytosol. A major cytosolic Hsp70 isoform, Hsc70-4, and the cytosolic E3 ubiquitin ligase, CHIP, were shown to mediate the targeting of these missorted preproteins for degradation (Figure 6A) [185]. Recognition of the cytosolic preproteins was mediated by Hsc70-4 binding to a motif within the transit peptides, providing a potential mechanism for the detection of mistargeted chloroplast proteins for delivery to the UPS [185]. Down-regulation of Hsc70-4 in Arabidopsis also led to the detectable accumulation of chloroplast

preproteins in the cytoplasm [185]. The UPS involving the CHIP E3 ligase also degrades the chloroplast FtsH1 protease under normal conditions when its precursor form appears in the cytoplasm [186]. Proteomics analysis of the Arabidopsis *ppi2* mutant demonstrate that several chloroplast preproteins are N-acetylated when they accumulate in the cytoplasm, and it has been proposed that this modification also might function as a signal for degradation by the UPS [184].

Taken together, these studies provide compelling *in vitro* and *in vivo* evidence for a cytosolic quality control mechanism that monitors the efficiency of protein targeting to chloroplasts (Figure 6A). The recognition of preproteins by the Hsp70-guidance complex involved in preprotein targeting and the Hsc70-CHIP system for quality control are very similar, and it remains to be determined if they represent separate systems or a single, integrated quality control system that assists with targeting to the TOC complex under normal conditions and triggers targeting to the UPS system for degradation when preproteins fail to engage the import system.

A recent study of the suborganellar distribution of the major soluble ClpP protease of the chloroplast and its association with Hsp93/ClpC suggests that quality control via regulated proteolysis might also play a role in monitoring the status of newly imported preproteins at the TIC complex [187]. The chloroplast ClpP protease was shown to associate with the inner envelope membrane in a manner that is dependent upon Hsp93/ClpC, and quantitative analysis suggested that Hsp93/ClpC and ClpP associated with the membrane in stoichiometric amounts. Although no evidence exists to directly connect protein import and the protease, the fact that membrane-bound Hsp93/ClpC appears to be occupied by ClpP raises the intriguing possibility that the Clp system could function as part of a quality control system at the TIC complex in addition to aiding in membrane transport (Figure 6B) [187]. The Clp/Hsp100 chaperones (e.g. ClpC) function in cooperation with the Clp proteases (ClpP) to constitute a major component of the proteolytic machinery in a wide array of microbes and organelles, including plastids [188-191]. Clp/Hsp100 chaperones select substrates and serve as molecular unfoldases to feed polypeptides into the ClpP proteolytic chamber for degradation. Based on these known activities, the Clp system operating at the TIC complex could function to monitor and eliminate newly imported proteins that fail to fold or engage suborganellar targeting systems before they accumulate and potentially aggregate in the stroma (Figure 6B). This hypothesis is consistent with genetic data indicating that the stromal cpHsp70 and Hsp93/ClpC function in distinct pathways [164], and suggest that cpHsp70-Hsp90C primarily comprises the translocation motor and the Clp system constitutes an essential proteolytic quality control system. At this point, a quality control system for newly imported proteins involving a chloroplast inner-membrane-associated Clp protease machinery remains speculative; however this is an attractive proposal to explain the presence of seemingly redundant, multiple chaperones in association with the import complex.

Role of distinct translocons in the diversity of plastid function

During the evolution of specialized cells and organs in land plants, the variety of plastid types expanded to provide numerous functions beyond their primary role in photosynthesis

[192]. The diversification of plastid types was accompanied by expansion of the number of genes encoding the TOC GTPase receptors [18,193]. The different receptors assemble with the single Toc75 channel component, resulting in structurally and functionally distinct translocons [105,110-113,184,194,195].

The Toc159 gene family consists of four genes in Arabidopsis, with several members of the family exhibiting distinct functions in plastid biogenesis [105,110,111,113,194,195]. Two classes of receptors within the Toc159 family in Arabidopsis have been studied in considerable detail. AtToc159 is the major isoform found in chloroplasts, and the *ppi2* null mutant lacking the receptor exhibits an albino, seedling lethal phenotype [105]. Overexpression of a second Toc159 family member, atToc90, can partially rescue the albino phenotype of the *ppi2* mutant, and therefore appears to have a partially overlapping function with atToc159 [110]. The relatively high expression levels of atToc159 at the early stages of chloroplast development and the observation that the seedling lethal phenotype of *ppi2* is rescued with supplemental sucrose led to the proposal that the receptor was primarily responsible for the import of nuclear encoded photosynthetic proteins [105]. Although direct binding and yeast two-hybrid studies indicate that the atToc159 receptor preferentially binds to several photosynthetic compared to constitutively expressed plastid preproteins [58,194,195], it has become clear from gene expression and yeast two-hybrid studies that atToc159 is involved in the import of a broad range of plastid proteins, and therefore its critical role in chloroplast biogenesis is not limited to the import of photosynthetic proteins [184,194].

atToc120 and atToc132 comprise a second distinct set of receptors within the Arabidopsis Toc159 family. These two receptors have overlapping functions and are expressed at similar levels in all tissues examined [111,113]. Overexpression of atToc132 cannot rescue the seedling lethal phenotype of the *ppi2* mutant [111], indicating that the receptor is functionally distinct from atToc159. Furthermore, the two receptors form complexes with Toc75 that are distinct from TOC complexes containing atToc159 [111]. atToc159 and atToc132 preferentially bind to different types of preproteins *in vitro* [58,194,195], and mutants deficient in atToc132 and atToc120 lead to severe or lethal phenotypes that cannot be rescued by sucrose or expression of atToc159 [111,113]. This has led to the hypothesis that the atToc120 and atToc132 receptors function in the import of proteins required for essential functions in all plastid types. Domain swapping experiments between atToc159 and atToc132 demonstrate that exchanging the highly variable A-domains of the two receptors can largely interconvert their functions *in vitro* and *in vivo* [195]. These data indicate that the A-domains of these receptors are major contributors to the distinct specificities of the TOC complexes. The A-domain also is highly phosphorylated, suggesting that its function in regulating the specificity of preprotein recognition could be regulated by post-translational modification [196].

Some plant species also contain multiple genes encoding receptors of the Toc34 family [113,115]. In Arabidopsis, atToc159 and atToc132/120 preferentially assemble with atToc33 and atToc34, the two Toc34 isoforms, respectively [111]. This has led to the hypothesis that the Toc159 and Toc34 family members assemble in a combinatorial manner to generate translocons with distinct binding specificities [20]. Mutants deficient in atToc33

(*ppi1*) or atToc34 (*ppi3*) exhibit different phenotypes, and some differential effects on the import of specific preproteins [48,112,114,115]. However, over-expression of atToc34 rescues the pale phenotype of the atToc33 *ppi1* mutant, suggesting that the differences are largely due to differential expression of the two receptors. Furthermore, some plant species (e.g. rice) contain multiple Toc159 receptor isoforms, but only a single Toc34 gene. Therefore the contributions of the Toc34 receptors to the preprotein binding specificities of distinct TOC complexes appear to be more nuanced than those of the Toc159 family members.

Although it is clear that the different TOC receptors mediate the import of distinct sets of preproteins, defining the exact substrates for each TOC pathway will be challenging. Proteomics and transcriptomics analyses of the *ppi2* mutant and atToc159 co-suppression lines indicated that a large set of photosynthetic proteins were down-regulated, however the expression of a significant number of proteins associated with photosynthesis were unaffected or up-regulated in these plants [184]. Furthermore, proteins associated with other distinct metabolic pathways were negatively impacted. A recent study also demonstrated that atToc132 binds many photosynthetic as well as non-photosynthetic preproteins in a yeast-two hybrid system [194]. The tissue-specific expression levels of the GTPase receptors vary considerably, but all receptor isoforms are expressed in all tissues examined [48,111-113]. These observations challenge models in which different receptors function exclusively in the import of proteins involved in distinct, tissue-specific functions (e.g. photosynthesis).

Li and Teng [197] have proposed a ‘multi-selection and multi-order’ model for transit peptide design on the basis of studies demonstrating differential import of specific classes of preproteins and evidence for the existence of motifs within transit peptides for their recognition by different import components. In this model, they hypothesize that different classes of transit peptides have evolved to contain combinations of recognition motifs that are required for temporally or developmentally controlled import in concert with specific import receptors or translocon components. This model would account for the unusual size range and complexity of plastid transit peptides, and also is consistent with distinct translocons that have evolved divergent, but not entirely exclusive preprotein specificities. Interestingly, studies of the import of an array of chloroplast preproteins revealed significant differences in the relative import efficiencies of preproteins depending on the developmental stage of chloroplasts [22]. Three classes of preproteins were identified, corresponding to those preferentially imported into young, developing chloroplasts, older, maturing chloroplasts or those whose import was insensitive to the developmental stage of chloroplasts. Although import of each class was not directly attributed to specific TOC pathways, the differences were specific to the transit peptide, suggesting a role for distinct GTPase receptors in mediating differential import.

The precise roles of distinct TOC complexes remain to be fully defined, but the expansion of the receptor GTPase families in conjunction with the evolution of functionally diversified plastid types in land plants indicate that the variety of TOC import pathways are an important element of plastid biogenesis, particularly during developmental transitions when plastids convert from one form to another [198,199]. The importance of multiple TOC

import pathways in plastid development was highlighted by a recent study demonstrating that the turnover of TOC complexes is controlled by the UPS [21]. Members of both receptor GTPase families and Toc75 were shown to be ubiquitinated via an E3 ubiquitin ligase, SP1, which is anchored in the outer envelope membrane by two transmembrane helices and contains a RING-type ubiquitin ligase motif exposed to the cytoplasm. In this study, it was shown that the turnover of atToc132 and atToc120 was increased during the etioplast to chloroplast transition (i.e. de-etiolation), and an *sp1* mutant delayed this transition, whereas over-expression of SP1 accelerated de-etiolation and senescence. These data are consistent with the hypothesis that TOC complexes are remodeled to accommodate changes in the types and abundance of preproteins and provide additional evidence that the relative proportions of distinct TOC complexes play a role in the transitions between different plastid types.

Concluding remarks

Recent progress on understanding the composition and activities of the TOC-TIC systems has revealed a protein targeting system that is equal in complexity to the diverse architecture and functions of plastids themselves. The list of TOC-TIC components continues to expand and many basic mechanistic questions remain to be answered. In particular, the nature of the TIC translocon and the relationship between the Tic110 and 1 MDa complexes need to be resolved. Although, the model presented in this review proposes that these components work in concert, it is possible that they represent distinct complexes with specialized functions. This could include a scenario similar to the roles of the mitochondrial Tim23 and Tim22 complexes in mediating the translocation of proteins into the matrix vs. insertion of polytopic membrane proteins at the inner membrane, respectively [200].

Another exciting challenge is to understand the functions of the diverse TOC translocons and their roles in coordinating protein import with the changes in gene expression that accompany developmental and physiological changes in plastids. This will be no small feat, as we have limited knowledge of the biogenesis of plastids other than chloroplasts, and we will need to further develop and refine experimental systems to investigate these diverse organelles. In addition to studies directed at the mechanism of import, investigating the interface between the protein import and quality control systems will be important in gaining insight into how cells control TOC complex diversity and monitor the early and late stages of import to ensure balanced and efficient targeting of import substrates.

Future work aimed at dissecting the specific functions of each chaperone in the import associated chaperone network will be important for understanding their relative contributions to the import motor and/or their roles in protein folding and homeostasis once preproteins emerge into the stroma. It will be interesting to test whether individual chaperones act downstream of the import motor to assist in protein folding directly or by delivering proteins to the Cpn60 chaperonin, and how they participate in the interface between protein import and subsequent suborganellar targeting pathways to ensure efficient hand-off of newly imported proteins to the sorting machineries at the inner membrane and thylakoids.

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Abbreviations

AAA+	ATPases associated with various cellular activities
BamA	β -barrel assembly machinery A
BPTI	bovine pancreatic trypsin inhibitor
CGE	chloroplast GrpE homologue
DHFR	dihydrofolate reductase
GAD	GTPases activated by dimerization
GAP	GTPase activating protein
GEF	GDP/GDP exchange factor
GTPase	guanosine triphosphatase
Hip	hsp70 interacting protein
Hop	HSP70/HSP90 organizing protein
OMP85	outer membrane protein 85
ppi	plastid protein import
POTRA	polypeptide-transport associated
PTS	peroxisomal targeting signal
RNC	ribosome-nascent chain
Sam50	sorting and assembly machinery 50
SRP	signal recognition particle
SPP	stromal processing peptidase
Sti1	stress inducible protein 1
TOC	translocon at the outer envelope membrane of chloroplasts
TIC	translocon at the inner envelope membrane of chloroplasts
TPR	tetratricopeptide repeat
TpsB	two-partner secretion B
TRAFAC	translation factor-related
UPS	ubiquitin-proteasome system

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Highlights

- Protein import into plastids is mediated by TOC and TIC translocons
- Distinct TOC receptors balance import of diverse substrates during plastid biogenesis
- TIC assembles in response to import to allow translocation across both membranes
- An ATP-dependent stromal chaperone network drives membrane translocation
- Quality control systems function to ensure efficient import of preproteins

Figure 1.

Role of cytoplasmic chaperone systems in targeting preproteins to the core TOC complex. Three pathways have been proposed for the targeting of newly synthesized preproteins to the TOC system. A. Cytoplasmic Hsp90 in conjunction with the HOP and immunophilin FKB73 co-chaperones bind preproteins and dock at the outer envelope membrane via an interaction with the cytoplasmically exposed tetratricopeptide repeat (TPR) domain of Toc64, an integral outer membrane protein [26,27]. Toc64 is proposed to deliver preproteins to the core TOC complex by interacting with Toc34 and transferring the preprotein to the Toc34 and Toc159 receptors. B. Cytoplasmic Hsp70 and a 14-3-3 protein of unknown identity form a guidance complex, which binds to the transit peptide and mature regions of newly synthesized preproteins and facilitates targeting to the TOC receptors [25,37]. Binding of the guidance complex is proposed to be regulated by reversible phosphorylation of the transit peptide although *in vivo* evidence in support of a phosphorylation cycle is lacking. The mechanism of preprotein transfer from the guidance complex to the TOC receptors is unknown, but might be facilitated by OEP61, an Hsp70 receptor at the outer envelope membrane. C. Cytoplasmic preproteins also can bind directly to the Toc34 and Toc159 receptors at the core TOC complex. Genetic studies suggest that the Hsp90 and Hsp70 chaperone systems are not essential for protein import, but could increase the efficiency of targeting by preventing mistargeting or misfolding of preproteins prior to binding at the TOC complex (for review see [24]).

Figure 2.

A GDP/GTP regulated switch model for the initiation of import at the TOC complex. A. The core TOC complex consists of two membrane bound GTPase receptors, Toc159 and Toc34, and the β -barrel channel protein, Toc75. The receptors are proposed to exist in their GDP-bound states in the absence of protein import. B. Toc34 and Toc159 can simultaneously recognize the transit peptides of cytoplasmic preproteins at the chloroplast surface via binding sites within their GTPase domains (G-domains). C. Transit peptide binding dissociates receptor homodimers, triggering the exchange of GDP for GTP, and promotes Toc34-Toc159 heterodimerization. This step corresponds to an 'activated' complex, which is primed to initiate transfer of the preprotein into the TOC channel. D. GTP hydrolysis at the receptors initiates preprotein insertion across the outer membrane via Toc75, and across the inner membrane via the TIC complex. The binding of molecular chaperones to the preprotein in the intermembrane space (IMS) and stroma would drive unidirectional translocation across the envelope. E. The core TOC complex would reset to its GDP-bound 'resting' state following the completion of translocation.

Figure 3.

Three-dimensional model of the Toc75 β -barrel channel of the TOC complex. A. The structural model of Toc75 illustrates the C-terminal domain consisting of a predicted 16-stranded β -barrel membrane channel and an N-terminal domain consisting of three repeats of POTRA (POlypeptide-TRAnsport Associated) domains [89]. The composite structure was generated by homology modeling to the crystal structure of the β -barrel membrane channel of the BamA component (PDB ID: 4K3B) of the β -barrel translocase of *Neisseria gonorrhoeae* [86] and the crystal structure of the three POTRA domains from the closely related OMP85 protein from the cyanobacterium, *Anabaena* sp. PCC 7120 (PDB ID: 3MC8) [88]. B. and C. Crystal structures of BamA (PDB ID: 4K3B) of *Neisseria gonorrhoeae* [86] and the TpsB transporter, FhaC, of *Bordetella pertussis* (PDB ID: 2QDZ) [84], respectively. The first and last β -strands of the β -barrel domains of the predicted structure of Toc75 and the known structures of BamA and FhaC are illustrated in green. The individual POTRA domains of each structure are color-coded and labeled P1-P5. Toc75 contains three POTRA domains. BamA and FhaC contain five and two POTRA domains, respectively. Crystal structures of the NgBamA (Barrel assembly machinery A; PDBID: 4K3B) and POTRA1-3 of *Anabaena* Omp85 (PDBID: 3MC8) were obtained from RCSB (<http://www.rcsb.org/pdb>) and used as templates for modeling the β -barrel and POTRAs of Toc75, respectively. The final alignment was used to construct the model using the software Modeller (version 9.12) [201]. A set of 200 models was generated, from which the lowest energy structure was used. The RMSD values of the β -barrel and POTRA domains are 1.48 $^{\circ}$ A and 1.07 $^{\circ}$ A, respectively. The initial low energy model obtained from Modeller was validated by using PROCHECK [202] and VERIFY_3D [203] servers.

Figure 4.

Model for the coordinate function of proposed TIC components in protein import across the inner envelope membranes. Two inner membrane protein complexes have been implicated as core components of the TIC complex. A. A complex composed of the integral membrane proteins, Tic110, Tic40, and Tic20, associate with molecular chaperones in the intermembrane space (Tic22) and the stroma (cpHsp70, Hsp90C and Hsp93/ClpC). Tic22 is proposed to facilitate transit of the preprotein between the TOC and TIC complexes and prevent mistargeting to the IMS. Tic40 contains TPR and Sti1 domains characteristic of chaperone organizing proteins and is proposed to coordinate the assembly of the stromal chaperones to form the import motor [127,158]. The C-terminal stromal domain of Tic110 serves as the scaffold for assembly of the stromal chaperones [122] and interacts with Tic20 and Tic21, two constituents of the TIC membrane channel [120,130,134]. B. A 1 MDa complex consisting of Tic20/21, Tic56, Tic100 and Tic214 also has been shown to associate with preprotein import intermediates and is proposed to constitute the TIC translocon [92,145]. C. An alternative model is proposed in which the Tic110 (A) and 1 MDa (B) complexes would associate dynamically to form the functional TIC complex at the inner envelope membrane. In this model, the two complexes would associate to form the translocation channel via the common components, Tic20/21. Interaction of the preprotein with either individual complex as it emerges across the TOC complex into the intermembrane space could trigger assembly of the functional TIC complex. The stromal chaperones docked at Tic110 would form the ATP-dependent translocation motor to drive import of the preprotein into the stroma.

Figure 5.

Alternative functions of the import associated chaperone network in the stroma. A. Tic110 and Tic40 play key roles in assembling the complex of stromal chaperones that constitute the import motor of the TIC complex. Binding of the stromal domain of Tic110 to the transit peptide of a preprotein as it emerges from the TIC membrane channel triggers binding of the Hsp93/ClpC chaperone to Tic110 with the aid of the TPR and Sti1 chaperone organizing domains of the Tic40 co-chaperone [122,158]. Tic40 also is proposed to facilitate the assembly of the cpHsp70 and Hsp90C chaperones at Tic110. At least two models have been proposed to account for the individual contributions of cpHsp70, Hsp90C and Hsp93/ClpC to protein import. B. In the first model, ATP-driven binding and release of the preprotein from cpHsp70 would constitute the rate limiting step in the import motor [169]. CpHsp70, Hsp90C and Hsp93/ClpC would act coordinately or in sequence to facilitate membrane translocation. C. In the second model, the cpHsp70-Hsp90C complex and Hsp93/ClpC would constitute separate motors with preferences for preproteins with different physical properties [164].

Figure 6.

Models for quality control systems operating in the cytoplasm and stroma to monitor preprotein import and folding. A. The capacity of the TOC-TIC system is sufficient to ensure efficient import of newly synthesized preproteins under normal conditions. When the expression of preproteins exceeds the capacity of the import system, preproteins misfold prior to engaging the TOC receptors, or the TOC-TIC system is damaged, preproteins can accumulate in the cytoplasm and potentially form harmful aggregates. The cytoplasmic Hsp70-4 chaperone recognizes the transit peptides of these excess preproteins and facilitates their degradation by the ubiquitin-proteasome system (UPS) via the activities of the E3 ubiquitin ligase, CHIP, and the 26S proteasome [185]. B. The ClpP protease associates with the inner envelope membrane in stoichiometric amounts relative to the Hsp93/ClpC chaperone [187]. This raises the possibility that Hsp93/ClpC and ClpP function to degrade newly imported proteins that do not fold or assemble properly during or shortly after import. In this model the Hsp93/ClpC docked at the TIC complex would have dual functions as a component of the import motor and in the quality control of newly imported proteins.