Published in FEBS Journal 276, issue 5, 1156-1165, 2009
which should be used for any reference to this work

Protein transport in organelles: The Toc complex way of preprotein import

Birgit Agne and Felix Kessler
Laboratoire de Physiologie Végétale, Université de Neuchâtel, Switzerland

Most of the estimated 1000 or so chloroplast proteins are synthesized as cytosolic preproteins with N-terminal cleavable targeting sequences (transit peptide). Translocon complexes at the outer (Toc) and inner chloroplast envelope membrane (Tic) concertedly facilitate post-translational import of preproteins into the chloroplast. Three components, the Toc34 and Toc159 GTPases together with the Toc75 channel, form the core of the Toc complex. The two GTPases act as GTP-dependent receptors at the chloroplast surface and promote insertion of the preprotein across the Toc75 channel. Additional factors guide preproteins to the Toc complex or support their stable ATP-dependent binding to the chloroplast. This minireview describes the components of the Toc complex and their function during the initial steps of preprotein translocation across the chloroplast envelope.

Keywords
chloroplast; outer membrane; preprotein; translocon

The key players

The first plastid import studies were performed with isolated chloroplasts from pea (*Pisum sativum*). Initially, the energetics of preprotein translocation were addressed and three major steps were identified [1,2]: (a) reversible binding to the surface of the outer chloroplast membrane in the absence of added nucleotides; (b) stable binding of preproteins to the outer chloroplast membrane in the presence of 100 μM ATP (subsequently, an additional requirement for GTP was demonstrated); and (c) translocation into the chloroplast stroma requiring the presence of at least 1 mM ATP.

Manipulation of nucleotide concentrations and experimental conditions allowed the formation of stable preprotein translocation intermediates and the subsequent isolation and identification of components of the associated chloroplast protein import machinery [3–5]. Included among the first components of the chloroplast import machinery to be identified were the three main components of the Toc (translocon at the outer envelope membrane of chloroplasts) complex [4–7]. Two of these components were GTP-binding proteins, later termed Toc34 and Toc159 (where the numbers account for their molecular masses in kDa). Both Toc34 and Toc159 are exposed at the chloroplast surface. This is consistent with their role in precursor protein recognition and receptor protein function. Toc159 was first identified by chemical cross-linking at both the reversible and stable binding stages of preprotein import [2], suggesting, at the time, that it may function as the primary import receptor. The third component identified, the β-barrel membrane protein

Abbreviations
GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; Hsp, heat shock protein; POTRA, polypeptide-transport-associated; ppi, plastid protein import mutant; Tic, translocon at the inner envelope membrane of chloroplasts; Toc, translocon at the outer envelope membrane of chloroplasts; TPR, tetratricopeptide repeat.

Correspondence
F. Kessler, Laboratoire de Physiologie Végétale, Université de Neuchâtel, Rue Emile-Argand 11, CH-2009 Neuchâtel, Switzerland
Fax: +41 32 718 22 71
Tel: +41 32 718 22 92
E-mail: felix.kessler@unine.ch

Toc75, is deeply buried in the outer membrane [4,8]. This is consistent with its function as an outer membrane translocation channel [9,10]. Toc34, Toc159 and Toc75 together form a stable complex and are sufficient for translocation of a preprotein in artificial lipid vesicles [11,12]. Therefore, this complex is generally referred to as the Toc core complex [12]. Two additional components, Toc64 [13] and Toc12 [14], were identified later, and are implicated in preprotein targeting to the Toc complex and heat shock protein (Hsp) 70 recruitment to the inner surface of the outer membrane, respectively (Fig. 1). For reasons of clarity, Figs 2 and 3 only depict the Toc core complexes without accessory components.

Meanwhile, fully sequenced *Arabidopsis thaliana*, with its multitude of molecular genetic tools, has emerged as a new model system and revealed a surprising complexity of Toc components. The *Arabidopsis* genome encodes two paralogs of Toc34 (atToc33 and atToc34) [15,16], and four paralogs each of Toc159 (atToc159, atToc132, atToc120 and atToc90) [17–20] and Toc75 (atToc75-III, atToc75-IV, atToc75-I and atToc75V/atOep80) [21]. There is evidence that the different Toc GTPases paralogs assemble into variable Toc core complexes [19] (Fig. 2). These Toc complexes, containing a small (Toc34 or family member) and a large receptor GTPase (Toc159 or family member) plus the translocation channel Toc75 (atToc75-III), might be structurally similar, but differ in their substrate selectivity [19]. By contrast, organisms with a lower complexity of import substrates such as *Chlamydomonas reinhardtii* having only one homologue of each Toc34 and Toc159 appear to manage with only one 'general' Toc core complex [22].

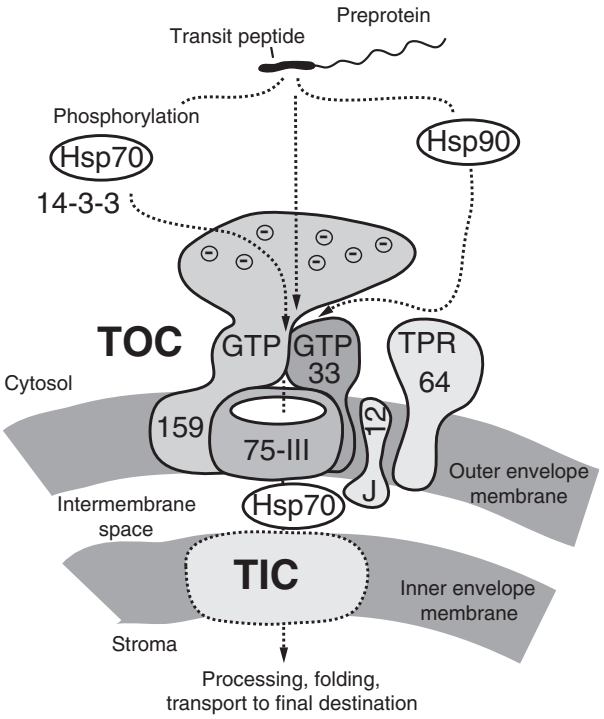

Fig. 1. Schematic representation of *A. thaliana* Toc proteins involved in preprotein translocation across the outer membrane of chloroplasts. The Toc core complex is formed by the two GTP-binding proteins atToc159 (159) and atToc33 (33) and the translocation channel atToc75-III (75). Note that the homologues of atToc159 (atToc90, atToc120, atToc132) and atToc33 (atToc34) may assemble with atToc75 into structurally similar but functionally distinct Toc core complexes (Fig. 2). In addition to its membrane-anchoring and GTP-binding domains, atToc159 has a highly charged acidic domain of unknown function. Some cytosolic preproteins are subject to phosphorylation and assemble into guidance complexes with cytosolic Hsp70 and 14-3-3 proteins before being transferred to the Toc GTPases. Preproteins that bind cytosolic Hsp90 may be targeted to the Toc GTPases via atToc64 (64). atToc64 is loosely associated with the Toc complex and contains three TPR motifs forming the docking site for Hsp90-bound preproteins. AtToc12 (12) exposes a J-domain (J) into the intermembrane space and has a role in anchoring Hsp70, thereby assisting in the transfer of preproteins to the translocase at the inner envelope membrane (Tic). The stoichiometry in actual Toc complexes may differ from the presented scheme.

Oligomeric composition and structure of the Toc core complex

The Toc core complex is often referred to as being trimeric. Moreover, distinct 'trimeric' *Arabidopsis* Toc complexes, atToc159/atToc33/atToc75 and atToc132 or −120/atToc34/atToc75, have been isolated. However, the exact number of each of the constituents of these complexes probably does not equal one. The masses (between 500 and 1000 kDa) that have been determined for the *P. sativum* Toc159/Toc34/Toc75 complex [23–25] indicate the presence of multiple copies of at least some of the components and that the Toc core complex is oligoheteromeric. A stoichiometry of the purified pea Toc core complex of 1 : 4–5 : 4 for Toc159/Toc34/Toc75 was reported [23]. Other Toc core complex stoichiometries determined are based on the quantification of the Toc components in chloroplasts or outer envelopes [24,26]. 2D structural analysis by electron microscopy of a stable Toc core complex from pea revealed approximately circular particles [23]. The particles had a diameter of 13 nm and a height of 10–12 nm and consist of a solid outer ring and a less dense central 'finger' domain. This finger domain divides the central cavity into four apparent pores. It is tempting to speculate that the four pores in the structure are formed by the individual Toc75 molecules that are associated with Toc34 surrounding just a

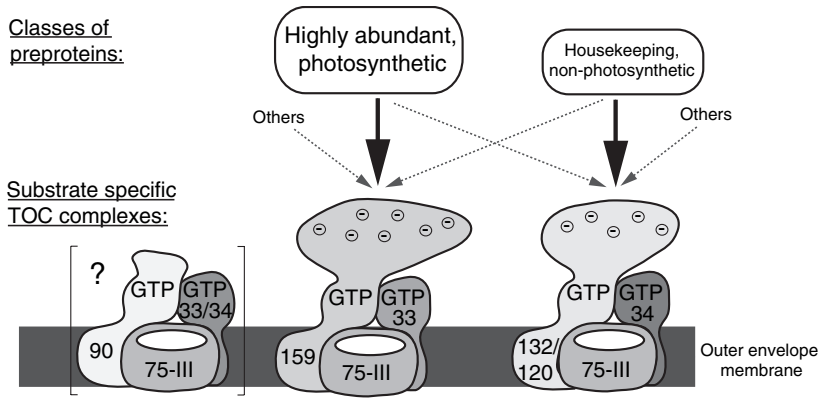

Fig. 2. Model for the assembly of the *Arabidopsis* Toc GTPases into substrate-specific core import complexes. Depending on the tissue and on the developmental stage, different Toc core complexes may be present in plastids to respond to changes in import substrate classes. The most abundant, largely co-expressed isoforms atToc159 (At4g02510) and atToc33 (At1g02280) assemble into Toc core complexes required for the accumulation of strongly expressed photosynthetic preproteins, whereas atToc132 (At2g16640) and/or atToc120 (At3g16620) preferentially assemble with atToc34 (At5g05000). AtToc120 and atToc132 are highly redundant and may be more selective for nonphotosynthetic, housekeeping preproteins. However, mutant analyses do not exclude a specificity overlap between atToc159/atToc33 and atToc132/atToc120/atToc34. So far, no information is available on Toc core complexes containing atToc90 (At5g20300), the only atToc159 isoform lacking an acidic domain.

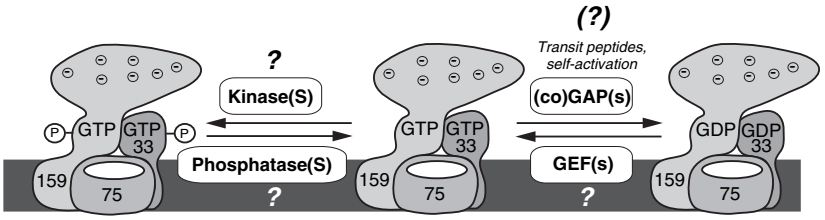

Fig. 3. Wanted! Factors likely to be involved in GTPase regulation at the Toc core complex but still awaiting identification. These are the kinase(s)/phosphatase(s) that phosphorylate/dephosphorylate atToc159 and/or atToc33, as well as factors that control the GTPase hydrolysis cycle by activation (GAPs) or facilitation of the nucleotide exchange (GEFs).

single copy of Toc159, which might contribute to the central 'finger' domain. Combining this structural information with the reconstitution of a chloroplast transport system, demonstrating that Toc159/Toc34/Toc75 are sufficient for GTP-dependent translocation of preproteins into proteoliposomes [12], it has been hypothesized that Toc159 acts as a dynamic component in the complex.

The translocation channel Toc75

Toc75, the major protein import channel across the plastid outer envelope membrane [4,8,9], belongs to the Omp85 superfamily of proteins. Omp85 is a protein present in Gram-negative bacteria and is required for the insertion of β-barrel proteins into the bacterial outer membrane, as well as for the transport of lipids to this membrane [27]. The yeast member of the family Tob55/Sam50 is part of the Tob/Sam complex and is involved in the insertion of β-barrel proteins into the outer mitochondrial membrane [28]. From an evolutionary point of view, it is likely that Toc75 has evolved from a cyanobacterial Omp85 homologue [29,30].

Pea Toc75 is predicted to have either 16 [31] or 18 membrane spanning β-strands [32]. In its N-terminal region, Toc75 possesses characteristic polypeptide-transport-associated (POTRA) domains [33]. POTRA domains are common to outer membrane β-barrel proteins and may confer additional chaperone-like or preprotein recognition functions to the translocation channel Toc75 [34]. Electrophysiological measurements in planar lipid bilayers demonstrated that reconstituted recombinant Toc75 forms a voltage-gated ion channel with properties resembling those observed for other β-barrel pores [10]. Studies of reconstituted Toc75 suggested the presence of a narrow, selective restriction zone (diameter 14 Å) and a 'wider pore vestibule' (diameter 26 Å). Selective interaction with a transit peptide suggests that Toc75 forms a channel specific for proteins to be imported into the chloroplast [9].

Toc75 is the only protein at the outer membrane known to be targeted by a cleavable targeting sequence. The targeting sequence is bipartite. Its N-terminal part functions as a classical transit sequence, whereas the bulk of the Toc75 molecule is retained at the outer membrane. The N-terminal part reaches the chloroplast stroma where it is cleaved by the stromal processing peptidase. The C-terminal part of the bipartite targeting sequence spans the intermembrane space and is cleaved by an envelope bound type-I signal peptidase. A polyglycine stretch in the C-terminal part appears to play an essential role in retaining Toc75 at the outer chloroplast membrane [35].

With the exception of atToc75-I (At1g35860/80), all *A. thaliana* Toc75 paralogs are expressed proteins. atToc75-I is a pseudogene containing a transposon as well as multiple nonsense mutations and stop codons [21].

Of the three remaining paralogs, atToc75-III (At3g46740) is the closest to pea Toc75 and is part of the *Arabidopsis* Toc core complex. T-DNA insertional mutants of atToc75-III are embryo lethal, indicative of a fundamental role in plastid development and differentiation [21,36]. In addition to its role in the import of chloroplast preproteins into the stroma, an additional one with respect to the insertion of the outer membrane protein Oep14 has been discovered [37]. This result suggests that multiple chloroplast targeting pathways may converge at Toc75.

atToc75-IV (At4g09080) is not essential for viability and has been shown to play a specific role in the development of plastids in the dark. AtToc75-V (At5g19620), also known as atOep80 [38], is the most distant paralog of Toc75 as well as that most closely related to Omp85 and Tob55/Sam50 [39]. By contrast to atToc75-III, atOep80 is not processed during membrane insertion, which depends on determinants contained within the protein sequence [38,40]. The expression level of atOep80, except for in embryos, is approximately 25% of that for atToc75-III [40]. The precise role of atOep80 is currently unknown, but an important role in the early stages of plastid development during embryogenesis has been demonstrated [40]. atOep80 is an excellent candidate for a channel component that is involved in the insertion of outer membrane β-barrel proteins.

Toc GTPases

The Toc GTPases, Toc34 and Toc159, are located at the chloroplast surface and interact directly with the transit sequences of preproteins to be imported (Fig. 1). Although their role in preprotein recognition is well documented, the details of the GTPase mechanisms in preprotein binding and outer membrane translocation turn out to be surprisingly complex. It is not entirely clear to what extent the Toc GTPase activity is either directly implicated in the translocation process or indirectly via the assembly of the Toc complex. In this context, the assembly of Toc159 into the outer membrane and the Toc complex has been shown to involve Toc34 (atToc33) in *Arabidopsis* [41–43]. All Toc GTPases are C-terminally anchored in the outer envelope membrane. The small Toc GTPases (in *Arabidopsis*, these are atToc33 and atToc34) have a short hydrophobic transmembrane sequence. The large Toc GTPases (atToc90, atToc159, atToc132, atToc120) have an unusually large C-terminal membrane anchoring domain (M-domain) which is largely hydrophilic in sequence. The GTP-binding domains (G-domain) are exposed to the cytosol. The large GTPases, with the exception of atToc90, have an additional, highly acidic N-terminal domain, designated the A-domain [44]. The function of the A-domain is not known and it appears to be dispensable for *Arabidopsis* Toc159 function [45]. Interestingly, the domain structure of the two Toc GTPases encoded by *Chlamydomas reinhardtii* (crToc159 and crToc34) is reversed with regard to the one of higher plants [22]. CrToc159 lacks the acidic N-terminal domain. By contrast, crToc34 has a longer and more acidic N-terminus than its higher plant counterparts. This suggests the requirement of an acidic stretch in at least one of the Toc GTPases present in the Toc complex.

The enigmatic Toc GTPase cycle

Toc GTPases share a highly conserved GTP-binding domain and belong to the superclass of P-loop NTPases. In this superclass, they can be assigned to the paraseptin subfamily of TRAFAC (after translation factor) GTPases [46,47]. Crystal structures have been reported for the G-domains of *P. sativum* (psToc34) [48] and its *Arabidopsis* functional homologue atToc33 in different nucleotide loading states [49]. Comparison with the minimal G-domain structure of Ras revealed that Toc GTPases, similar to other septin and paraseptin family members, have several insertions that enlarge the structure. Independent of its nucleotide loading-state (GDP or GMP-5′-guanylimidodiphosphate, a nonhydrolyzable GTP analog), psToc34 appears as a homodimer [49]. This, together with the findings of several *in vitro* studies, demonstrates that the G-domains of pea or *Arabidopsis* Toc34/Toc33 and Toc159 can homo- or heterodimerize [41–43,50–55]. Consequently, all current models of

the chloroplast protein import mechanism include the homotypic interaction of Toc GTPases as a key feature. PsToc34/atToc33 homodimerizes across the nucleotide binding cleft and the dimerization involves *inter alia* Toc specific insertions as well the bound nucleotides. Special attention was given to the positioning and function of an arginine residue (R133 in psToc34 and R130 in atToc33) contacting the β- and γ-phosphates of the nucleotide in the opposite monomer. This structural feature is reminiscent of an arginine finger of a GTPase activating protein (GAP) in complex with its GTPase [56]. Therefore, this configuration suggested cross-activation of one monomer by the other. The catalytic role of the presumed arginine finger has been addressed in structural and biochemical studies of mutant G-domains in which this residue was replaced by alanine (psToc34 R133A, atToc33 R130A) [49,51–53,57,58]. The mutation clearly affects dimerization [51–53,58], but has little or no effect on nucleotide binding and the overall structure of the monomer [53,58]. In favour of the arginine finger hypothesis is the observation made in some [48,51,53] but not all studies [52,58] demonstrating that the R133A/R130A mutation reduces GTP-hydrolytic activity and the observation of R133 dependent binding of aluminium fluoride to psToc34-GDP [58]. Aluminum fluoride can mimic the γ-phosphate of GTP, and its binding by GDP-bound GTPases requires the presence of a GAP. Other evidence argues against the theory of psToc34/atToc33 as self-activating GAPs: (a) the GTP-hydrolytic activity of the dimer is only slightly higher compared to the monomer; (b) dimerization does occur preferentially in the GDP-bound state; and (c) the structures of psToc34/atToc33 are similar in the GDP or GMP-5′-guanyl-imidodiphosphate-bound state and do not give any clues on the activation mechanism.

As a result of crystal and biochemical studies on the Toc33 homodimer, a significant advance in the understanding of Toc GTPases has been made. Of course, they do not yet deliver sufficient information to fully explain the unique Toc GTPase cycle, but clearly suggest the requirement of additional factors for activation. Requirements for activation could be Toc34/Toc33-Toc159 heterodimerization or the presence of an import substrate (precursor protein) or as yet unidentified GAP or co-activating GAP proteins [58] (Fig. 3). With respect to the GAPs [59], precursor proteins have already been demonstrated to stimulate the Toc GTPase hydrolysis rate, but this does not exclude the involvement of other factors. In addition, guanine nucleotide exchange factors (GEFs) could be required for nucleotide exchange and completion of the Toc GTPase cycle (Fig. 3).

Regulation of Toc GTPases by phosphorylation

Some of the Toc GTPases are subject to post-translational modification by phosphorylation [60,61]. For the small Toc GTPases psToc34 and its functional *Arabidopsis* homologue atToc33, *in vitro* phosphorylation sites could be determined at different locations in the G-domain: serine 113 in psToc34 [59] and serine 181 in atToc33 [50]. The G-domain of (pea) Toc159 can be phosphorylated *in vitro* as well [62]. Two phosphorylating activities could be located to the outer envelope [60,61], but the molecular identification of Toc GTPase specific kinases and phosphatases has not yet been accomplished (Fig. 3). Phosphorylation imposes a negative regulation because GTP and preprotein binding to *in vitro* phosphorylated psToc34/atToc33 are both inhibited [50,59,60]. The functional relevance of phosphorylation in *Arabidopsis* was studied by making use of a mutant mimicking phosphorylation (atToc33 S181E) [62–64]. AtToc33 S181E exhibits reduced GTPase activity and a reduced affinity for preproteins *in vitro* similar to the phosphorylated protein [64]. Complementation studies of the atToc33 knockout mutant [plastid protein import mutant (*ppi1*)] with the phospho-mimicking mutations atToc33 S181E and two other mutations of the same residue (S118A, S181D) demonstrated efficient complementation of the *ppi1* phenotype in all cases [63]; however, in a subsequent study, a slightly reduced photosynthetic performance of atToc33 S181E *ppi1* transgenic lines was observed at an earlier developmental stage under heterotrophic growth conditions [64]. More recently, an influence of atToc33 phosphorylation or phospho-mimicry on its homodimerization and heterodimerization with atToc159 and its assembly in the Toc complex was reported [62].

Specific functions of the *Arabidopsis* Toc GTPases

The diversity of the Toc GTPases, identified first in *Arabidopsis* but also present in other species, raises the question of their functions. Analysis of the Toc GTPase genes has begun to shed light on their roles in different tissues and plastid types. The knockout mutants of both atToc33 (*ppi1*) [15] and atToc159 (*ppi2*) [17] have pigmentation phenotypes: *ppi1* is pale green during early development but subsequently has wild-type levels of chlorophyll. The cotyledons of *ppi2* plants grown on soil almost completely lack chlorophyll and are therefore albino. Protein analysis in both the *ppi1* and *ppi2* mutants revealed a reduced

accumulation of many proteins involved in photosynthesis (termed 'photosynthetic proteins'), suggesting that both atToc33 and atToc159 are involved in the import of photosynthetic proteins. However, the reduced accumulation of photosynthetic proteins is also tied to a reduction in the expression of the corresponding genes [17,65]. Therefore, the extent of the physical involvement of the two receptors, atToc33 and atToc159, in the translocation of the photosynthetic preproteins (down-regulated in the mutants) is unclear. However, many proteins that are not involved in photosynthesis (termed 'housekeeping proteins') accumulate normally in both *ppi1* and *ppi2*. Their import thus requires neither atToc33, nor atToc159.

Recent research on the atToc159 paralogs, atToc90 [18], atToc120 and atToc132 [19,20], as well as on the atToc33 paralog atToc34 [16,66], has yielded insight on their distinct roles in protein import (Fig. 2). Unlike atToc159, which is highly expressed in green tissues, atToc120 and atToc132 are more uniformly expressed and levels are therefore relatively high in nonphotosynthetic tissues. Although neither of the single genes gives any particular phenotype, the double knockout resulted either in an albino phenotype resembling *ppi2* [20] or in embryo lethality [19]. Proteomics and transcriptomics analysis of the *toc132* mutant and comparison with *ppi1* demonstrated major differences in the expression and accumulation of chloroplast proteins, indicating a role for atToc132/atToc120 in the import of nonphotosynthetic proteins [65]. The single knockout of atToc90 (*ppi4*) had no visible phenotype [18,20]. A *ppi2/toc90* double knockout, however, resulted in a more pronounced albino phenotype, including a more strongly reduced accumulation of photosynthetic protein [18]. These data suggest that atToc90 may contribute to the import of photosynthetic proteins into chloroplasts.

Similar to atToc132 and atToc120, atToc34 is more uniformly expressed throughout the plant than atToc33, which is present at much lower levels in roots than in green tissue [66]. The knockout of atToc34 (*ppi3*) gave a mild phenotype in roots reducing root length, but had no effect in green tissue. Thus, in green tissue, the function of atToc34 may be masked by atToc33 and only revealed in nonphotosynthetic tissues. The double knockout of atToc34 and atToc33 (*ppi3/ppi1*) could not be isolated, suggesting embryo lethality and an essential role of the protein pair [36,66].

Biochemical experimentation also supports specific roles for the Toc GTPases. Immuno-isolation experiments demonstrated the existence of separate Toc complexes consisting of atToc159/atToc33 and atToc120-atToc132/atToc34, respectively [19]. Thus, the current state of knowledge is consistent with two largely separate import tracks containing different Toc GTPase components (Fig. 2). One of the tracks is specific for 'photosynthetic' proteins, whereas the other is specific for 'housekeeping' proteins [67,68]. How Toc GTPases distinguish between different classes of preproteins is currently not known, but this may be linked to subtle differences in the distribution of amino acids along the transit sequence. Recent studies have now classified transit sequences into different groups, which may help answer the questions regarding substrate specificity in chloroplast protein import [69].

Additional players – part I: targeting of cytosolic preproteins to the Toc complex

So far, two pathways targeting preproteins from the cytosol to the outer chloroplast membrane have been described: one involves cytosolic Hsp90 and the outer membrane protein Toc64 [13,70], the other involves cytoplasmic kinases for cytosolic preprotein phosphorylation and the subsequent action of a 'guidance complex' containing a 14-3-3 protein and a Hsp70 isoform [71] (Fig. 1). Toc64, an outer membrane protein, containing four tetratricopeptide repeats (TPR), was identified as a component dynamically associating with the Toc complex via Toc34 [13,70]. Toc64 functions as a receptor for Hsp90 carrying a cytosolic preprotein. In the pathway, Hsp90 docks to the TPR repeats of Toc64 before the preprotein is handed over to Toc34 [70]. Certain preproteins, such as the small subunit of Rubisco, may be phosphorylated at their transit sequence by a member of a small family of kinases that have recently been identified [72]. The phosphorylated preproteins are recognized by a cytosolic 14-3-3 protein contained in the 'guidance complex'. The phospho-preprotein/14-3-3/Hsp70 guidance complex is thought to dock directly to Toc34, without any requirement for the Toc64 receptor. Subsequently, the preprotein is dephosphorylated and passed on to Toc159 to allow progression of translocation across the outer membrane. Studies performed *in vivo* have shown that Toc64 is not an essential gene [73,74], suggesting the existence of alternative cytosolic targeting routes for nonphosphorylated preproteins.

Additional players – part II: recruitment of intermembrane space chaperones

Stable binding of preproteins to the outer chloroplast membrane requires low concentrations of ATP. It is

believed that ATP is hydrolyzed by an intermembrane space Hsp70 protein [75] (Fig. 1). Recently, Toc12 was identified as an outer membrane protein and as a component of the Toc complex [14]. Toc12 projects a DnaJ-like domain into the intermembrane space and was shown to interact with Hsp70 proteins. Toc12 may therefore serve to recruit the Hsp70 exit site of the Toc complex and thereby provide an explanation for the ATP requirement in stable preprotein binding.

Functional model

Recently, two functional models of protein translocation have been controversially discussed, the 'motor' and the 'targeting' hypotheses [68,76]. The main difference between those models is the nature of the primary receptor, namely Toc34 or Toc159 in the 'motor' and 'targeting' hypotheses, respectively. The 'motor' hypothesis proposes that Toc159 pushes the preprotein across the Toc75 channel. The 'targeting' model proposes a soluble cytosolic form of Toc159, the existence of which is contested. Despite the differences between the two models, there is a strong consensus on the composition of the Toc core complex and the role of the Toc GTPase interaction in its mechanism. The Toc GTPase interaction may be the reconciliatory element between the two models: the tight interaction between the two Toc GTPases is clearly required for preprotein insertion into the Toc75 channel and translocation across the outer membrane.

In a simple consensus model (Fig. 1), cytosolic Hsp70/14-3-3 and the Hsp90 guidance complexes (and possibly others still unknown) deliver preproteins to the two GTPases at the Toc complex. The GTP-bound G-domains of Toc159 and Toc34 co-operate to form a GTP-regulated gate at the Toc75 translocation channel. The transition of the receptors to their GDP-bound states and an ensuing conformational change in the GTPase pair pushes the preprotein into the Toc75 translocation channel. An intermembrane space Hsp70 may then contribute to translocation across the outer membrane. The recently discovered Toc12 may recruit the Hsp70 to the trans-side of the Toc complex by its J-motif. Finally, the Toc159 and −34 receptors are reset to their GTP-bound states and become ready for further translocation cycles.

Conclusions

Certainly, future biochemical, molecular genetic and structural experimentation will help to resolve the exquisitely complex details of the GTPase mechanism of protein recognition and translocation at the outer chloroplast membrane. Because preprotein recognition appears to require the tight, GTP-dependent co-operation between Toc159 and Toc34, it remains to be seen whether either one of the two comproses a certifiable primary preprotein receptor. Translocation at the Toc GTPases is regulated by GTP and phosphorylation. The factors implicated in these types of regulation are on the 'most wanted' list of the chloroplast import research community (Fig. 3): the list includes kinases and phosphates as well as co-GAPs and GDP/GTP GEFs. We expect that the available sophisticated molecular tools and sensitive instrumentation will reveal some of these players in the near future.

Acknowledgements

We thank our colleagues at the University of Neuchâtel for valuable discussion and the Swiss National Science Foundation (3100A0-109667), the University of Neuchâtel and the National Centre of Competence in Research (NCCR) Plant Survival for financial support.

References

1 Schnell DJ & Blobel G (1993) Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. *J Cell Biol* **120**, 103–115.
2 Perry SE & Keegstra K (1994) Envelope membrane proteins that interact with chloroplastic precursor proteins. *Plant Cell* **6**, 93–105.
3 Waegemann K & Soll J (1991) Characterization of the protein import apparatus in isolated outer envelopes of chloroplasts. *Plant J* **1**, 149–158.
4 Schnell DJ, Kessler F & Blobel G (1994) Isolation of components of the chloroplast protein import machinery. *Science* **266**, 1007–1012.
5 Kessler F, Blobel G, Patel HA & Schnell DJ (1994) Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science* **266**, 1035–1039.
6 Hirsch S, Muckel E, Heemeyer F, von Heijne G & Soll J (1994) A receptor component of the chloroplast protein translocation machinery. *Science* **266**, 1989–1992.
7 Seedorf M, Waegemann K & Soll J (1995) A constituent of the chloroplast import complex represents a new type of GTP-binding protein. *Plant J* **7**, 401–411.
8 Tranel PJ, Froehlich J, Goyal A & Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO J* **14**, 2436–2446.
9 Hinnah SC, Wagner R, Sveshnikova N, Harrer R & Soll J (2002) The chloroplast protein import channel

Toc75: pore properties and interaction with transit peptides. *Biophys J* **83**, 899–911.
10 Hinnah SC, Hill K, Wagner R, Schlicher T & Soll J (1997) Reconstitution of a chloroplast protein import channel. *EMBO J* **16**, 7351–7360.
11 Ma Y, Kouranov A, LaSala SE & Schnell DJ (1996) Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope. *J Cell Biol* **134**, 315–327.
12 Schleiff E, Jelic M & Soll J (2003b) A GTP-driven motor moves proteins across the outer envelope of chloroplasts. *Proc Natl Acad Sci USA* **100**, 4604–4609.
13 Sohrt K & Soll J (2000) Toc64, a new component of the protein translocon of chloroplasts. *J Cell Biol* **148**, 1213–1221.
14 Becker T, Hritz J, Vogel M, Caliebe A, Bukau B, Soll J & Schleiff E (2004b) Toc12, a novel subunit of the intermembrane space preprotein translocon of chloroplasts. *Mol Biol Cell* **15**, 5130–5144.
15 Jarvis P, Chen LJ, Li H, Peto CA, Fankhauser C & Chory J (1998) An *Arabidopsis* mutant defective in the plastid general protein import apparatus. *Science* **282**, 100–103.
16 Gutensohn M, Schulz B, Nicolay P & Flugge UI (2000) Functional analysis of the two *Arabidopsis* homologues of Toc34, a component of the chloroplast protein import apparatus. *Plant J* **23**, 771–783.
17 Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D & Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* **403**, 203–207.
18 Hiltbrunner A, Grunig K, Alvarez-Huerta M, Infanger S, Bauer J & Kessler F (2004) AtToc90, a new GTP-binding component of the *Arabidopsis* chloroplast protein import machinery. *Plant Mol Biol* **54**, 427–440.
19 Ivanova Y, Smith MD, Chen K & Schnell DJ (2004) Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. *Mol Biol Cell* **15**, 3379–3392.
20 Kubis S, Patel R, Combe J, Bedard J, Kovacheva S, Lilley K, Biehl A, Leister D, Rios G, Koncz C *et al.* (2004) Functional specialization amongst the *Arabidopsis* Toc159 family of chloroplast protein import receptors. *Plant Cell* **16**, 2059–2077.
21 Baldwin A, Wardle A, Patel R, Dudley P, Park SK, Twell D, Inoue K & Jarvis P (2005) A molecular-genetic study of the *Arabidopsis* Toc75 gene family. *Plant Physiol* **138**, 715–733.
22 Kalanon M & McFadden GI (2008) The chloroplast protein translocation complexes of *Chlamydomonas reinhardtii*: a bioinformatic comparison of Toc and Tic components in plants, green algae and red algae. *Genetics* **179**, 95–112.
23 Schleiff E, Soll J, Kuchler M, Kuhlbrandt W & Harrer R (2003a) Characterization of the translocon of the outer envelope of chloroplasts. *J Cell Biol* **160**, 541–551.
24 Kikuchi S, Hirohashi T & Nakai M (2006) Characterization of the preprotein translocon at the outer envelope membrane of chloroplasts by blue native PAGE. *Plant Cell Physiol* **47**, 363–371.
25 Chen KY & Li HM (2007) Precursor binding to an 880-kDa Toc complex as an early step during active import of protein into chloroplasts. *Plant J* **49**, 149–158.
26 Vojta A, Alavi M, Becker T, Hormann F, Kuchler M, Soll J, Thomson R & Schleiff E (2004) The protein translocon of the plastid envelopes. *J Biol Chem* **279**, 21401–21405.
27 Gentle IE, Burri L & Lithgow T (2005) Molecular architecture and function of the Omp85 family of proteins. *Mol Microbiol* **58**, 1216–1225.
28 Paschen SA, Neupert W & Rapaport D (2005) Biogenesis of beta-barrel membrane proteins of mitochondria. *Trends Biochem Sci* **30**, 575–582.
29 Reumann S, Davila-Aponte J & Keegstra K (1999a) The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog. *Proc Natl Acad Sci USA* **96**, 784–789.
30 Bolter B, Soll J, Schulz A, Hinnah S & Wagner R (1998b) Origin of a chloroplast protein importer. *Proc Natl Acad Sci USA* **95**, 15831–15836.
31 Sveshnikova N, Grimm R, Soll J & Schleiff E (2000b) Topology studies of the chloroplast protein import channel Toc75. *Biol Chem* **381**, 687–693.
32 Schleiff E, Eichacker LA, Eckart K, Becker T, Mirus O, Stahl T & Soll J (2003c) Prediction of the plant beta-barrel proteome: a case study of the chloroplast outer envelope. *Protein Sci* **12**, 748–759.
33 Sanchez-Pulido L, Devos D, Genevrois S, Vicente M & Valencia A (2003) POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. *Trends Biochem Sci* **28**, 523–526.
34 Ertel F, Mirus O, Bredemeier R, Moslavac S, Becker T & Schleiff E (2005) The evolutionarily related beta-barrel polypeptide transporters from *Pisum sativum* and Nostoc PCC7120 contain two distinct functional domains. *J Biol Chem* **280**, 28281–28289.
35 Inoue K & Keegstra K (2003) A polyglycine stretch is necessary for proper targeting of the protein translocation channel precursor to the outer envelope membrane of chloroplasts. *Plant J* **34**, 661–669.
36 Hust B & Gutensohn M (2006) Deletion of core components of the plastid protein import machinery causes differential arrest of embryo development in *Arabidopsis thaliana*. *Plant Biol (Stuttg)* **8**, 18–30.
37 Tu SL, Chen LJ, Smith MD, Su YS, Schnell DJ & Li HM (2004) Import pathways of chloroplast interior

proteins and the outer-membrane protein OEP14 converge at Toc75. *Plant Cell* **16**, 2078–2088.
38 Inoue K & Potter D (2004) The chloroplastic protein translocation channel Toc75 and its paralog OEP80 represent two distinct protein families and are targeted to the chloroplastic outer envelope by different mechanisms. *Plant J* **39**, 354–365.
39 Eckart K, Eichacker L, Sohrt K, Schleiff E, Heins L & Soll J (2002) A Toc75-like protein import channel is abundant in chloroplasts. *EMBO Rep* **3**, 557–562.
40 Patel R, Hsu SC, Bedard J, Inoue K & Jarvis P (2008) The Omp85-related chloroplast outer envelope protein OEP80 is essential for viability in *Arabidopsis*. *Plant Physiol* **148**, 235–245.
41 Smith MD, Hiltbrunner A, Kessler F & Schnell DJ (2002) The targeting of the atToc159 preprotein receptor to the chloroplast outer membrane is mediated by its GTPase domain and is regulated by GTP. *J Cell Biol* **159**, 833–843.
42 Hiltbrunner A, Bauer J, Vidi PA, Infanger S, Weibel P, Hohwy M & Kessler F (2001b) Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. *J Cell Biol* **154**, 309–316.
43 Bauer J, Hiltbrunner A, Weibel P, Vidi PA, Alvarez-Huerta M, Smith MD, Schnell DJ & Kessler F (2002) Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane. *J Cell Biol* **159**, 845–854.
44 Jackson-Constan D & Keegstra K (2001a) *Arabidopsis* genes encoding components of the chloroplastic protein import apparatus. *Plant Physiol* **125**, 1567–1576.
45 Lee KH, Kim SJ, Lee YJ, Jin JB & Hwang I (2003) The M domain of atToc159 plays an essential role in the import of proteins into chloroplasts and chloroplast biogenesis. *J Biol Chem* **278**, 36794–36805.
46 Leipe DD, Wolf YI, Koonin EV & Aravind L (2002) Classification and evolution of P-loop GTPases and related ATPases. *J Mol Biol* **317**, 41–72.
47 Weirich CS, Erzberger JP & Barral Y (2008) The septin family of GTPases: architecture and dynamics. *Nat Rev Mol Cell Biol* **9**, 478–489.
48 Sun YJ, Forouhar F, Li HM, Tu SL, Yeh YH, Kao S, Shr HL, Chou CC, Chen C & Hsiao CD (2002) Crystal structure of pea Toc34, a novel GTPase of the chloroplast protein translocon. *Nat Struct Biol* **9**, 95–100.
49 Koenig P, Oreb M, Hofle A, Kaltofen S, Rippe K, Sinning I, Schleiff E & Tews I (2008a) The GTPase cycle of the chloroplast import receptors Toc33/Toc34: implications from monomeric and dimeric structures. *Structure* **16**, 585–596.
50 Jelic M, Soll J & Schleiff E (2003) Two Toc34 homologues with different properties. *Biochemistry* **42**, 5906–5916.
51 Reddick LE, Vaughn MD, Wright SJ, Campbell IM & Bruce BD (2007) In vitro comparative kinetic analysis of the chloroplast toc GTPases. *J Biol Chem* **282**, 11410–11426.
52 Weibel P, Hiltbrunner A, Brand L & Kessler F (2003) Dimerization of Toc-GTPases at the chloroplast protein import machinery. *J Biol Chem* **278**, 37321–37329.
53 Yeh YH, Kesavulu MM, Li HM, Wu SZ, Sun YJ, Konozy EH & Hsiao CD (2007) Dimerization is important for the GTPase activity of chloroplast translocon components atToc33 and psToc159. *J Biol Chem* **282**, 13845–13853.
54 Becker T, Jelic M, Vojta A, Radunz A, Soll J & Schleiff E (2004a) Preprotein recognition by the Toc complex. *EMBO J* **23**, 520–530.
55 Wallas TR, Smith MD, Sanchez-Nieto S & Schnell DJ (2003) The roles of toc34 and toc75 in targeting the toc159 preprotein receptor to chloroplasts. *J Biol Chem* **278**, 44289–44297.
56 Scheffzek K & Ahmadian MR (2005) GTPase activating proteins: structural and functional insights 18 years after discovery. *Cell Mol Life Sci* **62**, 3014–3038.
57 Sun CW, Chen LJ, Lin LC & Li HM (2001) Leaf-specific upregulation of chloroplast translocon genes by a CCT motif-containing protein, CIA 2. *Plant Cell* **13**, 2053–2061.
58 Koenig P, Oreb M, Rippe K, Muhle-Goll C, Sinning I, Schleiff E & Tews I (2008b) On the significance of Toc-GTPase homodimers. *J Biol Chem* **283**, 23104–23112.
59 Jelic M, Sveshnikova N, Motzkus M, Horth P, Soll J & Schleiff E (2002) The chloroplast import receptor Toc34 functions as preprotein-regulated GTPase. *Biol Chem Hoppe Seyler* **383**, 1875–1883.
60 Sveshnikova N, Soll J & Schleiff E (2000a) Toc34 is a preprotein receptor regulated by GTP and phosphorylation. *Proc Natl Acad Sci USA* **97**, 4973–4978.
61 Fulgosi H & Soll J (2002) The chloroplast protein import receptors Toc34 and Toc159 are phosphorylated by distinct protein kinases. *J Biol Chem* **277**, 8934–8940.
62 Oreb M, Hofle A, Mirus O & Schleiff E (2008) Phosphorylation regulates the assembly of chloroplast import machinery. *J Exp Bot* **59**, 2309–2316.
63 Aronsson H, Combe J, Patel R & Jarvis P (2006) In vivo assessment of the significance of phosphorylation of the *Arabidopsis* chloroplast protein import receptor, atToc33. *FEBS Lett* **580**, 649–655.
64 Oreb M, Zoryan M, Vojta A, Maier UG, Eichacker LA & Schleiff E (2007) Phospho-mimicry mutant of atToc33 affects early development of *Arabidopsis thaliana*. *FEBS Lett* **581**, 5945–5951.
65 Kubis S, Baldwin A, Patel R, Razzaq A, Dupree P, Lilley K, Kurth J, Leister D & Jarvis P (2003) The *Arabidopsis* ppi1 mutant is specifically defective in the

expression, chloroplast import, and accumulation of photosynthetic proteins. *Plant Cell* **15**, 1859–1871.
66 Constan D, Patel R, Keegstra K & Jarvis P (2004a) An outer envelope membrane component of the plastid protein import apparatus plays an essential role in *Arabidopsis*. *Plant J* **38**, 93–106.
67 Kessler F & Schnell DJ (2006) The function and diversity of plastid protein import pathways: a multilane GTPase highway into plastids. *Traffic* **7**, 248–257.
68 Jarvis P (2008) Targeting of nucleus-encoded proteins to chloroplasts in plants. *New Phytol* **179**, 257–285.
69 Lee DW, Kim JK, Lee S, Choi S, Kim S & Hwang I (2008) *Arabidopsis* nuclear-encoded plastid transit peptides contain multiple sequence subgroups with distinctive chloroplast-targeting sequence motifs. *Plant Cell* **20**, 1603–1622.
70 Qbadou S, Becker T, Mirus O, Tews I, Soll J & Schleiff E (2006) The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64. *EMBO J* **25**, 1836–1847.
71 May T & Soll J (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. *Plant Cell* **12**, 53–64.
72 Martin T, Sharma R, Sippel C, Waegemann K, Soll J & Vothknecht UC (2006) A protein kinase family in *Arabidopsis* phosphorylates chloroplast precursor proteins. *J Biol Chem* **281**, 40216–40223.
73 Aronsson H, Boij P, Patel R, Wardle A, Topel M & Jarvis P (2007) Toc64/OEP64 is not essential for the efficient import of proteins into chloroplasts in *Arabidopsis thaliana*. *Plant J* **52**, 53–68.
74 Hofmann NR & Theg SM (2005b) Protein- and energy-mediated targeting of chloroplast outer envelope membrane proteins. *Plant J* **44**, 917–927.
75 Kessler F & Schnell DJ (2004) Chloroplast protein import: solve the GTPase riddle for entry. *Trends Cell Biol* **14**, 334–338.
76 Kessler F & Schnell DJ (2002) A GTPase gate for protein import into chloroplasts. *Nat Struct Biol* **9**, 81–83.