

Review

Protein import machineries in endosymbiotic organelles

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Abstract. Chloroplast and mitochondria, the two organelles with an accepted endosymbiotic origin, have developed multiple translocation pathways to ensure the subcellular allocation of proteins synthesized by cytosolic ribosomes, and to guarantee their assembly into functional complexes in coordination also with organellar-encoded subunits. The evolution

of different protein import machineries was thus essential for the development of these two organelles within cells. A general overview of the translocation machineries in chloroplast and mitochondrial membranes involved in targeting and import of nuclear-encoded proteins, with special focus on plant cells where the two organelles coexist, is expounded.

Keywords. Protein translocation, mitochondria, chloroplasts, thylakoids, dual-targeting, endosymbiotic organelles.

Endosymbiosis and the plant cell

It is widely accepted that mitochondria, found in eukaryotic cells, and plastids, just in plant cells, are organelles of endosymbiotic origin [1]. Two independent endosymbiotic events gave rise to the evolution of these organelles in a plant cell. First, mitochondria evolved from a single endosymbiotic event between a host cell and an aerobic α -proteobacterium. As a consequence, a diversity of heterotrophic eukaryotic lineages emerged [2]. Later, plastids were incorporated from a free-living photosynthetic prokaryote, an ancestor of contemporary cyanobacteria,

which was engulfed by a heterotrophic eukaryotic host that already contained mitochondria. The primary endosymbiotic event in plastid evolution gave rise to glaucophyta, rhodophyta and viridiplantae lineages [3]. Subsequent endosymbiotic events in plastid evolution have occurred [4].

With time, the majority of the genetic information from the endosymbionts was lost or transferred to the nucleus of the host cell, though some amount of information is still retained by the organelles, thus functioning as a semiautonomous system [5]. Because of the gene relocation in the host cell, several mechanisms were developed to re-target and efficiently transport the proteins acting in the organelles but now expressed in the host cytosol. Some of these machineries were adapted from bacteria; others show

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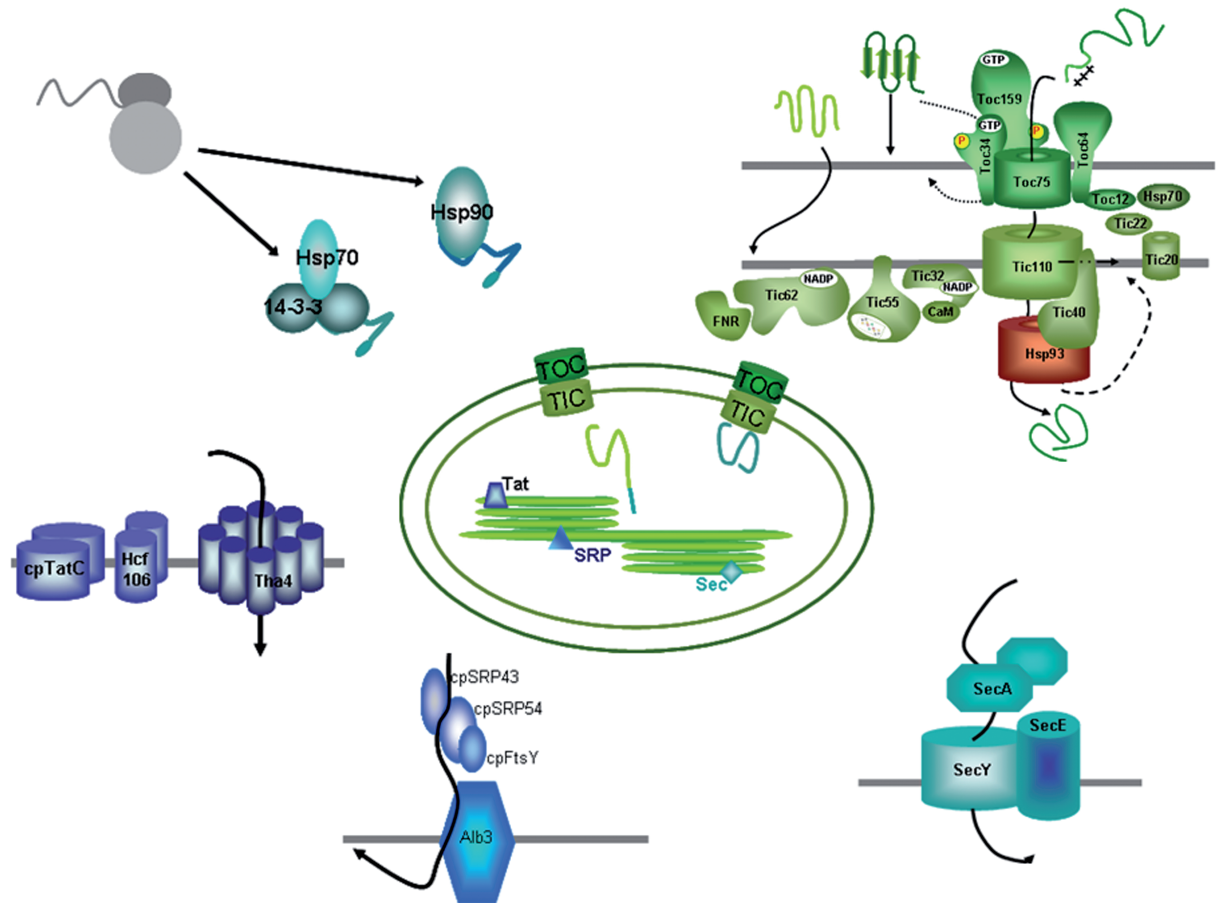


Figure 1. Overview of chloroplast translocation pathways. Indicated are the targeting pathways in the cytosol and all translocases present in chloroplast membranes. Complexes and their respective components are color coded (for easier identification). For details on composition, function and regulation please refer to the text.

no apparent homology to any bacterial secretion system but were newly developed, even though some constituents are of prokaryotic origin [6, 7]. In any case, all of them follow certain general principles [8]. With the exception of the Tat transport pathway in thylakoids (see below), the substrate has to be unfolded or loosely folded in order to be translocated. The substrates, in a complex with chaperones in an import-competent state (post-translational import) or nascent in ribosomes (co-translational import), bind to membrane receptors and are transferred to the membrane-embedded protein import channels. Once there, the substrates completely cross the membrane with the assistance of chaperones on the *trans* side or are laterally transferred into the lipid bilayer. Unlike the case in other eukaryotic systems, plant cytosolic proteins destined for the mitochondrion must be distinguished from those destined for the plastid. Moreover, given the presence of plastids in plant cells, it is not surprising that plant mitochondria have specific features, uncommon in other eukaryotes, to ensure their integration and communication within

the cell [9]. Herein we present a general overview of the translocation machineries in chloroplast and mitochondrial membranes involved in targeting and import of nuclear-encoded proteins, with special focus on plant cells where the two organelles coexist.

Protein import in chloroplasts

Most of the work in plastid-protein import has been carried out in chloroplasts, the most abundant type of plastids where photosynthesis occurs. It is believed that other plastids do contain identical sorting systems at the envelope level [10–12]. During the last years, one general import pathway in chloroplast envelopes has been described, constituted by the translocons at the outer and the inner envelope of chloroplasts (TOC and TIC, respectively) (Fig. 1). With the progress in plant genome information, gene multifamilies were recognized for some components of the envelope translocons.

Chloroplastic transit peptides

The majority of nuclear encoded proteins destined for an interior chloroplast compartment comprise an N-terminal transit peptide (TP), which is cleaved off by the stromal processing peptidase (SPP) during/after import [13,14]. In the early days of protein import research, people expected TPs to contain definite, clear motifs or structural features to ensure specific targeting and especially to avoid mistargeting to other organelles. Much to our surprise, it was found that TPs are heterogeneous in sequence and seem to be mostly unstructured [13, 14]. They vary in length (20–150 amino acids) and show no sequence conservation. The only general features are the lack of acidic residues, resulting in an overall positive charge, and the presence of several hydroxylated amino acids, especially serine. In these aspects, they resemble mitochondrial targeting sequences (see below). However, in contrast to mitochondrial targeting signals, chloroplast TPs do not adopt a secondary structure in aqueous solution [15]. Von Heijne and Nishikawa even proposed that TP features result in a “perfect random coil” [16]. This lack of secondary structure is supposed to play an important role in the recruitment of cytosolic factors upon completion of translation [17, 18]. For some TPs it has been demonstrated that they acquire a typical structure upon contact with the lipid environment of the chloroplast outer envelope [19, 20], which has a unique composition clearly distinguishing it from the outer mitochondrial membrane [14]. Thus, it could be speculated that only upon interaction with the outer envelope-specific galactolipids do TPs adopt a secondary structure which then furthers the import process [21]. Very recently, Lee et al. [22] conducted a detailed study using hierarchical clustering to define certain motives within TPs and so far, they could assign several chloroplast preproteins to seven different groups. Some TPs, however, still do not fit into any of the established groups, so that the authors speculate about there being additional criteria not yet recognized.

Moving from the cytosol to chloroplasts: Precursor protein delivery

As preproteins emerge from the translating ribosome into the cytosol, they encounter a molecular crowded environment where they need to be protected from misfolding/aggregation. Additionally, it is necessary to maintain preproteins in an import competent state, i. e. unfolded and extended. Chloroplast TPs comprise high affinity binding sites for Hsp70 proteins [18, 23], which generally bind to unfolded polypeptides, though deletion of these motifs does not affect translocation *in vitro* [24,25]. Besides keeping preproteins in an import favorable conformation, cyto-

solic components have been shown to be involved in the import process itself. Several plastid TPs comprise a binding motif for 14-3-3 proteins [17], known to mediate protein-protein interactions. These proteins specifically recognize phosphorylated residues within TPs [26,27] and together with Hsp70 build up a cytosolic guidance complex that escorts preproteins to chloroplasts only, since mitochondrial targeting sequences are not recognized by 14-3-3 proteins. Recently, an additional cytosolic system has been proposed: some preproteins have been shown to interact with Hsp90, rather than with Hsp70/14-3-3 [28]. These Hsp90 associated precursors first interact with the dynamically associated receptor Toc64 (see below) via its cytosolic tetratricopeptide repeat (TPR) domain and passes its cargo on to Toc34. *Toc64* deletion mutants failed to exhibit any effects in growth or import behavior, indicating that the first step of recognition at the chloroplast surface involving Toc64 can be bypassed and the precursor, even when bound to Hsp90, can be accepted by Toc34 directly [29, 30]. Thus, the pathway via Toc64 seems to represent a mechanism which renders *in vivo* import more efficient but is not absolutely essential.

Import machinery at the outer envelope membrane of chloroplasts: the TOC complex

The subunits that compose the TOC and the TIC complexes are named according to their molecular weight. The TOC complex is composed of a channel protein (Toc75), two GTPase receptors (Toc159 and Toc34) and two dynamically-associated components (Toc64 and Toc12) [31]. The protein import channel Toc75 is a highly conserved β -barrel protein evolutionarily related to Omp85, involved in the integration of proteins into the bacterial outer membrane in Gram-negative bacteria, and to the mitochondrial outer membrane protein Sam50/Tob55 family (see below) [32, 33]. Recently, a high resolution structure has been obtained for a member of the Omp85 superfamily, providing a general insight into the mechanism of the system [34]. The protein is composed of two domains: the N-terminal cytosolic region functions in recognition and as a complex assembly unit; the C-terminal membrane-embedded region forms the β -barrel-type protein-channel, itself modulated by the N-terminal region [35, 36]. In the *Arabidopsis* genome there are four Toc75 homologues: atToc75-I (a pseudogene), atToc75-III, atToc75-IV and atToc75-V, named according to the chromosome where they are encoded. AtToc75-III—the ortholog to Toc75 protein in *Pisum sativum*—is essential for the viability of plants from the embryonic stage, whereas atToc75-IV has a role during growth in the dark [37]. AtToc75-V, also known as OEP80, is the closer relative to the bacterial

Omp85 family and might have a more specialized role in biogenesis of a relatively small subset of proteins [38]. A multigene Toc75 family is also found in *Physcomitrella patens*, whereas in green algae just two isoforms are recognized, one related to atToc75-III/IV and another to atToc75-V [39]. Red algae lack the atToc75-V isoform and the only Toc75 sequence is related to atToc75-III/IV (unpublished data).

Toc34 is the major preprotein receptor of the Toc complex. It is anchored into the membrane by one transmembrane helix at the C-terminus and exposes a large GTPase domain to the cytosol [40–42]. Two Toc34 isoforms are found in *Arabidopsis*, atToc34 and atToc33. Although highly similar in sequence (60 % identity), they show different properties, e. g. atToc33, but not atToc34, can be specifically phosphorylated [43, 44], and they display different tissue distribution in the plant. Whereas atToc33 is mainly expressed in photosynthetic tissues, atToc34 is the dominant isoform in roots. Nevertheless, analysis of mutant plants shows that at least the atToc33 isoform is replaceable by the other [45], and both participate in the translocation of proteins into plastids. The cytosolic GTPase domain in Toc34 is evolutionarily related to that found in Toc159, and belongs to the septin family of GTPases [46].

The sequence of the members of the Toc159 family has a modular architecture: a large C-terminal membrane-anchored (M) domain, a cytosolic GTPase (G) domain and an N-terminal acidic (A) domain of unknown function whose length is reduced in the shorter versions of the Toc159 family [47]. Four orthologs (atToc159, atToc132, atToc120 and atToc90) form the Toc159 family in *Arabidopsis*. Many studies have related each of these isoforms with Toc75, pointing to a participation for all of the isoforms in the import process. Phylogenetically, they can be divided into three subfamilies: Toc159, Toc132/120 and Toc90 [47]. Functionally, they participate in at least two different import pathways. Whereas Toc159 and Toc90 seem to play a significant role in the import of photosynthetic proteins in green tissues, Toc132/Toc120 are important for import of essential house-keeping proteins, especially in non-green tissues [47, 48]. Reverse genetic studies have shown a functional redundancy between Toc132 and Toc120 [48]. Toc90 may have an accessory role in import. Moreover, their associations to Toc34 isoforms seem to respond specifically to functional demands: atToc33 associates preferably to atToc159 and atToc34 to atToc132/atToc120 [48]. Several isoforms of Toc34 or Toc159 that fall into the category of the atToc132/atToc120 subfamily are also found in *Physcomitrella* (Fig. 2), as previously found in green algae [39]. Three sequences in *Physcomitrella* belong to the Toc34 family [39], but

they diverged before the atToc34 and atToc33 branch (Fig. 2). Interestingly, just one copy of Toc34 is found in rice and *Chlamydomonas* [39]. Two homologous sequences to Toc159/Toc34 are found in red algae that phylogenetically seem to be closer to the Toc34 receptor type (Fig. 2). No bacterial or eukaryotic proteins are related to Toc159/Toc34 receptors [39], and this suggests that they appeared during evolution as a consequence of the development of chloroplasts, and that their specialization came with the evolution of vascular plants.

The third receptor, Toc64, has an N-terminal transmembrane anchor, a central amidase region, and a dicarboxylate clamp-type TPR domain at its C-terminus. It may act at both sides of the membrane, exposing the amidase region to the intermembrane space (IMS) and the TPR domain to the cytosol for specific interaction with cytosolic chaperones [49, 50]. The Toc64 family is composed of three isoforms in *Arabidopsis* (named according to the chromosome where they are encoded), atToc64-III, atToc64-IV and atToc64-I, but only atToc64-III participates in the import process in chloroplasts in association with Toc75 [51]. AtToc64-V is a mitochondrial protein also known as atOM64 [51] (see below). AtToc64-I, or atAMI1, is a soluble cytosolic protein. Recently, it has been proposed that Toc64 might not be strictly required for the efficient import of proteins into chloroplasts [29, 30], but may allow an efficient distribution of the precursor proteins within the cell [31]. No orthologs of atToc64-III are found outside land plants [39]. Toc12 contains a highly conserved DNA-J domain that is placed in the IMS of the envelopes, where it associates with the amidase module of Toc64 and Tic22 [52]. In the IMS it recruits a yet uncharacterized Hsp70 and activates its ATPase activity. Toc12 is anchored to the outer envelope membrane by a short hydrophobic motif at the N-terminus. Although longer in sequence, the *Arabidopsis* protein At1 g80920 might be related to Toc12 in pea. Similar proteins are found as well in *Physcomitrella* and rice, suggesting that Toc12 might be found exclusively in land plants.

Models of protein import through the TOC complex

Based on specific energy requirements, protein import in chloroplasts has been initially divided into three distinct steps/stages [53, 54]: (a) reversible binding to the chloroplast surface, which is independent of nucleotides; (b) stable binding/insertion at $\leq 100 \mu\text{M}$ ATP in the presence of GTP [55]. This intermediate step has very recently been analyzed in more detail and three distinct sub-stages have been defined [56]; (c) translocation and processing that requires $\geq 100 \mu\text{M}$ ATP [57, 58]. The recognition of preproteins by

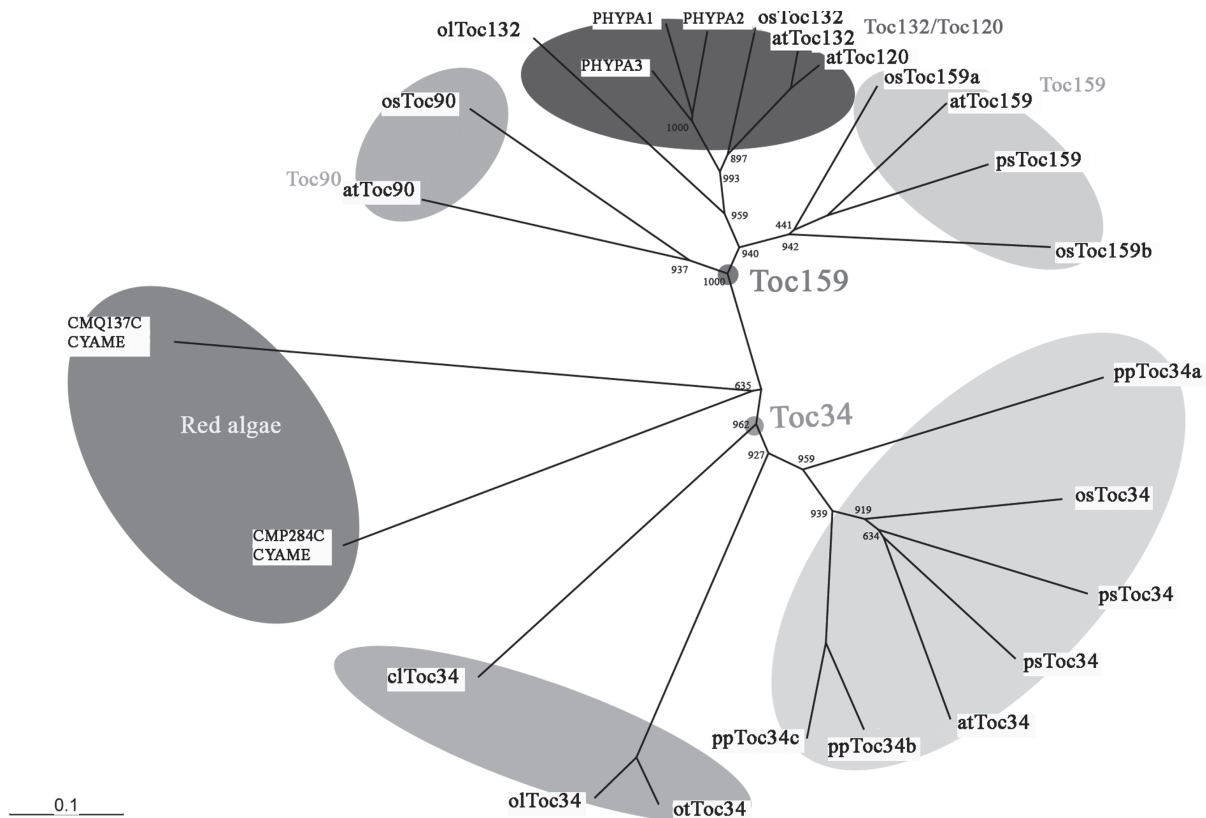


Figure 2. Unrooted phylogenetic tree of the Toc159/Toc34 families in chloroplasts. A multiple sequence alignment of members of the Toc159 and Toc64 family was performed by ClustalX v. 2.0.9. The phylogenetic relation between the Toc159 and Toc34 families was inferred by the neighbor-joining method implemented in ClustalX. The positions with gaps were excluded and 1000 bootstrap replicates were generated (values shown in the tree). The codes in the tree are as follows: clToc34, *Chlamydomonas reinhardtii* XP_001696644; olToc34, *Ostreococcus lucimarinus* XP_001417009; otToc34, *Ostreococcus tauri* CAL53037; ppToc34a, *Physcomitrella patens* PHYPA-DRAFT_113310; ppToc34b, *Physcomitrella patens* PHYPADRAFT_125509; ppToc34c, *Physcomitrella patens* PHYPADRAFT_211678; osToc34, *Oryza sativa* NP_001049508; atToc33, *Arabidopsis thaliana* NP_171730; atToc34, *Arabidopsis thaliana* NP_196119; psToc34, *Pisum sativum* Q41009; CMQ137C_CYAME, *Cyanidioschyzon merolae* CMQ137; CMQ284C_CYAME, *Cyanidioschyzon merolae* CMQ284; atToc90, *Arabidopsis thaliana* NP_197530; osToc90, *Oryza sativa* NP_001066359; olToc132, *Ostreococcus lucimarinus* XP_001421534; PHYPA1, *Physcomitrella patens* PHYPADRAFT_189669; PHYPA2, *Physcomitrella patens* PHYPADRAFT_216050; PHYPA3, *Physcomitrella patens* PHYPADRAFT_216964; osToc132, *Oryza sativa* NP_001065307; atToc132, *Arabidopsis thaliana* NP_179255; atToc120, *Arabidopsis thaliana* NP_188284; osToc159a, *Oryza sativa* NP_001054673; atToc159, *Arabidopsis thaliana* NP_567242; psToc159, *Pisum sativum* AAF75761; osToc159b, *Oryza sativa* NP_001051815. The organism name and the accession code for each sequence in the tree are indicated.

the Toc34 and Toc159 GTPases has been studied in detail, although the true sequence of events at the first contact remains a matter of debate. Two different models for the mode of precursor recognition have been proposed. The first model favors Toc159 as the initial TP receptor [59–61]. This process involves GTP hydrolysis and oligomerisation of both GTPases and results in transfer of the cargo protein to the Toc75 channel [62–64]. Transport across the membrane is then furthered by the IMS Hsp70 [52, 53]. This hypothesis has been challenged by the second model that proposes Toc34 to be the initial TP receptor [65–67]. Electron microscopy performed on purified TOC complex showed a toroidal structure built by four channels enclosing a central protruding domain [68]. Stoichiometric analysis revealed four Toc75 plus four

associated Toc34 molecules, forming the channel structures, and a single Toc159 subunit. It was speculated that a central Toc159 molecule represents the GTP-driven import motor that moves preproteins through the channel after receiving them from Toc34. Thus, one Toc159 molecule could alternately interact with the four Toc34 receptors by rotation and on-and-off hetero-oligomerisation [65, 68, 69]. Moreover, it has been demonstrated that Toc159 in concert with Toc75 as a minimal TOC complex is able to achieve precursor translocation into liposomes upon GTP hydrolysis. Findings from a different study led to the conclusion that the G-domain of Toc159 is not necessary for import *in vitro* or *in vivo* [70, 71]. Moreover, it has been shown that precursors can completely bypass the receptor stage and bind to

Toc75 directly [70]. Consequently, import can be achieved without the G-domain of Toc159. Recent findings show that when GTP hydrolysis on atToc159 is diminished, binding of precursors as well as import are increased, favoring a regulatory role of atToc159 in preprotein recognition [72]. Although the two models do agree about GTP-mediated regulation of the Toc34/Toc159 receptors and the central channel function of Toc75, it remains to be seen how their conflicting views can be matched to satisfactorily explain preprotein recognition at TOC.

Import machinery at the inner envelope membrane of chloroplasts: the TIC complex

The composition and stoichiometry of the membrane complex involved in the import into chloroplasts at the inner envelope membrane is less known than that of the TOC machinery. Seven or eight proteins have been proposed to form the TIC complex in higher plants: Tic110 and Tic20 as putative constituents of a translocon channel; the co-chaperone Tic40; and the translocon-associated Tic55, Tic32 and Tic62 subunits [73]. Tic21 is the most recently added putative component of the TIC translocon [74]. Its affiliation with TIC is, however, a matter of debate, since another group identified the protein as a metal permease [75]. Tic110 is the second most abundant protein of the inner envelope membrane and plays an essential role in plastid biogenesis, since knockout mutants of the single *tic110* gene in *Arabidopsis* are not viable [76]. It comprises two membrane-spanning α -helices in the extreme N-terminus, which are necessary for targeting to and insertion into the inner envelope [77, 78]. The carboxy-terminus is largely hydrophilic. According to one hypothesis, it forms a large globular domain on the stromal side where it recruits chaperones [79, 80]. The other hypothesis claims that regions of the C-terminal region are within the membrane and form part of the actual translocation pore [77, 81]. New evidence from our group combines both hypotheses for the C-terminal part of Tic110; that is, it contains regions in the IMS suitable to form supercomplexes with the TOC machinery and to receive the TP of preproteins, but also a large region in the stroma, capable of interaction with molecular chaperones as well as four amphiphatic helices which build up the channel [82]. Tic40 is anchored to the inner envelope membrane by its N-terminus [83]. The stromal C-terminal portion has a bimodular organization: a Hip/Hop domain functionally similar to the human co-chaperone Hsp70-interacting protein (Hip), and a TPR protein-protein interaction domain [84, 85]. Tic40 is present only in green plants, where it modulates the interaction of Tic110 and the Hsp93 chaperone, and an

Hsp100 homolog that acts as a *trans* translocation motor [86, 87].

Tic22 is a soluble protein in the IMS, peripherally associated to the TIC complex. It interacts with the TOC complex through Toc12 and Toc64 [52] and might participate in TIC-TOC supercomplex formation. Tic22 has been found intimately associated with Tic20, an integral membrane protein proposed to be a constituent of a translocation channel [88]. Tic20 has four predicted transmembrane helices and shares sequence similarities to bacterial amino acid transporters [7]. Four homologues are identified for Tic20 in *Arabidopsis*, but only two of them contain a predicted TP. The function and subcellular localization of the two Tic20 homologues with non-predicted TP are still unknown.

Tic55 is anchored to the inner envelope by two transmembrane helices at the C-terminus and it exposes a large soluble domain in the stroma that contains a Rieske-type iron-sulfur centre and a mononuclear iron-binding site [89]. Tic62 and Tic32 are classified as short-chain dehydrogenase-reductases, but their substrate specificity in chloroplasts is still unknown. They are peripherally attached to the inner envelope at the stroma side. Tic62 has a bimodular architecture: the N-terminus is involved in NADP(H)-binding; the C-terminus specifically interacts with ferredoxin-NADP-oxidoreductase (FNR) [90,91]. A full-length Tic62 is found only in vascular plants [92]. Tic32 also has a bimodular functional organization: an N-terminal NADP(H)-binding region and a C-terminal calmodulin-binding domain [93].

The TIC complex remains more of an enigma than TOC. Although several members have been identified to date (see above), their individual functions and cooperative mechanism are still mostly elusive. One of the most important parts of a translocon is the protein-conducting channel. In the case of the TOC translocon, Toc75 has been unequivocally identified as this essential protein. In case of the TIC translocon, discussion is still ongoing about the identity of the translocation channel. Several candidates have been proposed to fulfill this essential role: Tic110, Tic20 and Tic21 [74, 81, 94]. Tic20 and Tic21 show structural similarities to the translocon at the inner membrane of mitochondria (TIM) channel proteins Tim17 and Tim23 [74, 95], yet biochemical evidence for a channel function is lacking. Undoubtedly, Tic20 has an essential role in plant development, in spite of its low abundance at the protein level, since knockout plants are not viable [94]. Tic20 could very well be a specialized pore protein, but its candidacy as the general translocation channel is not built on biochemical evidence. Support for an important role for Tic20,

albeit not as a general import pore, comes from a recent publication by van Dooren and colleagues [96]. The authors hypothesize that Tic20 may have a regulatory role or might be involved in complex assembly. On the other hand, Tic110 features make it a good candidate for a translocation channel: it is conserved in different plastid types from multiple plant species [11]; it is expressed in cells in comparable amounts to Toc75 [97]; it shows channel activity *in vitro* [81]; it is found associated with precursor proteins and chaperones [77, 86]; it forms super-complexes with the TOC complex [98]; and it is essential for plant development [76]. In any case, Tic110 has an essential role in preprotein recognition on the *trans* side where it associates with Tic40 which links the central translocation channel to the import motor [99].

A detailed study of the different domains of Tic40 demonstrated that the Hip/Hop-like domain is indeed responsible for the co-chaperone capacity, namely the binding to Hsp93 and the stimulation of its ATPase activity [85, 100]. The TPR motifs were shown to procure the binding to Tic110. Intriguingly, it seems that upon binding of an incoming precursor at the TP, the interaction between Tic110 and Tic40 is favored, whereas the affinity of the channel to the precursor weakens. This leads to subsequent release of the precursor into the stroma, where it is processed by SPP [100]. The role of Tic40 is important for the efficiency of protein import, though not essential, because *tic40* mutants are pale but viable [84]. Very recently, it has been proposed that Tic40 is also involved in the reinsertion of proteins using the conservative sorting pathway, such as Tic110 and Tic40 itself [101]. In *tic40* deletion mutants, soluble intermediates accumulate in the stroma and are reinserted into the inner envelope more slowly compared to wild-type plants. The authors speculated that Tic40 together with Tic110 and Hsp93 might constitute the reinsertion complex or, alternatively, could be an accessory component of a yet unidentified specific reinsertion complex. In contrast to Tic40, Hsp93 is indispensable for chloroplast biogenesis [102].

Apart from the channel component(s) and the motor module, there are three regulatory members of the TIC complex: Tic62, Tic55 and Tic32. As described above, all of these have features rendering them perfect candidates for sensing and signaling the redox state of chloroplasts and thereby regulating protein import at the inner envelope according to the requirements of the plastid. It has been known for some time that at least two chloroplastic precursor proteins, the non-photosynthetic ferredoxin (FdIII) and the FNR isoform II (FNRII), are differentially imported in the light and in the dark [103]. Most likely, the different

redox states caused by photosynthetic activity are at least one of the reasons for this phenomenon. A good representative of the redox state is the NADPH/NADP⁺ ratio in the stroma. It has been demonstrated that this ratio influences TIC composition: under more oxidized conditions, Tic32 and Tic62 are closely associated with TIC, whereas reduced conditions lead to disassembly of the translocon [91, 104]. Tic62 even shuttles between a soluble stromal and a membrane-attached form, depending on the redox state [91]. The soluble form of Tic62 binds even more strongly to FNR, though the significance of this interaction is unknown. The role of Tic55 is still enigmatic, though it has been recently identified in a screen for thioredoxin targets [105]. Thus, translocation could be linked to thiol-mediated regulative processes but this has to be further investigated. Another means of regulation at the inner envelope was shown to involve calcium and calmodulin (CaM) [106]. The same authors later identified Tic32 as the CaM binding target [104]. Tic32, as described above, also contains an NADPH binding site and constitutes an active dehydrogenase. Binding of the nucleotide and CaM seems to be mutually exclusive, so that Tic32 might represent a scaffold of fine-tuning at TIC.

Outer envelope membrane protein insertion

Proteins localized in the outer envelope membrane of chloroplasts are mostly synthesized without a cleavable N-terminal TP and they are targeted to the envelope by internal signals residing within the mature protein [107]. Several members of the outer envelope protein (OEP) family (e.g., OEP14, Toc64, Toc34) have been analyzed concerning their integration pathways [108–110]. A common denominator, at least for proteins anchored to the membrane by α -helices, seems to reside within the hydrophobic transmembrane regions that carry the information for insertion into the lipid bilayer. The part adjacent to the transmembrane domain seems to determine the orientation of the OEP. This process was believed to be spontaneous and take place without assistance of other proteins. However, more recent results show that a proteinaceous machinery, as well as provision of energy in the form of nucleotides, facilitates the insertion into the outer envelope [109]. It has been demonstrated that OEP14 is in contact with Toc75 during insertion [111]. Similar findings have been published for the insertion of Toc34 [110], though it remains to be investigated if the same components that mediate insertion of OEP14 are involved. Recently a new player, the ankyrin repeat-containing protein AKR2, has been suggested to chaperone newly synthesized outer membrane proteins, such as OEP14, Toc64 and Toc34 proteins through the cytosol

to the chloroplast surface [112]. It was shown that AKR2 binds to the targeting signals located in the transmembrane regions as well as in the C-terminal parts via its ankyrin modules. It is not yet known if AKR2 is likewise involved in the targeting of other outer envelope proteins consisting of β -sheets rather than α -helices, and what kind of receptor it is using on the chloroplast surface [113]. In the case of β -barrel proteins, represented by the solute transporter family (*e.g.*, OEP21, OEP24), it is even less clear how they are integrated into the membrane. Experimental findings so far indicate that insertion is a spontaneous process, independent of a translocation machinery and energy [114, 115]. A notable exception in the family of β -barrel proteins is Toc75. It comprises a bipartite N-terminal TP [116]. The N-terminal portion directs the protein to the stroma via the general import pathway; the C-terminal part of the TP carries intra-organelle targeting information that seems to contain a stop-transfer signal and leads to arrest within the translocon/membrane. It remains to be investigated whether a special translocon exists that mediates the integration of β -barrel proteins into the outer envelope, comparable to the sorting and assembly machinery (SAM) in mitochondria (see below) [117]. One promising aspirant for this role would be Toc75-V, a member of the Omp85 family and distant relative of Toc75 [118].

Targeting to the intermembrane space

Information about components of the IMS is very scarce, since the compartment is hardly accessible in chloroplasts. Only two IMS proteins, Tic22 and MGD1, have so far been studied. MGD1 seems to engage TOC on its way across the outer envelope and it reaches the stroma at some point, since the TP can be cleaved by the SPP [119]. In contrast, Tic22 is not processed by the SPP and therefore probably does not cross the inner envelope at any point of translocation. It is controversial whether it uses components of TOC to travel across the outer envelope [119, 120]. The energy requirements of the two proteins also differ, suggesting that MGD1 would reach the stroma whereas Tic22 would not. Different pathways seem to exist for translocation to the IMS, at least in part, although the players involved and the mechanisms used are still elusive.

Inner envelope membrane protein insertion

Most inner envelope proteins carry N-terminal TPs and are imported via TOC/TIC but they differ in their final targeting to the inner envelope membrane. Some use the “stop-transfer” pathway, mediated by hydrophobic signals contained in the transmembrane regions that lead to arrest of the translocating protein

and lateral release into the lipid bilayer [121, 122]. Partly, these proteins carry a bipartite TP, which gives rise to a translocation intermediate that is already integral to the membrane. Other inner envelope components travel via the “conservative sorting” pathway. This is reminiscent of integration of inner membrane proteins in bacteria and involves a soluble stromal intermediate that is retargeted to the envelope by an unknown mechanism. Examples for proteins using this route are Tic110 [77, 123] and Tic40 [124]. These two again differ in their insertion characteristics, as Tic110 has one TP, and is cleaved only once by SPP, in contrast to Tic40. The latter comprises a bipartite TP and is cleaved twice, by SPP and a second protease of yet unknown identity, but which is most likely not the one responsible for the intermembrane cleavage of Toc75 [124]. Furthermore, Hsp93 has been shown to play a role in Tic110 reinsertion [123]. This feature has not yet been studied for the reinsertion process of Tic40.

Deviations from the general TOC/TIC import pathway

Proteomic studies revealed many proteins without a canonical TP in the chloroplasts [125]. The import properties of two of these proteins, Tic32 and cQORH, have been analyzed and the targeting information was found within the mature protein sequence [126, 127]. Although energy in the form of ATP is required for import, TOC/TIC components are not involved and their import mechanisms still await investigation. Another unexpected finding from this proteomic study was the identification of several plastid proteins carrying predicted signal peptides for the endoplasmic reticulum (ER). Some of these may represent contaminations by ER membrane vesicles but it seems improbable that all of them are. Physical contact and biochemical interactions of ER and chloroplasts have been repeatedly reported and may have essential functions [128]. Bearing in mind that in organisms containing complex plastids, vesicle-mediated targeting is an established system, it should not come as too big a surprise that three years ago a first chloroplast protein (CAH1) was found to be delivered to its destination by travelling as ER cargo [129]. More recently, a second protein (NPP1) that uses this pathway has been identified [130]. How these proteins cross the envelope membranes and if this involves vesicle fusion and/or TOC/TIC remains to be investigated.

The way to thylakoids

The thylakoid membrane is a highly specialized membrane harboring the photosystems and the ATPase complex where the photosynthetic electron

transfer is coupled with the chemiosmotic process. The abundant photosynthetic machinery in thylakoids is composed of subunits of both nuclear and chloroplast origin; in contrast, all the luminal proteins known so far are nuclear encoded. Proteomics and other analyses have demonstrated that systems homologous to the Sec, Tat and YidC machineries are found in thylakoids, the so-called cpSec, cpTat and Alb3 (Fig. 1) [131]. Some subunits that are transferred to thylakoids contain a bipartite TP at the N-terminus. The first part is used for translocation across the envelopes into the stroma and is cleaved off by SPP, resulting in an intermediate form of the protein. The second part guides the intermediate to thylakoids where it is processed by a thylakoidal processing peptidase (TPP), generating the mature form of the protein [132].

The Tat machinery in chloroplast thylakoids (cpTat)

In chloroplasts, the twin-arginine transport system (Tat) machinery is derived from the ancient cyanobacteria where it is responsible for the export of periplasmic proteins carrying cofactors [133]. It transports mainly folded substrates and likely relies completely on the proton motif force [134, 135], not requiring any form of nucleoside triphosphates [136]. It is still not totally clear whether the proton motif force is necessary in all organisms and under all *in vivo* conditions [137]. Although mainly involved in the transport of luminal proteins, two membrane proteins have been described that are inserted in the thylakoidal membrane by this pathway [138,139]. The TPs recognized by cpTat are characterized by an N-terminal charged domain (n-region), a twin arginine motif followed by a hydrophobic motif (h-region) and a polar C-terminal region (c-region) ending in the A-X-A motif, which is recognized by the TPP [140]. A special case is the Rieske Fe/S subunit of the cytochrome b(6)f complex that does not contain the typical twin arginine motif, though incidentally the Sec-machinery (see below) seems to be involved in the transport of this membrane protein [139].

The Tat system in thylakoids is conserved in plant and algal chloroplasts and consists of the Tha4, Hcf106 and cpTatC subunits that are homologous to TatA, TatB and TatC proteins respectively, found in bacteria [141]. The Hcf106 (19 kDa) and Tha4 (9 kDa) homologs are bitopic membrane proteins with an amphipathic helix positioned in the stroma; cpTatC (34 kDa) is predicted to contain six transmembrane helices. cpTatC and Hcf106 form a stable complex in the membrane, prepared for the reception of the preprotein [142]. The binding of the precursor protein to the receptor complex recruits Tha4 that assembles with the precursor-receptor complex and forms a pore

in the membrane, whose size is presumably dictated by the substrate [143, 144]. Afterwards, Tha4 dissociates and the TP is laterally transferred to the membrane where signal cleavage occurs [140]. The signal peptide is not always removed, as in the case of the Rieske Fe/S protein where it remains and constitutes the only anchor of the protein to the membrane [139]. The mechanism of translocation is not well defined and two different models have been proposed. Whereas one model proposes that oligomers of Tha4 in the membrane polymerize around the substrate to form a pore of variable size [145], another model considers a conformational change in Tha4 which, either through an infolding of the amphipathic helices or directly through a destabilized lipid bilayer mechanism, allows the passage of the precursor protein [144]. More studies are necessary to fully understand the mechanism by which the cpTat system operates.

The Sec machinery in chloroplast thylakoids (cpSec)

The Sec system in thylakoids, derived from the general secretory pathway (Sec system) in (cyano)bacteria, and consists of a membrane-embedded protein-conducting channel and a peripherally associated motor domain [146]. In thylakoids, cpSec is composed of the minimal required components, that is, the translocation channel made up of SecY [147] and SecE [148] subunits, and the ATPase SecA [149]. It works post-translationally for the transport of luminal proteins such as OE33 and plastocyanin, and membrane proteins such as cytochrome f and Psaf. It co-translationally cooperates with the SRP-dependent pathway (see below) for the biogenesis of the D1 subunit of photosystem II. The substrates are translocated in an unfolded state and the cofactors, as in the case of plastocyanin, are subsequently assembled in the lumen. The Sec-targeting signal peptides are analogous to those found in Tat-dependent signal peptides, with the exception of the twin arginine motif.

The cpSec machinery functions similarly to the bacterial Sec-pathway. SecY, a multispanning membrane protein, and SecE, with one transmembrane helix at the C-terminus, form a 180 kDa complex in the membrane that partially translocates the precursor. The helicase cpSecA, which contains a DEVD (Asp-Glu-Val-Asp) sequence in its Walker B motif, is functionally equivalent to SecA in bacteria and utilizes cycles of ATP hydrolysis to provide the driving force for translocation [150, 151]. It recognizes the signal peptide of precursor proteins in the stroma and attaches itself to the thylakoid membrane [152]. Upon ATP hydrolysis, the preprotein is partially released to the cpSecYE channel. Upon binding of SecA to SecY, ATP hydrolysis is stimulated, furthering the partial release to the cpSecYE channel. Subsequently, SecA

deinserts from the membrane and undergoes new cycles of insertion-deinsertion involving ATP hydrolysis. This, in concert with the proton motive force energy, results in the complete translocation of the preprotein through the channel.

The SRP machinery in chloroplast thylakoids (cpSRP)

The signal recognition particle (SRP) system is the major cellular machinery that mediates transport of proteins to the ER membrane or the bacterial/archaeal plasma membrane [153]. As a consequence of endosymbiotic events, a homologous system is found in thylakoids (cpSRP) [154]. cpSRP has a similar architecture to that found in bacteria, though the subunit composition and the mechanism of transport differs. The SRP in the stroma is a heterodimer that consists of cpSRP54 and cpSRP43 subunits, the latter being unique in green plant thylakoids [155]. Interestingly, it lacks the typical RNA-binding moiety found in cytosolic SRPs. Recently, the structure of cpSRP43 at atomic resolution was solved, demonstrating a comparable overall shape and charge distribution between cpSRP43 and the SRP RNA complex [156]. Moreover, a fraction of cpSRP54 free of any cpSRP43 exists in the stroma, capable of binding to 70S ribosomes [157]. As described below, this dual population of cpSRP54 in the stroma results in two different modes of translocation for cpSRP: the co-translational and the post-translational pathways. The membrane-bound receptor at the thylakoid membrane, cpFtsY, is homologous to bacterial FtsY [158]. The integral thylakoid membrane protein Albino3, or Alb3 [159], is homologous to YidC but lacks the first transmembrane helix found in bacteria, and therefore consists of 5 transmembrane helices with the N-terminus located in the thylakoid and a C-terminal soluble region oriented towards the stroma. As in bacteria, the transport process driven by the SRP system is GTP regulated through cpSRP54 and cpFtsY subunits. These GTPases comprise a unique subgroup in the GTPase superfamily [153] and reciprocally activate one another upon complex formation [160]. The complex is only stable when the two proteins are in the GTP-bound state; upon binding, GTP is hydrolyzed and the subunits dissociate [161]. In the co-translational insertion pathway, cpSRP cooperates with the Sec machinery in the insertion of some chloroplast-encoded proteins, such as the D1 protein, into thylakoids [162]. A transient binding between cpSRP54 and the nascent protein attached to the ribosome has been demonstrated during the early phase of elongation with the binding affinity decreasing as elongation proceeds [163]. The interaction of cpSRP54 with cpFtsY stimulates GTP hydrolysis and

the nascent chain is passed on to the Alb3/Sec translocon [162].

Still the most well known substrate for cpSRP is the family composed of some nuclear-encoded light harvesting chlorophyll a/b binding proteins (LHCP) [155]. The insertion mechanism proceeds post-translationally and is believed to be Sec-independent. The thylakoid translocation starts with the recognition of the signal peptide of LHCP in the stroma by the heterodimer composed of cpSRP54 and cpSRP43. Recently, the interaction of these two subunits was thermodynamically and structurally characterized [164]. The formation of the transit complex is initiated by the binding of LHCP to an ankyrin region in cpSRP43 [156]. Afterwards, the cpSRP54 contacts and binds LHCP. The transit complex formed by the substrate together with cpSRP54-GTP/cpSRP43 proteins is recognized by the receptor cpFtsY. Two mechanisms have been proposed: one is that FtsY, found in two pools, soluble and membrane associated like in bacteria, recognizes the complex in the stroma and shepherds it to thylakoids [165]; another relies on the fact that FtsY is not soluble at all in the stroma, but extrinsically attached to the thylakoid membrane [166]. The complex of cpSRP54/cpFtsY induces GTP hydrolysis and subsequently the heterodimer cpSRP54/cpSRP43 is released from cpFtsY [161]. The substrate is then targeted to Alb3 and laterally incorporated into the membrane. New proposals establish that cpSRP43 has capabilities in itself to drive LHC protein to Alb3 in the absence of cpSRP54 and cpFtsY [167].

Spontaneous insertion

A spontaneous insertion of a membrane protein in thylakoids, i.e. without the participation of any stromal factor, membrane protein translocator or nucleoside triphosphates (NTPs), was first described for Cfo-II [168]. Since then, some other proteins have been recognized that follow this method of insertion (e.g., SecE, PsaK, PsbS). The TP of this group of proteins is highly similar to that found in proteins following the other translocation pathways in thylakoids. However, some differences are noticeable, such as the occurrence of acidic residues in the c-region [169]. The presence of two hydrophobic helices surrounding the N-terminus of the mature protein seems crucial for this way of membrane insertion; one helix formed by the TP and another immediately after the hydrophilic N-terminal region. The insertion mechanism seems to proceed with the burial of the N-terminal hydrophilic region by the two hydrophobic helices during translocation across the membrane, forming a transmembrane loop intermediate [170]. Once the protein is inserted, the signal peptide might

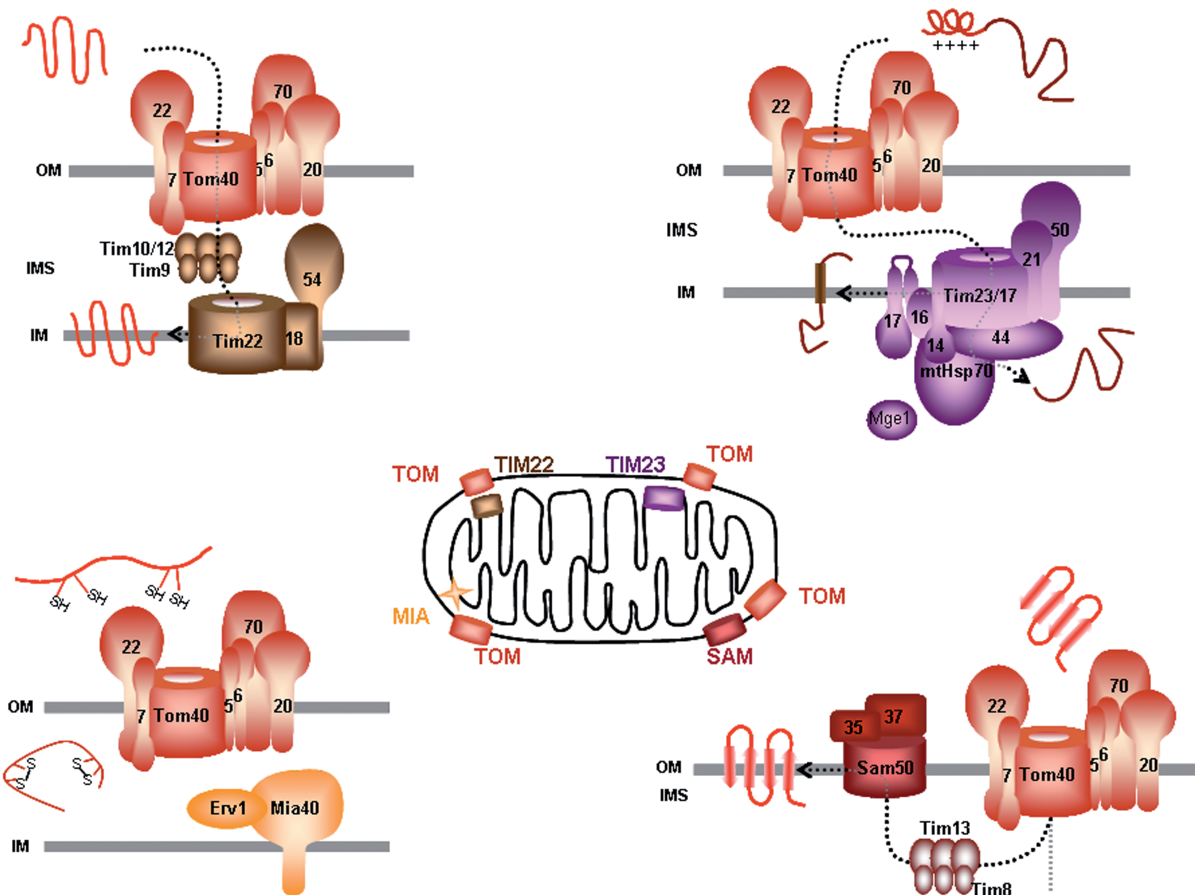


Figure 3. Overview of mitochondrial translocases. Different types of precursors and their targeting characteristics are indicated together with the model of TOM. Complexes and components are color coded for convenient allocation. For details please refer to the text.

be cleaved or not, in the latter case forming part of the membrane-spanning region of the protein.

Protein import in mitochondria

Most results concerning protein import into mitochondria have been obtained by working with yeast as a model organism. Plant mitochondria have slightly different components and, to some extent, a different mechanism.

Import of preproteins to mitochondria involves several membrane complexes: the translocases at the outer and inner membrane of mitochondria (TOM and TIM complexes, respectively); the sorting and assembly machinery (SAM) for the insertion of β -barrel proteins into the outer membrane, and the presequence translocase associated motor (PAM), for translocation into the matrix (Fig. 3) [171].

Targeting sequences

Most mitochondrial proteins comprise targeting sequences that share certain features with their chlor-

oplast counterparts, e.g. they are positively charged. The N-terminal targeting sequences are also called matrix-targeting sequences (MTSs), since they direct the protein into the mitochondrial matrix. They do not contain conserved primary sequences but they comprise a characteristic secondary structure: they form amphipathic helices, resulting in one positive and one hydrophobic surface [172]. This is crucial for their interaction with receptor proteins of the TOM machinery. A large number of mitochondrial proteins have, however, internal targeting signals whose characteristics are less well defined [171]. All outer membrane proteins belong to this class, but also several others which are sorted to the matrix or the inner membrane. Hydrophobic inner membrane proteins from the mitochondrial carrier family contain internal targeting information within their transmembrane domains [171]. All mitochondrial targeting signals are recognized by receptors of the TOM machinery and then guided further to their specific translocons. This will be discussed later.

Translocation through the TOM complex

En route into mitochondria, all proteins first encounter the TOM complex. The core module of this complex, 400 kDa in size, is made up of the integral membrane proteins Tom40 that form the protein channel, and the single α -helical transmembrane subunits Tom22, Tom5, Tom6 and Tom7. More loosely associated are the primary receptors Tom70 and Tom20 that are anchored in the outer membrane of mitochondria by a single transmembrane helix. As in the case of chloroplast subunits, the numbers indicate their molecular mass.

Tom40 is the major component of the TOM complex and constitutes the hydrophilic pore-forming subunit of the mitochondrial general import pore [173]. It is predicted to have an amphipathic β -barrel structure deeply embedded in the membrane, as does Toc75, the pore-forming protein in the outer envelope membrane of chloroplasts (see above). Topologically, Tom70 and Tom22 have a small N-terminal region in the IMS and a bigger C-terminal soluble domain exposed into the cytosol. This forms the receptor domain where TPR protein-protein interaction motifs, reminiscent of Toc64, are recognized [174]. However, the number and features of the TPR motifs differ, and this results in different, though partially overlapping substrate specificities: Tom70, with 11 TRP repetitions organized into a right-handed superhelix [175], preferably interacts with hydrophobic proteins destined for the mitochondrial inner membrane, as well as the cytosolic chaperones of the Hsp70 family. Tom20, where just one TPR is recognized, preferentially binds precursor proteins comprising MTSs [176–178]. Tom22, tightly associated with Tom40, cooperates in the transfer of preproteins from Tom70/Tom22 to Tom40. Additionally, it can act as a receptor for a small number of proteins [179]. Tom22 is described as a structural organizer of the TOM complex [180] that exposes the C-terminal region to the IMS and the N-terminal domain into the cytosol to dock the other receptors of the TOM complex. Its only transmembrane helix forms part of the core complex with Tom40 and is essential for the integrity of the complex. The region exposed to the IMS holds the presequence in the IMS after translocation [181] and also serves as a contact site with the TIM23 complex [182]. The Tom22 subunit is known as Tom9 in plants (see below). The other constituents, Tom5, Tom6 and Tom7, are located close to the translocation pore and regulate the interactions within the complex. Tom 5 accepts substrates from the receptors and mediates their insertion into Tom40 [183]; Tom6 and Tom7 modulate the dynamics of the TOM complex [184].

All substrates are recognized by either of the two primary receptors, Tom70 or Tom20. The TPR motifs of Tom70 and Tom20 are part of a hydrophobic groove that interacts with the hydrophobic signals or the hydrophobic surface of the amphiphilic MTSs [177, 178]. Upon binding, both receptors transfer their accepted cargo to the translocation pore, Tom40, via Tom22. Tom40, associated with the small helper compounds, Tom7, Tom6 and Tom5, mediates translocation of virtually all mitochondrial proteins including Tom40 itself. During translocation, Tom40 undergoes dynamic conformational changes essential for its function as a channel [185]. According to the binding chain hypothesis, the driving force for translocation through the TOM complex does not depend on ATP or the membrane potential across the inner membrane: the translocating substrates interact with several binding sites of increasing affinity that form a guidance system for preproteins across the outer membrane [186].

Outer membrane proteins and SAM

It was quite recently discovered that β -barrel proteins located in the outer membrane use a specialized pathway for their integration into the membrane. After transport through Tom40, β -barrel proteins are recognized by two small Tim proteins (Tim8 and Tim13) in the IMS (see below), which guide them to the SAM complex (also called TOB). This consists of the integral membrane protein Sam50 (also known as Tob55) and the peripheral subunits Sam35 (also known as Tob38) and Sam37 (also known as Tom37 or Mas37) [187–190]. Sam50 is a member of the Omp85 family that has been shown to be crucially involved in insertion of β -barrel proteins into the bacterial outer membrane [187]. Thus, Sam50 most likely represents the “integrator” for β -barrel proteins. The function of the other two SAM components is not completely clear yet. Sam35 might cooperate with Sam50, whereas Sam37 might be involved in the release of β -barrel intermediates to the membrane [117]. Protein integration into the outer membrane is independent on energy, indicating that the process itself is energetically favorable. The mechanistic action of SAM remains unknown to date. New roles in the biogenesis of α -helical proteins have been very recently ascribed to the SAM complex [191].

Proteins destined for the IMS

All proteins that reside in the IMS are nuclear encoded, but only a few of them carry MTSs. Two different pathways participate in the import of IMS proteins [192]. Mitochondrial IMS proteins with MTSs have a bipartite presequence, characterized by a hydrophobic sorting signal downstream of the

matrix targeting sequence. First the N-terminal region of the presequence directs the preprotein to the TIM23 complex (see below); then the hydrophobic sorting signal anchors in the inner membrane where, after a second processing step at the IMS and proteolytical removal of the presequence behind the sorting signal, the soluble mature protein is released to the IMS. Most of the IMS proteins, however, do not contain MTSs and they associate with specific factors in the IMS after TOM translocation. These specific factors act as folding catalysts, incorporating cofactors and/or promoting oxidative events, folding some substrates that “trap” them in the IMS. The IMS contains a complete machinery to catalyze the oxidative folding of proteins and reflects the evolutionary origin of this compartment from the periplasmic space of the prokaryotic ancestor of mitochondria [193]. The substrates of this specific intermembrane space pathway, called mitochondrial IMS import and assembly pathway (MIA), are proteins that carry conserved cysteine motifs. After passage through TOM, the substrates are intercepted by Mia40, a bitopic inner membrane protein with a large domain into the IMS, that interacts with the incoming precursors by forming intermolecular disulfide bridges [194]. Upon release from Mia40, the substrate proteins are oxidized and functionally folded. For reoxidation, Mia40 interacts with the sulfhydryl oxidase Erv1 [193], which itself is regenerated by transferring electrons to cytochrome *c* in the respiratory chain. This releases its electrons to molecular oxygen, thereby completing the electron transfer chain of the intermembrane space assembly pathway. Thus, the processes taking part in the MIA pathway seem to take the necessary energy from the formation and release of disulphide bonds.

Those substrates destined for the inner membrane or matrix are intercepted in the IMS by the small Tim proteins (Tim 8, Tim9, Tim10, Tim12 and Tim13) [195]. These small proteins fulfill crucial functions in the sorting of the incoming proteins. They form heterohexameric complexes [196, 197], Tim9/Tim10, Tim9/Tim10/Tim12 and Tim8/Tim13, that interact with precursor proteins and shelter them on their further way either to the SAM complex in the outer membrane or to the TIM complexes.

Matrix and inner membrane targeting

A distinction is made between two different protein import pathways in the inner membrane: the general import pathway for proteins that contain MTSs; targeted to the TIM23 complex; and the carrier import pathway for internal targeting sequences found in most inner membrane proteins, laterally inserted into the inner membrane by the TIM22

complex. On reaching the matrix space, the precursors are cleaved by the mitochondrial processing peptidase (MPP) and undergo chaperone-assisted folding and assembly to functional complexes.

TIM23

The TIM23 complex is responsible for translocation of matrix-targeted proteins as well as a limited number of inner membrane proteins and IMS proteins. Both classes of TIM23 substrates carry typical MTSs. Inner membrane proteins with a single transmembrane helix are imported by a stop-transfer mechanism, where the single transmembrane helix acts as stop-transfer signal. Inner membrane proteins with more than one transmembrane helix are first fully transported into the matrix, from where they are relocated to the inner membrane with the assistance of the essential Oxa1p, a YidC/Alb3 homolog [198]. This conservative sorting pathway has most probably been inherited from the bacterial ancestor and is reminiscent of the import mechanism of Tic110 in chloroplasts (see above). The Oxa1p complex is also involved in translocation of several mitochondria-encoded inner membrane proteins [154, 198]. The TIM23 translocase consists of several subunits that are dynamically associated depending on the substrate protein that is being translocated. The TIM23 constituents fall into two different classes: members forming the protein-conducting channel and those constituting the import motor (PAM). The first group is formed by Tim23, Tim17, Tim50 and Tim21, whereas Tim44, Tim16 (also known as Pam16), Tim14 (or Pam18), the mitochondrial Hsp70 chaperone (mtHsp70) and Mge1 belong to the latter group. Tim23 and Tim17 together form the actual pore [199, 200]. Both proteins have a similar size and a membrane topology that consists of four hydrophobic transmembrane helices, with the N- and C-termini in the IMS. Tim23 is different in that the longer N-terminus of Tim23 spans the IMS and protrudes into the cytosol, thus facilitating the transfer of precursor proteins [201].

In general, precursor proteins are handed over from TOM to TIM23, engaging the IMS parts of Tim50 and Tim23 [202]. At this step, the pH gradient across the inner membrane is required as the energy source. Once emerging from the TIM channel, matrix-destined precursors are caught by members of the PAM complex. First, Tim44, a largely hydrophilic matrix protein attached to the inner membrane [203], binds to the emerging precursor and passes it on to mtHsp70. With the help of Tim14 and Tim16 as co-chaperones [204, 205], their job most likely being the regulation of ATP hydrolysis, mtHsp70 binds tightly to the incoming precursor and detaches from Tim44. Translocation into the matrix is now furthered and

takes place in a stepwise manner, due to binding of more mtHsp70 molecules as the precursor moves along through the channel [206]. Mge1 acts as a nucleotide exchange factor, whose performance leads to decreased affinity of mtHsp70 to the precursor and eventually to its release into the matrix. The mechanism of how the motor works has been a matter of debate, but today results favor the described Brownian-ratchet mechanism over the power-stroke mechanism [207]. The form of TIM23 associated with the import motor PAM is called TIM23^{MOTOR} [208].

Another type of TIM23 mediates insertion of single-span inner membrane proteins. This form of TIM23 comprises Tim21 and was termed TIM23^{SORT} [208]. Tim21 was shown to interact with the IMS part of Tom22, thereby possibly mediating interaction of TOM and TIM23. Another proposed function of Tim21 was the regulation of import motor association. However, some groups suggested that Tim21 is replaced at some point by Tim14-Tim16, suggesting that the presence of Tim21 alone does not exclusively destine TIM23 as the sorting form of the translocase [182]. Consequently, the fine-tuning of complex composition as well as the details of the translocating mechanisms need to be clarified. A second class of precursor proteins engaging TIM23^{SORT} is represented by some IMS components that contain MTSs, as mentioned above. Still, the TOM23^{SORT} model has recently been challenged by evidence that Tim23 activity can be remodeled by association with different Tim polypeptides [209].

TIM22

The so-called carrier proteins are synthesized without a cleavable targeting sequence and characteristically have an even number of hydrophobic transmembrane helices, with their N- and C-termini oriented towards the IMS. Their internal sorting information leads them on to the TIM22-mediated pathway, which is accordingly termed the “carrier import pathway”. It not only involves the membrane-inserted compounds of TIM22 (Tim22, Tim18 and Tim54) but also includes the small Tim proteins Tim9, Tim10 and Tim12 in the IMS [210]. The voltage-dependent channel Tim22 is predicted to be made up of four transmembrane helices [211]. It resembles Tim23 and Tim17, with respect to structure and topology. The molecular functions of Tim18 and Tim54 in TIM22 are still unknown. New experiments suggest a role of Tim18 in TIM22 complex assembly, whereas Tim54, which exposes a large soluble domain into the IMS, might form the docking site for the small Tim proteins [212]. Import via TIM22 is subdivided into several steps: it starts with precursor recognition by Tom70 at the mitochondrial surface that essentially depends on the

presence of Hsp90/Hsp70 on the precursor [213]. Upon emerging from Tom40, carrier proteins are bound by the TIM10 complex formed by the hexameric Tim9/Tim10 assembly, which shield hydrophobic domains from unproductive interactions and guide precursor proteins to the TIM22 translocase. The insertion by TIM22 is strictly dependent on the membrane potential. Within the membrane, carrier proteins are then assembled to functional dimers [171].

Mitochondrial protein import in plant cells

Most of the knowledge about the mitochondrial import apparatus had been acquired in yeast or mammals. When people began looking closely at plant mitochondria they found that plant TOM, in particular, is slightly different from its non-plant counterparts [214]. Like in chloroplasts, many members of the TOM and TIM families are encoded by multiple gene families [215–217]. Only three TOM members are conserved throughout all species: the translocation pore Tom40, the organizer Tom22 and the small Tom7 protein. Two Tom40 isoforms, highly similar to those of fungi and mammals, are found in *Arabidopsis*. Tom22 is present in green plants (*Arabidopsis*, *Physcomitrella* and *Ostreococcus*) as a short version named Tom9. It lacks the large N-terminal acidic *cis*-receptor region in the cytosol that characterizes Tom22. This might have been an adaptive change associated with an increased requirement for selectivity in protein targeting [215]. In *Arabidopsis*, two Tom9 isoforms are present that differ in the *trans* domain; their significance is still unknown. Also, two Tom7 isoforms are recognized in *Arabidopsis* [215]. On the other hand, the receptor components Tom70 and Tom20 seem to be missing, at least if one looks on the sequence level. Intriguingly, a protein structurally and functionally similar to Tom20 was identified in plants [218]. However, though the individual structural domains of plant Tom20 are similar to e.g. rat Tom20, they occur in reversed order. Whereas atTom20 is anchored to the outer membrane via its C-terminus, rat Tom20 comprises an N-terminal membrane anchor. Sequence and structural analyses concluded that these proteins do not share a common ancestor but are the result of convergent evolution [218]. This points to the essential importance of this import receptor, since animal/plant mitochondria needed to “acquire” a protein with such a function to increase targeting specificity and efficiency of translocation. Four Tom20 isoforms are recognized in the *Arabidopsis* genome, though three isoforms (Tom20–2, Tom20–3 and Tom20–4) have been detected only by proteomics [216]. In the case of Tom70, its function might have been taken over by

OM64, a Toc64 homolog (see above) [51]. Additionally, Tom5 and Tom6 subunits have been experimentally identified in *Arabidopsis* [219].

In contrast to TOM, plant TIM complexes consist of homologous proteins highly similar to their yeast/mammal counterparts [220]. However, some TIM members differ in the distribution of certain motifs. Subunits Tim17, Tim23, Tim44 and Tim50 have been experimentally confirmed by proteomics [216]. Three different isoforms are encoded in the *Arabidopsis* genome for Tim17 and Tim23. In a manner analogous to yeast Tim23, plant Tim17 (isoforms atTim17-1 and atTim17-2, but not atTim17-3) comprises a long C-terminal extension that links the outer and inner membrane and is involved in TP binding [220]. In plants, however, Tim23 lacks this extension, which might explain the absence of Tim21 [217]. Proteomic analyses suggest a functional specialization in expression since Tim17-1, for example, is not present in non-green organs and its expression increases during development. Similarly, Tim23-3 and Tim44-1 are highly expressed in roots [216, 220]. The presence of Tim14 and Tim16 is concluded by sequence comparison [217]. Interestingly, many components of the carrier import pathway have not been identified at a sequence level in plants. This is the case for Tim18, Tim54 and the small subunit Tim12 [217, 220]. Besides, Tim22 and the small Tim proteins (Tim8/Tim13 and Tim9/Tim10) have been identified by proteomics [216]. It is presently unknown whether other proteins substitute Tim18 and Tim54 function in plants. Finally, just a few words to mention that other important sorting machineries such as SAM, MIA and Oxa1p are also present in plants [217, 221].

Dual targeting

Since researchers concentrated on looking for features rendering organelle-targeting specific, it came as a huge surprise that several proteins exist which are destined for both chloroplasts and mitochondria. Up until now approximately 50 dual-targeted proteins have been identified. Dual-targeting signals seem to be quite similar to chloroplast and mitochondrial ones. Some residues, e.g. arginine, seem to be more significant for mitochondrial recognition [222], whereas hydroxylated residues carry more weight in plastid import [223]. Interaction with cytosolic components might play a regulatory role in the distribution to the respective organelles. Another intriguing possibility is the “pre-sorting” by differential targeting of mRNA [224], but this remains elusive. In contrast to single-targeted proteins where the mature parts may influence import efficiency but not the targeting

itself, in the case of dual-targeting, the mature protein has a more decisive role [225]. Concerning the functions of proteins localized in both organelles, they seem to be particularly involved in basic processes such as the cell cycle and DNA and protein synthesis [223]. Thus, having proteins delivered to both chloroplasts and mitochondria may ensure their capability of performing essential functions co-ordinately and thereby may represent a manner of inter-organellar communication.

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