The Chloroplast Protein Import Apparatus, Its Components, and Their Roles

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Abstract According to the endosymbiont theory, an early eukaryotic cell engulfed the ancestor of present-day chloroplasts – a relative of extant cyanobacteria. This was the start of a new era for the chloroplast progenitor, because it was placed under the control of the host cell. Many of the genes found originally in the cyanobacterial genome are today present in the cell nucleus. Thus, over time, the chloroplast has learned to live with less and less "home-made" proteins. Nevertheless, the chloroplast retains many of the functions found in cyanobacteria (e.g., photosynthesis, fatty acid and amino acid production). To maintain these functions, many proteins have to be transported "back" to the chloroplast. An import machinery drives this transport process, and this consists of translocons located in the outer and the inner envelope membranes, called TOC and TIC (*T*ranslocon of the *O*uter/*I*nner envelope membrane of *C*hloroplasts). This chapter focuses on these translocons, and summarizes how they mediate import of nucleus-encoded proteins into the chloroplast from the surrounding cytoplasm.

1 Evolution of Chloroplasts and the Import Machinery

More than a billion years ago, an ancestral free-living cyanobacterium was taken up by a eukaryotic cell (Olson 2006). The resulting symbiosis gave the host cell access to valuable resources produced by photosynthesis (e.g., carbohydrates), and in return the cyanobacterium received a stable environmental milieu. Disadvantages of this arrangement included the tendency for oxidative damage to the organellar DNA and the absence of sexual recombination, rendering the plastome vulnerable to the accumulation of serious mutations (Lynch and Blanchard 1998; Martin and Herrman 1998). To avoid this scenario, many genes were transferred from the

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plastome to the nucleus, which also gave overall control to the host cell. Modern chloroplasts retain many metabolic pathways from the original endosymbiont, and so proteins that were previously synthesized inside the chloroplast are now transported "back" to the organelle to maintain its former functionality. The system required for the translocation of proteins into the chloroplast consists of at least 20 protein components in *Arabidopsis thaliana* (Jackson-Constan and Keegstra 2001; Jarvis 2008), called either TOC or TIC (*T*ranslocon of the *O*uter/*I*nner envelope membrane of *C*hloroplasts; Schnell et al. 1997).

In cyanobacteria, homologues for only a few of these proteins exist; e.g., Toc75, Tic20, Tic22 and Tic55 (Reumann et al. 1999; 2005). Interestingly, the related transport system in cyanobacteria (based on a homologue of the Toc75 channel) is proposed to mediate secretion from the cell, which is opposite to the direction of transport during chloroplast import. This directionality change might be related to the relocation of the Toc75 gene to the nucleus (von Heijne 1995). The transit peptide (TP) (which is needed to bring preproteins to the chloroplast) may be derived from a secretory peptide in the endosymbiont, which was recognized and secreted by the ancestral Toc75 (Reumann et al. 1999). In relation to the evolution of the import machinery, there are still many unanswered questions; for example, many components of the translocons are not present in cyanobacteria (e.g., Toc34, Toc159, and Tic110). However, this topic has been nicely reviewed by Reumann et al. (2005) and is not within the scope of this chapter.

2 Overview of Chloroplast Protein Import

Around 3,000 different proteins are predicted to exist within chloroplasts, and ~95% of these are nucleus-encoded and so must be imported post-translationally (Abdallah et al. 2000). Most imported proteins are thought to utilize the TOC and TIC translocons (Soll and Schleiff 2004; Bédard and Jarvis 2005; Reumann et al. 2005; Kessler and Schnell 2006). Interestingly, there appear to be at least two different TOC/TIC import pathways, and it is now clear that TOC/TIC-independent or "non-canonical" protein targeting to chloroplasts also occurs. Thus, the notion of a "general import pathway" for all chloroplast proteins is somewhat outdated.

All proteins that follow the TOC/TIC route have a cleavable, N-terminal TP. This acts as a targeting flag, directing the preprotein exclusively to the chloroplast (Smeekens et al. 1986). It has been suggested that the TP can be divided into three domains: the N-terminus is mainly uncharged and proposed to play a role in recognition; the central part lacks acidic residues and mediates translocation over the envelope; finally, the C-terminus is enriched in arginines and involved in TP cleavage inside the chloroplast (von Heijne et al. 1989; Rensink et al. 1998). Despite the presence of common, general features in TPs (a preponderance of hydroxylated residues and a deficiency in acidic residues, giving an overall positive charge), no consensus sequence or structure exists, making it difficult to predict chloroplast location by sequence analysis (Bruce 2000). However, in the last ten years several

algorithms have become available for predicting chloroplast localization with reasonable confidence; e.g., ChloroP (Emanuelsson et al. 1999), PSORT (Nakai and Horton 1999), TargetP (Emanuelsson et al. 2000) and Predotar (Small et al. 2004).

In the TOC/TIC pathway, the binding of the preprotein to the chloroplast outer envelope membrane (OEM) is mediated by the TP. In the absence of an energy source, binding to the import apparatus is reversible and no translocation will occur (Perry and Keegstra 1994). This step may also involve interactions between the TP and the outer envelope lipids (Bruce 2000). In the presence of GTP, and low concentrations of ATP (≤100 μM), the binding step is irreversible and an early import intermediate is formed (Olsen and Keegstra 1992; Young et al. 1999). At this stage, the preprotein has penetrated the OEM and is in contact also with the inner envelope membrane (IEM) (Wu et al. 1994; Ma et al. 1996). To achieve complete translocation, high ATP concentrations (>100 μM) are required in the stroma, and this is thought to be consumed by stromal molecular chaperones (Pain and Blobel 1987; Theg et al. 1989). In addition to the essential role of the TP, the mature part of the preprotein has also been reported to influence the interaction between the preprotein and the translocon (Dabney-Smith et al. 1999).

3 Initial Contact Between Preproteins and the Outer Membrane

Several hypotheses exist for the transport of nucleus-encoded proteins from the cytosol, where they are synthesized, to the chloroplast surface. One proposal involves a so-called "guidance complex," which brings the preprotein to the TOC components. A second hypothesis entails contact with OEM lipids, which might induce changes in the bilayer to facilitate contact with a nearby TOC complex (Bruce 2000). Another possibility is direct interaction with the TOC complex, mediated by membrane-integrated receptors (Toc34 or Toc159). A variation on the latter involves a soluble form of Toc159, which first recognizes the preprotein in the cytosol and, like the guidance complex, brings the preprotein to the TOC machinery (Fig. 1; Hiltbrunner et al. 2001b). Finally, a putative third TOC component, Toc64, has been suggested to act as a receptor for a subset of proteins pre-bound by Hsp90; however, the relevance of this idea is debated (Qbadou et al. 2006, 2007; Aronsson et al. 2007; see Sect. 4 for TOC receptors).

3.1 The Guidance Complex

The guidance complex consists of 14-3-3 and Hsp70 proteins; it interacts with the TP and targets it to the chloroplast OEM (May and Soll 2000). Formation of the guidance complex is proposed to be dependent on the phosphorylation of a serine or threonine residue within the TP (Waegemann and Soll 1996; May and Soll 2000). Use of the guidance complex might "fast track" certain preproteins (e.g.,

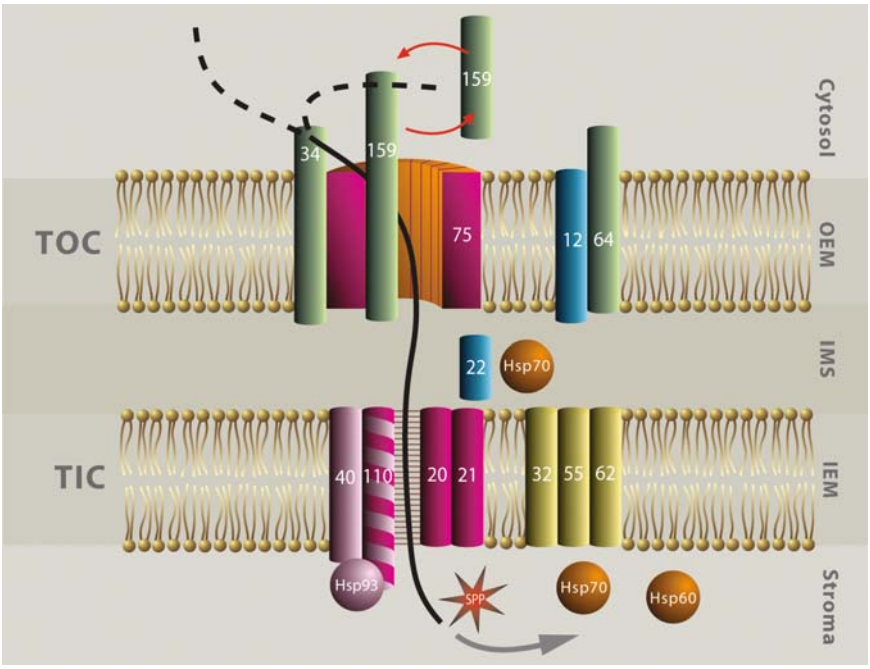

Fig. 1 *Chloroplast protein translocation machinery*. The preprotein (*black line*) approaches the outer envelope membrane (OEM) and is recognized by one of the TOC receptors (*green*): either (A) the membrane bound Toc34 receptor, or (B) a soluble form of the Toc159 receptor (*dashed lines*). Then, the preprotein enters the Toc75 channel (*magenta/orange*) and reaches the intermembrane space (IMS), where the TOC and TIC meet each other. IMS associated proteins (*blue*) mediate movement of the preprotein towards TIC channel components (*magenta*). Redox-related TIC subunits (*yellow*) may assist the transfer of some preproteins through the inner envelope membrane (IEM). Finally, the preprotein is driven into the stroma with help from a putative motor complex (*pink*), and the transit peptide (*grey arrow*) is removed by the stromal processing peptidase (SPP)

small subunit of Rubisco; pSS) that are needed at high levels, since import in the presence of the guidance complex becomes ~3–4-times faster (May and Soll 2000). However, only a few proteins have been experimentally tested for usage of the guidance complex (May and Soll 2000). Moreover, in a study by Nakrieko et al. (2004) using GFP-tagged pSS proteins, no effects of phosphorylation site mutations within the TP could be shown. Thus, the guidance complex is not a prerequisite for import; indeed, preproteins in many in vitro studies lack the guidance complex and still show functional import (Dabney-Smith et al. 1999; Aronsson et al. 2001).

3.2 The Involvement of Galactolipids

The galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are the most abundant, non-proteinaceous constituents of chloroplast membranes (Douce and Joyard 1990). The MGDG lipid has been

suggested to influence protein import into chloroplasts (for pSS and pFerredoxin) as well as insertion into the chloroplast OEM (for OEP7) (van't Hof et al. 1991, 1993; Chupin et al. 1994; Pilon et al. 1995; Pinnaduwage and Bruce 1996; Bruce 1998; Schleiff et al. 2001; Hofmann and Theg 2005a). However, other reports suggested no specific role for MGDG in import (Inoue et al. 2001; Schleiff et al. 2003b). Chloroplast protein import, or insertion into the OEM, in an *Arabidopsis* MGDG-deficient mutant, *mgd1*, showed equal performance as compared with wild type (Aronsson et al. 2008). Thus, in vivo data suggest that MGDG is not as important for chloroplast protein targeting as was implied by earlier in vitro studies. A major difference between the in vivo analysis and the previous studies was the use of intact chloroplasts instead of artificial lipid layers or vesicles that mimic OEM lipid composition (the latter lack the many proteins present in the OEM). Nevertheless, one must bear in mind that the *mgd1* mutant contains only ~40% less MGDG than wild type (Jarvis et al. 2000). A recently described MGD1 null mutant displays a very severe phenotype, and is so sick that it is unlikely it could be used for import studies (Kobayashi et al. 2007).

In contrast, similar experiments using the DGDG-deficient mutant, *dgd1*, did reveal a significant defect in protein import (Chen and Li 1998; Aronsson and Jarvis 2002). These results can be interpreted in two different ways. Firstly, they may indicate that DGDG is relatively more important for chloroplast import than MGDG. This possibility is supported by the observation that DGDG was the only galactolipid associated to the TOC complex (Schleiff et al. 2003b), and by the fact that in *mgd1* the level of DGDG is not altered (Jarvis et al. 2000). However, such an interpretation would be in disagreement with a host of in vitro studies, which suggested a particularly important role for MGDG (van't Hof et al. 1991, 1993; Chupin et al. 1994; Pilon et al. 1995; Pinnaduwage and Bruce 1996; Bruce 1998; Schleiff et al. 2001). An alternative explanation for the *dgd1* results is that they simply reflect the fact that this mutant has a much more severe lipid defect: the *dgd1* mutant shows a 90% reduction in DGDG levels (Dörmann et al. 1995).

4 Receptors at the TOC Complex

The Toc34 and Toc159 components are related GTPases, and were first identified in pea as being involved in preprotein recognition and binding (Hirsch et al. 1994; Kessler et al. 1994; Seedorf et al. 1995). Initially, Toc159 was seen as a fragment of 86 kDa, and so the name Toc86 was used. However, due to the identification of a larger homologue in *Arabidopsis*, the pea Toc86 protein was further scrutinized and shown to have a native size comparable to the *Arabidopsis* protein; the 86-kDa pea protein was produced by proteolytic degradation during the experiments (Bölter et al. 1998a; Chen et al. 2000). A putative third receptor, Toc64, was also identified in pea (Sohrt and Soll 2000), but its role is less well defined.

4.1 Toc34

So far, only one Toc34 isoform has been identified in pea (*Pisum sativum* Toc34, or psToc34), but two homologues exist in *Arabidopsis thaliana* (Table 1; atToc33 and atToc34); both *Arabidopsis* proteins are very similar to psToc34 (~60% identity) (Jarvis et al. 1998; Voigt et al. 2005). Toc34 consists of a cytosolic GTPase (G)

Table 1 The *Arabidopsis* proteins most closely related to putative or actual components of the pea chloroplast protein import apparatus, their proposed functions, and other homologues

Component (generic name)	Main isoform in *Arabidopsis*	AGI no.	Domains or motifs	Proposed function(s)	Additional homologues in *Arabidopsis*
Toc12	atToc12	At1g80920	DnaJ	Co-chaperone	None detected
Toc34	atToc33	At1g02280	GTPase	Preprotein receptor	atToc34
Toc64	atToc64-III	At3g17970	Amidase; TPR[a]	Receptor; unknown	atToc64-I, -V
Toc75	atToc75-III	At3g46740	POTRA[a]; ß-barrel	Import channel	atToc75-IV, -V
Toc159	atToc159	At4g02510	GTPase; A and M domains	Preprotein receptor; import motor	atToc90, 120, 132
Tic20	atTic20-I	At1g04940	Polytopic, α-helical TMDs[a]	Import channel	atTic20-II, -IV, -V
Tic21	atTic21	At2g15290	Polytopic, α-helical TMDs[a]	Import channel; permease	None detected
Tic22	atTic22-IV	At4g33350	None detected	TOC–TIC interaction	atTic22-III
Tic32	atTic32-IVa	At4g23430	NADPH-dependent short chain dehydrogenase	Redox/calcium sensing	atTic32-IVb[b]
Tic40	atTic40	At5g16620	TPR[a]; Sti1	Co-chaperone	None detected
Tic55	atTic55-II	At2g24820	Rieske iron-sulfur centre; mono-nuclear iron site	Redox sensing	None detected[b]
Tic62	atTic62	At3g18890	NAD(P)H dehy-drogenase; FNR-binding site	Redox sensing	None detected[b]
Tic110	atTic110	At1g06950	TP-binding site; Tic40-binding site	Import channel; Chaperone recruitment	None detected
Hsp93	atHsp93-V	At5g50920	Walker ATPase, ClpC/Hsp100	Import motor	atHsp93-III
SPP	atSPP	At5g42390	Zinc-binding, pitrilysin	TP cleavage	None detected

For a more comprehensive table, see Jarvis (2008)

[a] *TPR*, tetratricopeptide repeat; *POTRA*, polypeptide transport associated domain; *TMDs*, transmembrane domains

[b] Please also refer to Kalanon et al. (2008) and references therein

domain and a short, membrane-spanning helix at the C-terminal end (Fig. 1) (Kessler et al. 1994; Seedorf et al. 1995). Binding of GTP is thought to be necessary for Toc34 to receive incoming preproteins, and for it to act as a receptor (Kouranov and Schnell 1997; Sveshnikova et al. 2000b). According to one model for preprotein recognition, the structure of Toc34 changes upon GTP hydrolysis and the preprotein is released towards Toc159 and the Toc75 channel. In another model, Toc34 functions as a receptor for an incoming preprotein-Toc159 complex (Sect. 4.2).

Crystals of psToc34 in the GDP-bound state showed that the receptor can dimerize. It was suggested that each GTPase within a dimer acts as GTPase-activating protein (GAP) for the opposing monomer (Sun et al. 2002; Bos et al. 2007); however, it was subsequently reported that mutation of the putative arginine finger (R130 in atToc33; arginine fingers are critical mediators of GAP function) did not affect GTP hydrolysis (Weibel et al. 2003). Because Toc34 and Toc159 share significant homology within their G-domains, it has been suggested that both receptors dimerize, and, even more interestingly, that heterodimerization may be an important component of the import mechanism (Kessler and Schnell 2002). Assembly of the TOC translocon, as well as precursor transport, is also suggested to be dependent on heterodimerization (Wallas et al. 2003). However, there is considerable disagreement concerning the consequences of dimerization for receptor activity (Sun et al. 2002; Weibel et al. 2003; Reddick et al. 2007; Yeh et al. 2007). Interestingly, atToc33 was found to have unusual properties, exhibiting affinity for both GTP and XTP in its wild-type state (Aronsson et al. 2003a). Clearly, the structure of Toc34 and its GTPase activity are not yet fully understood.

The binding of GTP to Toc34 has been proposed to be controlled by receptor phosphorylation. When Toc34 is phosphorylated, GTP is not bound and the action of the receptor is inhibited (Sveshnikova et al. 2000b). The phosphorylation site has been identified as serine 113 in psToc34, and as serine 181 in atToc33 (Jelic et al. 2002; 2003). When S181 was substituted with alanine, aspartate or glutamate (predicted to create constitutively active or inactive mutants), the in vivo activity of the protein was not detectably altered (Aronsson et al. 2006). While a more recent study suggested that these mutations may have subtle consequences during the earliest stages of the development (Oreb et al. 2007), the fact remains that the role of the phosphorylation is far from clear. It could be more important at earlier stages of plant development when the need for import is higher (Dahlin and Cline 1991), but it is difficult to understand why the regulation of two orthologues (psToc34 and atToc33) might be mediated by phosphorylation at very different positions (both of which lack conservation in other species), and, presumably, through completely different mechanisms. A kinase was shown to phosphorylate Toc34 in vitro, but its identity is unknown (Fulgosi and Soll 2002).

The function of Toc34 in preprotein recognition is widely accepted, but whether Toc34 or Toc159 is the primary receptor for the preprotein is debated. Two models have been proposed: the "motor model" places Toc34 in this role, while the "targeting model" has Toc159 as the primary receptor (Sect. 4.2). Evidence arguing for Toc34 as the primary receptor includes: preprotein interaction with Toc34 in vitro (Jelic et al. 2002; Becker et al. 2004b; Reddick et al. 2007); greater quantities of Toc34 (versus Toc159) in the envelope membrane (Schleiff et al. 2003b; Kikuchi et al. 2006; Chen and Li 2007); proposed interaction with the guidance complex (Qbadou

et al. 2006); and, the lack of direct input from Toc34 during translocation over the membrane (Schleiff et al. 2003a).

Whether or not Toc34 is the primary receptor, it seems that different Toc34 isoforms have specific preferences for certain preproteins. Proteomic studies by Kubis et al (2003) on an atToc33 null mutant, named *ppi1* (*plastid protein import 1*; Jarvis et al. 1998), showed that photosynthetic proteins are specifically deficient in the mutant, whereas non-photosynthetic, housekeeping proteins are rather stable. The same is true for an atToc159 mutant, termed *ppi2* (Bauer et al. 2000), and so import into *Arabidopsis* chloroplasts is proposed to follow two different pathways (Sect. 4.2): atToc33 and atToc159 preferentially import photosynthetic proteins; atToc34 and atToc132/atToc120 import housekeeping proteins. Nevertheless, "cross-talk" between the pathways seems to occur (Hiltbrunner et al. 2001a).

While the atToc33 knockout mutant (*ppi1*) is pale (Jarvis et al. 1998), an atToc34 null mutant (*ppi3*) shows no obvious phenotypes in aerial, photosynthetic tissues, but its roots (which are non-photosynthetic) are shorter than normal (Constan et al. 2004b). In spite of their specialization, double-mutant studies on the homologues revealed a considerable degree of functional overlap: the atToc33/atToc34 double-null genotype is embryo-lethal, while plants homozygous for *ppi1* and heterozygous for *ppi3* are even paler than *ppi1* alone. Multiple Toc34 isoforms also exist in moss (*Physcomitrella patens*), maize and spinach, and in the latter case evidence suggests that the isoforms exhibit functional specialization (Voigt et al. 2005).

4.2 Toc159

The Toc159 family consists of four members in *Arabidopsis* – atToc159, atToc132, atToc120 and atToc90 (Table 1; Jackson-Constan and Keegstra 2001; Hiltbrunner et al. 2001a) – while in pea presently only psToc159 (formerly Toc86) has been identified (Hirsch et al. 1994; Kessler et al. 1994; Perry and Keegstra 1994). The psToc159 protein is most similar to atToc159 (48% identity), and so these two are believed to be functional orthologues (Bauer et al. 2000). Toc159 proteins have three domains: an N-terminal acidic (A) domain which is very sensitive to proteolysis, giving rise to an 86-kDa fragment (Hirsch et al. 1994; Kessler et al. 1994; Schnell et al. 1994); a central GTPase (G) domain related to the Toc34 G-domain; and, a hydrophilic M-domain that anchors atToc159 in the membrane (Bauer et al. 2000; Hiltbrunner et al. 2001a). The M-domain is a 52-kDa protease-resistant region that does not carry typical hydrophobic, transmembrane helices, and so is unusual (Hirsch et al. 1994; Kessler et al. 1994; Bauer et al. 2000).

In the "targeting model" of preprotein recognition, Toc159 acts as the primary receptor (Fig. 1). This is partly based on cross-linking studies using preproteins arrested during the binding step of import: under these conditions, Toc159 is the major TOC component cross-linked to the preprotein (Perry and Keegstra 1994; Ma et al. 1996; Kouranov and Schnell 1997). The blocking of early import intermediate formation by applying a Toc159 antibody also supports the primary receptor role of

Toc159 (Hirsch et al. 1994). Detection of an abundant form of atToc159 in the cytosol gave this model another dimension (Hiltbrunner et al. 2001b). This was seen in protoplasts by immunofluorescence microscopy, and supported by subfractionation studies in *Arabidopsis* and pea. It was proposed that Toc159 recognizes the preprotein in the cytosol, and then brings it to the TOC complex; in fact, soluble Toc159 was shown to interact exclusively with the TP of preproteins (Smith et al. 2004). This implies that Toc159 cycles between its soluble, cytosolic form and its membrane-integrated form (Fig. 1). The atypical hydrophilic M-domain of Toc159 may play a critical role in this integration/de-integration process. However, the relevance of cytosolic Toc159 has been questioned, and attributed to partial disruption of membranes due to the experimental procedures (Becker et al. 2004b). Whether this is true or not remains unanswered.

The "targeting model" is comparable to the co-translational translocation of proteins into the ER by the *s*ignal *r*ecognition *p*article (SRP) system. The latter starts with recognition of a nascent signal peptide, protruding from a ribosome, by the SRP (a GTPase). This complex carries the preprotein to the SRP-receptor (another GTPase) where GTP hydrolysis ensures preprotein transfer to the Sec translocase for transport over the membrane (Shan and Walter 2005; Bange et al. 2007). Toc159 is proposed to play a role analogous to that of SRP, while Toc34 may be analogous to the SRP-receptor. Intriguingly, a distant homology between TOC GTPases and chloroplastic SRP and SRP-receptors was recently presented (Hernández Torres et al. 2007). The SRP system can also act post-translationally, for example within chloroplasts for thylakoid targeting (Schuenemann 2004), which is more similar to the way in which chloroplast import occurs.

The Toc34 protein is believed to mediate the insertion of Toc159 in a GTPase-regulated fashion (Wallas et al. 2003). Hence, the initial binding to the membrane could occur by a heterodimerization between Toc34 and Toc159, both in the GTP state. That would perhaps induce GTP hydrolysis (each receptor acting as a GAP on the other), and thereby induce integration of the Toc159 M-domain. However, the role of heterodimerization in relation to GTP hydrolysis is debated (Sun et al. 2002; Weibel et al. 2003; Reddick et al. 2007; Yeh et al. 2007). Integration of a Toc159 receptor pre-bound to a preprotein client might simultaneously initiate the membrane translocation of the precursor (Fig. 1).

In the "motor model", where Toc34 is seen as the primary receptor (Sect. 4.1), Toc159 is permanently associated with the membrane and acts as a motor by driving the preprotein forward through the Toc75 channel (Fig. 1). The Toc159 motor action is powered by multiple cycles of GTP hydrolysis, each one pushing a new part of the preprotein into the channel (Schleiff et al. 2003a; Becker et al. 2004b). The model is based on several lines of evidence. Firstly, a minimal TOC complex consisting of a Toc159 fragment and Toc75 was able to mediate transport of preproteins into proteoliposomes at the expense of GTP hydrolysis (Schleiff et al. 2003a). Secondly, in isolated TOC core-complexes (whose stoichiometry was estimated to be 4–5:4:1 or 3:3:1 for Toc34:Toc75:Toc159), Toc34 and Toc75 were found in almost equal amounts (Schleiff et al. 2003b; Kikuchi et al. 2006). Also, crosslinking studies revealed that Toc159 is in close association with the preprotein

throughout OEM translocation (Kouranov and Schnell 1997). Studies using proteoliposomes containing the TOC core-complex showed that precursor binding could only be inhibited when Toc34 was blocked using a competitive TP fragment; similar inhibition of Toc159 did not interfere with binding (Becker et al. 2004b). This again supported the proposed role of Toc34 as the primary receptor.

In this model, Toc159 remains in close association with Toc75 via an interaction that is nucleotide-insensitive (Becker et al. 2004b). Firstly, Toc34 in its GTP-bound state binds to the C-proximal domain of an incident TP. Then, the binding of the N-terminal domain of the same TP to Toc159 (also in its GTP-state) causes the two receptors to associate closely. Next, Toc34 hydrolyses GTP, and so releases its grip on the TP such that it is transferred entirely to Toc159. The latter process promotes GTP hydrolysis by Toc159, which in turn drives the preprotein into the Toc75 channel (Fig. 1) (Schleiff et al. 2003a; Becker et al. 2004b). Following GTP hydrolysis, the association of Toc34 with the TOC complex is weakened. Further experimentation is required to determine which elements of the "targeting" and "motor" models most closely reflect the in vivo situation.

The atToc159 null mutant (*ppi2*) is more severely sick than the atToc33 null mutant (*ppi1*); it has an albino phenotype and cannot produce normal chloroplasts (Bauer et al. 2000; Smith et al. 2002). Like *ppi1*, it has reduced accumulation of photosynthetic proteins and low expression of the corresponding genes. However, many housekeeping genes are not affected. These results strengthen the hypothesis that atToc159 and atToc33 are part of a specific import machinery preferentially used by photosynthetic proteins. In the *ppi2* mutant, a photosynthetic-TP-GFP fusion-protein was not targeted efficiently to plastids, whereas a housekeeping-TP-GFP fusion-protein was imported normally (Smith et al. 2004).

Other *Arabidopsis* Toc159 homologues are believed to mediate import of housekeeping proteins (Bauer et al. 2000), as revealed by analyses of corresponding null mutants (Hiltbrunner et al. 2004; Ivanova et al. 2004; Kubis et al. 2004; Hust and Gutensohn 2006). In contrast with *ppi2*, mutants of atToc120 and atToc90 (*ppi4*) did not display any visible phenotypes, while atToc132 mutants expressed only a moderate yellow–green, reticulate phenotype (Kubis et al. 2004). Hence, none of these atToc159 homologues is essential, individually, for plant viability. However, atToc132 and atToc120 are highly redundant, since a double-knockout mutant displayed a severe, near-albino phenotype (Ivanova et al. 2004; Kubis et al. 2004); this phenotype could be abolished by overexpression of either atToc132 or atToc120 (Kubis et al. 2004). Interestingly, atToc159 overexpression could not complement the atToc132/atToc120 double mutant, supporting the notion that two different, functionally specialized import pathways exist (Kubis et al. 2004). In contrast, the role of atToc90 is less clear, and it does not share significant redundancy with the other Toc159 homologues (Kubis et al. 2004). However, its expression pattern resembles that of atToc159, and so it has been proposed to assist the function of atToc159 (Bauer et al. 2000; Hiltbrunner et al. 2004).

4.3 Toc64/OEP64

Analysis of the TOC complex from pea chloroplasts identified an unknown, co-purifying 64-kDa protein, later referred to as Toc64 (Sohrt and Soll 2000). The N-terminus of Toc64 contains a transmembrane anchor, the central part shares homology with amidases, and the C-terminal region has three tetratricopeptide repeats (TPRs) exposed to the cytosol. Cross-linking studies revealed that psToc64 is in close proximity with several TOC and TIC components as well as precursor proteins (Sohrt and Soll 2000; Becker et al. 2004a). In *Arabidopsis*, there are three genes for Toc64-like proteins: *atTOC64-I*, *atTOC64-III* and *atTOC64-V* (roman numbers indicate chromosome location) (Table 1; Jackson-Constan and Keegstra 2001).

The likely orthologue of psToc64 is atToc64-III, which is localized in chloroplasts (Chew et al. 2004; Qbadou et al. 2007). The atToc64-III transmembrane domain, and its C-terminal flanking region, are sufficient to mediate targeting to the OEM (Lee et al. 2004). In pea, Toc64 has been proposed to mediate docking of the "guidance complex" (Sect. 3), and to act as a receptor for preproteins delivered by Hsp90 (Sohrt and Soll 2000, Qbadou et al. 2006); in the latter model, the Toc64 TPR domain binds to the chaperone rather than to the preprotein itself. Despite its similarity with psToc64, a clear role for atToc64-III in chloroplast protein import could not be established (Aronsson et al. 2007). The atToc64-V protein is also very similar to psToc64, but it is localized in mitochondria and so was renamed mtOM64 (*mit*ochondrial *O*uter *M*embrane protein, 64 kDa) (Chew et al. 2004). The atToc64-I protein lacks both the TPRs and the transmembrane anchor, and is essentially just the amidase domain; this isoform seems to be cytosolic (Chew et al. 2004; Pollmann et al. 2006). Interestingly, atToc64-I was shown to have indole-3-acetamide hydrolase activity, relating the protein to auxin biosynthesis, and so its name was changed to Amidase 1 (AMI1) (Pollmann et al. 2006). However, an atToc64-I null mutant did not show significant growth phenotypes, and so more studies are needed to resolve its role (Aronsson et al. 2007).

Two Toc64-like proteins were identified in the moss, *Physcomitrella patens* (Hofmann and Theg 2005b). A double null mutant affecting these components showed no obvious phenotypes (except a slight chloroplast shape alteration). Most importantly, experiments revealed no defects in chloroplast protein import efficiency in the double mutant (Hofmann and Theg 2005b). This argues against a crucial role for Toc64 in import. Even in the higher plant, *Arabidopsis*, Toc64 (atToc64-III) null mutants are indistinguishable from wild type (Aronsson et al. 2007). Absence of defects in atToc64-III mutants raised the possibility of redundancy, but even triple mutants lacking all three isoforms appeared normal. In the absence of a clearly defined role for Toc64, it was suggested that it should be renamed using the general designation, OEP64 (*O*uter *E*nvelope *P*rotein, 64 kDa). Data supporting a role for Toc64 in import were generated biochemically, whereas those arguing against this role were derived in vivo, in *Arabidopsis* and *P. patens*.

The topology of Toc64/OEP64 is also disputed. In addition to the originally defined, N-terminal transmembrane region, two additional membrane spans have been suggested to exist (Qbadou et al. 2007). However, contradicting data on the

topology of Toc64 have been presented (Hofmann and Theg 2005c). If the more complex topology is correct, it may be that Toc64 acts on both sides of the OEM: in the cytosol, the TPR would facilitate docking of incident cargo proteins, which is consistent with the general role of TPRs as protein–protein interaction domains (Schlegel et al. 2007); in the intermembrane space (IMS), Toc64 would act as a "link" between TOC and TIC through interplay with Toc12, an IMS Hsp70 and Tic22 (Fig. 2a; Becker et al. 2004b; Qbadou et al. 2007).

However, preprotein translocation does not require a functional Toc64/OEP64 protein, since the TOC core-complex (Toc159, Toc75 and Toc34) was fully import-competent when reconstituted into proteoliposomes (Schleiff et al. 2003a). Moreover, knockout of a TOC protein involved in multifaceted activities on both sides of the OEM (as proposed for Toc64) would be predicted to have a clear phenotype (e.g., pale, albino, embryo-lethal), as found for other TOC components (Jarvis et al. 1998; Bauer et al. 2000; Baldwin et al. 2005). This is not the case for Toc64 (Hofmann and Theg 2005b; Aronsson et al. 2007), and so its role is far from clear. Nonetheless, the possibility remains that it acts as a "fine-tuner".

5 Channels and Intermediaries

Following recognition, preproteins are transferred to the OEM channel, of which Toc75 is the main component. Following OEM translocation, preproteins enter the IMS prior to their association with the IEM. Contact sites between the OEM

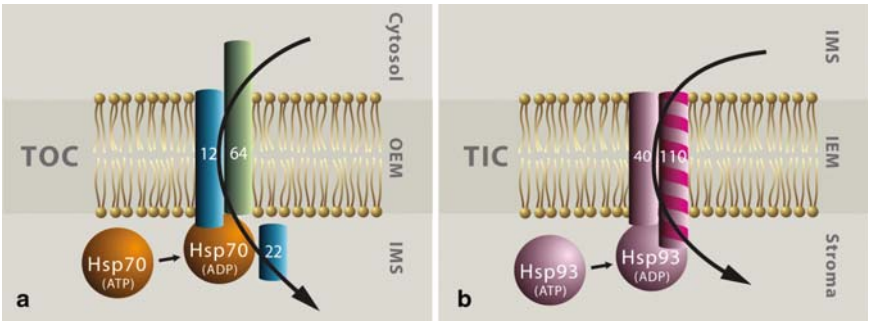

Fig. 2 *Putative motor complexes of the import machinery*. (**a**) Protein translocation across the outer membrane. The preprotein (*black line*) is recognized by Toc64 before being released to the TOC core-complex (not shown). Toc64 activates Toc12, which recruits Hsp70 in an ATP-bound state. Toc12 then stimulates ATP hydrolysis by Hsp70, enabling the chaperone to drive translocation of the preprotein in a ratchet mechanism. The preprotein is transferred to Tic22 for further movement to the TIC complex. (**b**) Protein translocation across the inner membrane. The preprotein (*black line*) moves through the TIC channel (not shown), binding at Tic110. This stimulates a Tic110–Tic40 interaction, which releases the transit peptide from Tic110 for association with Hsp93. The ATPase activity of Hsp93 is then stimulated by Tic40, which allows Hsp93 to complete import, possibly via a ratchet mechanism. OEM/IEM, outer/inner envelope membrane; TOC/TIC, translocon of the outer/inner envelope membrane of chloroplasts; IMS, intermembrane space

and the IEM are established to enable efficient translocation from TOC to TIC (Schnell and Blobel 1993; Perry and Keegstra 1994). Several components, including Toc12, an IMS Hsp70 and Tic22, are proposed to facilitate translocation across the IMS (Fig. 2a). On arrival at the IEM, the preprotein may contact Tic110 and/or Tic20, since both have been proposed to mediate channel formation in the IEM (Fig. 1).

5.1 Toc75

One of the first TOC components to be identified in pea was Toc75 (Waegemann and Soll 1991; Perry and Keegstra 1994; Schnell et al. 1994). Evidence for its role in import came from its proximity to preproteins engaged in import, as revealed by cross-linking (Perry and Keegstra 1994; Ma et al. 1996) and co-purification from solubilized envelopes (Schnell et al. 1994), and from the inhibition of import by Toc75 antibodies (Tranel et al. 1995). It is one of the most abundant proteins in the OEM (Cline et al. 1981), and can act as an aqueous ion channel in vitro, strongly suggesting that it forms the translocation pore (Fig. 1; Hinnah et al. 1997, 2002). As an integral membrane protein, Toc75 can withstand protease treatment and extraction by either salt or high pH (Schnell et al. 1994; Tranel et al. 1995). It is proposed to have either 16 or 18 amphiphilic β-strands that together make up a β-barrel domain (Sveshnikova et al. 2000a; Schleiff et al. 2003b), as often found in bacterial proteins (Gentle et al. 2005). The channel is estimated to be ~14-Å in diameter (Hinnah et al. 2002), which is sufficient only for largely unfolded proteins to pass. However, the import of a tightly folded substrate ~23-Å in diameter suggests a degree of elasticity (Clark and Theg 1997).

Interestingly, psToc75 shares 22% amino acid sequence identity with a cyanobacterial (*Synechocystis*) homologue, SynToc75 (Reumann et al. 1999). This homologue behaves as a channel protein with features resembling those of psToc75 (Bölter et al. 1998b). In pea, a second Toc75-related protein was identified on the basis of its similarity to SynToc75. This protein shares 31% identity with psToc75, is assumed to be a channel also, and was named psToc75-V due to the fact that the most similar *Arabidopsis* protein, atToc75-V, is encoded on chromosome 5 (Eckart et al. 2002). On the basis of phylogenetic studies, atToc75-V was proposed to be the most ancestral form of the Toc75 channel (Eckart et al. 2002). However, this was challenged by an idea that the two pea Toc75-like proteins each derived independently from cyanobacterial sequences (Inoue and Potter 2004).

Three Toc75-related proteins exist in *Arabidopsis*: atToc75-III, atToc75-IV and atToc75-V (Table 1; Jackson-Constan and Keegstra 2001; Eckart et al. 2002; Baldwin et al. 2005). The first of these, atToc75-III, is thought to be the true orthologue of psToc75, based partly on high sequence similarity (73% within the mature region) and similar developmental expression profiles (Tranel et al. 1995; Baldwin et al. 2005). The second protein, atToc75-IV, lacks a large N-terminal

domain, is only expressed at very low levels, and most likely plays a rather minor role (Baldwin et al. 2005). Interestingly, atToc75-IV and atToc75-V both differ from atToc75-III in respect of their membrane insertion requirements: neither appears to be processed during insertion, whereas the latter has a bipartite targeting sequence, like psToc75, which directs the protein firstly towards the stroma, and then in a second step mediates insertion into the OEM (Inoue and Keegstra 2003; Inoue and Potter 2004; Baldwin et al. 2005). The size of fully assembled atToc75-V was reported to be 80 kDa, which is somewhat larger than originally proposed (Eckart et al. 2002; Inoue and Potter 2004). Since a role in protein import has not been demonstrated, the Toc75-V protein was renamed with the general designation, OEP80 (*O*uter *E*nvelope *P*rotein, 80 kDa) (Eckart et al. 2002; Inoue and Potter 2004). The role of OEP80 is unknown, but it has been proposed to mediate the translocation of highly hydrophobic α-helical solute transporters of the IEM, and the biogenesis β-barrel proteins of the OEM (Eckart et al. 2002; Inoue and Potter 2004).

Null mutants have been identified for atToc75-III and atToc75-IV. Those affecting the former are embryo-lethal, while atToc75-IV knockouts exhibit no obvious defects except for inefficient de-etiolation, indicating a possible role in etioplasts (Baldwin et al. 2005). These data support the notion that atToc75-III is the true orthologue of psToc75, and the main Toc75 channel in *Arabidopsis*.

5.2 Outer Envelope Translocation Models

Several lines of evidence indicate that there is a motor activity at the OEM, and that TOC translocation is not simply driven by TIC-associated machinery (Guera et al. 1993; Scott and Theg 1996; Kovacheva et al. 2007). Different hypotheses exist for the mechanism of translocation through Toc75. One of these is the Toc159 "motor model", in which the receptor acts like a sewing machine to pushing the preprotein through the channel in cycles of GTP hydrolysis (Sect. 4.2) (Schleiff et al. 2003a). However, this model is inconsistent with data indicating that import can still proceed in the presence of non-hydrolyzable GTP analogues, or following removal of the Toc159 G-domain (Kessler et al. 1994; Young et al. 1999, Chen et al. 2000). The G-domain may instead function to place the M-domain of Toc159 in a position suitable for translocation; this idea is supported by the partial complementation of *ppi2* using the M-domain only (Lee et al. 2003).

Another possibility involves ATP hydrolysis and may be called the "chaperone model". Initial studies on import showed that formation of early import intermediates is ATP dependent (Olsen et al. 1989; Olsen and Keegstra 1992). Moreover, an Hsp70 chaperone has been identified in the IMS in close association with the TOC complex (Marshall et al. 1990; Schnell et al. 1994). In this model, Hsp70 acts as a molecular ratchet to ensure the unidirectional movement of the preprotein (Neupert and Brunner 2002). The recently identified component, Toc12, may act as a co-chaperone by controlling the ATPase activity of the IMS Hsp70 (Sect. 5.3) (Fig. 2a; Becker

et al. 2004a). In contrast with mitochondrial import, Hsp70 is proposed to mediate translocation over the OEM only; full transport into the stroma over the IEM seems to involve other chaperones (Sect. 6.2.3).

5.3 Intermediaries, with Emphasis on Toc12 And Tic22

Transport across the two envelope membranes takes place in concert (Schnell and Blobel 1993; Perry and Keegstra 1994; Schnell et al. 1994; Wu et al. 1994). Thus, the TOC and TIC translocons meet at so-called contact sites to enable efficient co-translocation (Schnell and Blobel 1993; Perry and Keegstra 1994). These contacts may be mediated by proteins present in the IMS (e.g., Toc12 and Tic22; Fig. 1).

Tic22 was identified by its interaction with preproteins arrested as early import intermediates (Ma et al. 1996; Kouranov and Schnell 1997). It was also demonstrated to lie mostly in the IMS and to be peripherally associated with the IEM. Its location led to the hypothesis that Tic22 is involved in the movement of preproteins from TOC to TIC (Kouranov et al. 1998, 1999). Tic22 has also been proposed to work in close association with Toc12 and Toc64 (Becker et al. 2004a).

The Toc12 protein is located in the OEM and is a DnaJ-like co-chaperone. Its C-terminal J-domain protrudes into the IMS, and is stabilized by an intramolecular disulphide bond, which might play a role in sensing the redox state of the chloroplast (Sect. 8) (Becker et al. 2004b). As mentioned earlier (Sect. 4.3), Toc12 is proposed to stimulate the ATPase activity of the IMS Hsp70, to assist the formation of early import intermediates. Together with Hsp70, Toc64 and Tic22, Toc12 was proposed to be part of an IMS translocase (Fig. 2a). In this model, Toc64 would coordinate preprotein arrival at the OEM with the readying or assembly of the IMS translocase for downstream steps. Tic22 would be the link to the TIC complex, so that upon preprotein arrival a contact site is induced (Becker et al. 2004a). This is an attractive hypothesis, but its relevance is unclear since the role of Toc64 is questionable (Hofmann and Theg 2005b; Aronsson et al. 2007). At present, no in vivo studies have been presented for Toc12 (Inoue 2007).

6 Translocation Events at the TIC Complex

Translocation over the IEM is less well characterized than OEM translocation. Nonetheless, IEM transport is proposed to involve: channel-forming protein(s); a motor complex that drives transport into the stroma; and, regulatory factors that control import by sensing plastid redox status (Fig. 1). Interestingly, there is considerable disagreement in the literature concerning the composition of the TIC machinery (e.g., the identity of the main channel component). This may reflect an inherent flexibility in the system, or indicate that multiple, different TIC complexes exist.

6.1 Candidate Channel Components of the TIC Complex

6.1.1 Tic110

As already mentioned, the identity of the TIC channel is uncertain, since three different components (Tic110, Tic20 and Tic21) have been proposed to perform this role (Fig. 1). The first of these, Tic110, was identified in pea by its close association with preproteins at a late stage of import (Schnell et al. 1994; Wu et al. 1994). Tic110 has an N-terminal membrane anchor of ~9 kDa comprising two helical membrane spans. The C-terminal bulk of the protein constitutes a ~98-kDa hydrophilic domain for which the location is debated. Initial reports that it protrudes into the IMS (Lübeck et al. 1996) have been corrected by more recent studies showing orientation towards the stroma (Kessler and Blobel 1996; Nielsen et al. 1997; Jackson et al. 1998; Inaba et al. 2003). Its location in the stroma is thought to be significant, enabling it to recruit stromal factors needed for import (Sect. 6.2).

When reconstituted into liposomes, the Tic110 C-terminal domain was reported to form a β-barrel structure; this was proposed to be part of the TIC channel, since it had a pore diameter of 15 Å and exhibited cation-selectivity (Heins et al. 2002). However, this model was disputed by others who proposed an alternative structure for the C-terminus. Upon overexpression in bacteria and plants, the domain was found to be soluble and to have an α-helical conformation (Inaba et al. 2003). Thus, any role of Tic110 as part of the TIC channel is most likely mediated by its N-terminal transmembrane spans, leaving the C-terminus free to manage later events of the import process; an important component of this role seems to be a domain that recognizes and binds to TPs (Inaba et al. 2003). It is clear that Tic110 is essential for the import mechanism, since reduced expression causes chlorosis and null mutations are embryo-lethal (Inaba et al. 2005; Kovacheva et al. 2005). Tic110 is found in most tissues and is distributed widely across different species (Davila-Aponte et al. 2003; Kovacheva et al. 2005).

6.1.2 Tic20

The Tic20 protein consists of four transmembrane α-helices and was first identified in pea by its association with arrested preproteins (Kouranov et al. 1998). In *Arabidopsis*, there are four paralogues: atTic20-I (the isoform most similar to psTic20; ~63% identity), atTic20-IV, atTic20-II and atTic20-V (Table 1). The latter three are progressively less similar to psTic20 (~20–30% identity). As mentioned earlier, Tic20 is thought to play a role in channel formation. Its weak homology and topological similarity with mitochondrial Tim23/22/17 preprotein translocase components support this notion (Rassow et al. 1999; Reumann et al. 1999). Expression of atTic20-I occurs in most tissues, just as for Tic110, and is highest in young tissues (Chen et al. 2002). By analyzing antisense lines, reduced expression of atTic20-I was correlated with a pale-yellow phenotype, which in turn was linked

to a chloroplast import defect at the level of IEM translocation (Chen et al. 2002). These data support the role of Tic20 as a key player for IEM translocation.

6.1.3 Tic21

The most recently identified putative channel component is Tic21 (also called *C*hloroplast *I*mport *A*pparatus 5; CIA5), which resembles Tic20 in terms of size and topology. This protein was identified in a forward-genetic screen for *Arabidopsis* mutants with defects in the chloroplast import of a selectable marker protein (Sun et al. 2001; Teng et al. 2006). The expression pattern of atTic21 is similar to those of Toc75 and Tic110, except for during germination where it is lower. Thus, it was suggested that it mainly functions at later developmental stages; since Tic20 has a more pronounced role during earlier stages, Tic21 might take over from Tic20 later on. Chloroplasts from mutant *cia5* plants display an IEM translocation deficiency, while binding to the OEM is unaffected. An atTic21 null mutant is albino, and accumulates unprocessed preproteins (Teng et al. 2006). However, the role of this component has been debated, since Duy et al. (2007) believe that it acts as an iron transporter and regulator of cellular metal homeostasis. Evidence for this comes from the accumulation of ferritin clusters and the differential regulation of genes involved in iron stress or transport in the mutant. An alternative name for the protein was therefore suggested: *P*ermease *I*n *C*hloroplasts *1* (PIC1). Further studies are needed to resolve these different ideas about Tic21.

6.1.4 PIRAC

Electrophysiological studies led to the identification of a *P*rotein *I*mport *R*elated *A*nion *C*hannel (PIRAC), and this has been proposed to be involved in preprotein translocation at the IEM (van den Wijngaard and Vredenberg 1997). The channel was blocked by preproteins and could also be inactivated by antibodies against Tic110, suggesting its close association with the TIC complex (van den Wijngaard and Vredenberg 1997; 1999). One problem with the putative involvement of PIRAC in preprotein translocation relates to the fact that TPs are positively charged, and so would have difficulty to pass through an anion channel. It was therefore proposed that PIRAC adapts to a cation channel structure upon preprotein arrival (van den Wijngaard et al. 2000). However, this idea, together with the molecular nature and significance of PIRAC needs to be established in future studies.

6.2 A Motor Complex of the TIC Machinery

Complete preprotein translocation over the IEM consumes large quantities of ATP in the stroma (Pain and Blobel 1987; Theg et al. 1989). This ATP consumption is attributed to stromal chaperones as part of a motor complex. Components of this

putative motor include Tic110, Tic40 and Hsp93 (Fig. 2b). The first of these, Tic110 (Sect. 6.1.1), plays a dual role. First, it possesses a TP recognition site located close to the TIC channel exit; this is proposed to prevent preproteins from sliding back into the IMS after passing through the channel (Inaba et al. 2003). Second, the Tic110 stromal domain probably also acts to recruit molecular chaperones (Hsp93). The second component, Tic40, seems to act as a co-chaperone, and is proposed to regulate the activity of Hsp93. The close cooperation of these three components is supported by genetic interaction data (Kovacheva et al. 2005). The fact that they are expressed in all tissues suggests that they likely mediate import into all plastid types.

6.2.1 Tic40

The Tic40 protein is encoded by a single-copy gene in pea and *Arabidopsis* (Stahl et al. 1999; Chou et al. 2003). It was first characterized biochemically in pea, and shown to closely associate with preproteins at the IEM (Wu et al. 1994; Ko et al. 1995). It possesses one transmembrane α-helix at its N-terminus, while its large C-terminal, hydrophilic domain protrudes into the stroma (Chou et al. 2003). Tic40 and Tic110 can be cross-linked to each other implying that they work in close association (Stahl et al. 1999). A co-chaperone role of Tic40 was suggested by limited homology with *H*sp70-*i*nteracting *p*rotein (Hip) and *H*sp70/Hsp90-*o*rganizing *p*rotein (Hop) in a short C-terminal region, termed the Sti1 domain. Like Hip and Hop, Tic40 also seems to possess a TPR protein–protein interaction interface, upstream of the Sti1 domain (Stahl et al. 1999; Chou et al. 2003). Furthermore, a recent study showed that the putative Tic40 Sti1 domain could be functionally replaced with the equivalent region of human Hip, strongly supporting the notion of a co-chaperone role for Tic40 (Bédard et al. 2007). In contrast with Tic110, Tic40 null mutants in *Arabidopsis* are not lethal, but instead show a strongly pale phenotype that correlates with import defects (Chou et al. 2003; Kovacheva et al. 2005). This indicates that Tic40 function is not essential for the import mechanism, but rather serves to increase the efficiency of import.

6.2.2 Hsp93 (ClpC)

The Hsp93 chaperone is also known as ClpC, due to homology with the bacterial *C*aseino*l*ytic *p*rotease (Clp) ATPase (Shanklin et al. 1995). Like the bacterial Clp ATPase, Hsp93 is proposed to form a protease holocomplex with the proteolytic subunit, ClpP (Sokolenko et al. 1998). In addition to this stromal proteolytic role, Hsp93 associates with the import apparatus at the IEM. Initial support for its involvement in the TIC complex came from cross-linking studies (Akita et al. 1997). Interaction with the TIC complex occurred even in the absence of preprotein, and was destabilized by ATP (Nielsen et al. 1997; Kouranov et al. 1998).

In *Arabidopsis*, there are two Hsp93 genes: *atHSP93-V* and *atHSP93-III* (Table 1; Jackson-Constan and Keegstra 2001). These genes display slightly different expression patterns, but the mature proteins share ~92% identity and so are believed to act redundantly (Kovacheva et al. 2005). Null mutants of atHsp93-V and atHsp93-III have been characterized by several groups (Constan et al. 2004a; Park and Rodermel 2004; Sjögren et al. 2004; Kovacheva et al. 2005, 2007). The atHsp93-V mutants display a chlorotic phenotype and have underdeveloped chloroplasts. Interestingly, import efficiency in *hsp93-V* mutant chloroplasts was found to be reduced in two studies (Constan et al. 2004a; Kovacheva et al. 2005), but normal in another (Sjögren et al. 2004). Knockout mutants of atHsp93-III showed no phenotypic differences from wild-type plants, suggesting possible redundancy with atHsp93-V (Constan et al. 2004a; Kovacheva et al. 2005). This was confirmed by complementation analysis, and by the demonstration that the double-null genotype is embryo lethal (Kovacheva et al. 2007).

6.2.3 A Model for the Operation of the Motor Complex

At the early import intermediate stage, TOC–TIC supercomplexes are formed and the preprotein is in contact with the TIC machinery (Akita et al. 1997; Kouranov and Schnell 1997; Nielsen et al. 1997; Kouranov et al. 1998; Inaba et al. 2003). Such supercomplexes already contain Hsp93, so the preprotein is able to move quickly to the next stage of translocation. The IEM motor complex then comes into play, driven by stromal ATP. The close association of Tic110 and Tic40 has been firmly established (Stahl et al. 1999; Chou et al. 2003, 2006; Bédard et al. 2007). The Tic110–Tic40 interaction is stimulated when a preprotein is present in the Tic110 TP binding site, and is proposed to be mediated by the TPR domain of Tic40 (Chou et al. 2006). This interaction triggers the release of the TP from Tic110, and enables the preprotein to associate with Hsp93 (Fig. 2b). The Sti1 region of Tic40 then stimulates the ATPase activity of Hsp93, enabling the chaperone to complete the translocation process, presumably through a molecular ratchet mechanism (Neupert and Brunner 2002). Thus, in this model (Fig. 2b), the role of Tic40 is to organize the last steps of envelope translocation, by regulating the interaction of the preprotein with Tic110 and Hsp93, and by controlling the activity of Hsp93. The net result is to enhance the efficiency of translocation (Chou et al. 2006).

Some aspects of the model are unexpected. It is surprising that the TPR region of Tic40 interacts with Tic110, since the TPR domains of Hip and Hop interact with their chaperone partners (Frydman and Höhfeld 1997; Abbas-Terki et al. 2001). That the Sti1 region should stimulate ATPase activity of Hsp93 is also unexpected, since this is not one of the proposed functions of Hip/Hop Sti1 domains; Bédard et al. (2007) showed that the Sti1 region of Tic40 is functionally equivalent to the Hip Sti1 domain. Nonetheless, the model is a useful subject for future refinement.

7 SPP

Once inside the stroma, the TP of the preprotein is cleaved off by the *s*tromal *p*rocessing *p*eptidase (SPP) (Fig. 1; Richter and Lamppa 1999). This peptidase is important for plastid biogenesis, since its down regulation by antisense technology causes albino or lethal phenotypes. Chloroplast import efficiency is negatively affected by the reduction of SPP levels (Wan et al. 1998; Zhong et al. 2003). This may be an indirect effect due to inefficient processing and assembly of TOC/TIC components, or reflective of a closely integrated role for SPP at the TIC complex.

The SPP protein has a zinc-binding motif, found also in other pitrilysin metalloendopeptidases like the enzyme responsible for cleaving mitochondrial presequences (VanderVere et al. 1995; Roth 2004), and it is essential for catalytic activity (Richter and Lamppa 2003). The peptidase binds to the C-terminal ~10–15 residues of the TP. The consensus of the cleavage site is rather weak (Emanuelsson et al. 1999), and some data suggest that recognition of physicochemical properties is more important than a specific sequence of residues (Rudhe et al. 2004). After cleavage, the TP remains attached to SPP for another round of proteolysis, and is then released into the stroma to be further degraded by a presequence protease (Moberg et al. 2003; Bhushan et al. 2006). The TP can be accessed by SPP even while the preprotein's C-terminus is attached to the TOC complex, indicating that cleavage occurs soon after entry to the stroma (Schnell and Blobel 1993). Newly imported proteins are likely folded with help from the stromal chaperones, Hsp70 and Hsp60 (Tsugeki and Nishimura 1993; Jackson-Constan et al. 2001). The association of Hsp60 with the TIC complex in an ATP-dependent manner suggests that translocation and folding may take place in concert (Kessler and Blobel 1996).

8 Fine-Tuning the TIC Complex, with Emphasis on Redox

It is well documented that chloroplast gene expression is regulated by redox status, due to the importance of photosynthesis. Three putative TIC complex components, Tic55, Tic62 and Tic32, have been identified as possible sensors of chloroplast redox state, suggesting that import is subject to similar regulation (Fig. 1; Caliebe et al. 1997; Küchler et al. 2002; Hörmann et al. 2004). All three components were first identified in pea: Tic55 and Tic62 were detected through blue native PAGE analysis of the TIC complex (Caliebe et al. 1997; Küchler et al. 2002), whereas Tic32 was found to tightly associate with the N-terminal transmembrane part of Tic110 (Hörmann et al. 2004). Additionally, Toc12 has features that could respond to changes in redox state, since its IMS J-domain is stabilized by an intramolecular disulphide bond (Becker et al. 2004b). There is some evidence to suggest that redox changes do have an effect on import. In the presence of light, the non-photosynthetic ferredoxin III preprotein is translocated only as far as the IMS, whereas in the dark it enters the stroma (Hirohashi et al. 2001). However, so far no translocon component has been shown to be responsible for this "mistargeting" phenomenon. One possibility

is that, under reductive conditions, Toc12 loses its disulphide bond, impairing its ability to stimulate Hsp70 in the IMS and leading to the disrupted passage of preproteins to the TIC complex.

8.1 Tic55

The Tic55 component is believed to be an integral IEM protein, and its characteristic features are a Rieske-type iron-sulphur centre (these normally function in electron transfer) and a mononuclear iron-binding site, which are also found in bacterial oxygenases (Caliebe et al. 1997). It was reported that *die*thyl*p*yro*c*arbonate (DEPC) treatment of pea chloroplasts inhibits protein import, and this was attributed to the disruption of histidine in the Tic55 Rieske centre, thereby supporting its role as part of the TIC complex (Caliebe et al. 1997). However, the possibility that other proteins were also affected by DEPC was not ruled out. To address this issue, we used null mutants of the *Arabidopsis* Tic55 orthologue (atTic55-II); when treated with DEPC, wild-type and *tic55* mutant chloroplasts were equally affected in terms of import capacity (Boij P, Patel R, Jarvis P, Aronsson H, unpublished data). Thus, the exact role of Tic55 is not clear. Furthermore, other groups have been unable to find Tic55 associated within TOC/TIC complexes (Kouranov et al. 1998, Reumann and Keegstra 1999). So far, no data have been reported for *Arabidopsis* Tic55 null mutants, but we observe that homozygous lines show no phenotypic deviance from the wild type (Boij P, Patel R, Jarvis P, Aronsson H, unpublished data). A weak homologue of Tic55 in *Synechocystis* was suggested to have a role as a cell death suppressor (Mason and Cammack 1992).

8.2 Tic62

Tic62 has been identified partly on the basis of its co-purification with Tic55. This component shares homology with eukaryotic NAD(P)H dehydrogenases and Ycf39-like proteins in cyanobacteria and non-green algae (Küchler et al. 2002). Circular dichroism measurements show that Tic62 is composed of two structurally different domains (Stengel et al. 2008). The NAD(P)H binding site lies in the N-terminal part, while the stromal-facing C-terminal part interacts with a *f*erredoxin-*N*AD(P)$^+$ oxido*r*eductase (FNR). FNR normally mediates electron transfer, during oxygenic photosynthesis, from ferredoxin to NADP$^+$ at the thylakoids. The FNR-binding domain of Tic62 proteins is thought to be a relatively recent development in vascular plants, with no sequence similarity to other known motifs (Balsera et al. 2007); it may be crucial for its function in the TIC complex. Reagents that interfere with either NAD binding or have an effect on the ratio of NAD(P)/NAD(P)H influence the import of leaf-specific FNR isoforms differently, suggesting that Tic62 regulates import through redox sensing (Küchler et al. 2002). Localization of

Tic62 (partitioning between the stroma and the IEM), and its association with the TIC complex and FNR, are all influenced by redox status, including the $NADP^+$/NADPH ratio in the stroma (Stengel et al. 2008).

8.3 Tic32

The Tic32 protein shares homology with *s*hort-chain *d*ehydrogenase/*r*eductase (SDR) proteins, and behaves as an integral membrane component. It was reported to associate with several other TIC components after co-immunoprecipitation (e.g., Tic22, Tic40, Tic62 and Tic110), and is proposed to play a role late in the translocation process. Null mutations for one of two *Arabidopsis* Tic32 homologues appear to be embryo lethal, suggesting an essential role for the protein (Hörmann et al. 2004). Interestingly, import of preproteins with a cleavable TP was reduced in the presence of calcium or calmodulin inhibitors (Chigri et al. 2005); in contrast, the import of other proteins with no cleavable TP (Sect. 9.3) was not affected. This calcium regulation was suggested to occur within the IMS or at the IEM, possibly involving TIC components and calmodulin. A subsequent study using affinity chromatography found Tic32 to be the predominant IEM protein bound to calmodulin, and this interaction was calcium dependent (Chigri et al. 2006). In the same study, Tic32 was shown to have NADPH-dependent dehydrogenase activity; moreover, NADPH (but not NADH or $NADP^+$) affected the interaction of Tic32 with Tic110. Binding of NADPH and calmodulin to Tic32 were mutually exclusive processes, suggesting that Tic32 is involved in sensing and integrating redox and calcium signals at the TIC complex (Chigri et al. 2006).

9 Targeting to the Envelope System

9.1 Outer Envelope Membrane Targeting

Several mechanisms exist for protein targeting to the OEM (Hofmann and Theg 2005c). Nonetheless, many OEM proteins have intrinsic, non-cleavable targeting information, consisting of a hydrophobic transmembrane span adjacent to a C-terminal positive region; the latter prevents such proteins from entering the endomembrane system, since the former are similar to signal peptides for ER translocation (Lee et al. 2001). Recently, an *a*n*k*yrin *r*epeat protein (AKR2) was identified as a cytosolic mediator of OEM targeting (Bae et al. 2008; Bédard and Jarvis 2008). It acts by binding to the targeting signals of client proteins, preventing their aggregation, and by docking at the OEM surface. Interestingly, AKR2-deficient mutants have reduced levels of many chloroplast proteins, not only OEM proteins. The internal chloroplast defects in these mutants may be indirectly related to defective OEM

biogenesis, but it is interesting that AKR2 can bind to 14-3-3 proteins, since this raises the possibility that AKR2 is also a component of the "guidance complex" (May and Soll 2000). Another recent development indicates that the TOC channel protein, Toc75, is involved in OEM insertion (Tu et al. 2004).

Interestingly, Toc75 is unusual amongst OEM proteins, since it has a bipartite, cleavable targeting signal. The first part is a standard TP, while the second downstream part is an intraorganellar targeting signal that mediates "stop-transfer", release from the translocon, and membrane integration (Inoue and Keegstra 2003).

9.2 Inner Envelope Membrane and Intermembrane Space Targeting

Most proteins destined to the IMS or IEM have a cleavable TP. Information on targeting to the IMS is limited and based on MGD1 and Tic22, which are both located at the IEM and sit facing the IMS (Kouranov et al. 1999; Vojta et al. 2007). While MGD1 seems to use the TOC/TIC machinery for its translocation, it is less clear whether this is true for Tic22. Furthermore, the TP of Tic22, unlike that of MGD1, does not seem to be cleaved by SPP; instead, it is removed by an unidentified protease in the IMS. Thus, two pathways for IMS targeting seem plausible. Proteins of the IEM follow two routes: in the "stop-transfer" route, hydrophobic transmembrane domains induce lateral exit from the TIC machinery and membrane integration; in the "post-import" route, the preprotein first enters the stroma prior to second-step IEM integration (Li and Schnell 2006; Tripp et al. 2007).

9.3 Inner Membrane Targeting without a Transit Peptide

Unlike most proteins targeted to the IEM, Tic32 is imported without a cleavable TP. Instead, ten N-terminal amino acids hold the essential targeting information. Furthermore, neither recognition nor translocation of Tic32 involves the standard import components, Toc34, Toc159 and Toc75, and the energy requirement is low (<20-μM ATP), suggesting stromal chaperones are not involved. Crosslinking to Tic22 suggests that Tic32 may be assisted by Tic22 during its targeting through the IMS, prior to assembly into the IEM (Nada and Soll 2004).

Another protein targeted in a non-canonical manner is *c*hloroplast *e*nvelope *Q*uinone *O*xido*r*eductase *H*omologue (ceQORH). This protein does not carry a predicted TP, but still it is found in the IEM. In contrast with Tic32, the N-terminus of ceQORH is not needed for targeting. Instead, ~40 central residues are required for proper targeting. Like Tic32, its import is not mediated by the standard TOC components, but the energy requirement for import is higher for ceQORH than for Tic32 (Miras et al. 2007).

10 Alternative Import Pathways

Identification of multiple TOC receptor isoforms in *Arabidopsis* cast new light on the previous view of a standardized "general import pathway" (Sect. 4). Studies on *Arabidopsis* mutants for these receptors indicated the existence of different sub-pathways (e.g., photosynthetic versus non-photosynthetic, abundant versus non-abundant) (Bauer et al. 2000; Kubis et al. 2003), and it has even been proposed that cell-specific import pathways may operate (Yu and Li 2001). Whether different combinations of TOC components also attract different TIC complex combinations is an open question, although at least some key TIC components appear to be constitutive (Kovacheva et al. 2005). Bearing in mind that ~3,000 different proteins need to be imported, it is not surprising that variations on the basic import theme exist. Remarkably, it is now clear that chloroplast protein targeting is even more complex. Some proteins are targeted without cleavable TPs (Sect. 9), while others are transported via the endomembrane system.

10.1 Is There a Substrate-Dependent Import Pathway?

In chlorophyll biosynthesis, NADPH:protochlorophyllide oxidoreductase (POR) catalyzes the reduction of protochlorophyllide (Pchlide) to chlorophyllide in a strictly light-dependent manner. There are two main isoforms of POR in several species: PORA (responsible for reduction in illuminated, dark-grown material) and PORB (responsible for reduction in green material) (Aronsson et al. 2003b). It has been suggested that the import of PORA can only occur in the presence of its substrate, Pchlide. Pchlide accumulates in etioplasts but is found only in small amounts in chloroplasts. Thus, in barley, PORA was reported to be imported into etioplasts due to the presence of envelope-bound Pchlide, but not into chloroplasts (Reinbothe et al. 1995, 1997). This implies that a novel import pathway exists. However, this hypothesis has been challenged by several laboratories, since the import of PORA in various homologous systems, including *Arabidopsis* (Jarvis et al. 1998), pea (Aronsson et al. 2000, 2001) and wheat (Teakle and Griffiths 1993), was not dependent on Pchlide. Furthermore, barley PORA was also imported independently of Pchlide (Aronsson et al. 2000, Dahlin et al. 2000).

An explanation for these different results was offered by showing that the import of PORA in *Arabidopsis* was substrate-dependent in cotyledons only, and not in true leaves (Kim and Apel 2004). Such organ-specific import of PORA might help control the light-dependent transformation of a storage organ into a fully photosynthesizing leaf. More recently, putative components of the proposed PORA-specific import machinery have been identified (e.g., Toc33 and OEP16) (Reinbothe et al. 2004). However, these components have been demonstrated not to be of importance for PORA import in vivo (Kim et al. 2005; Philippar et al. 2007). Thus, the relevance of the substrate-dependent PORA import pathway model is unclear. Interestingly, the envelope translocation of a light-harvesting chlorophyll *a*/*b* binding protein

(LHCP) has also been proposed to be regulated by a pigment (chlorophyll) in *Chlamydomonas reinhardtii* (Eggink and Hoober 2000). Thus, pigments should not be excluded as possible factors for import.

10.2 Interactions with the Endomembrane System

Close associations between the *e*ndoplasmic *r*eticulum (ER) and the chloroplast envelope have been long established (Crotty and Ledbetter 1973). Recently, specific regions of the ER, termed the *pl*astid-*a*ssociated *m*embrane (PLAM), were identified as sites where a strong physical link exists (Andersson et al. 2007). Whether or not a vesicle transport system operates at or near these PLAM contacts remains to be established. Interestingly, indirect evidence for protein transport to chloroplasts through the ER has existed for some time, since glycoproteins and proteins with ER targeting signals have been identified inside plastids (Gaikwad et al. 1999; Chen et al. 2004; Asatsuma et al. 2005). While plastid protein transport through the ER is common in organisms with complex plastids with more than two envelope membranes (Nassoury and Morse 2005), it was only recently shown to exist in angiosperms.

The *c*arbonic *anh*ydrase 1 (CAH1) protein has an ER targeting signal and was detected in the chloroplast stroma. This protein cannot be imported directly by chloroplasts, but instead has to be imported into the ER first of all. Moreover, CAH1 is found in glycosylated form in the stroma, arguing that the transport pathway involves the Golgi apparatus where glycosylation occurs. Most likely, transport occurs through vesicles, since the inhibition of vesicle formation blocked further transport of CAH1 into chloroplasts (Villarejo et al. 2005). Like CAH1, the *n*ucleotide *p*yrophosphatase/*p*hosphodiesterase 1 (NPP1) protein has been shown to use an ER-to-chloroplast transport pathway (Nanjo et al. 2006). So far, no data on how these proteins enter the chloroplast exist. Perhaps vesicles fuse with the OEM to release their contents into the IMS, and then the proteins are translocated through the TIC complex or an unknown translocon; alternatively, onward transport may involve further vesicle formation at the IEM.

11 Conclusion

Substantial progress has been made in the last two decades. Most components of the canonical TOC/TIC machinery have been identified, and many of their individual roles have been at least partially characterized. Nevertheless, many questions still remain to be solved, as was discussed in each of the relevant sections above. Perhaps the most significant recent development has been the realization that the targeting of proteins to chloroplasts is not nearly as simple or as standardized as was once thought. It has become clear that multiple mechanisms operate to ensure

that the many diverse proteins that must be transported to chloroplasts arrive safely and without undue delay.

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