Chapter 12

The Ins and Outs of Chloroplast Protein Transport

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Summary

Much of the chloroplast proteome is encoded in the nuclear genome and needs to be imported post-translationally. Information for the organellar targeting of these imported proteins lies in an N-terminal leader sequence, the transit peptide, which is specifically bound by receptor components at the chloroplast surface. These receptor components are part of the TOC (*t*ranslocon at the *o*uter envelope membrane of *c*hloroplasts) complex, which, together with the TIC (*t*ranslocon at the *i*nner envelope membrane of *c*hloroplasts) machinery, mediates the translocation of precursor proteins into chloroplasts. Apart from the receptors, these complexes incorporate channel, motor and regulatory functions. Many components of this TOC/TIC apparatus have been identified. Multiple isoforms of the TOC receptors (and possibly of some other components) enable the operation of different import pathways with different substrate preferences, perhaps so that non-abundant proteins can be imported without serious competition from highly-abundant proteins of the photosynthetic apparatus. The different import pathways might also play a role in the differentiation of different plastid types. While much research has focused on these canonical TOC/TIC-mediated import routes, a number of studies have revealed alternative protein transport pathways to chloroplasts that employ different mechanisms; one of these passes through the endoplasmic reticulum and the Golgi apparatus. Other recent studies have revealed several protein targeting pathways leading to the envelope itself.

I. Introduction

A. Chloroplasts and Protein Import

Chloroplasts are the most prominent and intensively-studied members of a diverse group of organelles, the plastids, found ubiquitously in plants and a variety of algae (Whatley 1978; Keeling 2010). They contain chlorophyll and are responsible for the light and carbon reactions of photosynthesis, as well as many important biosynthetic functions (Nelson and Ben-Shem 2004; López-Juez and Pyke 2005). Other plastid types include the amyloplasts, which amass large quantities of starch and play important roles in energy storage and gravitropism, and the chromoplasts, which accumulate carotenoid

Abbreviations: aaRS – Aminoacyl-tRNA synthetase; AKR2A – Ankyrin repeat-containing protein 2A; APG1 – Albino or pale green mutant 1; BamA – β-barrel assembly machinery A; CAH1 – Carbonic anhydrase 1; ceQORH – Chloroplast envelope quinone oxidoreductase homologue; CIA2 (-5) – Chloroplast import apparatus 2 (-5); ClpC – Caseinolytic protease, subunit C; Com44/Cim44 – Chloroplast outer/inner membrane proteins, 44 kD; Cpn60 – Chaperonin, 60 kD; DEPC – Diethylpyrocarbonate; Fd – Ferredoxin; FNR – Ferredoxin-$NADP^+$ reductase; GAP – GTPase activating protein; GEF – Guanine nucleotide exchange factor; Hip – Hsp70-interacting protein; Hop – Hsp70/Hsp90-organizing protein; Hsp70 (-93, -100) – Heat-shock protein, 70 kD (93 kD, 100 kD); IDP – Intrinsically disordered protein; LHCII – Light-harvesting complex protein of photosystem II; MGD1 – Monogalactosyldiacylglycerol synthase 1; OEP–Outerenvelopeprotein,kD;PAGE–Polyacrylamide gel electrophoresis; PIC1 – Permease in chloroplasts 1; POTRA – Polypeptide transport associated; *ppi1* (*-2*, *-3*) – *Plastid protein import 1* (*-2*, *-3*); PreP – Presequence protease; SAM (Sam) – Sorting and assembly machinery; SP1 (*sp1*) – Suppressor of *ppi1* locus 1; SRP – Signal recognition particle; SSU (pSSU) – Rubisco small subunit (precursor of); Sti1 – Stress-inducible 1; Tat – Twin-arginine translocase; TIC (Tic) – Translocon at the inner envelope membrane of chloroplasts; TIM (Tim) – Translocase of the inner mitochondrial membrane; TOC (Toc) – Translocon at the outer envelope membrane of chloroplasts; TOM (Tom) – Translocase of the outer mitochondrial membrane; TPP – Thylakoidal processing peptidase; TPR – Tetratricopeptide repeat; TROL – Thylakoid rhodanese-like protein; VIPP1 – Vesicle-inducing protein in plastids 1

pigments and act as attractants in flowers and fruits (Neuhaus and Emes 2000; López-Juez and Pyke 2005).

Like mitochondria, chloroplasts evolved through endosymbiosis. They are believed to be descendent from an ancient photosynthetic prokaryote related to extant cyanobacteria (Larkum et al. 2007; Reyes-Prieto et al. 2007). While the modern organelle retains a fully-functional, endogenous genetic system, the organellar genome is greatly reduced and typically encodes just ~100 different proteins (Martin et al. 2002; Timmis et al. 2004). As a result, most chloroplast proteins must be imported from the cytosol. Because all plastids within a particular organism contain the same small set of organellar genes, it are the imported proteins that control the functions and developmental fate of each organelle (López-Juez 2007).

Approximately 3,000 different proteins are needed to develop a fully-functional chloroplast, and most (>90%) of these are encoded in the nucleus and synthesized on free cytosolic ribosomes (Keegstra and Cline 1999; Leister 2003). Typically, chloroplast proteins are made in precursor form, each one having an amino-terminal targeting signal called a transit peptide. These precursors, or preproteins, are then transported into the organelle in an energy-consuming, post-translational targeting process termed chloroplast protein import. Import is mediated by hetero-oligomeric protein complexes in the outer and inner envelope membranes called TOC and TIC (*t*ranslocon at the *o*uter/*i*nner envelope membrane of *c*hloroplasts), respectively (Fig. 12.1) (Soll and Schleiff 2004; Bédard and Jarvis 2005; Kessler and Schnell 2006; Inaba and Schnell 2008; Jarvis 2008; Li and Chiu 2010). The various components of the TOC and TIC complexes are discussed in detail in the following sections; for a list of the components, the reader is referred to Table 2 of Jarvis (2008). Chloroplast import is somewhat similar to mitochondrial protein import, which is mediated by the functionally analogous TOM and TIM (*t*ranslocase of the *o*uter/*i*nner *m*itochondrial membrane) complexes (Neupert and Herrmann 2007; Schmidt et al. 2010). In both cases, preproteins are threaded through the membranes in unfolded conformation. However, the main components of the chloroplast and mitochondrial protein import machineries are not closely related. Once a chloroplast preprotein reaches the organellar interior (the stroma), the transit peptide is removed, allowing the remaining part of the protein to fold into its functional conformation or engage one of several internal sorting pathways (see Sect. IV) (Jarvis and Robinson 2004; Gutensohn et al. 2006; Schünemann 2007; Cline and Dabney-Smith 2008; Jarvis 2008; Li and Chiu 2010).

B. Transit Peptides

Most nucleus-encoded proteins of the chloroplast interior require an amino-terminal transit peptide to gain access to the organelle (Bruce 2000, 2001; Armbruster et al. 2009). Transit peptides are functionally analogous to the cleavable, amino-terminal presequences that mediate protein import into mitochondria (Neupert and Herrmann 2007; Schmidt et al. 2010). While the mature part of a chloroplast preprotein can influence import efficiency (Dabney-Smith et al. 1999; Rial et al. 2002), it is the transit peptide that specifically interacts with the import machinery (Sveshnikova et al. 2000; Hinnah et al. 2002; Inaba et al. 2003; Smith et al. 2004). In fact, transit peptides are very effective at mediating the import of heterologous passenger proteins into chloroplasts (Schreier et al. 1985; Van den Broeck et al. 1985; Lee et al. 2006, 2009a).

In spite of the apparent specificity of the chloroplast import process, transit peptides are remarkably heterogeneous in relation to amino acid sequence and total length (Bruce 2000, 2001). They possess no readily discernable blocks of sequence conservation, and lengths vary from 20 to >100 residues. Superficially, their only common characteristics appear to be an abundance of hydroxylated residues (serine in particular) and a deficiency of acidic residues, giving them a net positive charge. In this respect, transit

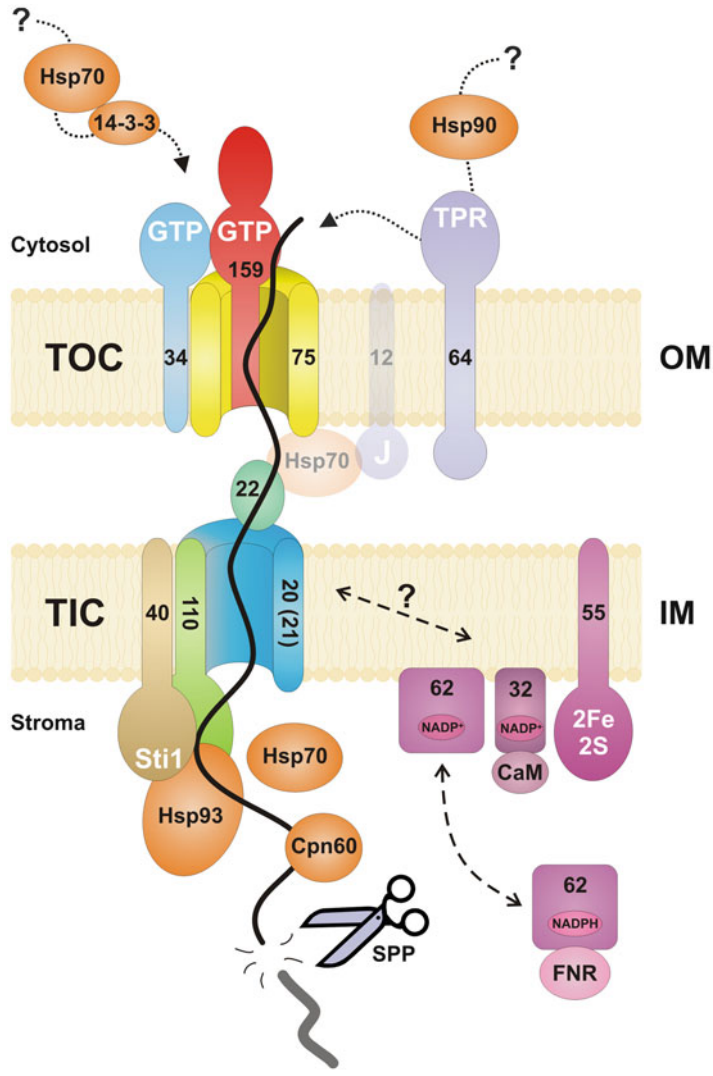

Fig. 12.1. The TOC and TIC complexes of the chloroplast protein import apparatus. An illustration showing the TOC and TIC translocons in the outer and inner envelope membranes (OM and IM, respectively). Individual components are identified by their molecular weights (*black text*), while some key functional domains are indicated (*white text*). Toc159, Toc34 and Toc75 together form the core TOC complex in the outer membrane. While Toc159 and Toc34 are responsible for preprotein recognition, Toc75 forms the outer envelope channel. The 14-3-3, Hsp70 and Hsp90 chaperones are proposed to interact with unfolded preproteins (forming so-called "guidance complexes") to maintain their import competence and direct them to the Toc34 or Toc64/OEP64 receptors. Tic22 is thought to provide a link between the TOC and TIC complexes, facilitating preprotein passage through the intermembrane space; however, the existence of Hsp70 and Toc12 in the intermembrane space is in doubt, as recent evidence indicates that both proteins are stromal. Inner envelope channels might be formed by Tic110 and/or Tic20/Tic21, or by cooperation between these components (as indicated). Tic110 also works together with Tic40 in the recruitment and regulation of stromal chaperones, such as Hsp93 and Hsp70; together, these components form a motor complex for protein import propulsion. SPP cleaves the transit peptide on the stromal side, while other chaperones (e.g., Cpn60 and Hsp70) facilitate protein folding or aid intraorganellar routing. A redox-regulator, comprising Tic32, Tic62 and Tic55, might be involved in fine-tuning the import process, working in conjunction with Ferredoxin-$NADP^+$ reductase (FNR) and calmodulin (CaM). (This figure has been adapted from Jarvis (2008).)

peptides are remarkably similar to mitochondrial presequences, and so it is not entirely clear how organellar specificity is achieved in plants (Macasev et al. 2000; Chew and Whelan 2004; Bhushan et al. 2006). In fact, a substantial number of preproteins are purposely "dual-targeted" to both chloroplasts and mitochondria (see Sect. V) (Silva-Filho 2003; Duchêne et al. 2005; Carrie et al. 2009), which clearly illustrates the functional similarities between the two types of targeting sequence.

Although mitochondrial presequences do not share a conserved consensus sequence, they do possess a characteristic secondary structure: they have the capacity to form amphipathic helices, and this is important for their interaction with receptors of the TOM complex (Brix et al. 1997; Abe et al. 2000). However, chloroplast transit peptides do not appear to possess secondary structure in aqueous solution (Krimm et al. 1999; Wienk et al. 2000). Instead, it has been suggested that they evolved specifically to have properties of a "perfect random coil", perhaps to aid interaction with cytosolic factors and/or the import machinery (von Heijne and Nishikawa 1991). It was recently reported that a minimal length of 60 N-terminal residues in unfolded conformation is required for efficient translocation, and, in cases where the transit peptide is shorter than this, the N-terminal part of the mature protein must also be unfolded (Bionda et al. 2010). An alternative possibility is that transit peptides adopt a characteristic structure only upon interaction with the outer envelope membrane, which is the only membrane containing galactolipids exposed to the cytosol (Krimm et al. 1999; Wienk et al. 2000; Bruce 2001). Indeed, transit peptides seem to interact strongly with artificial membranes containing chloroplast lipids in vitro (Bruce 1998), while an *Arabidopsis thaliana* mutant deficient in a chloroplast-specific galactolipid exhibited inefficient chloroplast protein import (Chen and Li 1998). Nonetheless, the role of envelope lipids in the import mechanism, if any, remains to be established.

Transit peptides have been dissected in a variety of mutational and deletion studies, leading to hypotheses that they possess a number of functional domains or motifs (Reiss et al. 1989; Pilon et al. 1995; Rensink et al. 2000; Lee et al. 2006, 2008, 2009a). However, no consensus of opinion has emerged from this work, and so the functional, defining features of a chloroplast transit peptide remain elusive. In spite of this, numerous computer programs are available that can be used to identify transit peptides with reasonable accuracy (Emanuelsson et al. 2007; Nakai and Horton 2007). Analysis of the Arabidopsis genome sequence using such tools yielded chloroplast proteome estimates ranging from ~2,000 to >4,000 proteins (Leister 2003; Richly and Leister 2004; Haas et al. 2005).

II. Events at the Outer Envelope Membrane

A. TOC Complex Composition

1. Identification of TOC Components

Attempts to find the envelope components involved in chloroplast protein import started in earnest about 20 years ago, and since then extensive biochemical studies have been performed with isolated *Pisum sativum* (pea) chloroplasts. In the early studies, thermolysin was found to be useful as it digests only those protein domains normally exposed to the cytosol at the chloroplast outer membrane. Significantly, this treatment can inhibit the ability of chloroplasts to bind precursors, which implied the existence of functional receptor proteins at the outer envelope surface involved in the import process (Cline et al. 1984, 1985; Friedman and Keegstra 1989). Quantitative analysis of precursor binding gave further evidence that chloroplast protein import is mediated by membrane-localized receptors, since the binding is saturable (Friedman and Keegstra 1989). There then followed attempts to isolate components of the protein import apparatus.

In the early 1990s, the core components were first detected in pea chloroplasts (Waegemann and Soll 1991; Perry and Keegstra 1994). It was not long before researchers from several laboratories actually identified the main proteins of the TOC and TIC complexes (Hirsch et al. 1994; Kessler et al. 1994; Perry and Keegstra 1994; Schnell et al. 1994; Wu et al. 1994; Seedorf et al. 1995; Lübeck et al. 1996). Among the earliest components identified, three are of the outer membrane and one is of the inner membrane. They are named according to their molecular weights, thus: Toc34, Toc75, Toc159 and Tic110 (Schnell et al. 1997). It should be noted that the Toc159 protein was initially identified as an 86 kD proteolytic fragment, termed Toc86. It was not until the *Arabidopsis thaliana* genome was sequenced that it was recognized that Toc86 is only a part of a much larger full-length protein (Bölter et al. 1998; Chen et al. 2000).

Among these first-identified components, Toc34 and Toc159 are both GTPases, while Toc75 possesses properties of a channel; these three components are all integral proteins of the outer envelope membrane. Precursor binding and outer envelope translocation are driven by a TOC complex comprising these three proteins, as was shown in an in vitro assay using a reconstituted translocation system in lipid vesicles (Schleiff et al. 2003a). Further analyses like density gradient centrifugation, gel filtration, and blue native PAGE confirmed that the TOC core complex consists of Toc34, Toc75 and Toc159, and showed that it was between 500 kD and 1 MD in size, in pea and Arabidopsis (Schleiff et al. 2003b; Kikuchi et al. 2006; Chen and Li 2007). Moreover, the stoichiometry of the TOC complex components was reported to be 4–5:4:1 (Schleiff et al. 2003b) or 3:3:1 (Kikuchi et al. 2006), between Toc34, Toc75 and Toc159, respectively. Differences between these stoichiometric estimates may be due to the dynamic nature of complex composition (Becker et al. 2004a), the formation of a TOC complex superdimer of 800–1,000 kD, the presence of additional, unidentified components (Kikuchi et al. 2006), the degradation of Toc159 (in the ~500 kD complex, Toc159 was present as the 86 kD fragment) (Schleiff et al. 2003b), or the application of different techniques. To date, there is still no consensus on the true constitution of the TOC complex.

The structure of the core TOC complex was elucidated by electron microscopic analysis, which revealed a toroid shape with a thick ring surrounding a central cavity, and a finger domain in the center which separates the central cavity into four pore-like structures (Schleiff et al. 2003b). It was speculated that each pore-like structure is made up of one Toc34 molecule and one Toc75 molecule, and that the central finger is formed by one Toc159 molecule; this corresponded to the proposed stoichiometry of 4–5:4:1. The complex particle was estimated to have a diameter of 13 nm and a height of 10–12 nm (Schleiff et al. 2003b).

As the identity of the main components of the TOC complex became clear (Fig. 12.1), researchers concentrated more on investigating the specific functions of individual Toc proteins. Since the completion of the genome sequencing project (Arabidopsis Genome Initiative 2000), Arabidopsis has increasingly been adopted as a model system for plant development and cell biology research. In the import field, this allowed in vivo studies to be performed (Jarvis et al. 1998; Bauer et al. 2000; Gutensohn et al. 2000), which led to a better understanding of the mechanisms of chloroplast protein import.

2. The Receptor Proteins, Toc34 and Toc159

The Toc159 and Toc34 proteins are integral membrane proteins of the outer envelope membrane, and both are substantially exposed to the cytosol. Each protein has a C-terminal membrane anchor and a homologous GTP-binding domain. They are responsible for preprotein recognition; both proteins can bind preproteins directly and thus they are considered to be receptors (Perry and Keegstra 1994; Sveshnikova et al. 2000; Smith et al. 2004).

a. Toc34

Toc34, together with Toc159, was first identified by its association with precursors bound to isolated pea chloroplasts (Kessler et al. 1994; Schnell et al. 1994). Toc34 has two domains: a GTPase domain which is exposed in the cytosol, and a very short, C-terminal hydrophobic membrane span for localization in the outer membrane. Crystal structure analyses have been performed on *P. sativum* Toc34 and on atToc33, a Toc34 homologue in *A. thaliana* (note that the "at" prefix denotes species of origin). An internal cavity in atToc33 was identified, which might act as an interaction site for precursor binding (Sun et al. 2002; Koenig et al. 2008). Importantly, these studies showed that Toc34 can homodimerize through its GTPase domain, a fact also suggested by gel filtration and pull-down assays (Sun et al. 2002; Weibel et al. 2003; Yeh et al. 2007). An interesting possibility is that Toc34 also heterodimerizes with Toc159, through interaction of the homologous GTPase domains; indeed, such Toc34-Toc159 interactions were detected in biochemical analyses (Bauer et al. 2002; Smith et al. 2002; Wallas et al. 2003) and in yeast (Aronsson et al. 2010), and were verified in planta (Rahim et al. 2009). In other GTPase systems, GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) are usually involved in the transitions between the active and inactive forms of the protein, by stimulating GTP hydrolysis and replacement of GDP with GTP (Bourne et al. 1991); however, so far no such factors have been identified in the chloroplast import system.

The dimerization of Toc34 revealed by the crystal structure suggested that each single Toc34 molecule within a dimer might act as a GAP on the interacting monomer (Sun et al. 2002). This is similar to the regulation that occurs between the signal recognition particle (SRP) and its receptor of the ER translocation system (Keenan et al. 2001). However, the consequences of TOC receptor dimerization have been tested, using mutants, with variable results (Weibel et al. 2003; Yeh et al. 2007). Weibel et al. found that atToc33-R130A abolishes dimer formation without changing the efficiency of GTP hydrolysis, while Yeh et al. observed that the same mutation leads to a significant decrease in GTPase activity. The latter study found that the dimerization property is influenced by protein sample aging in vitro, a phenomenon that was also observed by Koenig et al. (2008), and this might explain the differing results from the two groups. However, Koenig et al. observed only minor GTPase activation upon dimerization, which led them to propose that the homodimer requires an additional factor as a co-GAP. However, a more recent report suggested that Toc34 homodimerization limits the nucleotide exchange rate, instead of stimulating hydrolysis, and that preprotein binding disrupts the dimer in order to promote GDP-GTP exchange (Aronsson and Jarvis 2011; Oreb et al. 2011). This contrasted somewhat with earlier studies showing that preprotein binding strongly stimulates GTP hydrolysis (Jelic et al. 2002, 2003), which has led to the suggestion that transit peptides perform a GAP function (Reddick et al. 2007). Regardless of these inconsistencies, studies on dimerization-defective atToc33 point mutants in organello, using chloroplasts from transgenic Arabidopsis plants, showed that the dimerization is important for the initiation of the preprotein translocation process (Lee et al. 2009b). However, such mutations do not obviously affect chloroplast development in planta or plant growth (Aronsson et al. 2010). Thus, the exact function of Toc34 dimerization remains to be determined.

Functional analysis of Toc34 has also been performed with Arabidopsis mutants, showing its important role in plastid import in vivo. There are two Toc34 homologues in Arabidopsis, termed atToc33 and atToc34. The *plastid protein import 1* (*ppi1*) mutant, lacking atToc33, was the first protein import apparatus mutant to be found, and its analysis was significant as it illustrated that a translocon component identified by biochemical approaches is actually functional in vivo (Jarvis et al. 1998). Such *ppi1* mutants

show a striking chlorotic phenotype, altered chloroplast ultrastructure, and compromised protein import in vitro (Jarvis et al. 1998; Gutensohn et al. 2004). On the other hand, the atToc34 mutant, *ppi3*, shows no obvious phenotype in aerial parts, but significant growth defects have been observed in the roots (Constan et al. 2004a). Collectively, these data demonstrate that the Toc34 receptor plays a central role in plastid protein import. The functions of atToc33 and atToc34 are partially redundant, as indicated by the fact that the double mutation, *ppi1 ppi3*, is embryo lethal (Constan et al. 2004a; Hust and Gutensohn 2006), and by the demonstration that the *ppi1* phenotype can be recovered by the overexpression of atToc34 (Jarvis et al. 1998). However, it is also suggested that atToc33 acts more specifically in the import of precursors of the photosynthetic apparatus (so-called photosynthetic preproteins), whereas atToc34 is involved more in non-photosynthetic preprotein import (Kubis et al. 2003) (Fig. 12.2; discussed in detail in Sect. II.D).

b. Toc159

Like Toc34, Toc159 is also regarded as a receptor, but it has a more complex structure: in addition to the central GTPase (G) domain, which shares about 30% identity with that of Toc34, it possesses a large acidic (A) domain at the N-terminus, and a large C-terminal membrane (M) domain. The A-domain is extremely unstable and thus Toc159 was identified initially as an 86 kD fragment lacking the entire A-domain (Hirsch et al. 1994; Bölter et al. 1998). The function of the A-domain is unclear; Toc159 protein lacking the A-domain can efficiently complement the atToc159 knockout mutant (*ppi2*) phenotype in Arabidopsis (Lee et al. 2003; Agne et al. 2009, 2010), indicating that the A-domain is not essential in vivo. However, isolated chloroplasts with intact Toc159 did perform more efficient preprotein import than those in which Toc159 A-domain had been proteolysed (Bölter et al. 1998), indicating that this non-essential domain does play a role in the import process. A recent report suggested that the A-domain can exist in a free, highly-phosphorylated form in the cytosol, separate from the other Toc159 domains, although the biological significance of this is presently unclear (Agne et al. 2010).

The A- and G-domains of Toc159 are exposed to the cytosol. Toc159 was degraded to a 52 kD M-domain fragment after protease treatment of isolated chloroplasts, a treatment which removes the exposed parts of outer membrane proteins (Hirsch et al. 1994; Kessler et al. 1994; Chen et al. 2000). This also indicated that the M-domain is membrane-embedded. Interestingly, unlike other membrane-spanning protein domains, the Toc159 M-domain lacks a clear hydrophobic stretch and is rather hydrophilic. In vitro import experiments showed that preproteins can be efficiently imported into isolated chloroplasts even when Toc159 has been degraded to just the 52 kD M-domain (Chen et al. 2000). Moreover, using a protoplast transient expression system as well as transgenic Arabidopsis plants expressing a series of deletion mutants, the M-domain was found able to complement the import defect associated with the loss of Toc159, and to partially recover the mutant phenotype of *ppi2* plants (Lee et al. 2003). This suggests that the M-domain is the minimal domain required for Toc159 function.

Toc159 has been reported to exist in both soluble and membrane-bound forms (Hiltbrunner et al. 2001; Ivanova et al. 2004), implying that the receptor might bind preproteins in the cytosol and target them to the chloroplast membrane. However, whether this soluble form exists or is relevant is in doubt, as it has been reported that it is no longer found after higher-speed centrifugation is used to isolate Toc159-containing membranes (Becker et al. 2004a), and that it may in fact simply correspond to the free A-domain, as discussed earlier (Agne et al. 2009, 2010).

In Arabidopsis, the Toc159 receptor is encoded by a gene family with four members, and the corresponding proteins are termed atToc159, atToc132, atToc120 and atToc90

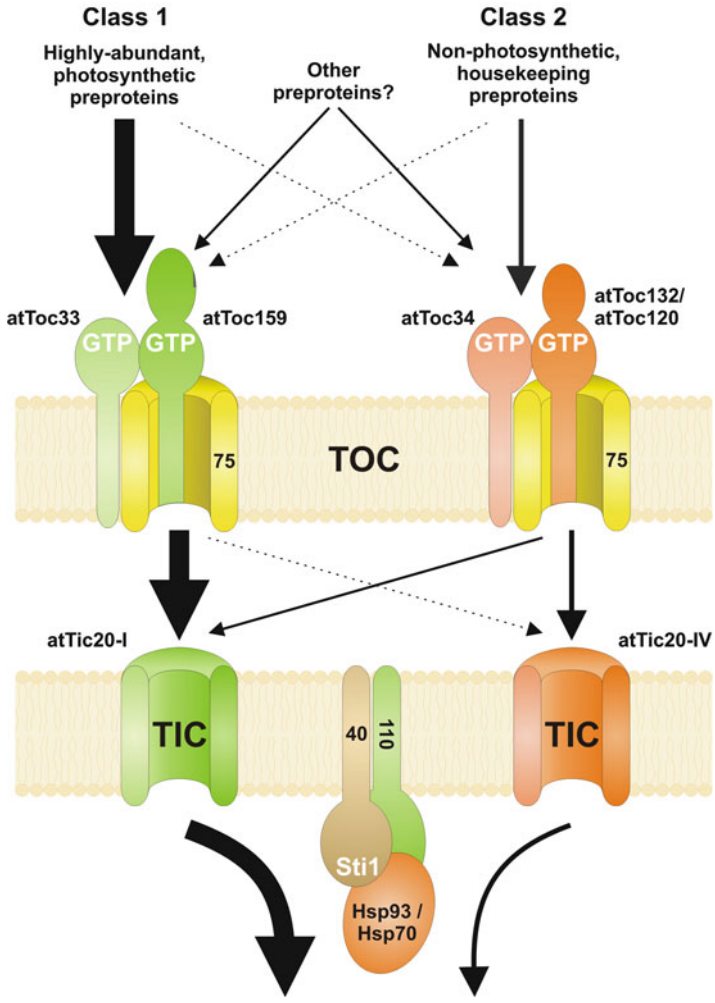

Fig. 12.2. Substrate-specific pathways for protein import into chloroplasts. Multiple isoforms of the TOC receptors exist in Arabidopsis and other species. These are believed to associate differentially to form distinct translocon complexes with different substrate (preprotein) specificities. The most abundant isoforms of the Toc159 and Toc34 receptors in Arabidopsis (atToc159 and atToc33, respectively) associate to form a TOC complex with specificity for highly-abundant, photosynthetic preproteins (*Class 1*). By contrast, the less abundant isoforms, atToc132/120 and atToc34, associate to form a different TOC complex with specificity for lower abundance, non-photosynthetic or housekeeping preproteins (*Class 2*). The *dotted crossing arrows* indicate that the aforementioned substrate specificities are not absolute, while there may even be a third class of preproteins that do not show a particular preference for either pathway. Recent evidence suggests that these different, substrate-specific import pathways may extend down to the level of the inner membrane. It is hypothesized that the two principal Arabidopsis isoforms of the putative channel protein, Tic20, form distinct TIC complexes with different properties: the atTic20-I complex having specificity for photosynthetic preproteins, and the atTic20-IV complex having specificity for non-photosynthetic proteins. A common motor complex, comprising Tic110, Tic40 and Hsp93/Hsp70, provides the driving force for translocation through both pathways. (This figure has been adapted from Jarvis (2008) and Hirabayashi et al. (2011).)

(Bauer et al. 2000; Ivanova et al. 2004; Kubis et al. 2004). The different isoforms share significant similarity in the G- and M-domains, but are rather divergent in the A-domain. Among them, atToc90 has only a truncated A-domain, while atToc132 and atToc120 are more closely related to each other than to atToc159 (Ivanova et al. 2004).

Genetic analyses showed that they do perform important roles in chloroplast protein import in vivo. The Arabidopsis atToc159 mutant, *ppi2*, possesses the strongest phenotype, and is albino due to severely disrupted chloroplast development (Bauer et al. 2000; Kubis et al. 2004). While the atToc132 and atToc120 single mutants have only weak or no mutant phenotypes, respectively, the double mutant exhibits an albino phenotype almost as severe as that of *ppi2*, indicating these two proteins together play an important role in chloroplast biogenesis (Ivanova et al. 2004; Kubis et al. 2004). Moreover, it has been proposed that atToc159 functions more specifically in photosynthetic protein import (like atToc33), and that atToc132/120 is involved more in non-photosynthetic protein import (like atToc34) (Fig. 12.2; discussed in detail in Sect. II.D). The function of atToc90 has not been fully clarified as yet, and its knockout mutant does not show any obvious mutant phenotypes (Hiltbrunner et al. 2004; Kubis et al. 2004). However, it seems that atToc90 is partially functionally redundant with atToc159; it was reported that the phenotype of *ppi2* can be partially recovered by the overexpression of atToc90, and that *toc90* knockout mutations can slightly enhance the phenotype of *ppi2* (Hiltbrunner et al. 2004; Infanger et al. 2010). It will be interesting to elucidate whether atToc90 is functionally related to atToc132/120 as well.

3. The Channel Protein, Toc75

Toc75 is generally regarded as the translocation channel at the outer envelope membrane, where it is the most abundant protein (Eckart et al. 2002; Vojta et al. 2004). It is deeply embedded within the outer membrane and possesses a typical β-barrel structure (Schnell et al. 1994; Tranel et al. 1995; Hinnah et al. 1997), as well as the ability to bind transit peptides directly (Hinnah et al. 2002). At the beginning, it was thought that the full length of Toc75 contributes to the β-barrel structure, but later it became clear that it has a more complex structure. Toc75 belongs to the conserved BamA (β-*b*arrel *a*ssembly *m*achinery A) family of proteins (Ertel et al. 2005; Gentle et al. 2005; Hsu and Inoue 2009) The BamA family can trace its origin to gram-negative bacteria, and it also exists in the mitochondria of eukaryotes. Like other family members, Toc75 can be divided into two parts: a C-terminal β-barrel domain as predicted previously, and an N-terminal domain with three POTRA (*po*lypeptide *tra*nsport associated) repeats (Sánchez-Pulido et al. 2003; Ertel et al. 2005; Gentle et al. 2005). The β-barrel domain contains 16–18 transmembrane strands for channel formation (Hinnah et al. 2002; Ertel et al. 2005), while the POTRA domain may be involved in preprotein recognition and/or complex assembly (Ertel et al. 2005). Using artificial lipid membranes, Toc75 was found to form a voltage-sensitive channel with a pore diameter of about 14–26 Å (Hinnah et al. 2002). Nonetheless, the exact topology of Toc75 remains unclear. Interestingly, a fraction of the Toc75 protein pool has been reported to exist in free form outside of TOC complexes (Kouranov et al. 1998), indicating that Toc75 may possess some functions beyond standard preprotein import (Tu et al. 2004). However, another report argued that such free Toc75 actually does not exist, or at least not in photosynthetic plastids (Kikuchi et al. 2006).

In the Arabidopsis genome, there are at least three Toc75-homologous sequences, *atTOC75-III*, *atTOC75-IV*, and *atTOC75-I*, which are named according to their chromosomal locations (Jackson-Constan and Keegstra 2001). In addition, there is a less related homologue, termed OEP80 (*o*uter *e*nvelope *p*rotein, 80 kD), formerly known as atToc75-V (Eckart et al. 2002; Inoue and Potter 2004). Based on sequence similarity and expression levels, atToc75-III is believed to be the major orthologue of pea Toc75, serving as the main channel of the Arabidopsis TOC complex. Its importance in Arabidopsis has also been verified in that *toc75-III* knockout mutations lead to embryo lethality (Baldwin et al. 2005; Hust and Gutensohn 2006). In fact, embryo development in *toc75-*

III null mutants is arrested at an extremely early stage, demonstrating the necessity of plastid protein import in early embryo development. Knockdown of *atTOC75-III* expression by RNA interference (RNAi) and a hypomorphic *toc75-III* mutant allele both produce plants with reduced chlorophyll content (Stanga et al. 2009; Huang et al. 2011), indicating an important role for atToc75-III in chloroplast biogenesis beyond embryogenesis. By contrast, atToc75-IV is expressed at very low levels and its knockout mutant does not show any obvious abnormal phenotypes under normal growth conditions. However, altered etioplast ultrastructure and a reduced de-etiolation efficiency indicated that atToc75-IV has some roles during dark growth of plants (Baldwin et al. 2005). The third homologue, *atTOC75-I*, proved to be a pseudogene with no expression, due to a gypsy/Ty3 transposon insertion (Baldwin et al. 2005).

Phylogenetic analyses suggested that OEP80 and Toc75 belong to distinct families, and it was for this reason (combined with a lack of information on its function) that the former was renamed from atToc75-V to OEP80 (Inoue and Potter 2004). OEP80 is not considered to be a TOC component, although it may be responsible for the insertion of β-barrel proteins (e.g., Toc75) into the outer membrane, with a similar function to its mitochondrial homologue, Sam50/Tob55 (Gentle et al. 2004; Inoue and Potter 2004; Huang et al. 2011). Paralleling the situation for atToc75-III, Arabidopsis *oep80* knockout mutations are embryo-lethal, indicating an important role for OEP80 in embryo development, while RNAi-mediated knockdown of *AtOEP80* expression causes chlorosis in plants (Patel et al. 2008; Huang et al. 2011). Differences in the stage of embryo arrest between the *toc75-III* and *oep80* knockout mutants (the latter abort considerably later in development), and in the severity of chlorosis in the knockdown lines (atToc75-III RNAi plants are much paler than AtOEP80 RNAi plants), may indicate a more specialized role for OEP80 (Patel et al. 2008; Huang et al. 2011).

4. Other TOC Components

More recently, Toc12 and Toc64 were identified as putative new components of the TOC complex. However, their exact roles in the import apparatus have yet to be determined.

Through proteomic studies of the outer membrane of pea chloroplasts and co-immunoprecipitation assays, Toc12 was identified as a new protein associated with the TOC apparatus (Becker et al. 2004b). It was described as an integral outer membrane protein with a large soluble part facing the intermembrane space. It is a DnaJ-like protein with a conserved J-domain that can interact with Hsp70 and enhance its ATP hydrolysis activity. The protein was shown to be associated with Toc64 and Tic22, and thus was proposed to form part of an intermembrane space complex acting as a "bridge" between the outer and inner membrane translocons. It was hypothesized that Toc12 facilitates translocation across the intermembrane space by stimulating an intermembrane space-localized Hsp70 (imsHsp70) (Qbadou et al. 2007). However, more recently, this hypothesis has been challenged by a study which demonstrated that the originally-found Toc12 in pea is actually a truncated form of a pea DnaJ-J8 protein (Chiu et al. 2011). In Arabidopsis, DnaJ-J8 seems to be a stromal protein with a transit peptide. Moreover, Arabidopsis *DnaJ-J8* T-DNA insertion mutants do not show any obvious defects in chloroplast protein import (Chiu et al. 2011). Further doubt is cast on the aforementioned TOC-TIC bridging model by the fact that a gene coding for the imsHsp70 has never been found in Arabidopsis (Ratnayake et al. 2008; Su and Li 2008). Thus, the mechanism of translocation through the intermembrane space remains to be established.

Toc64 was first identified in the isolated pea TOC complex after cross-linking (Sohrt and Soll 2000). It is suggested to dynamically associate with the complex, contrasting with the other stably-present core TOC components (Schleiff et al. 2003b). Topology studies showed that Toc64 is anchored in the outer membrane by three transmembrane

spans, thereby presenting a C-terminal TPR (*t*etratrico*p*eptide *r*epeat) domain to the cytosol and a central domain (with amidase homology) to the intermembrane space (Qbadou et al. 2007). In vitro biochemical studies indicated that these two domains may enable Toc64 to perform bipartite functions: the TPR domain might serve as a receptor for preproteins carried by the cytosolic factor, Hsp90 (see Sect. II.B) (Qbadou et al. 2006, 2007); the intermembrane space domain might assist the translocation of preproteins across the intermembrane space, together with other components (see previous paragraph on Toc12) (Qbadou et al. 2007). However, in vivo studies do not support the importance of Toc64 in protein import. There are three Toc64 isoforms in Arabidopsis: atToc64-III, atToc64-I and atToc64-V. The first of these was shown to localize in chloroplasts, and it shares the highest sequence identity with the original Toc64 isolate from pea (Chew et al. 2004); on the other hand, atToc64-I (AMI1) is a cytosolic protein acting as a typical amidase (Pollmann et al. 2003, 2006), while atToc64-V (mtOM64) is localized in the mitochondrial outer membrane, perhaps replacing a receptor of the TOM translocon, Tom70, that is found in yeast and mammals but not in plants (Chew et al. 2004; Lister et al. 2007). Surprisingly, even the triple mutant of Arabidopsis Toc64 homologues does not display any abnormal phenotypes, as judged by a variety of criteria (Aronsson et al. 2007), clearly showing that Toc64 is not essential for protein import in Arabidopsis. Moreover, knockouts lacking Toc64 in moss also do not present any obvious defects, with the possible exception of a slight deformity in chloroplast shape (Hofmann and Theg 2005b). However, because its presumed mitochondrial counterpart, Tom70 (the role of which is well established), is also not essential for cell survival in yeast (Hines et al. 1990), further work will be necessary before a final conclusion can be reached on Toc64 participation in import. It has been proposed that Toc64 should be renamed as OEP64 (*o*uter *e*nvelope *p*rotein, 64 kD) until its function has been clearly determined (Hofmann and Theg 2005b; Aronsson et al. 2007).

B. Cytosolic Factors

Protein translocation pathways into organelles can be grouped according to the use of two fundamentally different mechanisms: co-translational transport, which happens when translocation is closely linked to translation, as in SRP-dependent transport into the ER; and, post-translational transport, in which cytosolic factors may be required to assist precursor targeting to the organellar membrane, as in mitochondrial or peroxisomal import (Wickner and Schekman 2005). Since the import of chloroplast proteins is generally considered to be a post-translocation process, it is suggested that various cytosolic factors are involved. Several clues support this notion: the fact that precursors (especially of hydrophobic membrane proteins) produced in the cytosol are not in their final conformation and thus tend to aggregate or be degraded (Wickner et al. 1999; Lee et al. 2009c); the differing requirements for functionality of transit peptides between in vivo and in vitro conditions (Rensink et al. 1998; Lee et al. 2002); the differences in import behaviour seen in vitro when using precursors translated in rabbit reticulocyte or wheat germ lysates (May and Soll 2000; Schleiff et al. 2002). Indeed, in vitro evidence suggests that several chaperone proteins form "guidance complexes" that facilitate preprotein targeting to the chloroplast. However, the conditions under which such complexes exhibit significance in vivo remain to be established. Quite recently, the light-harvesting complex protein, LHCII, was reported to be translated near the border of chloroplasts in the green alga, *Chlamydomonas reinhardtii*, suggesting mRNA targeting combined with cotranslational transport as an alternative mechanism for protein import into chloroplasts (Uniacke and Zerges 2009).

1. Hsp70

Cytosolic Hsp70 (*h*eat-*s*hock *p*rotein, 70 kD) is one of the chaperones proposed to facilitate chloroplast protein transport. Interestingly, over 75% of chloroplast transit peptides are

predicted to contain at least one Hsp70 binding site (Rial et al. 2000). Direct interactions between Hsp70s and transit peptides have also been demonstrated both in vitro (Ivey et al. 2000; Rial et al. 2000; Zhang and Glaser 2002) and in vivo (Lee et al. 2009c). This supports the notion that the cytosolic chaperone is involved in keeping preproteins in an unfolded, competent form, which is important for protein import (Walker et al. 1996). However, the consequences of Hsp70 binding for protein import are still unclear. It has been shown that the unfolding process is not strictly linked with Hsp70 (Ruprecht et al. 2010). Moreover, recent evidence shows that a cytosolic Hsp70 in Arabidopsis can associate with accumulated cytosolic precursors that are targeted for degradation through the 26S ubiquitin proteasome system (Lee et al. 2009c); this indicates that the binding of Hsp70 might not simply escort the preproteins to the chloroplast membrane. Nonetheless, Hsp70 does seem to play a role in protein translocation in cooperation with other cytosolic factors, such as 14-3-3.

2. 14-3-3

The 14-3-3 family of proteins are regulatory molecules and chaperones that specifically bind to phosphorylated proteins to mediate a variety of signal transduction processes, as well as protein translocation (Gokirmak et al. 2010). Many transit peptides contain a phosphopeptide binding motif for 14-3-3 proteins. It was reported that 14-3-3 can form a "guidance complex" together with Hsp70 and preproteins, which can significantly increase in vitro import efficiency for certain phosphorylatable precursors (May and Soll 2000). The "guidance complex" containing 14-3-3 was also suggested to be important for determining the specificity of import to chloroplasts versus mitochondria in plants, since 14-3-3 cannot interact with plant mitochondrial preproteins (May and Soll 2000). However, mutation of the putative 14-3-3-binding phosphorylation site in transit peptides does not affect import efficiency and fidelity in vivo (Nakrieko et al. 2004; Lee et al. 2006), indicating that this "guidance complex" system is not essential.

Reflecting the unique problem faced by plant cells in differentiating between two different endosymbiotically-derived organelles, the protein import receptor components of mitochondria in plants are significantly different from those in other organisms (i.e., yeast or animals), as well as from those in chloroplasts (Macasev et al. 2000; Schleiff and Becker 2011). In spite of these receptor differences, some chloroplast preproteins can be efficiently imported into pea mitochondria in vitro, but not in vivo (Cleary et al. 2002). This indicates that special mechanisms must be utilized to achieve the specificity of import in vivo, and that components of such mechanisms are absent or inactive in vitro. Apart from the aforementioned 14-3-3 guidance hypothesis, another strategy that may be employed to achieve targeting specificity is transport of mRNA towards the destination organelle, such that preproteins are produced at the periphery of the correct organelles (Marc et al. 2002; Chew and Whelan 2004; Uniacke and Zerges 2009). However, there is no evidence yet concerning whether this is a general phenomenon for chloroplast protein import in plants.

3. Hsp90

In animals, Hsp90 and/or Hsp70 chaperones carry some preproteins to mitochondria via the Tom70 receptor (Young et al. 2003). Likewise, Hsp90 is proposed to deliver preproteins to chloroplasts in plants as part of another "guidance complex" (Qbadou et al. 2006, 2007). There are two major differences between the two plastidic "guidance complexes": one is that Hsp90 binds to preproteins that are not necessarily phosphorylated; the other is that, unlike 14-3-3, which carries preproteins directly to Toc34, Hsp90 makes use of Toc64 as the initial docking site and then afterwards passes the preproteins on to Toc34 (Qbadou et al. 2006). However, the precursor of the 33 kD subunit of the oxygen evolving complex, OE33, a protein which was shown to be transported to Toc64 by Hsp90 in vitro (Qbadou et al. 2006), is imported with normal efficiency into chloroplasts of *toc64* knockout mutants (Aronsson et al. 2007);

this indicates that the putative Hsp90-Toc64-based targeting mechanism is also not essential. Therefore, alternative systems differing from those described above might also be present, employing different components such as the newly-identified TPR-containing chaperone receptor, OEP61 (*o*uter *e*nvelope *p*rotein, 61 kD) (Kriechbaumer et al. 2011).

4. Actin

Recently, actin has been found to interact directly with Toc159 on the cytosolic side of the outer envelope membrane. In fact, many TOC/TIC components and VIPP1 (*v*esicle-*i*nducing *p*rotein in *p*lastids 1) were found associated with actin in a co-immunoprecipitation assay (Jouhet and Gray 2009a). It was proposed that this actin-TOC-TIC-VIPP1 complex may facilitate the trafficking of cytosolic preproteins to the thylakoid membrane (Jouhet and Gray 2009b). This is interesting, as the use of actin as a transport "highway" might well resolve the problems of targeting specificity and efficiency discussed above. However, myosin, the essential "motor" protein for actin-based motility (Ross et al. 2008), has not been found to interact with the chloroplast envelope (Jouhet and Gray 2009a), and so the significance of these observations remains to be established.

C. Models for Protein Translocation Through the TOC Complex

There are two main models for the mode of action of the TOC complex: the "targeting model" and the "motor model". The main difference between these two models is the role played by Toc159 in the translocation process.

The "targeting model" was originally proposed based on the finding that Toc159 exists in a free, cytosolic form as well as at the outer membrane. Based on this observation, cytosolic Toc159 was proposed to serve as a soluble receptor for preproteins, targeting them to the chloroplast surface (Hiltbrunner et al. 2001; Bauer et al. 2002; Smith et al. 2002, 2004). In the model, docking of Toc159 at the outer membrane is mediated by its interaction with Toc34, through their G-domains, and is dependent on GTP binding and hydrolysis activity (Bauer et al. 2002; Smith et al. 2002). Once docked, the GTPase activity of Toc159 and/or Toc34 was suggested to promote insertion of the preprotein into the channel protein, Toc75. Finally, Toc159 would become dissociated from the TOC complex to enable another targeting cycle. In this model, Toc159 plays two roles, as a preprotein carrier and in the triggering of translocation, while Toc34 acts as the docking site for the Toc159-preprotein complex. Evidence supporting the model includes: first, Toc159 was found to be the main component (along with Toc75) associated with preproteins during the early stages of import in cross-linking experiments (Perry and Keegstra 1994; Ma et al. 1996; Akita et al. 1997); second, the insertion of Toc159 into the membrane depends on the formation of a heterodimer with Toc34 (Bauer et al. 2002; Smith et al. 2002). However, as mentioned above (see Sect. II.A.2), the existence of the cytosolic form of Toc159 has been challenged (Becker et al. 2004a; Agne et al. 2009, 2010). Nonetheless, Toc159 may still act as a membrane-bound receptor in the model, as suggested by the fact that Toc159 can bind precursors at the envelope of isolated chloroplasts in import assays (Smith et al. 2004).

In the "motor model", membrane-bound Toc34 acts as the first receptor for the preprotein, and it dynamically associates with Toc159 in a manner regulated by its GTPase activity. By contrast, Toc159 plays a major role as a GTP-dependent motor in the translocation process (Schleiff et al. 2003a; Becker et al. 2004a). This model was suggested by experiments using the TOC translocon reconstituted into artificial membranes, which implied that Toc34 recognizes preproteins initially, while the minimal functional unit comprises just Toc159 and Toc75 (i.e., the motor and the channel) (Schleiff et al. 2003a; Becker et al. 2004a). This model is consistent with the stoichiometric and structural

analyses of the TOC complex described earlier (see Sect. II.A.1), which suggested that Toc159 is located in the center of the complex as a monomer, where it may serve as a catalytic motor and a docking site for four surrounding Toc34 molecules (Schleiff et al. 2003b). However, observations that Toc159 lacking the G-domain can still promote preprotein import (Chen et al. 2000; Lee et al. 2003) conflict with the proposed central role of Toc159 GTPase activity in this model. Moreover, recent studies on transgenic plants expressing Toc159 G-domain point-mutants imply that Toc159 acts more as a molecular switch than as a motor (Wang et al. 2008; Agne et al. 2009).

The two models differ considerably in detail. Nonetheless, it seems possible that what actually occurs in vivo shares elements with both models. For example, both receptors might be involved in the recognition and translocation processes of protein import, perhaps acting in a partially redundant way (Inaba and Schnell 2008; Jarvis 2008; Aronsson and Jarvis 2011). One feature that both models possess is a key role for GTP cycling at the receptors, which functions to control the interactions of the receptors and the preprotein, and to initiate or drive preprotein insertion into the channel. The importance of receptor GTP cycling has been shown by several G-domain mutation studies in vitro (Chen and Schnell 1997; Bauer et al. 2002; Wallas et al. 2003), and also suggested by the observation that the non-hydrolysable GTP analogues can inhibit the import process (Schnell et al. 1994; Young et al. 1999). However, the mechanism of import through the TOC apparatus may be more complicated, as indicated by recent studies which investigated the role of GTPase function in vivo (Wang et al. 2008; Agne et al. 2009; Lee et al. 2009b; Aronsson et al. 2010). Surprisingly, the transgenic expression of atToc159 mutants with defects in GTP binding and/or hydrolysis can efficiently complement the *ppi2* mutant phenotype (Wang et al. 2008; Agne et al. 2009); in addition, non-hydrolysable GTP analogues still strongly inhibit protein import in these transgenic plants, indicating that GTPases other than Toc159 are involved in the import process. Similarly, GTPase- and dimerization-defective mutants of atToc33 are effective at complementing the *ppi1* mutant phenotype (Lee et al. 2009b; Aronsson et al. 2010). The fact that G-domain-defective forms of both atToc159 and atToc33 retain in vivo functionality raised the possibility that the two GTPases might be partially redundant; however, complementation analysis shows that is not the case (Aronsson et al. 2010). An alternative explanation is that the presence of both receptor types is required to maintain the structural integrity of the complex, but that only one of the two receptors needs to have normal GTPase functionality in order for import to proceed (Aronsson et al. 2010). To better elucidate the functions of the receptors, additional experimentation will need to be performed, such as the mutation of the G-domains of both main receptors simultaneously.

D. Regulation of Import Through Substrate-Specific Pathways at the Outer Membrane

As mentioned above (in Sect. II.A.2), one of the notable characteristics of the TOC receptors in Arabidopsis is that they are encoded by small gene families, which is in contrast with the Toc75 channel protein (as it is generally regarded that only atToc75-III plays a significant role in canonical import pathways), and with most TIC components. In this regard, it is relevant that not only highly-abundant, photosynthetic preproteins need to be imported, but also lower abundance (but nevertheless essential) non-photosynthetic or housekeeping preproteins. Considering these facts, it is easy to imagine that import is organized by using separate TOC complexes with different substrate-specific receptors (Fig. 12.2), in order to ensure the efficient import of all proteins and avoid potentially damaging competition effects between precursors of widely differing abundances. Further significance of such specificity might be in controlling the differentiation of different plastid types; consistent with this notion, it

has been shown that while non-photosynthetic proteins can be imported into both chloroplasts and root plastids, their photosynthetic counterparts are generally more readily imported into chloroplasts (Wan et al. 1996; Yan et al. 2006). As detailed below, an accumulating amount of evidence has shown that such regulatory mechanisms do operate, and that different groups of receptors are responsible for importing specific preprotein substrates.

Firstly, the phenotypic analysis of Arabidopsis TOC receptor mutants indicated that the receptors may form two groups: one comprising atToc33 and atToc159, involved in the import of photosynthetic preproteins; and another comprising atToc34 and atToc132/120, involved in the import of non-photosynthetic, housekeeping preproteins (Jarvis et al. 1998; Bauer et al. 2000; Kubis et al. 2003; Constan et al. 2004a; Ivanova et al. 2004; Kubis et al. 2004). As mentioned earlier, the *ppi1* (or *toc33*) mutant has a striking chlorotic leaf phenotype, whereas *ppi3* (*toc34*) has only a specific defect in root development (Jarvis et al. 1998; Kubis et al. 2003; Constan et al. 2004a). Accordingly, *ppi2* (*toc159*) exhibits strong defects in chloroplasts and only mild defects in root plastids, while *toc132 toc120* exhibits the strongest phenotypes in root plastids (Yu and Li 2001; Kubis et al. 2004). Furthermore, it has been shown that atToc132 is involved in root gravitropism (Stanga et al. 2009).

Secondly, while atToc159 and atToc33 are highly expressed in leaves, the expression levels of atToc132, atToc120 and atToc34 are higher in roots. Moreover, in general, atToc159 and atToc33 are the dominant isoforms in their families (Kubis et al. 2003; Vojta et al. 2004). These tissue-specific expression patterns fit well with the proposed substrate specificities of the receptors, and also help to meet the huge demand for import capacity during the light-dependent biogenesis of highly-abundant photosynthetic proteins in chloroplasts.

Thirdly, transcriptomic and proteomic studies performed on the aforementioned TOC receptor mutants were consistent with the model. For example, the *ppi1* (*toc33*) and *ppi2* (*toc159*) mutants displayed down-regulated expression of photosynthetic genes/proteins, but relatively normal expression of non-photosynthetic genes/proteins (Bauer et al. 2000; Kubis et al. 2003, 2004; Kakizaki et al. 2009). These observations suggested that photosynthetic proteins could not be efficiently imported into plastids in these mutants, and that plastid-to-nucleus signaling pathways were consequently activated to prevent the futile expression of further organellar components that would be unable to reach their final destination.

Fourthly, a range of different experiments provided more direct evidence of specificity for different types of preprotein. For example, in vitro import assays using isolated *ppi1* chloroplasts showed that atToc33 is preferentially involved in the import of photosynthetic preproteins (Kubis et al. 2003). Moreover, in vivo targeting studies revealed similar functional preferences for atToc159 (Smith et al. 2004). In vitro pull-down experiments and functional assays supported the notion that this specificity is due to the preferential interaction of both receptors with photosynthetic precursors (Jelic et al. 2003; Smith et al. 2004).

Finally, co-immunoprecipitation data indicated that atToc159 and atToc132/120 exist in two distinct complexes. Furthermore, atToc159 exhibited preferential association with atToc33, while atToc132/120 was more prone to interact with atToc34 (Ivanova et al. 2004).

Although it seems that the specificity is related to the interaction between the transit peptide and the receptors (Smith et al. 2004; Yan et al. 2006), the mechanistic detail has yet to be clarified. No obvious defining features could be recognized upon examining the two transit peptide groups (Kubis et al. 2004; Vojta et al. 2004). However, some specific motifs in the transit peptide of the Rubisco small subunit precursor (pSSU) have recently been shown to be linked to the atToc159-dependent import pathway (Lee et al. 2009a). In addition, a recent report indicated that the Toc159 A-domain plays a significant role in defining

substrate-specific import pathways in vivo and in vitro (Inoue et al. 2010). Remarkably, transgenic overexpression of atToc132 lacking the A-domain (which may consequently lack selectivity), but not the full-length atToc132, can partially recover the phenotype of *ppi2* (*toc159*) Arabidopsis plants, indicating that the A-domain is responsible for selectivity. Interestingly, spectroscopic studies showed that the Toc159 A-domains display characteristics of intrinsically disordered proteins (IDPs), which usually function in highly dynamic protein-protein interactions (Richardson et al. 2009). However, the physiological significance of the Toc159 A-domain remains unclear, as it is not essential for plant development (Lee et al. 2003; Agne et al. 2009, 2010).

A recent work by Ling et al. (2012) has shed new light on how the protein import machinery is dynamically regulated to control the differentiation of different plastid types. A forward genetic screen in Arabidopsis identified a ubiquitin E3 ligase embedded in the plastid outer envelope membrane. This protein, termed SP1 (for suppressor of *ppi1* locus 1), was shown to mediate the ubiquitination of TOC proteins (particularly the receptors), thereby promoting their turnover by the ubiquitin-proteasome system (UPS). Mutant *sp1* plants were found to complete developmental transitions that involve plastid type interconversions (e.g., de-etiolation, when etioplasts transform into chloroplasts) inefficiently, implying an important role for SP1, the UPS, and the TOC machinery in governing organellar proteome changes. It was proposed that SP1 allows for the rapid replacement of TOC receptors of one type with different receptor isoforms, in order to accommodate and orchestrate changing protein import requirements (Ling et al. 2012).

Other regulatory mechanisms act to control the TOC components, in order to meet changing demands for protein import during different developmental stages and under different growth conditions. For example, the expression levels of atToc33 and pea Toc75 are much higher during early developmental stages (Tranel et al. 1995; Jarvis et al. 1998), presumably to fulfil the massive requirement for protein import at such times when chloroplast biogenesis activity is intense (Dahlin and Cline 1991). On the other hand, under temperature-stress conditions, import rates are down-regulated, which correlates with declining expression of TOC/TIC components (Dutta et al. 2009); this may serve to decelerate photosynthetic activity in order to avoid production of reactive oxygen species which might otherwise cause damage to the plant (Apel and Hirt 2004). However, the mechanisms underlying such regulatory expression changes are still largely unknown. To date, only one transcription factor with a role in chloroplast protein import has been identified: CIA2 (*c*hloroplast *i*mport *a*pparatus 2) was found to promote the expression of chloroplast translation components, and to regulate import by controlling the expression levels of certain TOC components, thereby helping to fulfil the vast demand for proteins in developing chloroplasts (Sun et al. 2001, 2009). Clearly, it will be of considerable interest to identify more regulators involved in chloroplast protein import in the future.

III. Events at the Inner Envelope Membrane

A. Overview of the Inner Membrane Translocation Machinery

While translocation through the outer envelope membrane is proposed to happen at multiple different TOC complexes, depending on preprotein specificity, it has generally been assumed that these different import pathways converge at the inner envelope membrane with a single TIC translocon (Kovacheva et al. 2005; Jarvis 2008). This translocon is composed of a channel, formed by Tic110 and/or Tic20 and Tic21, a motor complex comprising Tic110, Hsp93 and the Tic40 co-chaperone, and possibly also a redox-regulator with three components, Tic55, Tic32 and Tic62 (Fig. 12.1) (Jarvis 2008; Kovacs-Bogdan et al. 2010). Tic22 is

localized in the intermembrane space and is thought to link the TOC complex to the TIC complex, perhaps aiding formation of a TOC/TIC supercomplex. Such supercomplexes might form at contact sites between the outer and inner envelope membranes, where the distance from the chloroplast surface to the stroma is minimized and the import path is shortest (Schnell and Blobel 1993; Perry and Keegstra 1994); preproteins would then be able to pass through the outer and inner membrane channel components at the same time.

As soon as the transit peptide of a preprotein emerges from the TIC channel, it is bound and cleaved by the stromal processing peptidase (SPP), a zinc-binding metalloendopeptidase of the M16/pitrilysin family (Schnell and Blobel 1993; Richter and Lamppa 1998; Richter et al. 2005). Thereafter, the transit peptide is degraded and the mature protein is released and allowed to adopt its three-dimensional structure with the help of molecular chaperones (Jackson-Constan and Keegstra 2001), or engage downstream, intraorganellar targeting pathways (see Sect. IV) (Jarvis and Robinson 2004; Gutensohn et al. 2006; Schünemann 2007; Jarvis 2008). Evidence suggests that Cpn60 (*c*ha*p*eroni*n*, 60 kD) and Hsp70 are amongst the chaperones that facilitate protein folding or intraorganellar routing following import (Yalovsky et al. 1992; Madueño et al. 1993; Kessler and Blobel 1996).

Chloroplast inner envelope membranes are very similar to thylakoid membranes in terms of lipid composition. While outer envelope membranes are rich in phosphatidylcholine, like all extrachloroplastic membranes, inner envelope membranes as well as thylakoids contain much reduced amounts of phosphatidylcholine but increased amounts of monogalactosyldiacylglycerol (Block et al. 1983). Inner envelope membranes and thylakoids are developmentally related, and derive evolutionarily from the cyanobacterial chloroplast progenitor. Similarly, many components of the TIC machinery (namely, Tic20, Tic21, Tic22, Tic32, Tic55 and Tic62) have a cyanobacterial origin, and so are likely to have acquired new functions as they were recruited to the translocon (Reumann and Keegstra 1999; Reumann et al. 2005; Kalanon and McFadden 2008; Gross and Bhattacharya 2009). It has even been suggested that these components have retained their original functions in addition to their newer roles in protein import (Gross and Bhattacharya 2009); thus, Tic55 might be involved in chlorophyll *a* degradation, Tic32 and Tic62 might have metabolic dehydrogenase activities with specific substrates, Tic20 might be an ion channel, while Tic21 may function as an iron transporter (Gross and Bhattacharya 2009). In contrast, Tic110 has no cyanobacterial homologues and is therefore thought to have a eukaryotic origin. Its ubiquity amongst plastid-containing species suggests that it evolved very early on, and that it formed part of an ancient host-specific translocon together with Toc34 and Toc75. Tic40 is proposed to be a more recent acquisition, as it is less widely distributed, has eukaryote-derived co-chaperone activity, and seems to play an auxiliary role by accelerating the process of import (Kalanon and McFadden 2008; Gross and Bhattacharya 2009).

B. Energy Requirements and Different Stages of Translocation

Based on energetic requirements determined in vitro using isolated chloroplasts, protein import can be divided into several discrete steps. With an ATP requirement of 100 μM in the intermembrane space (along with a requirement for GTP, as used by the TOC receptors) (Olsen et al. 1989; Olsen and Keegstra 1992; Young et al. 1999), so-called "early import intermediates" are formed. Under such conditions, preproteins are inserted through the TOC complex and establish contacts with the intermembrane space exposed TIC components (Kouranov et al. 1998). In vitro, preproteins can be arrested at this stage by limiting ATP supply or by employing fully-energized chloroplasts at low temperatures (Leheny and Theg 1994); such manipulation enabled the further

subdivision of this early import intermediate step into three discrete sub-stages (Inoue and Akita 2008). After their emergence from the Toc75 channel, Tic22 might be one of the first points of contact for preproteins with the TIC machinery. Tic22 is a soluble protein with a largely unknown function, but it may be involved in guiding preproteins to the inner membrane channel (Kouranov et al. 1998). A putative intermembrane space HSP70 (imsHsp70) component was suggested to deliver the energy for progression to the early import intermediate step, stimulated by the J-domain co-chaperone component, Toc12 (Marshall et al. 1990; Becker et al. 2004b). However, as was discussed in Sect. II, the relevance of this hypothesis is in considerable doubt, as a gene for an imsHsp70 has never been identified (Ratnayake et al. 2008; Su and Li 2008), and Toc12 seems to be a truncated form of the stromal protein, DnaJ-J8 (Chiu et al. 2011). Thus, the question of how ATP is actually used in the intermembrane space remains unanswered.

Preproteins are subsequently threaded though the inner envelope membrane channel, composed of either Tic110 or Tic20/Tic21, or perhaps even a combination of these (Kouranov et al. 1998; Heins et al. 2002; Teng et al. 2006; Balsera et al. 2009). For complete translocation through the inner envelope channel, about 1 mM ATP is needed in the stroma (Pain and Blobel 1987; Olsen et al. 1989; Theg et al. 1989). This is thought to be used by stromal chaperones (heat shock proteins), such as Hsp93 and Hsp70, that cooperate with Tic110 and the Tic40 co-chaperone (Kessler and Blobel 1996; Akita et al. 1997; Nielsen et al. 1997b; Shi and Theg 2010). The mechanism of translocation has been suggested to be of a thermal ratchet type, with chaperones binding to freshly exposed parts of the translocating preprotein, thus preventing backward movement (Glover and Tkach 2001; Neupert and Brunner 2002; Kovacheva et al. 2005; Chou et al. 2006). In contrast with mitochondrial protein import, chloroplast import does not employ a transmembrane protonmotive force (Pain and Blobel 1987; Theg et al. 1989).

C. Translocation Through the Inner Membrane Channel

Tic110 is one of the most abundant components of the TIC apparatus (Vojta et al. 2004), and, based on electrophysiological studies, a role for the protein as a translocation channel in the inner membrane has been suggested (Heins et al. 2002; Balsera et al. 2009). This notion is supported by the fact that the single-copy gene in Arabidopsis is essential (as is the gene for the main TOC channel, atToc75-III), with the knockout mutants aborting at the globular stage with a raspberry-like embryo phenotype (Inaba et al. 2005; Kovacheva et al. 2005). Tic110 has been proposed to form a cation-selective, β-barrel channel with a pore diameter of 1.7 nm in liposomes in vitro (Heins et al. 2002). However, there has been considerable disagreement concerning the structural nature of this protein, as another investigation showed that it is composed primarily of α-helices, and that it is anchored in the inner membrane by two N-terminal hydrophobic transmembrane domains (Inaba et al. 2003). In the latter topology, a large hydrophilic domain is oriented towards the stroma and is thought to recruit Tic40 and stromal chaperones for the propulsion of preprotein import (Kessler and Blobel 1996; Inaba et al. 2003; Chou et al. 2006). A later study attempted to resolve these inconsistencies, and concluded that the large hydrophilic part contains four amphipathic helices that contribute to the channel (Balsera et al. 2009). Loops protruding into the intermembrane space might interact with Tic22 and TOC components to aid formation of TOC/TIC supercomplexes at contact sites.

Another component that has been suggested to form the TIC channel is Tic20 (Kouranov et al. 1998). It was originally proposed to share weak homology with bacterial amino acid transporters and mitochondrial Tim proteins; however, a careful analysis of consensus sequences recently concluded that Tic20 is not evolutionarily related to these other proteins (Kasmati et al. 2011). Tic20 possesses four α-helical transmembrane domains, similar to the

mitochondrial inner membrane channel components, Tim22 and Tim23 (Rassow et al. 1999; Kalanon and McFadden 2008; Kasmati et al. 2011). Tic20 has been found to interact with preproteins at a slightly later stage than Tic22 in cross-linking studies, and to be part of TOC/TIC supercomplexes (Ma et al. 1996; Kouranov and Schnell 1997; Kouranov et al. 1998). Antisense down-regulation of the main Tic20 isoform in Arabidopsis generated plants with defects in chloroplast biogenesis and protein import (Chen et al. 2002), while complete knockout mutants are albino and severely impaired in the import of photosynthetic preproteins (Teng et al. 2006; Kikuchi et al. 2009; Hirabayashi et al. 2011; Kasmati et al. 2011). In Arabidopsis, Tic20 is encoded by four genes that fall into two distinct groups based on sequence similarity and phylogenetic considerations (Kasmati et al. 2011). The atTic20-I and atTic20-IV proteins, together with the original Tic20 isolated from pea, are part of the main group with demonstrable importance in chloroplast biogenesis. It is proposed that atTic20-I is primarily responsible for the import of photosynthetic preproteins in shoots, and that atTic20-IV imports mainly non-photosynthetic preproteins in roots (Fig. 12.2) (Kikuchi et al. 2009; Hirabayashi et al. 2011). Double *tic20-I tic20-IV* knockout mutations are gametophytic- and embryo-lethal, indicating that atTic20-IV can partially compensate for the loss of atTic20-I in the *tic20-I* mutant (Hirabayashi et al. 2011; Kasmati et al. 2011); this demonstrates the partially redundant and essential functions of atTic20-I and atTic20-IV. In the second group, neither atTic20-II nor atTic20-V seems to be vital for chloroplast biogenesis, since *tic20-II tic20-V* double mutants are indistinguishable from wild type (Kasmati et al. 2011).

Interestingly, even though Tic20 is essential for protein import and plant viability, it appears to be much less abundant than the other putative or actual channel components in Arabidopsis, namely atToc75-III and atTic110 (Vojta et al. 2004). This suggests that Tic20 associates with only a subset of translocon complexes, and that its role might also be fulfilled by other components, such as Tic21, which has been proposed to perform a similar channel function during later development (Teng et al. 2006; Gross and Bhattacharya 2009). However, blue native PAGE analyses indicated that the Tic20 protein is present in a large, 1 MD complex together with Tic21 and preprotein (Kikuchi et al. 2009); Tic21 was only loosely associated with the complex, whereas Tic20 seemed to be a core component. Interestingly, Tic110 was not present in the 1 MD complex, and was instead present in a distinct, smaller complex of 200–300 kD. Thus, it was suggested that Tic20 together with Tic21 functions in a large channel complex (perhaps including other, unidentified components), whereas Tic110 instead acts later on in the import process, functioning in association with Tic40 and chaperones in a distinct motor complex (Fig. 12.2) (Kikuchi et al. 2009). Arabidopsis knockout mutants lacking either the main Tic20 isoform (atTic20-I) or Tic21 display similar defects in the import of photosynthetic preproteins, supporting the notion that the two proteins function together (Kikuchi et al. 2009).

Tic21 was identified as CIA5 (*c*hloroplast *i*mport *a*pparatus 5) in a screen for Arabidopsis plants defective in the import of a selectable marker into chloroplasts (Teng et al. 2006). The *tic21* knockout displays inefficient chloroplast protein import, with precursors seen to accumulate in the cytosol. The mutant has an albino phenotype, and so is viable only on an external carbon source. Interestingly, the *tic21 tic20-I* double mutant showed no additive phenotypic effects, relative to the single mutants, supporting the aforementioned hypothesis that Tic21 works in conjunction with Tic20 (Teng et al. 2006). It was suggested that Tic20 might play a channel role in early plant development, with Tic21 taking over the same function in later development (Teng et al. 2006); however, as already discussed, this idea is inconsistent

with the fact that the two proteins have been found together in the same complex (Kikuchi et al. 2009).

A protein called PIC1 (*p*ermease *i*n *c*hloroplasts 1) was described as a possible iron channel in the inner envelope membrane, and was found to be identical to Tic21 (Duy et al. 2007). Arabidopsis *pic1* mutants accumulated ferritin in the chloroplasts, which is a protein that binds iron in order to prevent iron loss or oxidative stress caused by free iron ions. Similarly, ferritin expression was up-regulated in the mutants, and other proteins related to iron stress and metabolism were differentially regulated. A yeast iron uptake defective mutant could be complemented with PIC1, further supporting a role of PIC1/Tic21 in iron transport across the inner envelope membrane (Duy et al. 2007). Consistently, plants overexpressing PIC1 accumulated free iron ions in the stroma, leading to oxidative stress, iron accumulation in flower tissue, and differential expression of genes associated with metal transport (Duy et al. 2011). It is known that protein components containing iron are important for chloroplast protein import, since diethylpyrocarbonate (DEPC), a chemical that inactivates iron-sulfur proteins, is reported to have a negative effect on protein import (Caliebe et al. 1997; Row and Gray 2001; Boij et al. 2009). Therefore, it is feasible that a block in iron import, and therefore in the biogenesis of iron-sulfur clusters, would affect protein import indirectly, similar to DEPC. However, it was found that genes related to iron homeostasis, encoding ferritin and copper superoxide dismutases, are not only up-regulated in PIC1/Tic21 mutants, but also in other pale mutants with defects in chloroplast biogenesis, such as *tic20-I* and *alb3* (Kikuchi et al. 2009). Thus, further investigation is required to determine whether disturbed iron homeostasis leads to diminished protein import, or diminished protein import leads to disturbed iron homeostasis. An alternative possibility is that the PIC1/Tic21 protein has a dual role, and that it acts in both of these processes (Gross and Bhattacharya 2009).

D. Import Propulsion at the Inner Envelope Membrane

A large portion of the C-terminal domain of Tic110 is oriented towards the stroma and has been reported to bind transit peptides as they emerge from the pore (Jackson et al. 1998; Inaba et al. 2003). Molecular chaperones are recruited to this Tic110 stromal domain, and these are believed to consume the energy, in the form of ATP, that is used to drive protein import at the stromal side, and to participate in the folding of newly-imported proteins (Kessler and Blobel 1996; Akita et al. 1997; Nielsen et al. 1997a; Chou et al. 2006). In mitochondria, it is well established that a matrix Hsp70 ATPase (mtHsp70) delivers the energy for preprotein translocation at the site of the inner membrane translocon (Neupert and Brunner 2002). However, even though chloroplast-localized Hsp70 does exist, it has generally been suggested that Hsp93 is the principal component of the TIC motor complex, since it associates with Tic110 (Akita et al. 1997; Nielsen et al. 1997a). The role of chloroplast Hsp70 (cpHsp70) has long been unclear, until recent studies clarified its function (Shi and Theg 2010; Su and Li 2010).

In Arabidopsis, knockouts of two cpHsp70 isoforms, *hsp70-1* and *hsp70-2*, were both shown to be defective in protein import (Su and Li 2008, 2010). Moreover, the phenotypes of *hsp70-1 hsp93-V* and *hsp70-1 tic40* double mutants were found to be more severe than those of the corresponding *hsp93-V* and *tic40* single mutants, respectively (Su and Li 2010) (note that the *hsp93-V* mutant lacks the main Hsp93 isoform in Arabidopsis; see below); this suggests that cpHsp70-1 has an important role in protein import propulsion, partially overlapping with the parallel system of Tic40/Hsp93. Since the *hsp70-1 tic40* genotype is lethal, whereas *hsp93-V tic40* causes only a pale phenotype, it seems that, in the *tic40* knockout background, cpHsp70-1

but not Hsp93-V becomes an essential and limiting factor for protein import propulsion. The cpHsp70-2 protein might be a minor isoform in Arabidopsis, partially redundant with cpHsp70-1 because the double knockouts are lethal (Su and Li 2010). Similarly, in moss, cpHsp70-deficient mutants displayed inefficient chloroplast protein import, as did another mutant with a deficiency in chloroplast-localized isoforms of the GrpE co-chaperone (Shi and Theg 2010). Related GrpE proteins promote nucleotide exchange at Hsp70 in prokaryotic systems, and play a well-established role in mitochondrial protein import in conjunction with mtHsp70 (Neupert and Brunner 2002). Moreover, immunoprecipitation studies showed that moss cpHsp70 associates with preproteins in a complex with Hsp93 and Tic40 (Shi and Theg 2010).

Hsp93, also named ClpC, is part of the Hsp100 family of molecular chaperones. Besides its function in protein import, it is also part of the Clp protease complex in chloroplasts, recognizing and unfolding substrate proteins that are destined for degradation (Shanklin et al. 1995). It forms hexameric rings in the presence of ATP, through which clients (either preproteins engaged in import, or other proteins targeted for degradation) may be threaded, thereby moving them towards their stromal destination or the Clp proteolytic core (Schirmer et al. 1996; Jackson-Constan et al. 2001). In Arabidopsis, two isoforms of Hsp93 exist, called atHsp93-V (ClpC1) and atHsp93-III (ClpC2). The former has a much higher expression level than atHsp93-III, and *hsp93-V* knockout mutants are pale and have a reduced protein import capacity, while *hsp93-III* knockout mutants are indistinguishable from wild type (Constan et al. 2004b; Sjögren et al. 2004; Kovacheva et al. 2005, 2007). Since *hsp93-III hsp93-V* double mutants are embryo lethal, and because the two proteins are very similar at the amino acid level (91% identical) (Kovacheva et al. 2007), these Arabidopsis homologues are believed to have largely redundant, overlapping functions, with atHsp93-V being the major isoform and atHsp93-III partially compensating for its loss in the *hsp93-V* mutant.

Tic40 is a homologue of the Com44/Cim44 protein originally identified in *Brassica napus*, and can be covalently linked to Tic110 via a disulfide bridge under oxidizing conditions (Stahl et al. 1999). It is anchored in the chloroplast inner envelope membrane by its N-terminal transmembrane domain, and projects a large hydrophilic C-terminal domain into the stroma, similar to Tic110 (Stahl et al. 1999; Chou et al. 2003). This stromal region contains a TPR domain through which it can interact with Tic110, as well as an Sti1-like co-chaperone domain of the type found in eukaryotic Hip/Hop co-chaperones (Chou et al. 2003, 2006; Bédard et al. 2007). It has been shown that Tic40 is in a complex not only with Tic110 but also with Hsp93, and that these three proteins all function at similar times in the import process, thus establishing a link between Tic110 and the energy-delivering chaperones (Chou et al. 2003). The current model suggests that Tic40 binds favourably to Tic110 when a transit peptide is bound to the stromal domain of Tic110 (Inaba et al. 2003; Chou et al. 2006). The transit peptide is then released from Tic110, upon binding of Tic40, and passed to hexameric Hsp93 which pulls the preprotein through the central pore. The Sti1 domain of Tic40 can stimulate the ATPase activity of Hsp93 and, thus, also the process of threading. Interestingly, the Sti1 domain of Tic40 can be functionally replaced in planta with the Sti1 domain of mammalian Hip (*H*sp70-*i*nteracting *p*rotein), for which an ATPase-stimulating function has never before been reported (Bédard et al. 2007).

Recently, it has been found that a variety of preproteins destined for the chloroplast inner envelope membrane (including Tic110, Tic21 and also Tic40 itself) accumulate as soluble, stromal intermediates in *tic40* mutant chloroplasts following in vitro import (Chiu and Li 2008). It has therefore been suggested that Tic40 has an additional function in the post-import re-insertion of certain

proteins that are destined to the inner envelope membrane (see Sect. IV) (Li and Schnell 2006; Tripp et al. 2007; Vojta et al. 2007b; Viana et al. 2010).

E. Redox Regulation of the TIC Machinery

A considerable body of literature indicates that the TIC translocon is regulated by the redox status of the chloroplast (Fig. 12.1) (Balsera et al. 2010). Light induces photosynthetic electron transfer which leads to an overall reduced state of the stroma during the day as NADPH accumulates. Consumption of NADPH at night leads to an oxidized stroma with a higher concentration of $NADP^+$. The import machinery might be directly regulated by the metabolic state of the stroma via the $NADP^+$/NADPH ratio (Stengel et al. 2009; Balsera et al. 2010). In maize chloroplasts, precursors of different isoforms of ferredoxin (Fd) and ferredoxin-$NADP^+$ reductase (FNR) were imported differentially under light or dark conditions: photosynthetic isoforms, pFdI and pFNRI, were equally imported in light- and dark-exposed chloroplasts, whereas non-photosynthetic isoforms, pFdIII and pFNRII, were mis-sorted to the intermembrane space under light conditions (Hirohashi et al. 2001). This suggests that the non-photosynthetic isoforms might interfere with photosynthesis and, therefore, that systems have evolved to prevent their import under light conditions.

Two proteins that associate with the TIC machinery and have the ability to bind NADPH, namely Tic32 and Tic62, have been described (Küchler et al. 2002; Hörmann et al. 2004). These components are thought to modulate protein import in response to changing $NADP^+$/NADPH ratios in the stroma. Tic32, a member of the family of short chain dehydrogenases, associates with Tic110 and with Tic40 at the stromal side of the inner envelope membrane, and has binding sites for both NADPH and calmodulin (Hörmann et al. 2004). Association of Tic32 with the TIC apparatus is dependent on the $NADP^+$/NADPH ratio, with Tic32 binding under oxidized conditions (high $NADP^+$/NADPH ratio) and dissociating from the TIC translocon under reduced conditions (low $NADP^+$/NADPH ratio) (Chigri et al. 2006; Kovacs-Bogdan et al. 2010). A reduction of protein import in the presence of ophiobolin A and ionomycin, which both disrupt calcium signaling, has been attributed to Tic32 (Chigri et al. 2005, 2006). Interestingly, calmodulin and NADPH binding to Tic32 are mutually exclusive, suggesting that a calcium signal is relayed via calmodulin only under oxidizing conditions when Tic32 is associated with the TIC apparatus (Chigri et al. 2006). The reported influence of calcium on Tic110 channel activity in vitro allows speculation that Tic32 delivers calcium via calmodulin directly to Tic110 under certain redox conditions in order to modulate protein import activity (Balsera et al. 2009).

Tic62 has several properties that are very similar to those of Tic32. It binds Tic110 and preproteins at the stromal side of the envelope, and has an N-terminal binding site for NADPH (Küchler et al. 2002). Its association with the TIC machinery is dependent on the $NADP^+$/NADPH ratio, with Tic62 being bound under oxidized conditions and dissociating under reduced conditions, just like Tic32 (Stengel et al. 2008). Like TROL (*t*hylakoid *r*ho*d*anese-*l*ike protein), which tethers FNR to thylakoids for the reduction of $NADP^+$ in the terminal step of photosynthetic electron transport, Tic62 possesses a C-terminal FNR-binding site (Küchler et al. 2002; Juric et al. 2009; Alte et al. 2010). Under reducing conditions in the stroma, Tic62 dissociates from the TIC apparatus, thereby increasing its affinity for FNR and leading to its preferential association with the thylakoids (Stengel et al. 2008). Interestingly, the Tic62-bound FNR appears not to be involved in photosynthetic electron transport even when bound to the thylakoids (Benz et al. 2009). Whether Tic62 has two distinct functions, one at the thylakoids and another at the TIC complex, or the capacity to relay thylakoid signals to the TIC translocon, is currently unknown (Benz et al. 2009).

Thioredoxins are small proteins that mediate the formation of disulfide bridges upon redox changes. Thioredoxin isoforms have been found associated with the inner envelope membrane, leading to the suggestion that the TIC machinery might be subject to thiol-based redox regulation (Ferro et al. 2003). N-ethylmaleimide, a compound that modifies cysteines and therefore interferes with thiol-based redox regulation, has been found to influence chloroplast protein import (Row and Gray 2001). Similarly, a variety of reducing agents such as glutathione and dithiothreitol can influence protein import, suggesting that regulatory disulfide bridges may exist within or between translocon components, including Toc159, Toc34 and Toc75 (Pilon et al. 1992; Seedorf and Soll 1995; Stengel et al. 2009). Hsp93, both from cyanobacteria and from higher plants, contains conserved cysteines with the potential to form intra- or intermolecular disulfide bridges (Mata-Cabana et al. 2007; Balsera et al. 2009); these may occur in the nucleotide binding domains of Hsp93, possibly leading to modulation of ATPase activity upon disulfide bridge formation (Balsera et al. 2009). Tic110 is able to form intramolecular disulfide bridges under oxidizing conditions, probably mediated by a stromal thioredoxin (Balsera et al. 2009). Disulfide bridges between Tic110 and Tic40 have also been found under oxidizing conditions (Stahl et al. 1999); Tic40 has only one cysteine at its C-terminus that might stabilize its binding to Tic110 and/or Hsp93 (Bédard et al. 2007; Balsera et al. 2010). Since these three components interact to form the motor complex, it can be speculated that disulfide bridges play a role in stabilizing or destabilizing complex formation under certain redox states in the chloroplast.

Tic55 was discovered in a complex with Tic110, and was found to associate with translocating preproteins along with other translocon components (Caliebe et al. 1997). It can bind to thioredoxins and contains some conserved cysteines that have the potential to form disulfide bridges (Bartsch et al. 2008). Additionally, Tic55 contains a Rieske-type iron-sulfur center. Rieske centers are known to be inhibited by DEPC, and it has been reported that DEPC treatment leads to a reduced import efficiency of pSSU; thus, Tic55 might be involved in the regulation of protein import via an electron transfer process, or act as a sensor of oxidative stress (Caliebe et al. 1997). However, knockout mutants of Arabidopsis atTic55-II, which is the orthologue of the originally-described pea Tic55, displayed neither visible abnormalities nor defects in chloroplast protein import (Boij et al. 2009). Moreover, the aforementioned negative effect of DEPC on protein import could also be observed in the *tic55-II* mutant, and so it can be concluded that, at least in Arabidopsis, DEPC has a chloroplast target different from Tic55 (Boij et al. 2009). Additional doubts over the participation of Tic55 in protein translocation were raised when two independent laboratories failed to detect the protein in import complexes (Kouranov et al. 1998; Reumann and Keegstra 1999).

F. Processing of Preproteins in the Stroma

Soon after the N-terminal part of the preprotein has emerged from the TIC machinery, the transit peptide is removed by the stromal processing peptidase (SPP), a metalloendopeptidase of the M16 family (other family members include subunit β of the mitochondrial processing peptidase, MPP, and *Escherichia coli* pitrilysin) (Vandervere et al. 1995; Richter and Lamppa 1998; Richter et al. 2005). SPP recognizes a stretch of basic residues with weak sequence homology or conservation of physicochemical properties near the C-terminus of the transit peptide (Emanuelsson et al. 1999; Richter and Lamppa 2002; Rudhe et al. 2004). It cleaves the transit peptide from the mature sequence using the catalytic activity of its zinc-binding domain, and then an additional proteolytic step releases transit peptide fragments from SPP; these are then degraded by a presequence protease termed PreP (Richter and Lamppa 2002, 2003; Moberg et al. 2003).

The SPP protein is evolutionarily well conserved, as related sequences are found in algae and malaria parasites (Richter et al. 2005). An ancestral activity was probably inherited with the original endosymbiont, as SPP-related sequences even exist in cyanobacteria. Interestingly, SPP is encoded by a single-copy gene in Arabidopsis, and so it must be able to bind to a wide range of transit peptides with highly variable sequences (Richter and Lamppa 1998; Bruce 2001; Jarvis 2008).

Antisense-mediated down-regulation of *SPP* gene expression in Arabidopsis or tobacco plants led to a variety of abnormal phenotypes, ranging from albinism to seedling lethality (Wan et al. 1998; Zhong et al. 2003). Chloroplasts of the antisense lines displayed abnormal ultrastructure and were less abundant than those in wild-type cells (Wan et al. 1998; Zhong et al. 2003). Similarly, in rice, a point mutation affecting a conserved glutamate residue of SPP caused visible chlorosis associated with small, abnormal chloroplasts (Yue et al. 2010). Interestingly, chloroplast protein import efficiency was compromised in the SPP antisense lines (Wan et al. 1998; Zhong et al. 2003); this might reflect the fact that most TIC components and Toc75 possess a transit peptide, and so rely on SPP for their correct maturation, or indicate that transit peptide cleavage is an integral component of the chloroplast import mechanism. Complete loss of SPP in Arabidopsis knockout mutants led to embryo abortion at the 16-cell stage, further emphasizing the importance of transit peptide cleavage for organelle development and plant growth (Trösch and Jarvis 2011).

IV. Intraorganellar Protein Transport Pathways

A. Internal Sorting of Plastid Proteins

Chloroplasts are complex organelles comprising several distinct suborganellar compartments; as a consequence, the internal routing of chloroplast proteins is necessarily a complex process. While envelope proteins may utilize variations of the TOC/TIC import pathway to reach their final destination (see Sects. IV.B and IV.C below), proteins destined for the thylakoid membrane or lumen additionally employ one of four distinct targeting pathways. In spite their undoubted importance, these thylakoidal pathways are beyond the scope of this chapter, and so the following paragraph provides a brief overview only, for the sake of completeness. Readers are referred to the following reviews for more detailed information (Jarvis and Robinson 2004; Gutensohn et al. 2006; Schünemann 2007; Cline and Dabney-Smith 2008).

Proteins destined for the thylakoid lumen engage the Sec ("Secretory") and Tat (*t*win-*a*rginine *t*ranslocase) pathways, and possess an additional cleavable targeting signal, just downstream of the transit peptide. Unlike transit peptides, such lumenal targeting peptides are similar to the signal peptides that mediate inner membrane transport in bacteria; they are removed by a type I signal peptidase termed thylakoidal processing peptidase (TPP). The Sec pathway is powered by ATP hydrolysis at the SecA motor protein, and accepts only unfolded proteins. By contrast, the Tat pathway uses the thylakoidal proton gradient as its energy source, and is able to deliver folded proteins; the importance of this pathway may relate to the transport of proteins that acquire their final conformation (through co-factor binding or oligomerization) in the stroma. On the other hand, thylakoid membrane proteins utilize either the SRP-dependent pathway or the so-called "spontaneous" insertion pathway. The former consumes GTP as a result of a critical interaction between the SRP and its membrane receptor (both are GTPases), and is mainly concerned with the insertion of polytopic light-harvesting complex proteins. The "spontaneous" pathway, however, seems to proceed without energy consumption or the involvement of a proteinaceous transport machinery.

In contrast with the TOC/TIC pathway, at least three of these thylakoidal pathways are closely related to protein transport systems in bacteria, which gives rise to the concept of conservative sorting: i.e., the transport of nucleus-encoded, thylakoid proteins occurs in two sequential, independent translocation steps, at the envelope and at the thylakoids, with the latter having been retained (or conserved) from the prokaryotic ancestor of the organelle. Recent evidence suggests that similar conservative sorting may operate at the level of the inner envelope membrane (see Sect. IV.C).

B. Sorting to the Outer Envelope Membrane

Most outer envelope membrane proteins do not possess a transit peptide; rather, they are directed to the membrane by intrinsic targeting information. There are several different mechanisms for targeting to the outer membrane (Hofmann and Theg 2005c), and the most prominent of these is probably that used by proteins such as OEP7/14 (*o*uter *e*nvelope *p*rotein, 7/14 kDa) and Toc64/OEP64. Targeting information in such proteins lies within an amino-terminal transmembrane domain, which bears superficial resemblance to signal peptides for ER translocation (Lee et al. 2001; Hofmann and Theg 2005c). Adjacent to the transmembrane domain there is a charged region that seems to play a crucial role in differentiating these proteins from those destined for the ER. Despite initial suggestions that such proteins insert "spontaneously" into the membrane, it now seems clear that they utilize a proteinaceous import machinery and consume nucleoside triphosphates during insertion (Tu and Li 2000; Hofmann and Theg 2005a). In fact, competition studies (with preproteins possessing transit peptides), and cross-linking results, revealed that Toc75 is employed during outer membrane insertion (Tu et al. 2004); the relevant Toc75 may correspond to a fraction that is disassociated from other TOC components (Kouranov et al. 1998). Toc75 involvement in outer membrane insertion parallels the situation in mitochondria, where the TOM channel, Tom40, is similarly employed (Rapaport 2005). More recently, the cytosolic protein, AKR2A (*ank*yrin *r*epeat-containing protein 2A), was identified as a cytosolic sorting factor in this targeting pathway (Bae et al. 2008; Bédard and Jarvis 2008). The AKR2A protein is proposed to act as a chaperone, preventing aggregation of client proteins and guiding them to the envelope membrane. Interestingly, AKR2A was also reported to be involved in the insertion of a peroxisomal membrane protein (Shen et al. 2010), suggesting that it may be important for the targeting of a broad class of membrane proteins (Zhang et al. 2010).

Targeting of Toc34 is also mediated by an intrinsic signal, but in this case the relevant transmembrane domain is situated at the C-terminus (i.e., it is a tail-anchored protein). As with OEP7/14 and similar proteins, insertion appears to require both envelope proteins and an energy source (Tsai et al. 1999). Moreover, competition studies suggest that Toc34, OEP7/14 and Toc64/OEP64 may all employ the same insertion mechanism (Tu and Li 2000; Hofmann and Theg 2005a, c). Toc34 insertion has been reported to depend on previously-inserted Toc34, as well as on membrane lipids, and to follow a different pathway from that used by another tail-anchored protein, OEP9 (a 9 kD outer envelope protein of unknown function) (Qbadou et al. 2003; Dhanoa et al. 2010). Quite a different targeting mechanism is employed by the Toc159 protein, a fact which may be related to its large, atypical M-domain. Membrane insertion of Toc159 is thought to involve a homotypic G-domain interaction with Toc34 already integrated into the membrane, as well as the channel protein, Toc75 (Bauer et al. 2002; Smith et al. 2002; Wallas et al. 2003). The G-domain interactions and protein insertion via this pathway are controlled by GTPase cycling at the receptors. That the M-domain alone could associate with the

outer membrane in vivo suggests that some targeting information must reside in this domain (Lee et al. 2003). As there are no typical transmembrane spans within the M-domain, its association with the membrane is most likely dependent on the TOC complex, although a short hydrophobic segment near the C-terminus could interface with the core of the lipid bilayer (Inaba and Schnell 2008).

Toc75 employs an even more unusual mechanism for membrane insertion. This protein possesses an unusually large, bipartite targeting signal: the N-terminal domain is a standard transit peptide, while the C-terminal domain mediates intraorganellar targeting (Tranel et al. 1995; Tranel and Keegstra 1996). The second domain contains a poly-glycine stretch that arrests translocation so that the preprotein can disengage from the translocon and undergo membrane integration (Inoue and Keegstra 2003). The transit peptide domain is cleaved by SPP as normal, whereas the second domain is removed by a TPP-related type I signal peptidase that resides in the envelope as well as in the thylakoids (where it additionally plays a role in the maturation of thylakoidal proteins) (Inoue et al. 2005; Shipman and Inoue 2009; Shipman-Roston et al. 2010). How Toc75 becomes integrated into the outer membrane following maturation is unclear. Insertion and topogenesis of similar β-barrels in the mitochondrial outer membrane is mediated by the sorting and assembly machinery (SAM) (Neupert and Herrmann 2007; Schmidt et al. 2010). At the core of the SAM complex is the BamA homologue, Sam50, and it is hypothesized that a related protein may have an analogous role in chloroplasts, with one candidate being OEP80 (Eckart et al. 2002; Inoue and Potter 2004; Gentle et al. 2005; Patel et al. 2008).

C. Sorting to the Intermembrane Space and Inner Envelope Membrane

Most proteins destined for the intermembrane space or inner envelope membrane possess a cleavable, amino-terminal targeting sequence. Thus, targeting to these destinations is quite different from outer membrane insertion.

Targeting to the intermembrane space has been studied for two components, Tic22 and MGD1 (*m*onogalactosyl*d*iacylglycerol synthase 1), which employ different targeting pathways to reach their destinations (Kouranov et al. 1999; Vojta et al. 2007a). While both proteins have a targeting sequence, only that of MGD1 seems to be cleaved by SPP; together with the energetic requirements for its import, this indicates that MGD1 partially enters the stroma. By contrast, Tic22 is cleaved by an as yet unknown protease in the intermembrane space, implying that it does not pass through the TIC channel; in fact, there is even uncertainty over the involvement of the TOC machinery during Tic22 transport.

For targeting to the inner envelope membrane, two pathways exist. In the stop-transfer pathway, proteins do not completely enter the stroma; instead, a hydrophobic transmembrane domain arrests translocation in the channel, leading to lateral release of the protein into the membrane (Brink et al. 1995; Knight and Gray 1995; Tripp et al. 2007). This pathway might be especially important for polytopic proteins that are prone to aggregation, such as the triose phosphate/phosphate translocator. By contrast, in the post-import pathway, proteins insert into the inner envelope membrane only after complete translocation into the stroma, where they form a soluble intermediate (Lübeck et al. 1997; Li and Schnell 2006; Tripp et al. 2007). Similar mechanisms operate in mitochondria, where targeting to the inner membrane employs stop-transfer and conservative sorting pathways (Neupert and Herrmann 2007); the latter pathway is analogous to the post-import pathway, and its name is a reference to the fact that it employs machinery that is at least partly of prokaryotic origin.

For both pathways, stop-transfer and post-import, a cleavable transit peptide is required, implying initial involvement of the TOC/TIC

apparatus (Knight and Gray 1995; Lübeck et al. 1997; Stahl et al. 1999). Recent studies on the inner envelope protein, APG1 (*a*lbino or *p*ale *g*reen mutant 1), which employs the stop-transfer pathway, revealed that membrane targeting information lies exclusively in the transmembrane domain, and that this domain alone is sufficient to direct stop-transfer insertion (as opposed to the use of the post-import route), even in the context of heterologous passenger proteins (Viana et al. 2010).

Tic40 and Tic110 have been used to study the post-import pathway (Li and Schnell 2006; Tripp et al. 2007). Both proteins are anchored in the inner membrane by N-terminal transmembrane spans, projecting large C-terminal domains towards the stroma. During transport, they form post-SPP intermediates lacking the transit peptide in the stroma (Inaba et al. 2005; Bédard et al. 2007). Actually, Tic40 possesses a bipartite targeting signal, although the role of the second domain is uncertain, as a serine/proline-rich region of the mature protein and the adjacent transmembrane domain seem to control membrane insertion (Li and Schnell 2006; Tripp et al. 2007). The former may interact with the latter to form a membrane insertion loop, while in Tic110 a similar structure may be formed by the two, closely-juxtaposed transmembrane helices. Efficacy of the targeting signals in Tic40 is dependent upon context within the protein sequence, suggesting that post-import pathway signals are complex, which is perhaps necessary to avoid stop-transfer insertion (Viana et al. 2010). Stromal events in the post-import pathway may be assisted by the Hsp93 chaperone (Vojta et al. 2007b), while reinsertion is dependent on proteinaceous membrane components of unknown identity (Li and Schnell 2006). Bearing in mind the previously-described conservative sorting pathways at the thylakoids and in mitochondria, it is intriguing that a second Sec translocase (in addition to the well-characterized thylakoidal system) was recently identified in chloroplast envelopes (Skalitzky et al. 2011). Evidence has also been presented that resident Tic40 (and possibly also Tic110) plays a role in inner membrane protein insertion (Inaba et al. 2005; Chiu and Li 2008).

V. Dual-Targeting and Non-canonical Protein Transport to Chloroplasts

A. Dual-Targeting of Proteins to Chloroplasts and Other Organelles

While the majority of chloroplast proteins are targeted quite specifically to plastids, in recent years it has become increasingly apparent that a significant number of proteins make their way to more than one destination (Peeters and Small 2001; Silva-Filho 2003; Mackenzie 2005; Carrie et al. 2009). Transport to both chloroplasts and mitochondria is the most common form of dual-targeting (with ~50 proteins having been reported to do this), but there are also proteins that reside in the nucleus, ER or peroxisomes as well as in chloroplasts (Levitan et al. 2005; Sapir-Mir et al. 2008; Krause and Krupinska 2009). The prevalence of such multi-destination transport suggests that protein targeting is not as inflexible as was once thought, and has been taken as evidence in support of a hypothesis that accounts for the evolutionary relocation of organellar genes to the nucleus, a model which depends on the "minor mistargeting" of large numbers of proteins to multiple destinations (Martin 2010).

Although there are exceptions (Ueda et al. 2008), dual-targeting to chloroplasts and mitochondria typically involves one of two different mechanisms (Peeters and Small 2001). In the first of these, alternative transcript splicing and/or differential transcriptional or translational initiation is employed to produce proteins that possess different N-terminal leader sequences with distinct targeting properties. Alternatively, a single mRNA may be produced encoding a single protein which possesses an ambiguous leader

sequence, that is competent for import into both chloroplasts and mitochondria. The functions of dual-targeted proteins include DNA and RNA synthesis and processing, protein synthesis, and cellular stress response (Mackenzie 2005; Carrie et al. 2009). The most striking example of dual-targeting occurs amongst the aminoacyl-tRNA synthetases (aaRSs), where 17 of the 24 organellar proteins in Arabidopsis are targeted to both chloroplasts and mitochondria (Duchêne et al. 2005); only two are uniquely chloroplastic. Plant cells originally inherited three aaRS genes, one from each of the three ancestral genomes (nuclear, mitochondrial and plastidic). Remarkably, there are no examples in Arabidopsis where all three still coexist, indicating that extensive exchange and loss of aaRS genes has occurred during evolution, such that the proteins are now shared between two, or even all three, of the compartments that possess translational machinery.

The ambiguous transit peptides of preproteins that are dually targeted to both endosymbiotic organelles have been examined in some detail (Peeters and Small 2001; Pujol et al. 2007; Berglund et al. 2009a, b). In general, they seem to have properties that are very similar to, but intermediate between, those of proteins targeted exclusively to either chloroplasts or mitochondria. In the N-terminal region, serine content is more similar to that in chloroplast transit peptides, while arginine content is more similar to that in mitochondrial presequences. Dual targeting peptides also show enrichment of phenylalanine and leucine residues, but they seem to lack a shared or common functional-domain architecture (Berglund et al. 2009a, b). Evidence also suggests that the extent to which a given protein is dual-targeted is influenced by the mature domain of the preprotein, as well as by developmental factors (Mackenzie 2005; Carrie et al. 2009). Software has been developed for the in silico prediction of ambiguous targeting peptides, and its use suggests that as many as ~400–500 proteins may be dual-targeted to chloroplasts and mitochondria in Arabidopsis and other plants (Mitschke et al. 2009). Competition assays suggest that dual-targeted proteins employ the same organellar import machineries as organelle-specific proteins (Berglund et al. 2009b).

B. Non-canonical Protein Transport to Chloroplasts

Until quite recently, transit peptide-dependent import was considered to be the sole protein transport route leading to the chloroplast interior. However, it is now clear that several alternative targeting signals and pathways exist (Radhamony and Theg 2006; Jarvis 2008). In fact, a recent study estimated that over 10% of the chloroplast proteome comprises proteins that lack a typical transit peptide (Armbruster et al. 2009). Proteomic studies played an important role in the identification of these non-canonical pathways (Kleffmann et al. 2004). For example, a protein named ceQORH (*c*hloroplast *e*nvelope *q*uinone *o*xido*r*eductase *h*omologue) was identified in the Arabidopsis envelope proteome, and was found to associate with the inner envelope membrane in spite of the fact that it lacks a transit peptide. In fact, its extreme amino-terminus is not required for import; instead, an internal sequence of ~40 residues controls localization (Miras et al. 2002). The protein does require proteinaceous components and ATP for its targeting, but the canonical TOC/TIC apparatus is not involved (Miras et al. 2007). Another inner membrane protein, Tic32/IEP32 (*i*nner *e*nvelope *p*rotein, 32 kD), was similarly found to lack a transit peptide (Nada and Soll 2004), and it too could localize properly without assistance of the TOC machinery. Competition analysis suggested that ceQORH and Tic32 follow different import pathways (Miras et al. 2007).

Proteomic analysis also led to the identification of a large number of chloroplast proteins with predicted signal peptides for ER translocation (Kleffmann et al. 2004). Chloroplast protein traffic through the endomembrane system is well documented in

organisms that have complex plastids, such as algae and apicomplexan parasites (Nassoury and Morse 2005). Such organelles are derived from secondary endosymbioses, and consequently are surrounded by multiple membranes including remnants of the secondary endosymbiont's cell membrane and the host ER. It is therefore inevitable that chloroplast protein traffic passes through the endomembrane system in these species, and it does so under the guidance of bipartite leader sequences comprising a signal peptide for ER transport followed by a transit peptide for chloroplast import. However, plant chloroplasts are not surrounded by such extra membranes, and so until recently similar trafficking was not thought to occur in plants. However, physical and functional associations between the ER and the outer envelope membrane are well documented in plants (Crotty and Ledbetter 1973; Whatley et al. 1991; Benning et al. 2006; Andersson et al. 2007). Moreover, indirect evidence for chloroplast protein transport through the ER and Golgi in plants has existed for some time, with glycoproteins and proteins with apparent signal peptides having been found in plastids (Gaikwad et al. 1999; Chen et al. 2004; Asatsuma et al. 2005).

Firm evidence for such targeting was provided recently by thorough analyses of proteins such as Arabidopsis CAH1 (*c*arbonic *an*hydrase 1) (Villarejo et al. 2005; Nanjo et al. 2006). This stromal protein was strongly predicted to have a signal peptide; accordingly, it could not be imported directly by isolated chloroplasts, but instead was taken up co-translationally by ER microsomes and processed to its mature size (Villarejo et al. 2005). In addition, glycosylated CAH1, as well as several other glycoproteins, was identified in the chloroplast stroma, implying that some proteins are transported through the Golgi en route to the chloroplast. Indeed, application of brefeldin A (a chemical that interferes with Golgi-mediated vesicle traffic) obstructed CAH1 transport within the endomembrane system. It is not clear how proteins following this pathway enter the chloroplast, as they do not seem to possess bipartite targeting signals. Some data suggest that the signal peptide itself provides the necessary targeting information (Chen et al. 2004), while others have argued that surface characteristics of the mature protein play a role (Kitajima et al. 2009). The proteins may be released into the intermembrane space, following vesicle fusion with the outer membrane, thereafter entering an unknown translocon, the TIC machinery (Scott and Theg 1996), or vesicles that pinch off from the inner envelope membrane (Benning et al. 2006; Benning 2009). Such vesicle fusion would inevitably deliver lipids to the chloroplast as well, although the significance of this in the context of other mechanisms of lipid transport remains to be seen.

VI. Concluding Remarks

Research on chloroplast protein import has provided a wealth of data in recent years, leading to a greatly enhanced understanding of the molecular steps underlying the process, but also to a certain amount of confusion over conflicting results. A major challenge for future research will be to construct consensus models that rationalize the contradictory results and explain as much of the available information as possible. While our knowledge concerning G-domain function in the TOC receptors has increased considerably thanks to recent work, the precise mode of action is still debated. The ATP requirement in the intermembrane space, a long-standing fact, will require renewed attention now that doubt has been cast on the existence of ATP-processing components previously assumed to reside in the intermembrane space. We have learnt much about the function, regulation and topology of several putative inner envelope channel proteins, but there is still no agreement concerning their capabilities for preprotein transport, or indeed their functional interactions with each other. Moreover, while a consensus view is emerging concerning the existence of substrate-specific import pathways, the molecular basis for TOC receptor (and possibly also TIC channel) selectivity will

require further work. It is commonly agreed that a complex and important process like chloroplast protein import must be tightly regulated, and a lot of work has been done to unveil putative redox-regulatory processes at the TIC apparatus. However, the mechanistic details of such regulatory networks are currently lacking. We hope and expect that future research focusing on these fascinating challenges will bring the consensus of opinion we are waiting for, increasing our knowledge about chloroplast protein import which is such a crucial process in plant life.

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