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Review

Protein import into chloroplasts—How chaperones feature into the game ☆

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ABSTRACT

Chloroplasts originated from an endosymbiotic event, in which an ancestral photosynthetic cyanobacterium was engulfed by a mitochondriate eukaryotic host cell. During evolution, the endosymbiont lost its autonomy by means of a massive transfer of genetic information from the prokaryotic genome to the host nucleus. Consequently, the development of protein import machineries became necessary for the relocation of proteins that are now nuclear-encoded and synthesized in the cytosol but destined for the chloroplast. Organelle biogenesis and maintenance requires a tight coordination of transcription, translation and protein import between the host cell and the organelle. This review focuses on the translocation complexes in the outer and inner envelope membrane with a special emphasis on the role of molecular chaperones. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

Chloroplasts are highly specialized organelles, which perform essential functions such as photosynthesis, nitrogen and amino acid metabolism. Like mitochondria they evolved through an endosymbiotic event from once free living prokaryotic cells [1]. To gain control over its newly enslaved component, the majority of the genes were transferred from the endosymbiont to the host genome, leaving the evolving chloroplast with only about 100 protein encoding genes [2]. Although this process allowed the cell to supervise the functions and biogenesis of the organelle, complex mechanisms had to be developed to transport approx. 3000 proteins into the chloroplast. The import process is additionally challenged by the complex organization of the chloroplast sub-compartments,

☆ This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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since the organelle is enclosed by two distinct membranes and a third independent membrane system, the thylakoids, which harbor the photosynthetic complexes. This results in three separated soluble compartments: the intermembrane space, the stroma and the thylakoid lumen. The plant cell is faced with several obstacles during the targeting of preproteins: (1) specific targeting to the chloroplast or/and other organelles, such as mitochondria or peroxisomes has to be ensured, (2) transport across the outer and the inner envelope membrane and (3) correct targeting and assembly inside the chloroplast, i.e. stroma, thylakoid and thylakoid lumen. Therefore most chloroplast targeted proteins are synthesized as precursor proteins and equipped with an N-terminal transit sequence, which serves as an entry ticket for the designed organelle and is cleaved after the protein has reached its destination [3]. Once the precursor protein has been guided to the chloroplast, a process involving several cytosolic chaperones, the precursor interacts with receptors on the chloroplast membrane surface and is transported through the membranes in a GTP and ATP dependant manner. Two multi-protein translocon complexes (the TOC and TIC complex) facilitate the transport across the outer (TOC—**t**ranslocon at the **o**uter membrane of **c**hloroplasts) and inner (TIC—**t**ranslocon at the **i**nner membrane of **c**hloroplasts) envelope membranes of most preproteins [4,5]. This import system is up to date the best characterized route, although alternative pathways have been described, especially for proteins of the outer and inner envelope. In a second step proteins designated for the thylakoid membrane or the thylakoid lumen are targeted with the help of specific signal peptides. The mechanisms involved derived from the chloroplasts bacterial ancestor and are beyond the scope of this article, yet a number of excellent reviews are available on that subject [6,7]. Since the biogenesis and function of chloroplasts is a very dynamic and adaptive process, the import mechanism of its constituents also provides a powerful way to act as a regulatory element. Regulation of protein import can occur at several steps, starting with the formation of cytosolic chaperone complexes, the involvement of several isoforms of the Toc subunits and a redox-mediated control at the stage of import through the inner envelope.

In this review we will especially focus on targeting of the proteins to the chloroplast and the process taking place at the envelope membranes as well as the regulation of protein import. The function of cytosolic as well as chloroplast chaperones is especially emphasized.

2. Targeting to the chloroplast

The most simplified way to imagine efficient sorting of proteins to their respective organelles would include targeting peptides with distinct features allowing a clear classification. Yet, such a scheme cannot be applied for the identification of chloroplast transit peptides. Although several programs (e.g. Target P [8]) are able to predict the localization of a nuclear encoded protein with reasonable success, transit peptides of chloroplasts do not show very conserved features [3,9]. Instead of being significantly different in comparison with mitochondrial targeting sequences, they even prove to be quite alike in their features. Both, chloroplast and mitochondrial sequences, show an abundance of hydroxylated amino acids (serine) and very few acidic residues, resulting in an overall positive charge of the signaling peptides [3]. This leaves the vital question, how proteins are really sorted, unanswered up to date. Despite the diversity of sequence motifs Lee and coworkers [10] tentatively defined seven subgroups of transit peptides by hierarchical clustering. When taking a closer look at the secondary structures of mitochondrial and chloroplast transit peptides, some differences become evident. Whereas mitochondrial presequences are capable of forming amphipathic helices [11,12], no secondary structure is formed by chloroplast transit peptides; they even have been proposed to form a perfect random coil [13]. This might play a role in their association with molecular chaperones, such as Hsp70, Hsp90 or 14-3-3 proteins, a topic that will be discussed below in detail.

To add to the complexity, there are a number of proteins which are targeted to more than one organelle, exerting similar functions in both organelles. Three mechanistic possibilities have been described, which allow targeting to both, plastids and mitochondria. The destiny of a precursor can be altered on RNA level, when alternative splicing of the transcript generates different transit signals or different start codons are used for translation of the preprotein leading to different N-terminal sequences. However, some proteins possess ambiguous targeting signals [14–17], raising the question how the distribution between the organelles is monitored and regulated on protein level.

3. Cytosolic components

In the past decade several novel cytosolic components have been assigned to play a role in protein targeting to the chloroplast in addition to the detailed investigation of the translocon complexes. Among these are mainly proteins functioning as chaperones, which associate with the freshly synthesized precursor proteins, thus keeping them in an import competent state and preventing aggregation. Their possible roles in regulation of protein import or discrimination between ambiguous transit signals, however, remain to be established. Most precursor proteins, either chloroplast or mitochondrial targeted, have a potential to bind the heat shock protein Hsp70, which is a highly conserved chaperone, with well described features in regard to its ATP-dependent and co-chaperone mediated assistance in protein folding [18]. Binding of cytosolic Hsp70 to mitochondrial and chloroplast precursor proteins has been shown in several in vitro experiments [19–22]. Since ca. 80% of the chloroplast transit peptides have an Hsp70 binding site [21,23] and Hsp70 was shown to bind to the transit peptides of the small subunit of RubisCo as well as FNR [19,21], binding in the N-terminal region of the precursors is likely. However, binding to the mature part of preproteins has also been observed [24]. Even 97% of the mitochondrial signal peptides contain binding motifs for Hsp70 and they have been shown to play a role in Hsp70 binding *in vitro* [25,26]. Additionally, a Hsp70 bound to the outer envelope membrane, facing the cytosol was identified in spinach, *com*70 [27], which has a potential to interact with precursor proteins as well.

Two further cytosolic components were identified in association with Hsp70s. A 14-3-3 dimer was shown to bind to the transit peptide of the small subunit of RubisCo and other precursors. Binding occurs at a phosphorylated 14-3-3 binding site, which was detected in these precursors [24]. The kinase responsible for the phosphorylation of these precursors could be identified and isolated from *Arabidosis* cytosol preparation. It belongs to a family of three homologous pant specific STY-kinases, containing a serine/threonine as well as a tryrosine phosphorylation domain [28]. The formation of this so-called guidance complex might well have a regulatory or discriminative function, since mitochondrial precursors do not form such complexes. Increased import efficiencies were shown for complexed preproteins in comparison with free precursors [24]. However, targeting was not affected by removal of the phosphorylation sites *in vivo* [29].

Apart from binding of 14-3-3, another major chaperone is involved in guiding loosely folded precursors to the chloroplast. Some preproteins were found to associate with Hsp90 in addition to Hsp70. Like Hsp70, Hsp90 is a well-described chaperone in other prokaryotic and eukaryotic organisms, where it is mainly known to assist the folding of transcription factors and protein kinases with the assistance of several co-chaperones [30]. These functions, however, have so far not been described in plants in great detail. The *Arabidopsis* genome encodes for 7 isoforms of Hsp90, four of which are localized in

the cytosol [31]. Down-regulation of all cytosolic Hsp90 forms in *Arabidopsis* indicates significant and diverse roles of these proteins in plant development and responsiveness to external stimuli [32]. Binding of Hsp90 to precursor proteins in the plant cytosol does not only prevent those proteins from aggregation, but also mediates docking of the bound precursors to an outer membrane protein, Toc64, via its cytosolic exposed TPR domains [33,34]. After this initial contact with the chloroplast the proteins are passed on towards the general import pathway via Toc34 and Toc75. Interestingly, precursor complexes were only found associated with Hsp90 in an experimental setup using wheat germ lysate for the *in vitro* translation of proteins and not reticulocyte lysate. This shows that binding of Hsp90 to chloroplast precursors is plant specific, again indicating a distinct role in the sorting process. However, mutants lacking the Toc64 protein in *Arabidopsis* do not show any visible phenotype [35], indicating that precursors can alternatively be directly accepted by Toc34 as an initial receptor. Toc64 seems to play a role analogous to that of Tom70 in the outer membrane of mammalian mitochondria. Tom70 and Tom20 are involved in precursor recognition in mitochondria of mammals and yeast, where Tom70 likewise binds to precursor chaperone complexes associated with Hsp70 and Hsp90 via a TPR domain [36,37]. Surprisingly, plant mitochondria lack a Tom70 homologue, indicating that receptor recognition is very divergent in plant mitochondria; a fact that might result from the specific environment of plant cells, since targeting to two different organelles has to be dealt with. However, a homologue of Toc64 is found in the mitochondrial membrane, which might replace Tom70 in plants and be involved in precursor recognition [17]. Its substrate selectivity or ability to bind to chaperone remains to be established.

A further cytosolic factor is represented by an *Arabidopsis* ankyrin repeat protein (AKR2A) and was shown recently to bind to outer envelope membrane proteins [38]. The data suggest an involvement of AKR2A in targeting of OEP7 to the outer membrane. Since an RNAi knockdown of AKR2A has a very pleiotropic effect on plant growth and was also shown to interact with the peroxisomal ascorbate peroxidase 3 [39], a broader function of AKR2A is likely.

4. Recognition at the Toc receptor

The Toc complex in the outer envelope directly mediates precursor translocation into the chloroplast (Fig. 1). Its core components are Toc159, Toc34 and Toc75. When it comes to precursor recognition at the chloroplast surface, two of these components play an essential role: the receptor proteins Toc34 and Toc159. Toc34 and Toc159 are anchored in the outer envelope membrane with their C-termini, leaving their large N-terminal portions with GTP-binding domains exposed at the chloroplast surface. Either of the two proteins is likely to recognize the precursor proteins directly, thus potentially acting as a receptor [40–43]. Toc75, the third component of the Toc core complex, is a β-barrel protein, deeply embedded into the membrane and acting as a translocation channel across the outer membrane [44,45]. All these components were initially discovered in pea and in *in vitro* experiments with lipid vesicles demonstrated these three

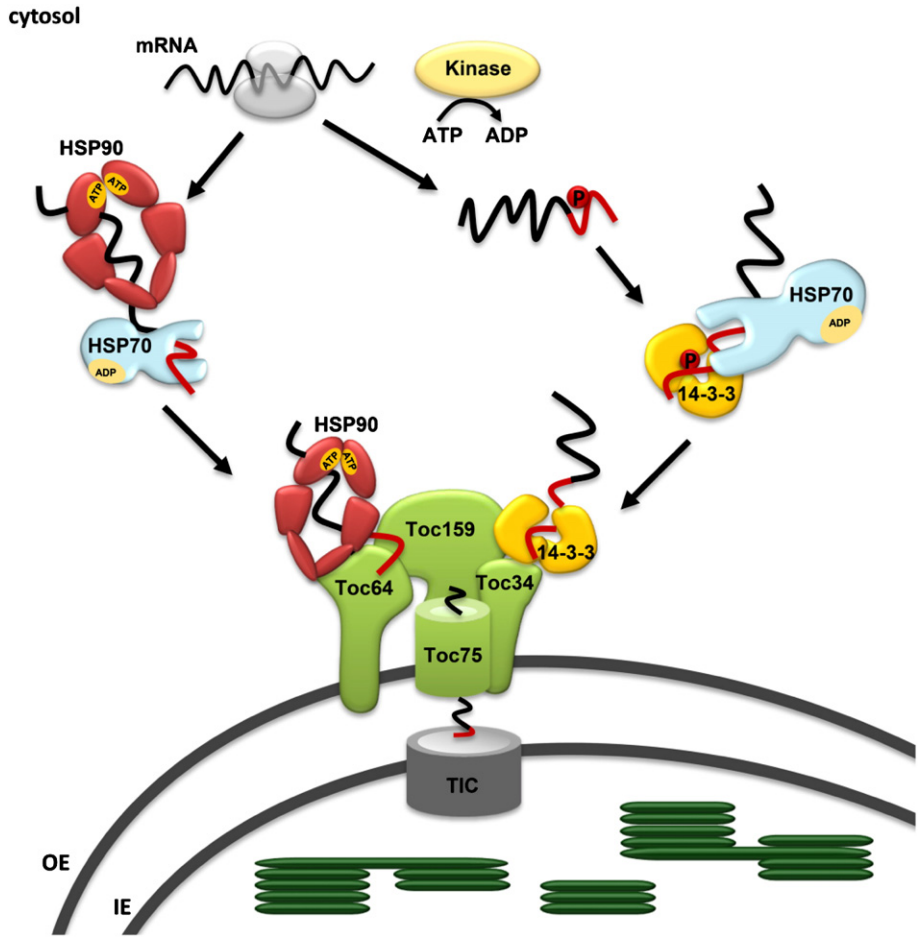

Fig. 1. Cytosolic components of the import pathway. Precursors which are synthesized in the cytosol are recognized by Hsp70, Hsp90 and 14-3-3 proteins. 14-3-3 binding precursors are initially phosphorylated by a specific kinase and further guided to Toc34. In contrast Hsp90 binding precursors use Toc64 as a first docking station, from where they are passed on to Toc34 and Toc75.

proteins are sufficient for protein translocation forming a voltage sensitive channel interacting with precursors [46].

Sequencing of the *Arabidopsis* genome added more complexity to the picture of the import apparatus, since mostly more than one isoform is present for each protein. In the case of Toc34, *Arabidopsis* encodes two isoforms, atToc33 and atToc34, and for Toc159 four isoforms are found (Toc159, Toc132, Toc120 and Toc90) [47]. *Arabidopsis* deletion mutants of the major isoforms atToc33 (*ppi1*) [48,49] and atToc159 (*ppi2*) [50] have severe phenotypes (pale green and albinotic, respectively), clearly depicting their essential roles in chloroplast biogenesis.

Protein translocation across the outer membrane requires GTP, which is hydrolyzed by the major receptor proteins of the Toc159 and Toc33/34 families. Whether Toc34 or Toc159 is the initial receptor for preproteins and the exact role of the GTP hydrolysis cycle still remains a matter of debate. Two models are currently proposed for translocation across the outer membrane, where either Toc159 or Toc34 could act as an initial receptor. A large conformational change of Toc159 either driven for example by GTP hydrolysis or by interaction with the preproteins facilitates the translocation through the Toc complex in a reconstituted system [25,156]. Deletion of the cytosolic domain in planta indicates that the so-called M-domain (membrane anchor domain of Toc159) is essential for the functional assembly of a rudimentary Toc complex, which allows plant growth [51]. This indicates, that chloroplast prercursors might also translocate in a Brownian motion mode as proposed for mitochondrial proteins [52].

5. The channel protein Toc75

Toc75 is of prokaryotic origin, as it is part of the Omp85 family. Other members of this family are found in gram negative bacteria and mitochondria, where they are responsible for the integration of β-barrel proteins into the outer membrane [53]. Intriguingly, Toc75 is the only outer envelope protein known so far with an N-terminal targeting sequence, also in contrast to bacterial and mitochondrial members of the Omp85 family, which do not contain a signaling sequence at all [54]. Even more unusual is the composition of this bipartite sequence. The first part is a stromal targeting signal, where the second partion only reaches the intermembrane space and is cleaved by a plastid type-I signal peptidase [44,54,55]. Four paralogues of the channel protein Toc75 are found in the *Arabidopsis* genome Toc75-III, Toc75-IV, Toc75-V and Toc75-I, where the latter represents a pseudogene [56]. Of the remaining three Toc75-III most likely is the only one, which is part of the Toc complex. T-DNA insertion lines are embryo lethal, thus emphasizing its vital role in protein import [57].

6. Phosphorylation and dimerization of the receptor proteins

Both, Toc33 and Toc159, are phosphorylated in their GTP binding domains by kinases located in the outer envelope [58–61]. Although *in vivo* mutational analysis with *Arabidopsis* knockout mutants could show that the phosphorylation is not essential [62], *in vitro* analyses indicated, that phosphorylation plays a role in binding of GTP as well as precursor to the receptor [60]. Additionally, a phospho-mimicry mutant of atToc33 was reported to be affected in its photosynthetic performance [63]. Phosphorylation has likewise been reported for Toc159 [58]. In addition to the G-domain (GTPase domain) and the membrane anchoring M-domain, the N-terminal domain of the large GTPases (Toc159 and its *Arabidopsis* isoforms, with the exception of Toc90) is highly acidic and termed A-domain. In contrast to the G- and M domain, which are essential for proper functioning, the A-domain is dispensable [64]. Recent results demonstrate, that the A-domain is also hyperphosphorylated, a fact, that might play a regulatory role in protein import [65]. Additionally, recent in vitro as well in vivo analysis of chimeric Toc159 family proteins could demonstrate an involvement of the A-domain in precursor substrate selectivity [66].

Another mode of regulation is represented by the ability of the GTPases to homo- and heterodimerize. The homodimerization of atToc33 and atToc34 has been investigated in great detail. A conserved arginine has been identified, which inserts into the catalytic domain of the opposite monomer, thus leading to dimerization [67]. A possible activation of the GTPase through this arginine was discussed studying mutated proteins. No effect of monomerization on GTP binding and hydrolysis on the homodimeric Toc33 was reported [68], but activation seems to occur between heterodimers of Toc33 and Toc159 [69]. However, GTPase activity of the dimer most likely requires additional factors. Complementation analysis of atToc33 (*ppi1* mutants) *Arabidopsis* knockout plants with several mutated forms of the atToc33 protein have shown that neither dimerization nor GTP binding is essential for chloroplast development *in vivo*. Import efficiencies, however, were reduced in the complemented plants [70,71].

7. Substrate specific recognition of preproteins

The existence of several isoforms of the import components in *Arabidopsis* and other organisms seems to enable the plants to regulate protein import. It has been suggested that Toc complexes with different compositions of the isoforms are able to regulate protein import by selectively binding to different classes of preproteins [72–74]. In order to maintain the required levels of preproteins in the organelle it is proposed that a complex assembly containing atToc120 or at atToc132 and atToc34 preferentially recognizes housekeeping proteins, whereas the complex containing atToc159 and atToc33 is responsible for the import of a second class of proteins including for example photosynthetic genes, which are more flexibly regulated. This correlates with the differential expression of the isoforms, where atToc120, atToc132 and Toc90 are more uniformly expressed in all plant tissues than atToc159, which is highly expressed in green and rapidly expanding tissues [75]. A double knockout of atToc120 and atToc132 also results in an albinotic phenotype, whereas the single mutants only have a mild phenotype, indicating their redundancy, as well as their possible involvement in protein import [72]. How and to what extent the different isoforms manage to distinguish selectively between preproteins remains to be shown.

Apart from the Toc core complex, further components have been identified at later stages. Toc64 is loosely associated with the core complex and is likewise involved in preprotein recognition. As mentioned above, Toc64 recognizes precursor proteins associated with Hsp90 via its C-terminal three tetratricopeptide motifs (TPR) mediating the protein-protein interaction [34]. Facing the other side of the membrane, however Toc64 is also part of a complex in the intermembrane space containing Hsp70, Tic22 and Toc12 (see below). In planta, Toc64 is not essential [35]. While it is easy to imagine that on the cytosolic side Toc34 and Toc159 could directly interact with precursor proteins to complement for Toc64 function, it is unknown how and if the putative intermembrane space complex is formed in the absence of Toc64.

8. Alternative pathways

Although the Toc/Tic translocon is undoubtedly the major import pathway into the chloroplast other, not yet identified pathways are likely to exist. Especially for proteins which do not contain a cleavable transit peptide alternative routes into the chloroplast are discussed. Two chloroplast inner envelope proteins, Tic32 and the quinone oxidoreductase (ceQORH) were described as two proteins lacking a N-terminal transit peptide [76,77], although in the case of Tic32 the first 10 most N-terminal amino acids were shown to be essential for

targeting and import [77]. Both proteins do not seem to find their way across the outer envelope membrane through the TOC complex, since no competition occurs with other chloroplast proteins using this route, and inhibitors of the channel proteins do not interfere with import of Tic32 and ceQORH.

9. Crossing the intermembrane space

The first soluble compartment encountered by an incoming precursor protein is the intermembrane space between the outer and inner chloroplast envelope. Knowledge about its proteome is scarce, but three proteins were identified that form (parts of) an IMS translocation complex: Toc12, and IMS Hsp70 and Tic22. Toc12 interacts with the amidase-like domain of Toc64 and is anchored to the outer envelope by a short hydrophobic domain in the N-terminus [34,78]. Noteworthy, Toc12 shows sequence similarity to J-domains and seems to interact with an IMS residing Hsp70 isoform. The existence of this IMS Hsp70 is controversially discussed [78–80], but the biochemical data for its existence are quite numerous and strong [78,79]. The pea form of Toc12 has been characterized in some detail and it was shown that it contains a conserved CXGXXC motif, potentially contributing to a regulatory disulfide bridge. Mutation of the cysteine from this region resulted in decreased functionality of this small Toc component, opening the possibility that translocation of preproteins might be regulated by thiols in the IMS. Orthologues of Toc12 are also found in *Arabidopsis*, *Zea*, *Medicago* and *Physcomitrella*, though these proteins seemed to have a C-terminal extension not present in the pea form. However, a thorough analysis of a pea EST database (A. Weber, Düsseldorf) revealed that psToc12 is of the same length as all the other Toc12 proteins (Fig. 2), including a highly conserved tryptophan-rich motif in the C-terminus [81]. The role of this domain has not been investigated yet, most probably due to it allegedly missing in the pea protein. In *Arabidopsis*, Toc12 was designated as a J-domain protein named AtJ8 [82]. Localisation studies in that work lead to the assumption that AtJ8 is a soluble component of the chloroplast stroma; however, the experimental setup was not designed to enable differentiation between stroma and intermembrane space. Knockout mutant plants of AtJ8 did not show a visible phenotype, but further analysis revealed decreased activity of Rubisco, which was most likely due to down-regulated Rubisco activase. Furthermore, the stability of PSII seemed to be effected. Investigation of several photosynthetic parameters disclosed only a slight influence of AtJ8 deletion. Two other J-proteins, analysed in the same study, showed a more enhanced effect in photosynthesis. This might indicate that these two indeed have a photosynthesis related function, whereas AtJ8 (= atToc12) is involved in protein import. This notion is as compatible with the presented results as the proposal of AtJ8 being a stromal chaperone. This will have to be clarified in the future. A recent study by Ruprecht et al. [83] investigated the unfolding force of the chloroplast translocon. They made use of two titin-fusion proteins as well as various mutants thereof and came to the conclusion that their data strongly imply a decisive role of an IMS Hsp70 in this process. This is in contrast to the mitochondrial system, where unfolding is mediated by a matrix Hsp70.

The third component of the IMS complex is Tic22, which might function as a linker between the two translocation machineries. It was identified in a cross-link approach in connection with an incoming preprotein together with Tic20 [84], though its exact role in preprotein transport is still elusive. It is one of the rare evolutionary conserved Tic components, being of cyanobacterial origin [85,86]. Interestingly, synTic22 is located mainly in the thylakoid lumen and only to a small extent in the periplasmic space [87]. Its allocated functions include protein transport and electron transfer, though no biochemical evidence has been provided for either. Remarkably, the amount of Tic22 in cyanobacterial cells seems to be dependent on their redox state. The elimination of synTic22 results in lethality [87]. *Arabidopsis* comprises two Tic22 isoforms: Tic22-III and Tic22-IV, the latter being more closely related to the pea protein. Like Toc12, Tic22 has cysteine residues conserved in plants, which might imply redox-dependent regulation. This intriguing idea needs further investigation to verify if Tic22 indeed represents a “thiol”-chaperone [81].

10. Translocation across the inner envelope: the Tic complex

Having crossed the intermembrane space, the precursor encounters the Tic translocon (Fig. 3). The first point of contact is most likely Tic110, which was characterised as a major import pore [88–90]. Tic110 is the most abundant Tic component and was the first to be identified, almost two decades ago [91]. Its important role in protein import became evident very early on, though its exact function and topology is still a matter of debate. In all plant genomes analysed so far, it is coded for by a single copy gene, with the exception of *Physcomitrella patens*, which contains two copies due to recent genome duplication [85]. In *Arabidopsis*, Tic110 is essential—even the heterozygous knock-out plants display a mild phenotype [92]. Functional studies of pea and *Arabidopsis* Tic110 revealed that it mediates precursor binding and chaperone recruitment on the stromal side of the inner envelope membrane [93], as well as constitutes a protein translocation channel [88,89]. These diverse allocated functions reflect the dispute about the topology of this versatile protein. It is widely accepted that the N-terminus of Tic110 comprises two hydrophobic α-helices, which are responsible for membrane targeting and anchoring [88,90]. The controversy pertains to the large C-terminal part: some experimental evidence indicates that it forms a globular domain in the stroma where it interacts with molecular chaperones [93–95]. Other results demonstrate that the largely α-helical C-terminus features four additional transmembrane domains, consisting of amphipathic α-helices [88]. This was corroborated by protease treatment of inner envelope vesicles, which clearly resulted in proteolytic degradation of Tic110 from the IMS side. Electrophysiological measurements of the heterologously expressed C-terminus showed that this part of Tic110 alone, without the two hydrophobic α-helices, is able to form a cation-selective, calcium-sensitive channel. The model generated according to these results combines all previously published data and is in line with all proposed functions of Tic110. Remarkably, Tic110 contains a number of conserved cysteine residues distributed throughout the sequence, most of which are likely to reside on the stromal side. Experimental evidence suggests that at least two of those cysteines form a regulatory disulfide bridge, which can be modulated by stromal thioredoxins. Fascinatingly, Tic110 seems to be in a reduced state in the dark and to get oxidized in the light, which might indicate that the

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psToc12_old MAATTTAGVIGGIGSGVSWMQFGRKEKKQNKMNTVTVCCSSYSSSVTDPYKILKVQPDAS
psToc12_new MAATTTAGVIGGIGSGVSWMQFGRKEKKQNKMNRVRVCCSSYSSSVTDPYKILKVQPDAS

psToc12_old ESDVRKAFRQLALQYHPDVCRGKDCDVQFHVINEAYVVSYYYL----------------
psToc12_new ESDVRKAFRQLALQYHPDVCRGKDCDVRFHVINEAYVVAITNLREEAKKRETYEKEKKKK

psToc12_old ------------------------------------------
psToc12_new RCYDDEPFRGKNDPGWGYWEEWMGYEGAGISNSDFSNHINPFI
```

Fig. 2. Alignment of the old psToc12 and the new sequence (psToc12 new) deduced from a pea cDNA database. Identical residues are marked in blue.

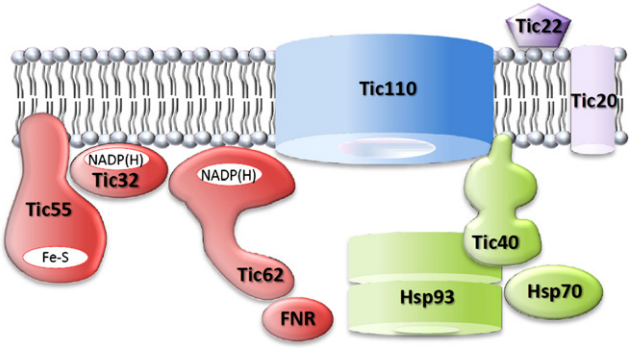

Fig. 3. Model of the TIC translocon. The components are named according to their molecular weight. The redox regulon is depicted in red color, the proteins with chaperone function in green. The Tic110 channel was colored in blue and the two proteins of yet unclear function, Tic20 and Tic22, in lilac. For details see text.

disulfide bridge forming is a stress related phenomenon in Tic110 [88]. If and how this affects translocation remains to be investigated in more detail.

The second component with a proposed channel function is Tic20 [84]. In the *Arabidopsis* genome two close homologues to the pea Tic20 are found as well as two more distantly related genes. Since knockout plants of Tic20-I, the closest relative of the pea protein, have a very severe phenotype and do not grow to maturity, import studies have been performed with antisense plants containing low levels of Tic20 [96]. Because some preproteins were imported at a decreased rate and topological predictions for Tic20 revealed four putative hydrophobic α-helices, a channel function for Tic20 was postulated. A study applying BN-PAGE with pea chloroplasts revealed the existence of a 1MDa complex consisting mainly of Tic20, which was also found to contain radioactively labelled preprotein [97]. This was interpreted as an indication for Tic20 functioning as an import channel. Support for this notion comes from a study in the complex plastid of *Toxoplasma gondii*, where Tic20 proved to be essential for protein import [98]. Importantly, according to these findings Tic20 was eliminated as a candidate for the general import pore and assigned an accessory or even regulatory role. Thus, Tic20 might form an alternative/specialized import channel for specific subsets of proteins, but biochemical evidence for channel function of Tic20 is still lacking. Reinforcing Tic110 as the general import pore is also the fact that molecular chaperones have only ever been found associated to Tic110, not to Tic20. Since a motor function for driving the import process is absolutely essential and ATP consumption most likely by chaperones inevitable, it is feasible to assume that the chaperones associate with the main channel protein. Tic110 was shown to interact with Hsp93, a AAA-ATPase family protein (synonymous to ClpC) [92,99–101] and Cpn60, the chloroplast homologue of the bacterial GroEL [102]. While Cpn60 is most likely involved in protein folding/assembly of mature proteins like RuBisCO, Hsp93 is a valid candidate for being the motor chaperone. It was demonstrated to bind to the very C-terminus of Tic110 in addition to interacting with another translocon component, Tic40 [92,100]. Interestingly, the integral membrane protein Tic40 comprises conserved domains placing it in the family of Hip/Hop co-chaperones (Hsp70-interacting protein/Hsp90-organizing protein) [103–105]. While a single hydrophobic α-helix in the N-terminus anchors Tic40 in the inner envelope membrane, the soluble C-terminal domain contains motifs similar to Sti1, the Hip co-chaperone interacting with Hsp70 in yeast. The presence of a degenerated TPR domain was also discussed [104], though recent database alignments argue against this notion. It rather seems that two Sti1 domains in tandem are located at the C-terminus [81]. In vitro analyses revealed that the alleged TPR domain interacts with Tic110 [104], while the Sti1 motif is responsible for binding Hsp93 as well as stimulating its ATPase activity [106]. The interaction with Tic110 has been found after cross-linking of inner envelope vesicles with copper chloride, which connects two juxtaposed cysteines by oxidation. Surprisingly, the only cysteine present in Tic40 lies within the alleged Hsp93-interacting domain. Puzzling is our observation that Tic40 forms a homodimer upon treatment of inner envelope with the oxidising agents copper chloride or diamide (B. Bölter, unpublished data). These seemingly contradictory results will have to be addressed in the future. Notably, the Tic40 Sti1-domain is functionally equivalent to the Hip/Hop family of Co-chaperones. This was demonstrated by complementation of Δ*tic40* plants with Tic40 containing the human hip Sti1 domain [103]. Plants lacking Tic40 have a pale, slightly chlorotic phenotype, and plastids isolated from them exhibit slower import rates of some precursor proteins [104]. These findings indicate that Tic40 rather mediates/regulates import efficiency than fulfils an essential function.

In contrast, Hsp93 is essential for plant viability. In *Arabidopsis*, two isoforms are encoded in the genome (Hsp93-V and Hsp93-III), the one on chromosome five being the dominant form [100,107]. Knockout mutants of *hsp93-V* are pale and smallish, whereas single knockout plants of *hsp93-III* have the same appearance as wild type plants [107]. Double mutants are not viable, indicating that at least a certain level of Hsp93 is absolutely required. In general, members of this superfamily of molecular chaperones use ATP to mediate protein folding/unfolding [108]. They either operate independently as chaperones or act as regulatory associates of the ClpP protease complex. Usually, Hsp93 is active as a hexamer [109], which has only be shown in chloroplasts from maize [110]. In *Arabidopsis* and pea, mostly the dimeric form has been found in the stroma [111]. Thus, it remains to be shown what the functionally active assembly is. Apart from Hsp93, there is evidence for two stromal Hsp70 isoforms [112]. Surprisingly, none of them had been detected in association with translocon components or incoming precursor proteins until very recently. All methods applied so far failed to provide evidence for cpHsp70 playing a role in chloroplast protein import. All the more fascinating was a recently published study in *Physcomitrella*, presenting first experimental evidence for an import-related function of Hsp70 [113]. In this study, it was demonstrated that two Hsp70 isoforms from *Physcomitrella* are imported into chloroplasts and that one of them, Hsp70-2, is essential for viability. By utilizing a conditional Hsp70 knockdown mutant for in vitro experiments after heat shock it was shown that plastids containing the temperature-sensitive Hsp70 mutant protein have a reduced import capacity. Furthermore, the homologue of the nucleotide exchange factor GrpE, a partner of bacterial Hsp70 (DnaK), also proved to be essential. In this case, a knockdown mutant exhibited decreased import rates, further indicating the importance of fully functional Hsp70-2 for protein translocation. Co-immunoprecipitation experiments found incoming precursor proteins on Hsp70-2 as well as on Hsp93, implying a coordinated action of both chaperones in translocation. It is intriguing to see that now in higher plant chloroplasts an import related role of Hsp70 could be detected [114]. By using a different antibody raised against stromal Hsp70 instead of anti-S78 [99], incoming precursor proteins as well as Tic110 could be co-immunoprecipitated with the chaperone from *Arabidopsis* chloroplasts. Moreover, double mutants of *tic40* and *hsp70-1* proved to be lethal, which indicates that these proteins have an overlapping, essential function. If Hsp70 was merely responsible for post-import folding or proper assembly of Tic110, the *tic40*/*hsp70-1* mutant would show the same phenotype as the single *tic40* mutant or the additive phenotype of *tic40* and *hsp70* plants [114]. If Hsp70 and Hsp93 work in parallel or are involved in different pathways remains to be established. It is, however, tempting to speculate that precursor specific chaperones not only exist in the cytosol but likewise in the stroma.

Tic110, Tic40 and Hsp93 could comprise a minimal functional Tic unit consisting of a translocation channel and chaperones for providing the translocation driving force. In addition, regulatory subunits are dynamically associated, namely three proteins forming

the so-called redox regulon: Tic32, Tic55 and Tic62 [115]. Functionally, Tic55 is the least well characterized of these. Sequence analyses revealed that it contains a Rieske–iron–sulphur centre as well as a mononuclear iron-binding site [116]. It is most probably anchored in the inner envelope membrane by two α-helices in the C-terminus, exposing the bulk of the protein into the stroma. It was originally found in complex with precursor protein, Tic110 as well as Tic62. Import experiments in the presence of the Rieske-inhibitor DEPC (diethylpyrocarbonate) suggested an important role in the import process. However, a recent study presented evidence that Tic55 is not the target of DEPC, since knockout mutants show the same import block after DEPC treatment [117]. These mutant plants grow normally with no recognizable phenotype, supporting the theory that it is a regulatory component. Tic55 was recently identified as a potential thioredoxin target by affinity chromatography on a Trx-column [118]. While further biochemical indications for this need to be obtained, it is in line with the presence of a CXXC motif within the sequence. Tic55 is an ancient protein with orthologues found in cyanobacteria and it shows homology to the LLS1 (lethal leaf spot 1) family of oxygenases [119].

Tic62 and Tic32 belong to the extended and classical family or short chain dehydrogenases, respectively, and both bind NADP(H) [74,120,121]. Though Tic32 originally was thought to be essential, this notion was corrected by the authors some time later [122]. For both proteins an electron transfer activity was demonstrated in vitro, though no endogenous substrates have been identified so far. Intriguingly, a dynamic association of the extrinsic membrane proteins Tic32 and Tic62 with the "core" translocon represented by Tic110 was observed, which was dependent on the metabolic redox state in the stroma, reflected by the $NADP^+$/NADPH ratio [120]. A more oxidized environment led to pronounced association with the translocon, whereas more reduced conditions resulted in detachment of Tic62 and Tic32. The latter was also shown to bind Calmodulin, which is in line with the observed Ca^{2+}-dependence of preprotein import. It seems that binding of Calmodulin and NADP(H) is mutually exclusive, suggesting that two controversial regulatory mechanisms convene at Tic32, one being of prokaryotic origin (redox regulation), the other coming from the eukaryotic host (calcium signalling) [123]. Tic62 is a multifaceted protein: it has a two-domain structure with a conserved NADP(H) binding-site in the N-terminus and an evolutionary new FNR binding domain in the C-terminal part. This domain consists of a repetitive pro/ser-rich motif, which occurs exclusively in vascular plants [74]. In addition to the two-sided sequence, Tic62 was shown to have a triple localization in chloroplasts: it is not only found at the inner envelope membrane where it was originally detected, but–depending on the redox state–also in the stroma as well as at the thylakoid membrane [124,125]. A recent study revealed that the role of thylakoid-associated Tic62 consists of "storing" the FNR in high-molecular-weight complexes [124]. It was in fact shown that Tic62 constitutes one of the long searched-for FNR-binding partners, the other being a newly identified thylakoid membrane protein named Trol [126]. Intriguingly, Trol likewise comprises a pro/ser-rich motif, which is highly similar to the ones found in Tic62. Thus, the role of a thylakoid/stromal pool of Tic62 was clarified, while the function within the framework of the Tic translocon remains to be further investigated. It is tempting to speculate that redox signals from the thylakoids, generated e.g. by photosynthetic activity, are somehow transferred by Tic62 travelling to the inner envelope where it conveys the signals via electron transfer to Tic32/Tic55, but experimental evidence for this is still lacking. None of the members of the redox regulon is essential for viability, insinuating that they fulfil an accessory function in regulating import efficiency rather than being strictly required for the translocation process.

The most recently added putative Tic component is Tic21, which was proposed to form another channel protein [127]. A follow-up study found Tic21 as a minor constituent of a 1 MDa complex consisting mainly of Tic20, leading to the conclusion that a Tic20/Tic21 complex functions in between Toc and Tic110 complexes [97]. However, the same protein was identified in an earlier study as the metal permease Pic1 [128], and thus affiliation as part of the Tic translocon remains to be based on further experimental verification.

11. Regulation of protein import

Since plants need to adapt to changing environmental conditions, which lead to different distinct metabolic activities within the chloroplast being active or dormant, regulation of the import process is mandatory. To date, several levels of regulation have been suggested affecting either the activity of Toc or Tic. The first mode of regulation described at the outer envelope is mediated by GTP (see above).

A second mode of regulation at the Toc complex is thiol mediated (Fig. 4). It was shown already in the early days of protein import research that the cysteine modifying agent NEM drastically reduced binding of preproteins to receptors [129], whereas incubation of chloroplasts with DTT enhanced import rates [130]. This was later confirmed by Stengel et al. [131]. Further analyses revealed that oxidizing compounds such as copper chloride result in the formation of a cross-linked complex consisting of Toc159, Toc75 and Toc34, which can be reversed by the addition of DTT [43,131]. Obviously, formation of intermolecular disulfide bridges decreases import activity, presumably by blocking binding sites at receptor components as well as the channel, while breaking of the disulfide bridges results in optimal confirmation for effective import [131].

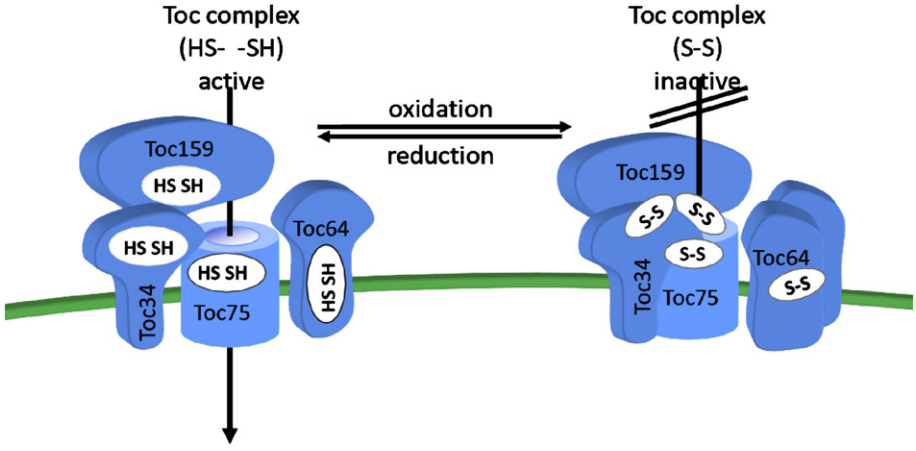

Fig. 4. Thiol-dependent regulation of the TOC complex. Under reduced conditions, cysteines in Toc components comprise free thiol groups, rendering the translocon in an active state. Oxidation of cysteines to inter- or intramolecular disulfide bridges results in decrease of transport activity.

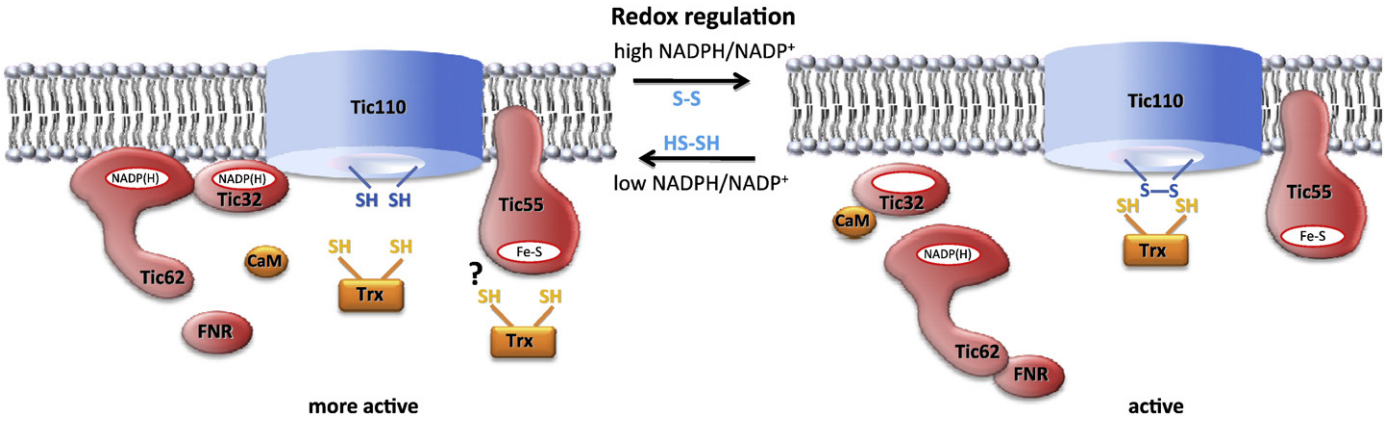

Fig. 5. Redox regulation of the TIC complex. An oxidized metabolic redox state, represented by a low NADPH/NADP ratio, within the stroma leads to attachment of regulatory subunits as Tic62 and Tic32 to the core translocon, rendering the complex more active (left side). The channel protein Tic110 is subject to thiol regulation and can be modulated by thioredoxins, where the reduced form of Tic110 is likely to be more active (left side). Upon the stroma becoming more reduced, the redox regulon members detach from the translocon, resulting in decreased import activity for a certain subset of precursor proteins (active, right side).

Concerning regulation at the intermembrane space nothing is known except that Toc12 is active upon formation of the intramolecular disulfide bridge [78] (see above). Indications for a redox-controlled disulfide relay system as described for mitochondria have not been found in plastids yet [132]. Future research will have to provide evidence if additional regulatory steps take place between the envelope membranes or if Toc and Tic are regulated separately and then somehow communicate their received signals.

The Tic translocon seems to be regulated from the inside of the chloroplast. A recent report showed that the metabolic redox state of the stroma, represented by the NADP/NADPH ratio, influences the import rate of a subset of preproteins [131]. This is most likely the result of Tic62/Tic32 associating with Tic upon oxidized conditions, leading to increased import of many precursors. A more reducing environment has no or even a slightly negative effect on import efficiency, indicating that the core translocon without the regulatory subunits is active to a certain extent. It is still unclear how this redox signalling works in detail, accordingly this needs to be a focus of future research. Additionally, thiol-mediated regulation seems to be involved at the level of Tic: Tic110 was identified as a thioredoxin target by a biochemical approach, while Tic55 was found to bind to a Trx-affinity column [88,118]. An indication for Trx being associated to the inner envelope comes from proteomic studies [133] (Fig. 5). Whether there are specific Trx isoforms targeting Tic110 and Tic55 and if these then act in concert or independent from each other will be intriguing to determine.

12. Transport into the inner membrane

All data published so far imply that proteins destined for the inner envelope membrane engage the Toc complex at the initial import step [134]. At the level of the inner envelope proteins travel one of two possible pathways: stop transfer or conservative sorting, depending on targeting information contained within their mature parts [134,135]. The first protein characterized in that respect was the triosephosphate/phosphate translocator [136,137]. In this case, the signal for arresting the protein during translocation is contained within the hydrophobic N-terminal domain. Other proteins taking this pathway are Arc6, IEP37 and some hypothetical proteins [134,135].

The post-import route includes a complete translocation into the stroma and re-targeting to the inner envelope. Examples for proteins travelling via this pathway are Tic110 and Tic40 [135,138]. It is not clear how the soluble intermediates are recognised in the stroma and re-inserted into the membrane, though some evidence was provided that Hsp93 and Tic40 are involved in these processes [135,139,140]. For Tic40 the re-insertion signal was allocated to a ser/pro-rich domain in the N-terminus, while in case of Tic110 the membrane targeting is determined by two N-terminal hydrophobic helices as well as at least partly by the first of four amphipathic helices in the C-terminus [88,138]. Very recently, a study on targeting and insertion of Tic40 and APG1 (identical to IEP37) revealed that for APG1 the single transmembrane domain represents the stop-transfer signal and likewise determines membrane topology [141]. The post-import targeting of Tic40 seems to be more complicated—replacing the stop-transfer domain of APG1 with the re-insertion signal of Tic40 leads to arrest of the chimeric protein in the translocon. This result suggests that the re-insertion signal of Tic40 functions only within the correct context of the native mature protein. It remains to be determined if this also holds true for other substrates of the post-import pathway.

13. Conclusions on the role of chaperones in chloroplast protein import

Molecular chaperones have versatile roles in cellular processes. Concerning chloroplast protein import, they are critically involved in several central steps. First, specific recognition and targeting to the organelle is mediated by different complexes including Hsp70 and Hsp90 with their respective co-chaperones. The presence of an Hsp70 isoform in the intermembrane space is still a matter of debate; some experimental evidence surely hints at the chaperone being localised in the IMS, but since it is not unequivocal, further studies are necessary to clarify this point. On the stromal side, two different chaperones seem to be involved: Hsp93 and Hsp70. Recently, evidence accumulated that indeed both fulfil a role in translocation across the inner envelope membrane. It will be intriguing to see if this is another way to confer specificity or if both chaperones are acting generally.

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