Biochimica et Biophysica Acta 1833 (2013) 332–340

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Molecular chaperone involvement in chloroplast protein import ☆

Úrsula Flores-Pérez, Paul Jarvis *

Department of Biology, University of Leicester, Leicester LE1 7RH, UK

ARTICLE INFO

Article history:
Received 18 January 2012
Received in revised form 16 March 2012
Accepted 31 March 2012
Available online 12 April 2012

Keywords:
Chaperone
Chloroplast
Hsp70
Hsp93
Protein transport
TOC/TIC machinery

ABSTRACT

Chloroplasts are organelles of endosymbiotic origin that perform essential functions in plants. They contain about 3000 different proteins, the vast majority of which are nucleus-encoded, synthesized in precursor form in the cytosol, and transported into the chloroplasts post-translationally. These preproteins are generally imported via envelope complexes termed TOC and TIC (*T*ranslocon at the *O*uter/*I*nner envelope membrane of *C*hloroplasts). They must navigate different cellular and organellar compartments (e.g., the cytosol, the outer and inner envelope membranes, the intermembrane space, and the stroma) before arriving at their final destination. It is generally considered that preproteins are imported in a largely unfolded state, and the whole process is energy-dependent. Several chaperones and cochaperones have been found to mediate different stages of chloroplast import, in similar fashion to chaperone involvement in mitochondrial import. Cytosolic factors such as Hsp90, Hsp70 and 14-3-3 may assist preproteins to reach the TOC complex at the chloroplast surface, preventing their aggregation or degradation. Chaperone involvement in the intermembrane space has also been proposed, but remains uncertain. Preprotein translocation is completed at the trans side of the inner membrane by ATP-driven motor complexes. A stromal Hsp100-type chaperone, Hsp93, cooperates with Tic110 and Tic40 in one such motor complex, while stromal Hsp70 is proposed to act in a second, parallel complex. Upon arrival in the stroma, chaperones (e.g., Hsp70, Cpn60, cpSRP43) also contribute to the folding, assembly or onward intraorganellar guidance of the proteins. In this review, we focus on chaperone involvement during preprotein translocation at the chloroplast envelope. This article is part of a Special Issue entitled: Protein Import and Quality Control in Mitochondria and Plastids.

1. Introduction

Plastids are a diverse group of organelles found ubiquitously in plant cells [1,2]. Chloroplasts, the most prominent members of the plastid family, contain the green pigment chlorophyll and are responsible for the reactions of photosynthesis, as well as sundry important biosynthetic functions. Plastids entered the eukaryotic lineage through endosymbiosis, and have evolved from an ancient photosynthetic prokaryote similar to extant cyanobacteria [3,4]. While plastids retain a functional endogenous genetic system, the plastid genome is greatly reduced and typically encodes just ~100 different proteins [5,6]. Most (>90%) of the ~3000 different proteins that are needed to develop a fully-functional chloroplast are encoded in the nucleus and synthesized on free cytosolic ribosomes [7,8].

Typically, nucleus-encoded chloroplast proteins are synthesized in precursor form, each one having an amino-terminal targeting signal called a transit peptide. These precursors, or preproteins, are transported into the organelle post-translationally, in an energy-consuming process termed chloroplast protein import. Import is mediated by hetero-oligomeric protein complexes in the outer and inner envelope membranes that surround each plastid; these complexes are termed, respectively, TOC and TIC (*T*ranslocon at the *O*uter/*I*nner envelope membrane of *C*hloroplasts) [9–12]. Once a preprotein arrives in the chloroplast interior (the stroma), the transit peptide is proteolytically removed by the stromal processing peptidase (SPP), allowing the protein to assume its functional conformation or engage one of several internal sorting pathways [12–14].

Chloroplast import bears considerable similarity to mitochondrial protein import, which is mediated by translocon complexes termed TOM and TIM (*T*ranslocase of the *O*uter/*I*nner *M*itochondrial membrane) [15–17]. In both cases, preproteins are threaded through the membranes in an unfolded state, amino-terminus first. Both import systems comprise multiple preprotein receptors that project large domains into the cytosol, both possess channel components in the outer and inner membranes, and both are powered, to a greater or less extent, by ATP hydrolysis (see below). However, the principal components of the TOC/TIC and TOM/TIM systems are not closely related. The core components of the TOC complex are Toc159, Toc34 and Toc75 (the numbers indicate size in kD). The first two are receptor components that mediate transit peptide recognition via their cytosolically-oriented GTPase domains, while Toc75 forms a β-barrel channel for preprotein conductance. Electrophysiological analysis of the Toc75 channel indicated a narrow pore ~14 Å in diameter, flanked on either side by

☆ This article is part of a Special Issue entitled: Protein Import and Quality Control in Mitochondria and Plastids.

* Corresponding author. Tel.: +44 116 223 1296; fax: +44 116 252 3330.
E-mail address: rpj3@le.ac.uk (P. Jarvis).

0167-4889
doi:10.1016/j.bbamcr.2012.03.019

two wider vestibules [18]. Such a pore would be wide enough only to accept largely unfolded preprotein clients. However, the successful import of a 6.5 kD (23 Å in diameter) tightly-folded, internally-crosslinked protein domain [19] suggests either that the pore is somewhat larger than the aforementioned estimate, or that the channel has a degree of flexibility. Critical components of the TIC apparatus include Tic110 and Tic40, the roles of which will be discussed later.

As already mentioned, protein import into chloroplasts is an energy-dependent process. According to energy requirements determined in vitro, three distinguishable stages of import have been defined. Firstly, the binding of preproteins with the TOC receptors is a reversible and energy-independent step called energy independent binding [20]. Subsequently, initial translocation leads to the formation of an early import intermediate. This irreversible second step requires GTP (for the receptors) and a low concentration of ATP ($\leq$100 μM) in the intermembrane space [21–23]. Finally, preproteins are completely translocated into the stroma at the expense of high concentrations of ATP (~1 mM) in the stroma [24]. The latter energy requirement is attributed to stromal ATPases [25].

The presumed need for preproteins to be in a largely unfolded state during import is dictated by physical characteristics of the import machinery, as discussed earlier, and this in turn necessitates the involvement of molecular chaperones — a diverse group of factors that facilitate folding processes and conformational changes in other proteins [26]. In fact, a variety of different chaperones are required during chloroplast protein import, and these are employed at different stages in the process: in the cytosol following ribosomal release, to prevent misfolding or aggregation of preproteins and to guide them to the chloroplast surface; during the import process itself, to maintain translocation competence of the preproteins and to drive transport at the expense of ATP hydrolysis; and, following the completion of import, to assist with folding, assembly or onward transport to internal destinations. In this review, we will touch on all of these aspects, focusing in particular on chaperone involvement during envelope translocation.

2. Chaperone involvement in the cytosol

Notwithstanding recent evidence that some chloroplast proteins are translated near the border of chloroplasts in the green alga, *Chlamydomonas reinhardtii*, suggesting mRNA transport as a component of the overall targeting scheme [27], chloroplast protein import is generally considered to be a post-translational process (in contrast with signal recognition particle [SRP]-dependent translocation into the endoplasmic reticulum, for example, which is co-translational). Thus, cytosolic factors are required to facilitate the passage of preproteins from the ribosome to the chloroplast surface, and to prevent their aggregation or premature degradation [28–30]. The transit peptide, as the first part of the preprotein to emerge from the ribosome, plays a critical role in the interactions with such components.

Transit peptides are to a large extent responsible for the targeting properties of chloroplast preproteins. Indeed, they are very effective at mediating the import of heterologous passenger proteins into chloroplasts [31,32]. And yet, despite the apparent specificity of the chloroplast import process, transit peptides are remarkably diverse in both length and sequence [33,34]. They vary from 20 to >100 residues, are rich in hydroxylated residues, and are deficient in acidic residues giving them a net positive charge. In this respect, transit peptides are rather similar to the functionally-analogous presequences of mitochondrial preproteins (raising puzzling questions about how organellar targeting specificity is achieved in plants [35,36]). While mitochondrial presequences share a characteristic secondary structure (they form amphipathic helices that are important for interaction with receptors of the TOM machinery [37]), chloroplast transit peptides do not seem to possess this property [38,39]. Instead, it has been hypothesized that they specifically evolved to have "perfect random coil" properties, perhaps to aid interaction with cytosolic factors [40].

Hsp70 (*H*eat-shock *p*rotein, *70* kD) is one of the chaperones thought to facilitate the cytosolic phase of chloroplast protein transport. Most chloroplast transit peptides are predicted to possess at least one Hsp70 binding site, while direct interactions between Hsp70s and transit peptides have been demonstrated [41–44]. However, the importance of such Hsp70 binding for protein import remains uncertain, as it is not essential for protein translocation in vitro [45,46]. Moreover, a recent study showed that cytosolic Hsp70 associates with accumulated precursors that are targeted for degradation via the ubiquitin proteasome system [30], indicating that Hsp70 binding does not necessarily serve to escort preproteins to the chloroplast surface. Nevertheless, Hsp70 does appear to play a role in protein import in cooperation with other cytosolic factors, such as 14-3-3 (see below; Fig. 1). It is conceivable that different isoforms of Hsp70 are responsible for these different functions.

The 14-3-3 protein family includes regulatory molecules and chaperones that specifically bind to phosphorylated proteins in order to mediate various signal transduction processes, as well as protein translocation [47]. Many chloroplast transit peptides contain a 14-3-3-binding phosphopeptide motif [28,48]. It was reported that 14-3-3 can form a "guidance complex" together with Hsp70 and preproteins, and that this significantly increases in vitro import efficiency for certain, phosphorylatable preproteins [28]. The 14-3-3-containing guidance complex was also hypothesized to play a role in determining the specificity of targeting to chloroplasts versus mitochondria in plants, as 14-3-3 cannot bind plant mitochondrial preproteins [28]. However, mutation of the putative 14-3-3-binding site in transit peptides did not affect import efficiency or fidelity in vivo [49,50], indicating that the 14-3-3 guidance complex system is dispensable. It is possible that this mechanism is important only under certain conditions; it was recently reported that the loss of a kinase thought to be responsible for transit peptide phosphorylation results in an inefficient de-etiolation response [51].

Differentiating between two distinct, endosymbiotically-derived organelles (i.e., chloroplasts and mitochondria) is a unique problem faced by protein transport systems in plant cells. Related to this issue, perhaps, is the fact that the protein import receptors in plant mitochondria are significantly different from those in yeast or animal mitochondria, as well as from those in chloroplasts [17,36]. In spite of these receptor differences, some chloroplast preproteins can be efficiently imported into plant mitochondria in vitro, but not in vivo [52]. This implies that special mechanisms are employed to achieve import specificity in vivo, and that components of such mechanisms are absent or inactive in vitro. Aside from the 14-3-3 guidance hypothesis discussed above, one strategy that might contribute to targeting specificity is mRNA transport towards the target destination, such that preproteins are produced only at the periphery of the correct organelle [27,53,54]. However, the general significance of mRNA targeting in plants remains to be seen.

In mitochondrial protein import in animal cells, Hsp90 is an additional chaperone involved in cytosolic guidance, directing some preproteins to the Tom70 receptor [55]. Similarly, Hsp90 has also been implicated in the delivery of certain preproteins to chloroplasts as part of a second guidance complex, which was recently reported to also involve the cochaperone Hop (*H*sp70/Hsp90-*o*rganizing *p*rotein) and the immunophilin FKBP73 [56–58]. There are two important differences between this guidance complex and the one discussed earlier: firstly, Hsp90 binds to preproteins that are not necessarily phosphorylated; secondly, unlike the 14-3-3 complex which carries preproteins directly to the Toc34 receptor, Hsp90 employs Toc64 (see below) as an initial docking site before preproteins are passed on to Toc34 [56] (Fig. 1). However, preproteins proposed to follow the Hsp90–Toc64 pathway were found to be imported with normal efficiency into chloroplasts that lack Toc64 protein [59,60], indicating that this putative targeting mechanism is also not essential. It is conceivable that such

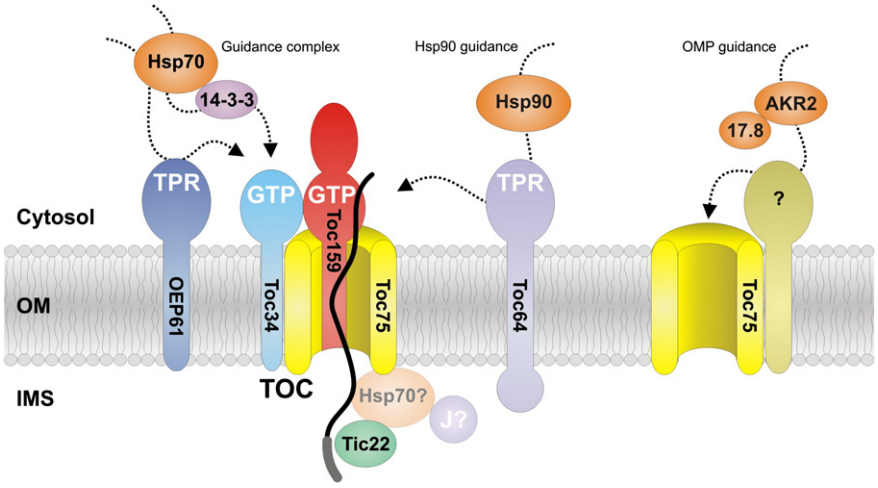

Fig. 1. Chaperone involvement in the cytosol and intermembrane space during protein transport to chloroplasts. Nucleus-encoded proteins destined for the chloroplast are recognized in the cytosol by soluble factors such as Hsp70, Hsp90, 14-3-3 and AKR2. Hsp70 may form a guidance complex together with 14-3-3 that delivers phosphorylated preproteins to the Toc34 receptor. Hsp70 is also proposed to deliver preproteins to the OEP61 protein. An alternative Hsp90 complex is reported to deliver a different subset of preproteins (that need not be phosphorylated) to the peripheral TOC component Toc64, before their onward passage to the core TOC machinery. The AKR2 protein guides outer membrane protein (OMP) clients to the chloroplast surface, with assistance from Hsp17.8 and possibly other, unknown factors, whereupon OMP insertion into the membrane is facilitated by Toc75. Transport of preproteins through the intermembrane space was proposed to involve imsHs70 and a J-protein called Toc12. However, the existence of imsHsp70 is now uncertain, while evidence suggests that "Toc12" is simply a truncated form of the stromal protein DnaJ-J8; whether some other J-protein acts in the IMS remains to be seen. A translocating preprotein is represented by a wavy black line, while its transit peptide is shown as a thick gray line. Functional domains of the proteins are indicated in white text. TOC, translocon at the outer envelope membrane of chloroplasts; OM, outer envelope membrane; IMS, intermembrane space.

guidance systems are only important under specific conditions that have so far eluded analysis [49,59,60], or that a certain amount of redundancy between these (and perhaps other) guidance systems exists such that loss of one does not have an appreciable effect.

The Toc64 protein was identified by its co-purification with the TOC complex after chemical cross-linking [61]. It is described as a peripheral component that dynamically associates with the complex, in contrast with the stably-associated core components [62]. The protein is proposed to have three transmembrane spans, thereby presenting a carboxy-terminal TPR (*T*etratrico*P*eptide *R*epeat) domain to the cytosol and a central domain (with amidase homology) to the intermembrane space [57]. Biochemical studies indicated that the two domains might enable bipartite functionality, the TPR domain acting as a receptor for the Hsp90 guidance complex [56,57], and the central domain aiding transport through the intermembrane space (see Section 3) [57]. However, as already mentioned, in vivo studies using knockout mutants did not support the importance of Toc64 for protein import [59,60]. Nonetheless, because its supposed mitochondrial counterpart, Tom70 (a TPR-domain receptor of well-established function), is also non-essential [63], further work is needed before a final conclusion can be reached on Toc64's role. Interestingly, one of the three Toc64 homologues in *Arabidopsis* (atToc64-V/mtOM64) is localized in the mitochondrial outer membrane, perhaps replacing the Tom70 receptor which is absent in plants [64,65]; but like its chloroplast relative, this protein too is dispensable [60]. More recently, an additional protein was identified that shares features with Toc64: the OEP61 (*O*uter *E*nvelope *P*rotein, *61* kD) protein possesses an amino-terminal TPR domain with similarity to that of Toc64, and it is localized in the chloroplast outer envelope membrane by a single carboxy-terminal span [66,67]. This protein was shown to interact with chloroplast preproteins and Hsp70, but not Hsp90, suggesting that it might be an additional docking site at the chloroplast surface.

Another cytosolic factor involved in chloroplast protein targeting is AKR2 (*An*K*yr*in *R*epeat-containing protein *2*), which was identified as having an important role in the insertion of outer envelope membrane proteins [68,69]; such outer membrane proteins do not have transit peptides, and follow a pathway different from the canonical TOC/TIC route discussed above [70] (Fig. 1). The AKR2 protein is proposed to act as a chaperone, preventing aggregation of these client proteins and guiding them to the envelope membrane, where Toc75 is thought to assist their insertion [71]. It was recently shown that this guidance function of AKR2 is assisted by Hsp17.8, which is a member of the small heat shock protein (sHsp) family [72]. Interestingly, AKR2 is also reported to have a role in the insertion of peroxisomal membrane proteins [73], suggesting that it may be important for the targeting of a broad class of membrane proteins [74].

3. Crossing the intermembrane space

To complete their translocation across the two envelope membranes, preproteins must traverse the intermembrane space (IMS) after their recognition at the TOC complex. Four components have been proposed to assist such transfer through the IMS: Toc64, Toc12, Tic22 and imsHsp70, with the latter putatively accounting for the ATP requirement in the IMS [75,76] (Fig. 1). However, the localization and functions of several of these proteins are controversial.

Toc64 was proposed to participate in the formation of an IMS complex through its central amidase domain, and to directly interact with Toc12 [57]. However, as described earlier, this protein does not seem to play an appreciable role in protein transport in vivo [59,60]. The Toc12 component was first described as a J-domain protein anchored to the outer membrane by its amino-terminal end, and with its carboxy-terminal J-domain localized in the IMS. In the original model, the latter domain would specifically stimulate the ATPase activity of an Hsp70 chaperone (imsHsp70) to facilitate its interaction with translocating preproteins. The preproteins would then complete their transport across the intermembrane space after contacting the soluble Tic22 protein, which is peripherally associated with the outer face of the TIC complex [57,75,76].

Toc12 was first identified by proteomic analysis of the outer envelope of pea chloroplasts. Through immunoprecipitation experiments, it was found that Toc12 interacts with Toc64 and core TOC components, Tic22 and imsHsp70, but not with other TIC complex components such as Tic110 [75]. However, more recent evidence indicates that Toc12 is an unlikely participant in IMS transport. It was found that Toc12 is a truncated form of a larger protein (DnaJ-J8) in pea [77]. Moreover, protein import analyses indicated that Toc12/DnaJ-J8 possesses a cleavable

transit peptide, and that the imported mature protein is soluble and localized in the stroma [77]. In *Arabidopsis*, the AT1G80920 gene was proposed to encode the orthologue of pea Toc12 [11]. Recent studies designated At1g80920 as AtJ8, and showed that it too is a soluble protein of the chloroplast stroma [77,78]. Furthermore, AtJ8 T-DNA insertion mutants did not show any defect in the import of various chloroplast preproteins, suggesting that AtJ8 is unlikely to be involved in protein import [77].

Another critical component of the putative IMS complex is the imsHsp70. The existence of three pea chloroplastic Hsp70 isoforms was originally reported: two soluble proteins located in the stroma, and one isoform tightly associated to the outer membrane but not exposed at the outer surface of the chloroplasts [79]. This latter imsHsp70 isoform is supposed to interact with translocating preproteins in pea chloroplasts [75,80]. Recent studies aimed to identify the *Arabidopsis* homologue of the imsHsp70, and described the subcellular localization of the three putative chloroplastic Hsp70 proteins. Two of them, AtHsp70-6 and AtHsp70-7 (alternatively called cpHsc70-1 and cpHsc70-2, respectively, for *c*hloro*p*last *H*eat *s*hock *c*ognate protein, *70* kD; [81]) were found to be localized in the soluble fraction of chloroplasts while the third, AtHsp70-8, was not even imported into chloroplasts in vitro [81,82]. Thus, the gene that encodes the imsHsp70 remains unknown.

As described above, the involvement of Hsp70 in the translocation steps that mediate passage through the IMS is still inconclusive. However, recent evidence does support the participation of Hsp70 in chloroplast protein import in the stroma, as detailed in Section 4 below.

4. Chaperone involvement in the stroma

4.1. Inner membrane components

Preprotein translocation is completed by passage through the TIC complex of the inner envelope. The putative or actual members of the TIC machinery that have been identified to date are Tic110, Tic62, Tic55, Tic40, Tic32, Tic22, Tic21 and Tic20 (reviewed in [11,83]); it should be noted that Tic21 was alternatively identified as an iron transporter, PIC1 (*P*ermease *I*n *C*hloroplasts *1*) [84,85]. Three components (i.e., Tic110, Tic20 and Tic21/PIC1) have been proposed to contribute to channel formation at the inner membrane [76,86–88]; however, evidence suggests that Tic110 is not present in the same complex as Tic20 and Tic21, and so it is unlikely that the three proteins cooperate in this function [89] (Fig. 2). The Tic40 protein, together with the stromal chaperone Hsp93, may constitute a motor providing the driving force for protein import dependent upon ATP hydrolysis (discussed in detail in the next section) (Fig. 2). Finally, Tic62, Tic55 and Tic32 constitute a putative redox-regulator of the TIC apparatus [11,83].

Tic110 is the second most abundant protein of the inner envelope membrane [90], and it plays an essential role in plastid biogenesis since knockout mutants abort during embryogenesis [91,92]. The amino-terminus of Tic110 contains two membrane-spanning α-helices, while its largely hydrophilic carboxy-terminal region is oriented towards the stroma [92–94]; alternatively, the latter domain may contain four amphipathic transmembrane helices that enable it to form a cation-selective channel [88]. The conformation of Tic110 results in one or more regions facing the IMS, that might interact with Tic22 and/or TOC components enabling the formation of TOC–TIC supercomplexes, or receive the transit peptides of incoming preproteins, as well as a large region facing the stroma that may interact with molecular chaperones such as Hsp93 and Cpn60 (*Cha*peroni*n*, *60* kD) [88,95] (Fig. 2).

Tic40, another inner envelope membrane protein, has been proposed to function as a cochaperone in the stromal complex that facilitates protein translocation across the inner membrane [96]. It possesses a single α-helical transmembrane span at its amino-terminal end, while the rest of the protein is hydrophilic and oriented towards the stroma [96,97]. This stromal part contains a putative TPR domain (in a central region of the sequence) followed by a C-terminal domain with sequence similarity to the eukaryotic cochaperones Hip (*H*sp70-*i*nteracting *p*rotein) and Hop/Sti1 [96,98]. While the so-called Sti1 (Hip/Hop) domain can be functionally replaced by that of human Hip [98], the identity of the central region as a true TPR domain has been drawn into question by the alternative suggestion that this region of Tic40 contains a second Sti1 domain [99]. Regardless of this issue, the central (putative TPR) region of Tic40 is involved in its binding to Tic110, which is stimulated in the presence of a preprotein, while the C-terminal Sti1 domain engages with Hsp93 [100].

Cross-linking, pull-down, yeast two-hybrid, and bimolecular fluorescence complementation assays all demonstrated the close proximity of Tic110 with Tic40 [97,98,100]. Additional crosslinking showed that Tic110 and Tic40 associate with preproteins at a late stage in the import mechanism, similar to Hsp93 [96]. In *Arabidopsis*, *tic40* null mutants are pale due to retarded chloroplast biogenesis, and display defects in protein import at the level of translocation across the inner envelope membrane [96]. *Arabidopsis* double mutants (i.e., *tic40 tic110* and *tic40 hsp93*) did not display additive effects, supporting the idea that these proteins cooperate functionally in vivo [91].

4.2. Protein import motors

4.2.1. Hsp93 as the driving force

Until recently, stromal ATP consumption in the protein import motor was attributed primarily to the chaperone Hsp93. Immunoprecipitation analysis, in the presence or absence of cross-linkers, revealed

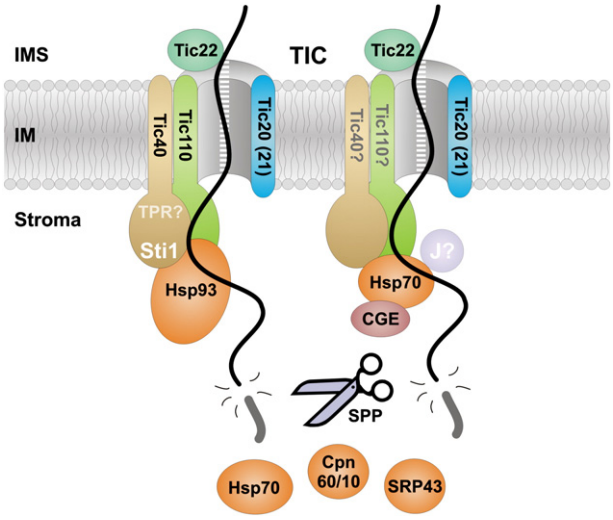

Fig. 2. Chaperone involvement in the stroma during chloroplast protein import. Preproteins arriving from the intermembrane space pass through the TIC channel, which may be formed by Tic110, Tic20 and/or Tic21; the white, vertical dotted line through the center of the channel indicates that Tic110 and Tic20/21 are unlikely to cooperate in channel formation. It is proposed that passage through the import machinery is facilitated by two different ATP-driven, stromal motor complexes. Both are associated with the TIC apparatus, and it is suggested that they act in parallel. In the first of these motors, Tic110 cooperates with Tic40 and the Hsp100-type chaperone, Hsp93. The Tic40 cochaperone associates with Tic110 via its putative TPR domain, while its Sti1 (Hip/Hop) domain engages Hsp93 to stimulate ATP hydrolysis, such that the preprotein is pulled into the stroma. In the second import motor, Hsp70 supplies the driving force for protein import. Whether Tic110 and Tic40 are functional components of this motor remains to be seen. Evidence suggests that Hsp70 activity is facilitated by the nucleotide exchange factor CGE, but a possible J-domain protein has not yet been identified. Upon arrival in the stroma, the transit peptide is cleaved by the stromal processing peptidase (SPP). Folding, assembly or onward transport inside the chloroplast is facilitated by other chaperones, including Hsp70, chaperonins (Cpn60/10) and cpSRP43. Translocating preproteins are represented by wavy black lines, while their transit peptides are shown as thick gray lines. Functional domains of certain proteins are indicated in white text. TIC, translocon at the inner envelope membrane of chloroplasts; IMS, intermembrane space; IM, inner envelope membrane.

the interaction of Hsp93 with Tic110 and components of the TOC complex [101,102]. The Hsp93 protein was also found in precursor-containing complexes under limiting ATP conditions, and could be immunoprecipitated with proteins that utilize the TOC/TIC import apparatus, but not with an outer membrane protein that does not use this import machinery [102]. In addition, a stable association of Hsp93 with transit peptides in vitro has been described [103]. It was recently shown that it is the amino-terminal region of Hsp93 that is important for its association with the inner envelope membrane in vivo [104].

The involvement of this chaperone in protein import has been supported by genetic studies. In *Arabidopsis*, there are two isoforms of Hsp93, termed Hsp93-V and Hsp93-III (or ClpC1 and ClpC2, respectively). The two proteins share very high levels of amino acid sequence identity, but the expression of Hsp93-V is much higher than that of Hsp93-III [91,105]. *Arabidopsis hsp93-V* knockout plants are pale, with underdeveloped chloroplasts containing fewer thylakoid membranes and displaying reduced protein import efficiency. In contrast, *hsp93-III* knockout mutants are indistinguishable from wild type; this can be explained by redundancy, as *hsp93-III hsp93-V* double mutants are embryo lethal and overexpression of Hsp93-III can complement *hsp93-V*, suggesting that the two proteins have overlapping functions and are able to partially substitute for each other in the single mutants [91,105–107].

Hsp93/ClpC is a member of the Hsp100 family of chaperones, which itself belongs to the broader AAA+ (*A*TPases *A*ssociated with various cellular *A*ctivities) superfamily. Hsp100 proteins contain one or two AAA+ domains, and typically assemble into hexameric rings with a central pore through which substrate proteins can be threaded (reviewed in [26,108]). Hsp100 proteins mediate ATP-dependent unfolding of proteins, in processes linked to protein degradation, protein disassembly, or protein trafficking across membranes [109]. In chloroplasts, Hsp93 can form part of the Clp protease complex (in a second role, additional to that in preprotein import), which recognizes and unfolds substrate proteins that are destined for degradation [110]; interaction of Hsp93 with the proteolytic ClpP core is ATP dependent [111]. The Hsp93-V/ClpC1 isoform has been identified as a dimeric complex of ~200 kD in the stroma of *Arabidopsis* chloroplasts [112], but the hexameric state has proved difficult to detect in vivo [113,114]. Recently, recombinant Hsp93-III/ClpC2 in solution was shown to be in dimeric form; upon addition of ATP, the hexamer state was observed [103].

The current model for protein import assumes that, as the transit peptide of an importing preprotein emerges from the TIC channel, it binds to the stromal domain of Tic110. This binding causes a conformational change in Tic110 and enables recruitment of Tic40, which binds Tic110 via its putative TPR domain [94,100]. The Tic110–Tic40 interaction triggers the release of the transit peptide from Tic110 and enables the association of the preprotein with Hsp93. Tic110 may dissociate from Tic40 when there is no transit peptide bound. The Tic40 Sti1 domain then stimulates ATP hydrolysis by Hsp93, which acts to pull the preprotein into the stroma using the released energy [100]. Subsequently, Hsp93-ADP may dissociate from Tic40. In this model, Tic40 seems to arrange the last steps of envelope translocation, by regulating the interaction of the preprotein with Tic110 and Hsp93, and by controlling the activity of Hsp93 [96,100] (Fig. 2).

By analogy with other Hsp100 proteins, TIC-associated Hsp93 may act as a hexamer, and by threading incoming preproteins through the axial channel of the complex. Such a threading mechanism would be facilitated by oscillating loops within the central channel (reviewed in [108,109]). Structural and functional studies on other Hsp100s led to the proposal that such loops translocate bound sections of a client protein axially down the channel, in response to the hydrolysis of ATP, thus applying a mechanical pulling force; this pulling action is associated with an unfolding force, since the substrate protein is forced to enter a narrow channel [26,108]. Such a model would make sense in relation to chloroplast import, considering the involvement of an Hsp100 protein (Hsp93) and that preproteins presumably emerge from the TIC channel in a largely unfolded state.

Based on genetic evidence derived using mutant yeast, an Hsp100 protein (Hsp78) was also proposed to act in mitochondrial protein import, by substituting for the main motor chaperone (mtHsp70; see Section 4.2.2) under certain conditions [115]. However, later analyses supported an alternative interpretation of the data that did not link Hsp78 to import [116].

4.2.2. Hsp70 as the driving force

Unlike Hsp100s, Hsp70-type chaperones do not oligomerize. They transiently associate with exposed hydrophobic segments of client proteins via a carboxy-terminal substrate binding domain (SBD), thereby preventing aggregation and promoting proper folding. Binding of ATP to the Hsp70 amino-terminal nucleotide binding domain (NBD) induces conformational changes in the adjacent SBD, opening up the substrate binding pocket, whereas ATP hydrolysis leads to closure of the pocket and stabilizes the client interaction [108]. Chloroplast stromal Hsp70s are believed to exist in a variety of plant species, including *Arabidopsis*, pea, poplar, rice, sorghum and moss [81]. And yet, the involvement of Hsp70 chaperones in chloroplast protein import was, until recently, only suggested to occur in the cytosolic and IMS compartments, as discussed above. This is because an early study could detect Hsp93, but not Hsp70, in association with translocating preproteins [102]; the failure of this early work to detect Hsp70 in import complexes was recently attributed to an inadequacy of the antibody originally used [117].

As was discussed earlier, most chloroplast transit peptides have the capacity to bind Hsp70s [42–44]. The interaction between stromal Hsp70 and the transit peptide, however, seems not to be crucial for protein import, since preproteins with decreased Hsp70 binding affinity in their transit peptides are efficiently imported into pea chloroplasts in vitro [45,46]. Nonetheless, recent studies provided genetic and biochemical evidence for the participation of stromal Hsp70 in the process of protein import [117,118] (Fig. 2).

Shi and Theg [118] identified three stromal Hsp70s in the moss *Physcomitrella patens*. The Hsp70-2 isoform is essential for moss viability, as Hsp70-2 knockout plants could not be isolated; this also indicated that neither of the other two isoforms, Hsp70-1 and Hsp70-3, can substitute the loss of Hsp70-2 despite their high degree of similarity. In order to study the participation of Hsp70-2 in protein import, a temperature-sensitive *hsp70-2* knockout mutant was generated. After heat-shock treatment, it was observed that isolated chloroplasts containing the temperature-sensitive Hsp70-2 protein display lower import competence when compared with wild-type chloroplasts [118]. In addition, moss Hsp70-2 could be immunoprecipitated together with preproteins arrested as early import intermediates, as well as with other translocon components (i.e., Hsp93 and Tic40), supporting the participation of Hsp70 in the TOC/TIC import pathway [118].

It is well established that Hsp70s invariably require a J-domain protein and, almost always, a nucleotide exchange factor (e.g., GrpE) as partners in order to be completely functional [119]. Thus, a second strategy in the aforementioned moss study [118] was to generate a mutant (*lcge*) with significantly reduced levels of stromal cochaperones termed CGEs (*C*hloroplast *G*rp*E* homologues). Chloroplasts isolated from the *lcge* mutant were defective in protein import, strongly suggesting that one of the CGE chaperone partners (i.e., an Hsp70) has an essential role in protein import [118] (Fig. 2).

Studies in *Arabidopsis* and pea also supported the involvement of stromal Hsp70s in the protein import mechanism. Protein import assays using chloroplasts isolated from the *Arabidopsis* Hsp70 knockout mutants, *cpshsc70-1* and *cphsc70-2*, showed that stromal Hsp70 is important for the import of both photosynthetic and non-photosynthetic precursor proteins, especially at early developmental stages [117]. What is more, pea cpHsc70 could be immunoprecipitated together

with the newly-imported preproteins and translocon components (such as Tic110), in the presence or absence of cross-linkers, strongly suggesting that cpHsc70 is part of the translocon [117].

The relationship between Hsp70 involvement and the Hsp93/Tic40 system discussed earlier was also assessed genetically in *Arabidopsis* [117]. The function of Hsp70 seems to be related to that of Hsp93 in driving translocation, as *cphsc70-1 hsp93-V* double mutants show stronger defects in chlorophyll content and import efficiency than single mutants. More strikingly, the *cphsc70-1 tic40* double mutation is lethal, indicating that Hsp70 and Tic40 share an overlapping and essential function. Perhaps Hsp70 and Hsp93/Tic40 perform the same function in parallel during protein import. Were the role of cpHsc70 only to facilitate the assembly or functioning of Hsp93/Tic40, then the *cphsc70-1 tic40* double mutant would be expected to display the same phenotype of the *tic40* single mutant, not lethality.

Considering all of the in vivo and in vitro data presented above, it seems that the stromal Hsp70 system is involved in protein translocation into chloroplasts, and that this mechanism is conserved from moss to higher plants [117,118]. It is not surprising that this Hsp70 function exists in chloroplasts, as the protein import motor in mitochondria is driven by a matrix Hsp70 chaperone termed mtHsp70. Like the TIC complex, the presequence translocase in mitochondria consists of several subunits, which contribute to the import channel complex (e.g., Tim23 and Tim17) or act as an import motor (Tim44, Tim16, Tim14, mtHsp70, Mge1) [15–17]. The resemblance between the Hsp70-driven motors in chloroplasts and mitochondria is evident from the following. After initial translocation through the Tim23/Tim17 core complex, mitochondrial preproteins contact subunits of the PAM (*P*resequence translocase-*A*ssociated import *M*otor) machinery, which provides the driving force for import. The Tim44 component is an essential protein peripherally associated with the inner surface of the inner membrane, and it serves to recruit mtHsp70 from the matrix to the import complex. Once recruited, the mtHsp70 chaperone binds to an emerging precursor, and then hydrolyses ATP, which provides the driving force for import, as discussed below. Additional PAM components are Tim14 and Tim16 (also called Pam18 and Pam16, respectively), which are integral inner membrane cochaperones that regulate the ATP-hydrolysis cycle of mtHsp70: Tim14 presents a J-domain at the matrix side of the inner membrane that, in cooperation with Tim16, stimulates the ATPase activity of mtHsp70. The nucleotide exchange factor Mge1 (*M*itochondrial *G*rp*E*-related protein *1*) is also required in this process.

There are competing models for the delivery of power in the mitochondrial import motor [120,121]. In the Brownian ratchet model, precursor-bound mtHsp70-ADP immediately dissociates from Tim44 so that random motion (Brownian oscillations) may cause a segment of the precursor to move forwards into the matrix. A second mtHsp70 associated with Tim44 then passively traps the newly-translocated segment, preventing backsliding into the channel. The alternative power-stroke model suggests that the import motor acts as a mechanical machine that actively pulls on the incoming precursor. According to this model, ATP hydrolysis induces a conformational change in mtHsp70 that generates inward movement of the bound precursor. It is possible that elements of both models are correct, or that the two mechanisms cooperate as follows: for loosely-folded or unfolded preproteins, the ratchet mechanism could be sufficient, whereas for preproteins with stably-folded domains an additional pulling force might be required [120,121].

Stromal Hsp70 might provide the driving force in chloroplast protein import in a similar fashion to mtHsp70 in the mitochondrial import motor. However, the extent to which the chloroplast Hsp70 system requires similar cochaperones needs to be established: while the involvement of the Mge1-related nucleotide exchange factor CGE1 has been shown in moss [118], information on J-protein involvement remains elusive [77]. Moreover, it remains to be determined whether a recruiting protein, similar to Tim44 for mtHsp70, is involved. Despite the evident similarities between the chloroplast and mitochondrial motors, a unique feature of the chloroplast system is the fact that there are two chaperone systems working in parallel (Fig. 2). The biological meaning of the presence of two motors has yet to be established, but it may relate to the fact that the two chaperones types (i.e., Hsp100 and Hsp70) have very different modes of action, as was discussed earlier. Perhaps the two chaperone systems provide necessary versatility to the organelle and its import machinery [117]. It is well known that both Hsp93 and Hsp70 have other functions in chloroplasts. Hsp93/ClpC acts as a regulatory chaperone in the Clp protease. Several genetic and biochemical studies have shown that the Clp protease is essential for chloroplast development and plant viability [122]. Hsp70 also displays other essential roles in chloroplast development [123]. Whether the Hsp93 and Hsp70 motors have different preprotein binding preferences, or are required in different plastids or developmental conditions, remains to be seen.

4.3. Chaperone involvement following the completion of import

After preproteins delivered into the stroma have been processed by SPP, they may require assistance to fold properly into their functional conformation, or to reach their final intraorganellar destination. The stromal molecular chaperones Hsp70, Cpn60 and Cpn10 are all believed to mediate the folding or onward guidance of newly-imported polypeptide chains [29,124] (Fig. 2). In addition, it has been reported that Hsp93 facilitates the stromal passage of imported proteins destined to the inner envelope membrane via the post-import (or conservative sorting) pathway [125].

Chloroplast Cpn60 is a homologue of the bacterial Hsp60-type chaperone GroEL [26,108]. Such chaperones assemble into two stacked heptameric rings, and cooperate with a cochaperone termed Cpn10 (an Hsp10/GroES homologue), which may form heptameric caps at either end of the Cpn60 tetradecamer. Client proteins enter a central cavity in the Cpn60 complex, providing a protected environment in which folding can take place, which is controlled by the nucleotide-dependent cycling of Cpn60 subunits between binding-active and folding-active states [26,108]. The role of the Cpn60/Cpn10 chaperonin system in the folding of Rubisco subunits has been well documented [124]. Cpn20 is a chloroplast-specific cochaperone comprising two, tandemly-arranged Cpn10-like units; while in vitro assays have shown that it is a functional homologue of Cpn10, its specific role in vivo remains uncertain [126].

Immunoprecipitation experiments revealed that Cpn60 is in close proximity with Tic110 [95]. This interaction was disrupted in the presence of ATP, while import experiments revealed a transient association of mature, newly-imported proteins with the Cpn60–Tic110 complex, suggesting that Tic110 can recruit Cpn60 in an ATP-dependent manner for the folding of proteins upon their arrival in the stroma. Biochemical data also support the interaction of newly-imported proteins with stromal Hsp70, either before or after the interaction with Cpn60 [127,128]. It has been suggested that stromal Hsp70 and Cpn60 act sequentially to assist the maturation of imported proteins, particularly those destined for the thylakoid membranes. Another factor that facilitates the passage of new proteins through the stroma to the thylakoids is cpSRP (*c*hloro*p*last *S*ignal *R*ecognition *P*article), the clients of which are light-harvesting chlorophyll-binding proteins. In fact, one of the cpSRP components, a protein termed cpSRP43, was recently reported to have unique chaperone-like properties [129,130] (Fig. 2).

5. Conclusions

Molecular chaperones participate in a host of different and essential processes in cells. Their flexibility allows them to act at various, distinct stages throughout the process of chloroplast protein import, where they perform a diversity of roles that include guidance, maintenance of

structural competence for transport, and provision of a driving force. Cytosolic chaperones may be the first contact for chloroplast preproteins following their emergence from the ribosome, and these are proposed to assist docking at different receptors at the chloroplast surface. Although the involvement of Hsp70 in the intermembrane space remains inconclusive, the participation of other molecular chaperones at the stromal side of the envelope, in so-called motor complexes, is strongly supported by different lines of evidence. While Hsp93 was for many years considered to be the primary chaperone of stromal motor complexes, recent genetic and biochemical studies strongly suggest that stromal Hsp70 also has a significant role to play. Challenges for the future include the elaboration of cytosolic events leading to docking, identification of the putative intermembrane space ATPase, elucidation of the mechanisms that enable two different stromal motor complexes to operate, and the definition of the specific biological conditions during which the different motors operate.

Acknowledgements

We are grateful to the Biotechnology and Biological Sciences Research Council (BBSRC; grant no. BB/F020325/1) for financial support. We thank Qihua Ling for helpful comments on the manuscript.

References

[1] J.M. Whatley, A suggested cycle of plastid developmental interrelationships, New Phytol. 80 (1978) 489–502.
[2] E. López-Juez, K.A. Pyke, Plastids unleashed: their development and their integration in plant development, Int. J. Dev. Biol. 49 (2005) 557–577.
[3] A. Reyes-Prieto, A.P. Weber, D. Bhattacharya, The origin and establishment of the plastid in algae and plants, Annu. Rev. Genet. 41 (2007) 147–168.
[4] A.W. Larkum, P.J. Lockhart, C.J. Howe, Shopping for plastids, Trends Plant Sci. 12 (2007) 189–195.
[5] W. Martin, T. Rujan, E. Richly, A. Hansen, S. Cornelsen, T. Lins, D. Leister, B. Stoebe, M. Hasegawa, D. Penny, Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12246–12251.
[6] J.N. Timmis, M.A. Ayliffe, C.Y. Huang, W. Martin, Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes, Nat. Rev. Genet. 5 (2004) 123–135.
[7] K. Keegstra, K. Cline, Protein import and routing systems of chloroplasts, Plant Cell 11 (1999) 557–570.
[8] D. Leister, Chloroplast research in the genomic age, Trends Genet. 19 (2003) 47–56.
[9] J. Soll, E. Schleiff, Protein import into chloroplasts, Nat. Rev. Mol. Cell Biol. 5 (2004) 198–208.
[10] F. Kessler, D.J. Schnell, The function and diversity of plastid protein import pathways: a multilane GTPase highway into plastids, Traffic 7 (2006) 248–257.
[11] P. Jarvis, Targeting of nucleus-encoded proteins to chloroplasts in plants (Tansley review), New Phytol. 179 (2008) 257–285.
[12] H.M. Li, C.C. Chiu, Protein transport into chloroplasts, Annu. Rev. Plant Biol. 61 (2010) 157–180.
[13] M. Gutensohn, E. Fan, S. Frielingsdorf, P. Hanner, B. Hou, B. Hust, R.B. Klösgen, Toc, Tic, Tat et al.: structure and function of protein transport machineries in chloroplasts, J. Plant Physiol. 163 (2006) 333–347.
[14] K. Cline, C. Dabney-Smith, Plastid protein import and sorting: different paths to the same compartments, Curr. Opin. Plant Biol. 11 (2008) 585–592.
[15] W. Neupert, J.M. Herrmann, Translocation of proteins into mitochondria, Annu. Rev. Biochem. 76 (2007) 723–749.
[16] O. Schmidt, N. Pfanner, C. Meisinger, Mitochondrial protein import: from proteomics to functional mechanisms, Nat. Rev. Mol. Cell Biol. 11 (2010) 655–667.
[17] E. Schleiff, T. Becker, Common ground for protein translocation: access control for mitochondria and chloroplasts, Nat. Rev. Mol. Cell Biol. 12 (2011) 48–59.
[18] S.C. Hinnah, R. Wagner, N. Sveshnikova, R. Harrer, J. Soll, The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides, Biophys. J. 83 (2002) 899–911.
[19] S.A. Clark, S.M. Theg, A folded protein can be transported across the chloroplast envelope and thylakoid membranes, Mol. Biol. Cell 8 (1997) 923–934.
[20] S.E. Perry, K. Keegstra, Envelope membrane proteins that interact with chloroplastic precursor proteins, Plant Cell 6 (1994) 93–105.
[21] L.J. Olsen, S.M. Theg, B.R. Selman, K. Keegstra, ATP is required for the binding of precursor proteins to chloroplasts, J. Biol. Chem. 264 (1989) 6724–6729.
[22] L.J. Olsen, K. Keegstra, The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space, J. Biol. Chem. 267 (1992) 433–439.
[23] M.E. Young, K. Keegstra, J.E. Froehlich, GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts, Plant Physiol. 121 (1999) 237–244.
[24] S.M. Theg, C. Bauerle, L.J. Olsen, B.R. Selman, K. Keegstra, Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes, J. Biol. Chem. 264 (1989) 6730–6736.
[25] D. Pain, G. Blobel, Protein import into chloroplasts requires a chloroplast ATPase, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 3288–3292.
[26] M.P. Mayer, Gymnastics of molecular chaperones, Mol. Cell 39 (2010) 321–331.
[27] J. Uniacke, W. Zerges, Chloroplast protein targeting involves localized translation in *Chlamydomonas*, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 1439–1444.
[28] T. May, J. Soll, 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants, Plant Cell 12 (2000) 53–64.
[29] D. Jackson-Constan, M. Akita, K. Keegstra, Molecular chaperones involved in chloroplast protein import, Biochim. Biophys. Acta 1541 (2001) 102–113.
[30] S. Lee, D.W. Lee, Y. Lee, U. Mayer, Y.D. Stierhof, G. Jurgens, I. Hwang, Heat shock protein cognate 70-4 and an E3 ubiquitin ligase, CHIP, mediate plastid-destined precursor degradation through the ubiquitin-26S proteasome system in *Arabidopsis*, Plant Cell 21 (2009) 3984–4001.
[31] G. Van den Broeck, M.P. Timko, A.P. Kausch, A.R. Cashmore, M. Van Montagu, L. Herrera-Estrella, Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase, Nature 313 (1985) 358–363.
[32] D.W. Lee, S. Lee, Y.J. Oh, I. Hwang, Multiple sequence motifs in the rubisco small subunit transit peptide independently contribute to Toc159-dependent import of proteins into chloroplasts, Plant Physiol. 151 (2009) 129–141.
[33] B.D. Bruce, Chloroplast transit peptides: structure, function and evolution, Trends Cell Biol. 10 (2000) 440–447.
[34] B.D. Bruce, The paradox of plastid transit peptides: conservation of function despite divergence in primary structure, Biochim. Biophys. Acta 1541 (2001) 2–21.
[35] S. Bhushan, C. Kuhn, A.K. Berglund, C. Roth, E. Glaser, The role of the N-terminal domain of chloroplast targeting peptides in organellar protein import and miss-sorting, FEBS Lett. 580 (2006) 3966–3972.
[36] D. Macasev, E. Newbigin, J. Whelan, T. Lithgow, How do plant mitochondria avoid importing chloroplast proteins? Components of the import apparatus Tom20 and Tom22 from Arabidopsis differ from their fungal counterparts, Plant Physiol. 123 (2000) 811–816.
[37] Y. Abe, T. Shodai, T. Muto, K. Mihara, H. Torii, S. Nishikawa, T. Endo, D. Kohda, Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20, Cell 100 (2000) 551–560.
[38] H.L. Wienk, R.W. Wechselberger, M. Czisch, B. de Kruijff, Structure, dynamics, and insertion of a chloroplast targeting peptide in mixed micelles, Biochemistry 39 (2000) 8219–8227.
[39] I. Krimm, P. Gans, J.F. Hernandez, G.J. Arlaud, J.M. Lancelin, A coil-helix instead of a helix-coil motif can be induced in a chloroplast transit peptide from *Chlamydomonas reinhardtii*, Eur. J. Biochem. 265 (1999) 171–180.
[40] G. von Heijne, K. Nishikawa, Chloroplast transit peptides. The perfect random coil? FEBS Lett. 278 (1991) 1–3.
[41] R.A. Ivey III, B.D. Bruce, In vivo and in vitro interaction of DnaK and a chloroplast transit peptide, Cell Stress Chaperones 5 (2000) 62–71.
[42] R.A. Ivey III, C. Subramanian, B.D. Bruce, Identification of a Hsp70 recognition domain within the rubisco small subunit transit peptide, Plant Physiol. 122 (2000) 1289–1299.
[43] D.V. Rial, A.K. Arakaki, E.A. Ceccarelli, Interaction of the targeting sequence of chloroplast precursors with Hsp70 molecular chaperones, Eur. J. Biochem. 267 (2000) 6239–6248.
[44] X.P. Zhang, E. Glaser, Interaction of plant mitochondrial and chloroplast signal peptides with the Hsp70 molecular chaperone, Trends Plant Sci. 7 (2002) 14–21.
[45] D.V. Rial, J. Ottado, E.A. Ceccarelli, Precursors with altered affinity for Hsp70 in their transit peptides are efficiently imported into chloroplasts, J. Biol. Chem. 278 (2003) 46473–46481.
[46] D.V. Rial, A.K. Arakaki, A.M. Almará, E.G. Orellano, E.A. Ceccarelli, Chloroplast Hsp70s are not involved in the import of ferredoxin-NADP$^+$ reductase precursor, Physiol. Plant. 128 (2006) 618–632.
[47] T. Gokirmak, A.L. Paul, R.J. Ferl, Plant phosphopeptide-binding proteins as signaling mediators, Curr. Opin. Plant Biol. 13 (2010) 527–532.
[48] T. Martin, R. Sharma, C. Sippel, K. Waegemann, J. Soll, U.C. Vothknecht, A protein kinase family in *Arabidopsis* phosphorylates chloroplast precursor proteins, J. Biol. Chem. 281 (2006) 40216–40223.
[49] K.A. Nakrieko, R.M. Mould, A.G. Smith, Fidelity of targeting to chloroplasts is not affected by removal of the phosphorylation site from the transit peptide, Eur. J. Biochem. 271 (2004) 509–516.
[50] D.W. Lee, S. Lee, G.J. Lee, K.H. Lee, S. Kim, G.W. Cheong, I. Hwang, Functional characterization of sequence motifs in the transit peptide of Arabidopsis small subunit of Rubisco, Plant Physiol. 140 (2006) 466–483.
[51] G. Lamberti, I.L. Gugel, J. Meurer, J. Soll, S. Schwenkert, The cytosolic kinases STY8, STY17, and STY46 are involved in chloroplast differentiation in Arabidopsis, Plant Physiol. 157 (2011) 70–85.
[52] S.P. Cleary, F.C. Tan, K.A. Nakrieko, S.J. Thompson, P.M. Mullineaux, G.P. Creissen, E. von Stedingk, E. Glaser, A.G. Smith, C. Robinson, Isolated plant mitochondria import chloroplast precursor proteins in vitro with the same efficiency as chloroplasts, J. Biol. Chem. 277 (2002) 5562–5569.
[53] P. Marc, A. Margeot, F. Devaux, C. Blugeon, M. Corral-Debrinski, C. Jacq, Genome-wide analysis of mRNAs targeted to yeast mitochondria, EMBO Rep. 3 (2002) 159–164.
[54] O. Chew, J. Whelan, Just read the message: a model for sorting of proteins between mitochondria and chloroplasts, Trends Plant Sci. 9 (2004) 318–319.
[55] J.C. Young, N.J. Hoogenraad, F.U. Hartl, Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70, Cell 112 (2003) 41–50.

[56] S. Qbadou, T. Becker, O. Mirus, I. Tews, J. Soll, E. Schleiff, The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64, EMBO J. 25 (2006) 1836–1847.
[57] S. Qbadou, T. Becker, T. Bionda, K. Reger, M. Ruprecht, J. Soll, E. Schleiff, Toc64 — a preprotein-receptor at the outer membrane with bipartide function, J. Mol. Biol. 367 (2007) 1330–1346.
[58] C. Fellerer, R. Schweiger, K. Schongruber, J. Soll, S. Schwenkert, Cytosolic HSP90 cochaperones HOP and FKBP interact with freshly synthesized chloroplast preproteins of Arabidopsis, Mol. Plant 4 (2011) 1133–1145.
[59] N.R. Hofmann, S.M. Theg, Toc64 is not required for import of proteins into chloroplasts in the moss *Physcomitrella patens*, Plant J. 43 (2005) 675–687.
[60] H. Aronsson, P. Boij, R. Patel, A. Wardle, M. Töpel, P. Jarvis, Toc64/OEP64 is not essential for the efficient import of proteins into chloroplasts in *Arabidopsis thaliana*, Plant J. 52 (2007) 53–68.
[61] K. Sohrt, J. Soll, Toc64, a new component of the protein translocon of chloroplasts, J. Cell Biol. 148 (2000) 1213–1221.
[62] E. Schleiff, J. Soll, M. Küchler, W. Kuhlbrandt, R. Harrer, Characterization of the translocon of the outer envelope of chloroplasts, J. Cell Biol. 160 (2003) 541–551.
[63] V. Hines, A. Brandt, G. Griffiths, H. Horstmann, H. Brutsch, G. Schatz, Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70, EMBO J. 9 (1990) 3191–3200.
[64] O. Chew, R. Lister, S. Qbadou, J.L. Heazlewood, J. Soll, E. Schleiff, A.H. Millar, J. Whelan, A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor, FEBS Lett. 557 (2004) 109–114.
[65] R. Lister, C. Carrie, O. Duncan, L.H. Ho, K.A. Howell, M.W. Murcha, J. Whelan, Functional definition of outer membrane proteins involved in preprotein import into mitochondria, Plant Cell 19 (2007) 3739–3759.
[66] O. von Loeffelholz, V. Kriechbaumer, R.A. Ewan, R. Jonczyk, S. Lehmann, J.C. Young, B.M. Abell, OEP61 is a chaperone receptor at the plastid outer envelope, Biochem. J. 438 (2011) 143–153.
[67] V. Kriechbaumer, A. Tsargorodskaya, M.K. Mustafa, T. Vinogradova, J. Lacey, D.P. Smith, B.M. Abell, A. Nabok, Study of receptor–chaperone interactions using the optical technique of spectroscopic ellipsometry, Biophys. J. 101 (2011) 504–511.
[68] W. Bae, Y.J. Lee, D.H. Kim, J. Lee, S. Kim, E.J. Sohn, I. Hwang, AKR2A-mediated import of chloroplast outer membrane proteins is essential for chloroplast biogenesis, Nat. Cell Biol. 10 (2008) 220–227.
[69] J. Bédard, P. Jarvis, Green light for chloroplast outer-membrane proteins, Nat. Cell Biol. 10 (2008) 120–122.
[70] N.R. Hofmann, S.M. Theg, Chloroplast outer membrane protein targeting and insertion, Trends Plant Sci. 10 (2005) 450–457.
[71] S.L. Tu, L.J. Chen, M.D. Smith, Y.S. Su, D.J. Schnell, H.M. Li, Import pathways of chloroplast interior proteins and the outer-membrane protein OEP14 converge at Toc75, Plant Cell 16 (2004) 2078–2088.
[72] D.H. Kim, Z.Y. Xu, Y.J. Na, Y.J. Yoo, J. Lee, E.J. Sohn, I. Hwang, Small heat shock protein Hsp17.8 functions as an AKR2A cofactor in the targeting of chloroplast outer membrane proteins in Arabidopsis, Plant Physiol. 157 (2011) 132–146.
[73] G. Shen, S. Kuppu, S. Venkataramani, J. Wang, J. Yan, X. Qiu, H. Zhang, Ankyrin repeat-containing protein 2A is an essential molecular chaperone for peroxisomal membrane-bound ascorbate peroxidase3 in *Arabidopsis*, Plant Cell 22 (2010) 811–831.
[74] H. Zhang, X. Li, Y. Zhang, S. Kuppu, G. Shen, Is AKR2A an essential molecular chaperone for a class of membrane-bound proteins in plants? Plant Signal Behav. 5 (2010) 1520–1522.
[75] T. Becker, J. Hritz, M. Vogel, A. Caliebe, B. Bukau, J. Soll, E. Schleiff, Toc12, a novel subunit of the intermembrane space preprotein translocon of chloroplasts, Mol. Biol. Cell 15 (2004) 5130–5144.
[76] A. Kouranov, X. Chen, B. Fuks, D.J. Schnell, Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane, J. Cell Biol. 143 (1998) 991–1002.
[77] C.C. Chiu, L.J. Chen, H.M. Li, Pea chloroplast DnaJ-J8 and Toc12 are encoded by the same gene and localized in the stroma, Plant Physiol. 154 (2010) 1172–1182.
[78] K.M. Chen, M. Holmstrom, W. Raksajit, M. Suorsa, M. Piippo, E.M. Aro, Small chloroplast-targeted DnaJ proteins are involved in optimization of photosynthetic reactions in *Arabidopsis thaliana*, BMC Plant Biol. 10 (2010) 43.
[79] J.S. Marshall, A.E. DeRocher, K. Keegstra, E. Vierling, Identification of heat shock protein hsp70 homologues in chloroplasts, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 374–378.
[80] D.J. Schnell, F. Kessler, G. Blobel, Isolation of components of the chloroplast protein import machinery, Science 266 (1994) 1007–1012.
[81] P.H. Su, H.M. Li, Arabidopsis stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds, Plant Physiol. 146 (2008) 1231–1241.
[82] R.M. Ratnayake, H. Inoue, H. Nonami, M. Akita, Alternative processing of Arabidopsis Hsp70 precursors during protein import into chloroplasts, Biosci. Biotechnol. Biochem. 72 (2008) 2926–2935.
[83] E. Kovacs-Bogdan, J. Soll, B. Bölter, Protein import into chloroplasts: the Tic complex and its regulation, Biochim. Biophys. Acta 1803 (2010) 740–747.
[84] D. Duy, R. Stube, G. Wanner, K. Philippar, The chloroplast permease PIC1 regulates plant growth and development by directing homeostasis and transport of iron, Plant Physiol. 155 (2011) 1709–1722.
[85] D. Duy, G. Wanner, A.R. Meda, N. von Wiren, J. Soll, K. Philippar, PIC1, an ancient permease in *Arabidopsis* chloroplasts, mediates iron transport, Plant Cell 19 (2007) 986–1006.
[86] L. Heins, A. Mehrle, R. Hemmler, R. Wagner, M. Küchler, F. Hörmann, D. Sveshnikov, J. Soll, The preprotein conducting channel at the inner envelope membrane of plastids, EMBO J. 21 (2002) 2616–2625.
[87] Y.S. Teng, Y.S. Su, L.J. Chen, Y.J. Lee, I. Hwang, H.M. Li, Tic21 is an essential translocon component for protein translocation across the chloroplast inner envelope membrane, Plant Cell 18 (2006) 2247–2257.
[88] M. Balsera, T.A. Goetze, E. Kovács-Bogdán, P. Schürmann, R. Wagner, B.B. Buchanan, J. Soll, B. Bölter, Characterization of Tic110, a channel-forming protein at the inner envelope membrane of chloroplasts, unveils a response to Ca^{2+} and a stromal regulatory disulfide bridge, J. Biol. Chem. 284 (2009) 2603–2616.
[89] S. Kikuchi, M. Oishi, Y. Hirabayashi, D.W. Lee, I. Hwang, M. Nakai, A 1-megadalton translocation complex containing Tic20 and Tic21 mediates chloroplast protein import at the inner envelope membrane, Plant Cell 21 (2009) 1781–1797.
[90] A. Vojta, M. Alavi, T. Becker, F. Hörmann, M. Küchler, J. Soll, R. Thomson, E. Schleiff, The protein translocon of the plastid envelopes, J. Biol. Chem. 279 (2004) 21401–21405.
[91] S. Kovacheva, J. Bédard, R. Patel, P. Dudley, D. Twell, G. Ríos, C. Koncz, P. Jarvis, In vivo studies on the roles of Tic110, Tic40 and Hsp93 during chloroplast protein import, Plant J. 41 (2005) 412–428.
[92] T. Inaba, M. Alvarez-Huerta, M. Li, J. Bauer, C. Ewers, F. Kessler, D.J. Schnell, Arabidopsis Tic110 is essential for the assembly and function of the protein import machinery of plastids, Plant Cell 17 (2005) 1482–1496.
[93] D.T. Jackson, J.E. Froehlich, K. Keegstra, The hydrophilic domain of Tic110, an inner envelope membrane component of the chloroplastic protein translocation apparatus, faces the stromal compartment, J. Biol. Chem. 273 (1998) 16583–16588.
[94] T. Inaba, M. Li, M. Alvarez-Huerta, F. Kessler, D.J. Schnell, atTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts, J. Biol. Chem. 278 (2003) 38617–38627.
[95] F. Kessler, G. Blobel, Interaction of the protein import and folding machineries of the chloroplast, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7684–7689.
[96] M.L. Chou, L.M. Fitzpatrick, S.L. Tu, G. Budziszewski, S. Potter-Lewis, M. Akita, J.Z. Levin, K. Keegstra, H.M. Li, Tic40, a membrane-anchored co-chaperone homolog in the chloroplast protein translocon, EMBO J. 22 (2003) 2970–2980.
[97] T. Stahl, C. Glockmann, J. Soll, L. Heins, Tic40, a new "old" subunit of the chloroplast protein import translocon, J. Biol. Chem. 274 (1999) 37467–37472.
[98] J. Bédard, S. Kubis, S. Bimanadham, P. Jarvis, Functional similarity between the chloroplast translocon component, Tic40, and the human co-chaperone, Hsp70-interacting protein (Hip), J. Biol. Chem. 282 (2007) 21404–21414.
[99] M. Balsera, J. Soll, B.B. Buchanan, Protein import in chloroplasts: an emerging regulatory role for redox, Adv. Bot. Res.: Oxid. Stress Redox Regul. Plants 52 (2009) 277–332.
[100] M.L. Chou, C.C. Chu, L.J. Chen, M. Akita, H.M. Li, Stimulation of transit-peptide release and ATP hydrolysis by a cochaperone during protein import into chloroplasts, J. Cell Biol. 175 (2006) 893–900.
[101] M. Akita, E. Nielsen, K. Keegstra, Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking, J. Cell Biol. 136 (1997) 983–994.
[102] E. Nielsen, M. Akita, J. Davila-Aponte, K. Keegstra, Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone, EMBO J. 16 (1997) 935–946.
[103] G.L. Rosano, E.M. Bruch, E.A. Ceccarelli, Insights into the CLP/HSP100 chaperone system from chloroplasts of *Arabidopsis thaliana*, J. Biol. Chem. 286 (2011) 29671–29680.
[104] C.C. Chu, H.M. Li, The N-terminal domain of chloroplast Hsp93 is important for its membrane association and functions in vivo, Plant Physiol. 158 (2012) 1656–1665.
[105] S. Kovacheva, J. Bédard, A. Wardle, R. Patel, P. Jarvis, Further *in vivo* studies on the role of the molecular chaperone, Hsp93, in plastid protein import, Plant J. 50 (2007) 364–379.
[106] L.L. Sjögren, T.M. MacDonald, S. Sutinen, A.K. Clarke, Inactivation of the *clpC1* gene encoding a chloroplast Hsp100 molecular chaperone causes growth retardation, leaf chlorosis, lower photosynthetic activity, and a specific reduction in photosystem content, Plant Physiol. 136 (2004) 4114–4126.
[107] D. Constan, J.E. Froehlich, S. Rangarajan, K. Keegstra, A stromal Hsp100 protein is required for normal chloroplast development and function in Arabidopsis, Plant Physiol. 136 (2004) 3605–3615.
[108] B. Bukau, J. Weissman, A. Horwich, Molecular chaperones and protein quality control, Cell 125 (2006) 443–451.
[109] R.T. Sauer, D.N. Bolon, B.M. Burton, R.E. Burton, J.M. Flynn, R.A. Grant, G.L. Hersch, S.A. Joshi, J.A. Kenniston, I. Levchenko, S.B. Neher, E.S. Oakes, S.M. Siddiqui, D.A. Wah, T.A. Baker, Sculpting the proteome with AAA+ proteases and disassembly machines, Cell 119 (2004) 9–18.
[110] J. Shanklin, N.D. DeWitt, J.M. Flanagan, The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease, Plant Cell 7 (1995) 1713–1722.
[111] T. Halperin, O. Ostersetzer, Z. Adam, ATP-dependent association between subunits of Clp protease in pea chloroplasts, Planta 213 (2001) 614–619.
[112] J.B. Peltier, D.R. Ripoll, G. Friso, A. Rudella, Y. Cai, J. Ytterberg, L. Giacomelli, J. Pillardy, K.J. van Wijk, Clp protease complexes from photosynthetic and non-photosynthetic plastids and mitochondria of plants, their predicted three-dimensional structures, and functional implications, J. Biol. Chem. 279 (2004) 4768–4781.

[113] A. Sokolenko, S. Lerbs-Mache, L. Altschmied, R.G. Herrmann, Clp protease complexes and their diversity in chloroplasts, Planta 207 (1998) 286–295.
[114] A.K. Clarke, T.M. MacDonald, L.L.E. Sjögren, The ATP-dependent Clp protease in chloroplasts of higher plants, Physiol. Plant. 123 (2005) 406–412.
[115] M. Schmitt, W. Neupert, T. Langer, The molecular chaperone Hsp78 confers compartment-specific thermotolerance to mitochondria, J. Cell Biol. 134 (1996) 1375–1386.
[116] M. Moczko, B. Schonfisch, W. Voos, N. Pfanner, J. Rassow, The mitochondrial ClpB homolog Hsp78 cooperates with matrix Hsp70 in maintenance of mitochondrial function, J. Mol. Biol. 254 (1995) 538–543.
[117] P.H. Su, H.M. Li, Stromal Hsp70 is important for protein translocation into pea and *Arabidopsis* chloroplasts, Plant Cell 22 (2010) 1516–1531.
[118] L.X. Shi, S.M. Theg, A stromal heat shock protein 70 system functions in protein import into chloroplasts in the moss *Physcomitrella patens*, Plant Cell 22 (2010) 205–220.
[119] H.H. Kampinga, E.A. Craig, The HSP70 chaperone machinery: J proteins as drivers of functional specificity, Nat. Rev. Mol. Cell Biol. 11 (2010) 579–592.
[120] W. Neupert, M. Brunner, The protein import motor of mitochondria, Nat. Rev. Mol. Cell Biol. 3 (2002) 555–565.
[121] M. van der Laan, D.P. Hutu, P. Rehling, On the mechanism of preprotein import by the mitochondrial presequence translocase, Biochim. Biophys. Acta 1803 (2010) 732–739.
[122] A.K. Clarke, The chloroplast ATP-dependent Clp protease in vascular plants — new dimensions and future challenges, Physiol. Plant. 145 (2011) 235–244.
[123] M. Latijnhouwers, X.M. Xu, S.G. Moller, Arabidopsis stromal 70-kDa heat shock proteins are essential for chloroplast development, Planta 232 (2010) 567–578.
[124] R.S. Boston, P.V. Viitanen, E. Vierling, Molecular chaperones and protein folding in plants, Plant Mol. Biol. 32 (1996) 191–222.
[125] L. Vojta, J. Soll, B. Bölter, Requirements for a conservative protein translocation pathway in chloroplasts, FEBS Lett. 581 (2007) 2621–2624.
[126] C. Weiss, A. Bonshtien, O. Farchi-Pisanty, A. Vitlin, A. Azem, Cpn20: siamese twins of the chaperonin world, Plant Mol. Biol. 69 (2009) 227–238.
[127] F. Madueño, J.A. Napier, J.C. Gray, Newly imported Rieske iron–sulfur protein associates with both Cpn60 and Hsp70 in the chloroplast stroma, Plant Cell 5 (1993) 1865–1876.
[128] R. Tsugeki, M. Nishimura, Interaction of homologues of Hsp70 and Cpn60 with ferredoxin-NADP+ reductase upon its import into chloroplasts, FEBS Lett. 320 (1993) 198–202.
[129] P. Jaru-Ampornpan, K. Shen, V.Q. Lam, M. Ali, S. Doniach, T.Z. Jia, S.O. Shan, ATP-independent reversal of a membrane protein aggregate by a chloroplast SRP subunit, Nat. Struct. Mol. Biol. 17 (2010) 696–702.
[130] S. Falk, I. Sinning, cpSRP43 is a novel chaperone specific for light-harvesting chlorophyll a, b-binding proteins, J. Biol. Chem. 285 (2010) 21655–21661.