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Minireview

Chloroplast precursor protein translocon

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Abstract Chloroplasts are believed to have originated from a photosynthetic, prokaryotic ancestor. As the result of endosymbiotic evolution, most of the genes of the endocytobiont were displaced to the host nucleus. Today's chloroplasts must import most of their proteins from the cytosol as precursors. Oligomeric protein complexes in the chloroplast outer and inner envelope membranes are responsible for the specific recognition and membrane translocation of precursor proteins. The translocon at the outer membrane of chloroplasts and the inner membrane of chloroplasts act jointly during the import process. Several translocon subunits have been partially characterized in their molecular structure and function. Initial evidence indicates the prokaryotic origin of some chloroplast translocon components.

Key words: Chloroplast; Endocytobiont; Inner envelope; Outer envelope; Protein translocon

1. Precursor protein targeting to chloroplasts

1.1. Stroma targeting signals

Proteins destined for various chloroplast subcompartments are synthesized on ribosomes in the cytosol as precursor proteins with a cleavable, amino-terminal extension called presequence or transit-peptide. The transit-peptide is both sufficient and necessary to accomplish precursor protein translocation across the chloroplast envelope membranes [1] in a post-translational event. The transit-peptide functions as an envelope transfer, stroma targeting domain. It is variable in size (20–120 amino acids) and contains no obvious blocks of conserved amino acids or secondary structure, but behaves as an ideal random coil [2]. In general, the amino-proximal portion lacks both positively charged residues as well as glycine and proline. The carboxy-terminal domain is predicted to form an amphiphilic β-strand in a hydrophobic environment (see below). Between these surrounding domains, the central portion lacks mainly acidic residues and is rich in hydroxylated amino acids like serine and threonine [3].

1.2. Cytosolic interactions

A cytosolic protein kinase phosphorylates a serine or threonine residue within a loosely conserved motif in the transit-peptides of chloroplast precursor proteins, but not in mitochondrial or peroxisomal pre-proteins of plant origin [4]. Phosphorylation of the precursor protein is not a prerequisite for translocation, but dephosphorylation is required before a phosphorylated precursor can be imported. This leads to the suggestion that a cycle of phosphorylation/dephosphorylation might be a regulating switch in the translocation pathway of chloroplast precursor proteins.

Chaperones of the Hsp70 family were shown to stimulate the import efficiency of the light harvesting chlorophyll a/b-binding protein in vitro [5], but were not required for the translocation of soluble, stromal proteins such as pre-ferredoxin (preFd) or rubisco small subunit precursor (preSSU) [6]. Until now, no further cytosolic proteins have been identified to be involved in the guidance of precursor proteins to the chloroplast surface or in the maintenance of an import competent conformation.

2. Pathway for plastid entry

The major route for precursor protein entry into plastids is the translocation through the translocon at the outer membrane of chloroplasts (Toc) and translocon at the inner membrane of chloroplasts (Tic) complex and leads into the stroma. This is termed the general import pathway (Fig. 1). All known precursor proteins use this common machinery no matter what their final chloroplast destination. While the import machinery of chloroplasts and other organelles may operate by similar principles, none of the components identified yet shares sequence homologies with those of other eukaryotic import/export systems, with the exception of the involvement of different chaperones, e.g. the Hsp70, Hsp100 and the GroEL/GroES homologue proteins. Intra-organellar transport processes into and across the thylakoids, which are not in the focus of this review, are of prokaryotic origin and their mechanisms are based on bacterial export systems (for a review [7]).

Recently, several investigators have proposed a role for transit-peptide interactions with galactolipids specific for the outer envelope membrane as the initial step of precursor-binding to the chloroplast surface (for a review [8]). The hydrophobic membrane environment would induce a regular structure in the transit sequence such as an amphiphilic β-strand (see above), which might be a relevant step in precursor-receptor recognition of the Toc complex. This hypothesis could be proven for two chloroplast precursor proteins, preFd [9] and preSSU [10]. It is supported by the observation that an *Arabidopsis thaliana* mutant deficient in digalactosyldiacylglyceride, which represents 20% of the chloroplast outer envelope lipids, exhibits a defect in chloroplast protein import [11]. Presently, it is not possible to discriminate between an instability of the Toc complex due to the different membrane lipid

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Abbreviations: Com, chloroplast outer envelope membrane; preSSU, precursor form of ribulose-1,5-bisphosphate carboxylase; preFd, precursor form of ferredoxin; Toc, translocon at the outer membrane of chloroplasts; Tic, translocon at the inner membrane of chloroplasts

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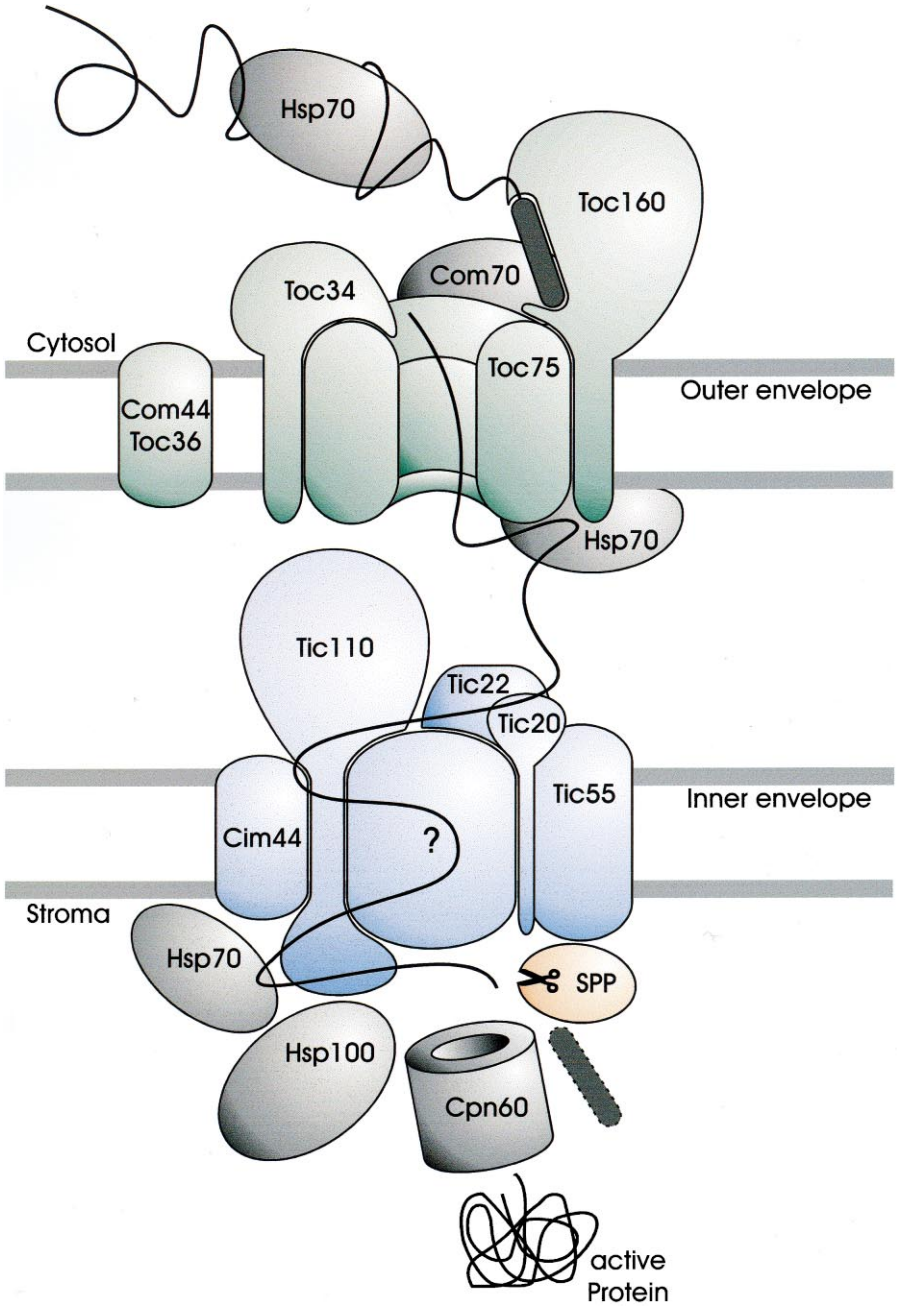

Fig. 1. Pathway for plastid entry. The general import machinery of the chloroplast envelope membranes. The components are explained in the text.

composition or an inability of the precursor to interact with the altered lipid surface in the mutant.

The initial interaction of the transit-peptide with a chloroplast surface protein is a reversible, energy-independent step [12–14]. Tight binding of the precursor to the import machinery requires the hydrolysis of ATP (<50 μM) [15] either at the outer envelope or in the intermembrane space [16,17] and might be regulated by GTP [18]. Complete translocation into the organelle cannot take place under these conditions, however, precursor proteins are inserted into the general import machinery [18] and can be co-fractionated with components of the Toc and Tic complex [19,20]. Recent biochemical studies indicate a stable association of the Toc-Tic complex independent of the presence of a translocating precursor protein [21–23]. However, at the moment, it is not determinable if contact sites seen in electron microscopy pictures [24] are actively involved in import by representing the Toc and Tic complex or have other functions, e.g. metabolic channelling.

Translocation into the organelle is accomplished only in the presence of higher ATP concentrations (>0.1 mM) in the stroma [25]. Stromal ATP hydrolysis is the only energy source required for membrane translocation. Concomitantly or shortly after translocation, the transit sequence is removed by a soluble stromal processing metallo-endopeptidase [26] and the mature form of the protein acquires biological activity after folding and assembling into suitable structures [27].

2.1. The Toc complex

The outer envelope translocon has to achieve three major functions: (1) specific recognition of the transit-peptide, (2) initiation of membrane translocation and (3) transfer of the precursor to the inner envelope translocon. Within the Toc complex, three prominent components involved in the tasks mentioned above have been identified: Toc160, Toc75 and Toc34.

Toc160 was originally identified as a 86 kDa polypeptide, Toc86 [18,28]. Recent studies have shown that Toc86 is only a carboxy-terminal proteolytic fragment of a larger polypeptide now named Toc160 [29]. Toc86 was identified as a receptor component for precursor proteins based on the following findings: Toc86 is exposed to the cytosolic surface of the outer membrane and is highly susceptible to proteolytic treatment [30–32]. It is the first binding partner of preSSU upon import into chloroplasts as shown by label-transfer crosslink experiments [32]. Additionally, Fab fragments of antibodies against Toc86 inhibit both binding and translocation [28]. Together, these data indicated that Toc86, i.e. Toc160, might function at a very early stage of translocation, most likely as a receptor for precursor proteins. However, the proposed functions for Toc86 need to be re-investigated for Toc160, although import studies demonstrate that protein translocation efficiency correlates well with the presence of an intact 160 kDa protein [29] and therefore it is most likely that Toc160 continues to serve as an import receptor. The extreme protease sensitivity together with the unidentified nature of the protease will hamper further analysis of the full-length Toc160 in situ.

Toc160 and Toc34 represent new types of GTP-binding proteins, as shown by specific photoaffinity labelling with [α-^{32}P]GTP and GTPase activity assays with heterologously expressed Toc34 [18,33]. Toc160 and Toc34 reveal considerable sequence homologies also outside the nucleotide-binding motifs. Furthermore, both proteins are phosphorylated by a protein kinase, which co-purifies with outer envelope membranes [34]. Nucleotide-binding and phosphorylation might represent further regulatory checkpoints during the translocation event.

Under oxidizing conditions, a disulfide bridge is formed between Toc34 and Toc75 as well as between Toc86 and Toc75 in vitro demonstrating their close physical proximity in situ [35]. Recently, Toc34 was shown to form a crosslink to a precursor protein in the abscence of added nucleoside triphosphates, but in the presence of the non-hydrolyzable GDP analogon, GDP-β-S. In the presence of GDP or GTP, a crosslink of Toc34 to a precursor could not be detected [36]. These experimental conditions could have resulted in an accumulation of precursor proteins at different locations of the Toc complex, which fortuitously leads to unspecific crosslink products. However, if Toc34 has its own precursor recognition site or is involved in another step in translocation, e.g. gating of Toc75 (see below), needs to be established.

Recently, an *A. thaliana* mutant defective in plastid biogenesis was shown to lack a new component of the Toc complex, Toc33. Toc33 shares 61% identical amino acids with Toc34 indicating a functional similarity. Mutants lacking Toc33 could be complemented by the expression of Toc34 [37]. This mutant appeared uniformly pale during the first 2 weeks of the life cycle, the oldest leaves of mature plants frequently had an appearance closer to that of the wild-type. It was shown that Toc33 operates during early stages of the plastid and leaf development when Toc33 mRNA is most abundant, but its expression declined rapidly as the age of the plant increased. Yet, Toc34 mRNA remained at a steady low level. These data demonstrate the in vivo role of a translocon component in the plastid protein import and lead to the suggestion that the composition and abundance of the import machinery during differentiation of chloroplasts is regulated on the basis of gene expression.

Translocation across the outer membrane requires an aqueous channel to conduct polypeptides across the chloroplast outer membrane. The major component of the outer envelope, Toc75, represents the translocation pore. The function of Toc75 as a translocation channel was verified with heterologously expressed Toc75 reconstituted in lipid bilayers [38], where Toc75 forms a cation-selective channel. Import competent precursor protein, but not the mature form, was able to interact specifically with Toc75 and decreases the conductance of the channel indicating the presence of precursor-binding site(s) in Toc75. This is corroborated by biochemical findings which show that (1) Toc75 is the most prominent crosslinked product under conditions which favor precursor protein-binding and partial translocation [32] and (2) that Toc75 has a distinct precursor-binding site [14]. The estimated pore diameter ≥ 2 nm of Toc75 suggests that the transfer of precursor proteins across the outer envelope membrane occurs in a mainly unfolded conformation. Nevertheless, Clark and Theg (1997) [39] reported that at least some domains of a fusion protein between prOE17 (precursor of the 17 kDa subunit of the photosystem II oxygen evolving complex) and bovine pancreatic trypsin inhibitor (18 Å diameter) can maintain a non-linear structure during their translocation into chloroplasts.

Two members of the Hsp70 chaperone family might participate in the translocation process at the outer envelope membrane [19,40,41]. Com70 (Com = chloroplast outer envelope membrane protein) is associated with the Toc complex at the cytosolic site and generates crosslink products with precursor proteins during early stages of translocation [41]. A second member of the Hsp70 family is localized at the intermembrane space [19,40]. Both chaperones might prevent folding and stabilize precursor proteins in an import competent form analogous to the role of chaperones in other cellular protein transport mechanisms like in mitochondrial import and ER sequestration. Therefore, they could maintain a productive transport cycle across the outer envelope membranes.

Another putative component of the Toc complex is Toc36, a member of the Cim/Com44 family [42]. Toc36 was proposed to be localized in both the outer and inner envelope membranes. Its interaction with precursor protein upon translocation into the chloroplast was indicated by crosslink experiments [43], but the exact function is still unclear and remains obscure, e.g. Toc36 was shown to complement a bacterial SecA mutant [44], though it lacks any sequence homology to soluble SecA.

2.2. The Tic complex

Translocation of precursor proteins occurs through the joint interaction of the Toc and Tic machinery. Translocation intermediates are generated in intact chloroplasts which engage both complexes [19,20]. Tic110 was the first component identified as a Tic subunit by virtue of its co-purification with Toc components [21,45]. Although Tic110 is the main constit-

uent of the inner envelope translocon, a direct precursor interaction could not be detected until now, but Tic110 was shown to interact with stromal Hsp100 [22,46] and Cpn60 [45]. Although the topology of Tic110 is not resolved [21,47], in addition of being a docking partner for stromal chaperones, it could be involved in the formation of joint translocation sites with the Toc complex.

Tic55 could be co-immunoprecipitated with Tic110, indicating that both are in the same protein complex [48]. It was possible to co-fractionate Tic55 with Tic110, Hsp100 and the major components of the Toc complex, Toc160, Toc75 and Toc34 by affinity chromatography under conditions which trapped a polyhistidine-tagged precursor protein in translocation. Furthermore, it was possible to isolate a mixed Toc-Tic complex by blue native gel electrophoresis [48]. Tic55 contains a Rieske-type iron-sulfur centre and a mononuclear iron-binding site. It could be envisioned that the redox state of Tic55, analogous to the signal transduction properties of SoxR [49], might regulate the protein translocation through the Tic complex, e.g. by influencing the Tic complex stability and assembly.

Recently, two new components were identified by the virtue of crosslinking to pre-proteins trapped during the translocation process [23]. Tic20 is an integral membrane protein and might be part of the inner envelope translocation channel, while Tic22 is peripherally associated to the inner membrane at the intermembrane space and might be a functional link between the translocon complexes of outer and inner envelope membranes.

3. The origin of the precursor protein translocon

A prerequisite for a successful gene transfer from the genome of the endocytobiont to the host nucleus was the capability to translocate the nuclear gene product, i.e. the polypeptide, back into the new organelle. While protein export and secretion systems are prominent in the bacterial plasma membrane as well as in the outer membrane (for a review [50]), protein uptake systems have not been described. Therefore, the protein import machinery of plastids and mitochondria seemed to be a new invention of endocytobiosis. Recently, an open reading frame (ORF) (slr1227) was found in the *Synechocystis* PCC6803 genome which has a significant homology to peaToc75 in the primary and predicted secondary structure [51,52]. The ORF encodes a protein called synToc75 which is located in the outer membrane of *Synechocystis* [51]. The functional similarity between synToc75 and peaToc75 could be verified by electrophysiological measurements. SynToc75 forms an aqueous channel with similar properties as peaToc75. While the exact function of synToc75 in the cyanobacterial outer membrane is unknown so far, it is related to a group of specific prokaryotic secretion channels, which transfer virulence factors, e.g. hemolysins and adhesins, across the outer membrane of Gram-negative bacteria. Although sequence comparisons of other Toc components did not reveal any further similarities, homologues to subunits of the pea Tic complex were found in the *Synechocystis* genome: Tic55 (slr1747), Tic20 (sll1737) and Tic22 (slr0924) [52,53]. The localization of encoded proteins, probably in the plasma membrane of Gram-negative bacteria, and the functions in vivo are unknown and remain to be established. These results lead to the conclusion that the import machinery of chloroplasts probably derived from an ancestral peptide transport pathway of prokaryotes and became, due to the addition of further components, the highly specific protein import complex in the chloroplast outer membrane we know to date. These results give new insights into the evolutionary origin of the chloroplast protein import machinery and the outer envelope membranes of plastids.

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