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Plastid protein import and sorting: different paths to the same compartments

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Summary

Chloroplasts contain several thousand different proteins, of which more than 95% are encoded on nuclear genes, synthesized in the cytosol as precursor proteins, and imported into the organelle. The major pathways for import and routing have been described; a general import apparatus in the chloroplast envelope and several ancestral translocases in the thylakoid membranes. In this update we focus on some interesting and emerging areas: the Tat translocase, which operates in parallel with the Sec system but transports folded proteins; different routes to the envelope membranes, which promises an understanding of the ways the Tic apparatus sorts transmembrane domains (TMDs) and may also uncover developmental relationships between envelope and thylakoids; and novel routes for proteins into chloroplasts including delivery from the secretory system.

Introduction

Chloroplasts evolved from an endosymbiotic cyanobacterium. Initially, the endosymbiont contained ~3000 genes and synthesized and localized its proteins by means of protein translocases i.e. enzymes that translocate proteins across or into membrane bilayers. This starting point for the chloroplast explains in many ways the organization of protein and lipid trafficking pathways we observe today. Most endosymbiont genes were ultimately transferred to the host nucleus, yet chloroplasts still carry out many prokaryotic functions by ancestral proteins located in topologically comparable compartments. Thus the chloroplast must correctly localize these proteins, even though they are now synthesized in the cytosol. Chloroplasts generally achieve this goal with novel translocases in the chloroplast envelope that import cytosolically synthesized precursor proteins and deliver them to ancestral protein translocases located in specific chloroplast compartments for proper sub-organellar localization. This routing process is known as conservative sorting [1].

As shown in Fig 1 the Toc (Translocon at the outer chloroplast envelope) and Tic (Translocon at the inner chloroplast envelope) are novel translocases that import most chloroplast proteins and deliver them to the stroma. The thylakoid cpSRP (chloroplast Signal Recognition Particle),

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cpSec, and cpTat (Twin arginine translocation) pathways are ancestral translocases (Fig. 2). The Toc and Tic systems have been intensively studied and several excellent reviews cover their modes of action and current controversies [2–4]. Only a general description of their operation will be given here. Similarly, we will focus only on novel aspects of thylakoid sorting.

I. The Toc–Tic system

Proteins targeted to the Toc-Tic system have cleavable amino terminal ‘transit peptides’. The Toc-Tic system has been called the general import apparatus and is responsible for import of most plastid proteins. Toc and Tic are separate translocases but appear to act in concert at sites where envelope membranes come in contact. Toc, as well as Tic, transports proteins in an unfolded conformation. Similar systems possess three types of essential components: receptors, protein conducting transmembrane channels, and motor proteins to drive preprotein movement through the channel. Toc undisputedly contains two membrane integrated GTPases, Toc159 and Toc34, that specifically bind transit peptides and are considered receptors, and Toc75, a β barrel membrane protein [2,4]. Concerted interactions among the two GTPases and the transit peptide deliver precursors to the Toc75 channel. The translocation motor for Toc is uncertain and at least two models have emerged [reviewed in 2,4].

The Tic translocase is less well characterized. Three different components have been suggested to be the channel for the Tic translocase: Tic20, Tic21, Tic110 [2,3,5]. All three candidates interact with the inner membrane by hydrophobic TMDs, suggesting that the channel has a significant hydrophobic character; an important concept because it appears that Tic can route different TMDs to different sorting pathways (see below). Transit peptides of precursor proteins emerging from the Tic channel bind to a stroma-facing domain of Tic110 [6], which also binds putative components of the Tic translocation motor, the chaperone Hsp93 and co-chaperone Tic40 [7,8], which is thought to function similarly to the mitochondrial and ER Hsp70 based *trans*-located motors [9].

II. Conservative sorting to the thylakoid lumen

Two thylakoid pathways transport proteins to the lumen; the cpSec pathway in the cpSecA-cpSecYE configuration and the cpTat pathway (Fig. 2) [10]. Proteins are targeted to these pathways by cleavable hydrophobic signal peptides. cpTat-directed signal peptides have an essential twin arginine (RR) motif just before the hydrophobic domain. The cpSecA-cpSecY/E system appears to operate much like the bacterial system in which SecYE is the channel for unfolded protein substrates and the SecA ATPase is a pushing translocation motor [see 11].

The cpTat pathway

The cpTat pathway was discovered in chloroplasts, but is widely represented among bacteria and even some archaea [10]. Tat is a unique translocation system that transports folded proteins that vary from ~2 kD to greater than 100 kD, using only the proton gradient as an energy source. Clues to cpTat mechanism have come from analysis of the energetics of cpTat transport and from studies of component and precursor interactions. A controlled counterflow of protons occurs during transport and this proton flow appears to drive the transport reaction [12]. Braun and Theg [13] recently showed that both energetic components of the proton gradient, i.e. the $\Delta\Psi$ and ΔpH , can drive protein transport. These findings imply that the cpTat system contains a proton well, a membrane-imbedded species that carries protons as part of the reaction mechanism [discussed in 10,13]. Thus we should be looking for a proton carrier among the components of the cpTat translocase.

cpTat has three membrane protein components of machinery: Tha4, Hcf106, and cpTatC (Fig 2B and C). cpTatC and Hcf106 comprise a large (700 kD) receptor complex that specifically

binds cpTat-directed precursors [14]. The RR region of the bound precursor is in close contact with cpTatC and can maintain that contact throughout the transport reaction [15]. Tha4 exists as a separate seemingly homo-oligomeric complex.

The more interesting aspect of the cpTat system is that precursor binding in the presence of the proton gradient triggers assembly of Tha4 with the receptor complex [16]. The timing of this assembly process suggests that the cpTatC-Hcf106-Tha4 complex is the translocase, which assembles anew for each translocation event. When Tha4 oligomers dock with the receptor complex, they undergo a conformational switch that brings their carboxyl tails into close proximity [17]. Although the conformation adopted by Tha4 is still unclear, the switch evokes a model in which Tha4 only acquires channel-forming capability upon docking with an occupied receptor complex. Another interesting observation is that a conserved transmembrane glutamate residue of Tha4 is essential for its ability to facilitate protein transport [17]. One possibility is that the Tha4 glutamate serves as the proton well.

Recent studies have investigated the nature of the hypothetical cpTat channel with synthetic precursors that range in length and diameter [18]. The cpTat system tolerated significant variation in substrate diameter during a single translocation event, but was limited by the substrate length. In addition, substrates that stalled rapidly lost contact with translocase components. This differs strikingly from translocases that transport unfolded proteins, i.e. Toc-Tic, Tom-Tim, and Sec, where stalled substrates remain in the channel. These observations suggest a highly dynamic and transient translocation channel, if it is a channel in the conventional sense.

III. Conservative sorting to the thylakoid membrane

Previous work characterized the integration of relatively simple membrane proteins by cpSecA-cpSecYE, cpTat, and the 'spontaneous' pathway, and integration of trispanning LHC proteins by the posttranslational cpSRP pathway (Figs. 1 & 2)[19,20]. Novel aspects of the cpSRP pathway are that it can function post-translationally, which differs from all other SRPs, and that, although the cpSRP targeting chaperone contains the conserved 54 kD GTPase, it lacks the SRP RNA required of all the other SRPs. The ability to function post-translationally has been attributed to cpSRP43, a novel subunit that binds to an 18 residue signature domain of LHCs [20]. Recently, Shan and colleagues [21] showed that cpSRP54 can function without RNA because it binds to its receptor cpFtsY with high affinity and stimulates the GTPase activity essential for recycling. The bacterial SRP absolutely requires its RNA component for these activities.

Nonetheless, there are many thylakoid membrane proteins for which very little is known about their localization pathways. Some are plastid-encoded, multi-spanning proteins that make up core subunits of photosynthetic complexes; some are nuclear-encoded. The nuclear-encoded core subunits of translocases, cpSecY, cpTatC, Alb3, are of particular importance because they are highly hydrophobic and control the protein composition of thylakoids. These proteins have not been amenable to in vitro reconstituted assays and the challenge is to devise methods that decipher their targeting pathways in vivo. Progress has recently been made with protein interaction assays [22], mutant selection [23,24], and co-immunoprecipitation experiments that implicate components involved in integration and assembly of specific substrates [reviewed in 10,20] and suggest that components of the cpSec and cpSRP systems may operate in a modular fashion similar to bacteria [25]. For example, a separate population of cpSRP54 lacking cpSRP43 is associated with plastid ribosomes and appears to be involved in co-translational integration of the multi-spanning thylakoid protein D1 in concert with cpSecY [10,20]. Other data suggest that the post-translational cpSRP pathway integrase component

Alb3, which is homologous to bacterial YidC and mitochondrial Oxa1p integrases, functions in the assembly of proteins into PSI and PSII complexes [22–24,26].

The recent identification of a second Alb3 protein [26,27] and a second cpSecY protein add to the complexity of what promises to be a fascinating story of biogenesis of chloroplast membranes. The two Alb3 and SecY proteins appear to have non-redundant functions. For example, knockout of one *Chlamydomonas* Alb3 has a mild phenotype limited to LHC proteins, whereas knockdown of the other results in severe reductions of photosynthetic complexes and is ultimately lethal, even under heterotrophic growth conditions [26,28]. Similarly, whereas knockout of cpSecY1 produces a seedling lethal phenotype characterized by loss of thylakoid membranes in maize [29] and *Arabidopsis* (Courtney A. Skalitzy, MS thesis, University of Wisconsin-Madison, 2006), disruption of cpSecY2 in *Arabidopsis* results in lethality at the globular embryo stage (Courtney A. Skalitzy, MS thesis, University of Wisconsin-Madison, 2006) [see also 10]. Certainly the additional Alb3 and cpSecY must be involved in integrating essential membrane proteins for general plastid functions, possibly even other translocase subunits.

IV. Sorting of proteins to the envelope compartment

A number of studies have addressed the insertion of single spanning outer membrane proteins and this topic has been recently reviewed [30]. However, recent work with a novel outer membrane pathway and an apparently conservative sorting inner membrane pathway are beginning to provide greater insight into subtle capabilities of the Tic system and also the relationship between the inner envelope and the thylakoids during development (Fig. 3).

A. Toc75

The insertion of the outer envelope β barrel Toc75 differs from other outer envelope proteins and may be a case of conservative sorting. Toc75 is synthesized as a precursor protein with a bipartite targeting signal that is removed in two steps. The amino terminal domain is functionally a stromal targeting transit peptide; the carboxyl proximal domain appears to be a retention peptide that stops transport at the level of the Tic complex [31]. Why would this protein need to go to the inter-envelope space? It may be because Toc75 has to enter a conserved β -barrel folding and insertion pathway [32]. Beta-barrel proteins are found only in the outer membranes of bacteria, chloroplasts and mitochondria. The β -barrel assembly pathway starts in the bacterial periplasm, which is equivalent to the plastid inter-envelope space [32]. Bacterial and mitochondrial β -barrel assembly requires a conserved outer membrane protein OMP85/SAM50 [33]. Interestingly, Toc75 is an OMP85 homologue. There are several Toc75 family members in *Arabidopsis* and Toc75-V/OEP80, which is the most similar to OMP85, is suggested to be involved in the plastid β -barrel assembly pathway [32].

Possibly the most intriguing aspect of the Toc75 assembly pathway is that a type I signal peptidase cleaves the 2nd domain of the targeting signal, thereby releasing Toc75 to the inter-envelope space (Fig. 3) [34]. Type I signal peptidases are of prokaryotic origin and cleave hydrophobic signal peptides of proteins that are transported by the Sec, Tat and 'spontaneous' protein transport systems. A knockout mutation of this enzyme was defective in processing both Toc75 and a thylakoid luminal protein [34]. This suggests interesting possibilities. The first is that the signal peptidase has a dual localization in envelope and thylakoids. The second is that during initial stages of development, when most of the Toc75 would be expressed and when thylakoids are largely absent, the enzyme is present in the envelope, but is relocated with development and the appearance of thylakoids.

B. Inner envelope

Virtually all inner envelope membrane proteins possess cleavable transit peptides and, where examined, the transit peptides are stroma-targeting [2]. Recent studies have shown that two such inner envelope proteins, Tic40 and Tic110, are completely imported into the stroma and then inserted into the inner envelope membrane from the stromal side [35–37]. Both proteins are integrally associated with the inner envelope by N-terminal TMDs and expose globular domains to the stroma. In vitro experiments provided clear evidence for productive stromal intermediates [36,37] and involvement of proteinaceous machinery in the insertion reaction [37].

This overall pathway is suggestive of conservative sorting to the envelope. The Tic40 stromal intermediate was correctly processed by a purified signal peptidase I in vitro [36]. If iTic40 is processed by a signal peptidase in vivo, this would constitute one component of conserved translocation machinery. Other components for such a conserved translocase remain to be determined. The core translocase subunits cpSecY1, Alb3, and cpTatC are virtually absent from the envelope in mature chloroplasts [27,38]. However, the sub-organellar location of cpSecY2 is not known and the studies of Inoue et al [34] suggest the possibility that thylakoid translocases could be present in the envelope in early developmental stages.

Studies of three inner envelope proteins, including ARC6, suggest that they follow a ‘stop transfer’ route, wherein their TMDs divert the protein laterally out of the Tic translocase into the inner membrane (Fig. 3)[36,39,40]. If some TMDs serve as envelope stop transfer signals, how can the envelope translocase system distinguish between stop transfer TMDs and conservative sorting TMDs of Tic40, Tic110, and all of the conservatively sorted thylakoid membrane proteins? In mitochondria, three sorting routes for inner membrane proteins have been described: a conservative sorting pathway, a stop-transfer pathway; and a third pathway in which multi-spanning metabolite carrier proteins of eukaryotic origin are diverted to a novel inner membrane integrase, the Tim22 complex [see 41,42 for reviews]. Whether plastids employ analogous sorting mechanisms or a novel mechanism should be interesting to determine. Plastids also have a number of similar multi-spanning metabolite carrier proteins that appear not to take the conservative sorting route [39,40]. Whatever the mechanism, this area of inner envelope (and thylakoid) membrane protein sorting promises to be a fertile area for obtaining deeper insight into translocases as well as mechanisms by which the endosymbiont became a chloroplast.

V. Alternative Routes to Chloroplasts

It is becoming clear that there are other routes for proteins into the chloroplast than the transit peptide Toc–Tic system. Two proteins, Tic32 [43] and ceQORH [44] are synthesized without transit peptides and are transported across the outer envelope membrane without involvement of the Toc apparatus. Proteomics analyses of chloroplasts found that ~30% of detected proteins did not possess identifiable transit peptides [45]. Interestingly, a large percentage of the transit peptide-less proteins had homologues in cyanobacteria [45]. How such proteins gain access to the chloroplast is not currently known. Even more puzzling was that a significant number of identified plastid proteins possess predicted signal peptides for entry into the secretory system [45]. Villarejo and colleagues [46] have provided compelling evidence that the plastid carbonic anhydrase, CAH1, which contains a signal peptide but lacks a discernable transit peptide, localizes to the chloroplast stroma via the endoplasmic reticulum and Golgi. How proteins can traffic from the Golgi to the stroma of higher plant plastid is unknown. A possibly analogous pathway involving travel through the ER and Golgi does occur in some algae and protists, such as *Euglena*, containing complex plastids with three or four outer membranes [47]. The key difference between CAH1 and proteins in *Euglena* is the absence of a discernable transit peptide

in CAH1. Identifying alternative routes for targeting proteins to the chloroplast is an emerging area of active investigation.

Perspectives

In this short review we highlight an emerging theme in chloroplast protein import and routing: there are parallel routes for proteins into the plastid as well as parallel routes into the different plastid compartments. In certain cases ancestral proteins are localized by ancient pathways with which they co-evolved before endosymbiosis. Some examples also suggest that routes taken depend on the needs of the substrate; whether it needs to be glycosylated or it is tightly folded; whether it is a beta barrel or TMD membrane protein and how many TMDs it contains. We are only just beginning to understand the connection between substrate and pathway. Continued investigation of these systems will not only solve interesting scientific puzzles, it will lay the foundation for sophisticated metabolic engineering of plants.

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Fig. 1. Major pathways for import and routing of nucleus-encoded plastid proteins

Most proteins are synthesized with amino-terminal stroma-targeting transit peptides, which direct import by the envelope Toc and Tic translocases. Upon transport across the inner envelope membrane the transit peptide is removed by the stromal processing protease. Proteins destined for the thylakoid system have additional targeting signals. Lumenal proteins and some membrane proteins have cleavable hydrophobic signal peptides that direct transport or integration by the chloroplast Sec, Tat, or 'spontaneous integration' pathways. The light harvesting chlorophyll a/b proteins are integrated by the chloroplast SRP pathway. Most outer envelope membrane proteins are synthesized without transit peptides and are inserted into the outer membrane by proteinaceous machinery. There are at least two pathways for sorting of inner envelope proteins. Recent studies provide compelling evidence that some plastid proteins are localized by non-classical pathways, e.g. by way of the secretory system.

Fig. 2. Thylakoid transport and integration systems

A. The characterized cpSec system consists of a transmembrane channel complex, cpSecYE, through which preproteins are transported in an unfolded conformation by cpSecA, an ATPase pushing motor. The cpSecA-cpSecYE system is primarily responsible for transport of luminal proteins, but also integrates several simple membrane proteins with large transported globular domains. The cpSRP system has been characterized for the post-translational insertion of the light harvesting chlorophyll a/b apoprotein family. cpSRP is a targeting chaperone and cpFtsY is its receptor. Alb3 is related to mitochondrial Oxa1p and bacterial YidC, proteins shown to facilitate insertion and assembly of membrane proteins. B. Components of the cpTat system. Hcf106 and Tha4 are homologous proteins that each contain an amino-proximal

transmembrane domain with a conserved glutamate residue (E), followed by a hinge region and an amphipathic helix (APH). C. Stages of cpTat transport determined by in vitro binding and transport assays. See text for details.

Fig. 3. Examples of protein sorting of envelope membrane proteins

Toc75 has a unique sorting pathway among outer envelope membrane proteins. It is synthesized with a bipartite targeting peptide in which the amino terminal domain is a stromal targeting transit peptide and the carboxyl proximal domain is an envelope retention signal. The precursor is transported across the outer envelope and appears to stall at the level of the Tic complex. The envelope retention domain contains a hydrophobic segment and an essential polyglycine tract. Although the hydrophobic segment is not essential for retention, it is possible that the Toc75 intermediate is briefly inserted in the inner envelope membrane before cleavage by a signal peptidase I (SP1) and release of Toc75 to the inter-envelope space. There are at least two pathways for inner envelope membrane sorting. Tic40 (and Tic110) is imported into the stroma and then inserts into the inner envelope by an unidentified integrase. The ARC6 protein appears to be imported to the Tic complex and is then diverted directly into the inner membrane by its TMD.