The complexity of pathways for protein import into thylakoids: it’s not easy being green

A. Di Cola, E. Klostermann and C. Robinson

Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, U.K.

Abstract
Numerous proteins are transported into or across the chloroplast thylakoid membrane. To date, two major pathways have been identified for the transport of luminal proteins (the Sec- and Tat-dependent pathways) and it is now clear that these protein translocases use fundamentally different transport mechanisms. Integral membrane proteins are inserted by means of at least two further pathways. One involves the input of numerous targeting factors, including SRP (signal recognition particle), FtsY and Albino3. Surprisingly, the other pathway does not involve any of the known chloroplastic targeting factors, and insertion is energy-independent, raising the possibility of an unusual ‘spontaneous’ insertion mechanism.

Introduction
Chloroplasts perform a variety of biochemical functions within plant cells, although these organelles are best known as the site of photosynthesis. Numerous plastid proteins are essential for the various physiological processes ongoing in this organelle. Although the chloroplast possesses its own genome, it encodes only a small proportion of the plastid proteins and the vast majority are nuclear encoded. These proteins are synthesized as precursors in the cytoplasm and targeted to the different intra-chloroplast destinations via specific routes.

Chloroplasts are formed of a ‘matrioska’ system of membranes separated by soluble intermembrane phases: from the outside cytosol moving inwards chloroplasts are bound by a double-membrane envelope enclosing an intermembrane soluble space and the inner major soluble phase, the stroma. Many essential pathways operate in the stroma, such as the Calvin cycle for carbon fixation, amino acid synthesis and transcription–translation for the synthesis of the chloroplast-encoded-proteins. Within the stroma, a further inner membrane system constitutes the thylakoid network, where light is captured and ATP is synthesized. The thylakoid membrane encloses another soluble phase, the thylakoid lumen, where soluble or loosely membrane-associated photosynthetic proteins are located.

Import into the chloroplast
Cytosolically synthesized chloroplast proteins are imported post-translationally into the organelle and a default translocation pathway involving distinct machineries in the outer and inner envelope membranes has been studied in some detail [1]. Many of the imported proteins remain in the stroma after import but others are targeted to the thylakoids by additional pathways that have been studied primarily using in vitro translocation assays. These studies have shown that four mainstream pathways are used for protein targeting to thylakoids, as detailed below.

The first stage for protein transport to the chloroplast is common to all the routes, involving the synthesis of cytosolic precursors and translocation across chloroplast envelopes. Precursors bear an N-terminal transit peptide containing stromal-targeting information, which is responsible for directing the precursor across the two chloroplast envelope membranes. In some cases, the signal peptide is bipartite and contains additional information for transport across the thylakoid membrane. The two signal peptides are removed by two different processing peptidases, one resident in the stroma (SPP, stromal processing peptidase) and the other in the thylakoid lumen (TPP, thylakoidal processing peptidase; for a detailed review, see [1]). Translocation across the chloroplast envelope is mediated by the Tic/Toc translocase. The process is ATP- and GTP-dependent and precursors are translocated in an unfolded state.

Targeting of thylakoid lumen proteins
Essentially, all thylakoid lumen proteins are initially synthesized with bipartite presequences that contain a transit peptide and signal peptide in tandem. The removal of the transit peptide by the SPP exposes the thylakoid signal peptide and generates a stromal intermediate form that can undertake one of the two possible pathways to reach the thylakoid lumen.

The Tat (twin-arginine translocation) pathway
Tat substrates are characterized by the presence of an invariant twin arginine motif located in the N-terminal region of the
signal peptide. This motif is essential for Tat-mediated translocation and gives the name to the pathway [2]. At the C-terminus of the peptide, an Ala-Xaa-Ala motif denotes the cleavage site for TPP allowing the release of the mature protein in the lumen [3].

One of the major characteristics of this thylakoid translocation system is that the translocation event is not driven by nucleoside triphosphate hydrolysis, but by the transmembrane pH gradient. For this reason the pathway is also known as the ΔpH-dependent pathway [4,5]. The second major and more unusual feature is that this system is capable of transporting fully folded proteins [6].

It is now known that three proteins (Tha4, Hcf106 and cpTatC) are constituents of the Tat system in plants [7–9]. Their discovery led to the identification of the homologous TatABC system in prokaryotes [10]. In bacteria, a number of periplasmic proteins containing cofactors have been shown to be Tat substrates, and the fact that these cofactors are enzymatically inserted only in the cytoplasm, led to the conclusion that Tat passenger proteins must translocate in an extensively folded form [11]. However, little is known about the actual translocation mechanism at the present time.

Previously, a detailed in vitro cross-linking study revealed that in isolated thylakoids, Tat subunits are found in two distinct subcomplexes: a cpTatC–Hcf106 complex and a Tha4 complex. Tat substrates preferentially bind to the first one that would work as precursor receptor, this interaction and the presence of ΔpH triggers the recruitment of Tha4 which assembles with cpTatC–Hcf106 subcomplex to form the translocation complex [12,13].

Other aspects of the system remain to be studied in detail and this applies to the functioning of the system in vivo. A recent study analysed the targeting of different Tat substrates in vivo in Chlamydomonas reinhardtii and found that in this case the Tat system is not dependent on the ΔpH [14]. More recently, these data have been revisited suggesting that the transmembrane Δψ can also power the Tat translocase [15]; another possibility is that the reconstituted in vitro import system is lacking some ‘crucial’ yet unidentified factors, which are normally present in planta. Overall, these findings have opened a new area of debate where still little is known and more study is needed.

The Sec pathway

The second mainstream pathway for protein transport into the thylakoid lumen is the Sec pathway. This is related to Sec-type translocation pathways in bacteria, which are responsible for protein export across the plasma membrane to the periplasm or medium. In bacteria, the Sec complex is formed by the SecA subunit and the heterotrimeric SecYEG translocation channel. SecA is an ATPase that acts as the translocation motor pushing segments of precursor proteins across the channel (reviewed in [16]).

For the chloroplast system, cpSecY [17], cpSecE [18] and cpSecA have been identified [19–21]. Antibodies against cpSecY inhibit cpSecA-dependent protein translocation, suggesting that cpSecA and cpSecY work in concert [22] as in bacteria. cpSecA has been demonstrated to promote ATP-dependent transport of OEC 33K and plastocyanin into thylakoids [21].

The bacterial SecYEG complex transports substrates in an unfolded state and the energy for translocation comes from ATP hydrolysis. Analogously, when DHFR (dihydrofolate reductase) is fused to an Sec signal peptide and assessed for import into thylakoids in vitro, its translocation is completely blocked in the presence of folate analogues that are known to bind to the precursor and stabilize it in a tightly folded form. These findings strongly suggest that Sec-mediated translocation can occur only when substrates are in an unfolded state [23]. A more recent observation seems to confirm this conclusion: EGFP (enhanced green fluorescent protein) targeted to the thylakoid can be efficiently transported via the Tat pathway, while transport mediated by Sec is very inefficient. The rapid folding of the precursor EGFP in the stroma appears to prevent its transport through the Sec translocase [24]. Figure 1 illustrates the salient features of the Tat- and Sec-dependent pathways.

Thylakoid membrane proteins

The SRP (signal recognition particle) pathway

The SRP pathway is used to direct a subset of hydrophobic proteins into the thylakoid membrane. In eukaryotes, prokaryotes and archaea a related SRP pathway mediates co-translational protein transport and insertion into the membranes (reviewed in [25]). In plants, the SRP pathway is best known for the post-translational targeting and membrane insertion of nuclear-encoded LHCPs (light-harvesting chlorophyll-binding proteins). For an extended review on chloroplast SRP, see [26]. In short, cpSRP54, a protein homologue of mammalian/bacterial SRP54 subunits, together with cpSRP43, a novel protein unique to this plant post-translational SRP pathway, forms the cpSRP that, in contrast with all other known SRPs, contains no RNA component. It binds to LHCP to form a ‘transit complex’, the soluble form of the hydrophobic LHCP for transport in the stroma. Lhcb1, Lhcb4.1 and Lhcb5 have been shown to use this pathway. For recent developments concerning the interactions within the transit complex, see [27,28]. Integration of LHCP also requires GTP, cpFtsY and Alb3. cpFtsY is thought to act as a receptor and form a complex with cpSRP and functional Alb3 [29]. The interaction of cpSRP with cpFtsY seems to promote GTP binding to the GTPases cpSRP54 and cpFtsY, which target the complex to Alb3 in the thylakoid membrane. Alb3 is homologous with the bacterial YidC and mitochondrial Oxa1 proteins that are known to assist in the insertion of membrane proteins into the plasma membrane or inner mitochondrial membrane respectively. cpSRP43 has no function in targeting to Alb3, but may be involved in the regulation of the GTPase cycle to minimize GTP hydrolysis [28,29]. Besides cpSRP and cpFtsY, cpSecY was localized in Alb3 complexes [29,30]. Studies suggest that it is not involved in post-translational LHCP integration [22,29]. But in a...
Figure 1 | Model of Tat and Sec targeting pathways for thylakoid lumen proteins
Thylakoid lumen proteins are synthesized in the cytosol with a bipartite presequence. After import into the chloroplast in an unfolded state, the first targeting signal is cleaved off and the proteins are then transported across the thylakoid membrane by means of either a Sec- or Tat-specific signal peptide. Proteins such as plastocyanin on the Sec pathway interact with SecA in the stroma, which hydrolyses ATP and effectively pushes the preprotein through a membrane-bound translocon in an unfolded state. The translocon is known to contain SecY and SecE but is otherwise poorly characterized. It is also unclear whether the substrate protein remains unfolded in the stromal phase, perhaps with the aid of (unidentified) chaperone molecules, or whether it is actively unfolded at the membrane. Substrates on the Tat pathway, for example PsbP or PsbQ, have been shown to refold in the stroma and are not believed to interact with any dedicated Tat components until they reach the membrane. Here, they are transported by a membrane-bound Tat system that contains Tha4, Hcf106 and TatC. The Tat system is able to translocate substrates in a fully folded state and the thylakoidal ΔpH is essential for translocation, at least in isolated thylakoids and intact isolated chloroplasts.

Figure 2 | Models for the SRP and ‘spontaneous’ targeting pathway for thylakoid membrane proteins
The SRP-dependent pathway is used by LHCPs such as Lhcb1. After import into the chloroplast, the substrate interacts with cpSRP, which comprises SRPS4 and SRP43 subunits, and its partner protein, FtsY. These factors direct Lhcb1 to the membrane and insertion occurs in a process that depends on membrane-bound Alb3. Other membrane proteins such as PsbW and Psak are inserted by pathways that do not require any known protein targeting apparatus, and these may therefore insert spontaneously. One pathway is used by a small number of thylakoid membrane proteins (to date, CfoII, PsbX, PsbW and PsbY) that bear cleavable signal-type peptides that somehow aid insertion. The other is used by the majority of thylakoid membrane proteins (Psak is shown as an example) and the protein inserts into the membrane as the mature-size form.

The ‘spontaneous’ pathway for thylakoid membrane insertion
So far, we have described thylakoid protein targeting pathways involving interactions with proteinaceous machineries. A fourth group of thylakoid proteins appears to behave differently. Proteins belonging to this group are all integral membrane proteins, and their main feature is that they do not require any known protein transport machinery, nor energy, for insertion into thylakoid membranes. This is an unusual pathway, which is unique to chloroplasts, and the absence of any known energy requirement or essential targeting factor suggests the possibility of a spontaneous insertion mechanism [33].
Examples of proteins that insert ‘spontaneously’ are the W and X subunits of Photosystem II (PsbW and PsbX), Psak from Photosystem I [34] and the SecE subunit (cpSecE) [35]. The first two bear a bipartite presequence resembling luminal proteins, but have been shown to insert into thylakoid membranes in the absence of SRP, NTPs or a functional Sec machinery. Furthermore, proteolysis of thylakoids or inactivation of Alb3 does not influence their insertion [36].

recently described co-translational cpSRP pathway [26] mediating transport of plastid-encoded proteins, elongating nascent chloroplast-encoded D1 protein has been shown to bind to ribosome-associated cpSRP54, and cpSecY transiently interacts with isolated D1 elongation intermediates [30a].
Further candidates for co-translational protein transport via cpSRP are the chloroplast-encoded proteins D2, CP43, PSI-A and CFI-III which have been shown to interact with Alb3 in a yeast two-hybrid system especially adapted to detect membrane protein interactions [31]. C. reinhardtii possesses two homologues of the alba gene. In an Alb3.1 deletion mutant it was found that D1 protein was inserted into the thylakoid membrane while assembly into the reaction centre complex was delayed, suggesting a role in complex assembly rather than as an integrase [32].
It has been proposed that after the removal of the transit peptide, the remaining N-terminal propeptide containing a hydrophobic region is necessary for the formation of a ‘helical hairpin’-type loop intermediate in the thylakoid membrane, upon interaction with the lipid bilayer of the thylakoid membrane [33]. Figure 2 depicts the operation of the SRP-dependent and ‘spontaneous’ insertion mechanisms.

References

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