Traffic 2001 **2:** 245–251
Munksgaard International Publishers

ISSN 1398–9219

Review

Multiple Pathways Used for the Targeting of Thylakoid Proteins in Chloroplasts

Colin Robinson*, Simon J. Thompson and Cheryl Woolhead

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom
**Corresponding author: Crobinson@bio.warwick.ac.uk*

The assembly of the chloroplast thylakoid membrane requires the import of numerous proteins from the cytosol and their targeting into or across the thylakoid membrane. It is now clear that multiple pathways are involved in the thylakoid-targeting stages, depending on the type of protein substrate. Two very different pathways are used by thylakoid lumen proteins; one is the Sec pathway which has been well-characterised in bacteria, and which involves the threading of the substrate through a narrow channel. In contrast, the more recently characterised twin-arginine translocation (Tat) system is able to translocate fully folded proteins across this membrane. Recent advances on bacterial Tat systems shed further light on the structure and function of this system. Membrane proteins, on the other hand, use two further pathways. One is the signal recognition particle-dependent pathway, involving a complex interplay between many different factors, whereas other proteins insert without the assistance of any known apparatus. This article reviews advances in the study of these pathways and considers the rationale behind the surprising complexity.

Key words: chloroplast, membrane, protein transport, tat, thylakoid

Received and accepted for publication 5 February 2001

The chloroplast is well known as the site of photosynthesis in plants but it also carries out many other critical functions and consequently contains a vast range of protein types – probably well over 1000. Some of these proteins, especially those involved in photosynthetic functions, are also present at very high concentrations, and the total protein concentration in the organelle is in the order of 300 mg/mL. A small proportion of these proteins are synthesised within the organelle, but the vast majority are encoded by nuclear genes and synthesised in the cytosol, with the net result that chloroplast biogenesis requires the import of a colossal amount of protein in a relatively short time. To complicate matters, this organelle is particularly complex in structural terms, comprising three separate membranes, which enclose three distinct soluble phases. The chloroplast is bounded by a double-membrane envelope, which encloses an intermembrane space, about which rather little is known. The major soluble phase is the stroma, which is the site of carbon fixation, amino acid synthesis and many other pathways, and the dominant membrane is the extensive interconnecting thylakoid network, where light is captured and ATP synthesised. Finally, the thylakoid membrane encloses a further soluble phase, the thylakoid lumen, which houses a number of extrinsic photosynthetic proteins as well as many others. Imported proteins are targeted into all six chloroplast subcompartments and the underlying mechanisms have been the focus of considerable attention over the past 25 years. Perhaps surprisingly, most proteins appear to be imported across the envelope membranes by a basically similar mechanism. This topic has been covered in a recent review (1) and will not be dealt with in detail here. Rather, the aim of this article is to review recent progress on the intraorganellar sorting of thylakoid proteins, where it is now clear that numerous pathways are utilised for the targeting of thylakoid lumen and membrane proteins. One of the lumen-targeting pathways, the Tat pathway, has been the centre of much interest since its discovery in bacteria, and the same applies to the Alb3-mediated pathway for membrane proteins. This review will focus on the mainstream pathways used for thylakoid proteins with an emphasis on providing a rationale for the surprising multiplicity.

Two Very Different Pathways for the Targeting of Thylakoid Lumen Proteins

Nearly all thylakoid lumen proteins are synthesised in the cytosol, and all of them appear to undergo a two-stage import pathway via stromal intermediate forms. They are initially synthesised in the cytosol as precursor proteins bearing bipartite N-terminal presequences which contain two targeting signals in tandem, after which they are transported across the envelope at contact sites between the two membranes. This import process requires ATP and probably GTP, and a key feature of the translocation mechanism is that the protein is transported in a largely unfolded form (reviewed in (1)).

Once in the stroma, the first 'envelope transit' domain of the presequence is removed by a stromal processing peptidase. The intermediate form is then transported across the thylakoid membrane by one of two distinct mechanisms. A subset

of lumenal proteins, including the 33-kDa photosystem II protein (33K), plastocyanin and others, are transported by a Sec pathway which closely resembles the well-characterised Sec-dependent export pathways in bacteria (reviewed in (2)). Many proteins are exported across the plasma membrane to the periplasm (or further) in bacteria, and until recently it was believed that this process was mediated almost exclusively by the Sec pathway. In this process, the substrate protein is synthesised with a 'signal' peptide comprising three domains: a basic N-terminal (N-) domain, hydrophobic core (H-) domain and more polar C-terminal (C-) domain. The export mechanism involves the threading of the protein through a SecYEG translocon, driven by the ATPase activity of a peripheral protein, SecA. Plant chloroplasts contain SecY, SecE and SecA homologues which are involved in targeting of thylakoid lumen proteins by a very similar mechanism (3–5), and this finding comes as no surprise since it is generally accepted that chloroplasts evolved from cyanobacterial-type organisms.

The discovery of a second mainstream pathway, on the other hand, did come as a surprise because at that time there were no indications of a second export system in bacteria. It was shown some time ago that other lumen proteins are transported by a mechanism that does not require SecA or nucleoside triphosphates (NTPs), but which relies totally on the ΔpH across the thylakoid membrane (6,7). Substrates for this pathway are also synthesised with bipartite presequences and the second, thylakoid-targeting domains resemble those of Sec substrates in many respects. However, these signals contain all of the information specifying translocation by the ΔpH-dependent pathway and are able to direct foreign proteins with high efficiency by this route (8,9). Closer examination of these signals showed that they contain an invariant twin-arginine motif that is critical for ΔpH-dependent targeting (10); a further important determinant is the presence of a highly hydrophobic residue two or three residues after this motif (11). These features are illustrated in Figure 1, which lists Sec- and ΔpH-dependent thylakoid-targeting signals of known lumenal proteins.

Figure 1: Targeting signals for thylakoid lumen proteins. Signal peptides specifying translocation by the Tat and Sec pathways in chloroplasts. The signals are shown for representative substrates from spinach (Sp), wheat (Wh), maize (Ma), *Arabidopsis* (Ara), barley (Bar) and Cotton (Cot). The list includes: proteins that have been experimentally shown to depend on the Tat-system and several that specify targeting by the Sec pathway. Hydrophobic (H-) domains are underlined, charged residues are shown in bold and twin-arginine motifs are shown in bold italics.

Discovery of a Related, Sec-independent Export Pathway in Bacteria

The major breakthrough in this field came with the characterisation of a maize mutant, termed *hcf106*, that was shown to be specifically defective in the ΔpH-dependent targeting pathway (12). Substrates for this pathway were shown to accumulate as intermediates in the stroma and the *hcf106* gene was later cloned, sequenced and shown to encode a thylakoid membrane protein (13). This work provided the next serious surprise because homologues of the *hcf106* gene were present in most sequenced bacterial genomes, all as unassigned open reading frames. In fact, bacteria usually contain two or more *hcf106* homologues and the gene organisation in *Escherichia coli* is shown in Figure 2, together with predicted basic structures and orientations of the encoded proteins. As discussed below, these genes do indeed encode components of second export pathway that is related to the thylakoid ΔpH-dependent pathway, and the genes have been termed *tat* genes (for *t*win-*a*rginine *t*ranslocase).

The *E. coli* genome contains three genes homologous to hcf106, two of which (*tatA and tatB*) form the first two genes of a four-gene operon. A third *hcf106* homologue, *tatE*, is monocistronic. Knockout studies have shown that disruption of either *tatB* or *tatA/E* leads to a complete block in the export of a range of periplasmic proteins (14,15). While all three genes are sequence-related to each other and to *hcf106*, they encode proteins with two distinct functions, and it is now clear that the export system basically requires TatB and either TatA or TatE. TatA is the more important of the latter two and its disruption has a far greater effect, but the *ΔtatA* cells can be complemented by overexpression of TatE (16). These Tat components (and Hcf106) are all strongly predicted to be single-span proteins with a very short N-terminal region in the periplasm/thylakoid lumen and a short domain in the cytoplasm/stroma (that of TatB being larger than TatA).

The next gene in the *tat* operon, *tatC* is also critical for Tat-dependent export and *E. coli* cells disrupted in this gene are completely defective in the Tat export pathway (17). The TatC protein is predicted to span the plasma membrane six times with the N- and C- termini in the cytoplasm. However, the *tatD* gene appears not be involved (or to only a minor extent). This gene encodes a soluble cytoplasmic protein and *ΔtatD* cells are unaffected in Tat-dependent export (18). The Tat

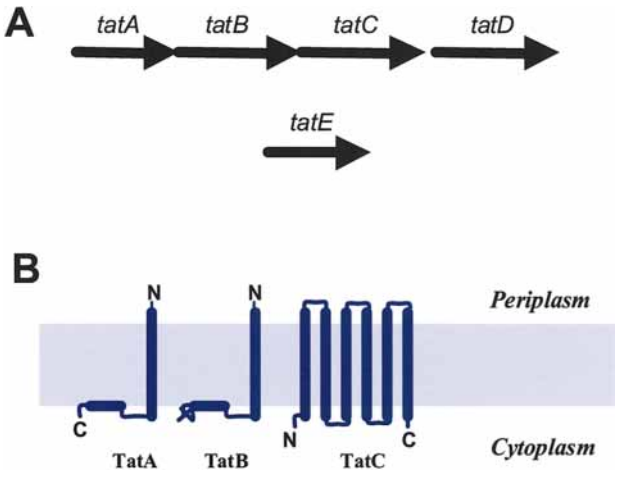

Figure 2: *tat* gene organisation in *E. coli* and the predicted structures of Tat proteins. A: Gene organisation. The primary known genes are organised in a *tatABCD* operon as shown. The *tatABC* genes encode membrane-bound components whereas the *tatD* gene product is a cytoplasmic protein which does not appear to be involved in Tat-dependent export. The monocistronic *tatE* gene encodes a TatA-type protein but this gene is expressed at low levels and the function of TatE is relatively unimportant. B: Predicted protein structure. TatA and TatB almost certainly contain a short periplasmic N-terminal domain and a single membrane-spanning region followed by a predicted short amphipathic region. TatA, TatE and TatB contain small cytoplasmic domains. TatC is predicted to contain 6 transmembrane helices and short interconnecting loop regions, with the N- and C-termini located in the cytoplasm. Chloroplasts contain homologues of all three Tat proteins.

pathway in bacteria has thus so far been shown to require the activities of three membrane-bound proteins, although the existence of further Tat subunits cannot be ruled out since the system has yet to be purified in a functional form. All of the indications are that the thylakoidal system is essentially identical; Hcf106 is believed to be a TatB homologue and homologues of TatA and TatC have been identified in higher plants (19,20).

Although this article is primarily concerned with thylakoid protein targeting, it is of passing interest to note that the targeting signals for bacterial Tat substrates are generally similar to thylakoid lumen proteins and they likewise contain the invariant RR-motif (21,22). They are furthermore able to direct proteins across the chloroplast thylakoid membrane with high efficiency (23–25), indicating that some features of the mechanism have remained very highly conserved. However, Gram-negative bacterial RR-signal peptides contain additional conserved features that are generally absent from thylakoid Tat signals; most contain a phenylalanine two residues after the RR-motif and many contain a lysine two residues later (21,22). The consensus motif for these bacterial signals is thus RRxFxK, but the precise significance of the conserved Phe and Lys residues is uncertain since they are not strictly required for efficient translocation (22).

Speciality of the Tat Pathway: the Translocation of Fully Folded Proteins

The targeting signals for the Sec- and Tat-type translocation pathways may be surprisingly similar but their mechanisms could hardly be more different, because there is now considerable evidence that the Tat system has the unique ability to translocate folded proteins across tightly sealed biological membranes. Two studies on the thylakoidal system have addressed this point directly using *in vitro* import assays. In the first (26), it was found that bovine pancreatic trypsin inhibitor could be transported across the thylakoid membrane by the Tat pathway using a Tat-specific signal, even after internal cross-linking to block any unfolding. The second study (27) showed that dihdyrofolate reductase could be transported by this pathway after binding a folate analogue in the active site, and that the ligand was almost certainly transported across the membrane with the protein, implying transport in a folded state. The two contrasting pathways used for the targeting of thylakoid lumen proteins are shown diagrammatically in Figure 3.

There is also a wealth of circumstantial, but equally compelling evidence for a similar transport mechanism in bacteria. Studies of the substrate specificity of the Tat system (21,28) indicate that the main substrates are those periplasmic proteins that bind any of a range of redox cofactors, including molybdopterin, FeS and others. There is good evidence that these cofactors can only be inserted in the cytoplasm and hence these proteins can only be exported after folding to a substantial degree. This is impossible by the Sec pathway, which can only transport proteins in an unfolded state, and hence one primary role of the Tat pathway is to transport proteins that are simply obliged to fold prior to translocation. However, there is good evidence that some Tat substrates do not bind cofactors. This applies particularly to substrates of the thylakoid Tat system, where only polyphenol oxidase of the group shown in Figure 1 is believed to bind such a cofactor (a multicopper centre (29)). Thus the second group of substrates is likely to comprise those proteins that either fold too quickly and/or too tightly for the Sec pathway to handle.

The Tat system is clearly capable of carrying out some remarkable translocation reactions. The largest known substrates in bacteria include TMAO reductase and formate dehydrogenase N, which have molecular masses of 83 and 132 kDa, respectively. If, as seems likely, these proteins are exported in a fully folded state, the Tat system must have a quite unique translocation mechanism because the transport of these relatively huge molecules must occur without substantial leakage of protons, since the system operates in energy-transducing membranes. All other protein translocases in such tightly sealed membranes (for example, the endoplasmic reticulum (ER) and bacterial Sec systems, and the chloroplast and mitochondrial envelope-localised translocases) operate by a 'threading' mechanism and it appears that the Tat system must have evolved to overcome the limitations of these systems. At present, however, we know very little

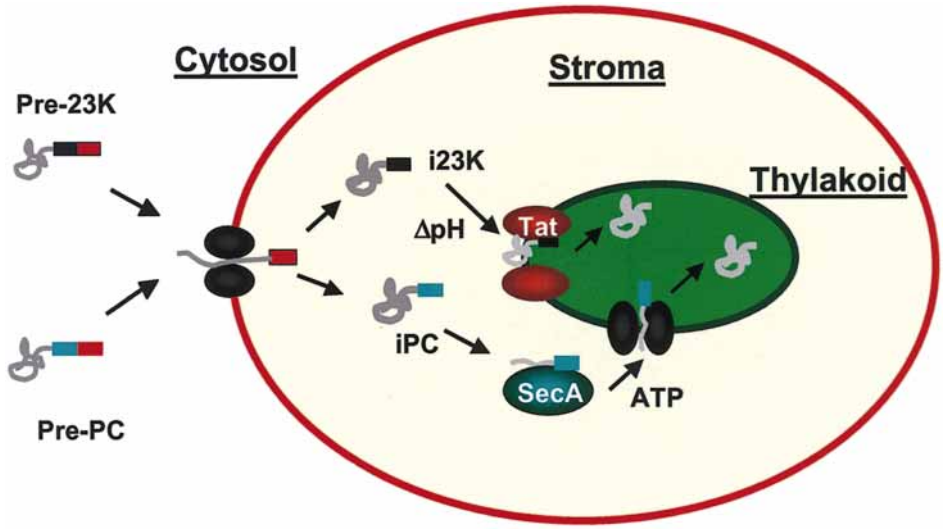

Figure 3: The Tat and Sec pathways for the targeting of thylakoid lumen proteins. Imported thylakoid lumen proteins such as plastocyanin (PC; a Sec substrate) and 23K (a Tat substrate) are synthesised with bipartite presequences containing envelope transit signals (red boxes) and thylakoid-targeting signals in tandem. Sec- and Tat-type thylakoid-targeting signals are depicted by green and black boxes, respectively. The envelope transit peptides of both precursors are recognised by a protein transport system in the envelope membranes which facilitates translocation across the envelope membranes in an unfolded state. The envelope transit signals are usually removed in the stroma. Proteins such as 23K are believed to refold in the stroma before being transported in a folded form by a ΔpH-driven Tat system; the translocation of Sec substrates involves stromal SecA, ATP hydrolysis and a membrane-bound Sec complex believed to be similar to prokaryotic SecYEG complexes. It is presently unclear whether these proteins fold in the stroma, but the later stages involve SecA/ATP-driven translocation of the intermediate in an unfolded state through the Sec translocon. After translocation, substrates on both pathways are processed to the mature forms by the thylakoidal processing peptidase.

about the structure or mechanism of this system. TatA and TatB have been found to co-immunoprecipitate with each other in a solubilized *E. coli* complex of about 600 kDa (30). One artificial Tat substrate has been found to lodge in a thylakoid Tat complex of about the same size (31), but the system has yet to be purified in a functional state or otherwise characterised in any detail.

Signal Recognition Particle-Dependent Insertion of Thylakoid Membrane Proteins: Another 'Inherited' Pathway

The thylakoid membrane houses integral membrane proteins of many types, from simple single-span proteins to complex multispanning, pigment binding proteins. Once again, most of these proteins are imported from the cytosol prior to being inserted into the thylakoid membrane, and the underlying mechanisms have been analysed in some detail, primarily using *in vitro* reconstitution assays. Much of the work has centred on a single model thylakoid membrane protein, the major light-harvesting chlorophyll-binding protein, Lhcb1. This protein spans the membrane three times, with the N-terminus located on the stromal side and the C-terminus in the lumen. As with almost all imported chloroplast proteins, Lhcb1 is synthesised with an N-terminal 'envelope transit' peptide which directs import into the organelle, but unlike the lumenal proteins discussed above there is no second cleavable thylakoid-targeting peptide. Instead, the thylakoid-targeting information is located within the mature Lhcb1 protein (32,33). Initial reconstitution assays showed that this protein undergoes a complex insertion pathway that requires a stromal signal recognition particle (SRP), FtsY, GTP and initially unidentified thylakoid-bound translocation machinery (34–36). These data clearly point to a prokaryotic origin for this pathway because SRP and FtsY have been shown to be involved in the insertion of several membrane proteins in bacteria (reviewed in (37,38)). Both of these factors hydrolyse GTP during their functioning and the emerging picture in bacteria suggests that SRP binds to highly hydrophobic regions, and hence selects mostly membrane proteins, whereas SecA is used for the targeting of soluble periplasmic proteins. Both pathways, however, appear to converge at the SecYEG translocon, at least in some cases (reviewed in (37)). A similar situation may well apply in chloroplasts but this important point has yet to be resolved.

Although the bacterial and chloroplastic SRP-dependent targeting systems are similar in several respects, it is interesting that the chloroplast SRP differs from other known SRPs. The eukaryotic SRP involved in targeting to the ER is a complex particle containing an RNA molecule and six protein subunits, while the *E. coli* particle is a simpler version containing an RNA molecule and one protein subunit, homologous to the 54-kDa signal peptide-binding subunit of the eukaryotic SRP (reviewed in (37,38)). In contrast, the chloroplast SRP contains the 54-kDa subunit together with another 43-kDa subunit that is completely novel (39). This arrangement may

have evolved to cope with the obligatorily post-translational mode of insertion for imported thylakoid membrane proteins, since the eukaryotic and bacterial SRPs may well bind to their cognate substrates in a cotranslational manner. However, further work is required before we can appreciate the global role of SRP in thylakoid membrane biogenesis, since Lhcb1 is the only cytoplasmically synthesised substrate identified to date. Moreover, there is evidence that the 43-kDa subunit specifically recognises a peptide sequence (denoted L18) that is present only in members of the light-harvesting chlorophyll-binding proteins family (40), which raises the possibility that SRP (or at least SRP43-containing SRP) is essentially dedicated to the biogenesis of this type of protein. Other studies strongly suggest a role for SRP in the insertion of one major chloroplast-encoded protein, D1 (41) but it is unclear whether the 43-kDa subunit is involved in this process.

The picture has also become more complex with the recent emergence of a critical new targeting factor that is used for the biogenesis of membrane proteins in many membrane systems. The Oxa1 protein is involved in the biogenesis of a range of mitochondrial inner membrane proteins, specifically those that insert from the matrix side of the membrane (42). An Oxa1 homologue, termed Alb3, is also essential for the insertion of Lhcb1 in thylakoids (43), and recent work has shown an *E. coli* homologue to be essential for the insertion of a range of membrane proteins in this organism (44). The latter work is particularly important because in this study it was found that both SRP-dependent and -independent proteins depend on the Oxa1 homologue, YidC. This raises the question as to how YidC (and Alb3) fit into the SRP-dependent and -independent insertion pathways. Some YidC is found tightly bound to the SecYEG translocon in bacteria (45), suggesting that it might participate in the insertion reaction either before or after SRP and FtsY deliver the membrane protein to the SecYEG translocon. However, YidC is also involved in SRP/Sec-independent insertion reactions, which suggests that it may form a separate translocon on its own or with other, as yet unidentified components. The precise role of Alb3 in thylakoid protein insertion is similarly unclear, but Figure 4 shows one possible model for the SRP-dependent targeting pathway in chloroplasts, in which Alb3 is involved in releasing the inserted protein from the translocon. Note, however, that the thylakoid SecYEG complex has not been shown to be involved in Lhcb1 insertion and this model is based partly on its involvement in the bacterial SRP-dependent pathway.

A Novel Insertion Mechanism for Other Thylakoid Membrane Proteins

The Sec-, Tat- and SRP-dependent targeting pathways described above were all apparently inherited from the cyanobacterial-type progenitors of higher plant chloroplasts, and this conservation of translocation machinery is of course logical in most respects. However, a further pathway has been identified for the targeting of thylakoid membrane proteins, and this particular pathway is not only virtually unique but also clearly more recent in origin. Initial studies showed that a range of single-span membrane proteins, including CF_oII, PsbW and PsbX, differ from other imported membrane proteins in that they are synthesised with clear bipartite presequences that strongly resemble those of imported lumenal proteins. However, these proteins insert into the thylakoid membrane in the absence of SRP, NTPs or a functional Sec machinery, and proteolysis of thylakoids, which destroys all of the known translocation apparatus, has no effect on their insertion (46–49). More recent studies showed that a further protein on this pathway, PsbY, is even more remarkable since this protein contains two cleavable signal peptides, which are cleaved after insertion to yield two small single-span mature proteins (50). In the absence of any identifiable insertion machinery or energy requirements, it has been proposed that these proteins may insert spontaneously, but the possible involvement of unknown machinery (in particular Alb3) cannot yet be ruled out. If this scenario is correct, however, it may be that the role of the signal peptide is to provide a second hydrophobic region which, in concert with that in the mature protein, may partition into the membrane and drive the overall insertion process. This pathway is shown in Figure 4.

Irrespective of the precise insertion mechanism, this is one pathway that appears not to have been inherited from cyanobacteria. Genes encoding proteins homologous to CF_oII, PsbX and PsbY are present in cyanobacteria and also in the plastid genomes of some eukaryotic algae, and in no case is the encoded protein synthesised with a signal peptide. In these organisms, the mature protein is clearly directed into the thylakoid membrane, which suggests that the requirement for cleavable signal peptides coincided with the transfer of the genes to the nucleus. Possibly, the signal peptides are needed for a rather different insertion mechanism that results from the much more complex pathway from cytosol to thylakoid.

Summarising Remarks

The biogenesis of thylakoids has turned out to be much more complex than originally anticipated, and even more targeting pathways may emerge in future studies. The underlying mechanisms are now understood in some detail and most of the known components have been identified and cloned. We also now appreciate the basic distinctions between the Sec- and Tat-dependent lumen-targeting pathways and the rationale behind their existence. However, many aspects of thylakoid proteins remain poorly understood. The Tat components have only recently been identified, we have essentially no idea how this remarkable system works, and the central reactions in the SRP pathway remain to be elucidated in real detail (this applies to bacteria too). Finally, the 'spontaneous' insertion pathway needs further study because it seems inherently unlikely that membrane-spanning proteins could re-

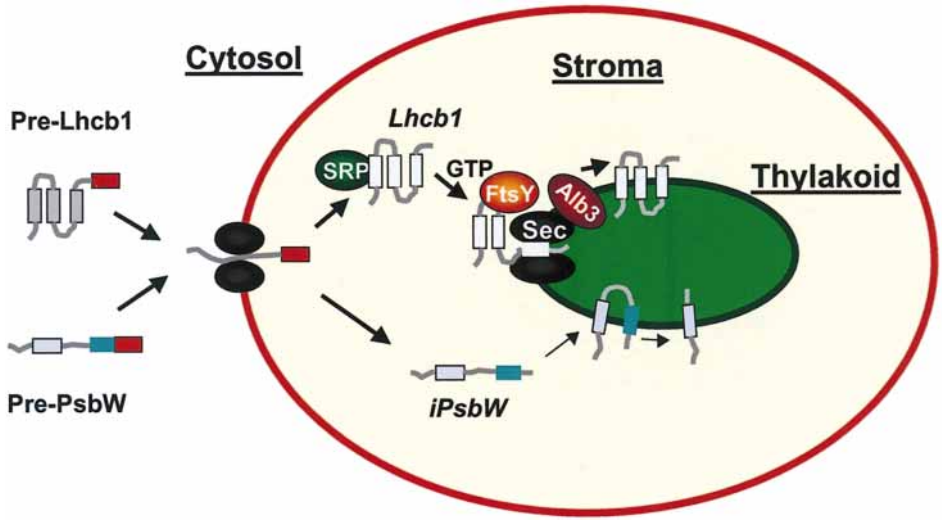

Figure 4: Models for the SRP-dependent and 'spontaneous' targeting pathways for membrane proteins. The SRP pathway: Lhcb1 is a chlorophyll binding protein that spans the membrane three times (membrane-spanning regions depicted as grey boxes). It is synthesised in the cytosol as a precursor containing a single envelope transit peptide. This directs the protein across the envelope membranes in an unfolded form, after which the presequence is removed and the protein is bound by stromal signal recognition particle, SRP (not necessarily in that order). SRP then delivers the protein to translocation apparatus in the thylakoid membrane (probably the SecYEG translocon, but this is not certain) and, together with FtsY, facilitates insertion by a GTP-dependent process. A further factor, Alb3, is required for full insertion. Spontaneous pathway: PsbW is a single-span protein synthesised in the cytosol with a bipartite presequence comprising an envelope transit peptide followed by a hydrophobic signal-type peptide. Translocation across the envelope probably takes place by the standard route, after which the transit peptide is removed and the protein then inserts into the thylakoid membrane in a loop conformation. The thylakoidal processing peptidase cleaves on the lumenal face of the membrane, generating the mature PsbW protein.

main soluble and insertion-competent for so long in the stroma. Nevertheless, the availability of so many genes encoding these components, together with the ability of thylakoids to import proteins so efficiently, means that we can look forward to continued progress in this field. This will be good for an understanding of this remarkable membrane system and also for an appreciation of the bacterial membranes from which it originated.

References

1. Heins L, Collinson IR, Soll J. The protein transport apparatus of chloroplast envelopes. Trends Plant Sci 1998;3:56–61.
2. Manting EH, Driessen AJ. *Escherichia coli* translocase: the unravelling of a molecular machine. Mol Microbiol 2000;37:226–238.
3. Yuan J, Henry R, McCaffery M, Cline K. SecA homolog in protein transport within chloroplasts: evidence for endosymbiont-derived sorting. Science 1994;266:796–798.
4. Laidler V, Chaddock AM, Knott TG, Walker D, Robinson C. A SecY homolog in *Arabidopsis thaliana*. Sequence of a full-length cDNA clone and import of the precursor protein into chloroplasts. J Biol Chem 1995;270:17664–17667.
5. Schuenemann D, Amin P, Hartmann E, Hoffman NE. Chloroplast SecY is complexed to SecE and involved in the translocation of the 33-kDa, but not the 23-kDa subunit of the oxygen-evolving complex. J Biol Chem 1999;274:12177–12182.
6. Mould RM, Robinson C. A proton gradient is required for the transport of two lumenal oxygen-evolving proteins across the thylakoid membrane. J Biol Chem 1991;266:12189–12193.
7. Cline K, Ettinger WF, Theg SM. Protein-specific energy requirements for protein transport across or into thylakoid membranes. Two lumenal proteins are transported in the absence of ATP. J Biol Chem 1992;267:2688–2696.
8. Robinson C, Cai D, Hulford A, Brock IW, Michl D, Hazell L, Schmidt I, Herrmann RG, Klösgen RB. The presequence of a chimeric construct dictates which of two mechanisms are utilized for translocation across the thylakoid membrane: evidence for the existence of two distinct translocation systems. EMBO J 1994;13:279–285.
9. Henry R, Kapazoglou A, McCaffery M, Cline K. Differences between lumen targeting domains of chloroplast transit peptides determine pathway specificity for thylakoid transport. J Biol Chem 1994;269:10189–10192.
10. Chaddock AM, Mant A, Karnauchov I, Brink S, Herrmann RG, Klösgen RB, Robinson C. A new type of signal peptide: central role of a twin-arginine motif in transfer signals for the ΔpH-dependent thylakoidal protein translocase. EMBO J 1995;14:2715–2722.
11. Brink S, Bogsch EG, Edwards WR, Hynds PJ, Robinson C. Targeting of thylakoid proteins by the ΔpH-driven twin-arginine translocation pathway requires a specific signal in the hydrophobic domain in conjunction with the twin-arginine motif. FEBS Lett 1998;434:425–430.
12. Voelker R, Barkan A. Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. EMBO J 1995;14:3905–3914.
13. Settles MA, Yonetani A, Baron A, Bush DR, Cline K, Martienssen R. Sec-independent protein translocation by the maize Hcf106 protein. Science 1997;278:1467–1470.
14. Weiner JH, Bilous PT, Shaw GM, Lubitz SP, Frost L, Thomas GH, Cole JA, Turner RJ. A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell 1998;93:93–101.
15. Sargent F, Bogsch EG, Stanley NR, Wexler M, Robinson C, Berks BC, Palmer T. Overlapping functions of components of a bacterial Sec-independent export pathway. EMBO J 1998;17:3640–3650.

16. Sargent F, Stanley NR, Berks BC, Palmer T. Sec-independent protein translocation in *Escherichia coli*. A distinct and pivotal role for the TatB protein. J Biol Chem 1999;274:36073–36082.
17. Bogsch EG, Sargent F, Stanley NR, Berks BC, Robinson C, Palmer T. An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. J Biol Chem 1998;273:18003–18006.
18. Wexler M, Sargent F, Jack RL, Stanley NR, Bogsch EG, Robinson C, Berks BC, Palmer T. TatD is a cytoplasmic protein with DNase activity. No requirement for TatD family proteins in Sec-independent protein export. J Biol Chem 2000;275:16717–16722.
19. Walker MB, Roy LM, Coleman E, Voelker R, Barkan A. The maize tha4 gene functions in Sec-independent protein transport in chloroplasts and is related to hcf106, tatA and tatB. J Cell Biol 1999;147:267–275.
20. Mori H, Summer EJ, Ma X, Cline K. Component specificity for the thylakoidal Sec and delta pH-dependent protein transport pathways. J Cell Biol 1999;146:45–55.
21. Berks BC. A common export pathway for proteins binding complex redox cofactors? Mol Microbiol 1996;22:393–404.
22. Stanley NR, Palmer T, Berks BC. The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in *Escherichia coli*. J Biol Chem 2000;275:11591–11596.
23. Mori H, Cline K. A signal peptide that directs non-Sec transport in bacteria also directs efficient and exclusive transport on the thylakoid delta pH pathway. J Biol Chem 1998;273:11405–11408.
24. Wexler M, Bogsch EG, Palmer T, Robinson C, Berks BC. Targeting signals for a bacterial Sec-independent export system direct plant thylakoid import by the ΔpH pathway. FEBS Lett 1998;431:339–342.
25. Halbig D, Hou B, Freudl R, Sprenger GA, Klösgen RB. Bacterial proteins carrying twin-R signal peptides are specifically targeted by the ΔpH-dependent transport machinery of the thylakoid membrane system. FEBS Lett 1999;447:95–98.
26. Clark SA, Theg SM. A folded protein can be transported across the chloroplast envelope and thylakoid membranes. Mol Biol Cell 1997;8:923–934.
27. Hynds PJ, Robinson D, Robinson C. The Sec-independent twin-arginine translocation system can transport both tightly folded and malfolded proteins across the thylakoid membrane. J Biol Chem 1998;273:34868–34874.
28. Santini CL, Ize B, Chanal A, Müller M, Giordano G, Wu LF. A novel Sec-independent periplasmic protein translocation pathway in *Escherichia coli*. EMBO J 1998;17:101–112.
29. Koussevitzky S, Ne'eman E, Sommer A, Steffens JC, Harel E. Purification and properties of a novel chloroplast stromal peptidase. Processing of polyphenol oxidase and other imported precursors. J Biol Chem 1998;273:27064–27069.
30. Bolhuis A, Bogsch EG, Robinson C. Subunit interactions in the twin-arginine translocase complex of *Escherichia coli*. FEBS Lett 2000;472:88–92.
31. Berghöfer J, Klösgen RB Two distinct translocation intermediates can be distinguished during protein transport by the TAT (delta pH) pathway across the thylakoid. FEBS Letters 1999;460:328–332.
32. Lamppa GK. The chlorophyll a/b -binding protein inserts into the thylakoids independent of its cognate transit peptide. J Biol Chem 1988;263:14996–14999.
33. Viitanen PV, Doran ER, Dunsmuir P. What is the role of the transit peptide in thylakoid integration of the light-harvesting chlorophyll a/b protein? J Biol Chem 1988;263:15000–15007.
34. Li X, Henry R, Yuan J, Cline K, Hoffman NE. A chloroplast homologue of the signal recognition particle subunit SRP54 is involved in the post-translational integration of a protein into thylakoid membranes. Proc Natl Acad Sci USA 1995;92:3789–3793.
35. Tu CJ, Schuenemann D, Hoffman NE. Chloroplast FtsY, chloroplast signal recognition particle, and GTP are required to reconstitute the soluble phase of light-harvesting chlorophyll protein transport into thylakoid membranes. J Biol Chem 1999;274:27219–27224.
36. Kogata N, Nishio K, Hirohashi T, Kikuchi S, Nakai M. Involvement of a chloroplast homologue of the signal recognition particle receptor protein, FtsY, in protein targeting to thylakoids. FEBS Lett 1999;329:329–333.
37. Dalbey RE, Robinson C. Protein translocation into and across the bacterial plasma membrane and the plant thylakoid membrane. Trends Biochem Sci 1999;24:17–22.
38. De Gier J-WL, Valent QA, von Heijne G, Luirink J. The *E. coli* SRP: preferences of a targeting factor. FEBS Lett 1997;408:1–4.
39. Schuenemann D, Gupta S, Persello-Cartieaux F, Klimyuk VI, Jones JDG, Nussaume L, Hoffman NE. A novel signal recognition particle targets light-harvesting proteins to the thylakoid membranes. Proc Natl Acad Sci USA 1998;95:10312–10316.
40. DeLille J, Peterson EC, Johnson T, Moore M, Kight A, Henry R. A novel precursor recognition element facilitates posttranslational binding to the signal recognition particle in chloroplasts. Proc Natl Acad Sci USA 2000;97:1926–1931.
41. Nilsson R, Brunner J, Hoffman NE, van Wijk KJ. Interactions of ribosome nascent chain complexes of the chloroplast-encoded D1 thylakoid membrane protein with cpSRP54. EMBO J 1999;18:733–742.
42. Herrmann JM, Neupert W, Stuart RA. Insertion into the mitochondrial inner membrane of a polytopic protein, the nuclear-encoded Oxa1p. EMBO J 1997;16:2217–2226.
43. Moore M, Harrison MS, Peterson EC, Henry R. Chloroplast Oxa1p homolog albino3 is required for post-translational integration of the light harvesting chlorophyll-binding protein into thylakoid membranes. J Biol Chem 2000;275:1529–1532.
44. Samuelson JC, Chen M, Jiang F, Moeller I, Wiedmann M, Phillips G, Dalbey RE. YidC mediates membrane protein insertion in bacteria. Nature 2000;406:637–641.
45. Scotti PA, Urbanus ML, Brunner J, de Gier JW, von Heijne G, van der Does C, Driessen AJM, Oudega B, Luirink J. YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. EMBO J 2000;19:in press.
46. Michl D, Robinson C, Shackleton JB, Herrmann RG, Klösgen RB. Targeting of proteins to the thylakoids by bipartite presequences: CF_0II is imported by a novel, third pathway. EMBO J 1994;13:1310–1317.
47. Kim SJ, Robinson C, Mant A. Sec/SRP-independent insertion of two thylakoid membrane proteins bearing cleavable signal peptides. FEBS Lett 1998;424:105–108.
48. Lorkovic ZJ, Schröder WP, Pakrasi HB, Irrgang K-D, Herrmann RG, Oelmüller R. Molecular characterisation of PSII-W, the only nuclear-encoded component of the photosystem II reaction centre. Proc Natl Acad Sci USA 1995;92:8930–8934.
49. Robinson D, Karnauchov I, Herrmann RG, Klösgen RB, Robinson C. Protease-sensitive thylakoidal import machinery for the Sec-, ΔpH- and signal recognition particle-dependent protein targeting pathways, but not for CF_0II integration. Plant J 1996;10:149–155.
50. Thompson SJ, Robinson C, Mant A. Dual signal peptides mediate the Sec/SRP-independent insertion of a thylakoid membrane polyprotein, PsbY. J Biol Chem 1999; 274:4059–4066.