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Protein Import and Routing Systems of Chloroplasts

Kenneth Keegstra[a,1] and Kenneth Cline[b]

[a]Michigan State University–Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

[b]Horticultural Sciences Department, University of Florida, Gainesville, Florida 32611

INTRODUCTION

Plastids are ubiquitous plant cell organelles that perform many essential functions. Chloroplasts are the most complex type of plastid, both structurally and functionally, containing six distinct compartments and performing essential processes, such as fatty acid and amino acid biosynthesis in addition to their well-known role in photosynthesis. The many plastid proteins needed for these and other essential metabolic pathways derive from two genetic systems. The plastid genome encodes ~100 proteins (Sugiura, 1989), but the vast majority of plastid proteins are encoded by nuclear genes and synthesized as precursors in the cytoplasm. Consequently, specific and reliable protein transport mechanisms are needed to direct these precursor proteins to their proper location within chloroplasts.

Most studies of protein transport to and within chloroplasts have been performed with isolated organelles, whereby the general process of protein transport, as schematically presented in Figure 1, can be broken down into three distinct phases: specific targeting of precursor proteins to plastids, transport across the two envelope membranes, and in many cases, sorting to the proper plastidic compartment. One major conclusion derived from these studies is that proper localization of cytoplasmically synthesized chloroplastic proteins is accomplished by specific interactions between targeting sequences contained within the precursor proteins and the transport machinery contained within chloroplasts. This review focuses on the transport process and the machinery that mediates it; it also includes a brief consideration of targeting sequences. Many excellent reviews have covered earlier progress in understanding chloroplastic protein transport (Keegstra et al., 1989; de Boer and Weisbeek, 1991; Schnell, 1995, 1998; Cline and Henry, 1996; Kouranov and Schnell, 1996; Fuks and Schnell, 1997; Lübeck et al., 1997a; Heins et al., 1998); these reviews can be consulted for additional details.

[1]To whom correspondence should be addressed. E-mail keegstra@pilot.msu.edu; fax 517-353-9168.

TARGETING SEQUENCES

Most precursor proteins are larger than their corresponding plastid-localized forms. The N-terminal transit peptide that is cleaved off the precursor protein upon entry into chloroplasts (Figure 1) usually contains stromal-targeting information that is necessary and sufficient for transport of precursors across the two envelope membranes (Keegstra et al., 1989; de Boer and Weisbeek, 1991). In some cases, the cleavable transit peptide is bipartite and contains additional targeting information, as is best exemplified by proteins destined for the thylakoid lumen. In addition to the stromal-targeting domain, bipartite peptides of lumenal proteins contain a second domain that directs transport across the thylakoid membrane (Figure 1; de Boer and Weisbeek, 1991; Cline and Henry, 1996). The two domains of these bipartite transit peptides are removed by two separate processing proteases (Figure 1, path 3): one in the stroma (VanderVere et al., 1995), and one in the thylakoid lumen (Chaal et al., 1998).

Many proteins, especially those destined for the inner envelope membrane or the thylakoid membrane, have both a cleavable stromal-targeting domain and additional targeting information retained within the mature protein (Figure 1, paths 2 and 4). A few precursors, especially those destined for the outer envelope membrane, do not have cleavable targeting sequences (Figure 1, path 6). In the latter case, information directing the protein to its proper location is contained within the mature protein (Cline and Henry, 1996).

Despite their common function, stromal-targeting domains from various precursor proteins may vary in length from 30 to >100 amino acids. Interestingly, they share minimal sequence similarity so that no consensus targeting sequences have been established, and efforts to identify common secondary structures have not been successful (Keegstra et al., 1989; von Heijne et al., 1989; von Heijne and Nishikawa, 1991). Most stromal-targeting domains are rich in serine and threonine but deficient in acidic amino acids (Keegstra et al., 1989). The lumenal-targeting domain from bipartite transit peptides has strong similarity to the hydrophobic signal sequences found in secreted proteins (Cline and Henry, 1996). The targeting information contained within mature proteins generally is located in membrane-spanning domains, where

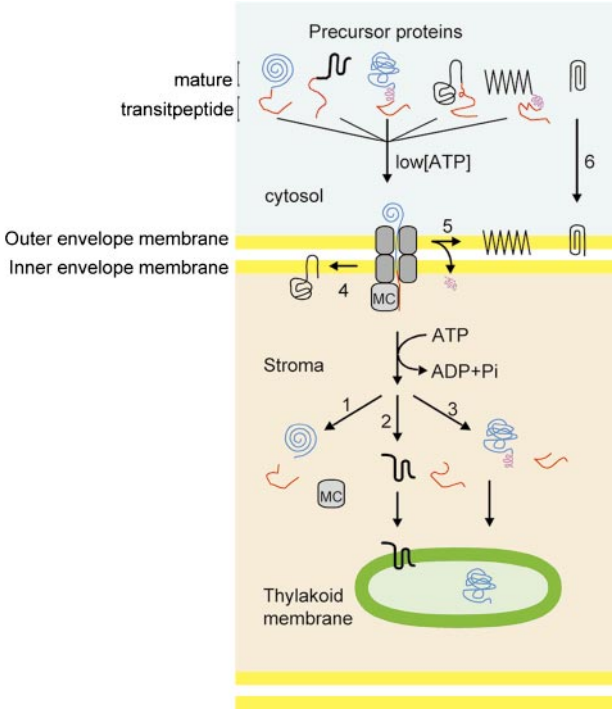

Figure 1. Schematic Representation of Pathways Responsible for Targeting Proteins to Their Proper Location within Chloroplasts.

The large majority of precursor proteins have a cleavable transit peptide (shown in red). In most cases, the transit peptide includes a stromal-targeting domain that initiates transport of the precursor through the general import pathway (shown in gray). The driving force for protein translocation is thought to be provided by molecular chaperones (MC) that pull precursor proteins into plastids. Precursors lacking additional targeting information are thus deposited into the stromal space, where the stromal-processing protease removes the transit peptide (path 1). For precursors destined for insertion into membranes, the additional targeting information generally is contained within the mature region of the protein (paths 2 and 4), although some proteins may require a stop-transfer signal for localization to the outer membrane (path 5). Precursors for some outer membrane proteins lack a cleavable transit peptide and are inserted directly into the outer membrane without using the general import pathway (path 6). Some precursors destined for the thylakoid lumen require a second targeting signal (shown in purple), which is cleaved as the proteins enter the lumen (path 3). See text for additional details.

it also can serve as a membrane insertion or a stop-transfer signal (Cline and Henry, 1996).

TRANSPORT ACROSS THE ENVELOPE MEMBRANES

Transport across the two envelope membranes, mediated by stromal-targeting sequences, is a common step for all proteins that enter plastids, regardless of their ultimate destination in the organelles. Most of the proteins studied to date are imported via a single transport system, which is sometimes referred to as the general import pathway (Cline and Henry, 1996; Heins et al., 1998). Although most of the work so far performed has focused on this pathway, it is possible, even likely, that other routes exist for entry into chloroplasts.

Stages of Envelope Membrane Transport

As detailed in Figure 2, transport across the envelope membranes can be divided into at least three stages. In the first stage, precursors associate reversibly with plastids (Figure 2, stage a). This interaction is thought to be mediated in part by protein–protein interactions and in part by protein–lipid interactions. Transit peptides interact specifically with lipids from the outer envelope membrane (Pinnaduwage and Bruce, 1996), and mutants deficient in galactolipids are impaired in their ability to import precursors (Chen and Li, 1998). One line of evidence for protein–protein interactions comes from cross-linking studies that are discussed in more detail below.

The next two stages in import usually are distinguished by their different energy requirements (Figures 1 and 2). Incubation of chloroplasts with precursor proteins in the presence of GTP or low concentrations of ATP ($\leqslant$100 μM) results in a stable association between precursors and the import machinery, but it leaves the precursors in a state in which they can be degraded by exogenous proteases (Olsen and Keegstra, 1992). This association often has been called binding but is more accurately described as an early translocation intermediate (Figure 2, stage b).

When adequate levels of ATP are present, complete translocation of precursors occurs, and processed forms accumulate inside the organelle (Figure 2, stage c). The ATP required for complete translocation of precursors is hydrolyzed within the organelle (Theg et al., 1989), presumably by molecular chaperones that pull precursors into the organelle (Figures 1 and 2). In contrast to mitochondria, where a proton-motive force often is required for protein import (Pfanner et al., 1997), a membrane potential is not needed for transport across the chloroplastic envelope membranes (Theg et al., 1989).

Components of the Translocation Machinery

Considerable progress has been made in identifying components of the envelope-based transport apparatus by means of two different biochemical strategies, chemical cross-linking and solubilization of translocons with mild detergents. Although variations of these two strategies have been used by multiple investigators, the results generally convey similar views of the translocation apparatus (summarized schematically in Figure 2). The components identi-

fied fall into three categories: outer membrane proteins, inner membrane proteins, and molecular chaperones. Each group is summarized below.

Components of the Outer Envelope Membrane

Three outer envelope membrane proteins have been identified as part of the Toc complex: Toc86, Toc75, and Toc34. (Toc is an acronym for translocon at the outer membrane of chloroplasts; the number designates the molecular mass of the given protein in kilodaltons; see Schnell et al. [1997] for details.) These three proteins interact with each other in the outer envelope membrane to form a complex even in the absence of preproteins that are in the process of translocation (Ma et al., 1996; Nielsen et al., 1997). Important unresolved questions regarding Toc complexes include the stoichiometry of individual components within the complex and the molar ratio of complexed components to components free to diffuse in the membrane.

Toc86 is a GTP-binding protein that is postulated to function as an import receptor (Hirsch et al., 1994; Perry and Keegstra, 1994). Although it lacks predicted membrane-spanning helices, it is an integral membrane protein, presumably anchored by transmembrane β-strands near the C terminus (Hirsch et al., 1994; Kessler et al., 1994). Toc86 is readily degraded when intact chloroplasts are subjected to mild protease treatment, indicating that it is exposed on the exterior chloroplastic surface. Recent evidence indicates that Toc86 is itself the result of proteolytic degradation (D. Schnell, personal communication).

Ordinarily, the 86-kD form of the putative receptor is the only one seen when isolated envelope membrane proteins are analyzed by SDS-PAGE, but when care is taken to prevent proteolysis, a 159-kD form also is detected. Apparently, the previously isolated cDNA clones were not full length, because the recently identified full-length pea cDNA clone predicts a protein of 159 kD (D. Schnell, personal communication). Schnell's data explain the puzzling observation that the Arabidopsis gene that encodes the Toc86 homolog (which is present on the bacterial artificial chromosome clones AF069298 and AC002330) is predicted to encode a protein of 159 kD.

The conclusion that Toc86 is a degradation product derived from a larger protein has profound implications for studies on the early stages of protein import. The extent to which Toc159 is converted to Toc86 may well vary in different preparations of intact chloroplasts, depending upon the procedures used to isolate intact organelles. Until it is known how this cleavage affects the activity of the putative receptor protein, previous conclusions regarding Toc86 function need to be interpreted cautiously.

Toc34, also a GTP-binding protein, has limited sequence similarity to the N-terminal region of Toc86 (Kessler et al., 1994). Toc34 is predicted to have a single membrane-spanning helix near its C terminus, with the majority of its hydrophilic domain exposed at the cytoplasmic surface of the outer membrane (Chen and Schnell, 1997; Li and Chen, 1997). Recent cross-linking studies have shown that Toc34 is also in close physical proximity to preproteins destined for import, but only very early in the translocation process (Kouranov and Schnell, 1997). Small quantities of ATP or GTP can cause rearrangements that prevent cross-linking of preproteins to Toc 34. Based on this evidence, it seems that Toc34 is also a good candidate for part of the receptor complex (Figure 2).

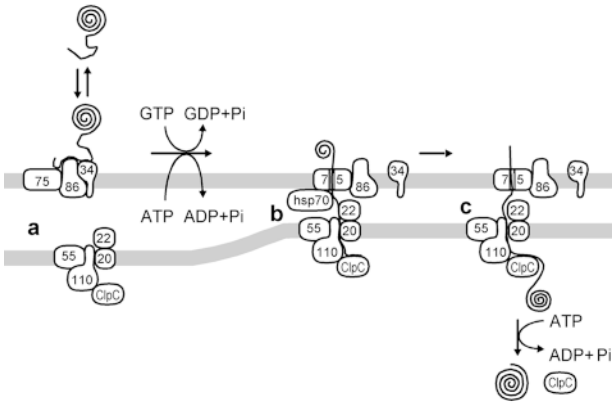

Figure 2. Schematic Representation of the Components and Stages Involved in the Translocation of Precursors across Plastid Envelope Membranes.

Details about each component and the stages in the translocation process are presented in the text. Although the Toc and Tic complexes show one molecule of each component, the stoichiometry of components in the complexes is not known. The composition of complexes may be dynamic, with certain components entering or leaving complexes during the course of translocation, as is shown here for hsp70 and Toc34.

Toc33, a second small GTP-binding protein with high sequence similarity to Toc34, recently was identified in Arabidopsis (Jarvis et al., 1998). The authors demonstrate that disruption of the Arabidopsis Toc33 gene causes defects in protein import, both in vivo and in vitro, providing the first in vivo evidence on the physiological relevance of the envelope translocation components. Interestingly, disruption of the gene encoding Toc33 was not lethal, presumably because of the presence of Toc34. However, gene disruption causes a severe phenotype early in development, a time when Toc33 is highly expressed and Toc34 is expressed at lower levels. The biological significance of the two genes and their differential expression is not understood, and it is not known whether other plant species also have two distinct versions of this import component.

Toc75, the most abundant protein in the outer envelope membrane, is postulated to form the channel through which

precursor proteins are transported across the outer membrane (Schnell et al., 1994; Tranel et al., 1995). Toc75 is deeply embedded in the membrane and is highly resistant to proteolysis from the outer surface (Tranel et al., 1995). Like Toc86, it lacks predicted membrane-spanning helices and is postulated to span the membrane with multiple β-strands. The best experimental evidence supporting its proposed function as a channel is from experiments demonstrating that highly purified Toc75, produced in *Escherichia coli*, is capable of forming a voltage-gated ion channel when reconstituted in a phospholipid membrane (Hinnah et al., 1997). Nevertheless, many questions remain unanswered about how Toc75 functions as a protein-conducting channel in the plastid outer envelope membrane. For example, how is channel opening and closing regulated in the outer envelope membrane, which lacks a membrane potential? Do other components contribute to channel function? These questions will perhaps be resolved after the reconstitution of entire Toc complexes in lipid bilayers.

Although most of the membranous translocation components of plastids lack sequence similarity to transport components found in the endoplasmic reticulum (ER) membrane or the mitochondrial transport apparatus, Toc75 has significant sequence similarity to an open reading frame from *Synechocystis* PCC 6803 (Reumann et al., 1999). Given the endosymbiont hypothesis, which posits that chloroplasts arose from free-living cyanobacteria, it is attractive to consider whether the *Synechocystis* gene might provide insight into the evolutionary origins of the protein transport apparatus. The precise function of the *Synechocystis* protein remains to be established, but one appealing hypothesis is that it represents part of a system used by Gram-negative bacteria to secrete proteins and that after endosymbiosis it was adapted for use in the protein import system (Reumann et al., 1999).

Components of the Inner Envelope Membrane

Several proteins have been identified as putative components of the Tic complex (for translocon at the inner membrane of chloroplasts). The best studied is Tic110, which has been identified independently by two groups (Kessler and Blobel, 1996; Lübeck et al., 1996). Although both groups agree that Tic110 has a membrane anchor near the N terminus, they come to opposite conclusions regarding its topology in the membrane. Lübeck et al. (1996) conclude that the large C-terminal hydrophilic domain extends into the intermembrane space, whereas Kessler and Blobel (1996) conclude that it extends into the stroma. Jackson et al. (1998) recently reinvestigated this issue and verified that the hydrophilic domain is in the stroma. Although the precise function of Tic110 remains unclear, Kessler and Blobel (1996) present evidence that the stromal hydrophilic domain interacts with molecular chaperones, specifically cpn60. Other studies do not detect an association of Tic110 with cpn60; rather, they observe an association with the chaperone ClpC, an interaction that may have important implications for the mechanism of import (Akita et al., 1997; Nielsen et al., 1997).

More recently, three other proteins have been identified as inner membrane translocation components. Tic55 is found associated with other translocation components by blue native gel electrophoresis (Caliebe et al., 1997). This protein is unusual among translocation components in that it contains an iron–sulfur center. The role for a putative redox center in protein translocation remains to be explained. However, it should be noted that Tic55 was not found in translocon generated with homobifunctional cross-linkers (M. Akita and K. Keegstra, unpublished observations) or in the translocon reported by Kouranov et al. (1998). Tic20 and Tic22 have been identified by a label-transfer cross-linking strategy, providing evidence that both are closely associated with preproteins undergoing translocation (Kouranov and Schnell, 1997). Tic20 is predicted to be an integral membrane protein, possibly part of the inner membrane translocation channel, whereas Tic22 is a peripheral membrane protein and possibly serves to connect inner membrane complexes to outer membrane complexes (Kouranov et al., 1998).

Finally, a 44-kD protein has been identified as part of the envelope-based translocation machinery (Ko et al., 1995). However, the role of this protein is unclear because it has been reported to be a component of both the inner and outer envelope membranes.

Molecular Chaperones and the Translocation Machinery

Molecular chaperones play essential roles in protein translocation into mitochondria (Pfanner et al., 1997) and during transport into the lumen of the ER (Walter and Johnson, 1994; see Vitale and Denecke, 1999, in this issue). It is likely that they also are involved in chloroplastic protein import, and chloroplasts have several molecular chaperones that are possible candidates. Com70, for instance, a heat shock protein (hsp) 70 found on the cytoplasmic surface of the outer envelope membrane, has been postulated to play a role in the early stages of protein import (Kourtz and Ko, 1997), although other investigators have not identified this hsp70 as a component of the Toc complex (Nielsen et al., 1997). A different hsp70, however, located on the inner side of the outer envelope membrane, has been identified as part of translocon solubilized with mild detergents (Figure 2, stage b; Schnell et al., 1994). This intermembrane-space hsp70 may be responsible for unfolding activity associated with the outer envelope membrane (Guéra et al., 1993; America et al., 1994). In this capacity, it may provide the driving force for preprotein transport across the outer envelope membrane and may account for the observed distinction between transport processes across the outer envelope membrane and those across the inner membrane, as reported by Scott and Theg (1996).

Molecular chaperones in the stromal space are thought to drive protein import into the stroma by pulling preproteins into chloroplasts through repeated cycles of binding and release. By analogy to the hsp70-dependent protein import of mitochondria (Pfanner et al., 1997), a stromal hsp70 might be expected to promote chloroplastic protein import. However, no stromal hsp70 could be found in the translocon (Nielsen et al., 1997). Rather, the translocon contains ClpC, a stromal protein of the hsp100 family of molecular chaperones (Akita et al., 1997; Nielsen et al., 1997). More work is needed to determine whether this chaperone is analogous to the mitochondrial hsp70 or whether it works in concert with the known stromal hsp70 in ways that still need to be described. In this respect, it is interesting to note that two separate functions have been described for hsp70 during mitochondrial protein import, namely, driving protein translocation and assisting in the early stages of preprotein folding (Pfanner et al., 1997). It is possible that ClpC and hsp70 cooperate to achieve these two different functions in chloroplasts.

Despite rapid progress in the identification of putative translocation components, much remains to be learned about their functions and how they interact with each other and with precursor proteins to accomplish protein translocation. Functions have not been established for several components, including Tic110, Tic55, Tic22, and Tic 20. Even for Toc86, Toc75, Toc34, and ClpC, where functions have been postulated, the experimental evidence supporting these hypotheses remains incomplete.

TARGETING TO THE ENVELOPE MEMBRANES

Protein Delivery to the Outer Envelope Membrane

At least two different pathways have been described for targeting proteins to the outer envelope membrane (Figure 1), and it is possible that others exist. The first (Figure 1, path 6) is used for the transport of various small proteins, such as Toc34, that are not synthesized with a cleavable N-terminal peptide (Li et al., 1991; Chen and Schnell, 1997; Li and Chen, 1997). Insertion of these proteins into the outer membrane of chloroplasts does not require hydrolysis of nucleoside triphosphates, nor does it appear to depend on proteins of the outer membrane. It seems likely that the partitioning into the lipid bilayer of hydrophobic domains contained within these proteins controls their insertion into the membrane (Li and Chen, 1996).

A second novel pathway (Figure 1, path 5) is used by the Toc75 precursor, which contains a bipartite transit peptide (Tranel and Keegstra, 1996). The N-terminal stromal-targeting portion of the transit peptide engages the components of the general import pathway and is removed by the stromal-processing protease. The resulting intermediate, still containing the second portion of the original bipartite transit peptide, can be detected by in vitro import assays and in vivo in young tissues (Tranel et al., 1995). It is hypothesized that the second domain of the transit peptide serves as a stop-transfer signal to halt translocation within the envelope membrane while Toc75 assumes its mature conformation (Tranel and Keegstra, 1996). The second portion of the bipartite transit peptide then is removed by an unidentified protease.

Protein Delivery to the Inner Envelope Membrane

Proteins destined for the inner envelope membrane have a transit peptide that consists of a stromal-targeting domain (Figure 1, path 4; Li et al., 1992; Brink et al., 1995). The information that directs these proteins to the inner envelope membrane, however, is contained within the mature region of the protein and has been localized by several studies to hydrophobic transmembrane domains (Brink et al., 1995; Knight and Gray, 1995; Lübeck et al., 1997b). Such envelope-targeting elements might function to promote insertion of the imported protein from the stroma into the inner envelope membrane. Such a mechanism has been proposed recently for Tic110 localization (Lübeck et al., 1997b).

Alternatively, hydrophobic segments within passenger proteins may function as stop-transfer sequences to induce premature release from the import machinery at the inner envelope membrane. Both mechanisms, that is, stop transfer and delivery to the inner membrane subsequent to import, operate in mitochondria (Hartl and Neupert, 1990; Glick et al., 1992). Regardless of which pathway is used in chloroplasts, the interesting question arises as to how specificity for envelope versus thylakoids is achieved. Many thylakoid proteins contain hydrophobic segments that must bypass any putative stop-transfer mechanism. On the other hand, hydrophobic domains of thylakoid proteins function as membrane insertion signals for thylakoid localization. Clearly, many interesting questions regarding the insertion pathway and membrane specificity need to be addressed.

TARGETING TO THE THYLAKOID MEMBRANE SYSTEM

Thylakoid proteins are imported into the stroma by the general import machinery and then are translocated into or across the thylakoid membrane (reviewed in Cline and Henry, 1996; Robinson and Mant, 1997; Schnell, 1998). Such a process is supported by several lines of evidence, including the identification of productive stromal intermediates and in vitro assays for translocation into isolated thylakoids. The availability of such reconstitution assays, in which experimental conditions can be varied so as to define the energy supply, stromal proteins, and so on, has led to greater insight into the thylakoid-based protein import mechanisms.

Multiple Precursor-Specific Pathways Target Proteins into Thylakoids

One unexpected result of studies into the transport of proteins into thylakoids is that precursor proteins fall into at least four distinct groups based on their translocation/integration requirements (Table 1). Three groups of precursor proteins display translocation requirements indicative of transport by proteinaceous machinery. A fourth group of thylakoid proteins, all integral membrane proteins, lacks obvious energy and protein requirements, suggesting the possibility of a spontaneous insertion mechanism (see Robinson and Mant, 1997, for discussion). Group-specific requirements imply that several distinct mechanisms are involved in the targeting and/or translocation steps. Competition studies with precursor proteins overexpressed in *E. coli* (Cline et al., 1993) confirm the conclusion that thylakoid proteins are specifically targeted to multiple distinct translocation pathways after their import into the stroma (Figure 3).

Isolation of Components Reveals that Pathways Are Derived from the Endosymbiont

As stated above, it generally is accepted that chloroplasts evolved from a prokaryotic endosymbiont similar to a cyanobacterium. Thus, it was anticipated that thylakoid protein transport pathways would be related to prokaryotic machinery that exports proteins from the cytoplasm. In addition to the work of Reumann et al. (1999) mentioned above, corroboration of this assumption comes from biochemical and genetic studies showing that thylakoid translocation components are homologous with those of the bacterial export machinery.

The Thylakoid Sec Pathway

Current evidence suggests a relationship between one thylakoid transport pathway and the bacterial Sec system, which consists of an azide-sensitive translocation ATPase, called SecA, and a heterotrimeric SecY/E/G membrane protein. SecA appears to undergo cycles of membrane insertion and de-insertion so as to push segments of precursor proteins across the lipid bilayer; SecY/E/G makes up part of the translocation channel (Economou, 1998). Transport characteristics of the 33-kD subunit of the oxygen-evolving complex (OE33), plastocyanin (PC), and subunit F of photosystem I (PSI-F) (Table 1), including sensitivity to sodium azide (Henry et al., 1994; Knott and Robinson, 1994), were among the preliminary indications that a SecA-like protein might be involved in thylakoid translocation.

A SecA homolog (cpSecA) subsequently was purified from pea chloroplast stroma and shown to promote ATP-dependent transport of OE33 and PC into washed thylakoids. However, cpSecA has no effect on the transport of OE23 and OE17 or on the integration of light-harvesting chlorophyll binding protein (LHCP) (Yuan et al., 1994). Concurrently, a cDNA clone for pea cpSecA was isolated, and antibodies, prepared to an expressed peptide, inhibited transport of OE33 but not OE23 (Nakai et al., 1994). Plant chloroplast homologs of SecY (Laidler et al., 1995) and SecE (H. Mori and K. Cline, unpublished data) also have been identified and shown to localize to thylakoids. An antibody prepared against a pea cpSecY peptide inhibits transport of the cpSecA-dependent precursors (H. Mori, E. Summer, and K. Cline, unpublished data), supporting the idea that cpSecA functions in concert with cpSecY (Figure 3).

Although much work remains to be done, present data suggest that the mechanism of the thylakoidal Sec pathway is similar to that of the prokaryotic system. CpSecA is present in both the stromal and thylakoid fractions (Nakai et al., 1994; Yuan et al., 1994), but functional interaction of cpSecA with precursor proteins appears to occur only on the membrane. A stable cpSecA–precursor complex forms on thylakoids when the system is depleted of ATP with apyrase (Haward et al., 1997; X. Ma and K. Cline, unpublished data). Upon addition of ATP, some of the bound precursor is transported into the lumen (Haward et al., 1997). Such complexes, moreover, are formed solely with precursor proteins

Table 1. Thylakoid Protein Translocation Requirements[a]

Precursor Protein	Destination	Stromal Protein(s)[b]	Thylakoid Protein(s)[b]	Energy[b]	
				NTP	ΔpH
OE33, PC, PSI-F[c]	Lumen	+	+	ATP	∼
OE23, OE17, PSII-T, PSI-N	Lumen	−	+	−	+
LHCP	Membrane	+	+	GTP	∼
PSII-W, PSII-X, CF_0-II, ELIP[d]	Membrane	−	−	−	−[e]

[a]Reviewed in Cline and Henry (1996), Robinson and Mant (1997), and Schnell (1998).

[b] + indicates a requirement for the component; − indicates no requirement; ∼ indicates a stimulatory effect.

[c]PSI-F is membrane anchored but faces the lumen.

[d]Kruse and Kloppstech (1992).

[e]A small ΔpH effect observed for CF_0II insertion. ΔpH requirement was not examined for ELIP.

that are transported by cpSecA (X. Ma and K. Cline, unpublished data).

Detailed characterization of the mechanism of the thylakoid system is required before further analogies can be drawn. However, one capability of the bacterial Sec machinery not shared by the thylakoid Sec machinery is the ability to translocate ΔpH pathway passenger proteins (see below). For instance, OE23, when fused to a Sec-compatible targeting signal, is efficiently transported by the *E. coli* Sec machinery in vivo, whereas the identical OE23 fusion protein is recalcitrant to transport by the thylakoid Sec machinery (Henry et al., 1997; see below.)

Evidence for the in vivo role of cpSecA comes from analysis of the maize mutant *tha1. tha1* is a seedling-lethal, high chlorophyll fluorescence *(hcf)* mutant that is selectively defective in transport of OE33, PC, PSI-F, and the plastid-encoded cytochrome *f* (Voelker and Barkan, 1995). The *tha1* gene encodes a protein highly related to pea and spinach cpSecA (Voelker et al., 1997). Subsequent in vitro analysis confirms that cytochrome *f* transport requires cpSecA (Nohara et al., 1996; Mould et al., 1997). The essential in vivo role of cpSecY also has been demonstrated recently with the isolation of a cpSecY deletion mutant (Roy and Barkan, 1998). This mutant also has a seedling-lethal phenotype but exhibits an even more severe defect in thylakoid biogenesis than does *tha1.*

Chloroplast Signal Recognition Particle–like Pathway

The LHCP integration pathway (Table 1) shares some features with the GTP-dependent signal recognition particle (SRP) systems of the ER and bacteria (Walter and Johnson, 1994). Upon import into the stroma, LHCP is assembled into a soluble 120-kD complex, called the transit complex, that maintains LHCP in a soluble and integration-competent form (Payan and Cline, 1991). Formation of the transit complex occurs spontaneously, but GTP is required for LHCP integration into the membrane (reviewed in Cline and Henry, 1996). Significantly, a chloroplast homolog of the mammalian SRP54 subunit (cpSRP54) appears to be necessary for both the formation of the transit complex and the integration of LHCP into the membrane. Immunoprecipitation and cross-linking studies reveal that cpSRP54 is intimately associated with LHCP in the transit complex (Li et al., 1995). Moreover, immunodepletion of cpSRP54 from stromal extracts eliminates the ability of stroma to promote transit complex formation and LHCP integration (Li et al., 1995). Thus, similar to the bacterial and ER SRPs, cpSRP54 appears to function as an essential chaperone or pilot for targeting preproteins to the membrane (Figure 3).

In other aspects, the chloroplast SRP-like system is unique. First, it can function post-translationally (Payan and Cline, 1991) and thus differs from the ER SRP, which is strictly cotranslational (Walter and Johnson, 1994), and the bacterial SRP, which also appears to function cotranslationally (Valent et al., 1998, and references within). Moreover, there are two populations of stromal cpSRP54 (Schuenemann et al., 1998), of which one is associated with chloroplast ribosomes and may participate in cotranslational insertion of plastid-encoded membrane proteins. The second population resides in a 200-kD particle that is involved in post-translational integration of LHCP. Recent evidence indicates that the 200-kD particle comprises cpSRP54 together with a novel 43-kD protein (cpSRP43) but lacks any associated RNA (Schuenemann et al., 1998), thereby differing from other SRPs that invariably possess associated RNAs (Walter and Johnson, 1994). In view of the fact that cpSRP43 is present only in the 200-kD particle, an attractive possibility is that cpSRP43 endows cpSRP54 with its post-translational capabilities.

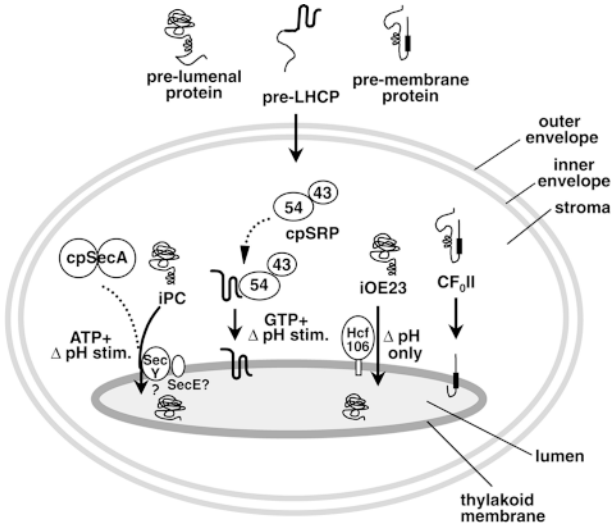

Figure 3. A Working Model for the Routing of Lumen-Resident and Integral Membrane Thylakoid Proteins via Four Precursor-Specific Pathways.

Proteins requiring cpSecA and ATP (e.g., PC [shown], OE33, PSI-F, and cytochrome *f*) are presumed to move across the membrane through a pore consisting of cpSecY and cpSecE. This pathway is referred to as the Sec pathway. Integration of the LHCP requires cpSRP, which consists of cpSRP54 and cpSRP43. Another stromal component and a membrane component (not shown) also are required. This pathway is referred to as the SRP-like pathway. OE23 (shown), OE17, PSII-T, and PSI-N require the membrane protein Hcf106p for transport. This pathway is called the ΔpH pathway because the transthylakoid ΔpH is the sole energy source for transport. A fourth pathway mediates the insertion of the membrane proteins CF_0II (shown), PSII-X, PSII-W, and ELIP, presumably by a spontaneous mechanism. Evidence for pathway-specific groupings is described in the text. Question marks denote components that are presumed but not yet demonstrated to operate on a pathway. iOE23 and iPC are the stromal intermediate forms of OE23 and PC, respectively. stim., stimulated.

Mechanistic details of the thylakoid SRP-like pathway are lacking, but the involvement of cpSRP54 and cpSRP43 is now established (Figure 3). A reconstituted complex solely consisting of recombinant cpSRP54 and cpSRP43 is sufficient to form the LHCP transit complex (Schuenemann et al., 1998). Both subunits are also necessary for integration, although they are not sufficient. An additional stromal component is required to reconstitute the full LHCP integration reaction (Schuenemann et al., 1998). One promising candidate for the additional factor would be a chloroplast homolog of the bacterial FtsY protein, which is itself homologous with the mammalian ER SRP receptor α-subunit and is required for bacterial SRP function (Valent et al., 1998, and references within). Clearly, additional studies are required to define precisely the function and substrates of the chloroplast SRP-like pathway. However, even at this preliminary stage, it is clear that chloroplasts have incorporated unique features not present in prokaryotes.

The ΔpH Pathway Is Related to a Previously Unrecognized Prokaryotic System

The pathway that uses the thylakoid pH gradient as sole energy source for transport of lumenal proteins (Table 1) is referred to as the ΔpH pathway (Figure 3). This pathway initially appeared to be a eukaryotic innovation because of its unique properties and the fact that proteins known to be transported by the ΔpH pathway are absent from cyanobacteria. This is in contrast to the thylakoid Sec pathway, which transports proteins that are also present in cyabobacteria (Robinson and Klosgen, 1994; Cline and Henry, 1996). However, identification of a component of the ΔpH pathway machinery indicates that it is of prokaryotic origin. Specifically, a maize mutant, *hcf106*, was shown to be selectively defective in the ΔpH pathway (Voelker and Barkan, 1995). The cloned *Hcf106* gene predicts a membrane protein possessing a single transmembrane domain near the N terminus, an amphipathic helix, and an acidic hydrophilic domain (Settles et al., 1997). Hcf106 is present in thylakoids and, to a lesser extent, in the chloroplast envelope (M. Settles, E. Summer, A. Yonetani, K. Cline, and R. Martienssen, unpublished data) and is oriented with its amphipathic helix and hydrophilic domain in the stroma, suggesting a role as a receptor (Settles et al., 1997). Although there is no direct evidence that Hcf106 functions as a receptor, recent experiments showing that antibodies to Hcf106 specifically inhibit transport on the ΔpH pathway argue that it is directly involved in the transport reaction (H. Mori, E. Summer, and K. Cline, unpublished data).

Of considerable interest is the striking homology between Hcf106 and predicted proteins of unknown function in a wide range of bacteria, leading Settles et al. (1997) to suggest that analogous transport systems are present in prokaryotes. Candidate protein substrates are periplasmic proteins, expressed under anaerobic or microaerobic conditions, that contain complex redox factors (Berks, 1996). Importantly, these proteins possess a conserved twin Arg motif in their signal peptides that, where examined, is essential for their translocation (Niviere et al., 1992; Dreusch et al., 1997). As discussed below, all ΔpH pathway substrates also possess the twin arginine motif in their signal peptides. Furthermore, it now has been shown that the signal peptide from *E. coli* hydrogenase 1 small subunit, which contains the twin arginine sequence, directs efficient and exclusive transport across thylakoid membranes on the ΔpH pathway (Mori and Cline, 1998).

Recent work substantiates the prediction of a ΔpH-like pathway in bacteria. Isolation of an *E. coli* mutant defective in membrane targeting of the twin arginine DMSO reductase and in periplasmic localization of two twin arginine-containing proteins identified the *mttA* (*tatB*) gene, a homolog of *Hcf106*, as an essential component of the export system (Weiner et al., 1998). Disruption of genes encoding two additional *E. coli Hcf106* homologs, *tatA and tatE*, causes overlapping defects in export of a range of periplasmic redox proteins (Sargent et al., 1998). The existence of three functional *Hcf106* homologs in *E. coli* is consistent with the presence of two *Hcf106* homologs in most bacterial genomes and the fact that there are at least two Hcf106-like proteins in maize. A maize mutant called *tha4* also is selectively defective in ΔpH transport (Roy and Barkan, 1998). The *Tha4* gene encodes a protein similar in structure and topology to Hcf106 (A. Barkan, personal communication). This raises the possibility that Hcf106-like proteins function in pairs, possibly as heterodimers.

Another potential component of the ΔpH machinery can be deduced from information regarding the bacterial transport machinery. The *E. coli tat* operon contains a gene, *tatC*, that encodes a protein with multiple membrane-spanning domains. Disruption of the *tatC* gene impairs export of a range of periplasmic redox proteins (Bogsch et al., 1998). Plant homologs of the TatC protein have not been identified. However, TatC is highly similar to predicted proteins present in the chloroplast genomes of two algae, *Porphyra purpurea* and *Odontella sinensis.* The extent of similarity between thylakoid and bacterial systems, both in components and mechanisms, remains to be determined. One notable difference between the systems regards substrate protein; there is no evidence that any of the ΔpH substrates bind a redox cofactor.

Do Pathways Converge at the Translocon?

Although the experimental evidence suggests that proteins are transported into thylakoids via a number of different pathways, an outstanding question is whether the various thylakoid pathways are completely distinct or have common steps. One attractive possibility is that the pathways converge at the translocon, the membrane machinery through which translocation occurs. A precedent exists in the yeast ER (Panzner et al., 1995) and in *E. coli* (Valent et al., 1998), where chaperone-mediated and SRP-mediated pathways

use similar or identical translocons. Experiments with signal peptide mutations in Chlamydomonas suggest that the cpSecA-dependent insertion of cytochrome *f* and cpSRP-dependent insertion of LHCP share at least one common component (Smith and Kohorn, 1994). The identity of that component is not known, but cpSRP54 is one possible candidate (see below); another is cpSecY/E. LHCP integration is not inhibited by azide and is therefore independent of cpSecA (Yuan et al., 1994). Thus, if these two pathways converge at the membrane, the situation would differ from that in *E. coli*, where the SRP pathway uses a translocon consisting of SecA as well as SecY/E/G (Valent et al., 1998).

Two lines of evidence argue against SecY/E serving as a common translocon for both the ΔpH/Tat and Sec pathways. First, trimethylamine *N*-oxide reductase of *E. coli*, the signal peptide of which contains a twin Arg, is exported across the cytoplasmic membrane at normal levels in a conditional SecY mutant strain at the nonpermissive temperature and in an *E. coli* strain depleted of SecE (Santini et al., 1998). Second, antibodies to pea cpSecY, in amounts that eliminate thylakoid Sec pathway transport, do not impair ΔpH pathway transport (H. Mori, E. Summer, and K. Cline, unpublished data).

These data raise the intriguing possibility that the two transport systems evolved independently. Current thinking is that protein export machinery evolved around hydrophobic domains that originally promoted spontaneous insertion (Schatz and Dobberstein, 1996). Both the Sec and ΔpH pathways use hydrophobic signal peptides for targeting (see below). Furthermore, recent experiments show that the ΔpH system initiates transport via a loop mechanism, which is a hallmark of export-type systems (Fincher et al., 1998, and references therein) and likely is the mechanism for spontaneous insertion (see Robinson and Mant, 1997, for discussion). Sequence similarity among Sec translocon components in bacteria, the ER, and thylakoids was a persuasive argument that export machinery arose from a common evolutionary precursor. That two different translocons could have evolved around hydrophobic targeting peptides that use loop-like insertion mechanisms is an exciting possibility that needs to be rigorously examined.

However, definitively identifying translocon components of a pathway is not a trivial endeavor. The genetic approach of assessing phenotypes in organisms in which genes encoding individual translocon components are deleted can produce equivocal results. For example, the recently described cpSecY null mutant (Roy and Barkan, 1998) is defective in transport on the Sec, SRP, and ΔpH pathways. But, because mutant chloroplasts are nearly devoid of internal membranes and are also impaired in plastid translation, the loss of ΔpH and SRP translocation is possibly due to secondary effects. Future experiments to address this point may require the application of alternative genetic strategies, such as the generation of conditional mutations, or biochemical strategies in which conditional inactivation of specific pathways is used.

Basis for Specific Targeting to Thylakoid Pathways

Thylakoid proteins are targeted by hydrophobic sequences, yet subtle differences must account for pathway specificity. Lumen-resident proteins have lumen-targeting domains with features of bacterial signal peptides, that is, charged N-terminal domains, hydrophobic core domains (Baillet and Kohorn, 1996; Henry et al., 1997), and hydrophilic domains for cleavage by the thylakoidal processing protease (Chaal et al., 1998). Domain-swapping experiments argue that pathway specificity is determined by the signal peptide (Henry et al., 1994; Robinson et al., 1994), whereas transport efficiency is dictated by the sequence of the passenger protein.

One invariant difference between Sec and ΔpH pathway signal peptides is the twin arginine motif in the N-terminal domain of ΔpH pathway precursors. The substitution of one or both arginines by lysine eliminates transport on the ΔpH pathway (Chaddock et al., 1995; Henry et al., 1997). The twin arginine motif is also compatible with the Sec system inasmuch as signal peptides that simultaneously direct efficient transport on both pathways have been constructed (Bogsch et al., 1997; Henry et al., 1997). Thus, although access to the ΔpH pathway requires twin arginines, exclusive targeting to the ΔpH pathway involves a Sec-incompatibility element(s).

Two hypotheses have been advanced regarding the identity of Sec-incompatibility elements in ΔpH signal peptides. The first suggests that the combination of twin arginines and a basic residue in the cleavage domain serves to avoid the Sec system (Bogsch et al., 1997). The second hypothesis holds that the hydrophobic region of the signal peptide is insufficient for Sec recognition (Henry et al., 1997). Indeed, the sequence of mature proteins can also influence targeting. For example, when placed behind Sec-targeting signal peptides, ΔpH pathway passenger proteins are transported very inefficiently, if at all (Clausmeyer et al., 1993; Henry et al., 1997). In one case, DT-23, which contains a Sec-compatible targeting sequence fused to the OE23 mature protein, does not even compete for cpSecA-dependent transport (X. Ma and K. Cline, unpublished data). This result indicates that the mature OE23 protein prevents proper engagement by the Sec machinery.

Targeting through the chloroplast SRP-like system may rely on the hydrophobicity of the targeting sequence. The feature essential for transit complex formation resides in the C-terminal third of LHCP (High et al., 1997). Interaction with cpSRP54, as determined with a nascent chain cross-linking assay, correlates with the hydrophobicity level of the peptides (High et al., 1997), as has been found for SRP54s in other systems. No cross-linking occurred to two lumenal proteins that do not require an SRP for transport. Interestingly, there was significant cross-linking to precytochrome *f*, which is known to use cpSecA. If these studies could be extended to verify that cytochrome *f* requires cpSRP54 for transport, they would provide the first example of thylakoid pathway convergence.

Nevertheless, the finding that there are two distinct populations of cpSRP54 (Schuenemann et al., 1998) begs the question of which cpSRP54 was cross-linked with nascent peptide chains. Future studies will need to examine the two populations separately. The ability of one population of cpSRP54 to interact post-translationally further implies substantial specialization in the recognition of protein substrates as opposed to the usual cotranslational mode of substrate recognition.

Why Are Multiple Pathways Necessary?

It is not clear why plants need multiple pathways for the translocation/integration of thylakoid proteins. Although it is possible that such redundancy provides a backup system, it is more likely that the different pathways exist to accommodate the specific assembly problems of the different groups of proteins that follow each pathway. For example, the chloroplast SRP system provides a means of keeping membrane proteins soluble during trans-stromal transport. In prokaryotes, the SRP system appears to be primarily dedicated to the assembly of cytoplasmic membrane proteins (Ulbrandt et al., 1997).

Similarly, for the lumenal proteins, the Sec pathway appears incapable of transporting protein substrates of the ΔpH pathway. It has been suggested that ΔpH passenger proteins, such as OE23, are tightly folded and that the ΔpH pathway, but not the Sec pathway, can transport folded proteins (Creighton et al., 1995). Recent studies support such a view. Clark and Theg (1997) showed that a tightly folded 6.5-kD bovine pancreatic trypsin inhibitor is efficiently translocated across the ΔpH pathway when placed at the C terminus of OE17. Moreover, Hynds et al. (1998) showed that the 22-kD dihydrofolate reductase, with bound methotrexate, is efficiently transported by the ΔpH pathway when it is placed at the C terminus of OE23, whereas it is not transported by the Sec pathway when fused to OE33. The apparent ability of the ΔpH pathway to transport folded proteins appears to be shared by the prokaryotic Tat system, in which precursor proteins appear to assemble with cofactors in the cytoplasm before their transport across the membrane (Berks, 1996). The possibility that large folded domains can indeed be transported via the ΔpH pathway has important implications for the mechanism of ΔpH transport because translocation of ΔpH substrates does not open ion-permeable channels across the thylakoid membrane (Teter and Theg, 1998).

IN VIVO ANALYSIS OF PROTEIN TOPOGENESIS

In most cases, the pathways for plastid protein transport and the components involved have been identified through in vitro studies. It is also likely that many of the precise mechanisms will be delineated through biochemical investigations. However, the tools are now available to analyze transport processes in vivo. One obvious and important question to address with in vivo studies is whether the components identified by biochemical methods are essential for viability of the plant, as would be expected. This question can now be addressed by strategies involving reverse genetics (Krysan et al., 1996). If plants survive the loss of genes that encode elements of the translocation apparatus, analysis of isolated plastids can determine how characteristics of the import process are affected. The power of combining genetic and in vitro approaches was beautifully demonstrated by investigations that showed the mitochondrial hsp70 to be the motor that drives protein import into mitochondria (Pfanner et al., 1997). Genetic studies also are needed to identify new components of the translocation machinery. Isolation of mutants defective in envelope translocation components was thought to be unlikely because of the essential nature of plastid functions, but recent results of Jarvis et al. (1998) have shown that it is feasible.

As described above, genetic approaches already have been applied to thylakoid biogenesis with impressive results. Not only have they tested the general applicability of biochemical models and identified new components, but genetic analyses are providing a window into the subtleties of protein localization that occur in an organismal context. For example, although the phenotypes of *hcf* mutants generally are consistent with in vitro studies, there are some differences. In particular, when the known components of a pathway are removed or disabled in vitro, the precursors do not enter alternative pathways. The *hcf106* and *tha1* mutants, although ultimately nonviable, nevertheless accumulate low levels of lumen-localized ΔpH pathway substrates and cpSecA pathway substrates, respectively (Voelker and Barkan, 1995; Roy and Barkan, 1998). Similarly, plants deficient in (Pilgrim et al., 1998) or completely lacking cpSRP54 (N. Hoffman, personal communication) or cpSRP43 (Klimyuk et al., 1999) are able to accumulate LHCP. Thus, in vivo, proteins apparently are being transported by mechanisms that are independent of essential factors in vitro.

Such genetic analyses provide the impetus for understanding alternative mechanisms and alternative pathways that exist in plants. One possibility is that the plant cell adapts to the loss of a translocation pathway by upregulating other components that can substitute for the missing component. For example, an increase in the chaperones hsp70 and ClpC accompanies the loss of cpSRP54 (Pilgrim et al., 1998), which reflects the situation when SRP54 is depleted from yeast (Hann and Walter, 1991). Another possibility to consider is that related components might substitute for each other, for example, the Tha4 protein for Hcf106p and vice versa.

One important distinction between in vitro and in vivo approaches that could explain the apparent presence of alternative protein transport mechanisms concerns the relationship between chloroplast development and protein lo-

calization mechanisms. For example, studies of thylakoid protein localization use plastids that contain nearly mature thylakoid membranes, whereas in vivo, thylakoid proteins are assembled concurrently with the deposition of new thylakoid bilayer. It has been suggested that vesicle flow from the inner envelope membrane accounts for the deposition of the thylakoid bilayer and that patches of thylakoid membrane might even be first formed in the envelope (Hoober et al., 1991; for a review of plastid development, see Pyke, 1999, in this issue). If this is true, then at early stages in chloroplast biogenesis, thylakoid precursor proteins might bypass stromal factors, such as cpSRP54, that are required for traversing the aqueous compartment. These possibilities and others can be examined best by a combination of in vitro and in vivo analyses. Certainly, future studies of plastid protein trafficking will use powerful combined approaches to dissect the varied mechanisms that are involved in protein translocation in plants.

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