IMPORT AND ROUTING OF
NUCLEUS-ENCODED CHLOROPLAST
PROTEINS

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ABSTRACT
Most chloroplast proteins are nuclear encoded, synthesized as larger precursor proteins in the cytosol, posttranslationally imported into the organelle, and routed to one of six different compartments. Import across the outer and inner envelope membranes into the stroma is the major means for entry of proteins destined for the stroma, the thylakoid membrane, and the thylakoid lumen. Recent investigations have identified several unique protein components of the envelope translocation machinery. These include two GTP-binding proteins that appear to participate in the early events of import and probably regulate precursor recognition and advancement into the translocon. Localization of imported precursor proteins to the thylakoid membrane and thylakoid lumen is accomplished by four distinct mechanisms; two are homologous to bacterial and endoplasmic reticulum protein transport systems, one appears unique, and the last may be a spontaneous mechanism. Thus chloroplast protein targeting is a unique and surprisingly complex process. The presence of GTP-binding proteins in the envelope translocation machinery indicates a different precursor recognition process than is present in mitochondria. Mechanisms for thylakoid protein localization are in part derived from the prokaryotic endosymbiont, but are more unusual and diverse than expected.

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INTRODUCTION

Plastids are developmentally related organelles in algae and higher plants. Among the differentiated plastids are the chloroplast, etioplast, chromoplast, and amyloplast. Plastid biogenesis, similar to mitochondrial biogenesis, requires the participation of two genetic systems. The plastid genome encodes \( \approx 100 \) plastid-localized proteins that are translated on 70S ribosomes and functionally assembled within the plastid. The remaining hundreds of plastid proteins are encoded in the cell nucleus and synthesized in the cytosol. Nucleus-encoded plastid proteins are initially synthesized as precursor proteins on free polyribosomes. Virtually all plastid precursor proteins possess transient amino-terminal transit peptides that govern their import into the organelle and occasionally direct their suborganellar routing (see Figure 1). Although the general features of the plastid protein import process have been known for over a decade, only recently have the detailed steps of translocation and the plastid machinery involved been described. Using an in vitro assay with intact chloroplasts, it has been shown that precursors bind to the chloroplast’s surface, insert into a proteinaceous outer envelope translocation complex, and then proceed across outer and then inner envelope membranes in an extended conformation. While these steps of the import process are similar to those for protein import into mitochondria, the translocation machinery consists of unique polypeptide components that include at least two GTP-binding proteins. These GTP-binding proteins appear to participate in early events of import and probably regulate precursor recognition and advancement into the translocation complex.

Import into the plastid is only half of the localization process because proteins must then be routed into their proper suborganellar compartment. The routing of imported chloroplast proteins is highly complex because of the multiplicity of compartments. Whereas all plastids have in common a double-membrane envelope and an aqueous matrix space called the stroma, the chloroplast uniquely
contains a closed internal thylakoid membrane system that surrounds an aqueous lumen. Thus precursor proteins must be specifically and faithfully localized to one of six different subcompartments. Remarkable progress has recently been made in defining the pathways and plastid machinery involved in routing to the thylakoid membrane and thylakoid lumen. Thylakoid proteins are localized by a two-step process that involves import across the envelope into the stroma and subsequent transport/integration into the thylakoids (Figure 1). Surprisingly, four distinct mechanisms have been described for thylakoid translocation; one is homologous to the bacterial Sec system; one homologous to the signal recognition particle (SRP) system of the endoplasmic reticulum (ER); one appears unique in its sole reliance on a trans-thylakoid ΔpH; and the last may be a spontaneous mechanism.


Figure 1  Localization pathways taken by nucleus-encoded proteins en route to their site of residence within the chloroplast.
PATHWAYS FOR PLASTID ENTRY AND SUBCOMPARTMENT LOCALIZATION

The first step toward understanding precursor protein localization is defining their routes into and within the plastid. Figure 1 summarizes the current state of knowledge for the pathways taken by proteins en route to the different chloroplast subcompartments.

Import into the stroma is the major route for protein entry into plastids and has been termed the general import pathway. Proteins destined for the chloroplast interior, i.e. stromal, thylakoid, and thylakoid lumenal proteins, are carried by common machinery as evidenced by common energy requirements, the need for proteinaceous components on the chloroplast surface (see de Boer & Weisbeek 1991), and the results of competition studies with overexpressed precursor proteins and with synthetic transit peptide fragments (see Gray & Row 1995). The in vitro rates of import on this pathway under precursor saturating conditions have been estimated to be 40–50,000 precursors per chloroplast per minute (Pilon et al 1992b, Cline et al 1993), which are consistent with estimates for the in vivo rates during peak periods of chloroplast development in plants (Pfisterer et al 1982). A subset of proteins imported into the chloroplast stroma are further directed to the thylakoid membrane and lumen (see below).

Pathways for localization of integral envelope proteins have not been fully elucidated. However, preliminary studies suggest that there are several routes into the envelope. A subgroup of outer envelope proteins (OEP), the Type A proteins, appears to insert directly into the outer envelope membrane. These proteins are small to moderate in size, contain at least one extended hydrophobic segment, and lack cleavable targeting peptides (e.g. Salomon et al 1990, Li et al 1991, Fischer et al 1994). Their insertion into the outer membrane may be spontaneous because there is no apparent energy requirement nor dependence on protease-sensitive components of the chloroplast surface (but see Seedorf et al 1995).

Type B OEPs, represented by two large polypeptides of the general import apparatus (OEP75 and OEP86), possess transit peptides and are localized by a process that is dependent on nucleoside triphosphates (NTPs) and protease-sensitive outer envelope components (Hirsch et al 1994, Tranel et al 1995). OEP75 appears to use at least part of the general import apparatus because its import is competed by the precursor to the small subunit of Rubisco (preSS), a stromal protein (Tranel et al 1995). Import of OEP86 is not competed by preSS, which suggests that it employs different localization machinery (Hirsch et al 1994). Both proteins, although tightly anchored in the outer membrane, are devoid of hydrophobic segments capable of spanning the bilayer as an α helix.
Inner envelope proteins (IEPs), e.g., IEP37, the triose phosphate/phosphate translocator, and Bt1p, are synthesized with transit peptides and possess one or more hydrophobic segments capable of spanning the bilayer. Their import and localization involves translocation machinery, probably the general import apparatus, because experiments with chimeric precursors show that their transit peptides are functionally identical to stroma-targeting transit peptides (see below) (Li et al. 1992, Brink et al. 1995, Knight & Gray 1995). Studies by Knight & Gray (1995) argue that inner envelope targeting is contained within a hydrophobic segment of the mature protein sequence of the triose phosphate/phosphate translocator. This envelope-targeting element might function as a re-export signal to promote insertion of the imported protein from the stroma into the inner envelope membrane. This type of mechanism is referred to as conservative sorting (Hartl & Neupert 1990). Alternatively, the hydrophobic segment might function as a stop-transfer sequence and induce premature exit from the general import translocation machinery at the level of the inner envelope. If hydrophobic segments of IEPs act as stop-transfer sequences, then a specific recognition mechanism must exist because many thylakoid proteins contain hydrophobic segments yet are imported across both envelope membranes (see Knight et al. 1993 for discussion). In mitochondria, both the conservative sorting pathway (Hartl & Neupert 1990) and the stop-transfer pathway (Glick et al. 1992) appear to operate for proteins of the inner membrane and intermembrane space.

THE GENERAL IMPORT PATHWAY

Stroma-Targeting Domains of Transit Peptides
Access to the general import pathway is governed by stroma-targeting domains (STDs) of transit peptides. Stromal protein precursors possess transit peptides that contain only an STD, whereas thylakoid luminal protein precursors have an additional targeting domain in their transit peptides (de Boer & Weisbeek 1991; Figure 2a). STDs are both necessary and sufficient for import of the passenger protein to the stroma and are usually specific for plastid protein import (but see Creissen et al. 1995). Nevertheless, the manner by which this specific targeting information is encoded has eluded investigators. STDs range in size from about 30 to 120 residues and have properties that superficially resemble presequences of mitochondrial precursor proteins; i.e., they are rich in hydroxylated residues and deficient in acidic residues. STDs tend to share several compositional motifs: an amino terminal 10–15 residues devoid of Gly, Pro, and charged residues; a variable middle region rich in Ser, Thr, Lys, and Arg; and a carboxy-proximal region with a loosely conserved sequence.
(Ile/Val-x-Ala/Cys’Ala) for proteolytic processing (von Heijne et al 1989, de Boer & Weisbeek 1991). However, there are no extended blocks of sequence conservation, nor any conserved secondary structural motifs. Theoretical analyses suggest that STDs adopt predominantly random coil conformation (von Heijne & Nishikawa 1991). Circular dichroism and tryptophan fluorescence quenching analysis confirm a general lack of secondary structure for the transit peptides of preferedoxin (Pilon et al 1992a) and preplastocyanin (prePC) (Endo

Figure 2  Domain structure and sorting elements of a transit peptide that directs protein import into the chloroplast and transport into the thylakoid lumen. (a) The transit peptide for the precursor to OE23 (23-kDa protein of the oxygen-evolving complex) contains a stromal targeting domain (STD) that directs import into the chloroplast stroma where the STD is cleaved as shown (Bassham et al 1991) by a processing protease. The lumen targeting domain (LTD) directs transport into the lumen where the LTD is removed by a second processing protease. The signal peptide motif (N, H, and C) is indicated, as is an acidic (A) domain. (b) Transport characteristics of precursors containing truncated LTDs demonstrate that the signal peptide motif of the LTD is necessary and sufficient for pathway-specific transport of OE23 and PC. A hybrid signal peptide that directs passenger proteins to both pathways shows that the N and H/C domains play a role in pathway-specific targeting. When directed by the hybrid signal peptide, transport of different passenger proteins demonstrates that delta pH mature domains were arrested in translocation across the Sec pathway (see text for discussion).
et al 1992) in aqueous solution. This general lack of structural motif differs from mitochondrial presequences, where an amphipathic helix is an essential structural element (von Heijne et al 1989). Thus interaction between STDs and plastids probably involves a subtle recognition event that could include several checkpoints to validate the targeting element.

One way that STDs may specifically interact with plastids is by inserting into the lipid bilayer. Biochemical studies of synthetic transit peptides and entire precursor proteins have shown that some transit peptides have a propensity to insert into membrane lipids (see Theg & Scott 1993). van’t Hoff et al (1993) demonstrated that preferedoxin or its transit peptide inserts into monolayers of plastid envelope lipids at physiological surface pressures. Interactions were predominantly with monogalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol, and phosphatidylglycerol. Interaction with the two former polar lipids is particularly notable because these lipids are present in eukaryotes only in plastid membranes. Interactions of the transit peptide and precursor with mitochondrial lipids occurred only below physiological surface pressures, indicating that the interactions with plastid lipids were specific. These studies suggest that precursors may initially bind to the lipids of the outer chloroplast envelope, but clearly more studies are required to establish definitively such an interaction during the import process. Furthermore, the generality of this model needs to be addressed, e.g. the PC (plastocyanin) STD appears not to interact with plastid lipids under comparable conditions (Endo et al 1992).

Evidence that STDs interact specifically with outer chloroplast envelope proteins includes the fact that synthetic STDs compete for a saturable component of the import apparatus (Gray & Row 1995) and the observation that STDs are in close contact with polypeptides of the translocation machinery at a very early stage in the import process (D Schnell, personal communication).

Steps of the Import Process

Defining the steps of the import process has been pivotal not only for understanding the progression of recognition and translocation but also for identification of translocon components (see Figure 3). Binding of precursors to the outer envelope membrane constitutes the first step and produces the first stable intermediate, the so-called early intermediate (Theg & Scott 1993, Schnell & Blobel 1993). Binding requires 50–100 µM of any of several NTPs and the presence of outer envelope membrane proteins (OEPs) (Olsen & Keegstra 1992, Theg & Scott 1993). Binding is saturable between 1500 and 3500 sites and represents an on-pathway step because bound precursors progress into the stroma when the ATP concentration is raised to approximately 1 mM (Theg & Scott 1993, Schnell & Blobel 1993). Such productive binding is mediated by the STD (Friedman & Keegstra 1989).
Early intermediate precursors have probably progressed deeply into the translocation machinery because they are irreversibly bound (Theg & Scott 1993) and frequently partially protected from protease treatment of chloroplasts and membranes (Freidman & Keegstra 1989, Waegemann & Soll 1991). The relatively large size of protected fragments raises the possibility that much of the early intermediate precursor has crossed the outer envelope membrane (see Figure 3).

Time-course analysis of precursor import from the bound state led to identification of a second or late intermediate (Schnell & Blobel 1993). Late intermediate proteins have translocated across the envelope sufficiently to be processed by the STD processing protease, yet are still exposed to the outside of the chloroplast as judged by susceptibility to exogenous protease. The STD processing protease is an ≈140-kDa metalloprotease that has recently been cloned (VanderVere et al 1995). Because this protease is released into the soluble fraction upon chloroplast lysis, it is believed to reside in the stroma. Thus the late intermediate protein probably represents a translocating precursor that spans both envelope membranes. In other words, similar to transport across mitochondrial membranes, polypeptide chain translocation proceeds amino-terminus first, in an extended conformation, and across both envelope membranes simultaneously. Consistent with this latter conclusion, Schnell & Blobel (1993) have presented electron microscopic as well as biochemical fractionation evidence that these early and late intermediates are preferentially located at
sites of close contact between inner and outer envelope membrane, i.e. contact sites.

The implication is that proteins cross the two envelope membranes in regions where they are attached. A similar conclusion was originally reached regarding protein import across outer and inner mitochondrial membranes (Schwaiger et al 1987). However, conditions that enhance the arrest of intermediates would also stabilize tight precursor-mediated outer envelope/inner envelope association. Thus the possibility that proteins can cross outer and inner envelope membranes independently cannot be discounted. Indeed, Scott & Theg (1996) provide compelling evidence that precursor proteins can cross outer and inner envelope membranes sequentially. By using reduced ATP concentrations and chloroplasts that were plasmolyzed with hypertonic osmoticum, they were able to capture full-size precursor that had apparently crossed the outer, but not the inner envelope membrane. This protease-protected precursor was subsequently imported into the stroma upon addition of larger amounts of ATP. These results imply that chloroplasts, similar to mitochondria (Hwang et al 1991, Segui-Real et al 1993), have independent outer membrane and inner membrane translocation machinery. When considered together, the results of Schnell & Blobel (1993) and Scott & Theg (1996) suggest that contact sites are formed under isotonic conditions when translocating precursor proteins engage the inner envelope translocation apparatus prior to completely crossing the outer membrane.

Envelope Translocation Machinery

Several early attempts to identify components of the import machinery were inconclusive or produced misleading identifications (see Gray & Row 1995 for discussion). However, in the past several years, converging investigations of four independent research groups have led to the identification of five OEPs and three IEPs that participate in precursor recognition and translocation (Figure 3). These studies, which relied primarily on early intermediate precursor, used chemical cross-linking, immunoaffinity chromatography of proteins associated with a protein A-containing chimeric precursor, and sedimentation-purification of detergent-solubilized translocation complexes. There are several excellent reviews of these translocon components (Gray & Row 1995, Schnell 1995, Soll 1995). The location and topology of each protein, as well as the manner of identification, suggest the order in which they participate in the import process. A cytosolically exposed outer envelope Hsp70 protein (Com70), which was cross-linked to a precursor protein intermediate (Wu et al 1994), may bind the precursor as it encounters the chloroplast surface and maintain it in an unfolded conformation. OEP86 appears to interact with precursor proteins at a very early stage of the import process, i.e. prior to formation of the early intermediate.
OEP86 was cross-linked to preSS in the absence as well as the presence of NTPs (Perry & Keegstra 1994). In addition, binding and import of preSS are inhibited by antibodies to OEP86 (Hirsch et al 1994). OEP75, OEP34, OEP86, and an inter-envelope space Hsp70 protein are considered to be components of the outer membrane translocation complex because they are co-isolated as a complex with early intermediate precursor (Waegemann & Soll 1991, Schnell et al 1994). OEP86 and OEP75 are intimately associated with precursor because they are directly cross-linked to preSS (Perry & Keegstra 1994). OEP75 is notable in that it is largely buried in the outer envelope membrane but possesses no extended hydrophobic sequences. It has been speculated that, because OEP75 is predicted to contain a substantial amount of $\beta$ structure, it may adopt a $\beta$ barrel conformation similar to bacterial outer membrane pore proteins, i.e. OEP75 may make up part of the protein translocating channel (Schnell 1995, Tranel et al 1995). The inner-envelope space Hsp70 appears to be integrally associated with the outer envelope membrane.

OEP86, OEP75, and OEP34 appear to form a complex prior to interaction with precursor. Seedorf et al (1995) showed that a substantial amount of OEP34 and OEP75 are connected by disulfide bridges and that addition of copper chloride to further oxidize sulfhydryl groups resulted in an OEP86, OEP75, OEP34 complex (Seedorf & Soll 1995). The fact that most of these polypeptides are located in the free outer envelope membrane subfraction on sucrose gradients (Cline et al 1985) suggests that precursor binding in the presence of NTPs is required to promote this complex into contact sites. This conclusion is further supported by the fact that OEP86 cross-linked to preSS in the absence of NTPs is located in an outer envelope-enriched fraction, but when cross-linked in the presence of NTPs, it is in the contact site-enriched fraction (Perry & Keegstra 1994).

Three inner envelope proteins, IEP97, IEP36, and IEP44, are thought to participate in translocation across the inner envelope membrane because they are only associated with translocating polypeptide in the later stages of translocation (Schnell et al 1994, Wu et al 1994).

Perhaps the most intriguing aspect of the outer membrane translocation machinery concerns OEP86 and OEP34, which have been shown to bind GTP (Kessler et al 1994). Additionally, OEP34 hydrolyzes GTP (Seedorf et al 1995). GTP-binding proteins are involved in protein targeting to the ER (Walter & Johnson 1994) and in the early steps of nuclear protein import (Sweet & Gerace 1995). In these systems, binding and/or hydrolysis of GTP appears necessary for commitment to the translocation site. The case of ER transport is particularly relevant. Here, GTP hydrolysis by the SR$\alpha$ subunit and GTP loading of the SRP54 polypeptide subunit is necessary for transfer of the nascent
chain:ribosome complex to the translocon. These roles are consistent with the known functions of other GTP-binding proteins as molecular switches that ensure directionality and accuracy of biological processes (Bourne et al 1991). Interestingly, SRP54 and SRα share significant sequence homology both within and flanking the GTP-binding domain (Walter & Johnson 1994), a characteristic that OEP86 and OEP34 also share (Kessler et al 1994, Seedorf et al 1995). It is likely that OEP86 and OEP34 act in concert, through GTP binding and hydrolysis, to recognize and then commit authentic plastid precursor proteins to the translocation complex of the outer envelope membrane, but this remains to be experimentally demonstrated. However, it does appear that GTP hydrolysis is necessary for engagement in the envelope translocation machinery because stable precursor binding to chloroplasts is inhibited by GTP-γ-S (Kessler et al 1994).

One surprise is that none of the chloroplast translocation components, with the exception of Hsp70, is homologous to the mitochondrial import apparatus (Ryan & Jensen 1995). It appears that chloroplast and mitochondrial import machinery have arisen independently and that mitochondria evolved a different means of recognizing targeting peptides. Another possibility is that in plant cells where mitochondrial targeting must be more stringent, mitochondria also employ a proof-reading system analogous to plastids.

What Drives Translocation?

NTPs appear to be the sole energy source for polypeptide translocation across the plastid envelope; transmembrane electrical or ion gradients are not involved in this process (de Boer & Weisbeek 1991, Theg & Scott 1993). ATP in the inter envelope space is required for precursor transport across the outer envelope membrane (Scott & Theg 1996); ATP in the stroma is necessary for translocation across the inner-envelope membrane (Theg & Scott 1993). This implicates trans-located ATPases in the translocation event.

There is now compelling evidence that protein translocation proceeds through a pore or channel through the membrane (Simon & Blobel 1991). Protein translocation channels may also exist in the plastid envelope. This is suggested by the observation that transit peptides mediate opening of aqueous channels across the chloroplast envelope, as assessed by patch-clamp studies (Bulychev et al 1994). Trans-located Hsp70 proteins facilitate translocation across the ER and mitochondrial membranes, presumably by a thermal ratcheting mechanism (Wickner 1994, Glick 1995), whereby successive binding and release of the polypeptide emerging from the channel serves as the thermodynamic driving force for chain movement. The presence of the inter-envelope Hsp70 in the early intermediate complex provides support for its role in transport across the outer envelope, and the observation that the stromal Hsp70 transiently binds
to newly imported proteins (Tsugeki & Nishimura 1993, Madueño et al 1993) suggests that it might be involved in inner envelope translocation. Nevertheless, these proposed roles remain to be verified experimentally. In addition, the possibility that other stromal peptide-binding proteins, e.g. the Hsp60 protein, a chloroplast SecA homologue (see below), and an SRP54 homologue (see below), are involved in translocation should be explored. In the latter case, one would expect that translocation across the inner envelope membrane would depend upon GTP.

An interesting example of a stromal requirement for transport has been recently described by Reinbothe et al (1995), in which translocation of the precursor to protochlorophyllide oxidoreductase requires the presence of the substrate, protochlorophyllide, inside the plastid. One interesting possibility is that the substrate drives the translocation step by inducing folding of the enzyme on the stromal side of the inner envelope membrane.

ROUTING OF PROTEINS INTO/ACROSS THE THYLAKOIDS

Thylakoid Proteins Are Localized by Sequential Protein Translocation Events

Thylakoid precursors are imported across both envelope membranes into the stroma and are then transported into/across thylakoid membranes by additional translocation machinery (see Figures 1 and 4). This model was initially adopted based on the transient appearance of a soluble, stromal form of an imported thylakoid protein (Smeekens et al 1986) and on the reconstitution of a thylakoid translocation step (Cline 1986). A thorough analysis of stromal intermediates has now authenticated this two-step localization process. Time-course analysis combined with rapid stopping methods demonstrated that the stromal intermediates accumulate with kinetics expected for pathway intermediates (Reed et al 1990, Cline et al 1992b). The intermediates accumulate to high levels in import assays when the thylakoid transport/integration step is inhibited (Cline et al 1989, Mould & Robinson 1991, Cline et al 1993, Konishi & Watanabe 1993, Henry et al 1994, Knott & Robinson 1994). And, most importantly, accumulated intermediates can be “chased” into thylakoids in vitro (Reed et al 1990, Cline et al 1992b 1993, Konishi & Watanabe 1993, Creighton et al 1995) and in vivo (Howe & Merchant 1993). In most cases, the intermediates had been processed to remove the STD (but see Bauerle et al 1991, Cline et al 1992b, Nielsen et al 1994). Thus lumenal protein intermediates are generally intermediate in size between the full-length precursor and mature protein, whereas most membrane protein intermediates are mature size (Figures 1 and 4).
is consistent with studies showing that thylakoid targeting is governed by the lumen targeting domain (LTD) of the transit peptide (Ko & Cashmore 1989, Hageman et al 1990; Figure 2 and see below) and by elements in the mature sequence of membrane proteins (de Boer & Weisbeek 1991, Madueño et al 1994). Robinson and colleagues (Halpin et al 1989) showed that LTDs are removed in the thylakoid lumen by a processing protease that has the identical reaction specificity as bacterial and ER signal peptidases.

Integration of the membrane protein LHCP (Cline 1986) and transport of the luminal protein OE33 (Kirwin et al 1989) into isolated thylakoids demonstrates that thylakoid localization can be uncoupled from import into chloroplasts. Subsequent studies have reconstituted thylakoid transport/integration of a variety of other thylakoid proteins (Robinson & Klösgen 1994). Pathway intermediates observed in organello (Cline et al 1992b), or produced in vitro (Viitanen et al 1988, Cline et al 1993, Hulford et al 1994), are efficiently transported/integrated in thylakoid transport assays, but the full-length precursors are also effective substrates (Robinson & Klösgen 1994). The fact that removal of the STD is not prerequisite to translocation is consistent with the loop model invoked for initiating transport of signal peptide precursors or signal anchor-bearing precursors (Kuhn et al 1994). These reconstituted thylakoid translocation assays provide the means for detailed analysis of the translocation process (see below).

There Are at Least Four Pathways for Protein Translocation Into or Across the Thylakoid Membrane

The discovery that different subgroups of precursors have different energy and soluble protein factor requirements for translocation suggested that more than one mechanism is involved (see Figure 4). Most striking was the fact that two luminal proteins, OE23 and OE17, are transported in the total absence of NTPs (Cline et al 1992a). These proteins rely entirely on the trans-thylakoid ∆pH as an energy source and have no soluble factor requirement (see Robinson & Klösgen 1994 for review). Equally intriguing is the finding that integration of LHCP requires GTP (Hoffman & Franklin 1994) rather than ATP, as originally thought (Cline 1986). A third subgroup (OE33 and PC) requires ATP and a soluble factor and is stimulated by ∆pH (Hulford et al 1994, Yuan & Cline 1994).

The implication of the above results, i.e. that there exists group-specific translocation machinery, was demonstrated by precursor competition studies (Cline et al 1993). In a novel in organello competition assay with intact chloroplasts, saturating amounts of iOE23 (the stromal intermediate) selectively competed with iOE17 for thylakoid transport, whereas iOE33 selectively competed with iPC. Neither iOE23 nor iOE33 competed for LHCP integration. The in
Figure 4  A working model for routing of lumen-resident and integral membrane thylakoid proteins via three precursor-specific pathways. Proteins requiring CPSecA and ATP are presumed to move across the membrane through a pore consisting of CPSecY and possibly a homologue of E. coli SecE. Cytochrome f, a plastid-encoded protein that also employ the CPSecA mechanism, is not shown. Integration of the light-harvesting chlorophyll-binding protein (LHCP) requires 54CP, a homologue of SRP54. 54CP is thought to pilot LHCP to the thylakoid in a targeting cycle that involves GTP hydrolysis (see text for discussion). A third pathway is unique and requires only ΔpH to power transport across the membrane. A fourth pathway that mediates the insertion of CF1II, presumably by a spontaneous mechanism, is not shown. Evidence for pathway-specific groupings is described in the text. Biochemical studies demonstrate that PS2T (Henry et al 1994) and PS1N (Mant et al 1994) are transported by the delta pH pathway. Question marks denote that these components are hypothetical and have not been identified.
organello assay was possible because the $V_{\text{max}}$ for import of preOE23, for example, into chloroplasts is 10 to 40 times the $V_{\text{max}}$ for transport into thylakoids, thereby allowing concentration of intermediates within the chloroplast to $\approx 100$ times the $K_m$ for thylakoid transport. Thus by use of precursor concentrations that are subsaturating for import, competition of imported proteins for thylakoid localization occurs in organello. Although this type of situation is probably more physiologically relevant, it does not permit determination of the specific site of competition. Competition assays with isolated thylakoids also produce the same groupings as those obtained by energy requirements (above), and additionally reveal that iOE23 and iOE17 compete for a thylakoid membrane component, possibly a receptor. Taken together, these results lead to a model for three mechanismically different pathways for thylakoid transport/integration (Figure 4). Analysis of chimeric precursor proteins (Henry et al 1994, Robinson et al 1994) show that commitment to a particular pathway is determined by elements in the LTD, rather than by the mature passenger proteins (but see below).

Recent analysis of two maize mutants provides in vivo evidence for operation of the above three pathways (Voelker & Barkan 1995). The maize mutant $\text{thal}$ is selectively defective in thylakoid transport of OE33 and PC and of another nucleus-encoded thylakoid protein, PS1F. Interestingly, $\text{thal}$ is also impaired in thylakoid transport of a chloroplast-synthesized protein, cytochrome f. Biochemical studies reinforce the conclusion that PS1F (Karnauchov et al 1994) and cytochrome f (R Mould & J Gray, personal communication) are transported on the same pathway as OE33 and PC. The mutant $\text{hcf106}$ has a complementary phenotype to $\text{thal}$; $\text{hcf106}$ plants are selectively defective in thylakoid transport of OE16 (OE17) and OE23. LHCP accumulated normally in both mutants.

Biochemical examination of the localization of CF0 II, an integral membrane protein with a bipartite transit peptide, suggests the existence of a fourth thylakoid pathway. CF0 II inserts into isolated thylakoids in the absence of $\Delta \text{pH}$, NTPs, and soluble factors and is unaffected by saturating quantities of pre-OE23 as a competitor (Michl et al 1994). CF0 II also inserts into protease-treated thylakoids (R Klösgen, personal communication), raising the possibility that it inserts spontaneously; however, this possibility remains to be firmly established.

Translocation Components Identify Two Thylakoid Pathways as Homologues of Bacterial and ER Systems

BACTERIAL SEC-HOMOLOGOUS PATHWAY FOR TRANSPORT OF PC, OE33, PS1F, CYTOCHROME F Chloroplasts are thought to have evolved from a prokaryotic endosymbiont similar to modern-day cyanobacteria. This suggests that a
conserved prokaryote-like protein transport mechanism would be operational within chloroplasts. The bacterial system for protein export across the cytoplasmic membrane employs the SecA translocation ATPase to initiate membrane insertion of precursor and a core translocon made up of the SecY/E/G polypeptides (Pugsley 1993, Wickner 1994). Translocation on this system requires ATP and a proton-motive force. Preliminary evidence for a Sec system operating in thylakoids includes the presence of secA- and secY-homologous genes in the chloroplast genomes of several algae (Robinson & Klösgen 1994), the appropriate energetics for OE33 and PC transport (Hulford et al 1994, Yuan & Cline 1994), and the fact that thylakoid translocation of OE33 and PC, but not OE23, OE17, or LHCP, are inhibited by azide (Henry et al 1994, Knott & Robinson 1994, Yuan et al 1994). Azide is a diagnostic inhibitor of *Escherichia coli* SecA (Oliver et al 1990).

A SecA-dependent thylakoid transport mechanism was demonstrated in pea chloroplasts by two approaches. In the first, antibodies prepared to a conserved region of a deduced algal SecA polypeptide were used to identify and purify a stromal 110-kDa SecA homologue, designated CPSecA (Yuan et al 1994). Purified CPSecA reconstituted transport of PC and OE33 across buffer (or urea) washed thylakoids in a concentration and azide-sensitive manner. Thus CPSecA accounts for the stromal factor requirement for PC and OE33 transport. CPSecA was unable to replace the stromal requirement for LHCP integration and neither simulated nor inhibited transport of OE23 and OE17. In a second approach, antibodies to a peptide deduced from a partial CPSecA cDNA inhibited thylakoid transport of OE33, but not OE23 (Nakai et al 1994). A similar reduction in OE33 transport occurred when stromal extract was immuno-depleted of CPSecA. Together, these results indicate that one thylakoid transport pathway employs a SecA-dependent translocation mechanism (see Figure 4).

Full-length cDNAs to two higher plant chloroplast SecAs have now been isolated (Berghofer et al 1995, Nohara et al 1995). The deduced protein sequence of these two nuclear-encoded proteins is strikingly homologous to that of cyanobacterial SecA. Recently, in vivo evidence for the selective operation of the SecA pathway has come from the identification of the maize *tha1* gene as a *secA* homologue (R Voelker & A Barkan, personal communication).

Laidler et al (1995) isolated a full-length cDNA to a chloroplast homologue of SecY (CPSecY) in *Arabidopsis*. Presumably, CPSecY functions in thylakoid transport in a manner similar to SecY in bacteria, forming part of the membrane translocon. Definitive evidence for CPSecY involvement in any of the thylakoid transport pathways is currently lacking. Based on the bacterial paradigm, CPSecY is expected to function with CPSecA in mediating transport of PC, OE33, PS1F, and cytochrome f (see Figure 4).
Chloroplasts also contain a localization system homologous to the SRP system used for protein targeting to the ER and the bacterial cytoplasmic membrane (see Walter & Johnson 1994). Eukaryotic SRP binds nascent protein chains and, in a targeting cycle that involves GTP binding and hydrolysis, pilots the entire SRP:ribosome:nascent chain complex to the ER membrane. Franklin & Hoffman (1993) identified a chloroplast homologue (54CP) of the SRP54 protein subunit and showed that the 54CP protein resides primarily in the stroma. Two observations implicated 54CP in LHCP targeting. First, the LHCP stromal intermediate is a soluble complex of ≈120 kDa, or about four to five times the size of the LHCP polypeptide (Payan & Cline 1991). This transit complex can be formed post-translationally by a proteinaceous stromal activity that shares similar properties with the stromal activity required for LHCP integration into isolated thylakoids (Payan & Cline 1991). Both activities co-elute from gel filtration columns with a peak of 54CP (R Henry & K Cline, unpublished data). The second correlative factor is that GTP is required for LHCP membrane integration (Hoffman & Franklin 1994).

Recent experiments more directly implicate 54CP in LHCP integration (Li et al 1995). First, 54CP was shown to be present in the transit complex by immunoprecipitation and also by chemical cross-linking. More importantly, 54CP was shown to be required for LHCP integration by immunodepleting 54CP from stromal extract. The depleted extract was unable either to form transit complex or to support LHCP integration. These results demonstrate that 54CP interacts with imported LHCP in the stroma and is required for its integration.

The precise role of 54CP in this process awaits purification of the active particle. Some observations suggest that there are important differences between the 54CP system and other SRP systems. The most obvious difference is that 54CP functions post-translationally, whereas all other SRPs appear to be strictly co-translational (Walter & Johnson 1994). Another difference is that all other SRPs contain an essential RNA component; RNA has not been found associated with 54CP.

However, the chloroplast SRP homologue also shows similarities with mammalian SRP (Walter & Johnson 1994), suggesting a model for its operation (see Figure 4). Mammalian SRP must be in a guanine nucleotide-free state in order to bind signal peptides (or signal anchors of membrane proteins). Similarly, 54CP must be in a nucleotide-free state to form a transit complex because GDPox, which covalently modifies guanine nucleotide-binding sites, prevents transit complex formation and LHCP integration (R Henry & K Cline, unpublished data). Certain observations indicate that the role of 54CP extends
beyond that of a molecular chaperone and argue for a role in targeting. For example, although 54CP can maintain LHCP solubility and integration competence for extended periods, it is not possible to bypass the stromal requirement for integration by urea-denaturation of LHCP or replace the stromal requirement with other chaperone proteins (Payan & Cline 1991, Yuan et al 1993). The GTP hydrolysis required for integration appears to occur at the thylakoid membrane because, although the non-hydrolyzable GTP analogue, GMP-PNP, inhibits integration (Hoffman & Franklin 1994), it does not inhibit transit complex formation (R Henry & K Cline, unpublished data). This is consistent with the mammalian SRP cycle, where GTP hydrolysis by SRP54 is necessary for release from the ER receptor and SRP recycling to the cytosol. A thylakoid receptor is implied by the fact that treatment of thylakoids with low levels of protease destroys their ability to integrate LHCP (C Dahlin & K Cline, unpublished data). Nevertheless, this targeting model for 54CP-LHCP needs to be experimentally examined.

It is clear that there are many unanswered questions regarding the chloroplast SRP-like system. The chloroplast 54CP particle is probably a multimeric complex because its native size is \( \approx 200 \text{ kDa} \) (R Henry & K Cline, unpublished data). What are the other components of the active particle and how do they function? The 54CP-binding segment of LHCP has not been identified; presumably, it is one or more of the three essential membrane-spanning (signal anchor?) domains. If so, why don’t the signal peptides of the luminal proteins (see below) also bind to 54CP? In addition, what other proteins utilize the SRP pathway? One attractive possibility is that the chloroplast SRP is dedicated to the localization of integral membrane proteins, both imported and plastid-encoded.

**DOES TRANSLLOCATION MACHINERY FACILITATE TRANSPORT ACROSS THE DELTA pH PATHWAY?** Because of the singular use of a pH gradient to power proteins (OE23, OE17, PS2T, PS1N) across the thylakoids, this pathway is referred to as the delta pH pathway. The fact that it requires neither soluble factors nor NTPs makes this translocation pathway unique and suggests a more rudimentary mechanism than the Sec and SRP pathways. Components of the delta pH machinery have not been identified. However, several observations imply the existence of proteinaceous translocation machinery. Precursor saturation of transport at the level of the membrane argues for a specific membrane component that recognizes the LTD of delta pH precursors (Cline et al 1993). Controlled protease pretreatments of thylakoids indicates that a proteinaceous thylakoid component is necessary for transport (Creighton et al 1995). Finally, the identification of the *hcf106* mutant deficient in transport on this pathway and localization of the *hcf106* gene product to the thylakoids support a protein-
mediated translocation process (Voelker & Barkan 1995; R Martienssen, personal communication).

DO PATHWAYS SHARE COMMON TRANSLOCON COMPONENTS? Although it is possible that the delta pH pathway employs a unique translocon, we think it more likely that it uses either the same translocon as the Sec pathway (a common pore) or a homologous one. Thylakoid transport via the delta pH pathway as well as via the Sec pathway employs hydrophobic targeting elements such as classical signal peptides and signal anchors (see below). The well-characterized signal peptide-based systems possess translocon components that are strikingly homologous at the sequence level; the ER Sec61α polypeptide is homologous to bacterial SecY, and Sec61γ is homologous to SecE (Dobberstein 1994). This points to a common evolutionary origin for such systems. Furthermore, where more than one pathway has been identified in the ER, the same core translocon is still utilized (Siegel 1995).

Definitive evidence for a common or homologous pore is lacking, but a recent study in *Chlamydomonas* points to the existence of common component(s) for at least two pathways. Smith & Kohorn (1994) used signal peptide mutations of cytochrome f to block its integration and isolate suppressors. One such signal peptide mutant, A15E, exhibited a dominant-negative effect on the accumulation of LHCP, the plastid-encoded D1 protein, and even a wild-type copy of cytochrome f. Although other thylakoid proteins appeared not to be affected as assessed by their steady-state levels, subtle effects on transport may have escaped detection. The identification of suppressors to A15E may shed light on which step of the translocation process is affected by this mutant. Suppressors isolated to a similar signal peptide mutation in *E. coli*, the lamB14D mutation, were alleles of secA, secY, and secE (Stader et al 1989). Because LHCP does not employ SecA, this analogy suggests that the A15E effect may be at the level of the translocon.

**A Rationale for the Operation of Dual Targeting Pathways Into the Thylakoid Lumen**

The underlying reason for multiple targeting pathways to the same membrane is not known for any system. However, analysis of lumen-targeting elements is beginning to shed light on this question for thylakoids. As mentioned, LTDs carry signaling information for exclusive transport in vitro via either the delta pH or the Sec pathway. They have in common the motifs of the classical signal peptides that direct targeting and transport across the ER and the bacterial cytoplasmic membrane: i.e. a charged (N) domain, a hydrophobic (H) domain, and a (C) domain for proteolytic processing (Figure 2). Extensive studies in bacteria have shown that N and H, but not C, domains are important for transport
(Pugsley 1993). Most LTDs also contain an acidic A domain amino-proximal to the signal peptide (Figure 2). Surprisingly, the A domain appears to be dispensable for pathway-specific thylakoid targeting because a truncated OE23 precursor lacking the A domain was transported exclusively on the delta pH pathway (Figure 2; R Henry et al, in preparation). This means that commitment to one of two different translocation systems relies totally on subtle differences between signal peptides.

Both the N and the H/C regions of thylakoid signal peptides play a role in pathway-specific targeting. Chaddock et al (1995) recently showed that an N domain double arginine (Arg-Arg), which is conserved in delta pH pathway LTDs, is essential for transport of delta pH precursors. Studies in our laboratory show that, although the Sec pathway can utilize Arg-Arg-containing N regions, the Sec pathway is incompatible with H/C regions derived from delta pH pathway precursors (R Henry et al, in preparation). One emerging conclusion is that exclusive sorting to the delta pH pathway is achieved with an N domain containing an Arg-Arg to gain access to that transport system and an H/C domain that is not recognized by the Sec system.

But why would delta pH pathway precursors need to avoid recognition by the Sec pathway? To address this question, a dual pathway targeting signal was constructed with an Arg-Arg-containing N domain and a Sec pathway H/C domain (Figure 2). This provided the ability to simultaneously test different passenger proteins for their ability to be transported across the two pathways. In this test, proteins normally transported by the Sec system were efficiently transported on either pathway (R Henry et al, in preparation). In contrast, proteins normally transported by the delta pH pathway were only transported by the delta pH pathway, even when purified CPsecA was added to boost Sec pathway transport. This agrees with previous studies of chimeric precursors showing that, even in organello, delta pH pathway proteins are very inefficiently translocated by the Sec pathway (Clausmeyer et al 1993, Henry et al 1994). In our experiments, delta pH pathway passenger proteins with a dual targeting peptide appeared to engage the Sec machinery but were arrested in the chain translocation step. One possible explanation for this is that the mature domains of delta pH proteins fold tightly (Creighton et al 1995) and cannot be unfolded by the Sec machinery. These results suggest that delta pH precursors have evolved a mechanism to avoid nonproductive interactions with Sec machinery.

The incompatibility of delta pH proteins with Sec machinery is surprising. The apparent simplicity of the delta pH mechanism, when compared with the ATPase-assisted Sec mechanism, might argue that the delta pH mechanism is a more primitive protein translocation system. Yet the above results suggest
otherwise, i.e. that the delta pH mechanism can accommodate a more diverse population of proteins than the Sec mechanism. In considering the presence of the delta pH pathway, it is interesting to note that proteins carried on this pathway do not appear to be present in modern-day cyanobacteria. Thus these proteins, as well as a compatible translocation mechanism, may have been recruited into the chloroplast after the endosymbiotic event. This possibility makes the identification of the components of the delta pH machinery and their comparison with Sec machinery an even more exciting and interesting endeavor.

FUTURE DIRECTIONS

In addition to the above-mentioned avenues for further investigation, we think that studies of the import and routing problem in other developmental plastid stages is a particularly important area for further research. An intriguing problem concerns the specific manner by which thylakoid proteins may be targeted very early in chloroplast development. The progenitor of chloroplasts, the proplastid, contains very little internal membrane. The deposition of thylakoid membranes during the proplastid-chloroplast progression is accompanied by invaginations of the inner envelope membrane and the accumulation of vesicles in the stroma (Whatley et al. 1982). It has long been theorized that the inner envelope directly gives rise to thylakoids. However, until recently there was little experimental evidence to support this hypothesis. Experiments in *Chlamydomonas reinhardtii* now show that at 38°C, photosynthetic complexes form rapidly following transfer to light. Extensive invagination of the envelope is observed concomitant with the acquisition of photosynthetic competence, but before any recognizable thylakoid membranes are present (Hoober et al. 1991). In another study, Hugueney et al. (1995) reconstituted plastid vesicle fusion in vitro. The vesicles, isolated from developing chromoplasts of *Capsicum* plants, fused in a stroma- and ATP-dependent manner. Purification of the stromal component defined a 72-kDa protein with homology to the N-ethylmaleimide-sensitive fusion protein (NSF) of yeast and animal cells. This protein is known to be involved in vesicle fusion. Importantly, the RNA for the plastid fusion protein also accumulated constitutively in green leaves. It is unlikely that thylakoid protein localization would differ fundamentally in the early stages of thylakoid formation, i.e. it would involve import into the stroma and subsequent translocation. But it is possible that the inner envelope membrane is the target for the final transport/integration step in early developmental stages and that this target site shifts to thylakoids as they mature. Thus it will be very important to learn how the components of the translocon are assembled in the membrane, when they are expressed, and in which plastid membrane they are located at various stages of development.
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