

Update on Chloroplasts

Mechanism of Protein Transport across the Chloroplast Envelope¹

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Photosynthetic eukaryotes have evolved an elaborate system of nuclear-chloroplast interdependence to ensure the coordinate regulation of photosynthesis and other cellular processes. One of the most dramatic consequences of the evolution of the chloroplast from original prokaryotic endosymbiont to a cellular organelle has been the transfer of the vast majority of genes encoding chloroplast polypeptides to nuclear chromosomes, thereby centralizing the control of many essential developmental and metabolic processes in the nucleus. Although the chloroplast has conserved its own genome and is able to synthesize a number of polypeptides, the vast majority of the chloroplast proteins (approximately 90%) are encoded in the nucleus and are translated on free cytoplasmic polysomes. Following the completion of translation, these polypeptides are imported into the chloroplast, directed to one of six suborganellar subcompartments, and assembled into functional proteins.

The central role of protein import in chloroplast biogenesis was recognized when the first nuclear gene encoding a chloroplast polypeptide was identified and sequenced (Dobberstein et al., 1977). A general overview of the import process during the past two decades is now available from the research of numerous laboratories. More recently, attention in the field has turned to identifying components of the import apparatus in hopes of defining the molecular mechanism of protein import. The results of these investigations have revealed a pathway of import that parallels the targeting and membrane translocation systems of other organelles, especially the mitochondrion. However, investigations of the mechanism of chloroplast protein import have revealed fundamental differences among the structure and functions of the import machinery of chloroplasts and other organelles. These differences provide important insights into the distinct evolutionary origins and mechanistic variations of different protein translocation systems. In this review we will provide a summary of our current

knowledge of the chloroplast protein import pathway, with an emphasis on the recently discovered import components and their functions in the import process. In conclusion, we will consider a working model for the molecular mechanism of targeting and import of nuclear-encoded chloroplast proteins into the interior of the organelle.

THE GENERAL PATHWAY FOR PROTEIN IMPORT

The import of nuclear-encoded precursor proteins into the internal compartments of the chloroplast can be viewed as a cascade of protein-targeting and assembly events that results in the localization of proteins to their proper functional location within the organelle (for review, see Cline and Henry [1996]). Proteins destined for the internal compartments of the chloroplast (i.e. the stroma, thylakoids, and inner envelope membrane) begin the import process by engaging a common recognition and translocation machinery at the chloroplast envelope. This machinery is referred to as the general import machinery (Kouranov and Schnell, 1996). With one known exception (Tranel et al., 1995), chloroplast outer envelope membrane proteins appear to use a mechanism of integration that does not involve the general import machinery (for review, see Cline and Henry [1996]). Targeting to the outer membrane will not be discussed here because of our limited knowledge of its mechanism.

All proteins destined for the general import pathway are synthesized as higher molecular weight precursors carrying an N-terminal domain called the transit sequence. The transit sequence is necessary and sufficient to direct the import of a polypeptide into the chloroplast and, therefore, represents the chloroplast-targeting signal (for review, see Keegstra and Olsen [1989]). Transit sequences vary greatly in length (approximately 30–75 amino acids) and primary structure, but they tend to have an abundance of hydroxylated residues and are deficient in acidic residues. Although the structural elements of the transit sequence that are necessary for targeting to the envelope have not been well defined, the transit sequences from different chloroplast precursor proteins are functionally interchangeable, making it likely that they are recognized by a single receptor system at the envelope.

Our current knowledge of the import process has been derived almost exclusively from *in vitro* biochemical assays with which the import of recombinant proteins into

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isolated intact chloroplasts from pea (*Pisum sativum*) or spinach (*Spinacia oleracea*) were studied. The import reaction can be divided into three steps based on analyses of the energetics of precursor protein binding and translocation at the envelope (Fig. 1). First, the cytoplasmic precursor specifically associates with the envelope membrane via the interaction of the transit sequence with proteinaceous receptors at the surface of the outer membrane (Perry and Keegstra, 1994; Ma et al., 1996). This interaction appears to be reversible, energy-independent, and takes place across the entire cytoplasmic face of the outer membrane. Recent studies suggest that the recognition of the precursor by outer membrane receptors may be facilitated by an initial lipid specific partitioning of the transit sequence into the unique lipid environment of the membrane (for a summary, see van't Hoff and de Kruijff [1995]). In fact, transit sequences have been shown to assume a regular secondary structure upon interaction with outer membrane lipids, suggesting that this may be an essential step in generating a regular structure that can be recognized by the binding site of a proteinaceous receptor.

Second, the precursor inserts across the protein-conducting machinery of the outer membrane (Cline et al., 1985; Friedman and Keegstra, 1989). This step requires the hydrolysis of low concentrations of both ATP and GTP ($<100 \mu\text{M}$) in the cytoplasm or in the interenvelope space (Olsen et al., 1989; Olsen and Keegstra, 1992; Kessler et al., 1994). Insertion across the outer envelope membrane triggers the association of the outer and inner import machineries and brings the transit sequence in close proximity to import components of the inner membrane (Ma et al.,

1996). Precursors at this stage in import have been designated early import intermediates. The functional association of the outer and inner membrane import machineries at this step in import occurs at specific envelope subdomains called contact sites, where the two membranes are held in close proximity (Schnell and Blobel, 1993). Outer membrane insertion is likely to represent the committal step in the import reaction because the interaction of the precursor with the envelope at this stage is irreversible (Friedman and Keegstra, 1989).

Finally, the precursor inserts across the inner membrane and translocation into the stroma proceeds simultaneously across both envelope membranes. This step requires the hydrolysis of ATP in the stromal compartment (Pain and Blobel, 1987; Theg et al., 1989). Proteins in transit across both envelope membranes have been isolated and are referred to as late import intermediates. A variety of ionophores show no effect on import, indicating that protein translocation at the chloroplast envelope does not involve an electrochemical potential (Pain and Blobel, 1987; Theg et al., 1989). This is in contrast to mitochondrial protein import or bacterial protein export, both of which require a membrane potential. However, like other translocation systems, envelope translocation does require a fully unfolded polypeptide chain (Pilon et al., 1992).

Recently, investigators have identified a strong polypeptide-unfolding activity associated with the outer membrane (Guéra et al., 1993; Walker et al., 1996) that may serve to induce or stabilize the unfolded import-competent conformation of the precursor. The nature of this unfolding activity is unknown, but along with cytoplasmic molecular

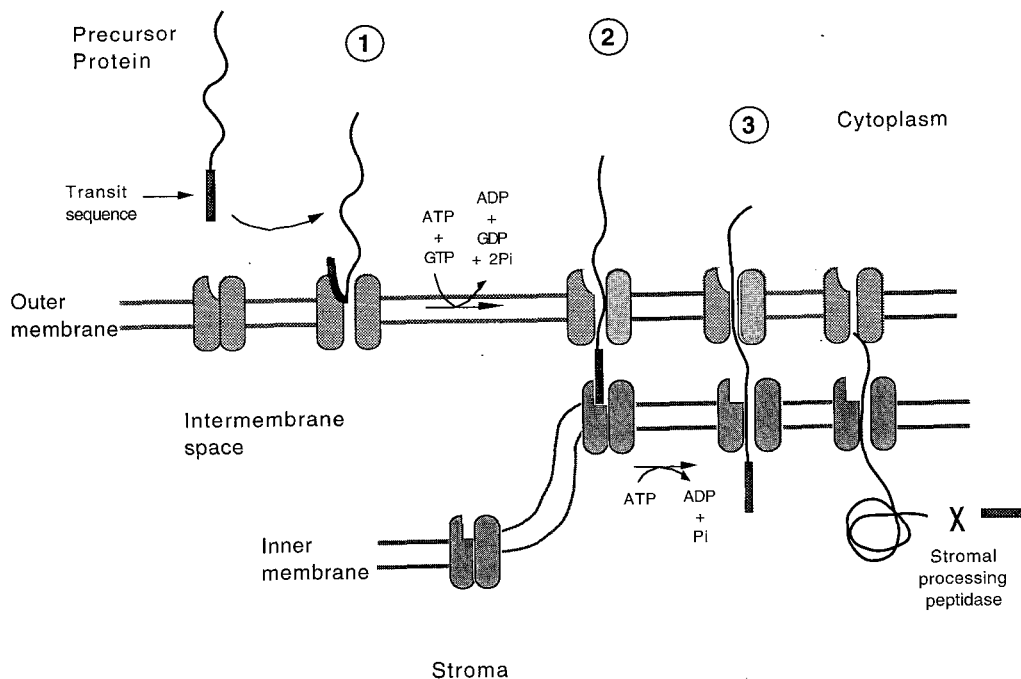


Figure 1. Schematic representation of the stages of protein import into chloroplasts. Stage 1, Energy-independent recognition of the amino-terminal transit sequence of the precursor by the outer membrane import complex. Stage 2, ATP/GTP-dependent insertion of the precursor across the outer membrane. Stage 3, ATP-dependent insertion of the precursor across the inner membrane resulting in simultaneous translocation across the double-envelope membrane.

chaperones (i.e. hsp70 homologs; Waegemann et al., 1990) it is likely to ensure the import competence of the cytoplasmic precursor protein *in vivo*. At the end of translocation or shortly after arrival in the stroma, the transit sequence is cleaved from the precursor by a stromal metalloendopeptidase called the general stromal processing peptidase (vanderVere et al., 1995), yielding a mature protein. The newly imported protein folds in the stroma with the assistance of molecular chaperones or undergoes further suborganellar targeting to the thylakoid membrane as directed by secondary intrinsic targeting signals.

IDENTIFICATION OF THE IMPORT COMPONENTS OF THE OUTER ENVELOPE MEMBRANE

The protein-import machinery of the outer envelope membrane performs critical functions, specific recognition of cytoplasmic chloroplast precursor proteins, and initiation of membrane translocation. In the last few years substantial progress has been made in identifying envelope components involved in protein import. The generation of import intermediates by varying the energy state of *in vitro* import reactions and the arrest of precursors at different stages of import have led to the identification of six components of the outer envelope import apparatus. Four of them have been identified based on the association of chloroplast envelope proteins with trapped early import intermediates (Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995). They have been termed the intermediate-associated proteins (IAPs) or the outer envelope-associated proteins (OEPs). These proteins are IAP34 (OEP34), Hsp70-IAP, IAP75 (OEP75), and IAP86 (OEP86), named according to their molecular masses. A second Hsp70 homolog (Com70) has been found to be associated with precursors arrested across the envelopes (Wu et al., 1994).

Three of these components, IAP34, IAP75, and IAP86, form a multisubunit import complex in the outer envelope that stably associates with early import intermediates. All of these proteins are integral membrane proteins (Waegemann and Soll, 1991; Schnell et al., 1994; Tranel et al., 1995). Subsequent experiments indicated that the three outer envelope IAPs appear to form this complex prior to the association of the precursor with the outer envelope import apparatus (Ma et al., 1996). Analysis of the primary structures of IAP34, IAP75, and IAP86 indicates that they are unique, with no comparable homologs in mitochondria. Thus, the components and underlying mechanisms of chloroplastic and mitochondrial import are likely to have separate evolutionary origins.

IAP34 and IAP86

IAP86 and IAP34 are specific GTP-binding proteins that are closely related in primary sequence (Hirsch et al., 1994; Kessler et al., 1994; Seedorf et al., 1995). The presence of two GTP-binding proteins in the outer envelope is consistent with the observation that nonhydrolyzable GTP analogs inhibit protein import into the chloroplast and that GTP is required for the formation of early import interme-

diates (Olsen and Keegstra, 1992; Kessler et al., 1994). Protease treatment of the chloroplast surface indicates that IAP34 and IAP86 are inserted in the outer membrane in an N-terminus_{out}-C-terminus_{in} orientation, with their N-terminal GTP-binding domains exposed to the cytosol. The topology and GTP-binding activity of these two components suggest that they function in regulating the recognition of the precursor protein at the outer membrane. Two pieces of experimental evidence support the involvement of IAP86 in precursor recognition. Anti-IAP86 IgGs block the binding of precursors at the envelope (Hirsch et al., 1994). In addition, covalent cross-linking studies indicate that IAP86, along with a second component, IAP75, are in intimate contact with the transit sequence of precursors during the initial energy-independent binding to the outer envelope (Perry and Keegstra, 1994; Ma et al., 1996). There is no direct experimental evidence for the function of IAP34 in import, but it has been proposed that it acts in concert with IAP86 to regulate the presentation of the precursor to the protein-conducting machinery of the envelope.

IAP75

IAP75 is deeply embedded in the outer membrane as indicated by its resistance to alkaline extraction and protease treatment in intact chloroplasts (Schnell et al., 1994; Tranel et al., 1995). It is surprising that analysis of its primary sequence indicates that the protein does not contain any typical membrane-spanning α -helices, but is overall hydrophilic in nature. Secondary structure predictions suggest that IAP75 could contain up to 26 β -strands (Schnell et al., 1994; Tranel et al., 1995). By analogy to the function of bacterial porins that form large aqueous pores through extensive membrane-spanning β -strands, it has been proposed that IAP75 could create a protein-translocating channel in the outer membrane. Antibodies to IAP75 block the import of precursor proteins into intact chloroplasts (Tranel et al., 1995). Furthermore, IAP75 is the major target of covalent cross-linking to precursors upon their insertion across the outer membrane (Perry and Keegstra, 1994; Ma et al., 1996). Both observations provide compelling, albeit indirect, evidence for a role for IAP75 in protein transport at the outer membrane.

Molecular Chaperones Associated with the Outer Membrane

The outer envelope also contains two associated molecular chaperones of the heat-shock protein 70 family, hsp70-IAP (Waegemann and Soll, 1991; Schnell et al., 1994) and Com70 (Wu et al., 1994). Com70 is loosely associated with the cytoplasmic surface of the outer membrane and can be covalently cross-linked to early import intermediates. Its role in protein import has been confirmed by the demonstration that Com70 antibodies inhibit protein import into the chloroplast. This protein is closely related in primary structure to the major cytoplasmic Hsp70s. It is tempting to speculate that Com70 may be responsible for the unfolding activity at the outer membrane surface and thereby acts to maintain the import competence of the precursor at the

cytoplasmic face of the outer membrane. This role would be similar to that proposed for the cytoplasmic hsp70s in translocation into mitochondria and ER. Unlike Com70 and other cytosolic chaperones, hsp70 IAP is tightly anchored to the outer membrane. In addition, it is insensitive to external protease treatments, suggesting that the protein is located in the interenvelope space between the outer and inner membrane (Schnell et al., 1994). It has been proposed that the hsp70-IAP binds to the precursor protein as it emerges across the outer membrane, thereby maintaining the import competence of the polypeptide until the transit sequence engages the inner membrane import machinery. The discovery of two hsp70 homologs associated with the outer membrane would account for the requirement of ATP during the formation of early import intermediates, and it has been proposed that the binding of chaperones to the early import intermediate is responsible for the irreversible association of the precursor with the envelope.

THE IMPORT COMPONENTS OF THE INNER ENVELOPE MEMBRANE

The same strategies used for outer envelope IAPs, e.g. cross-linking and immunoaffinity chromatography, have led to the identification of four components of the inner envelope machinery.

IAP100 and IAP36

Two proteins, IAP100 (IEP110/Cim97) (Schnell et al., 1994; Wu et al., 1994; Lübeck et al., 1996) and IAP36 (Schnell et al., 1994), are associated with late import intermediates, suggesting that these two polypeptides are components of the inner envelope import apparatus. IAP36 has not been characterized to date. An antibody specific to IAP100 has been used to confirm its inner envelope location (Kessler and Blobel, 1996; Lübeck et al., 1996). It is an integral membrane protein with one or, perhaps, two putative α -helical transmembrane domains at its extreme N terminus. Anti-IAP100 co-precipitates at least two additional chloroplast polypeptides with IAP100. One protein has a mobility similar to IAP36, although its identity remains unknown. The second protein is the stromal chaperonin, cpn60 (Kessler and Blobel, 1996). cpn60 is known to play a role in the folding and assembly of newly imported precursor proteins, prompting the proposal that IAP100 functions to localize the chaperonin at the site of translocation by serving as a membrane-docking site for cpn60. Recently, another group has identified a second molecular chaperone, ClpC, in a complex with IAP100 and bound precursor protein (Nielsen et al., 1997).

IAP21 and IAP25

Two additional components of the import machinery, IAP21 and IAP25, have been cross-linked to the transit sequence of an early import intermediate at envelope contact sites (Ma et al., 1996). These IAPs are not susceptible to protease treatments in intact chloroplasts, suggesting their location at the inner envelope membrane. The localization

of IAP21 has been confirmed to be the inner membrane. Although the function of these proteins remains to be investigated in detail, their close proximity to the transit sequence of the early import intermediate suggests that they may be receptors of translocation components at the inner membrane.

Com/Cim44

Another envelope component, Com/Cim44, has been identified in cross-linked complexes containing import intermediates (Wu et al., 1994; Ko et al., 1995). Molecular cloning of the protein indicates that it is a member of a family of proteins that associate with both the outer and inner envelope membranes. The function of Com/Cim44 in import remains to be investigated.

Molecular Chaperones Associated with the Inner Membrane

In a recent paper it was shown that ClpC, a stromal hsp100 homolog, is part of the multisubunit import complex that contains import components from both envelope membranes (Nielsen et al., 1997). This chaperone is found in stable association with the import complex in an ATP-dependent manner and it appears that its interaction with the complex decreases upon the release of the precursor in the stroma. A third stromal chaperone, the hsp70 homolog S78 (Marshall and Keegstra, 1992), can also associate with the precursor but the specificity of this interaction has not been established (Nielsen et al., 1997). The association of cpn60 with IAP100 suggests that this chaperone also is present at or near the site of membrane translocation (Kessler and Blobel, 1996). These observations make it clear that the release of the protein from the import machinery and its folding in the stroma require the action of more than one chaperone, as observed in several other systems (Schatz and Dobberstein, 1996).

A WORKING MODEL FOR ENVELOPE TRANSLOCATION

Based on the characteristics of the recently described import components, a working model can be proposed for the molecular mechanism of protein import at the chloroplast envelope (Fig. 2). In this model recognition and translocation of the cytoplasmic precursor at the outer membrane would be mediated by a multisubunit import complex composed of IAP34, IAP75, and IAP86. An initial receptor site for the transit sequence would be formed by IAP86 and, perhaps, IAP75. IAP34 and IAP86 would regulate the presentation of the cytoplasmic precursor to the protein-conducting machinery of the envelope through cycles of GTP binding and hydrolysis. This would serve in proofreading the transit sequence of the precursor to ensure its identity as a chloroplast-targeting signal prior to its insertion into the translocation channel. IAP75 would constitute at least part of the protein-conducting channel in the outer membrane. Com70 and hsp70-IAP would serve chaperone functions on either side of the outer membrane. Com70 would bind precursors on the cytosol-

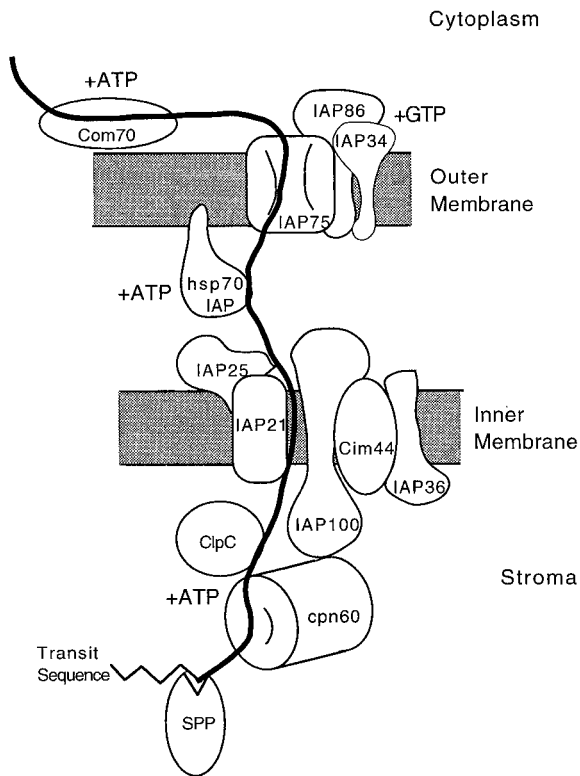


Figure 2. Model of the protein translocation apparatus at the chloroplast envelope. A detailed description of the proposed function of the import components is provided in the text. SPP, Stromal processing peptidase.

lic face of the outer membrane, maintaining the precursor in an unfolded, import-competent conformation. The hsp70-IAP would bind to the precursor as it emerges from the outer membrane channel into the intermembrane space, thereby ensuring that the precursor does not fold before engaging the translocation machinery of the inner membrane at contact sites. In a role similar to that proposed for the mitochondrial matrix hsp70, binding of the hsp70-IAP to the precursor would also provide the thermodynamic driving force for translocation across the outer membrane. Translocation across the inner membrane would be initiated by the interaction of the transit sequence with a second receptor system at this membrane. IAP21 and IAP25 are candidates for components of this receptor system. Envelope translocation would proceed across both membranes simultaneously through two distinct protein-conducting channels. The composition of the inner membrane translocation channel remains to be determined. Presumably, IAP100, IAP36, and Cim44 participate in translocation events at the inner membrane. IAP100 would serve as a membrane component that recruits molecular chaperones to the *trans* side of the inner membrane. Binding of chaperones such as ClpC and/or cpn60 to the translocating chain would drive transport across the inner membrane into the stroma and aid in folding and assembly of the newly imported protein.

PERSPECTIVES

One of the most intriguing challenges remaining to be addressed in both mitochondrial and chloroplast protein import is the nature of the interaction of the outer and inner membrane import machineries at contact sites. The structure and the formation of contact sites are still obscure, but recent experimental data suggest that they are dynamic structures. Although the outer and inner membrane import components have been shown to associate (Schnell et al., 1994; Nielsen et al., 1997), the major proportion of import components in the two membranes are not bound to one another (Kessler and Blobel, 1996). These observations suggest a dynamic equilibrium between individual envelope machineries and an import complex containing both translocation machineries at contact sites. This scheme may still be more complex because there is some evidence that the outer envelope can also import proteins independently of the inner envelope machinery (Scott and Theg, 1996), suggesting that the import capabilities of the outer and inner membrane are not a priori linked. Whether contact sites are formed by association of the import machineries of the outer and inner membrane in response to import or whether they exist as structures independent of the import machinery remains to be determined.

The tremendous progress in our understanding of chloroplast protein import during the past 2 years has provided a foundation for investigations over the next decade aimed at defining the precise molecular mechanism of precursor protein recognition and translocation. Several obvious challenges remain to be addressed, such as the exact roles of ATP and GTP in import and direct evidence for the proposed functions of the known import components. These challenges are substantial, and it is likely that partial or complete reconstitution of the import reaction using purified components will be necessary to provide definitive answers to these questions. In addition, the search for other players in the import process is ongoing, and it is becoming imperative that those of us in the field develop *in vivo* models to augment the elegant *in vitro* biochemical studies that have contributed to our understanding of the import reaction.

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