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Shedding Light on the Chloroplast Protein Import Machinery

Minireview

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The complex architecture of the chloroplast presents an intriguing challenge for investigators interested in the mechanisms of protein targeting and organelle biogenesis. Chloroplasts are subdivided by three noncontiguous membrane systems into at least six suborganellar compartments that serve to segregate and organize a number of essential metabolic functions, most notably the reactions of photosynthesis. The development and maintenance of this structure require an elaborate sorting system to ensure proper targeting and integration of resident proteins. This complexity is compounded by the fact that the vast majority of chloroplast polypeptides are nuclear-encoded and posttranslationally imported into the organelle from the cytosol. This cascade of targeting and assembly reactions is initiated by the recognition and translocation of precursor proteins at the double membrane of the chloroplast envelope.

After years of studies that have contributed to our understanding of the general pathway of import, recent investigations have begun to uncover the components that underlie the mechanism of targeting and translocation at the envelope. These studies support the existence of a single common mechanism of envelope transport for all cytosolically synthesized precursors that are destined for the internal compartments of the chloroplast. In particular, extensive progress has been made in understanding the import apparatus in the outer membrane of the envelope. There is now convincing evidence that the initial stages of envelope translocation are mediated by a complex of outer envelope membrane proteins (OEPs) that includes two GTP-binding proteins, a putative channel component, and at least two membrane-associated molecular chaperones. Several candidates for components of the inner membrane translocation machinery also have been identified, and their structures and specific functions are under investigation.

These recent discoveries are especially satisfying because they corroborate previous evidence supporting a single mechanism of envelope translocation. Moreover, the apparent activities of the components serve as the basis for the formulation of testable hypotheses that should greatly facilitate dissection of the mechanism of envelope translocation. This minireview will focus on these recent discoveries and their implications with respect to the mechanism of translocation in chloroplasts and other relevant systems. More detailed discussions of the import process can be found in recent review articles (Gray and Row, 1995; Keegstra et al., 1995).

Identification of Envelope Translocation Components

Chloroplast protein import can be divided into two distinct steps (reviewed by Keegstra et al., 1995). In the first step, the cytosolic precursor specifically associates with the outer membrane in a high affinity, essentially irreversible interaction. The establishment of this interaction is mediated by the amino-terminal transit sequence of the precursor and requires hydrolysis of low concentrations of nucleoside triphosphate (50–100 μM). This outer membrane–bound precursor has been designated an "early" import intermediate. In the second stage of import, the early intermediate is fully translocated into the stromal compartment. Membrane translocation requires higher concentrations of ATP (>1 mM) and takes place simultaneously across both outer and inner envelope membranes at contact sites where the two membranes are in close apposition. "Late" import intermediates representing polypeptides that are inserted across both the outer and inner membranes also have been identified. In contrast with mitochondrial import, a membrane potential is not involved at either step in chloroplast translocation. Upon translocation, the transit sequence is removed, and the newly imported polypeptide folds and assembles in the stroma or undergoes further targeting to another internal compartment.

In recent work, the findings of four independent groups have converged to provide compelling evidence for the participation of a set of five OEPs in precursor import (Table 1). In each case, the identifications were based on the association of the proteins with early import intermediates. These components have been named OEPs, chloroplast outer membrane proteins (COMs), or import intermediate-associated proteins (IAPs) followed by the molecular mass of the component in kilodaltons.

Table 1. Components of the Chloroplast Protein Import Machinery

Component	Location	Approximate Molecular Mass	Activities	Proposed Function
OEP/IAP34	Outer membrane (integral)	34 kDa	GTP binding	Precursor recognition
OEP/IAP86	Outer membrane (integral)	86 kDa	GTP binding	Precursor recognition
OEP/IAP75	Outer membrane (integral)	75 kDa	Unknown	Protein conducting channel
Hsp70	Outer membrane (integral)	75 kDa	Hsp70 homolog	Chaperone
Com70	Outer membrane (peripheral)	72 kDa	Hsp70 homolog	Chaperone
Cim44	Inner membrane	44 kDa	Unknown	Unknown
IAP100/Cim97	Inner membrane	100 kDa	Unknown	Unknown
IAP36	Unknown	36 kDa	Unknown	Unknown

Two groups have provided evidence that OEP/IAP34, OEP/IAP86, OEP/IAP75, and the Hsp70 IAP form a translocation complex in the outer membrane. Schnell et al. (1994) used an early import intermediate that was tagged with staphylococcal protein A to immunoaffinity purify directly a precursor–import complex containing these four proteins from detergent solubilized envelopes. Waegemann and Soll (1991) demonstrated that all four cosediment on sucrose gradients with an early intermediate following detergent treatment of envelope membranes. A third group has used covalent cross-linking to show that two members of this complex, OEP/IAP75 and OEP/IAP86, are in intimate association with an early import intermediate (Perry and Keegstra, 1994).

The analyses of the activities and primary structures of the components of this outer membrane complex have provided significant insight into the mechanics of import. OEP/IAP34 and OEP/IAP86 are specific GTP-binding proteins with extensive sequence similarity (Kessler et al., 1994). Both polypeptides are tightly anchored in the outer membrane with their GTP-binding domains exposed to the cytosol (Hirsch et al., 1994; Kessler et al., 1994; Seedorf et al., 1995). These observations immediately promote OEP/IAP86 and OEP/IAP34 as candidates for proteins involved in regulating the recognition of precursors at the chloroplast surface. This hypothesis is especially attractive when one considers the parallels to the involvement of GTP in regulating protein targeting to the endoplasmic reticulum by the signal recognition particle and its receptor (Miller et al., 1993).

OEP/IAP75 shows no similarity to any sequences in the databases (Schnell et al., 1994; Tranel et al., 1995). This component is deeply embedded in the outer membrane without a detectable cytosol-exposed domain. Secondary structure predictions of OEP/IAP75 suggest a propensity toward the formation of extensive β strands. Such structures are characteristic of the bacterial porins and have prompted Schnell et al. (1994) and Tranel et al. (1995) to propose that OEP/IAP75 functions as a component of the protein-conducting channel or pore for outer membrane translocation.

The Hsp70 IAP is a homolog of the Hsp70 family of molecular chaperones, but it has a number of unique characteristics that distinguish it from Hsp70s that are involved in other translocation reactions (references can be found in Schnell et al., 1994). The Hsp70 IAP exhibits the biochemical characteristics of an integral membrane protein, indicating that it is very tightly anchored to the outer membrane. The nature of the membrane anchor is not known, but it will be of great interest to determine whether it is due to a proteinaceous membrane domain or, perhaps, a lipid anchor. The Hsp70 IAP does not possess a cytosolically exposed domain, and the bulk of the molecule appears to lie predominantly in the intermembrane space between the outer and inner envelope membranes. The location and membrane association of the Hsp70 IAP distinguish it from the soluble cytosolic and matrix Hsp70s that are essential for protein import into mitochondria. Schnell et al. (1994) have proposed that it may serve as a chaperone for precursor proteins as they emerge from the outer membrane translocation channel into the intermembrane space (Schnell et al., 1994).

In addition to the Hsp70 IAP, Wu et al. (1994) have reported the involvement of a second outer membrane Hsp70 homolog, Com70, in precursor import. This group used chemical cross-linking and coimmunoprecipitation to show that Com70 was in close proximity to an import intermediate representing a partially imported precursor. Antibodies to Com70 inhibited precursor import, providing further evidence for its role in the import reaction. This chaperone is localized to the periphery of the outer membrane and is similar in sequence to the major soluble cytosolic Hsp70 (Ko et al., 1992).

Characterization of the inner membrane import machinery is less advanced than that of the outer membrane because of the inaccessibility of this membrane to direct biochemical manipulation and the difficulties encountered in isolating intermediates that span the inner membrane. Despite these technical challenges, at least three candidates for chloroplast inner membrane translocation components have been reported within the last year (Table 1). One of these components, named IAP100 or Cim97, has been shown to associate with a late import intermediate by covalent cross-linking (Wu et al., 1994) as well as by direct association with the translocating chain (Schnell et al., 1994). This evidence makes it the strongest candidate for an inner membrane import component. In addition, proteins of 36 kDa (Schnell et al., 1994) and 44 kDa (Wu et al., 1994) also have been shown to be present in complexes containing a late intermediate that spans the inner membrane. The primary structures of the three putative inner membrane components have yet to be reported, but their characterization should open a window into the process of inner membrane translocation.

With the exception of the two Hsp70 homologs, there are no apparent similarities in primary structures among the chloroplast import components and their mitochondrial counterparts. This is somewhat surprising considering that the pathways of precursor translocation across the chloroplast envelope and the double membrane of mitochondria appear to be analogous. Both systems require transport across a double membrane boundary at the expense of internal ATP with transport across two membranes facilitated by membrane contact sites. The sequences of the chloroplast outer membrane import components clearly indicate that the protein import machineries of these two organelles have separate evolutionary origins.

A Model for Recognition and Translocation of Proteins at the Chloroplast Envelope

A viable hypothesis for the coordinate function of the import machineries of the envelope can be proposed when the apparent activities of the newly described components is put in the context of the well-established characteristics of the import reaction (Figure 1). In this scenario, OEP/IAP34 and OEP/IAP86 would regulate the presentation of the cytosolic precursor protein to the protein conducting machinery of the envelope. A cycle of GTP hydrolysis by one or both of the proteins would be used in proofreading the transit sequence of the precursor prior to its insertion

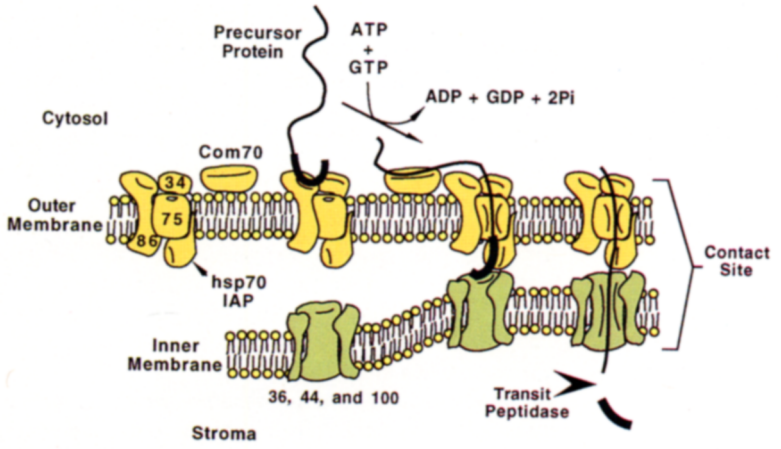

Figure 1. A Model for Protein Import into Chloroplasts

The known components of the envelope import machinery are indicated by their molecular masses in kilodaltons.

into the translocation channel. OEP/IAP75 would constitute at least part of the protein conducting channel. Com70 and Hsp70 IAP would serve chaperone functions on either side of the outer membrane. Com70 would bind precursors on the cytosolic 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 providing the thermodynamic driving force for translocation and ensuring that the precursor does not fold before engaging the translocation machinery of the inner membrane at contact sites. Translocation across the inner membrane would be initiated by the interaction of the transit sequence with a second receptor system at this membrane, and envelope translocation would proceed across both membranes simultaneously through two distinct protein-conducting channels. Presumably, IAP100/Cim97, IAP36, and Cim44 participate in translocation events at the inner membrane, although their functions remain to be determined.

There is convincing experimental evidence to support aspects of this hypothesis. OEP/IAP86 can be cross-linked to a precursor protein in a binding assay even in the absence of added nucleoside triphosphate (Perry and Keegstra, 1994). This suggests that OEP/IAP86 may be involved in a primary recognition event that does not require energy input. The addition of nucleoside triphosphate to the binding reaction results in the formation of the early import intermediate. This stable intermediate can be cross-linked to both OEP/IAP86 and OEP/IAP75, suggesting that energy is utilized to promote the precursor to a subsequent step in the import process (e.g., insertion into the channel). The model also is consistent with the rather complicated energetics of the formation of the early import intermediate. Olsen and Keegstra (1992) observed that either ATP or GTP support formation of the early intermediate, and Kessler et al. (1994) subsequently presented evidence that both nucleoside triphosphates are required for its formation. The presence of two GTP-binding proteins, OEP/IAP86 and OEP/IAP34, and two ATPases, the Hsp70 IAP and Com70, in the import complex accounts for this dual requirement. The essentially irreversible, high affinity interaction of the precursor in this intermediate state is consistent with an interaction with the Hsp70s.

Future Directions

The model in Figure 1 highlights a number of questions that now can be addressed with the tools provided by the core set of envelope translocation components. First, what is the master receptor(s) for precursor recognition at the chloroplast surface? The label transfer cross-linking results (Perry and Keegstra, 1994) and the observation that OEP/IAP86 antibodies block formation of the early import intermediate (Hirsch et al., 1994) make OEP/IAP86 the best candidate for a precursor receptor. However, there is no evidence that OEP/IAP86 interacts with the transit sequence of the precursor, an activity that, by definition, is required of an import receptor. Additional cross-linking experiments and direct binding experiments with transit sequence analogs should provide the evidence for or against this assignment. In addition, other unidentified components (e.g., soluble factors) also may play a role in precursor recognition. Second, what are the precise roles of GTP and ATP in the import reaction? The participation of GTP in precursor recognition and ATP in chaperone function are the most obvious possibilities. One interesting additional possibility is that hydrolysis of either ATP or GTP participates in the functional association of the outer and inner membrane translocation machineries at contact sites. The cross-linking of OEP/IAP86 to precursor detected by Perry and Keegstra (1994) in the absence of added energy was localized in regions of free outer membrane that are not engaged in contact sites. Remarkably, cross-linking of the early import intermediate to both OEP/IAP86 and OEP/IAP75 in the presence of ATP was located only in membrane fractions containing contact sites. These results suggest that energy is not only involved in promoting the precursor from an initial interaction with OEP/IAP86 to one that includes OEP/IAP75, but that it also may participate in the engagement of contact sites. Perhaps this activity involves the second GTP-binding component of the apparatus, OEP/IAP34. The physical nature of contact sites is not understood. Therefore, it is not clear whether the engagement of these structures results from migration of the import complexes to preexisting

contact sites or whether contact sites form as a consequence of import. Does OEP/IAP75 constitute a translocation pore in the outer membrane? Bulychev et al. (1994) recently have used patch–clamp methods to demonstrate the existence of a precursor responsive channel in intact chloroplasts. It will be quite interesting to test whether antibodies to OEP/IAP75 affect this channel activity. OEP/IAP75 antibodies have been shown to block translocation of precursors into the stromal compartment (Tranel et al., 1995).

It is likely that the chloroplast import components that have been described serve as the essential scaffold of the import machineries. Undoubtedly, other components will soon follow that participate directly in import as well as regulate the formation or engagement of contact sites. Thus far, the identification and characterization of the chloroplast protein import machinery have relied exclusively on biochemical approaches. This is in stark contrast with studies of protein sorting to mitochondria, peroxisomes, and the secretory pathway that have benefited tremendously through the complementation of biochemistry and molecular genetics. Mutations in the chloroplast envelope translocation machinery would likely yield pleiotropic, lethal effects due to the essential role of plastids in cell metabolism. Thus, genetic approaches will depend on our ability to design highly selective screens for conditional mutations. This is practically impossible in plant systems with our current technology. The greatest promise for developing in vivo model systems consisting of selected mutations in the near future relies on the use of the already cloned components to perform "reverse" genetics in transgenic plants or aquatic algae. Several groups are in the process of using antisense RNA and dominant negative mutations to manipulate the import machinery. These future studies will contribute to the developing picture of a dynamic process in which the machineries of two membranes cooperate to facilitate transport of proteins into the interior of the organelle.

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