Protein import into chloroplasts

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Chloroplasts have evolved an elaborate system of membrane and soluble subcompartments to organize and regulate photosynthesis and essential aspects of amino acid and lipid metabolism. The biogenesis and maintenance of organellar architecture rely on protein subunits encoded by both nuclear and plastid genomes. Import of nuclear-encoded proteins is mediated by interactions between the intrinsic N-terminal transit sequence of the nuclear-encoded preprotein and a common import machinery at the chloroplast envelope. Recent investigations have shown that there are two unique membrane-bound translocation systems, in the outer and inner envelope membranes, which physically associate during import to transport preproteins from the cytoplasm to the internal stromal compartment. This review discusses current understanding of these translocation systems and models for the way in which they might function.

The chloroplast contains at least six compartments that have evolved to compartmentalize specialized aspects of photosynthetic metabolism (Fig. 1). These include membrane and luminal compartments of the thylakoids that harbour the photosystems and electron-transport proteins that are responsible for light-driven ATP and NADPH synthesis. The thylakoid is surrounded by the stroma, which contains the soluble enzymes of reductive carbon assimilation. The envelope serves as the boundary membrane of the chloroplast, and the outer and inner envelope membranes regulate transport and communication between the organelle and cytoplasm. Targeting of nuclear-encoded preproteins to these compartments is directed by one or more distinct targeting signals that are decoded at the envelope and thylakoid membranes. Although these sorting reactions involve a number of distinct targeting pathways, the general import pathway across the envelope is the common gateway for all preproteins into the organelle.

Our knowledge of chloroplast protein import has been obtained largely by the biochemical analyses of in vitro import assays consisting of recombinant preproteins and isolated chloroplasts from pea seedlings. Within the past year, the biochemical methods have been combined with emerging in vivo model systems to provide an unprecedented insight into the mechanism of the import process. The early stages of protein import are mediated by direct association of the transit sequence with components of the translocon at the outer envelope membrane (Toc apparatus). In the absence of nucleoside triphosphates (NTPs), the preprotein binds to the Toc apparatus with low affinity (Fig. 2, stage 1). This early binding step is detected experimentally only when binding is trapped by covalent crosslinkers. In the presence of low concentrations of external ATP and/or GTP, the preprotein is promoted to a second stage in import, referred to as an early import intermediate (Fig. 2, stage 2). Recent covalent crosslinking and co-immunoprecipitation experiments have demonstrated that early import intermediates are partially inserted across the outer membrane and are in contact with components of the inner envelope membrane. It is generally believed that binding and early import intermediates represent sequential stages in import, corresponding to the initial recognition of the transit sequence by the import machinery and subsequent NTP-triggered insertion of the preprotein into the protein-conducting channel of the outer membrane.

Considerable effort has been expended in elucidating the structural elements of the transit sequences that confer specificity to the chloroplast import pathway. However, the great diversity in length and primary structure among the transit sequences from different preproteins has thwarted efforts to identify consensus elements that define a standard transit sequence. Despite this lack of structural similarity, reliable algorithms for predicting transit sequences (e.g. PSORT, http://psort.nibb.ac.jp) have been developed based on the length, overall basic charge, high proportion of hydroxylated amino acids and lack of acidic amino acids that characterize most transit sequences. The efforts to define structural motifs in the transit sequence have been complicated further by the observation that synthetic transit peptides form no regular secondary structure in aqueous solutions. Recently, several investigations have proposed a role for interactions between transit sequences and lipids in chloroplast targeting.

The transit peptides of two stromal proteins exhibit selective interactions with artificial monolayers or bilayers of similar composition to the outer envelope membrane. The interactions with lipids are most pronounced in membranes enriched in galactosyldiacylglycerols that are unique to the chloroplast envelope. Furthermore, conditions that presumably mimic the membrane environment induce a significant degree of α-helical structure in several transit sequences. This observation prompted the proposal that the early stages of targeting to the chloroplast involve selective partitioning of cytoplasmic preproteins into the lipid environment of the outer envelope membrane. The nonpolar environment would induce a regular secondary structure in transit sequences that could be recognized by a single receptor component of the Toc apparatus. Although
this model would account for the ability of a single receptor system to recognize the diverse range of transit sequences, it suffers from the fact that specific interactions between transit sequences and lipids have only been demonstrated for two chloroplast preproteins. Recently, this hypothesis has gained additional in vivo support from the observation that an Arabidopsis thaliana mutant defective in diagalactosyldiacylglyceride synthesis has a general defect in chloroplast protein import19.

Insertion of the early import intermediate across the outer envelope results in the association of the transit sequence with components of the translocon of the inner envelope membrane (Tic apparatus; Fig. 2, stage 2). At this stage in import, the Toc and Tic machineries are physically linked to form a Toc-Tic supercomplex14,15,20. The hydrolysis of stromal ATP results in the insertion of the preprotein into the protein-conducting machinery of the Tic complex, and translocation into the stroma proceeds simultaneously across both envelope membranes21 (Fig. 2, stage 3). Stromal ATP appears to be the only energy requirement for translocation through the inner membrane. Site-specific crosslinking studies with urea-denatured import substrates have demonstrated that translocation is a vectorial process proceeding from the N-terminus to the C-terminus with respect to the preprotein10. A regulated ion-channel activity of relatively large conductance is associated with translocation across the envelope, suggesting that selective opening of protein-conducting channels occurs22. At least one report suggests that a small, folded polypeptide can be imported across the envelope16. Therefore, the protein-conducting machinery of the import apparatus might be able to expand to a relatively large diameter while maintaining the permeability barrier of the inner membrane. Upon accessing the stroma, the transit sequence of the preprotein is removed by a specific stromal processing peptidase24. At this point, the newly imported protein folds and assembles in the stroma and integrates into the inner envelope membrane or undergoes targeting to the thylakoid membrane as directed by secondary intrinsic targeting signals24.

**The Toc apparatus**

The outer membrane translocon performs three crucial functions in the import process: specific recognition of preprotein transit sequences, initiation of membrane translocation and the formation of contact sites. Within the past year, considerable evidence has accumulated for the role of a complex of three integral membrane proteins in preprotein recognition and translocation at the outer membrane (Table 1). Two members of this Toc complex, Toc159 and Toc34, form the primary contacts with preproteins during energy-independent binding25 (Fig. 2, stage 1). Toc159 and Toc34 contain structurally related, membrane-embedded GTP-binding domains that define a distinct family of membrane GTPases16,26. Toc159 was identified originally as an 86-kDa polypeptide, designated Toc86, but recent studies have shown that Toc86 is a proteolytic fragment of the native 159-kDa protein27 (D. Schnell, unpublished). Several lines of evidence suggest that Toc159 participates in the initial transit sequence binding site for preproteins at the chloroplast surface. Foremost among these data are the observations that antibodies against Toc159 block preprotein binding to isolated chloroplasts18 and that the transit sequences of two preproteins crosslink predominantly to Toc15928. The third known component of the Toc complex, Toc75, also crosslinks to preprotein transit sequences at the early binding step19. Toc34 does not appear to interact selectively with the transit sequence of the preprotein, but its association with preproteins is strictly regulated by GTP binding at the Toc complex. On the basis of these observations, Toc34 does not appear to be a component of the transit sequence receptor but has been proposed to play a regulatory role in preprotein binding.

Considerable evidence indicates that Toc75 also constitutes the major component of the protein-conducting channel in the outer membrane (Fig. 2, stage 2). Consistent with this proposal is that early import intermediates inserted across the outer membrane crosslink to Toc7517,18. Recombinant Toc75 has recently been shown to possess voltage-sensitive ion conductance in reconstituted proteoliposomes29. This conductance was selectively regulated by the presence of an authentic chloroplast preprotein, thereby providing convincing evidence for transit-sequence-regulated channel activity. Whether or not Toc75 is the only constituent of the protein-conducting channel remains debatable. The
Hsp70–IAP
Preprotein
224
Initial binding is mediated by Toc159 and Toc75. In a subsequent reaction, the preproteins insert across the outer membrane. ATP hydrolysis is strictly required to stabilize early import intermediates. The site of ATPase activity at the envelope has not been established, but the involvement of molecular chaperones is one likely explanation. Two chaperones of the Hsp70 family are associated with the outer envelope membrane, and both have been implicated in import. Com70, Tic20, Com60, and ADP+P

FIGURE 2

Model for the role of import components at each stage in the import of preproteins into chloroplasts. Cytoplasmic preproteins bind to the surface of the chloroplast through direct interaction of their intrinsic transit sequences with components of the Toc complex at the outer membrane (stage 1). Initial binding is mediated by Toc159 and Toc75. In a subsequent reaction, the preproteins insert across the outer membrane (stage 2). Toc75 comprises the major component of a protein-conducting channel in the Toc complex. ATP hydrolysis at Toc159 and/or Toc34 regulates access of preproteins to the protein-conducting channel, and ATP-driven chaperones at the outer membrane (Com70 and Hsp70–IAP) stabilize preprotein insertion through the Toc complex. At this stage in import, the Toc and Tic components associate at envelope contact sites. Preprotein insertion across the inner membrane is mediated, at least in part, by Tic20 and Tic22. Stromal chaperones (ClpC and Cpn60) that are docked at Tic110 bind to preproteins as they emerge from the inner membrane translocon, and translocation proceeds simultaneously across both envelope membranes (stage 3). IM, intermembrane space; OM, outer envelope membrane; IMS, intermembrane space; OM, outer envelope membrane; Tic, translocon at the inner envelope membrane of chloroplasts; Toc, translocon at the outer envelope membrane of chloroplasts. Con70

Transit
intrinsic transit sequences with components of the Toc complex at the outer membrane (stage 1).

Toc36

Stroma

Toc159

Tic110

Tic20

Toc34

Tic59

Tic22

ClpC

Cpn60

IM

OM

ATP

ADP

P

P

i

GDP+GTP+2P

ATP

ADP

P

P

i

224

Although GTP hydrolysis is required, it is not sufficient for efficient insertion of preproteins across the outer membrane. ATP hydrolysis is strictly required to stabilize early import intermediates. The site of ATPase activity at the envelope has not been established, but the involvement of molecular chaperones is one likely explanation. Two chaperones of the Hsp70 family are associated with the outer envelope membrane, and both have been implicated in import. Com70, Tic20, Com60, and ADP+P

The Tic apparatus

Three inner membrane proteins, Tic110, Tic22 and Tic34, associate with the outer membrane translocon in a Toc–Tic supercomplex to form functional import sites (Table 1). In contrast to the Toc complex, a stable complex consisting exclusively of Tic components has not been detected. Consequently, the assembly of a functional inner membrane translocon appears to be mediated by the direct or indirect association with the Toc complex. Tic20 and Tic22 have been identified as candidates for core components of the inner membrane translocon based on covalent crosslinking data. Tic22 appears to be the first inner membrane protein that associates with preproteins upon insertion across the outer membrane.

The role of import components at each stage in the import of preproteins into chloroplasts.

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ATP

ADP

P

P

i

GDP+GTP+2P

ATP

ADP

P

P

i

224
Tic20 is integrated deeply in the membrane, with limited exposure of its structure to the stroma or intermembrane space. The predicted topology of Tic20 makes it a candidate for a component of the protein-conducting machinery of the inner membrane translocon (Fig. 2, stage 3). A role in protein conductance is supported by the observation that crosslinking of preproteins to Tic20 increases at the later stages of protein import when the translocating chain has inserted across the inner envelope membrane42. Tic110 was the first inner membrane protein to be implicated in protein import by virtue of its association with early and late import intermediates. Tic110 is a 110-kDa integral membrane protein, with the bulk of its mass (≥90 kDa) protruding into the stromal compartment43. Tic110 co-immunoprecipitates with two stromal chaperones: the Hsp100 homologue, ClpC2,11, and the GroEL homologue, cpn6011. These observations have led to the hypothesis that Tic110 acts as a stromal chaperone docking site, thereby facilitating the translocation and folding of preproteins as they emerge from the Toc–Tic supercomplex43 (Fig. 2, stage 3). The association of Tic110 with ClpC is direct and is not strictly dependent on the presence or absence of contact sites39. On the other hand, cpn60 interacts exclusively with the Tic–Tic supercomplex at functional import sites, and it is not clear whether its association with Tic110 is direct or indirect39. An additional inner membrane protein, Tic55, was shown to cofracionate with Tic110 on blue native gel electrophoresis43. Tic55 contains an iron–sulphur binding motif that is characteristic of redox proteins, but there is no evidence that it participates in import.

Envelope contact sites

For years, investigators noted, by electron microscopy, the existence of distinct envelope subdomains where the outer and inner membranes are in close contact. More recently, immunoelectron microscopy studies have shown that import intermediates are localized to these contact zones31 (Fig. 1), thereby supporting the proposal that they represent the sites of protein import (Fig. 2). It now appears that the Toc–Tic supercomplex that has been isolated biochemically corresponds to the envelope contact sites that have been defined morphologically. The stable physical association between the Toc and Tic apparatus and the fact that import intermediates are associated quantitatively with this Toc–Tic supercomplex provide strong support for this hypothesis1,3. In mitochondria, the formation of contact sites between the preprotein translocases in the outer and inner membrane appears to be mediated directly by the translocating preprotein44. By contrast, the presence or absence of envelope-bound preprotein does not detectably alter the amount or pattern of proteins in the Toc–Tic supercomplex. An interesting challenge for the future will be to define which of the Toc and Tic components interact directly in the supercomplex and are thus responsible for contact-site formation. Of equal interest is the mechanism that regulates the association/dissociation of the outer and inner membrane translocons. Mitochondrial contact sites are dynamic44, and several observations suggest that the Toc–Tic association also is regulated. Covalent crosslinking studies indicate that the initial binding of preproteins can occur at Toc complexes that are not associated with Tic components39, and there is evidence that import can proceed through Toc and Tic complexes independently when contact sites are disrupted by hypertonic treatments45. Furthermore, major fractions of Toc and Tic components are not stably associated in contact sites. It is intriguing to speculate that preprotein binding at the Toc complex might stimulate a GTP- or ATP-dependent switch that leads to contact site formation.

Developmental regulation of the import apparatus

Chloroplasts represent one member of a diverse group of essential organelles that are referred to collectively as the plastids. All plastid types develop...
from a precursor organelle, the proplastid, in response to the particular metabolic demands of the differentiated cell type in which they function. Despite the diversity in plastid morphology and function, the study of protein import has been restricted largely to chloroplasts. For the most part, the import apparatus was viewed as performing a housekeeping function for the cell, and consequently the regulation of import was not predicted to play a crucial role in plastid development. This viewpoint has been revised by the recent discovery that the composition of the import apparatus might be regulated differentially in response to specific developmental changes. These data raise the exciting possibility that regulation of the import apparatus plays an active role in plastid differentiation.

The concept of a single import apparatus was reinforced by the observations that Toc75 is expressed in all tissues of pea seedlings and that preproteins specific to a variety of plastid types are imported into isolated chloroplasts. However, the recent discovery of two differentially expressed Toc34-like genes in *Arabidopsis* confirms that the composition of the import apparatus varies during plastid development. The protein products of these genes, atToc33 and atToc34, exhibit 59% and 64% amino acid sequence identity to the original Toc34 isolate from pea, respectively, and 63% identity to one another. The atToc33 is expressed at high levels during the early stages of chloroplast development from proplastids, whereas atToc34 is expressed at constitutively low levels during all stages of leaf development. Mutant plants deficient in atToc33 are defective in protein import. However, the defect is only partial, implying a certain degree of functional redundancy between the two members of the Toc34 family. These observations led to the proposal that plastids possess Toc complexes of distinct composition and different, but overlapping, preprotein specificity. Variation in the expression of distinct Toc complexes could be an important element in regulating the rate or specificity of protein import during plastid development. In fact, differences in the efficiency of import have been observed among different plastid types, supporting the hypothesis of variability in the import apparatus. These results raise the possibility that the central protein-conducting machinery of the import apparatus, containing Toc75, is conserved, whereas the regulatory elements, such as Toc34, might vary in response to developmental signals.

**Concluding remarks**

The basic framework for the mechanism of protein import into chloroplasts is now in place. The emerging picture is of a dynamic set of outer and inner membrane translocon complexes that associate physically to facilitate import. We now have a reasonable concept of the stages of import, and a major focus in the future must be to demonstrate directly and define the molecular interactions that take place over the course of the import reaction. Although Toc159 and Toc75 clearly participate in preprotein recognition, direct binding of the transit sequence to either component remains to be demonstrated. Likewise, identifying the binding partners responsible for the Toc–Tic supercomplex is essential for determining the structure and regulation of contact site formation. Biochemical reconstitution of the import apparatus should also provide methods for elucidating the role of the GTPases in regulating preprotein recognition.

One of the most surprising revelations from the identification of Toc and Tic proteins is the lack of similarity between the chloroplast and mitochondrial import components. Both systems adhere to the general principles of translocating components of a stepwise assembly system that regulates the presentation of preproteins to a system of linked outer and inner membrane protein-conducting channels, yet the Toc and Tic components have no apparent sequence similarity to their Tom and Tim counterparts in mitochondria. Thus, endosymbiotic evolution appears to have resulted in the acquisition of mitochondrial and chloroplast import machineries from distinct origins. What is the evolutionary origin of the chloroplast import machinery? Genes encoding proteins with similar size and significant sequence similarity to Toc75, Tic20 and Tic22 (D. Schnell, unpublished) are found in the genome of *Synechocystis* sp., the photosynthetic cyanobacterium species that presumably resembles the original endosymbiont. The functions of the cyanobacterial proteins are unknown, but the similarities suggest that parts of the chloroplast protein-import machinery might have been adapted from existing bacterial membrane proteins. In fact, sequence comparisons of the cyanobacterial Toc75 homologue, SynToc75, to sequence databases indicates that this protein is structurally related to a family of prokaryotic secretion channels involved in the transport of virulence factors across the outer membrane. Moreover, SynToc75 exhibits ion-channel activity that is consistent with a function in membrane transport. The identification of potential bacterial homologues for some translocon components provides a powerful system to investigate the evolution and potential functions of the import apparatus. For example, the gene encoding SynToc75 is essential, raising the possibility of genetic complementation studies to explore the role of the homologous proteins in membrane transport.

Equally exciting is the emerging application of *Arabidopsis* as an in vivo model system for investigating the import mechanism. This system should be particularly valuable in identifying and characterizing components of the import apparatus that are not readily accessible to biochemical manipulation. Biochemical strategies to investigate the protein-import process in other plastid types have been difficult because of the challenges faced in isolating these fragile organelles. The in vivo molecular-genetic system might provide new insights into the exciting and somewhat unexpected role of protein import in plastid differentiation and the regulation of protein trafficking to plastids in different cell types.
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This antibody (p85-Fab) has been identified as a substrate for the caspase group of enzymes, which are known to be integral components of programmed cell death (apoptosis). Caspases cleave PARP into 116-kDa and 85-kDa fragments, and the presence of these two products in the cell is a classical hallmark of apoptosis. The new Anti-PARP p85 Fragment pAb from Promega is the first of an impressive series of antibodies that can completely distinguish the cleaved fragments of PARP from the uncleaved holoenzyme.

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