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Chloroplast biogenesis: diversity and regulation of the protein import apparatus

Felix Kessler[1] and Danny Schnell[2]

[1] Laboratoire de Physiologie Végétale, Université de Neuchâtel, Rue Emile-Argand 11, 2007 Neuchâtel, Switzerland

[2] Department of Biochemistry and Molecular Biology and Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, MA 01003, USA

Corresponding author: Schnell, Danny (dschnell@biochem.umass.edu)

The biogenesis of chloroplasts is dependent on the coordinate expression of genes encoded in both nuclear and plastid genomes. The chloroplast protein import machinery plays key roles in organelle biogenesis by mediating the import and assembly of thousands of nuclear-encoded proteins into the organelle. It is now apparent that multiple levels of control exist to integrate the activities of the protein import apparatus into the network of chloroplast-nuclear communication that is essential to maintain organelle homeostasis. The import apparatus has diversified into small, functionally specialized gene families to coordinate the import of distinct classes of differentially expressed proteins. Protein targeting to chloroplasts also has evolved regulatory mechanisms that respond to cellular developmental and physiological changes, including redox sensing, phosphorylation, and dual targeting. Recent studies also have revealed new components that could represent additional levels of control on the import process.

Introduction

Chloroplasts evolved from a photosynthetic bacterial symbiont [1] to participate in numerous essential metabolic and cellular processes in plants and algae, including photosynthesis, amino acid, and lipid metabolism, cell signaling, and host defense. As single-celled algae evolved into multicellular organisms, chloroplasts concomitantly evolved specialized functions in different tissues and cell types, giving rise to a diverse group of interrelated organelles called plastids [2,3]. In Arabidopsis, plastids rely on the import of ~3000 different nucleus-encoded proteins from the cytoplasm [4]. Three decades of research have unraveled complex communication networks that couple and integrate the physiological and biogenetic status of the chloroplast with nuclear gene expression [5,6]. More recent studies implicate the plastid protein import apparatus itself as a key regulatory entity in mediating plastid–nuclear interactions. As such, the protein import machinery plays a role in a network of processes that (1) control the overall levels of nucleus-encoded plastid proteins synthesized, (2) maintain the stoichiometry of multi-protein complexes that contain both plastid and nucleus-encoded subunits, (3) respond to organelle status or dysfunction (e.g. photosynthetic activity or organelle stress) and, (4) coordinate changes in protein profiles during developmental transitions from one plastid type to another. Several recent comprehensive reviews cover molecular mechanisms of protein import [7–9], and here we will focus on its control and integration with gene expression.

Overview of protein trafficking to chloroplast/plastids

The major pathway for protein import, in terms of sheer volume, is mediated by the TOC and TIC translocons (translocons at the outer and inner membrane of chloroplasts, respectively) [4,7]. The TOC-TIC pathway also represents the first step in the targeting of the majority of nucleus-encoded proteins to the thylakoid membrane and inner envelope membrane. TOC-TIC substrates are distinguished by the presence of an N-terminal targeting sequence, designated the transit peptide [10]. Although more than a dozen proteins are implicated in TOC-TIC function, experimental evidence, and the analysis of available algal and plant genomes identify a core subset of these components that are required for import *in vivo* [11,12,13••,14,15] and are conserved across the phylogenetic spectrum of the plant kingdom [16,17•].

The core TOC translocon consists of a β-barrel membrane channel (Toc75) that forms a stable complex with two membrane-bound GTPases, Toc34 and Toc159 [18,19]. The TOC GTPases represent the primary receptors for transit peptides at the chloroplast surface, and evidence supports roles for their intrinsic GTPase activities in controlling access of preproteins to the channel [20]. Once transferred from the receptors to Toc75, the preprotein translocates across the outer membrane in an ATP-dependent reaction that appears to involve an Hsp70-type molecular chaperone in the intermembrane space [21•].

The core TIC translocon physically associates with the TOC complex and preprotein translocation proceeds

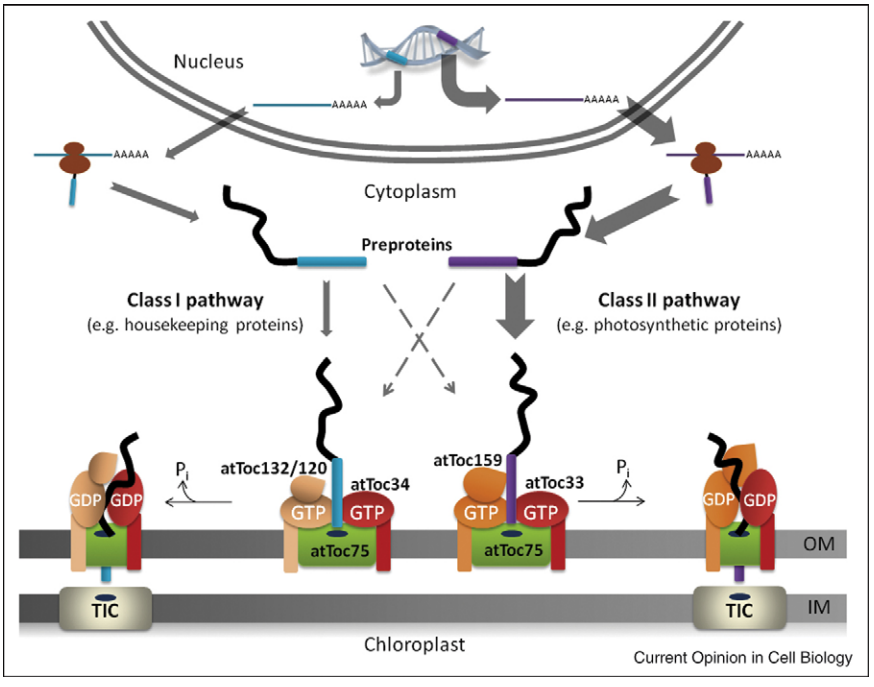
across the outer and inner envelope membranes via their linked channels. Although the precise nature of the TIC channel remains a matter of discussion, three conserved multi-spanning membrane proteins are implicated in inner membrane translocation (Tic110, Tic20, and Tic21) [22–24]. Tic110 also contains a binding site for the stromal chaperone, Hsp93, or ClpC [25], and the chaperone is proposed to bind to preproteins and provide the driving force for translocation through the TIC channel [26]. This appears to account for the additional ATP requirement for inner membrane translocation. The coordination of Tic110-Hsp93 activities appears to be mediated by Tic40, an inner membrane protein, via its intrinsic TPR and Hip/Hop domains [27].

Regulation at the TOC GTPase receptors

As with other membrane translocation systems, preprotein recognition and the initiation of membrane translocation at the TOC complex are the committed steps in the import process. Consistent with their roles as gatekeepers, the TOC GTPase receptors represent key functional and regulatory points in the pathway. All preproteins initially bind at the TOC receptors before translocation through the TOC channel. Interestingly, protein import in isolated chloroplasts can be driven by the internal ATP generated in the light via photosynthetic electron transport [21•]. A role for GTP in the import process is revealed only with the addition of non-hydrolyzable GTP analogs, which inhibit preprotein binding and translocation [21•]. This suggests an important role for TOC GTPase activities in regulating membrane transport. For the purposes of our discussion, we will consider the TOC GTPases as a functional regulatory unit and not concentrate on parsing the detailed mechanics of their individual activities. Although Toc34 and Toc159 both appear to be required *in vivo*, recent studies suggest that their GTPase activities might be partially overlapping [28,29••].

The foremost regulatory function of the TOC receptors appears to be as gatekeepers to the translocon channel (Figure 1) [28,29••]. Both receptors bind transit peptides and are in contact with preproteins at the initial stage of preprotein binding to the translocon. Transit peptide binding stimulates the GTPase activity of both receptors [30], and therefore has been proposed to control the GTP switch that opens the gate to the translocon and allows membrane translocation to proceed. The TOC receptors also interact directly with one another via their GTPase domains [31,32••], leading to the hypothesis that GTP-

Figure 1



The role of the TOC GTPase receptor families as gatekeepers to the TOC translocon. Members of the Toc159 (atToc159 and atToc132/120) and Toc34 (atToc33 and atToc34) preprotein receptor families in Arabidopsis mediate the recognition of nucleus-encoded preproteins by binding to preprotein transit peptides at the outer envelope membrane (OM) [20]. Preprotein binding is hypothesized to stimulate receptor GTPase activity [30], thereby controlling the molecular switch (changes in receptor dimerization) that provides preprotein access to the translocon channel (atToc75). The Toc159 and Toc34 family members assemble in combination to form distinct translocons with the Toc75 channel (atToc75). The different TOC receptor isoforms mediate the recognition of distinct classes of nucleus-encoded preproteins to maintain the proper levels of functional classes of proteins that are required for the biogenesis and homeostasis of the organelle [13••,36].

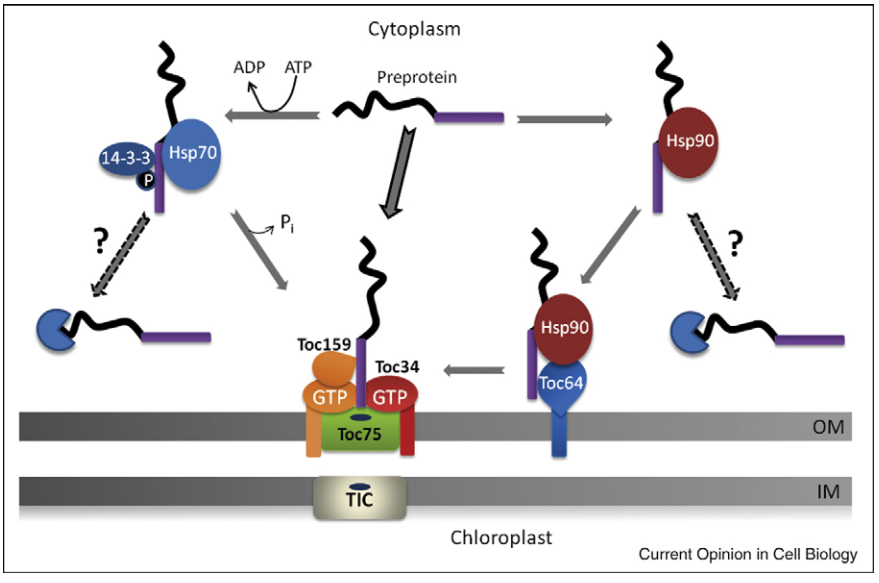
regulated TOC receptor interactions represent a gate across the translocon [33,34]. Although other modulators might exist (i.e. GAP or GEF proteins) and the stoichiometry of GTP hydrolytic cycles and preprotein translocation is unknown, the role of the transit peptide in controlling the GTPases is mechanistically satisfying and provides a link between recognition of the transit peptide and opening the translocon gate [30,35].

A second dimension to the TOC GTPase switch function has been revealed by the biochemical and molecular genetic analysis of the small multi-gene families encoding Toc159 and Toc34 receptors in Arabidopsis. These studies demonstrate that the receptors not only specifically recognize transit peptides but also discriminate between different types of transit peptides [36,37] (Figure 1). The size of the Toc34 family varies from one to two in higher plant species [16]. Individual null mutants of the two Toc34 genes in Arabidopsis (atToc33 and atToc34) exhibit distinct phenotypes [11]. However, this is probably due to differential expression of the two genes, and their functions appear to be largely redundant [11]. In higher plants, the Toc159 gene family consists of at least three or four genes [16]. In contrast to the Toc34-like genes, null mutants of individual atToc159 family members exhibit more pronounced differential effects on plastid biogenesis [13••,36]. At least three of the Arabidopsis receptors, atToc159, atToc132, and atToc120, bind distinct classes of preproteins. Furthermore, atToc159 mutants preferentially disrupt chloroplast biogenesis, whereas atToc120/132 mutants exhibit more general effects on plastid function. This leads to a novel hypothesis that includes not only the role of the Toc receptors in regulating translocon access but also their role in defining distinct import pathways. The Toc159 and Toc34 family members are envisioned to assemble in various combinations to generate translocons with distinct selectivities that would thereby control the type of cargo that gains access to the organelle [7,8]. This additional selectivity would be key to maintaining organelle homeostasis during development. In particular, it allows flux of preproteins within a specific class (e.g. photosynthetic proteins) to be controlled independent of other classes (e.g. housekeeping proteins), thereby avoiding competition at import sites (Figure 1). As such, the TOC receptors provide an additional level of control that balances protein import with the changes in transcriptional profiles that accompany physiological and developmental changes during plastid biogenesis. It will be important to more fully explore the mechanism of GTPase function and determine more complete substrate profiles of the receptors to more clearly define the roles of the distinct pathways in organelle development.

Regulation via cytosolic factors

Two complexes have been proposed to assist in targeting cytoplasmic preproteins to the TOC GTPase receptors (Figure 2). The 'guidance complex' consists of a 14-3-3

Figure 2



The potential role of cytosolic factors in targeting preproteins to the TOC complex. A cytosolic guidance complex (14-3-3 protein and cytosolic Hsp70) [38,39] and an Hsp90 complex (Hsp90 and Toc64) [40,41] are proposed to stimulate the delivery of preproteins to the TOC receptors. Association of the guidance complex with preproteins is controlled by transit peptide phosphorylation. Cytosolic Hsp90 is proposed to bind preproteins and deliver them to Toc64 at the chloroplast surface for subsequent transfer to the TOC translocon. The activities of neither complex are essential in Arabidopsis [42–44] suggesting that they play specialized roles in the targeting of specific preproteins. Alternatively, these factors could function as parts of a cytosolic quality control system (broken arrows) to avoid the accumulation of preproteins in the cytoplasm if the preprotein is damaged or the levels of translation exceed the capacity of the import apparatus.

protein and a cytoplasmic Hsp70 chaperone [38]. This complex increased the efficiency of protein import *in vitro*. Recognition of preproteins by the guidance complex centers on potential serine/threonine phosphorylation sites within transit peptides, suggesting that this interaction is controlled by a phosphorylation/dephosphorylation cycle [39]. The second cytoplasmic targeting complex involves cytoplasmic Hsp90 and a putative chloroplast outer membrane receptor, Toc64 [40,41]. Toc64 is proposed to function as an alternative preprotein receptor for preproteins bound to Hsp90. Both targeting complexes are proposed to subsequently deliver preproteins to the TOC complex for translocation.

Interestingly, neither the guidance complex nor Hsp90 complex appear to be essential for protein import *in vivo*. Many transit peptides lack the phosphorylation sites for guidance complex binding, and mutations in the sites in potential guidance complex substrates do not affect protein import *in vivo* [42]. Insertional mutants in the Toc64 genes in Arabidopsis and moss (*Physcomitrella patens*) exhibit no detectable physiological or protein import defects [43,44]. These observations suggest that their primary roles might be to assist the targeting of specific proteins, for example, highly abundant preproteins, such as the small subunit of rubisco. Although speculative, the guidance complex and Hsp90 complex could play roles in targeting to the specific pathways represented by different TOC GTPases (Figure 1) or participate in quality control systems that monitor these pathways (Figure 2). The activity of the Hsp90 complex is reminiscent of the role of members of the Hsp90 family in quality control systems that monitor protein levels or protein damage (e.g. misfolding) in other cellular compartments [45]. The proposed phosphorylation cycle regulating guidance complex binding also is consistent with a monitoring function. These systems could represent a mechanism to avoid the accumulation of newly synthesized preproteins in the cytoplasm under conditions when plastid integrity is compromised or when the levels of protein synthesis exceed the capacity of protein import.

Regulation at the level of dual targeting

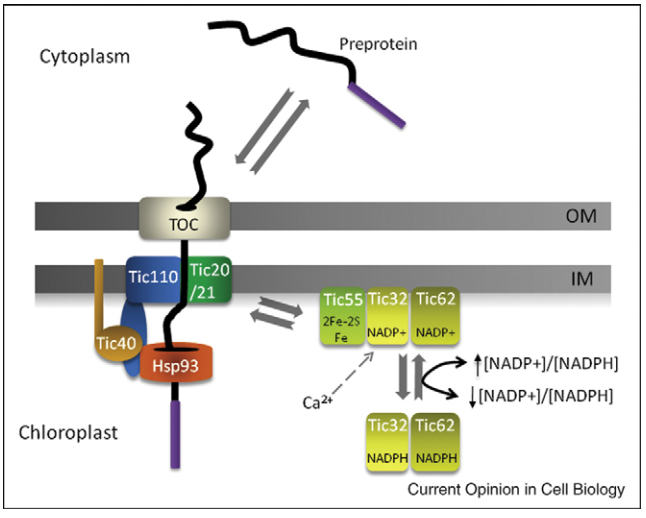
Dozens of different proteins have been shown to exhibit dual targeting to chloroplasts and mitochondria, and there have also been reports of dual targeting between chloroplasts and the nucleus, peroxisomes and the ER [46]. Many dual targeted proteins are those involved in shared processes within the different organelles, including transcription, translation, protein turnover, and shared metabolic or enzymatic activities. Dual targeting provides a mechanism for single genes to encode conserved functions within different organelles and is proposed to constitute a mechanism of inter-organellar regulation that coordinates these functions within the two organelles [46].

Dual targeting has been best characterized for chloroplasts and mitochondria and is achieved by a number of mechanisms. These include alternative mRNA splicing to generate a mitochondrial presequence or a chloroplast transit peptide, ambiguous (dual) targeting signals that target to both organelles, or the selection of alternative translational start sites [47,48,49•]. Although the mechanisms of regulating dual targeting have not been examined in detail, the distribution of these proteins between organelles appears to be closely monitored. General mechanisms of regulating alternative splicing are well known, and might apply to the generation of alternative targeting signals by this mechanism. Much less is known about the control of distribution of proteins carrying dual targeting signals. However, there are reports that the degree of dual targeting of specific proteins is cell type specific, indicating that the distribution is tightly regulated [50]. It also has been noted that many dual targeting signals contain potential phosphorylation sites, and this modification could regulate the interaction of the targeting signal with cytosolic factors or receptors at the organelle surface [46].

Regulation in response to organelle metabolic status

Redox regulation plays a key role in the biogenesis and metabolic regulation of chloroplasts [51]. More recently, the role of redox regulation in plastid function has been

Figure 3



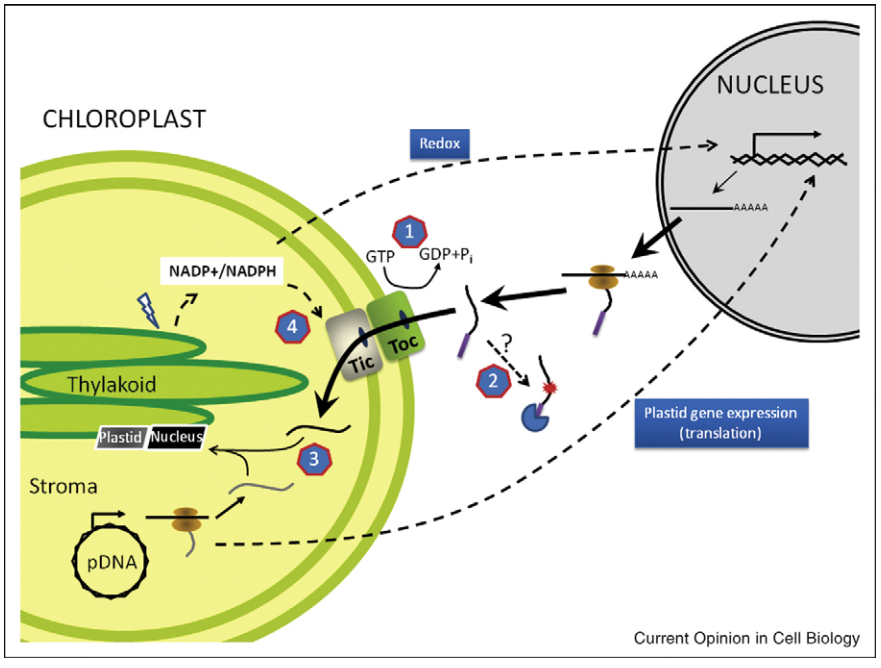
Potential redox control of protein import. Three membrane associated redox proteins (Tic55, Tic32, and Tic62) have been shown to associate with the Tic translocon [54,55]. The association of Tic62 and Tic32 with the inner envelope membrane appears to be controlled by their redox state [56•]. All three proteins contain redox cofactors, leading to the hypothesis that they modulate protein import in response to the redox state (i.e. photosynthetic activity) of the chloroplast. Tic32 also contains a potential calmodulin binding site [55]. Redox control would couple the import of photosynthetic preproteins with the photosynthetic activity of the organelle.

extended to the protein import apparatus (Figure 3). The import of two chloroplast redox proteins, ferredoxin FdIII and ferredoxin-NADP-oxidoreductase II, has been shown to be regulated in response to light [52,53]. This suggests that the import apparatus responds directly to the physiological state of the organelle, in particular, the level of photosynthesis as indicated by redox state. Three inner envelope proteins carrying redox cofactors, Tic32, Tic62, and Tic55, are associated with the TIC translocon [54,55], and evidence suggests that the association of Tic62 and Tic32 with the inner envelope is regulated by the NADP/NADPH ratio within the organelle [56•]. Although there is no direct evidence that Tic32, Tic62, and Tic55 modulate TIC activity or are involved in the regulation of ferredoxin FdIII or ferredoxin oxidoreductase II import, their dynamic association with the translocon provides a potential mechanism whereby protein import is modulated in response to the physiological status of the organelle. Redox is known to regulate aspects of mitochondrial protein import, suggesting a similar system for regulating trafficking in energy generating organelles [57].

Conclusions and perspective

Import through the TOC-TIC translocons is a common step in the expression, targeting, and assembly of thousands of distinct nucleus-encoded plastid proteins. A comparison of plastid to nucleus signaling pathways with the numerous potential control points in the import process begins to identify sites of regulatory integration between the processes (Figure 4). The overall levels and functional classes of proteins that accumulate within the organelle appear to be regulated not only at the level of nuclear transcription but also by a diverse set of import translocons that control the flux of these protein classes. This balance may be accompanied by monitoring systems in the cytoplasm (e.g. soluble factors) that ensure that excess or damaged preproteins are degraded and not targeted to the TOC-TIC system. The control of preprotein targeting at both of these levels would complement the signaling and control systems that regulate the translation of plastid proteins and influence nuclear gene expression [58], thereby ensuring the proper stoichiometry of proteins containing subunits encoded by both genomes. Redox also provides a mechanism of integrating chloroplast metabolic activity with gene expression and import. Numerous photosynthetic processes are regulated directly in response to the bioenergetic status of the organelle as reflected in the redox potential of the stroma. Redox signals also feedback on nuclear transcription to control the expression of a variety

Figure 4



Potential sites for integration of protein import into the known pathways of chloroplast nuclear signaling. (1) Preprotein import is controlled by the intrinsic GTPase activity of the TOC receptors. Distinct isoforms of TOC receptors provide a mechanism to modulate the levels and types of preproteins that accumulate within the organelle in response to physiological and developmental changes in gene expression [3]. (2) Cytosolic factors [38–41] could monitor the balance between the levels of preprotein translation and the capacity of the import apparatus to avoid the accumulation of preproteins in the cytosol. (3) The proper stoichiometry of proteins containing subunits encoded by both plastid and nuclear genomes would be maintained by the rate of preprotein import and signals from the chloroplast to the nucleus that report on the levels of plastid-encoded subunits [58]. (4) The import apparatus can be directly modulated by the redox state of the chloroplast [52,53]. Redox signals also control the levels of expression of specific nuclear genes [5,6]. This combined regulatory mechanism could couple the levels of preprotein expression directly to the activity of the import apparatus.

of plastid proteins [5,6]. The potential control of import via redox sensitive TIC components provides a potential mechanism to integrate metabolic status, gene expression, and protein import. The variety of known and potential regulatory sites of protein targeting to plastids was unimaginable when the import apparatus was initially envisioned as a housekeeping function for organelle biogenesis and maintenance. Research in the field will undoubtedly expand from basic mechanistic studies to include investigations of the extent of regulation and its role in plastid biogenesis and development.

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