

How do endosymbionts become organelles? Understanding early events in plastid evolution

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Summary

What factors drove the transformation of the cyanobacterial progenitor of plastids (e.g. chloroplasts) from endosymbiont to bona fide organelle? This question lies at the heart of organelle genesis because, whereas intracellular endosymbionts are widespread in both unicellular and multicellular eukaryotes (e.g. rhizobial bacteria, *Chlorella* cells in ciliates, *Buchnera* in aphids), only two canonical eukaryotic organelles of endosymbiotic origin are recognized, the plastids of algae and plants and the mitochondrion. Emerging data on (1) the discovery of non-canonical plastid protein targeting, (2) the recent origin of a cyanobacterial-derived organelle in the filose amoeba *Paulinella chromatophora*, and (3) the extraordinarily reduced genomes of psyllid bacterial endosymbionts begin to blur the distinction between endosymbiont and organelle. Here we discuss the use of these terms in light of new data in order to highlight the unique aspects of plastids and mitochondria and underscore their central role in eukaryotic evolution. *BioEssays* 29:1239–1246, 2007. © 2007 Wiley Periodicals, Inc.

Introduction

A hallmark feature of eukaryotic cells is the presence of *organelles*, discrete subcellular structures of specialized function usually bound by two or more membranes. Mitochondria and plastids are the classic examples of organelles, although the term is sometimes used in reference to other cellular structures such as the Golgi apparatus, flagellar basal bodies and centrioles. Mitochondria and plastids are, however, unique in that they evolved from free-living prokaryotes through the process of *endosymbiosis*. Given this fundamental distinction, here we use organelle to refer only to permanent cellular inclusions of exogenous origin. How many times have such organelles evolved during eukaryotic evolution? More specifically, what is known about the molecular and cellular events that accompanied the transformation of the prokaryotic ancestors of modern-day mitochondria and plastids from endosymbionts to organelles?

The existence of a protein import apparatus is often touted as *the* defining feature of an organelle of endosymbiotic origin.^(1,2) The mitochondria and plastids of extant eukaryotes are highly derived entities, with most of the genetic material present in their prokaryotic ancestors having been lost or transferred to the host cell nucleus. The mitochondrion predates the plastid and therefore was the first to evolve a sophisticated protein machinery for targeting nucleus-encoded gene products back to this compartment.⁽³⁾ Later the first algae independently developed a protein import apparatus to service the plastid (see below). Without protein import, the argument goes, gene loss and endosymbiotic gene transfer alone are insufficient to permanently cement the connection between endosymbiont and host, and for the transition from endosymbiont to organelle to be complete. Evolutionary analyses indicate that the protein import machineries of mitochondria and plastids, the TIM–TOM (translocators of the inner and outer mitochondrion membrane) and TIC–TOC (translocators of the inner and outer chloroplast membrane) complexes are mosaics of both endosymbiont- and host- cell-derived proteins,^(4,5) suggesting that both cellular partners were active players in organelle establishment. In the case of plastids, was the evolution of the TIC–TOC translocon *the* defining feature of organelle genesis

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or could plastids have initially used alternate import pathways that were critical for rendering permanent the endosymbiont—host cell relationship? Here we investigate this issue by reviewing and synthesizing recent literature on plastid protein import and explore the possibility that the evolution of the TIC–TOC import system is an *outcome of*—and not the *reason for*—plastid establishment.

Plastid origins: pattern and process

Unlike the origin of mitochondria,^(6–9) the basic characteristics of the cellular partners involved in the endosymbiotic origin of plastids are generally agreed upon. Early in the evolution of eukaryotes (but after mitochondria evolved), a heterotrophic eukaryote with a highly developed endomembrane system engulfed a cyanobacterium and retained it in its cytoplasm. Over time, the prokaryotic endosymbiont surrendered its autonomy and became the light-harvesting, carbon dioxide-fixing, oxygen-producing organelle present in modern-day eukaryotic photosynthesizers. The number of times this occurred is actively debated. Three eukaryotic lineages, the Glaucophyta (glaucophyte algae), Rhodophyta (red algae) and Viridiplantae (green algae and land plants), possess double-membrane-bound plastids of “primary” endosymbiotic origin^(10–13) but it is not known with certainty whether the plastids in these groups evolved once or multiple times. The answer to this question has a fundamental impact on our understanding of eukaryotic evolution.

On the one hand, if photosynthesis evolved in the Glaucophyta, Rhodophyta and Viridiplantae on three separate occasions, this would suggest that harnessing a cyanobacterium as a plastid is, in evolutionary terms, relatively straightforward. If, on the other hand, the plastids of these groups evolved only once in their common ancestor, then all autotrophic eukaryotes trace their ancestry to a serendipitous evolutionary event that ultimately gave rise to a vast assemblage of organisms that are one of the dominant groups on our planet and a driving force behind its climate, geochemistry, and ecology. Although not yet definitively proven, the majority of evidence suggests that primary plastids evolved only once in a common ancestor of the Glaucophyta, Rhodophyta and Viridiplantae and all plastids trace their ancestry (directly or indirectly) to this singular event (Fig. 1).^(7,11,14–20) Historically referred to as the Plantae, this assemblage is now also known as the Archaeplastida⁽²¹⁾ to recognize its position as the ancestral plastid-bearing eukaryotic lineage. Once these monumental innovations had occurred in the Plantae, the plastid and all of the nuclear genes that maintain it were subsequently passed on piecemeal (with subsequent modifications) to other eukaryotes through secondary (algal) endosymbioses (e.g. as in the chromalveolates, see Fig. 1).⁽¹⁵⁾

Several key steps were required however to bring the primary endosymbiotic origin of plastids to fruition. One of these was the establishment of a reliable connection between

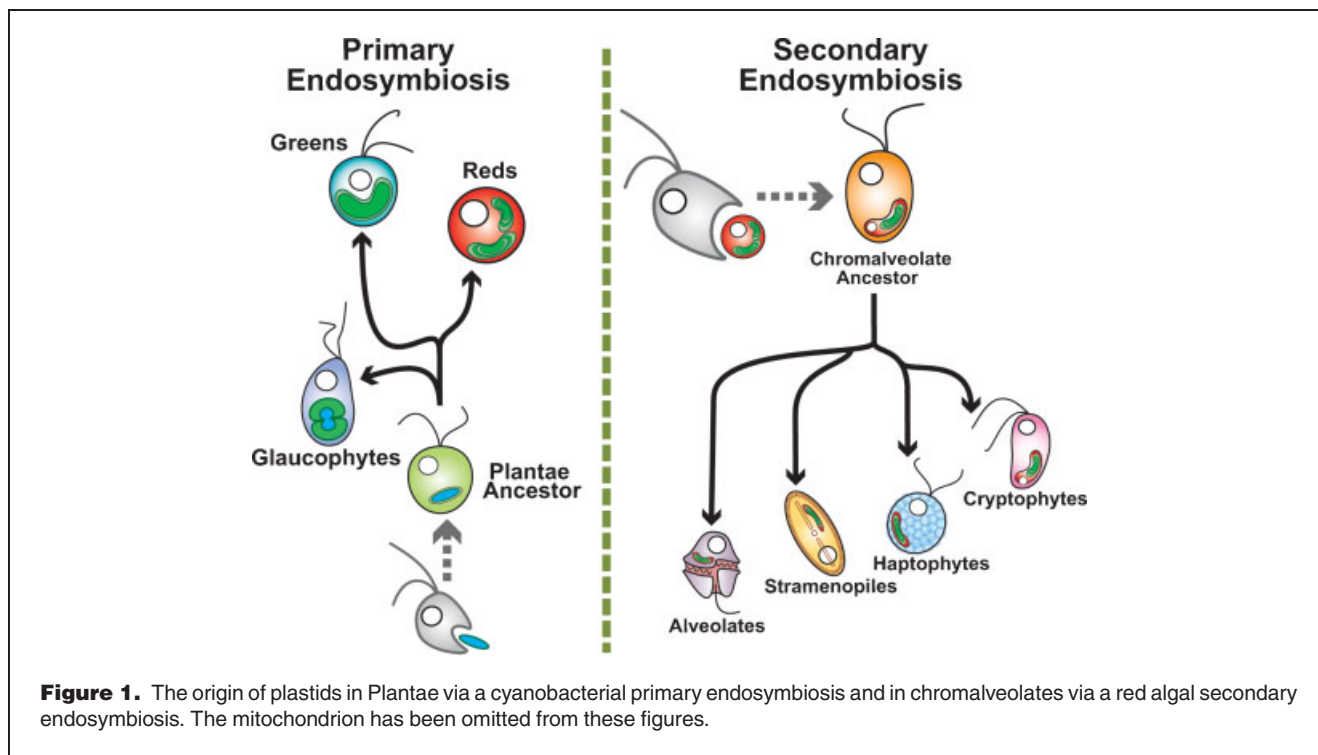


Figure 1. The origin of plastids in Plantae via a cyanobacterial primary endosymbiosis and in chromalveolates via a red algal secondary endosymbiosis. The mitochondrion has been omitted from these figures.

the host cell and endosymbiont allowing the controlled exchange of metabolic intermediates between the symbiotic partners. Metabolite antiporters are ideally suited for this purpose because the antiport function is dependent on the presence of a suitable counter-exchange substrate on the *trans*-site of the membrane. It was recently shown that several key plastid metabolite translocators (e.g. the phosphate translocator family) were established in the common ancestor of the red and green algae (and likely all members of the Plantae), allowing the first algae to profit from cyanobacterial carbon fixation.⁽²²⁾ This evolutionary step may have rendered irreversible the association between the incipient plastid and the host cell. The topology of the plastid phosphate/carbon antiporter tree suggests that the ancestral gene evolved from an existing endoplasmic reticulum (ER)/Golgi metabolite translocator protein family in the host cell that was recruited to the plastid envelope membrane to connect host and endosymbiont metabolism.⁽²²⁾ This is consistent with the idea that a well-developed endomembrane system was already present in the mitochondrial Plantae ancestor.

Another critical step in plastid establishment was the genetic integration of the endosymbiont and host. Assuming they each possessed a full suite of genes, two organisms living in close association could, in principle, live apart. However, if essential endosymbiont genes were transferred to the nucleus of the host and lost from its own genome, the endosymbiont would perish unless the transferred genes were expressed and their corresponding proteins somehow directed back to the endosymbiont. Both of these processes—endosymbiotic gene transfer and protein re-targeting—form the foundation of current models for organelle evolution and for good reason. Most free-living cyanobacteria have genomes ~2,000–4,000 kilobases (Kb) in size (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>), whereas plastid genomes are rarely >200 Kb and generally possess at less than 200 genes. The majority of the genes present in the cyanobacterial progenitor of plastids have been lost or transferred to the nucleus and the genes that remain are primarily involved in photosynthesis, transcription and translation.⁽²³⁾ Plastid-encoded genes are presumably under selection for transfer to the nucleus to avoid the consequences of “Muller’s ratchet”, the accumulation of deleterious mutations that occur in non-recombining genomes of small population size.⁽²⁴⁾ In light of Muller’s ratchet and the higher mutation rate caused by oxygen free-radicals in organelles,⁽²⁵⁾ it is surprising that so many genes are still encoded in plastid and mitochondrial DNA and, indeed, that organelles retain a genome at all. Discussion of various models for why some genes have not or cannot be transferred to the nucleus is beyond the scope of this review,^(26,27) but suffice it to say that endosymbiotic gene transfer and protein re-targeting are major hurdles that were overcome in the early stages of organelle establishment.

Plastid-containing eukaryotes have evolved a sophisticated system for targeting and importing the products of scores of endosymbiont-derived genes to the plastid. As will be elaborated upon below, plastid proteins synthesized on cytosolic ribosomes are targeted to the plastid with N-terminal targeting peptides and pulled across the two membranes through channels formed by the TOC and TIC protein complexes.⁽²⁸⁾ The conservation of many aspects of the plastid import machinery in diverse members of the Plantae supports the hypothesis of a single origin of plastids in their common ancestor.^(10,29) However, whereas the TIC–TOC import machinery is undoubtedly an important characteristic of present-day plastids, it does not necessarily follow that the evolution of this machinery was the defining event in plastid evolution, or that all photosynthetic organelles in eukaryotes must possess this feature. The enigmatic unicellular eukaryote *Paulinella chromatophora* is a possible case in point.

P. chromatophora is an autotrophic filose amoeba that has been known to science for more than 100 years⁽³⁰⁾ but whose potential to shed light on the origin of eukaryotic photosynthesis has only recently become dramatically clear. The organism harbors two kidney-shaped photosynthetic bodies that appear much more similar to cyanobacteria than to canonical plastids,⁽³¹⁾ a similarity that has been born out by partial genome sequencing. Remarkably, the *P. chromatophora* organelles appear to have been acquired much more recently than the plastids of all other eukaryotes through the uptake of a *Synechococcus*-type cyanobacterium.^(32,33) Some have questioned whether these cyanobacterial-derived bodies should even be called organelles,⁽²⁾ but the fact that they divide synchronously with, and cannot be cultured outside of, the host eukaryote suggests at least a minimum level of genetic and biochemical integration.^(33,34) Detailed study of *P. chromatophora* and its newly acquired “organelle” is in its very early stages, but promises to be tremendously insightful. In particular, an important question is whether a protein import apparatus exists in *P. chromatophora* and, if so, in what form. As we shall see below, detailed consideration of standard and non-standard mechanisms for protein import in canonical eukaryotic phototrophs challenge the notion that TIC–TOC-based protein import came first. Rather, evidence suggests that the initial system for protein trafficking to the endosymbiont was likely the existing endomembrane system of the eukaryotic host cell.

The canonical plastid protein import apparatus

The best-known plastid protein import system depends on the TIC–TOC multimeric protein complexes.⁽³⁵⁾ Comprehensive studies of this system in land plant plastids have resulted in a detailed understanding of the canonical import process.^(36–38) The plastid proteins imported by the TIC–TOC system usually have a cleavable amino-terminal extension (transit peptide)

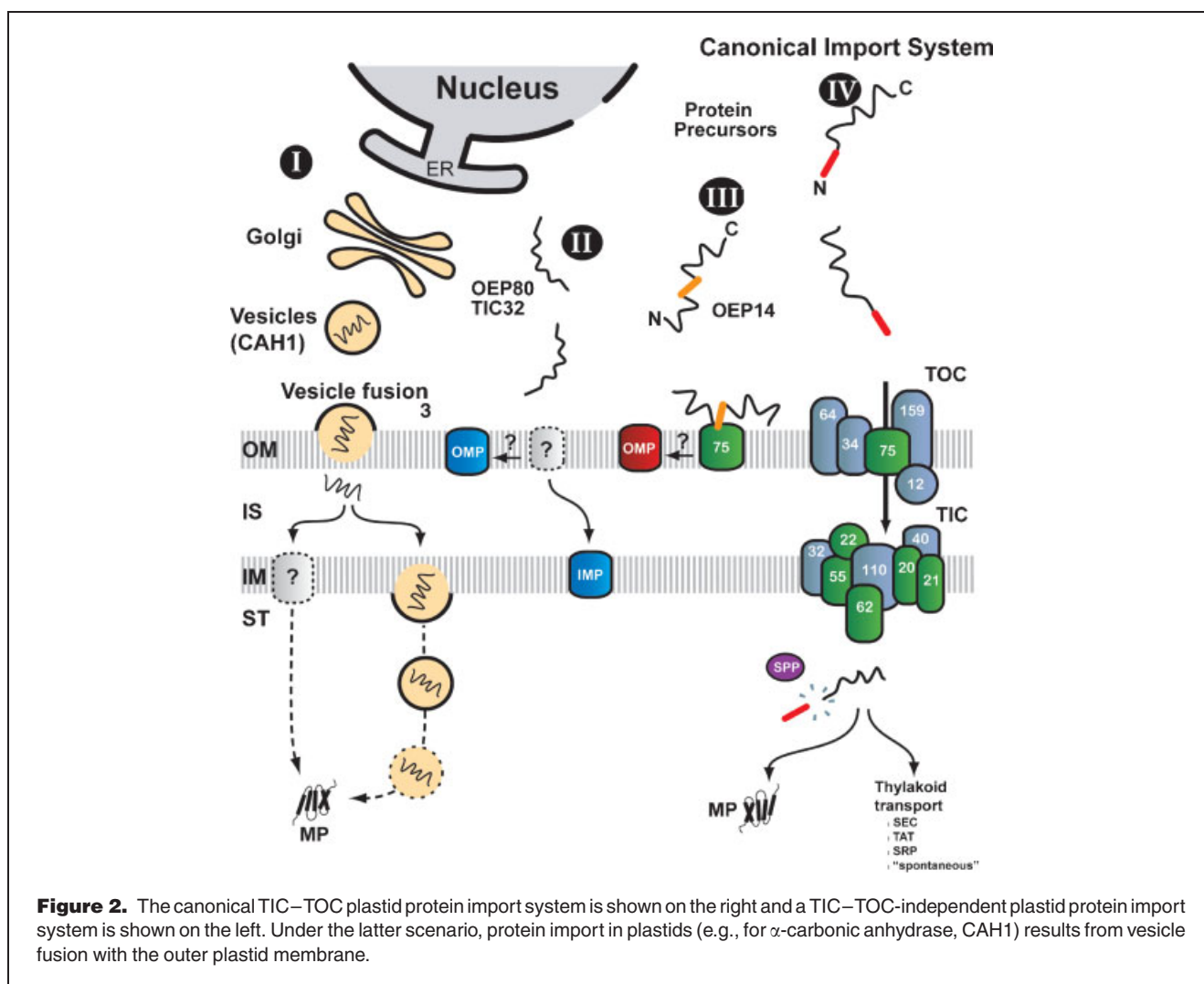


Figure 2. The canonical TIC–TOC plastid protein import system is shown on the right and a TIC–TOC-independent plastid protein import system is shown on the left. Under the latter scenario, protein import in plastids (e.g., for α -carbonic anhydrase, CAH1) results from vesicle fusion with the outer plastid membrane.

that is 30 to >100 amino acids in length.⁽³⁹⁾ Transit peptides are the transport signal to import the protein into the plastid (see Fig. 2). However, the amino acid sequence of signal peptides is not conserved. Structural and electrostatic characteristics of signal peptides are recognized by the plastid outer membrane receptors TOC159 and TOC34 (GTP-binding proteins) during the initial stages of protein import. The mechanism of action of TOC159 and TOC34 receptors is not completely understood, however, both proteins could participate in alternative ways during precursor binding-recognition and, in the case of TOC159, protein translocation.⁽³⁶⁾ Recent findings suggest the membrane protein TOC64 is also involved in both recognition (as a receptor) and translocation of particular precursors.⁽⁴⁰⁾ These and related processes are facilitated by cytosolic factors such as the molecular chaperone HSP90 and 14-3-3 proteins.

After binding of the transit peptide to the TOC receptors, protein precursors are transported across the channel-

forming protein TOC75 in a GTP-dependent process.⁽⁴¹⁾ Some experimental evidence suggests that TOC75 is sufficient for protein translocation in the absence of receptors. TOC75 could therefore be a feature of the first plastid protein import system.⁽⁴²⁾ Once the precursor emerges from the TOC complex, inter-membrane space proteins such as the HSP70-family chaperones, TOC12 and TIC22 mediate the TOC–TIC interaction for the subsequent translocation steps. The integral inner membrane proteins TIC20⁽⁴³⁾ and TIC110 are apparently involved in the formation of the TIC protein-conducting channel for precursor transport.^(44,45) Recently, another integral membrane protein, TIC21, has been proposed as a novel and major element of the protein-conducting channel.⁽⁴⁶⁾ Another presumed role of TIC110 is as a docking point for stromal factors (chaperones).⁽⁴⁷⁾ The TIC elements TIC55 (Fe-S protein), TIC32 and TIC62 [both NAD(P) binding proteins] have been associated with translocon redox regulation.⁽³⁸⁾ The role of the typical TIC component TIC40 is not

completely understood and several functions have been postulated, such as acting in a co-chaperone⁽⁴⁸⁾ or 'modulating' role.⁽⁴⁹⁾ The proteins imported by the TIC–TOC complex follow alternative transport systems depending on their final destination. Some proteins settle in the stroma, whereas others are re-targeted to thylakoidal membranes or the lumen by alternative protein-mediated routes (such as TAT, SEC-dependent or SRP-dependent) or via 'spontaneous' insertion (see Fig. 2).^(36,38,50)

Experimental and structural evidence indicates that the TIC–TOC system is a sophisticated outcome of the requirement for regulated protein import in plastids. Like other biochemical pathways in plastids^(20,51) the TIC–TOC complex comprises proteins of evolutionarily diverse origins.⁽³⁷⁾ Channel-forming TOC75 and the proteins TIC62, TIC55, TIC20, TIC22 and TIC 21 are likely of cyanobacterial (i.e. endosymbiotic) origin, whereas the remaining components (TOC159, TOC34, TOC64, TIC40, TIC110, TIC32) probably evolved from co-option of existing host genes or by horizontal gene transfer from bacterial sources. Therefore, considering the evolution of the complex signal recognition system, its energy requirements, and the redox regulation in the import process, we propose that the evolution of the canonical TIC–TOC machinery was not the initial driver in the establishment of the plastid. Instead, it is more reasonable to regard it as a subsequent development in organelle evolution. As discussed below, the initial system for protein trafficking to the endosymbiont was likely through the host endomembrane system and the subsequent recruitment of proteins of the outer (TOC75) and inner (TIC20, TIC21) envelope of the cyanobacterial endosymbiont facilitated the growth in complexity of the translocons.

As mentioned previously, elucidating the nature of host–organelle integration in organisms such as *P. chromatophora* has the potential to provide insight into the early stages of plastid establishment. Analysis of existing plastid proteins also provides clues to how the primitive protein import system could have evolved. For example, the protein OEP14 is inserted in the plastid outer envelope without a typical signal peptide mediated only by TOC75. This suggests that TOC75 alone may participate in the insertion of proteins into the plastid outer membrane, perhaps indicating an ancestral role during plastid evolution.⁽⁵²⁾ This observation supports the idea that plastid protein import could have taken place using a subset of the proteins in the TIC–TOC complex. However, other plastid outer membrane proteins, like OEP80 (a homolog of TOC75), are apparently imported without the typical N-terminal transit peptide but in a TOC-independent route (i.e. TOC75 is not involved).⁽⁵³⁾ Apparently, most of the plastid outer membrane proteins require diverse protein mediators to allow import but not the canonical TIC–TOC complex.⁽⁵⁴⁾ Another example is TIC32, which is imported into the plastid inner membrane without a canonical transit peptide and independent of any

known TOC element.⁽⁵⁵⁾ These examples clearly indicate that the TIC–TOC machinery is not the sole avenue for the transport of proteins into the plastid.

Non-canonical protein import in plastids

Given that plastid protein import can take place independent of the TIC–TOC machinery, the question arises whether the evolution of a protein translocation apparatus was a *condicio sine qua non* for organelle establishment or whether pre-existing protein secretion mechanisms of the host cell could have been used initially for protein targeting to the evolving organelle, and were gradually replaced over time with a more efficient and specific protein translocation system. If the latter were true, defining an organelle by the existence of a protein-import apparatus might be too restrictive, in particular if evidence exists in extant organisms for translocon-independent protein translocation to organelles.

Thanks to modern proteomics techniques, such evidence for non-canonical protein targeting to organelles, in particular to plastids, has recently emerged. Before large-scale proteomics became possible, the inventory of the organellar proteomes was largely based on bioinformatic analysis of the genomes of a few model plant species, such as *Arabidopsis*.^(56,57) Bioinformatic predictions of organellar proteomes use computational tools such as TargetP⁽⁵⁸⁾ that have been trained on a relatively limited set of well-characterized proteins to recognize the presence of organellar targeting sequences. Clearly, this approach towards generating inventories of organellar proteins is biased because only proteins following canonical import routes were used to train the prediction programs. Recent proteomics studies have generated experimentally validated inventories of the proteomes of various plastid subtypes and of mitochondria.^(59–68) These studies have led to the surprising discoveries that dual-targeting to both mitochondria and plastids is quite common and that a previously inconceivable number of proteins are present in plastids that apparently do not carry plastid-targeting signals.^(66–68) Of 604 proteins identified by mass spectrometry-based proteomics in *Arabidopsis* chloroplasts, only 62% (376 proteins) contained plastid-targeting signals (according to TargetP prediction), and 49 proteins were actually predicted to have ER signal sequences.⁽⁶⁴⁾ Another proteomics study of rice etioplasts found 224 nuclear-encoded plastidic proteins, 168 of which were predicted to carry plastid targeting sequences and 10 to be targeted to the secretory pathway (see Fig. 2).⁽⁶³⁾ In summary, these and other proteomics studies have provided evidence for protein targeting to the plastid in the absence of canonical targeting signals. Hence, protein targeting to chloroplasts might involve more pathways than previously assumed.^(28,68,69) However, proteomics neither provides insight into the mechanism of non-canonical protein targeting nor excludes the possibility that the canonical protein import apparatus is involved in the transport of proteins that do not carry targeting sequences.

Further insight came from detailed studies of individual proteins. Miras et al.⁽⁷⁰⁾ showed that a ceQORH-domain-containing protein is targeted to the inner chloroplast envelope membrane without carrying a canonical targeting sequence and without being processed. Even after truncation of the 59 amino-terminal residues, a C-terminal GFP fusion was still targeted to the chloroplast. Additional experiments showed that the information for targeting to chloroplasts is contained in the first 100 N-terminal amino acid residues and that the C terminus is not required for targeting to the chloroplast. This study thus demonstrated that translocation of proteins across the outer chloroplast membrane is possible without the requirement for a canonical targeting sequence. Another recent study provided direct and conclusive experimental evidence for import of α -carbonic anhydrase (CAH1) into the chloroplast stroma via the secretory pathway.⁽⁷¹⁾ CAH1 carries a short N-terminal extension that is predicted by TargetP as ER signal sequence and it apparently does not possess a canonical plastid-targeting signal. In a series of elegant experiments, CAH1 was localized to the chloroplast stroma and it was shown that CAH1 was entering the chloroplast through a TIC/TOC-independent pathway, likely by vesicular transport through the secretory pathway. Chloroplast CAH1 is *N*-glycosylated and transport of a CAH1-GFP to the chloroplast was inhibited by Brefeldin A, indicating that Golgi-mediated vesicular traffic is involved in routing CAH1 to the chloroplast stroma. This study also demonstrated that *Arabidopsis* chloroplasts contain multiple distinct *N*-glycosylated proteins, indicating that routing of proteins to the plastid via the secretory pathway is a relatively common feature that is not restricted to CAH1.⁽⁷¹⁾ Vesicular transport to the chloroplast would require physical contact between the endomembrane system and the chloroplast surface. Such direct physical interaction of ER-membranes with the surface of the chloroplast in living cells was recently demonstrated in a study that used optical tweezers and confocal fluorescence microscopy.⁽⁷²⁾

Conclusions

Taken together, findings from proteomics and detailed studies of individual proteins convincingly demonstrate the existence of TIC–TOC-independent, non-canonical protein import into plastids. This is important with respect to current use of the terms organelle and endosymbiont for the following reason. The import of proteins into extant plastids without the requirement for a functional TIC–TOC import apparatus is consistent with the hypothesis that sorting of proteins to the plastid initially occurred via the host protein secretion system and a specific and efficient protein sorting system evolved later. This hypothesis is further supported by the first step of protein targeting to plastids of “secondary” endosymbiotic origin, i.e. plastids that are the result of a primary plastid-containing alga taken up by a non-photosynthetic host eukaryote.⁽¹⁵⁾ In this case, protein targeting involves routing through

the secretory pathway of the host cell,^(29,73) thus recapitulating the process that we posit to have occurred during establishment of the primary plastid in the protoalga.⁽²⁹⁾ The finding that a protein crucial for photosynthate export from plastids, the plastidic triosephosphate/phosphate translocator, evolved from an ancient family of endomembrane-localized sugar-nucleotide transporters⁽²²⁾ lends additional support to the idea that canonical protein transport to the plastid evolved after the endosymbiont had established itself as a cellular organelle and argues against defining organelles solely by the presence of a protein translocation apparatus.

Finally, whereas this review has focused on the origin and early evolution of plastids, a recent discovery in the area of insect endosymbionts serves to further illustrate how the distinction between endosymbiont and organelle can become blurred. The genomes of aphid endosymbionts (e.g. members of the genus *Buchnera*) are well known to have reduced genomes relative to their closest free-living ancestors.⁽⁷⁴⁾ However, the recently described psyllid (plant louse) endosymbiont has taken bacterial genome reduction to a whole new level. The *Carsonella ruddii* genome is a mere 160 Kb (similar to many extant plastids), far and away the smallest prokaryotic genome known.⁽⁷⁵⁾ It encodes a mere 182 proteins—nowhere near enough to produce a functional bacterium. How does this organism still function? The answer to this question is not known, but the authors speculate that the ‘missing’ genes of *C. ruddii* have moved to the nuclear genome of its multicellular host.^(75,76) If so, and assuming the missing genes are essential for the survival of the endosymbiont, this means that the products of these genes are translated in the host cytoplasm and somehow redirected to the bacterium. Does this occur through passive diffusion or via active protein import? If the latter, this would represent the evolution of a novel protein import system in a multicellular animal. If this turns out to be true, is the *Carsonella* endosymbiont an ‘endosymbiont’ or an ‘organelle’? Obviously this example raises more questions than answers, but it serves to point out that given our incomplete knowledge of organelle evolution outside of the well-studied plastids and mitochondria, perhaps a strict definition based on a TIC–TOC type protein import system should not be applied a priori to a new evolutionary scenario, such as is playing out in *P. chromatophora*. We suggest therefore that it is premature to define strictly the terms ‘organelle’ and ‘endosymbiont’ until we have a better understanding of the *P. chromatophora* system as well as of alternative protein import pathways into canonical algal/plant plastids.

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