Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1743 (2005) 5–19

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

Review

Protein targeting to the chloroplasts of photosynthetic eukaryotes: Getting there is half the fun

Nasha Nassoury, David Morse*

Institut de Recherche en Biologie Végétale, Département de Sciences Biologiques, Université de Montréal, 4101 Sherbrooke est, Montreal, Quebec, Canada H1X 2B2

Received 27 May 2004; received in revised form 10 August 2004; accepted 17 September 2004
Available online 29 September 2004

Abstract

The plastids of many algae are surrounded by three or four membranes, thought to be a consequence of their evolutionary origin through secondary endosymbiosis between photosynthetic and non-photosynthetic eukaryotes. Each membrane constitutes a barrier to the passage of proteins, so protein targeting in these complex plastids has an extra level of difficulty when compared to higher plants. In the latter, protein translocation across the two membranes uses multi-protein complexes that together import proteins possessing an N-terminal leader sequence rich in serine and threonine (S/T). In contrast, while targeting to most complex plastids also involves an S/T-rich region, this region is preceded by an N-terminal hydrophobic signal peptide. This arrangement of peptide sequences suggests that proteins directed to complex plastids pass through the ER, as do other proteins with hydrophobic signal peptides. However, this simplistic view is not always easy to reconcile with what is known about the different secondary plastids. In the first group, with plastids bounded by three membranes, plastid-directed proteins do indeed arrive in Golgi-derived vesicles, but a second hydrophobic region follows the S/T-rich region in all leaders. In the second group, where four membranes completely surround the plastids, it is still not known how the proteins arrive at the plastids, and in addition, one member of this group uses a targeting signal rich in asparagine and lysine in place of the S/T-rich region. In the third group, the fourth bounding membrane is contiguous with the ER, but it is not clear what distinguishes plastid membranes from others in the endomembrane system. Knowing what to expect is important, as genomic sequencing programs may soon be turning up some of the missing pieces in these translocation puzzles.

Keywords: Secondary chloroplast; Protein targeting; Signal peptide; Transit peptide

The evolution of the molecular machinery for oxygenic photosynthesis in cyanobacteria-like cells more than 3.5 billion years ago [1] changed the form of life forever on this planet. Furthermore, these cells were so valuable as energy-producing machines that some eukaryotes enslaved them to form an endosymbiont. During time, the endosymbiont lost its autonomy and gradually transformed into an organelle. The host cell as well lost autonomy, since the partnership between the two formerly separate entities was beneficial for both. This symbiogenesis represents the merger of two cells to form a novel chimeric organism [2], on which natural selection would act to prevent the scattering of this amalgamation [3]. An additional mechanism to ensure the permanence of the arrangement is the transfer of genes from one partner to the other. However, gene transfer gives rise to a variety of new problems related to the evolution of a transport system for the proteins encoded by the transferred genes. The fundamental difference between an organelle and an intracellular symbiont is thus the existence of a specific mechanism that makes possible protein import to the organelle [4]. All these complications appear to have made symbiogenesis an infrequent evolutionary event in the several-billion-year history of cells [2], yet nonetheless essential for the formation of mitochondria and different types of plastids in photosynthetic eukaryotes.

* Corresponding author. Tel.: +1 514 872 9975; fax: +1 514 872 9406.
E-mail address: david.morse@umontreal.ca (D. Morse).

0167-4889/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.09.017

1. Symbiogenesis has happened infrequently

It is now generally accepted that mitochondria are derived from a single endosymbiotic event between a host cell and an α-proteobacteria very early in the evolution of eukaryotes. This is the only symbiogenic event that is broadly agreed to be of monophyletic origin [5]. The origin of chloroplasts is another indisputably symbiogenic event, but there are still unsettled issues about the number of times that this event has taken place. For example, plastids of chlorophytes (green algae, similar to plastids of land plants), rhodophytes (red algae) and the glaucophytes are generally termed "primary plastids", meaning that they have evolved directly from a symbiotic event between a photosynthetic prokaryote (cyanobacteria) and a eukaryote (Fig. 1). All primary plastids are surrounded by two membranes that correspond to the plasma membranes of the original eukaryotic and prokaryotic partners. Eventually, the prokaryote genes were transferred to the eukaryote genome and mechanisms to translocate proteins from the

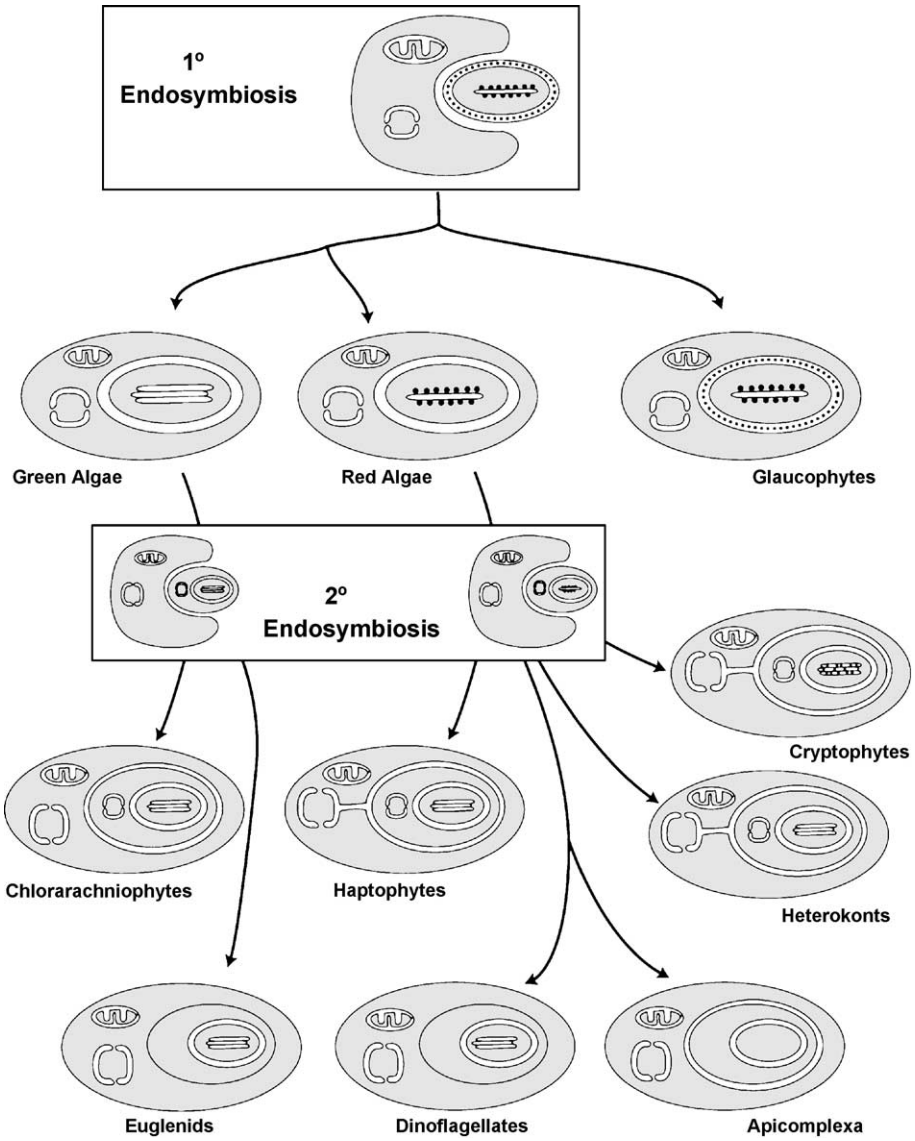

Fig. 1. Schematic representation of plastid evolution. A typical eukaryote, containing both a nucleus and mitochondria, is proposed to have engulfed a cyanobacterial-like cell in the primary endosymbiotic event that eventually gave rise to all plastids. This original endosymbiont, surrounded by two membranes and a peptidoglycan wall (dotted line), presumably contained thylakoids dotted with phycobilisomes. Three groups of extant primary plastids are distinguished, those of green alga and higher plants (which have lost their phycobilisomes), those of the red alga, and those of the glaucophytes (which have retained the peptidoglycan wall). All have lost the outer bacterial membrane. In the different secondary endosymbioses, a photosynthetic eukaryote (either a red or a green alga) instead of a cyanobacterial-like cell was engulfed by a new eukaryotic host. Extant secondary plastids are surrounded by either three or four membranes, and in some cases contain a residual nucleus termed a nucleomorph derived from the original eukaryotic host. In several cases the outer membrane of the secondary plastid is contiguous with the ER of the new host. No secondary plastids have phycobilisomes, although cryptophytes do have phycobilin pigments inside the thylakoid lumen.

transferred genes back to the prokaryote were developed [6,7].

But how many times had the transformation of endosymbionts to primary plastids actually taken place? The proponents of a monophyletic origin for the primary endosymbiosis have a strong case, based on plastid gene phylogenies, plastid gene order [8] and the similarity in gene content among plastid genomes [2,9,10]. To a first approximation, the phylogenetic relationships inferred between the chlorophytes, rhodophytes and glaucophytes are the same whether nuclear genes or plastid genes are used (Fig. 1). However, proponents of a separate origin for the host cells of red and green algae believe that the details of the reconstructions show inconsistencies between nuclear and plastid gene phylogenies, and that this is sufficient to cast doubt on the monophyletic origin idea [11]. With respect to the identities of genes retained, Stiller and colleagues have also shown that different plastid genomes have no more similarity to each other than they do to mitochondrial genomes once genes related to organelle-specific function are factored out. They suggest that the similarities in plastid genome contents can be explained by convergent evolution as a result of control on the gene loss rather than a shared evolutionary history [12].

Curiously enough, the first gene phylogenies tended to support a polyphyletic origin of the primary plastids, for example in the separate branches of red and green algae when Rubisco phylogeny is examined [13,14]. Later interpretations of these data invoked lateral gene transfer to account for the two different branches, thus allowing these data to peacefully coexist with the growing consensus for a monophyletic plastid origin [15] and with the puzzling presence of a form II Rubisco in the dinoflagellates [16]. However, a similar divergence is seen with chlorophyll a oxygenase (CAO) gene phylogenies. This component of the biosynthetic pathway for chlorophyll *b* synthesis is expressed in prochlorophytes and green algae but is not found in cyanobacteria, rhodophytes or glaucophytes [17]. To reconcile this with a monophyletic origin, either the gene was developed independently in chlorophytes or lost in cyanobacteria and two of the three primary plastid classes [12]. Given that, to date, plastids do not ally themselves with any particular group of cyanobacteria, there is still not sufficient data to exclude multiple endosymbiosis possibly from different groups of cyanobacteria [8,10,12].

As if the problems with primary plastids were not enough, there are also seven groups of "secondary plastids" (Fig. 1). These plastids all share a distinguishing feature of being surrounded by more than two membranes. Two groups harbor plastids surrounded by three membranes (Euglenoids and dinoflagellates), while the other five contain four membrane-bound plastids (Apicomplexa, Cryptophytes, Haptophytes, Heterokonts, and Chlorarachniophytes). These plastids, also known as complex or second-hand plastids, are thought to be the consequence of a secondary endosymbiosis between a photosynthetic and a non-photosynthetic eukaryote [18], and are principally responsible for the diversity of different algal groups. As with the primary plastids, the partnership has become irreversible due to the transfer of plastid genes to the new host nucleus. This adds a new layer of complexity to the return of nuclear-encoded genes, as will be discussed below. In addition, it might be expected that the different numbers of membranes around the plastid should hold important information about the evolutionary history of the organelle, but this is not the case at least for the triple membrane bound plastids of Euglena (where the plastid is derived from a green alga) and the dinoflagellates (where most of the plastids have a red algal origin).

Once again, the number of times that secondary symbiogenesis has happened is a key question. One viewpoint holds that each of these seven groups have obtained their plastids independently through a separate secondary endosymbiotic event [8,10]. An alternative view, based on complexity of symbiogenesis, is that only two major independent secondary symbiosis events have occurred, the engulfment of either a green alga or a red alga by another eukaryote [2,19]. Here, the chlorarachinophyte plastids, like those of the euglenophytes, are thought to have derived from a green algal precursor [20], despite the nuclear phylogeny that places the heterokonts (with their red-algal derived plastids) between them [21]. Furthermore, the secondary plastids of chromalveolates [22], a group comprised of haptophytes, dinoflagellates, apicomplexa, cryptophytes and heterokonts, are thought to trace back to a single common secondary symbiosis of a red alga [21,23–26] despite the nuclear gene phylogeny that places the euglenozoa and the chloroarachniophyta among them [21]. Thus, despite the importance of plastid origins in the analysis of protein targeting mechanisms, it is clear more work is required to resolve the differences between host cell and organelle phylogenies.

Lastly, it must be noted that there are also some cases of tertiary endosymbiosis, where secondary plastids have been replaced with secondary plastids from a different host [27]. This further complicates both evolutionary relationships and protein targeting mechanisms, but discussion of these is beyond the scope of this review.

2. Plastid membranes are biological barriers

We have mentioned plastids that are surrounded by two, three and sometimes even four membranes, but where do these membranes come from? It is generally accepted that new membranes are always formed by division or fusion of preexisting membranes, and they have only been "made from scratch" a few times in evolution [2,28]. Therefore, each membrane around a plastid must have its own evolutionary history. Cyanobacteria, of course, are Gram-negative, with an outer periplastic membrane in addition to the normal plasma membrane. The engulfment of these

bacteria might be expected to produce an organelle with three bounding membranes. The outermost membrane would then correspond to the phagotrophic vacuole of the host [19] and the innermost membrane to the cyanobacterial plasma membrane. Which of the three membranes was lost to produce two membrane-bound plastids? The inner membrane still seems similar to that of the cyanobacterial plasma membrane, so either the cyanobacteria escaped from the phagotrophic membrane and began dividing in the cytoplasm [19] or the original outer membrane of the cyanobacteria was lost during a organelle division [29]. Unfortunately, biochemical analyses of the outer membrane have failed to resolve this issue. On the one hand, the current outer membrane has high amounts of carotenoids and galactolipids [30] similar to those found in cyanobacterial outer membrane. These galactolipids in particular are reported to be involved in interactions between the transit peptide and the outer plastid membrane [31]. On the other hand, the outer membrane also has high levels of phosphatidylcholine [30]. This "eukaryotic" type lipid is transferred from the ER by lipid transfer proteins and is thought to have substituted the original bacterial liposaccharides [32]. The outermost membrane may thus be chimeric in nature [33]. As recently noted, however, regardless of which membrane was lost, that remaining functions as an acceptor for proteins and lipids from the two sides it has been separating [29]. The magic disappearing act of the third membrane is thus really nothing more than sleight of hand.

For plastids that are surrounded by four membranes, the engulfment of a eukaryote harboring a two membrane bound plastid can neatly account for the topology. In this case, the outermost membrane would correspond to the plasma membrane of the new host, while the membrane adjacent to it would represent the plasma membrane of the endosymbiont. In the case of the triple membrane bound plastids, one of the four membranes around these plastids has presumably been lost, perhaps in a manner analogous to the loss of the third membrane from the primary plastids. Again, the exact nature of the lost membrane is less important than its capacity to transport proteins. We will discuss below the evidence that the outer membrane in these plastids functions as part of the endomembrane system of the host.

3. Prokaryotes and organelles share two main types of protein translocation machinery

Independent of the evolutionary origin of the membranes, each constitutes a barrier to the movement of proteins. This would have become a severe problem following transfer of genes from the plastid to the host cell nucleus, as nuclear-encoded proteins synthesized in the cytoplasm would then have to pass these barriers to re-enter the plastids. In this context, membrane barriers are a strong driving force for the evolution of protein import pathways and, indeed, the development of a protein import machinery is proposed to be the key step for transforming the free-living cyanobacteria to an organelle dependent on the host [4]. The idea of a membrane as a barrier to movement of proteins is closely associated with the idea of compartments, as different compartments maintain their identity through their specific and conserved complement of proteins. The mechanisms that orchestrate protein movement through membrane barriers are thus of vital importance to the cell.

As will be discussed below, several evolutionarily unrelated mechanisms catalyze protein translocation across membranes, and this presumably reflects the importance of this process. However, the underlying logic of all protein translocation mechanisms is similar. For example, all mechanisms must have a method for identifying the protein to be translocated amongst the myriad of other proteins whose destiny is to remain where they are synthesized. Typically, this involves a peptide signal in the protein to be translocated, usually at its N-terminal end, and a receptor in the target membrane that recognizes and binds the signal. A useful analogy is to imagine the peptide signal as a key, designed to fit a particular protein receptor lock. The next element is the translocator itself, a normally impermeable channel extending through the membrane bilayer. To pursue the analogy, the channel is the door through which the proteins will pass. The receptor and channel can be, but are not necessarily, distinct molecules. Lastly, all translocation systems use energy to either push or pull the protein through the channel, and all have a means of ensuring the translocation occurs unidirectionally. This latter is usually ensured by the asymmetry of the energy generating machinery.

Prokaryotes possess two main types of protein translocation systems, required for exporting proteins to the periplasmic space (Table 1). The most basic and most highly conserved protein translocation machinery is the Sec61/SecY complex (Sec61 in eukaryotes and SecY in eubacteria and archea) [34]. The transmembrane component of this complex is a heterotrimeric protein that serves as a channel. It is formed from Sec61α (the homologous SecY in prokaryotes), Sec61γ (the homologous SecE in prokaryotes), and Sec61β (the homologous Secβ in archea or a non-homologous SecG in eubacteria).

The signal that identifies the protein to be translocated is a hydrophobic amino acid sequence at its N-terminal end, presented to the complex either as a nascent protein on a ribosome or in a complex with the soluble receptor protein SecA [35]. These two mechanisms are often termed signal recognition particle (SRP)-dependent and SRP-independent, respectively. SRP is a GTPase that, in its GTP bound form, can bind hydrophobic peptides on a protein as they emerge from a ribosome, and this binding often provokes translational arrest. Interaction of SRP with a membrane-bound SRP receptor brings the SRP–ribosome complex into proximity of a translocator, and it is believed that interaction

Table 1
Protein components to membrane translocation systems in bacteria and plastids

	E. coli		Chloroplast			
	SecY system	Tat/ΔpH	Outer membrane	Inner membrane	Thylakoid membrane	
					Sec	Tat/ΔpH
Signal	Hydrophobic	-RR- Hydrophobic	S/T-rich transit peptide	S/T-rich transit peptide	Hydrophobic	-RR- Hydrophobic
Signal receptors	SecY	TatB TatC	Toc33/34	Tic22	cpSecY	Hcf106 cpTatC
Translocator channel	SecY SecE SecG	TatA TatB TatC TatE	Toc75 Toc159	Tic20 Tic32? Tic62 Tic110	cpSecY cpSecE	Tha4 (TatA) Hcf106 (TatB) cpTatC
Soluble components	SecA		Toc159		cpSecA cpSRP54	
Accessory proteins	SecD SecF	TatD	Toc64	Tic55 Tic40? IAP100?		
Energy	SecA Ribosome	ΔpH	Toc34? Toc159?	Chaperone		ΔpH

between the translocator, the SRP and its receptor triggers GTP hydrolysis on SRP [36]. SRP in its GDP-bound form does not bind the hydrophobic peptides, which are then transferred to the translocator as synthesis restarts. The presence of ribosomes in the cytoplasm assures the directionality of translocation, and GTP hydrolysis during translation transport supplies the energy requirement for protein translocation.

In contrast to the SRP-dependent mechanism, an ATPase called SecA is also able to deliver cytoplasmic proteins to SecY. SecA binds to hydrophobic peptides and to a number of conserved residues on the cytoplasmic side of the channel complex that are proposed to act in signal recognition as well as in binding to SecA [37]. The cytoplasmic location and ATPase activity of SecA ensures the unidirectionality of protein translocation through the SecY complex and fulfills the energy requirement for posttranslational transport, respectively.

The SecY complex structure is known from X-ray diffraction of the archeal channel at 0.32-nm resolution [38]. The channel is formed from α-helices and has an hourglass shape containing two aqueous funnels and a constriction in the middle lined by hydrophobic residues. The hydrophobic residues in the constriction are proposed to act as a gasket, able to keep water out when closed and to form a watertight seal around translocating proteins. The structure also suggests that the pore may open only when a signal is bound to the complex, an aspect important to the maintenance of membrane impermeability.

In spite of the common evolutionary history of the Sec61/SecY complex in prokaryotes and eukaryotes, this protein translocation machinery does not find widespread use in protein import into organelles. One possible reason for this is that the direction of protein translocation depends on the topology of the transmembrane components of the SecY complex, which, in turn, depends on their site of synthesis. If SecY were encoded by the plastid, as is the case for non-green algae [39], the orientation of the protein would presumably be that designed for protein export from the plastid rather than protein entry. Alternatively, it may be that a mechanism different from that used for secreting proteins from the cell was required to distinguish plastid-targeted proteins. In any event, in all extant plastids the SecY complex is restricted to the thylakoid membranes and a different protein import mechanism has been developed for passing the bounding membranes of plastids.

In addition to the SecY complex, prokaryotes also possess a completely different protein export machinery, constructed from Tat gene products (for *t*win-*a*rginine *t*ranslocation) [40]. This name is derived from one of the two features that distinguish it from the SecY pathway: the signal identifying proteins translocated by the Tat pathway contain an obligatory pair of arginine residues immediately upstream of the hydrophobic region. The second distinguishing feature lies in the energy requirement for translocation: a ΔpH across the membrane is required while ATP or soluble proteins are not. In prokaryotes, there are five genes implicated genetically, four in the TatABCD operon in *E. coli* and another version of the TatA gene, called TatE, found elsewhere in the genome.

The structure of this protein complex is still unknown, as is the mechanism employed for translocation. However, in higher plants, it is restricted to protein translocation across the thylakoid membranes (like the SecY complex, and probably for the same reasons). Interestingly, this pathway was actually first identified in higher plants as the maize mutant Hcf106, defective in a pathway that uses a ΔpH as the sole energy source for translocating proteins into the thylakoid [41]. This protein is homologous to TatA, TatB and TatE genes, and is predicted to have a single membrane-spanning region close to the N-terminal end. Another maize mutant, Tha4, was also found to inhibit the same pathway

and is actually more closely related to TatA than is Hcf106 [42]. This suggests that two versions of the protein must work together in both higher plants and prokaryotes. The bacterial TatC gene is an integral membrane protein, and is thus a more likely candidate for the channel. Again like SecY, the TatC gene has homologues in the nuclear genome of *Arabidopsis*, and in the chloroplast genome of the red alga *Porphyra* and the diatom *Odontella* [43].

In addition to these well-studied pathways, other more poorly understood mechanisms are also present. For example, plastid thylakoid development can be inhibited by mutation in the Alb3 gene, which appears to encode a protein homologous to the YidC gene in bacteria and the Oxa1 gene in mitochondria [44]. Members of this family of proteins may act as integral membrane chaperones, which act to promote membrane insertion of proteins in a Sec-independent manner by recognizing and binding hydrophobic regions on the protein. Thus, these proteins appear to act in membrane insertion rather than in translocation.

It is also important to note that most of the studies on prokaryotic protein translocation have used non-photosynthetic organisms. The distinction is not negligible, as cyanobacteria have highly differentiated thylakoid membranes that are distinct from the plasma membrane. Some studies indicate that different signal peptides may impart a specific destination to a protein [45,46]. However, partially formed photosystems have been found in the plasma membrane, suggestive of transfer either to or from the thylakoids [47]. Perhaps protein targeting in cyanobacteria may use signals in the leader in some cases and signals in the mature protein in others as has been found for targeting to higher plant thylakoids [48].

4. The protein import machinery of primary plastids

The major components of the apparatus for importing proteins from the cytoplasm into the plastids of higher plants are now known in some detail and can serve as a basis for comparison with more complex plastid ultrastructures. The import machinery is found clustered in two protein complexes termed Toc and Tic (for *t*ranslocons for the *o*uter/*i*nner *c*hloroplast membranes) [49,50]. The Toc complex recognizes and binds plastid preproteins, and then translocates them across or inserts them in the outer membrane. Toc and Tic components connect at plastid envelope contact sites presumably by preprotein binding, and the protein is inserted into the Tic components of the inner membrane. Stromal chaperons then bind to the preprotein and pull it inside, acting to translocate the preprotein through the two membranes simultaneously.

The major Toc components identified to date are Toc159, Toc33/34, and Toc75 (Fig. 2) [51]. It is important to note that some of these components have homologues in cyanobacteria while others do not. Toc159 has GTP-binding motifs in a cytoplasmic domain which share homology with a distinct family of membrane GTPases [52,53]. This protein is presumed to be the main receptor for precursor proteins [51] and does not seem to have significant homology to any cyanobacterial protein [54]. Instead, it shows partial similarity to the α-subunit of the host SRP (signal recognition particle) receptor and may thus be of host origin [55]. Functionally, this protein appears to act like a SRP as well: it is found in comparable amounts soluble in the cytoplasm and integrated in the membrane, and it is proposed that Toc159 serves as a preprotein receptor by cycling between cytoplasm and the outer plastid membrane [56].

Toc33/34 also has a GTP-binding motif similar to that found in Toc159 [52], and shares limited sequence identity to cyanobacterial proteins. However, this low similarity is intriguing as the cyanobacterial proteins in question are the only small G proteins in the bacteria and the homologous region corresponds to the GTP-binding domain of Toc34 [54]. One proposed role for Toc34 is in recognition of preprotein sequences [52], as binding to Toc159 is enhanced by both GTP and preprotein binding [57]. Recently, it has been shown that the GTPase activity of Toc34 is necessary for docking of the receptor Toc159 at the Toc complex and that the interaction of Toc34 and Toc159 stimulates association with the translocon complex [58].

Toc75 is reported to be the most abundant protein of the chloroplast outer membrane [59,60] and is completely different from Toc159 and Toc34. It shows homology to a protein found in the outer membrane of all Gram-negative bacteria including cyanobacteria (SynToc75) that is reported to form a voltage-gated channel with high affinity for peptides in reconstituted liposomes [61]. These Gram-negative bacteria homologues of Toc75 are involved in protein export and are reported to secrete virulence factors across the outer membrane [62]. Interestingly, the Toc75 and its homologues in Gram-negative bacteria function in opposite directions compared to one another, with the nuclear-encoded Toc75 functioning in protein import and the synToc75 functioning in protein export. This apparent discrepancy may be related to the topology of the protein, as Toc75 is nuclear encoded in higher plants and may be inserted into the outer membrane in a direction opposite compared to that of synToc75. Alternatively, it may result from the unusual structure of the Toc75. Instead of the α-helical structure found for channels such as the SecY complex [38], both sequence [63] and functional analysis [64] suggest that Toc75 is a β-barrel. Indeed, it now appears that Toc75 belongs to a highly conserved Omp85 family of proteins also found in mitochondrial and bacterial outer membranes [65]. This structure is important as the larger and more rigid β-barrel structure offers less opportunity for regulating channel size than do the smaller and more flexible α-helical structures, and suggests that proteins might pass more easily in both directions. The unidirectionality of protein translocation observed in plastid Toc75 might result from accessory proteins that bind only to one

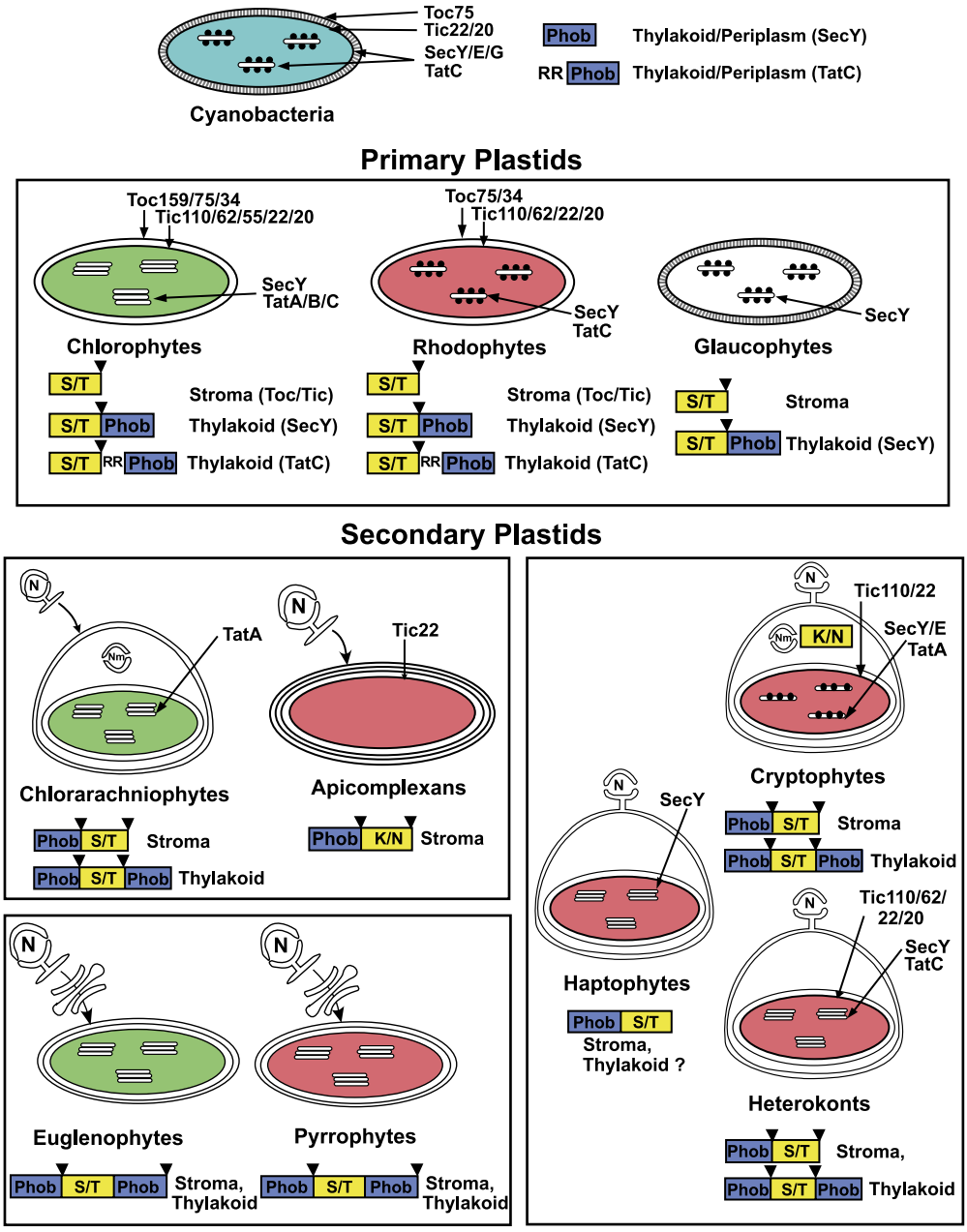

Fig. 2. Schematic views of primary and secondary plastids and the known translocator components. The structure of known peptide leader sequences, and the compartment to which they are directed, is shown immediately below each schematic of the plastids or cyanobacteria. The sequences marked S/T are rich in the hydroxylated amino acids serine and threonine, N/K are rich in asparagine and lysine, Phob are rich in hydrophobic amino acids and the black triangles represent sites for proteolytic cleavage. The known protein translocators, and the membranes in which they are found, are shown where known for each group. The translocators include Toc complex components in the outer membrane, Tic complex components in the inner membrane, and the Tat and SecY pathways for import into the thylakoids. Chlorarachniophytes and cryptophytes contain a nucleomorph (NM), the remnants of the original endosymbiont nucleus in addition to the new host cell nucleus (N). A cyanobacterial cell is shown at top for comparison. The three groups of secondary plastids are those with four membranes lacking CER (top left), with four membranes with CER (right) and those with three membranes (bottom left).

side of the channel or from an association with the inner membrane translocator.

The translocation across the inner membrane of higher plant plastids also uses a complex of several proteins, called the Tic complex, some of which are known to be of cyanobacterial origin. The major Tic proteins identified are Tic110, Tic 62, Tic55, Tic40, Tic32, Tic22 and Tic20 (Fig. 2) [51]. The first Tic component to be identified was Tic110, reported to be the most abundant translocation protein. The C-terminal of Tic110 can form a cation-selective high conductance ion channel and thus may be (or be a main part of) the import channel across the inner membrane [66]. Furthermore, the bulk of this integral membrane protein has been shown to function as a docking site for stromal chaperons assisting the translocation of the precursor proteins [67]. Tic110 shares no sequence homology with

any protein of known function and does not seem to have a cyanobacterial origin [51].

Tic62 is an integral membrane protein that coimmunoprecipitates with Tic110 (and Tic55), suggesting that it forms part of the translocator [68]. It is an integral membrane protein that interacts with ferredoxin-NADP oxidoreductase, suggesting it may be able to modulate protein import as a function of the organellar redox state. It is interesting that the N-terminal region of the protein has strong homology to putative proteins with unknown function in cyanobacteria, glaucophytes and cryptomonads with unknown function, although these proteins are most likely soluble and thus not functional homologues of Tic62. Perhaps this reflects conscription of a prokaryotic protein for a new function within the eukaryotic host.

Tic55 is another inner membrane component, and contains a Riesk-type iron–sulfur cluster and a mononuclear iron-binding site that are usually characteristic of redox proteins [69]. Tic55 appears to have homology with a hypothetical protein of unknown function in the cyanobacteria *Synechocystis*, hence suggesting it may have a symbiotic origin [54,69].

The role of a recently discovered Tic32 is not yet clear, although it associates with other Tic components in immunoprecipitation assays and with precursor proteins during translocation by cross-linking assays [70]. Tic32 appears essential for viability, as deletions of the gene in *Arabidopsis* are lethal during embryo development.

Tic22 is a peripheral inner plastid membrane protein and is thought to be the first protein that associates with precursor proteins as they emerge from the Toc complex and consequently directs them to the inner membrane translocon [71]. Tic22 is also considered to be the functional connection of the inner and outer membrane translocon. There is a cyanobacterial homologue of Tic22 but it has no known function [54].

Another component of the Tic apparatus with a homologue of unknown function in the cyanobacteria *Synechocystis* is Tic20. Tic20 is an integral protein that appears to have a role in protein conductance [71]. Tic20 was considered to be an ideal candidate for the inner membrane protein-translocating channel because of its structural similarities to prokaryotic amino acid transporters in *Bacillus subtilis* and *Methanococcus jannaschii* [54]. This is supported by the observation that *Arabidopsis* plants expressing Tic20 in antisense show a 50% decrease in the levels of protein import efficiency [72]. The exact role of Tic20 is uncertain, however, as Tic110 functions as an ion channel [73]. Tic110 protein levels do increase in antisense Tic20 plants, which might imply compensation for a defective inner membrane translocon [72]. It is possible that Tic110 and Tic20 form distinct translocation channels, or alternatively, act together to form the ion channel [73]. Actually, both Tic22 and Tic20 can associate with Tic110 and the main Toc proteins and so help to construct the Toc–Tic supercomplex [71]. This is important as the Toc–Tic supercomplex is deemed accountable for the regions of the plastid envelope where the inner and outer membranes are in close contact together. In fact, immunolocalization studies have restricted protein intermediates to these regions [74].

The chlorophytes have most of the protein translocators found in higher plants, with Tic 55 as the only exception. Similarly, with the exception of Toc159 and Tic55, homologues to higher plant protein translocators are also found in the genome of the red alga *Cyanidoschyzon* [75]. This strongly supports the monophyly of red and green algal plastids. So far, little is known about plastid protein translocators in the Glaucophytes.

5. Evolution of the plastid translocators

There are several different hypotheses for the evolutionary origin of the protein import apparatus. In one scenario, the synToc75 of *Synechocystis*, which initially secreted proteins across the outer membrane of cyanobacteria, was transferred to the nucleus of the host and formed the initial translocator channel capable of importing proteins after its insertion (in reverse) into the outer membrane of the plastid [62]. This suggests that the ancestral transit peptide may have evolved from the bacterial secretion signals that were the original substrates of SynToc75 [62], and would explain why the stromal peptidase that cleaves the leader peptide of plastid precursor proteins has sequence similarity to *Synechocystis* peptidases [54]. The inner membrane channel might then have been formed by modifying the amino acid-transporting Tic20 protein to increase the size of the proteins translocated and the efficiency of translocation [54]. Additional contemporary components of the protein import machinery may eventually have been added to optimize efficacy, especially in the aftermath of extensive gene transfer to the nucleus. Presumably, these additional components were derived from the host, as components like Toc159 and Tic110 do not have bacterial counterparts.

Another scenario, recently advanced by Kilian and Kroth [29], proposes that proteins produced from genes transferred to the nucleus might have used the secretory pathway in order to get to the phagotrophic vacuole in which the endosymbiont originally found itself. This clearly puts the outer membrane as part of the hosts' secretory system [29], and suggests that early transferred genes had to acquire a signal peptide in order for the proteins they encoded to access the secretory pathway and thus pass the outermost membrane of the plastid. Passage through the inner membrane would then take place using nonspecific ion channels [54] or by exploiting the ancestral Tic20 with its properties as amino acid transporters as described above. Interestingly, the secretory pathway proposal has the attractive feature of providing a plausible solution to the thorny issue of which came first, the translocator or the signal. A gene transferred from the endosymbiont to the

nucleus might find itself able to enter the plastid by exploiting mechanisms already in use in the host cell if it fortuitously acquired a signal peptide. And why was the original endosymbiont not digested if it ended up in a phagocytic vacuole? Recent work has provided strong evidence that ER membrane recruitment is largely responsible for forming the phagocytic membrane in macrophages [76]. This suggests that original symbiont may not have entered a phagosome at all, and that a process inhibiting phagocyte fusion to an ER-derived membrane, rather than the more complicated task of getting the original prey out of a phagocytic vacuole, may have been involved in endosymbiosis.

However, the proposed entry of plastid-directed proteins into the secretory pathway solves one problem by introducing another: how would ER proteins be directed to the plastid. This is not a trivial issue and it arises again when protein import mechanisms into complex plastids are considered (see below). One view is that the ER and outer plastid membranes are contiguous allowing proteins to simply diffuse from one to the other. A second option requires vesicular transport. Here, vesicles full of plastid-directed proteins might be recognized and targeted specifically to the organelle, by development of a modified pair of SNARE proteins, for example [77]. Alternatively, vesicles containing many different nucleus-encoded plastid proteins might fuse with plastids and other various compartments [29]. This form of transport would maintain plastid identity if the chloroplast proteins were specifically extracted from the mixture of proteins delivered to the intermembrane space. Kilian and Kroth propose that the N-terminal region of the protein, immediately following the signal peptide, became modified to function as a transit peptide. In this scenario, the transferred proteins were escorted by a bipartite presequence containing a signal peptide as well as a transit peptide (similar to present-day secondary plastids), and the removal of the signal peptide exposed the transit peptide. The translocon in the inner membrane (Tic) would ensure the correct and efficient protein transport into the endosymbiont of any proteins with this transit peptide. In the next logical step in this scenario, when a protein translocator became established in the outer membrane (Toc), the secretory pathway was of no further use and the now unneeded signal sequence could be lost. One must keep in mind that this tedious gain of the signal peptide, development of a transit peptide and final loss of the signal peptide are proposed to have happened only to the genes transferred to the host at the very primary stages of endosymbiosis [29]. This hypothesis nicely explains the presence of Toc159 in the outer membrane, as its similarity to host cell signal recognition particle receptor suggests it may have been introduced into the outer membrane to allow recognition of a hydrophobic signal peptide [78]. Presumably this role is accomplished by Toc34 in red alga where Toc159 is not found [75].

6. Evolution of the targeting sequence

These proposed gymnastics of the signal and transit peptides at the N-terminal end of the proteins underscore the vital relationship between the sequence key and the receptor lock that allows a protein passage through the translocator channel door. Indeed, while the development of the protein import apparatus is crucial in transforming the free-living endosymbiont to an organelle, and presumably an inevitable consequence of gene transfer from the endosymbiont genome to the host nucleus, this must occur in concert with the mechanism for discriminating which proteins should be reintroduced into the plastid. How might a targeting sequence be added to the N-terminal end of the protein? Some clues are available from studies of mitochondrial genes. One possible mechanism is exemplified by the rps14 gene of maize, which when transferred from the mitochondrion to the nucleus landed in the intron of the mitochondrial directed sdh2 gene [79]. Alternative splicing thus allows the rps14 gene to freeload on the sdh2 targeting system. In another example, the rps10 gene was transferred into a duplicate gene for mitochondrial hsp22 [80]. Thus, over evolutionary time scales, transfer of a given gene to the nucleus might have occurred many times, until eventually the gene found itself adjacent to a sequence that could serve as a targeting signal. The idea of repeated gene transfers has received experimental support from measured gene transfer rates to the nucleus from mitochondria [81] and chloroplasts [82], and of course any new gene fusions will be maintained if they provide a selective advantage for the host. Alternatively, the targeting signal could have been stitched onto the transferred genes through exon shuffling. This later idea has experimental support for the generation of plastid-directed and mitochondrial-directed proteins [83,84].

However it happens, the addition of targeting signals apparently happened early in evolution. The prototypical “transit peptide” found in chlorophytes and higher plants is a stretch of amino acids rich in hydroxylated amino acids such as serine and threonine and contains some basic but few acidic amino acids [85]. This same transit peptide is also used for translocation into rhodophyte [86] and glaucophyte [87] plastids, suggesting that the gene transfers and targeting signal acquisitions occurred prior to the divergence between the different primary plastid-containing lineages (compare Figs. 1 and 2). Furthermore, the same serine/threonine-rich sequence is also used for entry into the inner two membranes of dinoflagellate [88] and diatom [89] plastids, suggesting that gene transfer to the nuclei of the secondary plastid-containing lineages may have occurred after the first transit sequence was already in place. However, apicoplast targeting sequences in *Plasmodium* [90] and the transit peptides on proteins encoded by the cryptomonad nucleomorph [91] contain roughly much more lysine and asparagine as they do serine and threonine (Fig. 2). Whether these are derived modifications in these specific lineages or

a reflection of different protein translocation systems is an open question at this point.

7. Protein import into the secondary plastids with three membranes

Interestingly enough, the leader sequence of proteins entering the triple membrane bound plastids of Euglenids and dinoflagellates is similar to that predicted by the secretory membrane hypothesis [29]. The N-terminal domain is hydrophobic and is followed by a transit peptide domain rich in serine and threonine (Fig. 2). The only unusual feature of these leader sequences is the presence of a second hydrophobic region that follows the S/T-rich region.

Euglena plastids are undeniably the most extensively studied secondary plastids and, in fact, the passage of nuclear encoded plastid proteins through the secretory system was initially shown in *Euglena* by immunolocalization of the light-harvesting protein LHCPII at the EM level [92]. Several years later, it was shown that the presequence of the LHCPII did indeed have a functional ER targeting domain [93]. Furthermore, pulse-chase experiments have shown that newly synthesized pre-LHCPII is found first in the ER then in the Golgi apparatus before arriving in the chloroplasts [94]. All *Euglena*'s chloroplast protein presequences have an unusual second hydrophobic core located downstream of the transit peptide domain. This second hydrophobic region acts as a stop transfer sequence after the plastid proteins begin co-translational translocation to the ER, so that the C-terminal end of the protein is found in the cytoplasm. The first hydrophobic region is followed by a signal peptidase site, so that when synthesis is complete, the final product is a single pass membrane protein with the N-terminal transit peptide inside the ER lumen and the bulk of the protein in the cytoplasm [95]. These integral membrane proteins maintain their peculiar topology during vesicular transport to the plastid, so that following fusion with the outermost chloroplast membrane, the precursor is embedded in the outer membrane with a transit peptide dangling down into the intermembrane space. At this juncture the membrane-bound protein is presumably capable of moving laterally in the membrane until the transit peptide reaches import receptors located in the middle membrane. One likely scenario is that Toc complexes in the middle membrane, homologous to those in the outer membrane of primary plastids, would then bind and begin importing the protein. A potential contact between Toc and Tic complexes, as found in higher plant plastids, could then result in import into the plastid stroma. The inner membrane translocators are presumed here to be homologues of the Tic complex. Independent of their identity, concerted translocation across the three membranes would couple ATP hydrolysis to translocation using Hsp70-like proteins in the stroma. This is important as energy will presumably be required to pull the hydrophobic anchor free of the outer membrane [95].

Recently, a similar role as protein anchor has been demonstrated for the second hydrophobic core in the leader sequence of the dinoflagellate plastid-directed proteins [88]. The protease sensitivity of proteins synthesized by in vitro translation in the presence of microsomes shows that the bulk of the protein is also on the cytoplasmic side of the ER membranes, implying they will approach the plastid as Golgi-derived vesicles with only the transit peptide inside [88]. This is important because *Euglena* is phylogenetically unrelated to dinoflagellates. The similarities in protein translocation mechanisms thus indicate that they are a requirement of plastid ultrastructure, not phylogeny.

The crucial feature of this translocation mechanism is the second hydrophobic region in the leader sequence. As will be discussed below, four membrane bound plastids have targeting signals that differ by the absence of the second hydrophobic region. It seems reasonable that loss of one membrane from an initial four membrane bound plastid could be compensated for by accentuating any existing hydrophobic character in the targeting signal or even the mature protein sequence. One intriguing question is why the second hydrophobic region should be required at all. We have speculated that targeting to the plastid may not be specific [88], perhaps as a result of the protein having its bulk in the cytoplasm. This topology suggests that the proteins may lack the usual cytoplasmic sorting signals expected to associate with adaptors or coat recruitment proteins which charge the vesicles with the appropriate cargo [96]. The hydrophobic membrane anchor would anchor the proteins to the plasma membrane if accidentally secreted and could permit their recovery.

8. Protein import into the secondary plastids with four membranes and CER

The second hydrophobic region is never found in leader sequences that target proteins to the stroma of four membrane-bound plastids. Instead, the targeting signals contain only the hydrophobic ER-signal sequence followed by the transit peptide (Fig. 2). Despite this general similarity, details of the targeting mechanism differ in the two main groups of four membrane-bound plastids. The first group of four membrane-bound plastids is found in most heterokonts and cryptophytes, and is characterized by having ribosomes attached to their outer membrane (often called chloroplast ER, or CER) [97]. The evolutionary origin of this membrane is moot since it is thought to be derived from the food vacuole surrounding the endosymbiont and its modification to an ER membrane remains mysterious [98]. However, in several organisms the CER is reported to be continuous with the ER and the nuclear envelope [97,99]. Are the ER and CER functionally distinct

albeit their continuity? This seems likely in heterokonts, as GFP accumulates in the plastid when fused with a plastid preprotein signal peptide, whereas GFP accumulates in the ER when an ER-targeting signal peptide is used instead [100]. It has been suggested there is a subtle difference between the two signal peptides and their recognition sites [101], even though diatom plastid presequences do enter canine microsomes in vitro [102]. As a caveat, however, these observations do not exclude the possibility that some plastid-targeted proteins directed to the ER instead of the CER could then be transferred to the CER through the ER/CER lumenal connections [99]. A receptor/translocator could be used to sieve out proteins with a transit peptide from others in the general secretory pathway.

Regardless of the location of translation (i.e., ER or CER), the mechanism used to pass the three additional plastid membranes still remains unknown. Thus far, two models have been proposed. The first involves vesicular shuttling between the two middle membranes, and is supported by microscopic observation of vesicles in the space between them [97]. In this model, plastid-directed proteins with a signal peptide in their leader sequence exploit the secretory pathway of the host for targeting to the outer membrane. Once past this first barrier, proteins found themselves outside the host cell and at the exterior surface of a membrane topologically equivalent to the former plasma membrane of the endosymbiont. This membrane was presumably capable of endocytosis when the eukaryote endosymbiont was free-living, and if proteins delivered from the host cell were taken up by a similar mechanism, they would find themselves in vesicles between the two middle membranes. Vesicle fusion would then place the proteins in front of the innermost plastid membrane, where transport into the stroma could be mediated by a Tic transport system [29,98]. There is also some indirect support for the model, derived from the effects of Brefeldin A on plastid-directed protein transport in cryptomonads. Brefeldin A inhibits vesicular traffic in most eukaryotes [103], and in cryptomonads causes swelling of the space underneath the outermost membrane of the plastid where the ribosomes are found attached [104]. One interpretation of this result is that vesicular traffic inside the plastid is also sensitive to the drug allowing proteins to build up there. Interestingly, this model does not require Toc translocator components, in agreement with the lack of Toc homologues in the almost complete diatom nuclear genome sequence [105].

The second model for protein transport into the CER-containing plastids proposes the presence of a protein translocator, possibly a duplicate of the Toc complex, in both of the middle two membranes of the plastid [22,106]. In common with the previous model, the signal sequence would allow passage across the outermost membrane and the transit peptide would allow the protein to pass through a Tic complex of the innermost membrane [106]. However, the vesicular transport step, where proteins effectively vault over the middle two membranes, is replaced by true translocation through two successive Toc complexes, one on each of the membranes. Both of these two Toc complexes must be nuclear-encoded. While this is self-evident for most four membrane-bound plastids, it is not necessarily so for the cryptomonads and chlorarachniophytes that have a miniaturized nucleus termed a nucleomorph in between the middle two membranes [91,107]. The nucleomorph is thought to represent the remnants of the nucleus belonging to the host of the primary endosymbiont, as evidenced by phylogenetic analyses that place it among the algae with mainly primary plastids. However, the nucleomorph genome of the cryptomonad *Guillardia theta* does not encode any Toc complex components (although it does encode a protein homologous to Tic22 and a chaperone-binding Tic complex-associated protein called IAP100) [91]. The jury is still out on the chlorarachniophyte nucleomorph, as the full sequence is not yet available [108].

Interestingly enough, the cryptomonad nucleomorph genes encode either chloroplast-targeted proteins or are housekeeping genes required for expression of those plastid-directed genes [91]. Obviously, these nucleomorph-encoded plastid-directed proteins must have a targeting mechanism in order to cross the two membranes surrounding their plastid. As one might guess, these proteins do have an N-terminal extension reminiscent of the transit sequence of higher plants [91], but differ in that these leaders contain almost twice as much asparagine and lysine as serine and threonine (Fig. 2). The preparation of import-competent chloroplasts from cryptomonads, surrounded by only two membranes, allowed a direct test of the similarity between the nucleomorph N-terminal extension and higher plant transit sequences. While nucleomorph-encoded proteins were efficiently imported, nuclear-encoded proteins truncated to remove the hydrophobic signal peptide were not [104]. This result suggests there are two distinct import pathways for traversing the two inner membranes. Perhaps nuclear-encoded proteins employ vesicular shuttling while nucleomorph-encoded proteins require protein translocators in both of the two remaining membranes.

9. Protein import into the secondary plastids with four membranes and no CER

The other category of four membrane-bound plastids is found in the apicomplexans and the chlorarachniophytes. These plastids lack CER, meaning the outer most membrane is not continuous with the ER and ribosomes have never been observed. The phylum Apicomplexa is a group of obligate endoparasites with members such as *Toxoplasma* and *Plasmodium* that contain a non-photosynthetic secondary plastid termed an apicoplast [109,110] thought to be involved in fatty acid biosynthesis [111], isoprene formation and haem synthesis [112]. Chlorarachniophytes contain a nucleomorph between their two middle membranes which is a remnant of a green algal nucleus [113], but to date has not

yet been found to contain any protein translocator components [108].

The leader sequence of proteins targeted to the plastids of this group is not structurally different from that directing proteins to the plastids containing a CER, as it is composed of a signal peptide followed by a transit peptide. Most of the work in this group has focused on protein targeting to apicoplasts because they can be transformed and have an important impact on human health [114–116]. The leader sequence contains a typical hydrophobic signal sequence, suggesting that the plastid-directed proteins enter the secretory system through the ER, while the transit peptide contains an abundance of asparagine, lysine and basic amino acids [90]. This transport system differs from that used by the CER-type plastids, in which the ER and the outer membrane of the plastid are connected, in that the apicoplast targeted proteins require a separate step of vesicular transport to arrive at the apicoplast. These proteins must somehow be sorted from the rest of the secretory proteins and get transported to the outermost membrane of the apicoplast. Two different routes for protein trafficking to the apicoplasts have been proposed to account for this. One proposed route for protein transport considers the apicoplast to be an alternate end point of the secretory pathway. As a consequence of this, one might expect proteins to be transported in vesicles from the ER to the Golgi where they can be sorted from other proteins by an unknown transit-peptide recognition factor. However, so far there is no evidence that supports a role for the Golgi in the transport process. In particular, protein transport to the apicoplast is not blocked by Brefeldin A [117].

In the other proposed route, the apicoplasts are proposed to lie prior to the point where proteins flowing through the secretory pathway are sorted. This considers the apicoplast as a part of the default pathway, and suggests that all proteins in the secretory pathway must then pass through the outer membrane space of the apicoplast. Proteins lacking the transit peptide would continue their passage via vesicle budding from the apicoplast outer membrane, while proteins possessing the transit peptide will be drawn in. Recently, a rule-based predictor tool named PlasmoAP (*Plasmodium falciparum a*picoplast-targeted *p*roteins) has been designed in order to predict the apicoplast-targeted proteins from the *P. falciparum* genome. Unlike the hydroxylated amino acid-rich transit peptides of higher plants, the apicoplast targeting sequences are rich in asparagine, lysine and basic amino acids [90]. It has been suggested that this positively charged transit peptide is electrophoretically pulled into the apicoplast lumen by a series of negatively charged transmembrane pores (possible duplicate of the Toc protein complex) [90,106]. This characterization provides a useful tool to screen out possible protein translocators in the apicoplast. For example, the hypothetical *Plasmodium* protein (NP_703634) shares 26% sequence identity over 272 amino acids with Tic22 of *G. theta* and has an N-terminal hydrophobic signal peptide followed by a region rich in asparagines and lysine. Clearly, this is a good candidate for a plastid translocation complex component in these organisms. So far, however, no other unambiguous translocator components have come to light in the Apicomplexan genome, although 466 proteins from the total 5282 proteins of *P. falciparum* are predicted to be targeted to the apicoplast [90]. The closest match lies with a hypothetical *Plasmodium* protein (gi 23619599) that shares weak similarity (1 e^{-7}) to Toc159 from pea and has a leader sequence appropriate for plastid targeting. Genomic data mining thus has considerable potential to identify translocator candidates, although all candidates will require rigorous testing to establish a real involvement in translocation.

10. Conclusions and perspectives

Cells rely on protein translocators to maintain the functional identity of each of their different membrane-bound compartments. In simple systems, such as the two compartments of cyanobacteria, several protein translocators (the SecY and TatC complexes) and several types of targeting signals (hydrophobic regions in the leader sequence or other regions in the mature protein) are involved. In more complex systems, such as photosynthetic eukaryotes, the problem is even more acute as other compartments in the host could compete with those inside the plastid. Cells with primary plastids appear to have resolved this problem by conscripting cyanobacterial protein translocators (such as synToc75) and overlaying a transit signal rich in hydroxylated amino acids (such as the original synToc75 substrate) to the N-terminal end of plastid-directed proteins. This new protein translocation system for eukaryotes was distinct from the SecY and TatC systems used for targeting proteins to the thylakoids, allowing the elements of the thylakoid targeting mechanism used by the ancestral prokaryotic symbiont to be conserved. In an analogous manner, cells with secondary plastids have overlaid yet another targeting signal on to the N-terminal end of the protein. This latest system used the same hydrophobic signal and translocator complex initially used for protein export by the ancestral prokaryotic symbiont. The additional membranes separating the host cytoplasm from the thylakoids allowed this system to be used without any possible confusion as to the destination of the protein.

It seems likely, therefore, that a limited number of protein translocating systems have arisen throughout evolution. Genomic sequencing efforts should thus prove very informative with respect to determining which translocators will have homologues in the nuclear genomes of algae with secondary plastids. The characterization of the protein components to the import pathway will provide an unambiguous picture of which pathways are evolutionarily conserved and which have been derived de novo. However, some problems still remain to be resolved. First, for those plastids with CER, how do proteins get across the second

membrane? And for those plastids without it, how do protein get to the plastid from the ER? This later question is even more acute in the three-membrane bound plastids, where transport vesicles appear to have the bulk of the protein rather than a targeting signal in the cytoplasm. So, while we have come far in our understanding of protein import into complex plastids, there is still a long way to go. Fortunately, getting there really is half the fun.

Acknowledgements

We thank Drs. J. Palmer and P. Keeling for advice on phylogenic relationships derived from plastid and nuclear genes. Work in our laboratory has been supported by the National Science and engineering Research Council of Canada (NSERC).

References

[1] J.W. Schopf, Microfossils of the Early Archean Apex chert: new evidence of the antiquity of life, Science 260 (1993) 640–646.
[2] T. Cavalier-Smith, Membrane heredity and early chloroplast evolution, Trends Plant Sci. 5 (2000) 174–182.
[3] G.I. McFadden, Plastids and protein targeting, J. Eukaryot. Microbiol. 46 (1999) 339–346.
[4] T. Cavalier-Smith, J.J. Lee, Protozoa as hosts for endosymbioses and the conversion of symbionts into organelles, J. Protozool. 32 (1985) 376–379.
[5] B.F. Lang, M.W. Gray, G. Burger, Mitochondrial genome evolution and the origin of eukaryotes, Annu. Rev. Genet. 33 (1999) 351–397.
[6] W. Martin, R.G. Herrmann, Gene transfer from organelles to the nucleus: how much, what happens, and why? Plant Physiol. 118 (1998) 9–17.
[7] S.D. Dyall, M.T. Brown, P.J. Johnson, Ancient invasions: from endosymbionts to organelles, Science 304 (2004) 253–257.
[8] C.F. Delwiche, J.D. Palmer, The origin of plastids and their spread via secondary endosymbiosis, in: D. Bhattacharya (Ed.), The Origins of Algae and Their Plastids, Springer-Verlag, Vienna, 1997, pp. 53–86.
[9] W. Martin, B. Stoebe, V. Goremykin, S. Hapsmann, M. Hasegawa, K.V. Kowallik, Gene transfer to the nucleus and the evolution of chloroplasts, Nature 393 (1998) 162–165.
[10] J.D. Palmer, The symbiotic birth and spread of plastids: how many times and whodunit? J. Phycol. 39 (2003) 4–11.
[11] J.W. Stiller, B.D. Hall, Sequences of the largest subunit of RNA polymerase II from two red algae and their implications for rhodophyte evolution, J. Phycol. 34 (1998) 857–864.
[12] J.W. Stiller, D.C. Reel, J.C. Johnson, A single origin of plastids revisited: convergent evolution in organellar genome content, J. Phycol. 39 (2003) 95–105.
[13] W. Martin, C. Somerville, S. Loiseaux-de Goër, Molecular phylogenies of plastid origins and algal evolution, J. Mol. Evol. 35 (1992) 385–404.
[14] C. Morden, C. Delwiche, M. Kuhsel, J. Palmer, Gene phylogenies and the endosymbiotic origin of plastids, Biosystems 28 (1992) 75–90.
[15] C. Delwiche, J. Palmer, Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plasmids, Mol. Biol. Evol. 13 (1996) 873–882.
[16] D. Morse, P. Salois, P. Markovic, J.W. Hastings, A nuclear encoded form II rubisco in dinoflagellates, Science 268 (1995) 1622–1624.
[17] A. Tomitani, K. Okada, H. Miyashita, H.C. Matthijs, T. Ohno, A. Tanaka, Chlorophyll b and phycobilins in the common ancestor of cyanobacteria and chloroplasts, Nature 400 (1999) 159–162.
[18] S. Gibbs, The chloroplasts of *Euglena* may have evolved from symbiotic green algae, Can. J. Bot. 56 (1978) 2883–2889.
[19] T. Cavalier-Smith, The origins of plastids, Biol. J. Linn. Soc. 17 (1982) 289–306.
[20] K. Ishida, Y. Cao, M. Hasegawa, N. Okada, Y. Hara, The origin of chlorarachniophyte plastids, as inferred from phylogenetic comparisons of amino acid sequences of EF-Tu, J. Mol. Evol. 45 (1997) 682–687.
[21] S.L. Baldauf, A.J. Roger, I. Wenk-Siefert, W.F. Doolittle, A kingdom-level phylogeny of eukaryotes based on combined protein data, Science 290 (2000) 972–977.
[22] T. Cavalier-Smith, Principles of protein and lipid targeting in secondary symbiogenesis: euglenoid, dinoflagellate and sporozoan plastid origins and the eukaryotic family tree, J. Eukaryot. Microbiol. 46 (1999) 347–366.
[23] Y. Van de Peer, S.L. Baldauf, W.F. Doolittle, A. Meyer, An updated and comprehensive rRNA phylogeny of (crown) eukaryotes based on rate-calibrated evolutionary distances, J. Mol. Evol. 51 (2000) 565–576.
[24] N.M. Fast, J.C. Kissinger, D.S. Roos, P.J. Keeling, Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids, Mol. Biol. Evol. 18 (2001) 418–426.
[25] H.S. Yoon, J.D. Hackett, G. Pinto, D. Bhattacharya, The single, ancient origin of chromist plastids, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15507–15512.
[26] J.T. Harper, P.J. Keeling, Nucleus-encoded, plastid-targeted glyceraldehyde-3-phosphate dehydrogenase (GAPDH) indicates a single origin for chromalveolate plastids, Mol. Biol. Evol. 20 (2003) 1730–1735.
[27] J.F. Saldarriaga, F.J. Taylor, P.J. Keeling, T. Cavalier-Smith, Dinoflagellate nuclear SSU rRNA phylogeny suggests multiple plastid losses and replacements, J. Mol. Evol. 53 (2001) 204–213.
[28] G. Blobel, Intracellular protein topogenesis, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 1496–1500.
[29] O. Killian, P.G. Kroth, Evolution of protein targeting into "complex" plastids: the "secretory transport hypothesis", Plant Biol. 5 (2003) 350–358.
[30] J. Joyard, E. Teyssier, C. Miege, D. Berny-Seigneurin, E. Marechal, M.A. Block, A.J. Dorne, N. Rolland, G. Ajlani, R. Douce, The biochemical machinery of plastid envelope membranes, Plant Physiol. 118 (1998) 715–723.
[31] B.D. Bruce, The role of lipids in plastid protein transport, Plant Mol. Biol. 38 (1998) 223–246.
[32] T. Cavalier-Smith, The origins, losses and gains of chloroplasts, in: R.A. Lewin (Ed.), Origin of Plastids: Symbiogenesis, Prochlorophytes and the Origins of Chloroplasts, Chapman & Hall, 1993, pp. 291–348.
[33] T. Cavalier-Smith, The simultaneous symbiotic origin of mitochondria, chloroplasts, and microbodies, Ann. N.Y. Acad. Sci. 503 (1987) 55–71.
[34] T.A. Rapoport, B. Jungnickel, U. Kutay, Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes, Annu. Rev. Biochem. 65 (1996) 271–303.
[35] A.E. Johnson, M.A. van Waes, The translocon: a dynamic gateway at the ER membrane, Annu. Rev. Cell Dev. Biol. 15 (1999) 799–842.
[36] W. Song, D. Raden, E.C. Mandon, R. Gilmore, Role of Sec61alpha in the regulated transfer of the ribosome-nascent chain complex from the signal recognition particle to the translocation channel, Cell 100 (2000) 333–343.
[37] A. Economou, W. Wickner, SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion, Cell 78 (1994) 835–843.

[38] B. Van den Berg, W.M. Clemons Jr., I. Collinson, Y. Modis, E. Hartmann, S.C. Harrison, T.A. Rapoport, X-ray structure of a protein-conducting channel, Nature 427 (2004) 36–44.
[39] H. Vogel, S. Fischer, K. Valentin, A model for the evolution of the plastid sec apparatus inferred from secY gene phylogeny, Plant Mol. Biol. 32 (1996) 685–692.
[40] B.C. Berks, F. Sargent, T. Palmer, The Tat protein export pathway, Mol. Microbiol. 35 (2000) 260–274.
[41] R. Voelker, A. Barkan, Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid, EMBO J. 14 (1995) 3905–3914.
[42] M.B. Walker, L.M. Roy, E. Coleman, R. Voelker, A. Barkan, The maize tha4 gene functions in sec-independent protein transport in chloroplasts and is related to hcf106, tatA, and tatB, J. Cell Biol. 147 (1999) 267–276.
[43] E.G. Bogsch, F. Sargent, N.R. Stanley, B.C. Berks, C. Robinson, T. Palmer, An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria, J. Biol. Chem. 273 (1998) 18003–18006.
[44] A. Kuhn, R. Stuart, R. Henry, R.E. Dalbey, The Alb3/Oxa1/YidC protein family: membrane-localized chaperones facilitating membrane protein insertion? Trends Cell Biol. 13 (2003) 510–516.
[45] M.M. Mackle, B.A. Zilinskas, Role of signal peptides in targeting of proteins in cyanobacteria, J. Bacteriol. 176 (1994) 1857–1864.
[46] E. Spence, M. Sarcina, N. Ray, S.G. Moller, C.W. Mullineaux, C. Robinson, Membrane-specific targeting of green fluorescent protein by the Tat pathway in the cyanobacterium *Synechocystis* PCC6803, Mol. Microbiol. 48 (2003) 1481–1489.
[47] E. Zak, B. Norling, R. Maitra, F. Huang, B. Andersson, H.B. Pakrasi, The initial steps of biogenesis of cyanobacterial photosystems occur in plasma membranes, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 13443–13448.
[48] D.J. Schnell, Protein targeting to the thylakoid membrane, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 97–126.
[49] X. Chen, D.J. Schnell, Protein import into chloroplasts, Trends Cell Biol. 9 (1999) 222–227.
[50] D.J. Schnell, D.N. Hebert, Protein translocons: multifunctional mediators of protein translocation across membranes, Cell 112 (2003) 491–505.
[51] P. Jarvis, J. Soll, Toc, tic, and chloroplast protein import, Biochim. Biophys. Acta 1590 (2002) 177–189.
[52] F. Kessler, G. Blobel, H.A. Patel, D.J. Schnell, Identification of two GTP-binding proteins in the chloroplast protein import machinery, Science 266 (1994) 1035–1039.
[53] S. Hirsch, E. Muckel, F. Heemeyer, G. von Heijne, J. Soll, A receptor component of the chloroplast protein translocation machinery, Science 266 (1994) 1989–1992.
[54] S. Reumann, K. Keegstra, The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes, Trends Plant Sci. 4 (1999) 302–307.
[55] K. Keegstra, J.E. Froehlich, Protein import into chloroplasts, Curr. Opin. Plant Biol. 2 (1999) 471–476.
[56] A. Hiltbrunner, J. Bauer, P.A. Vidi, S. Infanger, P. Weibel, M. Hohwy, F. Kessler, Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane, J. Cell Biol. 154 (2001) 309–316.
[57] T. Becker, M. Jelic, A. Vojta, A. Radunz, J. Soll, E. Schleiff, Preprotein recognition by the Toc complex, EMBO J. 23 (2004) 520–530.
[58] T.R. Wallas, M.D. Smith, S. Sanchez-Nieto, D.J. Schnell, The roles of toc34 and toc75 in targeting the toc159 preprotein receptor to chloroplasts, J. Biol. Chem. 278 (2003) 44289–44297.
[59] E.J. Summer, K. Cline, Red bell pepper chromoplasts exhibit in vitro import competency and membrane targeting of passenger proteins from the thylakoidal sec and DeltapH pathways but not the chloroplast signal recognition particle pathway, Plant Physiol. 119 (1999) 575–584.
[60] J. Joyard, A. Billecocq, S.G. Bartlett, M.A. Block, N.H. Chua, R. Douce, Localization of polypeptides to the cytosolic side of the outer envelope membrane of spinach chloroplasts, J. Biol. Chem. 258 (1983) 10000–10006.
[61] B. Bolter, J. Soll, A. Schulz, S. Hinnah, R. Wagner, Origin of a chloroplast protein importer, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 15831–15836.
[62] S. Reumann, J. Davila-Aponte, K. Keegstra, The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 784–789.
[63] D.J. Schnell, F. Kessler, G. Blobel, Isolation of components of the chloroplast protein import machinery, Science 266 (1994) 1007–1012.
[64] S.C. Hinnah, R. Wagner, N. Sveshnikova, R. Harrer, J. Soll, The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides, Biophys. J. 83 (2002) 899–911.
[65] I. Gentle, K. Gabriel, P. Beech, R. Waller, T. Lithgow, The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria, J. Cell Biol. 164 (2004) 19–24.
[66] L. Heins, A. Mehrle, R. Hemmler, R. Wagner, M. Kuchler, F. Hormann, D. Sveshnikov, J. Soll, The preprotein conducting channel at the inner envelope membrane of plastids, EMBO J. 21 (2002) 2616–2625.
[67] F. Kessler, G. Blobel, Interaction of the protein import and folding machineries of the chloroplast, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7684–7689.
[68] M. Kuchler, S. Decker, F. Hormann, J. Soll, L. Heins, Protein import into chloroplasts involves redox-regulated proteins, EMBO J. 21 (2002) 6136–6145.
[69] A. Caliebe, R. Grimm, G. Kaiser, J. Lubeck, J. Soll, L. Heins, The chloroplastic protein import machinery contains a Rieske-type iron–sulfur cluster and a mononuclear iron-binding protein, EMBO J. 16 (1997) 7342–7350.
[70] F. Hormann, M. Kuchler, D. Sveshnikov, U. Oppermann, Y. Li, J. Soll, Tic32, an essential component in chloroplast biogenesis, J. Biol. Chem. (2004) (Electronic publication ahead of print).
[71] A. Kouranov, X. Chen, B. Fuks, D.J. Schnell, Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane, J. Cell Biol. 143 (1998) 991–1002.
[72] X. Chen, M.D. Smith, L. Fitzpatrick, D.J. Schnell, In vivo analysis of the role of atTic20 in protein import into chloroplasts, Plant Cell 14 (2002) 641–654.
[73] J. Soll, Protein import into chloroplasts, Curr. Opin. Plant Biol. 5 (2002) 529–535.
[74] D.J. Schnell, G. Blobel, Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites, J. Cell Biol. 120 (1993) 103–115.
[75] M. Matsuzaki, O. Misumi, I.T. Shin, S. Maruyama, M. Takahara, S.Y. Miyagishima, T. Mori, K. Nishida, F. Yagisawa, Y. Yoshida, Y. Nishimura, S. Nakao, T. Kobayashi, Y. Momoyama, T. Higashiyama, A. Minoda, M. Sano, H. Nomoto, K. Oishi, H. Hayashi, F. Ohta, S. Nishizaka, S. Haga, S. Miura, T. Morishita, Y. Kabeya, K. Terasawa, Y. Suzuki, Y. Ishii, S. Asakawa, H. Takano, N. Ohta, H. Kuroiwa, K. Tanaka, N. Shimizu, S. Sugano, N. Sato, H. Nozaki, N. Ogasawara, Y. Kohara, T. Kuroiwa, Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D, Nature 428 (2004) 653–657.
[76] M. Desjardins, ER-mediated phagocytosis: a new membrane for new functions, Nat. Rev., Immunol. 3 (2003) 280–291.
[77] T. Cavalier-Smith, Genomic reduction and evolution of novel genetic membranes and protein-targeting machinery in eukaryote–eukaryote chimaeras (meta-algae), Philos. Trans. R. Soc. Lond., B Biol. Sci. 358 (2003) 109–133 (discussion 133–4).
[78] L. Heins, J. Soll, Chloroplast biogenesis: mixing the prokaryotic and the eukaryotic? Curr. Biol. 8 (1998) R215–R217.

[79] N. Kubo, K. Harada, A. Hirai, K. Kadowaki, A single nuclear transcript encoding mitochondrial RPS14 and SDHB of rice is processed by alternative splicing: common use of the same mitochondrial targeting signal for different proteins, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 9207–9211.
[80] K.L. Adams, D.O. Daley, Y.L. Qiu, J. Whelan, J.D. Palmer, Repeated, recent and diverse transfers of a mitochondrial gene to the nucleus in flowering plants, Nature 408 (2000) 354–357.
[81] P.E. Thorsness, T.D. Fox, Escape of DNA from mitochondria to the nucleus in *Saccharomyces cerevisiae*, Nature 346 (1990) 376–379.
[82] C.Y. Huang, M.A. Ayliffe, J.N. Timmis, Direct measurement of the transfer rate of chloroplast DNA into the nucleus, Nature 422 (2003) 72–76.
[83] M. Long, S.J. de Souza, C. Rosenberg, W. Gilbert, Exon shuffling and the origin of the mitochondrial targeting function in plant cytochrome c_1 precursor, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7727–7731.
[84] F.P. Wolter, C.C. Fritz, L. Willmitzer, J. Schell, P.H. Schreier, rbcS genes in *Solanum tuberosum*: conservation of transit peptide and exon shuffling during evolution, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 846–850.
[85] K. Keegstra, Transport and routing of proteins into chloroplasts, Cell 56 (1989) 247–253.
[86] K.E. Apt, N.E. Hoffman, A.R. Grossman, The gamma subunit of R-phycoerythrin and its possible mode of transport into the plastid of red algae, J. Biol. Chem. 268 (1993) 16208–16215.
[87] J. Jakowitsch, C. Neumann-Spallart, Y. Ma, J. Steiner, H.E. Schenk, H.J. Bohnert, W. Loffelhardt, In vitro import of pre-ferredoxin-NADP+-oxidoreductase from *Cyanophora paradoxa* into cyanelles and into pea chloroplasts, FEBS Lett. 381 (1996) 153–155.
[88] N. Nassoury, M. Cappadocia, D. Morse, Plastid ultrastructure defines the protein import pathway in dinoflagellates, J. Cell. Sci. 116 (2003) 2867–2874.
[89] M. Lang, K.E. Apt, P.G. Kroth, Protein transport into "complex" diatom plastids utilizes two different targeting signals, J. Biol. Chem. 273 (1998) 30973–30978.
[90] B.J. Foth, S.A. Ralph, C.J. Tonkin, N.S. Struck, M. Fraunholz, D.S. Roos, A.F. Cowman, G.I. McFadden, Dissecting apicoplast targeting in the malaria parasite *Plasmodium falciparum*, Science 299 (2003) 705–708.
[91] S. Douglas, S. Zauner, M. Fraunholz, M. Beaton, S. Penny, L.T. Deng, X. Wu, M. Reith, T. Cavalier-Smith, U.G. Maier, The highly reduced genome of an enslaved algal nucleus, Nature 410 (2001) 1091–1096.
[92] T. Osafune, A. Yokota, S. Sumida, E. Hase, Immunogold localization of Ribulose-1,5-bisphosphate carboxylase with reference to pyrenoid morphology in chloroplasts of synchronized *Euglena gracilis* cells, Plant Physiol. 92 (1990) 802–808.
[93] R. Kishore, U.S. Muchhal, S.D. Schwartzbach, The presequence of Euglena LHCPII, a cytoplasmically synthesized chloroplast protein, contains a functional endoplasmic reticulum-targeting domain, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 11845–11849.
[94] C. Sulli, S.D. Schwartzbach, A soluble protein is imported into Euglena chloroplasts as a membrane-bound precursor, Plant Cell 8 (1996) 43–53.
[95] C. Sulli, Z. Fang, U. Muchhal, S.D. Schwartzbach, Topology of Euglena chloroplast protein precursors within endoplasmic reticulum to Golgi to chloroplast transport vesicles, J. Biol. Chem. 274 (1999) 457–463.
[96] T. Kirchhausen, J.S. Bonifacino, H. Riezman, Linking cargo to vesicle formation: receptor tail interactions with coat proteins, Curr. Opin. Cell Biol. 9 (1997) 488–495.
[97] S.P. Gibbs, The route of entry of cytoplasmically synthesized proteins into chloroplasts of algae possessing chloroplast ER, J. Cell. Sci. 35 (1979) 253–266.
[98] P.G. Kroth, Protein transport into secondary plastids and the evolution of primary and secondary plastids, Int. Rev. Cyt. 221 (2002) 191–255.
[99] K. Ishida, T. Cavalier-Smith, B.R. Green, Endomembrane structure and the chloroplast protein targeting pathway in *Heterosigma akashiwo* (Raphidophyceae, Chromista), J. Phycol. 36 (2000) 1135–1144.
[100] K.E. Apt, L. Zaslavkaia, J.C. Lippmeier, M. Lang, O. Kilian, R. Wetherbee, A.R. Grossman, P.G. Kroth, In vivo characterization of diatom multipartite plastid targeting signals, J. Cell. Sci. 115 (2002) 4061–4069.
[101] S.D. Schwartzbach, T. Osafune, W. Loffelhardt, Protein import into cyanelles and complex chloroplasts, Plant Mol. Biol. 38 (1998) 247–263.
[102] D. Bhaya, A. Grossman, Targeting proteins to diatom plastids involves transport through an endoplasmic reticulum, Mol. Gen. Genet. 229 (1991) 400–404.
[103] J.B. Helms, J.E. Rothman, Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF, Nature 360 (1992) 352–354.
[104] J. Wastl, U.G. Maier, Transport of proteins into cryptomonads complex plastids, J. Biol. Chem. 275 (2000) 23194–23198.
[105] G.I. McFadden, G.G. van Dooren, Evolution: red algal genome affirms a common origin of all plastids, Curr. Biol. 14 (2004) R514–R516.
[106] G.G. van Dooren, S.D. Schwartzbach, T. Osafune, G.I. McFadden, Translocation of proteins across the multiple membranes of complex plastids, Biochim. Biophys. Acta 1541 (2001) 34–53.
[107] P.R. Gilson, G.I. McFadden, Good things in small packages: the tiny genomes of chlorarachniophyte endosymbionts, BioEssays 19 (1997) 167–173.
[108] P.R. Gilson, G.I. McFadden, Jam packed genomes—a preliminary, comparative analysis of nucleomorphs, Genetica 115 (2002) 13–28.
[109] S. Kohler, C.F. Delwiche, P.W. Denny, L.G. Tilney, P. Webster, R.J. Wilson, J.D. Palmer, D.S. Roos, A plastid of probable green algal origin in Apicomplexan parasites, Science 275 (1997) 1485–1489.
[110] G.I. McFadden, M.E. Reith, J. Munholland, N. Lang-Unnasch, Plastid in human parasites, Nature 381 (1996) 482.
[111] R.F. Waller, P.J. Keeling, R.G. Donald, B. Striepen, E. Handman, N. Lang-Unnasch, A.F. Cowman, G.S. Besra, D.S. Roos, G.I. McFadden, Nuclear-encoded proteins target to the plastid in Toxoplasma gondii and *Plasmodium falciparum*, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 12352–12357.
[112] S.A. Ralph, G.G. Van Dooren, R.F. Waller, M.J. Crawford, M.J. Fraunholz, B.J. Foth, C.J. Tonkin, D.S. Roos, G.I. McFadden, Tropical infectious diseases: metabolic maps and functions of the *Plasmodium falciparum* apicoplast, Nat. Rev., Microbiol. 2 (2004) 203–216.
[113] Y. Van de Peer, S.A. Rensing, U.G. Maier, R. De Wachter, Substitution rate calibration of small subunit ribosomal RNA identifies chlorarachniophyte endosymbionts as remnants of green algae, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7732–7736.
[114] R.F. Waller, M.B. Reed, A.F. Cowman, G.I. McFadden, Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway, EMBO J. 19 (2000) 1794–1802.
[115] G.G. van Dooren, R.F. Waller, K.A. Joiner, D.S. Roos, G.I. McFadden, Traffic jams: protein transport in *Plasmodium falciparum*, Parasitol. Today 16 (2000) 421–427.
[116] A. DeRocher, C.B. Hagen, J.E. Froehlich, J.E. Feagin, M. Parsons, Analysis of targeting sequences demonstrates that trafficking to the Toxoplasma gondii plastid branches off the secretory system, J. Cell. Sci. 113 (Pt 22) (2000) 3969–3977.
[117] K.A. Joiner, D.S. Roos, Secretory traffic in the eukaryotic parasite Toxoplasma gondii: less is more, J. Cell Biol. 157 (2002) 557–563.