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# Protein Targeting into Secondary Plastids[1]

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ABSTRACT. Most of the coding capacity of primary plastids is reserved for expressing some central components of the photosynthesis machinery and the translation apparatus. Thus, for the bulk of biochemical and cell biological reactions performed within the primary plastids, many nucleus-encoded components have to be transported posttranslationally into the organelle. The same is true for plastids surrounded by more than two membranes, where additional cellular compartments have to be supplied with nucleus-encoded proteins, leading to a corresponding increase in complexity of topogenic signals, transport and sorting machineries. In this review, we summarize recent progress in elucidating protein transport across up to five plastid membranes in plastids evolved in secondary endosymbiosis. Current data indicate that the mechanisms for protein transport across multiple membranes have evolved by altering pre-existing ones to new requirements in secondary plastids.

**Key Words.** Protein transport, secondarily evolved plastids, secondary endosymbiosis.

MANY proteins synthesized in the cytoplasm are posttranslationally directed to their final destination by crossing membranes, which surround specific compartments. All of them have to be synthesized as preproteins with one or more topogenic signals, which act like addresses for identification and targeting by specific receptors. The complexes formed in this process are directed to the target compartment and then transported by way of compartment-specific translocons. During symbiogenesis, new compartments originated. As in mitochondria and plastids, symbiont nucleic acids were mixed intracellularly. Transport of nucleus-encoded proteins into the symbiont is a prerequisite for the maintenance of the partnership. In this review we summarize our knowledge of protein transport into different types of plastids and discuss whether the identified protein transport mechanisms can be used to reflect the phylogeny of phototrophic eukaryotes.

## PRIMARY EVOLVED PLASTIDS

The primary plastid derives its energy from solar radiation and serves its host as a powerhouse. It originated from an engulfed cyanobacteria-like cell, which was reduced to an organelle within a eukaryotic host cell. As a result of this evolution and reduction, its plastidial envelope is composed of two membranes, a feature characteristic of the plastids from red and green algae, land plants, and glaucophytes (Hempel et al. 2007). After this incorporation of the cyanobacteria-like cell into its eukaryotic host, the majority of the symbiont's coding capacity receded either by gene loss or by transfer of genetic material into the nuclear genome (Martin et al. 2002; Timmis et al. 2004). In the latter case, the reading frames mostly came to be equipped with an N-terminally located extension in their new genetic compartment, i.e. the transit peptide, which enables the re-import of the encoded proteins across the two membranes of primary plastids. The mechanistic and structural foundations of protein import into primary plastids have been investigated in land plants in detail. Research indicates that two translocons, translocator of the outer chloroplast membrane (Toc) and translocator of the inner chloroplast membrane (Tic), which are inserted into the outer and inner plastid membrane, respectively, are the major players for crossing the membrane barrier (Becker et al. 2004; Kessler and Schnell 2006; May and Soll 2000). After entering the stroma, the transit peptide is cleaved off and the proteins are targeted to their final subcompartimental destination. The underlying mechanisms and recent models of protein import into chloroplasts have recently been reviewed by Jarvis and Robinson (2004), Soll and Schleiff (2004) and Kessler and Schnell (2006).

Besides import mechanisms mediated by the Toc and Tic machinery, targeting to the plastids has also been shown to first be directed via the secretory pathway and subsequent sorting steps to the plastid (Villarejo et al. 2005). Therefore, at least two different import pathways exist and it has yet to be determined whether components such as the Tic translocon are used for both protein import mechanisms.

Homologs of the Toc and Tic translocons of land plants are also present in red algae (McFadden and van Dooren 2004). This suggests that the common ancestor of the Archaeplastida (i.e. green algae, land plants, red algae, and glaucophytes [Adl et al. 2005]) may have developed the protein transport systems for crossing two plastid membranes, and these have been retained in most modern organisms.

## SECONDARILY EVOLVED PLASTIDS

Other important algal groups harbor plastids surrounded by three or four membranes, the so-called complex or secondary plastids (McFadden 2001; Stoebe and Maier 2002). It is now generally accepted that secondary plastids evolved in a phagotrophic host by the incorporation of a phototrophic eukaryotic cell some time after the above-mentioned primary endosymbiosis. The subsequent reduction of the ''ingested'' eukaryote led to a plastid, surrounded by more than two membranes (Fig. 1).

At least three independent endosymbiotic events led to the formation of different groups of organisms with secondary plastids. Euglenophytes and chlorarachniophytes acquired their complex plastids from two independent incorporations of a green alga (Rogers et al. 2007). Another group has been united into the subclade of chromalveolates, including the heterokontophytes, haptophytes, cryptophytes, apicomplexans, and the peridinin-containing dinoflagellates (Cavalier-Smith 1999, 2003). All of these harbor a symbiont of red algal origin, which evolved from a single endosymbiotic event according the chromalveolate hypothesis (Archibald and Keeling 2002; Harper, Waanders, and Keeling. 2005; Rogers et al. 2006). In addition to organisms characterized by a secondary plastid, other groups, such as oomycetes and ciliates, may have acquired their plastid with the rest of the chromalveolates but subsequently lost it (Archibald 2008; Cavalier-Smith 2002; Reyes-Prieto, Moustafa, and Bhattacharya 2008; Tyler et al. 2006).

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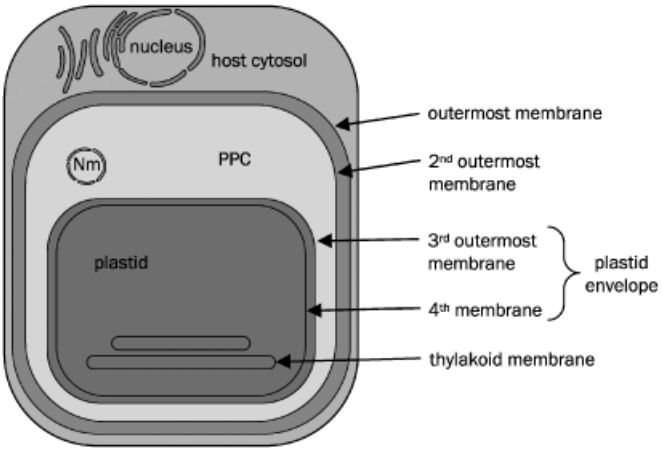


Fig. **1.** Schematic depiction of the membrane topology of an organism harboring a secondary plastid with four membranes. The complex plastid is surrounded by four membranes. The outermost membrane is consistent to the phagosomal membrane, which is interconnected with the host's ER in the cases of cryptophytes, heterokontophytes, and haptophytes. The converted plasma membrane of the enslaved alga is depicted as the second outermost membrane. Cryptophytes and chlorarachniophytes have retained the reduced cytoplasm (PPC) and the vestigial nucleus, i.e. nucleomorph (Nm), of the eukaryotic endosymbiont. The remaining membranes, the third outermost and the fourth membrane, may well correspond to the plastid envelope of the primary plastid of the eukaryotic symbiont.

An intermediary state of the evolution of the secondary endosymbiont within the eukaryotic host cell is found in cryptophytes and chlorarachniophytes. The ''plastids'' of these organisms maintain a vestigial cell nucleus, the nucleomorph, located in a remnant cytoplasm of the eukaryotic endosymbiont (Maier, Douglas, and Cavalier-Smith 2000). This compartment, the so-called periplastidal compartment (PPC), is located between the outer and inner pairs of membranes in the secondary plastid (Greenwood, Griffiths, and Santore 1977; Hibbert and Norris 1984) (Fig. 1).

Most of the plastid proteome of secondary plastids, as in primary plastids, is encoded in the host's nuclear genome (Armbrust et al. 2004; Douglas et al. 2001; Gilson et al. 2006; Rogers et al. 2006, 2007). Thus, hundreds of proteins needed for essential plastid functions must cross either three or four membranes to reach their final destination in the plastid stroma. The thylakoid membrane can be considered an additional membrane barrier, if the protein is destined for the thylakoid lumen. Thus, although protein import into complex plastids is much more complicated than import into primary plastids, recent investigations into the cellular mechanisms involved have nonetheless resulted in a preliminary albeit incomplete picture of the evolution of these import machineries.

## PROTEIN IMPORT INTO PLASTIDS I: CROSSING THREE MEMBRANES

Phototrophic euglenophytes and peridinin-containing dinoflagellates possess independently acquired plastids surrounded by three membranes. Euglenophytes harbor a secondary endosymbiont of green algal origin, whereas the peridinin-containing dinoflagellates integrated a symbiont of red algal origin, most likely via secondary endosymbiosis (Bachvaroff, Sanchez Puerta, and Delwiche 2005; Yoon, Hackett, and Bhattacharya 2002). Nevertheless, the similar morphological architecture of their plastids may also require analogous transport mechanisms (Nassoury, Cappadocia, and Morse 2003). For both groups of organisms, pre-protein classification is generally divided into three classes (Durnford and Gray 2006; Patron et al. 2005). Class I pre-proteins have an N-terminal topogenic signal consisting of a signal peptide at the N-terminus, followed by a transit peptide and a hydrophobic region (the stop transfer sequence) in front of the coding sequence for the mature protein. Class II pre-proteins, however, contain a bipartite signal sequence without the hydrophobic region. Despite the similarities in their sequences, pre-proteins crossing the thylakoid membrane, in addition to the three plastid-surrounding membranes, seemingly belonging to a third group, are nonetheless classified as Class Ib proteins in euglenophytes and Class III proteins in dinoflagellates (Durnford and Gray 2006; Patron et al. 2005). Proteins related to these latter classes possess an additional region, which seems to be required for import into thylakoids in euglenophytes at the least (Inagaki et al. 2000).

Class I proteins from dinoflagellates and euglenophytes were investigated for the initial steps of transport across the first plastid membrane (Inagaki et al. 2000; Nassoury et al. 2003; Slavikova et al. 2005; Sulli et al. 1999). These studies showed that the first step in both groups of organisms is co-translational transport into the ER lumen. The additional hydrophobic region would seem to be essential as a stop-transfer signal, anchoring the pre-protein in the ER-membrane. Further transport of Class I pre-proteins involves the Golgi apparatus, from which vesicles carrying the plastid pre-proteins bud off. The vesicles subsequently fuse with the outermost membrane of the plastid, most likely doing so independently of soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNAREs) in euglenophytes (Slavikova et al. 2005).

Import experiments of Class I and Class II proteins (i.e. precursors lacking the signal peptide) from euglenophytes and dinoflagellates into pea chloroplasts (Chaal and Green 2005; Inagaki et al. 2000) led to the conclusion that the transport across the plastid envelop as well as the intraplastidal transport may be directly comparable to the situation in primary plastids.

## PROTEIN IMPORT INTO PLASTIDS II: CROSSING FOUR MEMBRANES

The plastids of chlorarachniophytes, heterokontophytes, haptophytes, cryptophytes, and apicomplexa are surrounded by four membranes (Fig. 1). As in euglenophytes and peridinin-containing dinoflagellates, nucleus-encoded plastid proteins are expressed in these organisms as pre-proteins, which differ from the mature protein by an N-terminal bipartite topogenic signal sequence (BTS), composed of a signal peptide followed by a transit peptide (Apt et al. 2002; DeRocher et al. 2000; Gould et al. 2006a; Kilian and Kroth 2005; Lang, Apt, and Kroth 1998; Rogers et al. 2004; Waller et al. 1998, 2000; Wastl and Maier 2000).

With respect to morphology of the secondarily evolved plastids with four surrounding membranes, one major difference within the groups is the outermost membrane. In haptophytes, heterokontophytes, and cryptophytes, this membrane is studded with 80S ribosomes and interconnected with the host's ER membrane, which is not the case in the apicomplexans and chlorarachniophytes, indicating possible differences in the first steps of protein import (Fig. 2). Experimental data on the apicomplexans, heterokontophytes, and cryptophytes supports this postulate as discussed below.

## PROTEIN TRANSPORT ACROSS THE OUTERMOST MEMBRANE

*Plasmodium falciparum* and *Toxoplasma gondii* are the best-studied apicomplexans with respect to protein import into their

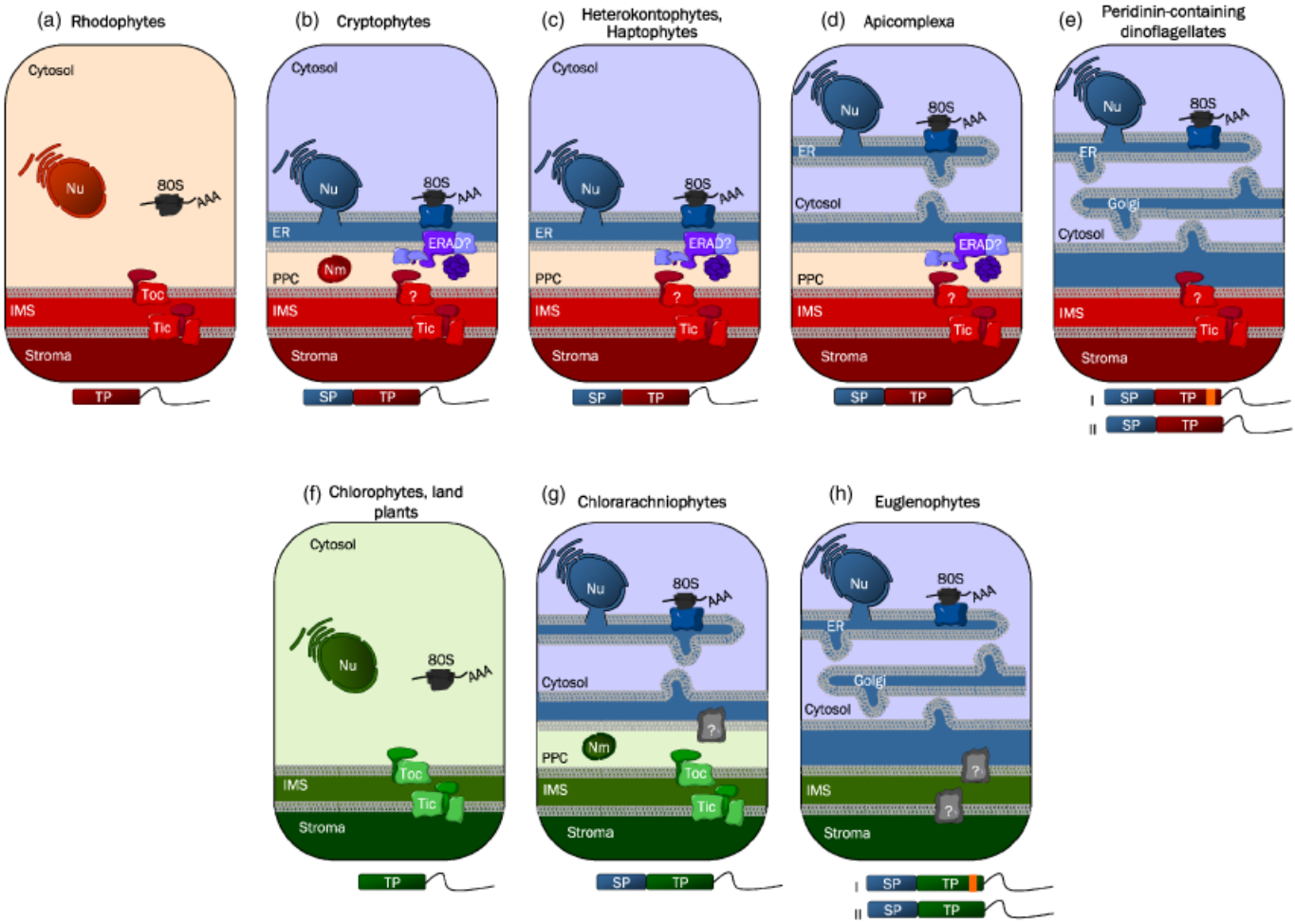


Fig. **2.** Schematic depiction of nucleus-encoded proteins targeting into different types of primary and secondary plastids. Synthesis of plastid proteins, encoded in the host's nucleus (Nu), begins in the host's cytoplasm. To reach their final destination, proteins are expressed as pre-proteins, possessing N-terminal extensions. These seem to function somewhat like an ''address'' to send the proteins to their destined compartment. Nucleus-encoded plastid proteins destined to be transported into a primary plastid have a transit peptide (TP) as topogenic signal in most cases, which is necessary for translocation across the plastid envelope by the translocator of the outer chloroplast membrane (Toc)/translocator of the inner chloroplast membrane (Tic) machinery (a, f). In the case of secondary plastids derived from red algal endosymbionts and green algal endosymbionts, nucleus-encoded proteins possess a bipartite topogenic signal (BTS), composed of a signal peptide (SP) followed by a transit peptide (TP) (b–e, g, h). **a–e:** Protein targeting into secondary plastids of red algal origin. The synthesis of nucleus-encoded stromal proteins starts at the ER-membrane. This compartment builds a continuum with the outermost membrane of the complex plastid in cryptophytes, heterokontophytes, and haptophytes. Therefore, transport across this membrane occurs co-translationally via the Sec61 complex (b, c). In the ER-lumen (ER), cleavage of the signal peptide occurs, leading to the exposure of the transit peptide for further targeting. Proteins of the complex plastids in apicomplexans and peridinin-containing dinoflagellates are transported via vesicles from the ER to the outermost membrane of the plastid, which has not been identified as being interconnected with the ER system of the host. In peridinin-containing dinoflagellates, the Golgi apparatus seems to be part of the vesicle transport (e), whereas in apicomplexans the transport might be directly from the ER (d). Transport across the second outermost membrane most probably occurs by way of an ER-associated degradation (ERAD)-derived system (b–d). A possible exception might be the peridinin-containing dinoflagellates, where the equivalent membrane was probably lost. Proteins transported from the periplastid compartment (PPC) with its nucleomorph (Nm) must cross the plastid envelope and the intermembrane space (IMS) with the help of the Tic/Toc machinery. Due to the fact that Toc components have yet to be found in any of these organisms, a still unknown mechanism for transport across the third outermost membrane might exist. **f–h**. Protein targeting into secondary plastids of green algal origin. Like those with red algal origin, targeting of nucleus-encoded stromal proteins starts cotranslationally into the ER. Within the ER lumen, cleavage of the signal peptide occurs and the pre-proteins are transported to the outermost membrane via vesicles. In chlorarachniophytes this pathway might be Golgi-independent as it is suggested for apicomplexans (g). In contrast, stromal proteins in euglenophytes cross this compartment before being transported to the outermost membrane of the plastid (h). Transport across the second outermost membrane is completely unclear in chlorarachniophytes, whereas in euglenophytes this membrane was probably lost. Crossing the plastid envelope (i.e. the third outermost and the fourth membrane) probably involves a Toc/Tic translocon machinery in chlorarachniophytes. In contrast, transport across the inner two membranes of the three-membrane-bound plastids in euglenophytes remains completely unknown. For further details please see text.

plastid, the apicoplast. Initial studies demonstrated that targeting signals fused to green fluorescent protein (GFP) are transported to the correct sub-cellular compartments (Waller et al. 1998). In vivo localisation studies showed that the signal peptide in the BTS of nucleus-encoded apicoplast proteins directs GFP to the secretory system, whereas the entire BTS (consisting of signal and transit peptide) leads to an apicoplast localization (Waller et al. 1998, 2000). These experiments demonstrated that the signal peptide is

necessary for protein import into the plastid compartment. The most likely model for the first steps in protein import is that the proteins first enter the secretory pathway. Support for this from recent experiments on *P. falciparum* suggest that the route of apicoplast trafficking is ER-mediated, after which vesicles bud off and are transported to the outermost membrane of the apicoplast without the participation of the Golgi-apparatus (Tonkin et al. 2006). A competing model suggests that the apicoplast itself resides within the ER lumen, implying that discrimination of apicoplast proteins from other secretory proteins might occur at the second outermost apicoplast membrane (Tonkin, Kalanon, and McFadden 2008). In this case, all secretory proteins would have to pass across the first apicoplast membrane.

In cryptophytes, haptophytes, and heterokontophytes, the outermost membrane is continuous with the host's ER, denoted by the occurrence of 80S ribosomes on the plasmatic face (Fig. 2). Nucleus-encoded plastid proteins must therefore be imported co-translationally into the lumen of the ER, a hypothesis, which was subsequently proven by in vitro and in vivo experiments. In vitro import assays were carried out using canine microsomes to mimic this import into the ER lumen (Bhaya and Grossman 1991; Gould et al. 2006a; Lang et al. 1998; Lang and Kroth 2001; Wastl and Maier 2000). This determined that the signal peptide is the imperative component of the BTS for microsomal import, which was further confirmed by in vivo experiments, in which signal peptides were fused to GFP and shown to be targeted to the ER-lumen or to the secretory system (Apt et al. 2002; Gould et al. 2006b Kilian and Kroth 2005). In the ER lumen, the signal peptide is cleaved off, thus exposing the transit peptide. All these experiments were performed with BTS sequences from heterokontophytes, cryptophytes, and apicomplexans, albeit not from a haptophytic source, but a similar result could be expected, because the morphology of the outer plastid membrane of haptophytes is similar to that of heterokontophytes and cryptophytes. Signal peptides of nucleus-encoded plastid proteins from haptophytes can be detected by commonly used search algorithms, such as SignalP (http://www.cbs.dtu.dk/services/SignalP/). Thus, one would expect that plastid proteins of haptophytes are also co-translationally transported across the outermost membrane.

Dissection of transport mechanisms in chlorarachniophytes would be very interesting, because these are the only known organisms with plastids of green algal origin surrounded by four membranes. Because of the occurrence of BTS sequences in nucleus-encoded plastid proteins (Rogers et al. 2004) and the morphology of the outermost plastid membrane, which is not connected with internal host membranes, one can assume that plastid import is initiated at the ER as it is in the apicomplexa (Fig. 2).

## CROSSING THE SECOND OUTERMOST MEMBRANE

All nucleus-encoded proteins of organisms with secondary plastids are characterized by an N-terminal bipartite targeting signal sequence within the pre-protein. As shown above, the signal peptide is most likely involved in co-translational import into the ER or the lumen between the outermost and second outermost membrane. Analogous to chloroplast import in seed plants (Jarvis and Robinson 2004; Kessler and Schnell 2006; Soll and Schleiff 2004; Stengel, Soll, and Bolter 2007), the transit peptide of the BTS should be necessary for crossing the plastid envelope. Thus, according to this model, there is no topogenic signal in nucleus-encoded plastid pre-proteins known to specify protein transport across the second outermost membrane. Several hypotheses were developed to explain the possible mechanisms of transport across the second outermost membrane, ranging from vesicle-mediated transport of proteins (Gibbs 1981; Kilian and Kroth 2005) to transport by an unknown pore (Kroth and Strotmann 1999). In addition, a relocated plastid or mitochondrial translocon component has been proposed to mediate protein transport across the second outermost membrane (Bodyl 2004; Cavalier-Smith 1999). However, genome projects of algae with a red algal secondary endosymbiont did not reveal any genes encoding relocated translocons of plastidal or mitochondrial origin (McFadden and van Dooren 2004).

Cryptophytes have played an important role in explaining secondary endosymbiosis. They harbor the remnant cytoplasm of the secondary symbiont; this PPC includes a small, vestigial nucleus, the nucleomorph, between the outer and inner membrane pairs of the plastid (Maier et al. 2000) (Fig. 2). A genome project on the nucleomorph genome of one representative organism indicated that this genome is highly reduced in size and gene content (Douglas et al. 2001; Zauner et al. 2000). Moreover, several genes encoding proteins essential for different PPC tasks (e.g. the maintenance of the nucleomorph and its expression machinery), are missing in the nucleomorph genome and were thus expected to be encoded on the host's nuclear genome.

To summarize, the following two groups of plastid proteins for the symbiont must be transported across the plastid membranes (Hempel et al. 2007): one group crossing at least four membranes to reach the stroma and the other traversing only two membranes on its way into the PPC. An inspection of the latter group demonstrated that these proteins are also encoded as pre-proteins with an N-terminal bipartite topogenic signal sequence, thus far only known in nucleus-encoded stromal proteins (Gould et al. 2006a, b). This means that not only nucleus-encoded stromal proteins but also proteins located in the PPC are equipped with both a signal peptide and a transit peptide. It was quite surprising to find that transit peptides appear to be essential for transport across the second outermost membrane, although they were only expected to mediate transport across the third outermost and the fourth membrane, which are most likely homologous to the primary plastid envelope. This feature of the cryptophyte transit peptide has since been noted in other nucleus-encoded PPC located proteins from other chromalveolates, like the heterokontophyte *Plasmodium tricornutum* and the apicomplexan *P. falciparum* (Sommer et al. 2007) (see later sections for details). Very recently, the Keeling lab investigated nucleus-encoded PPC proteins from chlorarachniophytes. Interestingly, they identified this group of proteins as using a bipartite targeting sequence with a composition similar to those from chromalveolates (Gile and Keeling 2008). Thus, targeting proteins into the PPC seems to utilize similar mechanisms in chlorarachniophytes and chromalveolates, indicating that like in the case of euglenids and dinoflagellates protein transport reflects cell biology but not phylogeny.

Because both PPC- and stroma-located proteins seem to share the same topogenic signal composition, the discrimination between both has to occur in the PPC. Recently one distinguishing feature was shown to be the first amino acid of the transit peptide of cryptophytes. Stromal proteins possess in most cases an aromatic amino acid at the +1-position, whereas PPC-located proteins seldomly bear such an aromatic amino acid (Gould et al. 2006a). Moreover, in vivo tests have demonstrated that this criterion can also be directly applied to diatoms and partially to apicomplexans (Gould et al. 2006b; Gruber et al. 2007; Sommer et al. 2007). Thus, the PPC has important functions not only in cryptophytes, but also in other chromalveolates, although their PPC is even more reduced than in cryptophytes. Despite these observations on diverse organisms, the aromatic amino acid at position +1 of the transit peptides in haptophytes seems not to be as conserved as it is in other chromalveolates (Patron and Waller 2007).

Cavalier-Smith (2003) proposed that a relocated Toc could be the translocon in the second outermost membrane. The identifi-

cation of a BTS in all known symbiont-targeted proteins is consistent with this hypothesis (Gould et al. 2006a). However, screening the genomic data of cryptophytes, diatoms, and apicomplexans has not yet identified any relocated Toc components (McFadden and van Dooren 2004). This suggests several explanations: that either all Toc components were lost, that appropriate components are just not present in the available genomic data, or that Toc components of secondary plastids evolved in such a way that they cannot be identified with the genomic analysis methods commonly used.

Once again, studies on cryptophytes generated new data, explaining how nucleus-encoded proteins may manage the transport across the second outermost membrane (Hempel et al. 2007; Sommer et al. 2007). In a re-screening for genes encoding putative protein translocons in the set of nucleomorph-encoded proteins of *Guillardia theta*, homologous components of an ER-associated degradation (ERAD) system (reviewed in Ismail and Ng 2006; Meusser et al. 2005) were identified (Sommer et al. 2007). This was both surprising and intriguing at first, because a central function of the ERAD machinery is the translocation of unfolded proteins across the ER membrane from the ER lumen into the cytosol. Except for the nucleomorph envelope, no ER membrane could be identified in the PPC. Furthermore, diatoms and the apicomplexan *P. falciparum* express a second symbiont-specific ERAD-derived system (sERAD) beside the host's own ERAD system, which seems to be PPC-specific judging by BTSs associated with it. This was even more surprising because the PPCs of these latter organisms have completely lost their endomembrane systems. Despite this, the BTSs of the sERAD components were able to target GFP into this compartment (Sommer et al. 2007). It was postulated that an ERAD-derived system is located within the second outermost membrane of the four membrane-bound secondary plastids in chromalveolates, and that this sERAD machinery is the actual translocator facilitating protein transport from the ER lumen into the PPC (Fig. 2). This result was supported by similar discoveries in other members of the chromalveolates (Sommer et al. 2007). It will be very exciting to clarify transport mechanism at the second outermost membrane of chlorarachniophyte plastids, which are of green algal origin.

After crossing the second outermost membrane, the transit peptides of PPC-located proteins are cleaved off and the proteins are folded to their mature conformations. Recently a transit peptide peptidase activity in the PPC was shown indirectly (Deschamps et al. 2006), but the molecular identity of this peptidase remains unclear. In contrast, stromal proteins remain intact, because they still require their transit peptide for crossing the plastid envelope.

## CROSSING THE PLASTID ENVELOPE (THIRD OUTERMOST AND FOURTH MEMBRANE)

It is generally accepted that the third outermost and the fourth membranes of secondary endosymbionts are homologous to the plastid envelope of primary plastids. Thus, one would expect translocons similar to Toc/Tic to mediate protein transport across these membranes into the stroma. However, as already mentioned, analysis of the genomic data from algae with secondary endosymbionts has indicated that in contrast to the situation in green and red algae, no Toc components have been identified in organisms with a red algal endosymbiont (presented in McFadden and van Dooren 2004). The failure to identify Toc components may be due to the complete loss of all Toc components, their absence in the available genomic data, or the low sequence conservation of these proteins in comparison to plant Toc components. The latter is obvious for *P. falciparum* where the high AT content of the genome is strongly correlated with the use of AT-rich codons (Szafranski et al. 2005).

If indeed no Toc complex exists for import into the plastids of red algal endosymbionts, then an unknown molecular machinery must exist to enable the transport of proteins across the third outermost membrane. Recently it was proposed that, similar to the situation at the second outermost membrane, an additional sERAD machinery is acting at the third outermost membrane (Gould, Waller, and McFadden 2008). This would indicate that two sets of such a system were encoded in the cell nucleus, which is the case for the Der1 protein (degradation at the ER), as shown by Sommer et al. (2007). However, for an ERAD system in the third outermost membrane, the translocation direction would have to be reversed, because proteins are transported from a plasmic into an extraplasmic space.

On the other hand, components of the Tic machinery have already been identified in crypthophytes, heterokontophytes, and apicomplexa (Douglas et al. 2001; McFadden and van Dooren 2004; van Dooren et al. 2008), which implies a translocon mechanism across the fourth membrane comparable to land plants.

The chlorarachniophyte *Bigelowiella natans* with a green algal endosymbiont on the other hand, encodes at least one central Toc component, Toc75, and a component of the Tic complex, Tic20, on its nucleomorph genome (Gilson et al. 2006). The existence of these two central translocon components indicates a conserved import mechanism across the appropriate membranes comparable to primary plastids.

## PROCESSING AND TARGETING WITHIN THE PLASTID

After reaching the plastid stroma, pre-protein transit peptides are cleaved off, most likely by a stromal processing peptidase, as known from plants (Richter and Lamppa 1998; VanderVere et al. 1995). A similar activity of such a peptidase was described for heterokontophytes (Chaal, Ishida, and Green 2003) and *P. falciparum* (van Dooren et al. 2002). Additionally, the zinc metalloprotease falcilysin may be involved in further degradation of apicoplast transit peptides (Ponpuak et al. 2007). After cleavage, stromal proteins are folded to their mature conformation and targeted to their final suborganellar destination. Some nucleus-encoded proteins must cross the thylakoid membrane in plastids of secondary endosymbionts. From work on higher plants it is known that luminal proteins are transported across the thylakoid membrane via the general secretory (Sec)- or pH-gradient dependent ($\Delta$pH) pathway (for review see Jarvis and Robinson 2004) and therefore harbor an additional topogenic signal. This was recently shown to be the case in organisms with secondary endosymbionts as well (Broughton, Howe, and Hiller 2006; Gould et al. 2007), in which thylakoid pre-proteins harbour an N-terminal tripartite signal sequence in some cases with a Sec targeting domain or a twin arginin targeting (TAT) motif located C-terminal to the BTS. Thylakoid import experiments performed with topogenic signals from cryptophytes in pea chloroplasts indicated that thylakoid import via the $\Delta$pH-pathway is similar at the least in all phototrophic eukaryotes (Gould et al. 2007).

In short, nucleus-encoded proteins have to traverse a long path through the cell before they are finally located in the thylakoid lumen: after crossing the outermost membrane via cotranslational import, the signal peptide is cleaved off. Translocation across the second outermost membrane then takes place, presumably mediated by a symbiont-specific ERAD-derived system. After crossing the third outermost membrane via an unknown mechanism, the fourth membrane is traversed most likely by a Tic translocon and the transit peptide is cleaved off. Finally, transport across the thylakoid membrane into the thylakoid lumen is mediated via the Sec system or the $\Delta$pH pathway. Additional topogenic signals, like the TAT motif, are processed in both cases, after the proteins have reached their final destination.

## CONCLUDING REMARKS

Although many efforts to map protein transport into complex plastids have been made, many details are still not understood. Transport within secondary plastids surrounded by three membranes and transport at the second and third outermost membranes in plastids surrounded by four membranes especially require further detailed study. New hypothetical models are being inspired by various genome projects, and these will be able to be tested with new cell biological techniques. Thus, there is good reason to believe that the molecular processes underlying protein trafficking will become much clearer in the near future.

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