The Apicoplast: A Plastid in *Plasmodium falciparum* and Other Apicomplexan Parasites

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Apicomplexan parasites cause severe diseases such as malaria, toxoplasmosis, and coccidiosis (caused by *Plasmodium* spp, *Toxoplasma*, and *Eimeria*, respectively*).* These parasites contain a relict plastid – termed 'apicoplast' – that originated from the engulfment of an organism of the red algal lineage. The apicoplast is indispensable but its exact role in parasites is unknown. The apicoplast has its own genome and expresses a small number of genes, but the vast majority of the apicoplast proteome is encoded in the nuclear genome. The products of these nuclear genes are post-translationally targeted to the organelle via the secretory pathway courtesy of a bipartite N-terminal leader sequence. Apicoplasts are non-photosynthetic but retain other typical plastid functions like fatty acid, isoprenoid and heme synthesis, and products of these pathways might be exported from the apicoplast for use by the parasite. Apicoplast pathways are essentially prokaryotic and therefore excellent drug targets. Some antibiotics inhibiting these molecular processes are already in chemotherapeutic use, while many new drugs will hopefully spring from our growing understanding of this intriguing organelle.

KEY WORDS: Apicoplast, Apicomplexa, Malaria, Evolution, Plastid division, Protein targeting, Chemotherapy

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I. Introduction

Plastids are cellular organelles that come in many forms and colors. The photosynthetically active plastids range from the green 'chloroplasts' of plants and green algae, to the bright red 'rhodoplasts' of red algae and the golden-brown 'chromoplasts' of algae like diatoms and dinoflagellates. The term plastid also embraces various non-photosynthetic versions of these organelles such as 'amyloplasts' and 'leukoplasts' of underground plant tissues and parasitic plants. Recently, one more variation on the theme was identified when a relict plastid was found in parasites of humans. Dubbed the 'apicoplast', this plastid can be found in obligatory parasites like *Plasmodium*, the causative agent of malaria, and *Toxoplasma*, a deadly disease affecting immune-compromised patients (Wilson *et al.*, 1994; McFadden *et al.*, 1996; Köhler *et al.*, 1997).

Plasmodium and *Toxoplasma* belong to the phylum Apicomplexa (formerly included in the phylum Sporozoa) and hence the term apicoplast. Virtually all members of the Apicomplexa are unicellular endoparasites and they infect a very wide range of host organisms. Many apicomplexan parasites cause serious diseases in livestock and humans, like cryptosporidiosis, coccidiosis, babesiosis (Texas cattle fever), theileriosis (East Coast Fever), toxoplasmosis and – worst of all – malaria. Malaria alone gives rise to untold human suffering in more than 300 million clinical cases every year, while at the same time claiming more than one million human lives. Malaria in humans is caused by four species of *Plasmodium*, i.e. *P. malariae*, *P. ovale*, *P. vivax*, and *P. falciparum*, the latter being the deadliest of these parasites. *Toxoplasma gondii* on the other hand is a very common human parasite that causes serious disease only in immune-compromised people. The economic loss and social burden caused by apicomplexan diseases in livestock are enormous.

The discovery of a plastid in apicomplexan parasites sent shockwaves through the fields of parasitology, protistology and malaria research in particular. It dramatically changed the way we look at these parasites and their evolutionary history – after all, we now know that one of the world's worst infectious diseases is caused by an organism that was once an alga. More importantly, hopes are high that the apicoplast will allow us to employ a whole range of new drugs against these serious diseases. It is hoped that these new drugs will have higher efficacy and fewer side-effects than those currently in use, which are becoming less and less useful because of spreading resistance in parasites. The apicoplast represents an ideal drug target for two reasons. Firstly, it has been shown to be vital for the survival of *Plasmodium* and *Toxoplasma*. Secondly, since all plastids ultimately trace back to cyanobacteria-like forebears (cyanobacteria are common photosynthetically active eubacteria), molecular and metabolic processes that occur in plastids are bacterial in nature, making the parasites susceptible to the action of certain antibiotics and herbicides. In contrast, many of these compounds are – in appropriate dosage – harmless to humans and livestock, because humans and animals do not have a plastid in their cells and altogether lack some of the metabolic pathways found in this organelle. Even where the parasitized host does share similar pathways, it often transpires that the host and plastid pathways or the enzymes involved are sufficiently different to allow differential inhibition. Such differences offer great potential for chemotherapeutic exploitation.

The realization that most apicomplexan parasites harbor a vestigial plastid is only recent and surprised parasitologists and botanists alike. Since then, new data and insights have been generated apace. Several authors have reviewed the status of apicoplast research at various times (McFadden *et al.*, 1997; Wilson and Williamson, 1997; Roos *et al.*, 1999a; Gleeson, 2000; Sato *et al.*, 2000; Marechal and Cesbron-Delauw, 2001) and these compilations served as an excellent springboard for this review. We will summarize what is known to date about the evolutionary origin and the division process of the apicoplast. We will look at protein targeting to this organelle, and we will explore its metabolic functions, the resulting indispensability for the parasite, and the action and mechanism of specific inhibitors. We will not re-tell the intriguing story leading up to this organelle's discovery and its recognition as a plastid, since this topic has been covered extensively before (McFadden *et al.*, 1997; Gleeson, 2000).

II. Evolutionary Origin

A. Plastids and Endosymbiosis

All plastids ultimately derive from cyanobacterial-like, prokaryotic organisms that were engulfed by a phagotrophic eukaryote (Gray, 1992). But instead of digesting the swallowed prokaryote and only enjoying a once-off feast, the host cell kept its new 'pet' in a mutually beneficial relationship (endosymbiosis) and could thus continually enjoy food scraps that the photoautotrophic guest was willing (or coerced) to share. Over time, this relationship grew closer, biochemical exchanges became more substantial, and the endosymbiont (the prokaryotic guest) started losing genes that were no longer needed in its sheltered new home. Gradually, other genes that were still essential for endosymbiont functions were transferred to the host cell nucleus (Palmer, 1991; McFadden and Gilson, 1995; Martin *et al.*, 1998; McFadden, 2001; Rujan and Martin, 2001). Eventually, the endosymbiont lost its autonomy and gained immortal status as an organelle instead. We refer to this acquisition of a plastid by engulfment of a cyanobacterium as a primary endosymbiosis, and it is believed that the plastids of the green algae and plants, of red algae, and of a small group of algae called glaucophytes derive from *one* such primary endosymbiotic event (Cavalier-Smith, 2000; Moreira *et al.*, 2000). However, numerous other plastid-containing organisms (from various photoautotrophic protist and algal groups like heterokonts, cryptophytes, chlorarachniophytes and euglenoids) are not direct descendants of the evolutionary line that established the primary endosymbiosis. Rather, these groups represent several branches of eukaryotic evolution, and for many of these organisms the only feature in common is a plastid – a plastid obtained second-hand from the primary endosymbiotic lineage. Instead of engulfing a cyanobacterial prokaryote, these 'late-comers' gained their plastids by taking up a eukaryote that had already acquired a plastid, typically either an organism from the green or red algal lineage. We refer to these plastid acquisitions as *secondary* endosymbioses (Gibbs, 1978; Delwiche, 1999; McFadden, 1999a, 2001; Moreira and Philippe, 2001). Again, genes were transferred to the new host nucleus, and the endosymbiont was heavily reduced over time, losing all of its organelles except for its plastid. Intriguingly, there are two instances,

cryptophytes and chlorarachniophytes, where vestigial components of the endosymbiont persist, including a miniature nucleus and modicum of cytoplasm along with the plastid (Gilson and McFadden, 1997; Zauner *et al.*, 2000; Douglas *et al.*, 2001; Gilson, 2001).

It is clear that the apicoplast is an example of secondary endosymbiosis but there are two pivotal questions concerning the origin of the apicomplexan plastid that remain to be answered. Firstly, was there only one origin or did the Apicomplexa acquire plastids multiple times? And secondly, what was the endosymbiont(s)?

B. Monophyly and Distribution of Apicomplexan Plastids

Three lines of evidence support the monophyly (single origin) of apicomplexan plastids. Firstly, researchers have found traces of a plastid (either at the ultrastructural level or by molecular means) in members of all major apicomplexan lineages (Coccidia, Haemosporida, Gregarinia and Piroplasmida) (McFadden *et al.*, 1997; Lang-Unnasch *et al.*, 1998; Gleeson, 2000). Secondly, tree topologies derived from phylogenetic analyses of plastid- and nuclear-encoded ribosomal RNA genes are strikingly similar. This congruity strongly suggests the co-evolution of these two organelles in apicomplexan parasites (a clear indicator for a common origin of the apicoplast in the various apicomplexans studied) (Lang-Unnasch *et al.*, 1998; Obornik *et al.*, 2002a). Thirdly, a comparison of parts of the apicoplast genomes from two coccidians (*Eimeria tenella* and *Toxoplasma gondii*), one haemosporidian (*Plasmodium falciparum*) and one piroplasmid (*Theileria annulata*) showed these genomes to be remarkably similar with regard to gene content and gene organization (Denny *et al.*, 1998), which again argues for a common origin of all apicomplexan plastids.

The only apicomplexans currently thought to lack a plastid are *Colpodella*, a phagotrophic flagellate with an apical complex (McFadden *et al.*, 1997), and *Cryptosporidium parvum* (Zhu *et al.*, 2000b), a parasite that some consider to represent a basal lineage within the Apicomplexa (Zhu *et al.*, 2000a) but others regard as an unusual coccidian. Also, no published evidence for a plastid in the bivalve parasite *Perkinsus* has yet emerged so it remains unclear as to whether or not a plastid exists in this organism (McFadden *et al.*, 1997; McFadden, 2000). Yet, despite earlier classification of *Perkinsus* as an apicomplexan parasite, this organism may not be a true member of the Apicomplexa (Siddall *et al.*, 1997; Noren *et al.*, 1999). Noren *et al.* (1999) suggest that *Colpodella* and *Perkinsus* (together with *Parvilucifera*, a parasite that infects dinoflagellates) represent a distinct group that combines characters of dinoflagellates and apicomplexans in a unique way, forming a 'missing link' between these two groups. They propose to consider this new group, the Perkinsozoa, a taxon on the same level as the Dinoflagellata, Apicomplexa and Ciliophora, thus adding a fourth phylum to the alveolates (Noren *et al.*, 1999). If *Colpodella, Perkinsus* and *Parvilucifera* lack a plastid it could be because they diverged from the lineage containing the Apicomplexa prior to plastid acquisition. The exact sequence of divergences between the four lineages of alveolates remains uncertain making it difficult to rationalize plastid presences and absences.

Cryptosporidium parvum has been suggested to lack a plastid, since concerted efforts to recover plastid DNA from this apicomplexan have yielded no genes (Zhu *et al.*, 2000b). The dilemma is, as ever, to decide when the lack of evidence is evidence of lack. Failure to recover genes by PCR is always a difficult criterion on which to base absence. Regrettably, *Cryptosporidium parvum* preserves very poorly for electron microscopy so an ultrastructural search has also been less than conclusive. If *Cryptosporidium parvum* indeed lacks a plastid, what is the significance to our question of plastid origin(s) in the Apicomplexa? Did *Cryptosporidium parvum* diverge before plastid acquisition or did it lose the plastid after acquisition? Again, *Cryptosporidium* proves to be less than cooperative in that its phylogenetic position within the Apicomplexa is difficult to resolve. Molecular phylogenies position it at the base of the Apicomplexa (Zhu *et al.*, 2000a) but morphological studies suggest it is a true, albeit aberrant, coccidian. If *Cryptosporidium parvum* is a coccidian and it lacks a plastid, one has to conclude that it has lost the plastid.

C. A Red Algal Origin of the Apicoplast, and the Idea of the Chromalveolata

If we accept a single origin for all plastids in the Apicomplexa, what was the source of the endosymbiont? The evolutionary origin of the endosymbiont that gave rise to the apicoplast has been widely debated. Argument pivots around whether the apicomplexan plastid is of green algal or red algal ancestry. Dinoflagellates, the sister group of the Apicomplexa (Barta *et al.*, 1991; Gajadhar *et al.*, 1991; Wolters, 1991; Cavalier-Smith, 1993; Escalante and Ayala, 1995; Fast *et al.*, 2002), are a diverse group of algae that also acquired their (typical peridinin-containing) plastid via secondary endosymbiosis (McFadden and Gilson, 1995; Palmer and Delwiche, 1996; Delwiche and Palmer, 1997; Douglas, 1998). These algae gained their plastid by uptake and retention of an organism from the *red* algal lineage (Durnford *et al.*, 1999; Zhang *et al.*, 1999, 2000). Because of the sister-relationship of dinoflagellates and apicomplexans, it was speculated very early that the plastids of both groups might share a common red algal origin (Palmer, 1992; Wilson *et al.*, 1994; McFadden and Waller, 1997). But the bench- and computational work was yet to be done.

Photosynthetic pigments (chlorophyll) and enzymes (e.g. Rubisco) are classic markers for the major plastid lineages, but the apparent absence of either from apicomplexan organisms forced researchers to resort to alternative avenues. Early on, phylogenies constructed from plastid-encoded genes of ribosomal RNAs and ribosomal proteins suggested an evolutionary affiliation of the apicoplast with euglenoid plastids (Howe, 1992; Gardner *et al.*, 1994; Egea and Lang-Unnasch, 1995), which belong to the green plastid lineage (Delwiche, 1999). A green algal ancestry was also suggested by sequence analysis of the *tufA* gene coding for the protein synthesis factor Tu, which is also located on the plastid genome (Köhler *et al.*, 1997). A common problem of these studies of primary sequence data, though, is the high A+T content of the apicomplexan plastid genomes that skews both ribosomal RNA sequences as well as the amino acid composition of proteins (Wilson *et al.*, 1996b; Köhler *et al.*, 1997). Furthermore, taxon sampling was always limited: none of these studies had plastid sequences from

dinoflagellates at their disposal, and some of the earlier analyses did not even include any sequences from the red plastid lineage (i.e. rhodophytes, dinoflagellates, cryptophytes or heterokonts).

On the other hand, the presence of a gene (ORF470/*ycf24*/*sufB*, see Section V.B.) on the *Plasmodium falciparum* and *P. berghei* plastid genomes that was otherwise only known from the plastid genome of red algae, led others to propose a red algal origin of the apicoplast (Wilson, 1993; Williamson *et al.*, 1994; Yap *et al.*, 1997). To minimize the limitations of single gene sequence data, three studies turned to the analysis of whole plastid genome arrangements. McFadden *et al.* (1997) and Blanchard and Hicks (1999) combined primary sequence data with a comparison of the apicoplast genome with other completely sequenced plastid genomes, focusing on patterns of gene loss and gene rearrangements. These authors concluded that the apicomplexan plastid is likely derived from outside the green plastid lineage. Blanchard and Hicks (1999) further concluded that the affiliation of apicoplasts with euglenoid plastids (Howe, 1992; Gardner *et al.*, 1994; Egea and Lang-Unnasch, 1995) is probably an artifact due to high A+T bias and convergent codon usage. Importantly, the data did not contradict the idea that apicomplexan and dinoflagellate plastids share a common origin. Gene-cluster analyses (McFadden *et al.*, 1997; Stoebe and Kowallik, 1999) clearly highlighted the similarities in the organization of the ribosomal protein gene cluster between the apicoplast genome of *P. falciparum* and various genomes from within the red plastid lineage (two rhodophytes, one cryptophyte and one diatom), again suggesting a red algal ancestry for the apicoplast.

Some crucial and desperately awaited sequences from dinoflagellate plastids were finally cast into the phylogenetic arena by Zhang *et al.* (1999; 2000). Their 23S rRNA trees indicate (with good bootstrap support) that apicomplexan plastids are most closely related to the (peridinin-containing) plastids of dinoflagellates (Zhang *et al.*, 2000). Trees based on 16S rRNA genes showed the same topology as the 23S rRNA trees when calculated by maximum likelihood methods, but were inconsistent using other methods. This finding indicated that the small subunit rRNA of plastids is sub-optimal for elucidating overall plastid phylogeny (at least in this context). In analogy to previous results by Egea and Lang-Unnasch (1995), who had based their analysis on 16S rRNA sequences, Zhang and coworkers found their trees to also show a close association of the dinoflagellate/apicomplexan clade with euglenoids, but this and other inconsistencies were attributed to base composition bias and long branch attraction (Felsenstein, 1978; Philippe, 2000). Based on the phylogenetic analysis of this recent sequence data from dinoflagellates, Cavalier-Smith (1999) also argued for a common origin of dinoflagellate and apicomplexan plastids. Cavalier-Smith (1999) went further and suggested that *all* secondary plastids of the red algal lineage derive from the very same secondary endosymbiotic event. If this hypothesis turned out to be true, heterokonts, cryptophytes and haptophytes (the Chromista) as well as apicomplexans, dinoflagellates and ciliates (!) (the Alveolata) would form one big monophyletic clade, for which the name 'Chromalveolata' has been suggested (Cavalier-Smith, 1999) (see Fig. 1). According to this hypothesis, the secondary plastid acquisition preceded the divergence of the chromistan and alveolate lines. Furthermore, the following organisms would have subsequently lost their ancestral, photosynthetically active plastid: all ciliates, the various apicomplexans, dinoflagellates and members of the Chromista that lack a plastid, and all

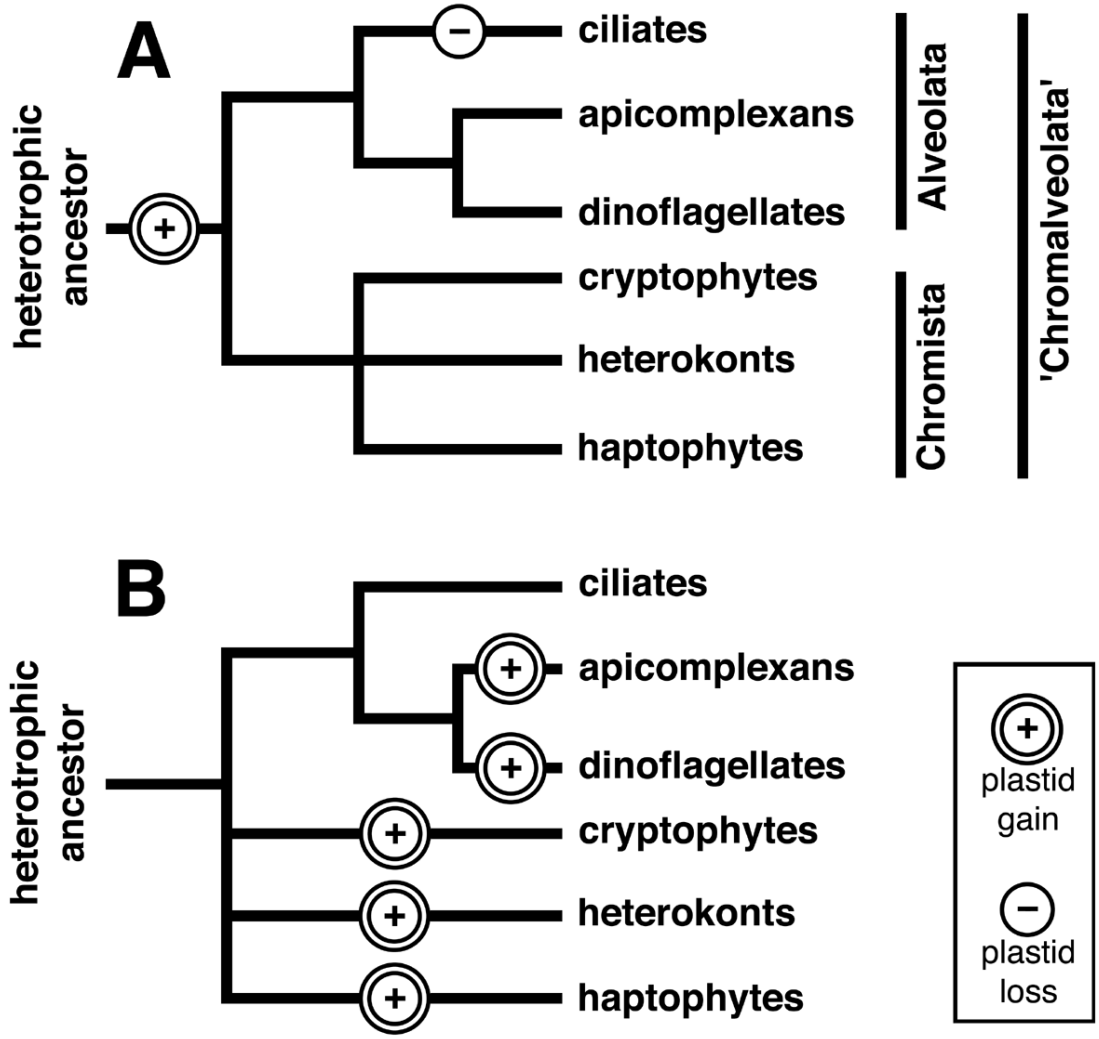

FIG. 1: Diagram outlining two competing hypotheses for plastid origin(s) in organisms containing plastids of secondary red algal origin. (A) The 'chromalveolate' hypothesis proposed by Cavalier-Smith (1999, 2000) suggests that one secondary endosymbiotic event involving a red alga and a heterotropic eukaryote gave rise to multiple evolutionary lines encompassing the cryptophytes (often also referred to as cryptomonads), the heterokonts, and the haptophytes (the Chromista), as well as the ciliates, apicomplexans and the dinoflagellates (the Alveolata). (B) A more traditional interpretation assumes that the various groups arose through independent secondary endosymbiotic events. The diagram does not indicate the various lineages that lack a plastid (e.g. some heterokonts and dinoflagellates) or the respective secondary plastid losses, except in the case of the ciliates. It also does not take into account those dinoflagellates that bear a plastid other than the peridinin-containing plastid. The symbols for plastid gains ('+' surrounded by a double circle) and losses ('–' surrounded by a single circle) are designed to indicate that the secondary acquisition of a plastid is a by far more complex evolutionary process than plastid loss (Cavalier-Smith, 2000). See Section II.C.

dinoflagellates that contain a plastid other than the typical peridinin-containing plastid (for an overview of Cavalier-Smith's work relating to this topic, see e.g. Cavalier-Smith, 1986, 1999, 2000, 2002).

Fast *et al.* (2001) have provided some experimental evidence supporting this 'chromalveolate scenario' (and a shared dinoflagellate/apicomplexan plastid origin in particular) by employing a novel and elegant approach. Instead of studying plastid-encoded genes or gene arrangements, these authors exploited the unusual evolutionary history of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), an enzyme with central metabolic function in glycolysis and the Calvin cycle. Plastid-bearing organisms have two versions of GAPDH: one is located in the cytosol, the other in the plastid. The plastidic GAPDH genes of plants, green algae and red algae are cyanobacterial-like, as one would expect for a plastid-targeted protein that is encoded in the nucleus. In dinoflagellates and cryptophytes on the other hand, the genes for plastidic GAPDH are more closely related to eukaryotic *cytosolic* genes. This situation is most easily explained by assuming that the plastidic GAPDH of dinoflagellates and cryptophytes originated by gene duplication of the cytosolic homologue and subsequent replacement of the cyanobacterial-like GAPDH gene of the red algal endosymbiont. Fast *et al.* (2001) then compared the cytosolic and plastidic GAPDH genes of the apicomplexan *Toxoplasma gondii* with many other GAPDH sequences by neighbor-joining analysis based on distances calculated by PUZZLE, as well as by comparison of specific, highly-conserved amino-acid substitutions and insertions. Their analyses show the apicomplexan plastidic and cytosolic GAPDH sequences to cluster with those genes from dinoflagellates, heterokonts and cryptophytes, and not with the sequences from plants, green algae and red algae. These results therefore support the red algal origin of the apicoplast as well as the common origin of all secondarily acquired plastids from the red algal lineage (the chromalveolate scenario). Further molecular support for multiple losses of secondary plastids during evolution (as implicated by the chromalveolate hypothesis) comes from the genes encoding 6-phosphogluconate dehydrogenase (*gnd*) in various eukaryotes. Andersson and Roger (2002) argue that the *gnd* homologues of plants, various algae groups, *Plasmodium*, and members of the exclusively heterotrophic amoeboflagellate Heterolobosea (phylum Percolozoa) are of plastid origin. Their report thus adds another example to the steadily growing list of taxa that have apparently lost a plastid. Some support for the chromalveolate lineage is also emerging from analysis of host genes. Baldauf *et al.* (2000) recovered a chromalveolate clade in a meta-analysis of nuclear genes.

The two glycolytic enzymes glucose-6-phosphate isomerase and enolase of *T. gondii* and *P. falciparum* (which appear to be located in the cytosol) were found to be plant-like, and it was suggested that they had been transferred to the parasite nucleus from the algal endosymbiont (Read *et al.*, 1994; Dzierszinski *et al.*, 1999). In particular, apicomplexan enolase genes share a pentapeptide insertion with their counterparts from land plants and green algae, but phylogenetic analysis using these proteins did not help in further elucidating the apicoplast's origin, mainly because such sequences were not available from any plastid-containing organism of the red algal lineage. Keeling and Palmer (2001) have given the enolase story an unsuspected and very interesting turn of general importance for studies in molecular evolution. These authors report that the presence of the pentapeptide insertion in the enolase gene across different lineages does

not match the phylogenic analysis inferred from the whole gene, and conclude that the apicomplexan enolase gene is not derived from the endosymbiont that gave rise to the apicoplast. Instead, they argue that the pentapeptide insertion in the apicomplexan gene is the result of a sub-genic integration event of only a fragment of an algal enolase gene (possibly following the uptake of a charophyte-like alga for food) into the apicomplexan host gene, resulting in a mosaic gene arrangement (Keeling and Palmer, 2001).

A patchwork scenario for the apicoplast genome itself has recently been suggested by Obornik *et al.* (2002). These authors report that three ribosomal protein genes (*rpl2, rpl14* and *rps2*) that are encoded on the apicoplast genome cluster with mitochondrial and not with plastid genes in phylogenetic analyses, suggesting that these three plastid genes may have been derived from the mitochondrial genome through lateral gene transfer (Obornik *et al.*, 2002b).

D. Apicoplast Membranes – a Convoluted Issue

A *secondary* endosymbiotic origin of the apicoplast is also consistent with and supported by the number of membranes surrounding this organelle. Despite our own early contention that the apicoplast in *Toxoplasma* is bounded by only three (or even two) membranes (McFadden *et al.*, 1996; McFadden *et al.*, 1997), it is nowadays commonly accepted (also by us) that there are in fact four membranes (Köhler *et al.*, 1997; McFadden and Roos, 1999; McFadden and Waller, 1999; Cavalier-Smith, 2000). In addition, electron micrographs of the apicoplast of *Garnia gonadati*, a haemosporine parasite of lizards, clearly show this organelle to be surrounded by four membranes (Diniz *et al.*, 2000). Four apicoplast membranes have also been reported for *Sarcocystis muris* and the fish coccidian *Goussia janae* (Radchenko, 1994; Obornik *et al.*, 2002a). However, in *Plasmodium* the situation might (literally) be a bit more convoluted. Hopkins *et al.* (1999) present a comprehensive, three-dimensional ultrastructural analysis of the apicoplast in *P. falciparum* based on serial sections of chemically fixed material (2.5% glutaraldehyde and 1% osmium tetroxide). Their images suggest that the malarial plastid is typically bounded by three membranes, while additional membranes in the form of whorls, loops and coils at times accompany and extend from the basic three membranes. The complexity and number (up to six in cross-section) of these additional membranes are reported to increase with parasite development throughout the intraerythrocytic life stages and to be more pronounced around the center of the elongated plastid in trophozoites. Hopkins *et al.* (1999) speculate that these distinct inner and outer membrane complexes might play a role in the transport of lipids that are produced in the apicoplast (see Section V.C.).

A scenario in which the apicoplast of *Plasmodium* and *Toxoplasma* are surrounded by a different number of membranes (three vs. four) is not very parsimonious. The number of organellar membranes has long been regarded as a very conservative character that does not change easily in evolution (Cavalier-Smith, 2000). Nevertheless, if the chromalveolate scenario (see Section II.C.) is correct, the peridinin-containing dinoflagellate plastid and the plastids of other chromalveolates might just present one good example for such a case (three vs. four plastid membranes within the

Chromalveolata). Still, one piece of evidence may argue directly against a difference in number of plastid membranes between *Plasmodium* and other apicomplexans. As will be discussed in Section IV., the N-terminal leader sequences for apicoplast targeting are complex, yet leader sequences from *T. gondii* target the reporter protein GFP to the plastid in *P. falciparum* and vice versa (Jomaa *et al.*, 1999; Waller *et al.*, 2000). This interchangeability strongly suggests that the targeting mechanisms are virtually identical in *Toxoplasma* and *Plasmodium*. Any difference in the number of membranes surrounding the apicoplast in these two apicomplexan genera needs to be reconciled with their similar targeting mechanisms (see discussion in Section IV.G.).

The fact that a hypothesis is less parsimonious than alternative views certainly does not preclude its correctness. But it does make one consider and scrutinize the supporting evidence more closely and carefully. It is in this context that it seems important to point out one problem that may unfortunately affect the careful three-dimensional ultrastructural analysis of Hopkins *et al*. (1999). Chemically fixed cells of *P. falciparum* are notorious for their bad ultrastructural preservation and lack of membrane clarity. In the case of Hopkins *et al*. (1999), this problem is manifest in the waviness and irregular nature of the membranes as well as the varying shape and thickness of the intermembrane spaces. From our own experience we know that even cryofixation does not easily overcome this problem, making an ultrastructural determination of membrane number a treacherous task. One way to conclusively resolve the membrane number issue in this genus may be to perform electron microscopy on apicoplasts from *Plasmodium* species that exhibit better ultrastructural preservation than *P. falciparum.*

Nevertheless, if the difference in plastid membrane number between different apicomplexans is substantiated, we will, as Hopkins *et al.* (1999) point out, face two obvious possibilities: (1) the common ancestor of all alveolates contained a plastid bounded by three membranes – a situation still encountered in *Plasmodium* and dinoflagellates today – and the fourth membrane found in *Toxoplasma* and *Garnia* (and other apicomplexans?) is all that remains of the membrane whorls acquired by early apicomplexans; or (2) the ancestral condition for the alveolate plastid is to be bounded by four membranes, and dinoflagellates and *Plasmodium* (and other apicomplexans?) independently lost one of these membranes, while the complex membrane whorls are a unique acquisition (autapomorphy) of *Plasmodium*. In light of the chromalveolate scenario (see Section II.C.), and considering that some haemosporidians like *Plasmodium* display a high molecular evolutionary rate (apparent in long branches in molecular phylogenies or the high A+T content of the apicoplast genome) as well as some rather unusual adaptations like a highly modified secretory system (see Section IV.F.), the latter of the two scenarios seems much more likely. Obornik *et al.* (2002) suggest that the presence of three bounding apicoplast membranes might be a general characteristic of haemosporidians (and not coccidians).

III. Apicoplast Division

A. Division of Bacteria and Plant Chloroplasts

The last few years have seen a dramatic increase in our knowledge about how plastids divide (McFadden, 1999b; Beech and Gilson, 2000; Kuroiwa, 2000; Margolin, 2000a; Osteryoung, 2000; Gilson and Beech, 2001). Since plastids derive from endosymbiotic bacteria, bacterial cell division was considered a model for plastid replication. And indeed, bacterial division proteins like FtsZ and MinD have been found in plants and photosynthetic protists, notably in red algae and a cryptophyte (Beech and Gilson, 2000; Margolin, 2000a; Osteryoung, 2000; Gilson and Beech, 2001). FtsZ, a tubulin-like molecule, is the most conserved cell division protein in bacteria (Margolin, 2000b). It is part of a ring ('Z-ring') that forms at the midcell point between dividing cells. It is not known exactly how FtsZ is involved in bacterial cytokinesis or whether it actually generates the contractile force that pinches the mother cell into the two daughter cells. Nevertheless, its pivotal role in the division of most bacteria is evident (Gilson and Beech, 2001). The *minicell* genes (*minC, minD, minE*) encode a collection of helper proteins that enable FtsZ to be positioned correctly (Margolin, 2000b).

In plants, a tripartite plastid-dividing ring that contains two different homologues of FtsZ (Vitha *et al.*, 2001) is located in the midpoint of dividing chloroplasts (Kuroiwa, 2000). Antisense repression and gene knockout experiments demonstrate that FtsZ plays an essential functional role in chloroplast division (Osteryoung *et al.*, 1998; Strepp *et al.*, 1998). A plant homologue of MinD has likewise been implicated in plastid division in plants (Colletti *et al.*, 2000; Kanamaru *et al.*, 2000). Other genes involved in the division of chloroplasts are the ARC genes (Pyke, 1999), *ftsH* (Itoh *et al.*, 1999; Adam *et al.*, 2001) and the dynamins (Kim *et al.*, 1999). These recent findings from a variety of plastid-bearing organisms (higher plants, mosses, red algae, cryptophytes) support the notion that the division machinery of various plastids is derived from the prokaryotic cell division apparatus and that essential compounds like FtsZ and MinD have been retained (Beech and Gilson, 2000; Margolin, 2000a; Osteryoung, 2000).

B. A Model for Apicoplast Division – and Some Conflicting Evidence

Most of our understanding of plastid division in the Apicomplexa to date comes from morphological and ultrastructural observations. Since the plastid in apicomplexan parasites is not pigmented, apicoplast division could not be observed directly in live cells until the advent of transgenic parasites expressing marker proteins such as GFP in the apicoplast (Striepen *et al.*, 2000; Waller *et al.*, 2000; He *et al.*, 2001b). The general morphology of apicoplast division in *Plasmodium falciparum* was first outlined by Waller *et al.* (2000) in striking fluorescence images depicting the asexual parasite cell cycle within erythrocytes (see Fig. 2). In ring stage parasites (the earliest stage after initial infection of red blood cells) the single apicoplast is crescent shaped. It subsequently rounds up into a sphere in early trophozoites and grows in size. As schizont formation begins (the stage at which the parasite begins to segment into multiple daughter

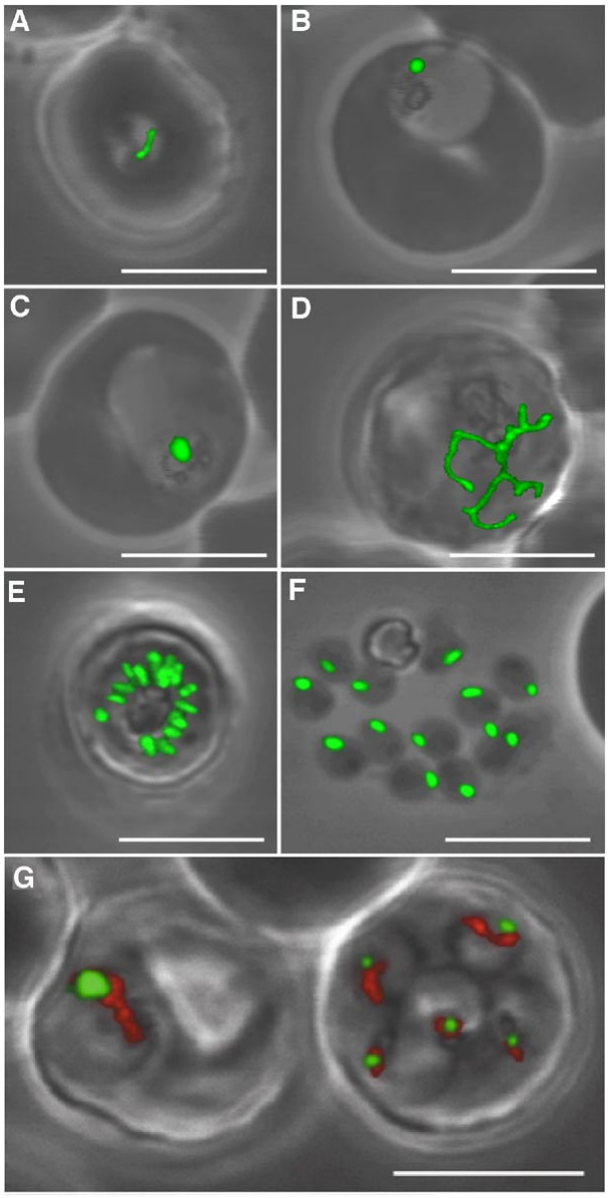

FIG. 2: Apicoplast morphology throughout the asexual life cycle of blood stage malaria parasites expressing apicoplast-targeted GFP. The images show red blood cells infected by *Plasmodium falciparum* in various life stages. The panels represent parasites (A) just after red blood cell invasion (ring form), (B, C) during the major growth phase (trophozoites), (D, E) during cell division (schizont stage), and (F) as free daughter cells that are ready to infect new red blood cells (merozoites). These images by Waller *et al.* (2000) for the first time showed the suprising morphology of the malarial apicoplast during cell division as an elaborately branched structure (D). (G) Costaining with Mitotracker Red shows that the apicoplast (green) is clearly distinct from but located in close proximity to the mitochondrion (red). The erythrocyte on the right contains multiple infections. Scale bars, 5µm. Reproduced from EMBO Journal, 2000, Volume 19, pp. 1796 and 1799, with kind permission of Oxford University Press.

parasites), the apicoplast surprisingly starts forming a reticulate, branched structure, which divides (apparently simultaneously) into as many plastids as there are daughter cells in the maturing schizont. After red blood cell rupture, every merozoite (the stage that travels through the plasma from the spent blood cell and invades a new host erythrocyte) contains one slightly elongated plastid (Waller *et al.*, 2000). In *P. falciparum* the number of daughter cells produced per schizont is variable and must obviously match the number of replicated nuclei (Read *et al.*, 1993). In addition, the division of the apicoplast and its genome must yield as many daughter plastids as there are merozoites being formed. This and other evidence (Speer and Dubey, 1999) suggested that nuclear division and plastid division and/or segregation are intimately linked in these parasites (Waller *et al.*, 2000). In *Toxoplasma*, such a connection has been convincingly demonstrated by Striepen *et al.* (2000) using fluorescence microscopy.

In contrast to *Plasmodium*, cells of *T. gondii* divide by endodyogeny, a process that results in only two daughter cells per mother cell. Despite this obvious difference, endodyogeny can be considered a simpler version of schizogony. In their elegant study, Striepen *et al.* (2000) show that in *Toxoplasma* the plastid genome is localized within the organelle as a distinct nucleoid. Once apicoplast division commences, the replicated genome segregates and the two nucleoids associate with the ends of the elongating plastid. Centrin- and α-tubulin-labeling as well as electron microscopic observations indicate that the two ends of dividing plastids are in close proximity to the centrosomes and the ends of the intranuclear mitotic spindle (as well as directly adjacent to the pellicle of the newly forming daughter cell buds). In fact, a close association of the centrosomes with the (posterior) end of plastids persists even in non-dividing apicoplasts during interphase. Finally, the apicoplast divides concurrently with the nucleus. Striepen *et al.* (2000) then made another striking observation using dinitroaniline herbicides like oryzalin and ethalfluralin that disrupt microtubule formation. In *T. gondii*, these drugs block nuclear division, thus generating cells containing multiple centrosomes and mitotic spindles (Morrissette and Roos, 1998; Shaw *et al.*, 2000). These 'artificial schizonts' contain either multiple plastids or just one single reticulate apicoplast (Striepen *et al.*, 2000) that looks strikingly similar to the elaborately branched plastids observed in schizonts of *P. falciparum* (Waller *et al.*, 2000). Multiple centrosomes can be seen in close association with such reticulate apicoplasts in *T. gondii* (Striepen *et al.*, 2000), making it tempting to assume that the same connection exists in *P. falciparum*.

Based on their data, Striepen *et al.* (2000) devise a model of apicoplast division for *T. gondii*, which is depicted and described in detail in Fig. 3. This model can easily explain how an apicomplexan cell, which may form a variable number of cells during schizogony, manages to distribute exactly one plastid per nucleus into the resulting daughter cells. At the same time, it highlights a couple of fundamental differences that appear to exist between the organellar division in *T. gondii* and chloroplast division in plants. Firstly, apicoplast division may take place without the involvement of a plastid division ring, a notion that is supported by the failure to find FtsZ homologues in any of the apicomplexan genomes (Striepen *et al.*, 2000) (our own observation). Secondly, apicoplast division is tightly linked to mitosis, even using components of the division machinery of the nucleus, while chloroplast division in plants is independent of mitosis.

But how then can the very different plastid division mechanisms in red algal plastids (and this includes the second-hand plastids of red algal origin in cryptophytes)

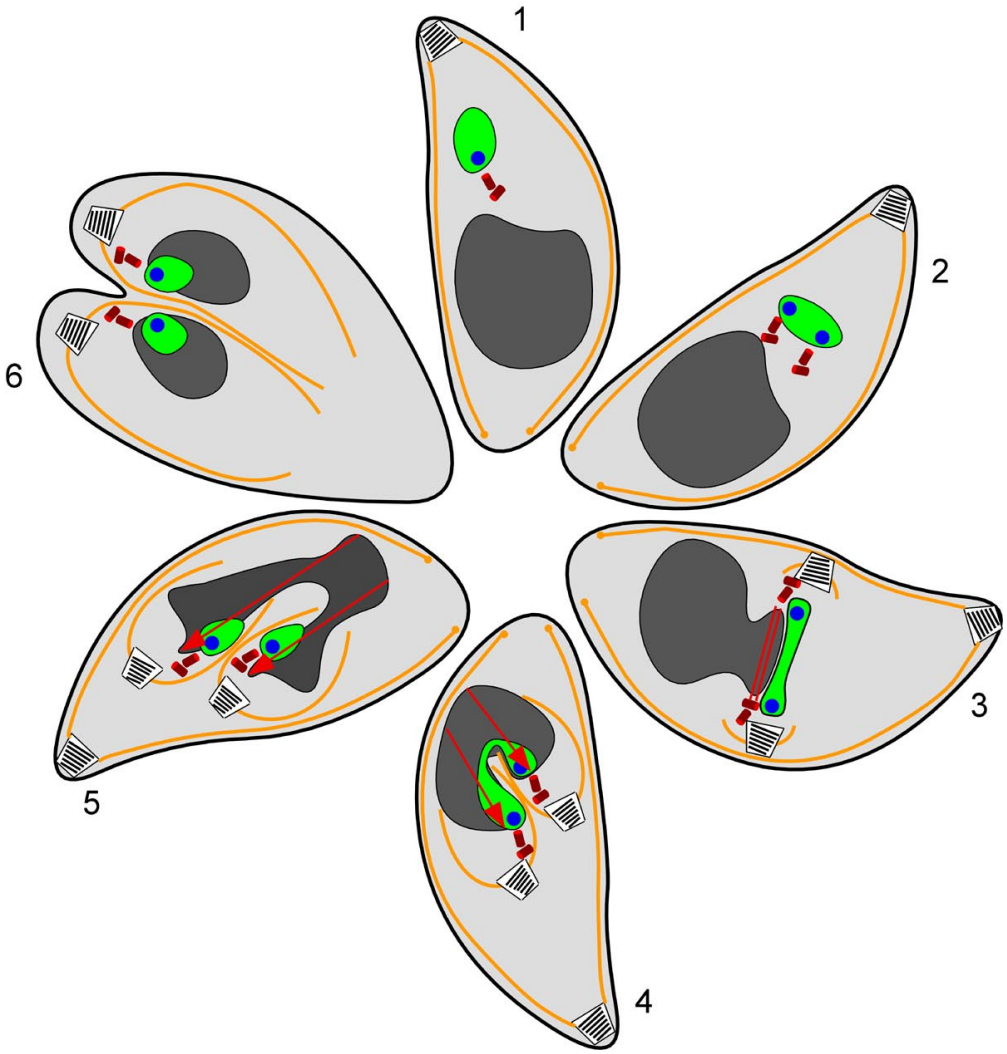

FIG. 3: Model of Striepen *et al.* (2000) for apicoplast division in *Toxoplasma gondii*. According to this model, the centriole (red), which is somehow linked to one end of the apicoplast (green) even during interphase (1), duplicates at the beginning of cell division. The second centriole also associates with the plastid (2), so that the two ends of the elongating organelle (which contain the two distinct nucleoids, blue) are in close proximity to the centrioles. The centrioles then move to opposite sides of the nucleus, pulling the plastid into a dumbbell shape (3). After formation of the intranuclear mitotic spindle, the centrioles link up with the newly formed inner-membrane complex (pellicle, yellow) of the forming daughter cells. As the centrioles move into the newly created daughter cell buds, the plastid ends are pulled along, while the extending pellicle cuts into the plastid and the nucleus, forcing them into a U-shape (4). Finally, the resulting shearing force between the pulling centrioles and the ingrowing pellicle is postulated to split the plastid (and the nucleus) in two (5, 6). Reproduced from The Journal of Cell Biology, 2000, Volume 151, p. 1432, by copyright permission of The Rockefeller University Press.

and the apicoplast be explained? Striepen *et al.* (2000) speculate that the apicoplast's division mechanism might derive from the endosomal system of the host cell that originally engulfed the red algal-like ancestor of the apicoplast. Since centrioles are known to associate with sorting endosomes, and since the plastid is located within the secretory system of the host cell (DeRocher *et al.*, 2000; Waller *et al.*, 2000), these sorting endosome-centrosome interactions might have been the origin of the centrioles' involvement in apicoplast division today (Striepen *et al.*, 2000). At face value the evidence suggests that apicoplast division has abandoned the bacterial mechanisms and hitched a ride on the division mechanism of the host cell. In some ways this is similar to division of mitochondria, which in lower eukaryotes seems to be bacterium-like but has adopted a dynamin (eukaryotic) based system in derived eukaryotic lineages such as animals and fungi (and perhaps plants) (Beech and Gilson, 2000; Gilson and Beech, 2001; Arimura and Tsutsumi, 2002).

While the model proposed by Striepen *et al.* (2000) is based on excellent microscopical analysis and answers some complex questions, it is in conflict with data presented by Matsuzaki *et al.* (2001). While the latter study confirms some of the findings of Striepen *et al.* (2000), it also suggests the involvement of a plastid division ring and even of a microbody- or peroxisome-like structure in apicoplast division (Matsuzaki *et al.*, 2001). Several electron micrographs show short, dumbbell-shaped apicoplasts without an obvious association with centrioles, nucleus or pellicle, suggestive of the central organellar constriction being caused by a specific molecular apparatus. Yet, an image of a putative plastid division ring is not very convincing because the ring-like structure depicted appears to lie in the wrong plane for fission (Fig. 5F in Matsuzaki *et al.*, 2001). The presence of a microbody-like structure adjacent to the site of constriction in several dumbbell-shaped apicoplasts on the other hand is curious. Considering the close association of a microbody with the dividing mitochondrion and chloroplast in a unicellular red alga (Miyagishima *et al.*, 1999), one may wonder whether apicoplast division in *T. gondii* is at least linked to the duplication of the microbody-like structure identified by Matsuzaki *et al.* (2001). Perhaps other organelles have 'learned' to hitch-hike on the mitotic division apparatus too.

C. Parasite Mutants Lacking the Apicoplast

A *Toxoplasma* mutant with disrupted plastid division was recently reported by He *et al.* (2001a). A construct consisting of GFP, an N-terminal plastid-targeting leader and a rhoptry targeting sequence at the C-terminus was transiently transfected into *T. gondii*. The pattern of GFP fluorescence after a brief period of expression revealed a remarkable phenomenon. Only one parasite per parasitophorous vacuole (all parasites within one vacuole are the clonal progeny of a single invasion) contained a plastid, which became progressively enlarged with each round of division. This single plastid was apparently expanding but never dividing, and was hence never partitioned into any daughter cells produced. Despite this apicoplast's inability to divide and segregate into the daughter cells, cell division (endodyogeny) and even replication of the plastid genome appeared not to be affected (He *et al.*, 2001b).

Plastid-deficient parasites were temporarily viable and continued to grow and divide seemingly unimpeded while within the initial infected host cell. However, these plastid-lacking mutants could not successfully re-infect another host cell, dying soon after re-invasion (He *et al.*, 2001b). This is the first report of apicomplexan parasites being cured of their apicoplast, albeit temporarily. Further observations revealed membranous inclusions inside the apicoplast containing cytoplasmic material, while GFP localized to only the periphery of the plastid (He *et al.*, 2001b; He *et al.*, 2001a) (in control cells transfected with GFP and the plastid-targeting leader alone, GFP is found throughout the lumen of the organelle). It appears that in these cells the fusion protein fails to be translocated completely across all four plastid membranes and gets stuck somewhere in between or perhaps across the bounding membranes. The membranous inclusions may be caused by bits of apicoplast membrane becoming internalized into the lumen of the organelle as the protein import machinery tries to translocate the jammed fusion protein. He *et al.* (2001a) speculate that the apicoplast mis-segregation may be due to the presence of these large inclusions, although it could also be that the organelle division apparatus is perturbed simply by the large amounts of fusion protein being stuck in or between some of the plastid membranes. Interestingly, replacement of the rhoptry protein fragment in the 'poison' construct with a conventional alpha-helical transmembrane domain or a GPI anchor abolished its ability to target efficiently to the apicoplast or to disrupt apicoplast division (He *et al.*, 2001a). He *et al.* (2001b) also report that none of their varied efforts to isolate the apicoplast from *T. gondii* by subcellular fractionation via density gradient ultracentrifugation was successful.

D. Apicoplast Genome Replication

Apicoplast genome copy numbers have been estimated to be between one and more than 15 for *P. falciparum*, and between six and 25 for *T. gondii* (Fichera and Roos, 1997; Köhler *et al.*, 1997; Matsuzaki *et al.*, 2001). The 35 kb apicoplast genome of *P. falciparum* consists mainly (> 90%) of circular molecules (Williamson *et al.*, 2001). Surprisingly, the apicoplast genome of *T. gondii* on the other hand has been shown to consist mostly of linear tandem arrays in multiples (between one and twelve) of 35 kb (Williamson *et al.*, 2001). These linear oligomers start and terminate in the center of the large inverted repeat of the 35 kb DNA. Based on the observed frequency distribution of linear oligomers of different sizes, Williamson *et al.* (2001) propose a rolling circle model for apicoplast genome replication in *T. gondii*, similar to the rolling circle replication encountered in chloroplasts of higher plants. Their model states that only 35 kb circles are capable of initiating replication, with the center of the large inverted repeat serving as the origin of replication (Williamson *et al.*, 2001). Their calculations indicate that replication only stops (due to successful processing) in about one third of cases after a given round of apicoplast genome replication. In the majority of cases replication continues, generating the observed linear tandem arrays of various sizes. Linear apicoplast DNA molecules have also been reported from *Eimeria tenella* (Dunn *et al.*, 1998) and *Neospora caninum* (Gleeson and Johnson, 1999).

IV. Protein Targeting and Import

A. Protein Targeting to Plastids of Plants and Algae

During the evolutionary development of plastids, many genes that were originally encoded in the organellar genome were transferred to the nucleus of the host cell. Clearly, nuclear-encoded plastid proteins had to be imported back into the organelle, which is achieved courtesy of N-terminal extensions called 'transit peptides'. Once inside the plastid, these targeting leaders are removed from the respective mature protein by a processing peptidase, and one loosely defined motif where this cleavage occurs has been identified in plants. The molecular protein import machinery of plant chloroplasts, which are bounded by two membranes, spans both the outer and the inner chloroplast membranes. Its components have been appropriately termed Toc and Tic complexes (acronyms for translocon at the outer/inner chloroplast membrane) (Keegstra and Froehlich, 1999; McFadden, 1999a, 1999b; Chen *et al.*, 2000; Vothknecht and Soll, 2000; Bruce, 2001; Jarvis and Soll, 2001).

In plastids of *secondary* endosymbiotic origin, protein targeting is a bit more complicated for two reasons. Firstly, plastids of secondary endosymbiotic origin were subject to a second round of gene transfer: many genes that encoded plastid proteins (both organelle- and nucleus-encoded) were again transferred, this time to the nucleus of the new, second host cell. Secondly, all second-hand plastids are bounded by more than two membranes. The additional one or two membranes are thought to derive from the phagosome of the second host cell (the outermost membrane) and – in those plastids that are surrounded by four membranes – from the plasma membrane of the secondary endosymbiont (the second membrane counting from the outside, also referred to as 'periplastid membrane') (McFadden, 1999a) (see Fig. 4 and Section IV.G.). Since all second-hand plastids therefore reside within the endomembrane system, proteins destined for this organelle are often trafficked using a two-part targeting tag: a classic 'signal peptide' that directs the protein into the endomembrane system, followed by a plant-like transit peptide that effects the translocation across the inner organellar membranes (Schwartzbach *et al.*, 1998; McFadden, 1999a; van Dooren *et al.*, 2001).

B. Protein Targeting to the Apicoplast is Via the Secretory Pathway

When it became clear that the plastid of apicomplexan parasites was derived from a *secondary* endosymbiont, it was obvious that protein-targeting to the apicoplast would be likely to follow a two-step mechanism. Indeed, several studies have shown that apicomplexan parasites target plastid-destined proteins via the secretory system (Waller *et al.*, 1998; Yung and Lang-Unnasch, 1999; DeRocher *et al.*, 2000; Waller *et al.*, 2000). In these studies, N-terminal extensions of putative apicoplast-targeted proteins were fused to GFP and expressed in either *Plasmodium falciparum* or *Toxoplasma gondii*. Fluorescence and electron microscopy revealed that these leader sequences are both

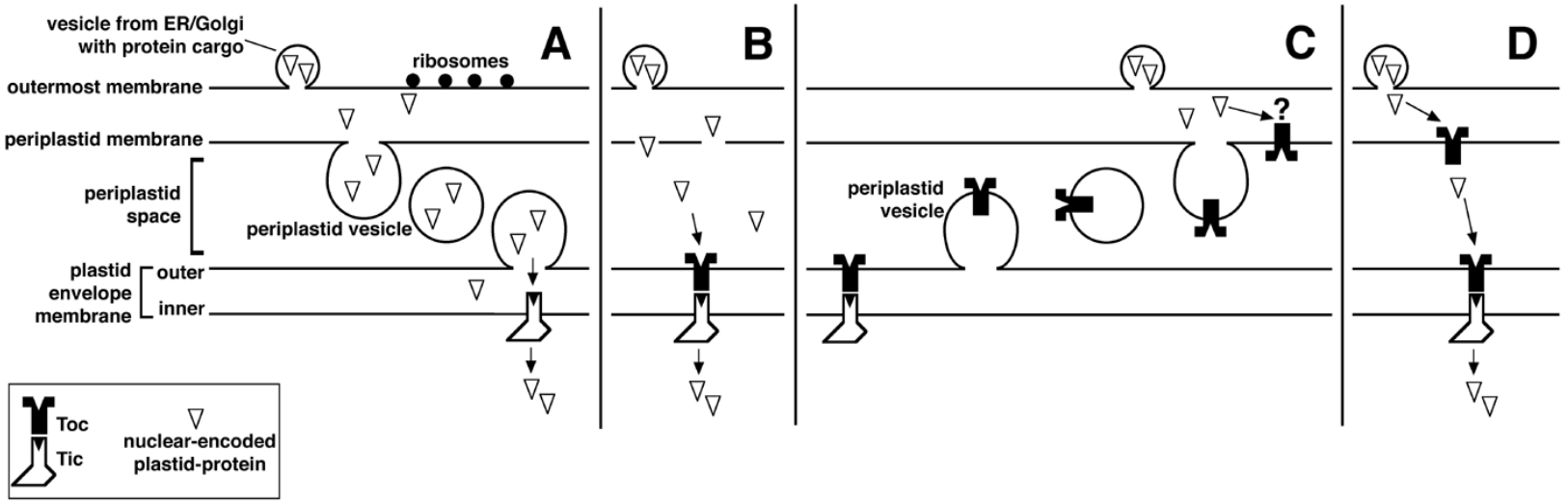

FIG. 4: Schema representing four models proposed to explain how nuclear-encoded proteins bound for the plastid lumen get across the periplastid membrane (second from the outside) in plastids with four membranes. Examples for organisms harboring such complex plastids of secondary endosymbiotic origin include the apicomplexan parasite *Toxoplasma gondii* and heterokont algae. In apicomplexans, proteins are thought to reach the outermost plastid membrane via vesicles that originate from somewhere along the secretory pathway (left side of panel A). In heterokont algae, nuclear-encoded plastid proteins may be translated on ribosomes present on the outermost plastid membrane itself, which is continuous with the ER and the nuclear envelope (right side of panel A, and omitted in other panels). For a detailed discussion of the four models, see Section IV.G. In short, (A) Gibbs (1981) suggested that vesicles originating from the periplastid membrane fuse with the outer plastid envelope membrane, thus carrying the protein cargo across the periplastid space. (B) The simplest scenario postulates the presence of unspecific pores in the periplastid membrane that allow proteins to freely cross this membrane (Cavalier-Smith, 1999; Kroth and Strotmann, 1999). (C) Cavalier-Smith's (1999) hypothesis suggests that periplastid vesicles may exchange (galacto)lipids as well as components of a transit peptide-recognition and protein transporter apparatus between the outer plastid envelope membrane and the periplastid membrane. The diagram shows how a directional import apparatus in the outer plastid envelope membrane would be inserted in the wrong orientation into the periplastid membrane, rendering the import apparatus nonfunctional. This reversal of orientation would also apply to a polar plastid envelope membrane consisting of two lipid monolayers that differ in their lipid composition. (D) Van Dooren *et al.* (2000) proposed dual targeting of the Toc complex components to both the outer plastid envelope membrane and the periplastid membrane. Good arguments have been made both for and against all of these models. The scarce research data currently available does not strongly favor any one scenario in particular (and in fact, none of these models may be correct), and it is not even clear whether the same translocation mechanism exists in all plastids with four membranes.

necessary and sufficient to direct import of the reporter protein into the plastid in both parasites. Further analysis of the leader sequences showed that they are, indeed, bipartite: the N-terminus starts with a typical hydrophobic signal peptide that can usually be recognized by a neural network (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen *et al.*, 1997), while the remainder of the N-terminal extension represents a plastid transit peptide. Deletion of just the transit peptide caused proteins, that now only contained an N-terminal signal peptide, to be secreted from the cell (into the parasitophorous vacuole) (DeRocher *et al.*, 2000; Waller *et al.*, 2000), while removal of the signal peptide alone led to accumulation of the protein in the cytosol (Waller *et al.*, 2000). Intron patterns found in genes encoding plastid-targeted proteins in *Plasmodium*, *Toxoplasma* and the plant *Arabidopsis* were consistent with the notion that the modular arrangement of signal peptide, transit peptide and mature protein commonly evolved through exon-shuffling (Waller *et al.*, 1998; Schaap *et al.*, 2001).

C. Apicoplast Transit Peptides

The region of the N-terminal apicoplast targeting leader immediately downstream of the signal peptide has been likened to the transit peptide domain of plant and algal primary plastids. Initially these comparisons, which were based mainly on similar amino acid composition, were somewhat tentative. This is because the plant transit peptide is itself a very poorly defined entity (Bruce, 2001). Chloroplast transit peptides vary greatly in length (between 20 and 150 amino acids) and primary sequence, and seem to lack a regular secondary or tertiary structure (von Heijne and Nishikawa, 1991) in an aqueous environment. Some recent evidence suggests that they are able to form helices in interaction with galactolipids of the plastid membrane, but whether this is a universal feature remains to be established (van't Hof *et al.*, 1991; Wienk *et al.*, 2000). Other transit peptide characteristics may include the capacity to interact with chaperones and peptidases (Richter and Lamppa, 1999; Rial *et al.*, 2000) and the presence of distinct subdomains (von Heijne *et al.*, 1989; Bruce, 2000). One well established key feature of transit peptides in plants is an enrichment in the hydroxylated amino acids serine and threonine (see below) and a net positive charge. Despite the lack of any consensus sequence or predictable structural motifs, the transit peptides of plants can be identified using computational approaches such as neural networks. The most widely applied prediction tool for plant transit peptides is ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson *et al.*, 1999), which can successfully identify the majority of chloroplast targeted proteins from their primary sequence (Peltier *et al.*, 2000; The Arabidopsis Genome Initiative, 2000).

The enrichment for serine and threonine and the net positive charge are also characteristic of transit peptide-like domains of *T. gondii*. Accordingly, some *Toxoplasma* apicoplast transit peptides are correctly recognized by ChloroP (DeRocher *et al.*, 2000) (our own observation). Transit peptides in *P. falciparum* on the other hand, while sharing the high basic/acidic residue ratio of chloroplast transit peptides (resulting in a net positive charge), are not enriched in serine and threonine and are only rarely recognized

by ChloroP (Waller *et al.*, 1998). The paucity of serine and threonine in *Plasmodium* transit peptides is contrasted by a marked enrichment in lysine and asparagine.

Despite this somewhat bewildering lack of any consensual features, it is becoming increasingly apparent that transit peptides from the bi-partite leaders of apicoplast targeted proteins are functionally equivalent to the transit peptides of plants and algae with primary plastids. Domain swapping experiments clearly demonstrate that apicoplast transit peptides can effect targeting into isolated pea chloroplasts (DeRocher *et al.*, 2000). Conversely, a plant transit peptide (in conjunction with a preceding signal peptide) can target GFP to the apicoplast in *T. gondii* (Foth *et al.*, manuscript in preparation). This interchangeability argues very strongly for conserved function of transit peptides, an argument that can be extended to cover the components of the import machinery that recognize and translocate the protein across the plastid membranes.

For plant transit peptides, phosphorylation of serine and threonine residues is known to be important, probably for transit peptide recognition by 14-3-3-type chaperones (Waegemann and Soll, 1996; May and Soll, 2000). Yet, transit peptides from *P. falciparum* are not particularly enriched in hydroxylated amino acids, and a mutated transit peptide bereft of any serines or threonines (two threonine residues were replaced by alanines) still directed GFP to the malarial apicoplast (Waller *et al.*, 2000). Unfortunately, this mutant transit peptide did still contain one tyrosine residue, and it is unknown whether this could have compensated for the lack of serines and threonines. Interestingly, Waegemann and Soll (1996) argue that phosphorylation of plastid-bound proteins might be important in discriminating between proteins destined for the plastid or the mitochondrion in plant cells. Mitochondrial targeting also uses a transit peptide to direct proteins to the mitochondrion, and plants and algae with primary plastids must be able to detect the difference between a mitochondrial transit peptide and a plastid transit peptide: phosphorylation of the plant transit peptides is perhaps a distinguishing feature. Considering that the apicoplast (in the endomembrane system) is spatially separated from mitochondria (in the cytoplasm) in apicomplexans, it is possible that these organisms have dispensed with phosphorylation as a means of distinguishing between plastid- and mitochondrial-targeting transit peptides. In this respect it is highly intriguing that the transit peptide of the apicoplast-targeted ribosomal protein S9 (rps9) directs GFP to the mitochondrion in *T. gondii* if the signal peptide component, which would normally direct S9 into the endomembrane system and away from the mitochondrion, is deleted (DeRocher *et al.*, 2000). It would be interesting to see whether mitochondrial transit peptides in conjunction with a signal peptide are able to route proteins to the apicoplast. Still, further experimentation is needed to show unambiguously whether transit peptide phosphorylation is necessary for protein import into the apicoplast or not, and experiments with *Plasmodium* transit peptides bare of any hydroxylated amino acids (serine, threonine *and* tyrosine) are currently underway in our laboratory.

D. Predicting the Apicoplast Proteome

In order to maximize our understanding of the apicoplast, it is desirable to identify the components of its proteome. The genome project for *Plasmodium falciparum* offers

an excellent opportunity to do this using a bioinformatic approach that seeks to identify apicoplast-targeted proteins by recognizing their N-terminal bipartite leader sequences. Clearly, better definition of transit peptides will facilitate more accurate predictions and a number of approaches have addressed this goal.

Deletion and insertional mutagenesis experiments with the transit peptide of the *T. gondii* apicoplast ribosomal protein S9 (rps9) indicated that apicoplast transit peptides – like their plant chloroplast counterparts – are very loosely defined (DeRocher *et al.*, 2000; Yung *et al.*, 2001). Substantial deletions of the rps9 transit peptide did not compromise its functionality. These experiments also suggested that apicoplast transit peptides contain some redundancy, and that the targeting information resides mostly in the N-terminus of the transit peptide (DeRocher *et al.*, 2000; Yung *et al.*, 2001). The relatively poor efficiency of *Plasmodium* transformation on the other hand has limited the ability to dissect transit peptide structure in this apicomplexan, and only a few strategic experiments have been done. Interestingly though, the somewhat aberrant nature of transit peptides in *Plasmodium* has made the identification of essential transit peptide elements a simpler task. As mentioned above, *Plasmodium* transit peptides are enriched in lysine and asparagine. We rationalize this enrichment as reflecting the high A+T content of the genome, which results in preferential use of amino acids (such as lysine and asparagine) that require minimal G's and C's in the DNA. This means we can now visualize some essential elements of transit peptides because much of the 'clutter' engendered by redundant characteristics of different amino acids has been dispensed with by a bias towards a limited set of amino acids. Accordingly, we are now able to predict the presence of transit peptides in *Plasmodium* protein sequences by employing a few simple 'if-then' rules pertaining to amino acid composition (Foth *et al.*, manuscript in preparation). By combining this prediction with an existing neural network tool known as SignalP we are able to identify likely apicoplast-targeted proteins from *P. falciparum* nuclear genome data (Foth *et al.*, manuscript in preparation).

Another approach also uses a neural network that has recently been trained specifically for the purpose of identifying apicoplast proteins in *Plasmodium* (Zuegge *et al.*, 2001). By combining these two bioinformatic tools, we will soon be able to make a well educated guess as to what proteins comprise the apicoplast proteome when the full *P. falciparum* genome is published in mid-2002. Such a first glance will obviously be speculative and will have to be revised and amended once experimental proteomics data on the apicoplast becomes available, an achievement that will depend largely on our ability to isolate this organelle. Isolation of the apicoplast is a difficult venture (He *et al.*, 2001a), yet one we are currently tackling in our lab. In the meantime, the bioinformatic approaches will supply us with a preliminary protein inventory for the apicoplast. From this inventory we will be able to extrapolate a great deal about apicoplast metabolism (see Section V.).

E. Processing of N-terminal Leaders

The processing of the bipartite leader sequences in apicomplexans does not appear to present us with major surprises. Based on models of signal peptide function it was

anticipated that the signal peptide would be removed by a signal peptidase during translocation across the ER, and Western blot analyses from *T. gondii* and *P. falciparum* suggest that this is exactly what occurs. The transit peptide domain is most likely removed once the protein has been translocated across the plastid's membranes, as occurs with transit peptides in plant chloroplasts (Waller *et al.*, 1998; Waller *et al.*, 2000; He *et al.*, 2001a; Vollmer *et al.*, 2001; Cheresh *et al.*, 2002; Van Dooren *et al.*, 2002). Van Dooren *et al.* (2002) report a gene in *P. falciparum* that encodes a plant-like stromal processing peptidase (SPP) homologue and propose that this enzyme is likely responsible for the cleavage of transit peptides off mature proteins inside the apicoplast. As expected for an enzyme that is active in the apicoplast, SPP contains a putative N-terminal bipartite leader consistent with plastid-targeting. Interestingly, this leader appears to be shared via alternative splicing with the gene for ALAD (delta-aminolevulinic acid dehydratase) ; (Van Dooren *et al.*, 2002), another likely apicoplast-targeted enzyme that is involved in heme biosynthesis. Pulse-chase labeling experiments indicate that it takes about 40 minutes for newly synthesized proteins to be trafficked into the apicoplast (Van Dooren *et al.*, 2002). This time frame is equivalent to that observed in *Euglena gracilis* (Sulli and Schwartzbach, 1996), an alga that also routes plastid-bound proteins via the secretory pathway (the *Euglena* chloroplast is also of secondary endosymbiotic origin).

Not much is known about the processing site where the transit peptide is cleaved from the mature protein. Only two fully processed apicoplast-targeted proteins (FabI and ACP) have so far been N-terminally sequenced and thus provided us with the transit peptide cleavage site (Surolia and Surolia, 2001; Van Dooren *et al.*, 2002). Unfortunately, no cleavage motif is obvious in those two sequences. It is interesting to note, though, that the fully processed ACP still contains a short N-terminal stretch of sequence (about 16 amino acids) that does not match its (cyanobacterial) homologues in the database and that consists of 50% lysine and asparagine residues (characteristic for *P. falciparum* transit peptides), making it look as if the cleaving enzyme had cut the protein '16 residues too far upstream' (Van Dooren *et al.*, 2002).

F. Routing Proteins from the ER to the Apicoplast

It is clear that the signal peptide directs proteins into the endomembrane system, and deletion and insertion experiments (see Section IV.C.) demonstrate that the transit peptide is sufficient to deliver proteins through the endomembrane system to the apicoplast. But how exactly do proteins reach the apicoplast from inside the ER?

In the secondary plastids of Chromistan algae (cryptophytes, heterokonts, and haptophytes), the outermost plastid membrane is continuous with the membrane of the ER, with ribosomes often decorating the outermost plastid membrane (the 'chloroplast ER') (Gibbs, 1981). Initially, this configuration was interpreted as a continuous sheet of ER enveloping the plastid, implying that the two outermost membranes (in plastids bounded by four membranes) are derived from ER (Gibbs, 1981). Today, this interpretation is generally regarded incorrect (see Sections IV.A. and IV.G., and e.g. Cavalier-Smith, 1999). In those cases where ribosomes are located on the outermost plastid membrane (Chromistan algae), proteins synthesized by these ribosomes are able

to reach the inner plastid membranes without further trafficking through the endomembrane system. But in apicomplexans, electron micrographs have revealed no continuity between the outer apicoplast membrane and the ER, nor are any ribosomes evident on the outermost plastid membrane, suggesting that transport between the ER and the outermost membrane occurs via vesicles (Bodyl, 1999). Some experimental support for this notion comes from apicoplast-deficient cells of *T. gondii*, in which plastid-targeted GFP has been observed in vesicles located in the apical region of the cell (He *et al.*, 2001a). Yet, it is unclear whether these observations made in parasites that completely lack the apicoplast correctly reflect the normal plastid-targeting pathway. Also, how do transit peptides induce packaging of apicoplast-bound proteins into the appropriate vesicles? Or alternatively, is there a simpler mechanism in operation? One proposal is that all secreted proteins have to 'wash past' the plastid by default, and those bearing transit peptides are sieved out by receptors on the apicoplast membranes (van Dooren *et al.*, 2000).

Another unresolved question is whether or not the Golgi apparatus is involved in protein-trafficking to the apicoplast. The frequently observed close proximity of Golgi and apicoplast (which was previously referred to as the 'Golgi-adjunct') is somewhat suggestive of a Golgi-to-apicoplast transport. If this is true, does the situation differ between *T. gondii* with its tightly stacked Golgi and *P. falciparum* with its 'unstacked' Golgi-clusters and unorthodox secretory pathway, part of which appears to even be located outside the parasite within the red blood cell cytoplasm (Bannister *et al.*, 2000; Adisa *et al.*, 2001; Nacer *et al.*, 2001; Wickham *et al.*, 2001)? Arguments against an involvement of the Golgi in apicoplast targeting comes from unpublished work on *Toxoplasma* (see Roos *et al.*, 2002). Roos *et al.* (2002) claim that the Golgi-disrupting agent Brefeldin A fails to ablate targeting or processing of apicoplast-targeted proteins, and that appending the C-terminal ER-retrieval signal HDEL to recombinant apicoplast-targeted proteins does not inhibit apicoplast targeting. If plastid-bound proteins in *T. gondii* are not trafficked via the Golgi, do they leave the ER before or after the nuclear envelope, which in this organism serves as an intermediate compartment between the ER and the Golgi (Hager *et al.*, 1999)?

Recently, Cheresh *et al.* (2002) have presented the first research paper that directly addresses the question of how proteins are trafficked from the ER to the apicoplast, adding some interesting pieces to the puzzle of apicoplast targeting. These authors report that trafficking to the apicoplast in *P. falciparum* proceeds via the parasitophorous vacuole (PV) (Cheresh *et al.*, 2002). In their intricate study, GFP was expressed using a promoter that is active only early on in the intraerythrocytic life cycle, causing a spike of GFP expression in ring-stage parasites that was then followed through the further development of the parasites. Data gathered from immunoelectron microscopy and from several lysis-experiments using low concentrations of saponin (which is thought to specifically lyse the red blood cell and PV membranes without affecting the parasite plasma membrane or any structures therein) suggests that GFP accumulates in the PV during the early part of the intraerythrocytic life cycle, whereas it is trafficked to the plastid (back into the parasite) and processed (cleavage of the transit peptide) between late ring and late trophozoite stage (Cheresh *et al.*, 2002).

These findings are somewhat surprising, and we wonder whether it is possible that GFP localized in the PV in ring stage parasites might reach this destination simply

because the apicoplast protein import apparatus in these early parasites is not yet fully developed and can therefore not cope with large amounts of overexpressed plastid-targeted GFP, resulting in the protein following the default secretory pathway into the PV. On the other hand, this scenario could not easily explain other data that directly suggests that GFP is transferred from the PV to the apicoplast in trophozoites (see Fig. 5 in Cheresh *et al.*, 2002). Fluorescence images of ring stage parasites, in which – according to saponin lysis-experiments (see e.g. Fig. 5B in Cheresh *et al.*, 2002) – GFP is localized mainly to the PV, demonstrate that GFP does not accumulate in the same compartment as the classic ER-marker BiP. Yet, we are surprised that this GFP-fluorescence appears to be *within* the parasite (mostly perinuclear) and not surrounding it, as one would expect if significant amounts of GFP were present in the PV: cf. Figs. 3A and 5Bii in (Cheresh *et al.*, 2002) with GFP fluorescence in the PV in Fig. 3E-H in (Waller *et al.*, 2000).

We are certainly curious and eager to see more research data on this exciting topic emerge in the future. Because of the inherent limitations of transgenic systems in general, one might anticipate that studies on native apicoplast-targeted proteins will provide the most unequivocal answers to the question of how exactly proteins are trafficked from the ER to the apicoplast.

G. Apicoplast Membranes and Protein Targeting: Another Convoluted Issue

The two part model (signal peptide/transit peptide) for apicoplast targeting is inadequate for explaining targeting across four membranes. While the signal peptide can mediate traffic across the outermost membrane (which is functionally part of the endomembrane system) and the transit peptide is thought to mediate traffic across the inner two membranes (which are thought to be homologous to the two membranes of primary plastids), no part of the model accounts for traffic across the membrane immediately subtending the outermost membrane. This second membrane (counted from the outside) is thought to be the relict plasma membrane of the eukaryotic endosymbiont and is also referred to as the 'periplastid membrane' (see Fig. 4) (Cavalier-Smith, 1999). Since a plant transit peptide in combination with a signal peptide from a secreted protein is sufficient to translocate GFP across all four plastid membranes in *Toxoplasma* (Foth *et al.*, manuscript in preparation), it is clear that there is no secret key for that elusive periplastid membrane hidden in the apicomplexan-type transit peptide itself. But how do proteins get across this membrane?

The literature on this intricate topic is – considering the scarcity of research data – quite abundant and rather speculative. We will therefore simply present the main hypotheses that have been put forward to explain how proteins bound for the plastid lumen may get across the periplastid membrane in secondary plastids with four membranes, as well as the most obvious counter-arguments. Beyond this overview, interested readers are invited to consult recent reviews dealing with this issue in detail (e.g. Cavalier-Smith, 1999; Kroth and Strotmann, 1999; van Dooren *et al.*, 2001).

Initially, it was suggested that proteins that reach the space between the outermost and the periplastid membrane may be packaged into periplastid vesicles that ferry across

the periplastid space and fuse with the outer plastid envelope membrane (Gibbs, 1981) (see Fig. 4A). This hypothesis was supported by the presence of vesicles and tubules (termed 'the periplastidal reticulum') within the periplastid space in various algae (Gibbs, 1981). Yet, at least two arguments can be made against this hypothesis. In many cases the periplastidal reticulum is found only in that area of the plastid that is directly apposed to the nucleus, the only part of the plastid surface that is *not* studded with ribosomes (e.g. in heterokont algae). If the function of these vesicles was indeed to carry protein cargo across the periplastid space, one might expect the vesicles to be located in the vicinity of the ribosomes, minimizing the distance that newly synthesized proteins needed to travel to be packaged into vesicles. Another argument against this scenario is that the proteins would be released into the space between the two innermost membranes, thus preventing the protein's transit peptide from interacting with the Toc complex/transit peptide receptors that are thought to be located on the cytosolic side of the outer plastid envelope membrane (the second membrane from the inside) (see Cavalier-Smith, 1999).

The simplest solution to the periplastid membrane-problem assumes that the periplastid membrane contains large pores through which proteins can freely pass (see Fig. 4B) (Cavalier-Smith, 1999; Kroth and Strotmann, 1999). In this simple scenario no targeting elements would be necessary to effect protein translocation across the periplastid membrane, while the transit peptides already present in the proteins would effect translocation across the two innermost membranes, just like in plastids of primary origin that are bounded by only two membranes. Despite its attractive simplicity, several arguments can also be made against this scenario (Fig. 4B). One counter-argument is that the proposed pores would lead to leakage of proteins from the periplastid space (in those cases where the outermost membrane is continuous with the ER; see Section IV.F.). In addition, one might wonder why an apparently non-functional membrane was not simply lost more often in the course of evolution.

In his very interesting paper, Cavalier-Smith (1999) presents another intriguing idea: two-way vesicle shuttling between the two middle membranes could have led to the insertion of the Toc complex from the outer plastid envelope membrane into the periplastid membrane (Fig. 4C). In theory, this vesicle shuttling, which itself would not be directly responsible for transporting the protein cargo across the periplastid space, could have inserted both transit peptide receptor as well as translocation apparatus of the Toc complex into the periplastid membrane, thus allowing transit peptides to direct protein translocation across both middle membranes courtesy of the same molecular machinery. According to Cavalier-Smith (1999), the proposed vesicle shuttling would also account for a similar (or identical) lipid composition of the two middle membranes – potentially an important point, considering that galactolipids in the outer membrane of plant plastids are thought to be necessary for transit peptides to assume their correct three-dimensional structure and therefore to be correctly recognized by the appropriate receptor (Douce and Joyard, 1990). In analogy, galactolipids that may occur naturally in the outer plastid envelope membrane but not in the periplastid membrane (which derives from the plasma membrane of the eukaryotic endosymbiont) could be integrated into the latter by the proposed vesicle shuttling (Fig. 4C), allowing transit peptides to take on their correct three-dimensional shape. In apicomplexans, the presence of galactolipids in any of the plastid membranes is so far merely speculative. One weighty problem with this hypothesis, though, is depicted in Fig. 4C. The vesicle shuttling between the two middle

membranes would insert the components of the Toc complex into the periplastid membrane in the wrong orientation. Transit peptide receptors would therefore be inaccessible to the transit peptides of proteins that encounter the periplastid membrane barrier after translocation across the outermost plastid membrane. Furthermore, it is likely that the lipid composition of the two leafleats of the outer plastid envelope membrane differ. In this case the problematic reversal of orientation caused by vesicle shuttling would not only apply to proteins, but to lipid molecules also.

Finally, van Dooren *et al.* (2000) suggest that Toc complex components may be targeted to both the outer plastid envelope membrane and the periplastid membrane. How this dual targeting might be accomplished is not specified. Nevertheless, if cells were to somehow achieve such dual targeting of the Toc components, the resulting situation would be quite simple and parsimonious (see Fig. 4D). Transit peptides would be sufficient to allow proteins to be translocated across the inner three membranes, without the requirement for any kind of vesicle shuttling. Yet, this proposal, too, is not complete: it does not account for different lipid composition of the two middle membranes as e.g. Cavalier-Smith's (1999) vesicle shuttling does (Fig. 4C). How likely is it that transit peptide/Toc complex interactions could be successful at the periplastid membrane in the absence of galactolipids? Furthermore, since targeting to the periplastid membrane would presumably have to proceed via the endomembrane system (i.e. from outside the plastid), one would have to make the unparsimonious assumption that all genes encoding the various Toc components would have to be among the first genes to be transferred to the nuclear genome during the evolution of these plastids (Cavalier-Smith, 1999; van Dooren *et al.*, 2000).

Obviously, many questions in regard to protein trafficking across four plastid membranes remain unanswered at this point, and none of the proposed models is able to give a complete explanation of this phenomenon. Importantly though, the latter three of the four proposed scenarios (Figs. 4B, 4C, 4D) could at least explain why N-terminal leaders of *T. gondii* could target GFP to the apicoplast in *P. falciparum* and vice versa, even in the case that the apicoplasts of *Toxoplasma* and *Plasmodium* are indeed bounded by four and three membranes, respectively (see Section II.D.). Assuming that the periplastid membrane and not the outermost membrane has been lost in *Plasmodium* – as is assumed for the plastids of dinoflagellates, which are bounded by three membranes (Cavalier-Smith, 1999) – these three models could reconcile the interchangeability of plastid-targeting leaders with a differing number of organellar membranes (as suggested by Hopkins *et al.*, 1999) with only minor modifications (e.g. the loss of vesicle shuttling in Cavalier-Smith's model, Fig. 4C).

V. Function

A. The Apicoplast is Indispensable, But is Not the Site of Photosynthesis

The apicoplast is essential for the survival of apicomplexan parasites (Fichera and Roos, 1997; McConkey *et al.*, 1997; McFadden and Roos, 1999; Law *et al.*, 2000; He *et al.*, 2001b) (see also Section VI.). It is therefore clear that it provides some essential

function(s) for the parasite. But what exactly does it do? Photosynthesis – the process by which plants and algae convert light energy into chemical energy by fixing CO_2 into carbohydrates – is the most prominent function of plastids. One publication actually reported the presence of small amounts of photosynthetic pigment in the apicoplast and presented a partial sequence for a photosynthetic gene (the D2 protein or PsbA) from apicomplexan parasites (Hackstein *et al.*, 1995). Today – seven years later – these 'provocative suggestions' (Wilson *et al.*, 1996b) remain unconfirmed and must be viewed with some skepticism. Indeed, neither the fully sequenced plastid genomes of *P. falciparum* (Wilson *et al.*, 1996b) and *T. gondii* (http://www.sas.upenn.edu/~jkissing/toxomap.html), nor the almost completely sequenced nuclear genome of *P. falciparum* or the many thousands of *T. gondii* ESTs have revealed any genes whose products are directly involved in photosynthesis. Furthermore, virtually all apicomplexans are intracellular parasites that live in dark, nutrient-rich environments. It therefore appears highly unlikely that any process directly related to photosynthesis still occurs in any extant apicomplexan organism. The antiquity of the apicomplexan lineage (Escalante and Ayala, 1995) and the apparent similarity of the apicoplast and its genome across different lineages within the Apicomplexa – e.g. in the Haemosporida (*Plasmodium*) and Coccidia (*Toxoplasma*) (see Section II.B.) – are consistent with the notion that photosynthesis was lost early in apicomplexan evolutionary history.

But photosynthesis is not the only metabolic process occurring in plastids. In plants and algae, plastids also produce fatty acids, isoprenoids, heme, starch, and branched chain and aromatic amino acids (Weeden, 1981; Hrazdina and Jensen, 1992; Emes and Neuhaus, 1997; Neuhaus and Emes, 2000). Some of these products of organellar metabolism are then exported from the plastid into the cytoplasm and other compartments in the cell. This is likely the reason why non-photosynthetic plant tissues and parasitic plants retain plastids in their cells. Intriguingly, plastids of non-photosynthetic organisms commonly contain an organellar genome of reduced size that lacks photosynthesis-related genes making an interesting parallel to apicoplasts (dePamphilis and Palmer, 1990; Siemeister *et al.*, 1990). Early speculation suggested that the apicoplast might perform one or more of these anabolic functions (Palmer, 1992; Wilson, 1993; McFadden *et al.*, 1997). The challenge was then to determine what function(s) the apicoplast serves.

B. Nuclear-Encoded Genes Point to Plastid Function

Analysis of the genes encoded by the apicoplast genomes of *P. falciparum* and *T. gondii* , which are very similar, has only offered us a small glimpse of what is going on inside the apicoplast. As is the case in non-photosynthetic plants and algae (dePamphilis and Palmer, 1990; Siemeister *et al.*, 1990), the compact apicoplast genome consists predominantly of genes involved in protein expression within the organelle (Wilson *et al.*, 1996b). Examples include ribosomal RNAs and proteins, transfer RNAs, an RNA polymerase and the translation elongation factor TufA. The only apicoplast-encoded genes with (potentially) different function are *clpC* and *sufB* (previously known as

ORF470 and *ycf*24), as well as seven (possibly redundant) short ORFs of unknown function (Wilson *et al.*, 1996b; Wilson and Williamson, 1997; Ellis *et al.*, 2001). The *clpC* gene probably encodes a molecular chaperone involved in protein import into the apicoplast, making it unlikely to be the *raison d'être* gene. *sufB* (*ycf*24) on the other hand is the apicomplexan homologue of a gene found in (cyano)bacteria and in the plastid genome of red algae, and its gene product has been shown to be essential for the growth of the cyanobacterium *Synechocystis* (Law *et al.*, 2000). An extensive comparison of bacterial operons carrying *sufB* combined with *Plasmodium* genome database searches unearthed potential malarial homologues of five proteins (SufA, C, D, S, and NifU) that are thought to be associated with SufB in bacteria, and at least one of these proteins contains a putative apicoplast-targeting leader sequence (Ellis *et al.*, 2001). Extrapolating from the function of Nifs and Sufs in bacteria, Ellis *et al.* (2001) propose that SufB and other malarial proteins may be functioning in the apicoplast in iron metabolism, [Fe-S] cluster formation, and/or in resistance to oxidative stress. While such conjectures from bacterial function to the role of gene products in eukaryotes are problematic, the discovery of a plant-like ferredoxin-$NADP^+$ reductase and ferredoxin (see Section V.E.) lends support to the function proposed by Ellis *et al.* (2001) for SufB in the apicoplast.

The protein synthesis machinery within the apicoplast has probably been retained to ensure the expression of SufB and the Clp chaperone (Wilson *et al.*, 1996b; Law *et al.*, 2000). Interestingly, both *sufB* (*ycf24*) and *clpC* have recently been used to elucidate the relationship between different species of the genera *Eimeria* and *Plasmodium*, respectively (Rathore *et al.*, 2001; Zhao and Duszynski, 2001).

As discussed earlier (see Sections II.A. and IV.A.), the bulk of proteins that are functionally active in the apicoplast's metabolism are encoded in the nuclear genome and post translationally imported into the apicoplast. Accordingly, one approach to elucidating apicoplast-localized metabolic pathways is to search for genes encoding plastid-targeted proteins in the nuclear genome and EST databases of apicomplexan parasites like *P. falciparum* and *T. gondii*. Candidates can be identified by the presence of an N-terminal extension similar to known bi-partite apicoplast targeting sequences (see Section IV.D.) and/or by an anticipated function common to other plastids. Having found candidates it is then necessary to validate the predicted subcellular localization and proposed activity. Verifying localization can include immunolocalization or *in vivo* expression of GFP-fusion proteins with leader elements. Demonstration of activity to verify predictions made on sequence similarity can involve *in vitro* assays using recombinant protein, complementation assays where the putative enzyme gene is transferred into a mutant to restore function (in yeast or *E. coli* for instance), metabolic labeling, or drug inhibition studies.

Several laboratories have successfully applied this approach over the past few years and demonstrated the presence of several key metabolic enzymes in the apicoplast (see Sections V.C.-V.F.). Thus, the apicoplast has been convincingly shown to be the site of *de novo* fatty acid (Waller *et al.*, 1998; Zuther *et al.*, 1999; Waller *et al.*, 2000; Jelenska *et al.*, 2001; McLeod *et al.*, 2001; Surolia and Surolia, 2001) and isoprenoid biosynthesis (Jomaa *et al.*, 1999; Wiesner *et al.*, 2000; Reichenberg *et al.*, 2001). It also appears to contain a ferredoxin-based redox system (Vollmer *et al.*, 2001) and part of a heme synthesis pathway (Sato and Wilson, 2002; Van Dooren *et al.*, 2002). However, the synthesis of chorismate – an essential precursor for the generation of aromatic amino

acids and other aromatic compounds – by the shikimate pathway does *not* occur in the apicoplast. Despite early assertions that this pathway is localized in the plastid of apicomplexan parasites (as it is in plants) (Roberts *et al.*, 1998), it has now been clearly demonstrated that chorismate is made in the cytosol in *P. falciparum* (Keeling *et al.*, 1999; Fitzpatrick *et al.*, 2001).

C. Fatty Acid Synthesis

De novo fatty acid synthesis (FAS) is a cyclical process whereby two-carbon precursors are iteratively assembled into fatty acids (typically 16 or 18 carbon atoms long). FAS involves multiple chemical reactions that are carried out by either one single multifunctional enzyme complex with several domains ('type I FAS') or by several individual enzymes assembled into a complex ('type II FAS'). Type I FAS occurs in the cytosol of fungi and mammals (Smith, 1994), while type II FAS is found in most bacteria and in the plastids of plants and algae (Harwood, 1996; Rock and Cronan, 1996).

It had long been held that *P. falciparum* does not synthesize fatty acids *de novo* (Holz, 1977; Matesanz *et al.*, 1999), but today, several lines of evidence clearly demonstrate the presence of a type II FAS pathway in the apicoplast of *P. falciparum* and *T. gondii*. Firstly, ACP (acyl carrier protein), FabH (β-ketoacyl-ACP synthase III) and an ACC (acetyl-CoA carboxylase) have been shown to be localized in the apicoplast (Waller *et al.*, 1998; Waller *et al.*, 2000; Jelenska *et al.*, 2001). Based on the presence of bipartite leader sequences, FabI (enoyl-ACP reductase) and FabZ (β-hydroxyacyl-ACP dehydratase) are also likely to be plastid-targeted (Waller *et al.*, 1998; McLeod *et al.*, 2001; Surolia and Surolia, 2001). Secondly, triclosan and thiolactomycin, two specific inhibitors of type II FAS enzymes, inhibit growth of apicomplexan parasites *in vitro* and, in select cases, *in vivo* (Waller *et al.*, 1998; McLeod *et al.*, 2001; Surolia and Surolia, 2001). In addition, herbicides of the 'fops' and 'dims' categories (aryloxyphenoxypropionates) inhibit growth of *T. gondii* apparently by targeting ACC (Zuther *et al.*, 1999) (see Section VI.D.). Thirdly, ^{14}C-acetate and ^{14}C-malonyl-CoA are incorporated into fatty acids by *P. falciparum*, and the resulting fatty acid chains have been shown to be 10, 12 and 14 carbons long (Surolia and Surolia, 2001).

Interestingly, Zhu *et al.* (2000c) have reported the presence of a cytosolic type I FAS and apparent lack of type II FAS in the apicomplexan *Cryptosporidium parvum*. This finding fits in nicely with other work of the same laboratory that suggests that *C. parvum* lacks an apicoplast altogether, although this conclusion is still preliminary (Blunt *et al.*, 1997; Tetley *et al.*, 1998; Zhu *et al.*, 2000b). However, if confirmed, the situation in *C. parvum* would be convincingly simple: no apicoplast, no type II FAS. In this case, the lack of a plastid and type II FAS (as well as other biochemical differences) (Zhu *et al.*, 2000c) might be explained by *Cryptosporidium*'s early evolutionary divergence from the main apicomplexan lineage (Van de Peer and De Wachter, 1997; Carreno *et al.*, 1999; Zhu *et al.*, 2000a; Zhu *et al.*, 2000c). It is possible that *Cryptosporidium* lost its plastid before the organellar type II FAS replaced the incumbent cytosolic type I FAS of the host cell. Furthermore, the presence of FAS machinery of type II and I in apicomplexan parasites with and without apicoplast, respectively, suggests that the *de novo* synthesis of

fatty acids is an essential metabolic pathway occurring in many if not all members of the Apicomplexa (Zhu *et al.*, 2000c). No doubt, further research into the special case of *Cryptosporidium* to corroborate the current evidence will provide more exciting insights into the early evolutionary history of the Apicomplexa and their plastid.

What then are the *de novo* synthesized fatty acids used for? In general, one might expect fatty acids to be incorporated into phosphoglycerides, sphingolipids and their glycosylated derivatives like glycosylphosphatidylinositols (GPIs) – all compounds that are produced in apicomplexan parasites (Dieckmann-Schuppert *et al.*, 1992; Elabbadi *et al.*, 1997; Gowda *et al.*, 1997; Florin-Christensen *et al.*, 2000; Gerold and Schwarz, 2001). In *P. falciparum* and *T. gondii*, large quantities of membrane lipids are required for establishing the parasitophorous vacuole surrounding the parasites after host cell invasion. It is still uncertain whether these lipids derive mainly from the host cell or the invading parasite or both (Lingelbach and Joiner, 1998). But if parasite lipids are indeed essential for the formation of a functional PV and therefore for successful invasion of the host cell, it might explain one curious phenomenon (Waller *et al.*, 1998). The disruption of apicoplast function or the complete absence of an apicoplast is not instantly lethal to apicomplexan parasites. Rather, it causes a peculiar 'delayed-death' phenotype instead: such parasites remain viable for a while and keep replicating but are unable to successfully re-invade another host cell and die soon thereafter (Fichera *et al.*, 1995; Fichera and Roos, 1997; He *et al.*, 2001b) (also see Section VI.A.).

D. Isoprenoid Synthesis

Isoprenoids are one of the largest and most diverse group of natural products and include sterols, carotenoids and terpenoids (Sacchettini and Poulter, 1997). Cells use two very different pathways to synthesize the C5 molecule isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), the sole precursors of all isoprenoids. Fungi, animals and certain bacteria and protozoa rely on a mevalonate-dependent pathway, whereas the so-called deoxyxylulose phosphate (DOXP) (non-mevalonate) pathway is found in plants, algae and many bacteria. In plants and algae the DOXP pathway is located in the plastid and is sometimes the organism's only source of IPP. In some algae and plants the plastid pathway is accompanied by a mevalonate pathway that is located in the cytosol or mitochondria (Ericsson and Dallner, 1993; Coppens and Courtoy, 1996; Lichtenthaler, 1999; Boucher and Doolittle, 2000; Eisenreich *et al.*, 2001; Schwender *et al.*, 2001; Steinbacher *et al.*, 2002). Several specific inhibitors have been used to study these two pathways: mevastatin (compactin), lovastatin (mevinolin) and simvastatin (synvinolin) inhibit a key enzyme involved in the synthesis of mevalonate and therefore interrupt the mevalonate-dependent pathway (Endo and Hasumi, 1993). On the other hand, the antibiotic/herbicide fosmidomycin (and its derivatives) block the DOXP pathway by targeting DOXP reductoisomerase (Lichtenthaler, 2000; Lichtenthaler *et al.*, 2000). In *Plasmodium*, the presence of a DOXP pathway has been clearly demonstrated, while reports on a possible mevalonate-dependent pathway are inconclusive.

The most impressive evidence for a functional DOXP pathway in apicomplexan parasites comes from Jomaa *et al.* (1999). Mice infected with *P. vinckei* – a rodent malaria that is lethal when untreated – could be completely cured by treatment with fosmidomycin and a derivative, FR-900098 (Jomaa *et al.*, 1999). These authors also showed that the two compounds inhibit growth of *P. falciparum* in vitro (with IC50 concentrations < 500nM), and that the *P. falciparum* DOXP reductoisomerase leader sequence is able to route GFP to the apicoplast in *T. gondii*. The presence of N-terminal leader sequences suggests that two other (nuclear-encoded) enzymes of the DOXP pathway, DOXP synthase and YgbB (IspF), are also targeted to the plastid in *P. falciparum* (Gardner *et al.*, 1999; Jomaa *et al.*, 1999; Rohdich *et al.*, 1999). Since these initial discoveries, Jomaa and coworkers were able to measure DOXP reductoisomerase activity in *P. falciparum* protein extracts (Wiesner *et al.*, 2000) and, more importantly, to modify the fosmidomycin derivative FR-900098 producing prodrugs with increased antimalarial activity after oral administration in mice (Reichenberg *et al.*, 2001).

Low levels of mevalonate and acetate incorporation into isoprenoids and some inhibition of parasite growth and development *in vitro* by mevastatin, lovastatin (mevinolin) and simvastatin also suggest the presence of a mevalonate-pathway in apicomplexan parasites (Vial *et al.*, 1984; Mbaya *et al.*, 1990; Grellier *et al.*, 1994; Chakrabarti *et al.*, 1998; Couto *et al.*, 1999). But unequivocal evidence for the presence of such a pathway in the Apicomplexa has not yet been presented. Furthermore, the almost completely sequenced genome of *P. falciparum* has not revealed any of the genes that are involved in the mevalonate-dependent pathway (S. Ralph, unpublished).

Possible end uses of IPPs include farnesyl and geranylgeranyl diphosphate which are often transferred onto proteins. Such (iso)prenylated proteins may be involved in signal transduction, protein-protein interactions and membrane-associated protein trafficking (Sinensky, 2000). Inhibitors of farnesylpyrophosphate synthase (FPPS) and protein prenyl transferases show antimalarial action, and prenyl transferase activity has been demonstrated in *P. falciparum* (Chakrabarti *et al.*, 1998; Martin *et al.*, 2001; Ohkanda *et al.*, 2001). However, these prenylation activities are most likely to occur in the cytosol and not in the plastid (Chakrabarti *et al.*, 1998). Whether or not IPP subunits from the apicoplast DOXP pathway are incorporated into these prenyl chains on cytosolic proteins remains an open question. Cells may also use IPPs for the formation of dolichols, non-sterol isoprene compounds that are required for N-glycosylation of proteins (Couto *et al.*, 1999). Again the role, if any, of the apicoplast is undetermined.

The recently discovered apicoplast-localized DOXP pathway of apicomplexan parasites certainly represents a promising drug target because it involves a different suite of enzymes to the host's mevalonate pathway (Lichtenthaler *et al.*, 2000; Vial, 2000).

E. Ferredoxin-Dependent Electron Transport

Another function of the apicoplast may be the generation of reducing power in the form of reduced ferredoxin (Vollmer *et al.*, 2001). In plants, a ferredoxin-based electron transfer system is present in plastids of both photosynthetically active and inactive tissues. During photosynthesis electrons derived from the splitting of water are

(eventually) transferred from the electron carrier ferredoxin onto the cofactor $NADP^+$ by ferredoxin-$NADP^+$ reductase (FNR). Reduced cofactors are then utilized in plastids for anabolic synthesis. In non-photosynthetic plastids, electrons go backwards being transferred from NADPH to ferredoxin by a ferredoxin-$NADP^+$ reductase (FNR) which is distinct from the FNR found in photosynthetically active plastids. The reduced ferredoxin may then serve as a reductant for various plastid-localized processes such as the desaturation of fatty acids. The presence of an FNR and a [2Fe-2S] ferredoxin (both plant-like) has been reported in the apicoplasts of *T. gondii* and *P. falciparum*, respectively (Vollmer *et al.*, 2001). The FNR found in *T. gondii* is more similar to the plant FNRs of photosynthetically inactive plastids than to those of photosynthesizing plastids and is recognized by antibodies raised against a spinach FNR. No evidence for the metabolic significance of this electron-transport mechanism in the apicoplast has been presented so far, but the confirmed presence of *de novo* fatty acid biosynthesis may suggest an involvement in fatty acid desaturation.

F. Heme Synthesis

Heme is the prosthetic group of oxygen-carrying (myo- and hemoglobin) and electron-transferring proteins (cytochromes). Several cytochromes are present in malarial parasites (Pandey *et al.*, 1986; Aldritt *et al.*, 1989; Feagin, 1992), and it appears that protein synthesis in *P. falciparum* is heme-dependent (Surolia and Padmanaban, 1991, 1992). One crucial precursor for heme biosynthesis is delta-aminolevulinate (ALA), which – like isoprenoid synthesis – has two different biosynthetic pathways. In the Shemin pathway of animals and fungi (which is located in the mitochondria) glycine and succinyl-CoA are converted into ALA by the action of delta-aminolevulinate synthetase (ALAS). The C5 or glutamate pathway on the other hand forms ALA from glutamate and is found in the plastids of algae and plants as well as in most eubacteria. In some organisms, e.g. the alga *Euglena gracilis,* both pathways are active at the same time, but in their respective compartments (Weinstein and Beale, 1983; Iida *et al.*, 2002). The two pathways converge when the enzyme delta-aminolevulinate dehydratase (ALAD) condenses two molecules of ALA yielding porphobilinogen. In plants, the gene for the plastidic ALAD is thought to derive from the plastid genome and is usually resident in the nucleus (Kaczor *et al.*, 1994).

Ironically, malarial parasites – which are inundated in heme from digested hemoglobin scavenged from the surrounding red blood cell – are dependent on their own *de novo* heme biosynthesis. It is thought that *Plasmodium* uses ALAS and the Shemin pathway in its mitochondria (Surolia and Padmanaban, 1992; Wilson *et al.*, 1996a; Srivastava and Pandey, 1998). ALAD has also been found in these parasites. While some evidence indicates that large amounts of the mammalian ALAD enzyme are imported from the red blood cell into the parasite cytoplasm (Bonday *et al.*, 1997; Bonday *et al.*, 2000; Padmanaban and Rangarajan, 2000), other authors have found that the *Plasmodium* genome encodes its own ALAD (Sato *et al.*, 2000; Sato and Wilson, 2002; Van Dooren *et al.*, 2002). This ALAD gene is transcribed in *P. falciparum* (Van Dooren *et al.*, 2002), and complementation of an *E. coli hemB* mutant demonstrated that it encodes a functional

enzyme (Sato and Wilson, 2002). Furthermore, it is likely that the malarial ALAD is active within the apicoplast for a couple of reasons: (1) the gene contains a putative plastid-targeting leader sequence (which it shares with another gene, see Section IV.E.) (Sato and Wilson, 2002; Van Dooren *et al.*, 2002), and (2) it exhibits an amino acid signature around its cation-binding site that is typical of plastidic ALADs (Sato *et al.*, 2000). It has been speculated that the malarial parasites synthesize ALA in the mitochondria and continue the biosynthetic pathway of heme with ALAD in the apicoplast, the close contact between these two organelles observed in various life stages supporting this scenario (Sato and Wilson, 2002; Van Dooren *et al.*, 2002). An alternative, and in our opinion less likely possibility is that, in addition to the Shemin pathway in the mitochondria, the C5-pathway of heme biosynthesis is active in the apicoplast, analogous to the situation found in *Euglena* (Iida *et al.*, 2002). However, no plastid-like ALAS is evident in the almost fully sequenced *P. falciparum* genome. In any case, the functional significance of ALAD imported into the malarial parasites from the mammalian host requires further clarification.

VI. Drugs

A. The Apicoplast As An Excellent Drug Target

The identification of a relict plastid in apicomplexans could have profound consequences for management of the diseases caused by these parasites. For drug purposes, we can think of the apicoplast as a mini-bacterium living inside the parasite. Normal cellular processes such as DNA replication, transcription, translation, post-translational modification, catabolism and anabolism characteristic of a free-living bacterium are likely occurring within the apicoplast. Each of these processes is bacterial in nature and potentially a drug target because they differ to the host processes, which are fundamentally eukaryotic.

Ablation of apicoplast function has been shown – particularly in *Toxoplasma* and *Plasmodium* – to lead to a 'delayed-death' phenotype where parasites stay alive and keep replicating as long as they do not leave the parasitophorous vacuole after drug administration. Growth inhibition and death typically occur with or after invasion of a new host cell (Fichera *et al.*, 1995; Fichera and Roos, 1997; Sullivan *et al.*, 2000; He *et al.*, 2001b). On the other hand, Surolia *et al.* (2002) point out that triclosan inhibition of *P. falciparum* (which is thought to interfere with fatty acid synthesis within the apicoplast, see Section VI.D.) occurs very rapidly (Surolia and Surolia, 2001), a finding that is not consistent with delayed-death kinetics. These authors therefore suggest the existence of two different categories of apicoplast functions: those that pertain to plastid housekeeping only (like DNA replication, transcription or protein translation) and whose inhibition leads to a delayed-death phenotype, and those functions that are essential for the survival of the whole organism (like fatty acid synthesis) and whose ablation rapidly leads to growth inhibition and a 'non-delayed' death (Surolia *et al.*, 2002). If this classification scheme generally proves to be true, it will be desirable to concentrate future drug development efforts more on the latter class of apicoplast functions.

For some of the drug targets described in the following sections, single point mutations may confer significant resistance to respective inhibitors. This has been shown in bacteria e.g. for FabI in combination with triclosan (McMurry *et al.*, 1998) (see Section VI.D.), and in *T. gondii* for the large ribosomal subunit RNA and clindamycin (Camps *et al.*, 2002) (see Section VI.C.) as well as for ACC and aryloxyphenoxypropionates (Zagnitko *et al.*, 2001) (see Section VI.D.). Future use of drugs targeting apicoplast functions will certainly have to take the possible development of resistance into account.

The fact that the combination of apicoplast-targeting and other drugs can have synergistic effects *in vivo* that are not necessarily observed *in vitro* is encouraging (Derouin, 2001).

B. DNA Replication and Transcription

The genome of the parasite plastid is circular (Wilson *et al.*, 1996b) (like most bacterial chromosomes) and a bacterial-type DNA gyrase is thought to be required for replication of the apicoplast genome in *P. falciparum* (Fichera and Roos, 1997). In support of this, the fluoroquinolone ciprofloxacin has been shown to inhibit apicoplast (prokaryotic) DNA replication but not nuclear (eukaryotic) replication in *P. falciparum* (Fichera and Roos, 1997). Additionally, Fichera and Roos (1997) demonstrated that ciprofloxacin inhibits apicoplast DNA replication in *T. gondii*, a close relative of *P. falciparum*. Various derivatives of quinolones and fluoroquinolones have been tested for their ability to inhibit apicoplast DNA replication and models for their mode of action proposed (Gozalbes *et al.*, 2000). These findings validate DNA replication as a viable therapeutic target in the apicoplast and point towards ciprofloxacin as a potential antimalarial.

Plastid transcription in plants and algae utilizes an RNA polymerase homologous to that of cyanobacteria and other bacteria, the so-called α_2, β, β' DNA-dependent RNA polymerase (Gray and Lang, 1998). This polymerase recognizes -10, -35 promoters courtesy of the σ factor and transcribes polycistronic RNAs from plastid DNA operons. The apicoplast genome encodes the β and β' subunits of the polymerase (*rpo*B, *rpo*C1, *rpo*C2 genes), strongly suggesting it uses a similar transcription system (Wilson *et al.*, 1996b; Köhler *et al.*, 1997). The α subunit (*rpoA*) and the σ factor (*rpoD*) are not encoded by the apicoplast DNA, so these genes are likely to be encoded by the nucleus and the products targeted into the plastid as occurs in plants and algae for the *rpoD* gene. The α_2, β, β' polymerase of bacteria and plastids is highly sensitive to rifampicin, and antimalarial activity of rifampicin suggests this drug blocks apicoplast transcription (Wilson *et al.*, 1996b). A derivative of rifampicin, rifabutin, also shows activity against *T. gondii* in *vitro* and *in vivo* (Araujo *et al.*, 1994; Olliaro *et al.*, 1994). Plant plastids also use a second, phage-like RNA polymerase that is related to the RNA polymerase used by most mitochondria (Gray and Lang, 1998). A phage-like RNA polymerase was recently identified in *P. falciparum* but it is believed to be the mitochondrial polymerase (Li *et al.*, 2001). No drugs specific to phage type polymerase are known to us, and they would likely affect the host mitochondrial RNA polymerase (very similar phage type).

C. Protein Translation

Many antibacterial agents work by specifically inhibiting bacterial protein translation. Translation almost certainly occurs in the apicoplast, but has never been formally demonstrated. Numerous drugs that block prokaryotic translation systems are parasiticidal (McFadden and Roos, 1999; Ralph *et al.*, 2001). Some of these drugs (including doxycycline, clindamycin and spiramycin) are used clinically for the treatment of malaria and toxoplasmosis, so further knowledge of their modes of action in parasites would be extremely valuable.

Lincosamides (lincomycin and clindamycin) and macrolides (erythromycin and azithromycin) block protein synthesis by interacting with the peptidyl transferase domain of bacterial 23S rRNA (Steigbigel, 1990). These antibiotics inhibit growth of apicomplexan parasites such as *P. falciparum*, *T. gondii, Cryptosporidium parvum* and *Babesia microti* (cited in Jeffries and Johnson, 1996; Blunt *et al.*, 1997; Gleeson, 2000), and in *T. gondii* there is strong circumstantial evidence implicating blockage of apicoplast translation (Pfefferkorn and Borotz, 1994; Beckers *et al.*, 1995; Fichera *et al.*, 1995; Tomavo and Boothroyd, 1995). Clones of *T. gondii* exhibiting strong clindamycin resistance *in vitro* were recently shown to contain the same, single point mutation in the gene encoding the large subunit rRNA on the apicoplast genome, corresponding to a position in the *E. coli* gene predicted to bind clindamycin (Camps *et al.*, 2002). The development of these clindamycin resistant clones through strict selection but without overt mutagenesis bodes ill for spontaneous genesis of similar drug resistance *in vivo*. Curiously, hypersensitivity to doxycycline (which targets the small subunit) was also observed in these mutants (Camps *et al.*, 2002).

Certain thiopeptide antibacterials (thiostrepton and micrococcin) are potent inhibitors of *P. falciparum* growth *in vitro* (McConkey *et al.*, 1997; Rogers *et al.*, 1997; Rogers *et al.*, 1998) and *in vivo* (Sullivan *et al.*, 2000). Thiostrepton likely binds to the guanosine triphosphatase (GTPase) binding domain of the large subunit rRNA blocking apicoplast translation (Clough *et al.*, 1997; McConkey *et al.*, 1997; Rogers *et al.*, 1997; Rogers *et al.*, 1998). Only the plastid rRNA has the thiostrepton-susceptible genotype in *P. falciparum,* so this drug is unlikely to affect cytosolic or mitochondrial rRNAs. The apicoplast rRNA of *T. gondii* does not have the susceptible genotype, and *T. gondii* growth is not inhibited by this drug (D. Roos, personal communication).

D. Fatty Acid Synthesis

Another drug target in apicoplasts is fatty acid biosynthesis. Bacteria and chloroplasts synthesize fatty acids using a type II FAS (fatty acid synthase) that utilizes several distinct enzymes for the steps of the fatty acid biosynthetic pathway, while humans and other plastid-lacking eukaryotes possess a single multi-functional type I FAS that apparently derives from gene fusion(s) of the separate type II proteins into a single multifunctional protein. Malaria parasites have long been held to be unable to synthesize fatty acids *de novo,* but emerging evidence indicates that the apicoplast harbors type II

FAS machinery (see Section V.C). Several genes encoding apicoplast-targeted homologues of bacterial type II FAS enzymes were recently identified (Waller *et al.*, 1998; McLeod *et al.*, 2001; Surolia and Surolia, 2001). One of these enzymes, ß-ketoacyl-ACP synthase (FabH), is the target for the antibiotic thiolactomycin, and thiolactomycin displays *in vitro* inhibition of *P. falciparum* (Waller *et al.*, 1998). Another member of type II FAS, enoyl-ACP reductase (FabI), has also been identified in the *P. falciparum* apicoplast (Surolia and Surolia, 2001). FabI catalyses the NADH-dependent reduction of enoyl-ACP into a saturated acyl chain (Heath and Rock, 1995) and is the target of the broad spectrum antibacterial triclosan (Levy *et al.*, 1999). Triclosan binds to and inhibits purified *P. falciparum* enoyl-ACP reductase (Perozzo *et al.*, 2002), as well as inhibiting parasite growth *in vitro* and *in vivo* (McLeod *et al.*, 2001; Surolia and Surolia, 2001). Furthermore, *in vivo* experiments using 40mg/kg triclosan on blood stage malaria parasites in mice showed no evidence of side effects (Surolia and Surolia, 2001), suggesting that triclosan is nontoxic. Other evidence for non toxicity comes from the safe, widespread use of triclosan in consumer products such as plastid food wraps, toothpastes, deodorants, soaps, detergents, and anti-acne preparations (Bhargava and Leonard, 1996). Taken together, these observations point towards triclosan as a promising antimalarial (Perozzo *et al.*, 2002; Surolia *et al.*, 2002) although the spectre of triclosan resistance needs to be examined (Ralph *et al.*, 2001).

The rate-limiting step of fatty acid synthesis in plants is the supply of malonyl-CoA by the chloroplast acetyl-CoA carboxylase (ACC), an enzyme targeted by the aryloxyphenoxypropionate class of anti-graminicidal herbicides ('fops' and 'dims') (Golz *et al.*, 1994; Konishi and Sasaki, 1994). Although the chloroplast ACC is a single large polypeptide in the grasses, in other plants it is made up of four separate subunits that are insensitive to aryloxyphenoxypropionates. *T. gondii* possesses an apicoplast-targeted ACC carboxylase similar to the multi-domain chloroplast enzyme found in diatom algae and grasses (Jelenska *et al.*, 2001), and *Toxoplasma* is likewise sensitive to aryloxyphenoxypropionate herbicides (Zuther *et al.*, 1999). Several of these herbicides also inhibited *T. gondii* growth in human fibroblasts (Zuther *et al.*, 1999). A likely apicoplast-targeted ACC is also found in *P. falciparum* (Jelenska *et al.*, 2001). Some apicomplexan parasites also appear to possess cytosolic isoforms of the enzyme, which, as with those isoforms in plants, are expected to be herbicide-insensitive (Zuther *et al.*, 1999; Jelenska *et al.*, 2001). Another class of herbicides targeting the ACC of grass, the cyclohexanediones (see Golz *et al.*, 1994 for review), did not inhibit the *T. gondii* ACC (Zuther *et al.*, 1999). Progress is being made towards an understanding of the specific interaction between herbicides and ACC (Nikolskaya *et al.*, 1999; Jelenska *et al.*, 2001), which may pave the way for the rational design of more potent ACC inhibitors.

E. Isoprenoid Synthesis

Other anabolic pathways in the apicoplast are also potential drug targets. Isopentenyl diphosphate (IPP) is the precursor for the structurally diverse isoprenoid class of compounds. In animals and plants, IPP is synthesized via the classical mevalonate pathway, but an alternate pathway proceeding via 1-deoxy-D-xylulose 5-phosphate

(DOXP) has recently been elucidated in chloroplasts and bacteria (Lichtenthaler *et al.*, 1997). Enzymes from this pathway have recently been identified in *P. falciparum* (Jomaa *et al.*, 1999) and one of these enzymes, DOXP reductoisomerase, is the target of the antibiotic fosmidomycin (see Section V.D.). Fosmidomycin inhibits growth of *P. falciparum* in culture, and cures malaria in a mouse model (Jomaa *et al.*, 1999), and recombinant *P. falciparum* DOXP reductoisomerase is inhibited by fosmidomycin (Jomaa *et al.*, 1999; Wiesner *et al.*, 2000), all of which points to fosmidomycin or derivatives (Reichenberg *et al.*, 2001) as excellent leads for new chemotherapy of malaria.

F. Synthesis of Aromatic Amino Acids

The shikimate pathway synthesizes aromatic (essential) amino acids. In plants and algae the shikimate pathway is located in the plastid. Animals lack a shikimate pathway, which is why aromatic amino acids are essential in their diet. *Plasmodium* has been presumed to be auxotrophic for aromatic amino acids, but this paradigm has recently been called into question with the discovery of a shikimate pathway in *Plasmodium, Cryptosporidium* and *Toxoplasma* (Roberts *et al.*, 1998). Glyphosate, a common herbicide also known as RoundUp™, Zero™, or Tumbleweed™, blocks the activity of 5-enopyruvyl shikimate 3-phosphate synthase, and evidence indicates that the parasite version of this enzyme is moderately sensitive to glyphosate (Roberts *et al.*, 1998; Roberts *et al.*, 2002). However, unlike plants and most algae it is now apparent that the *P. falciparum* shikimate pathway is located in the parasite cytosol (Keeling *et al.*, 1999; Fitzpatrick *et al.*, 2001) and not in the apicoplast. Nevertheless, shikimate enzymes may be useful targets for parasiticidal drugs.

G. Heme Synthesis

The Shemin pathway, which synthesizes heme from glycine and succinyl-CoA, is well known in animals. However, plastids (and their cyanobacterial forebears) use an unusual pathway starting with glutamate ligated to tRNA-Glu (Kannangara *et al.*, 1988). The Shemin pathway apparently occurs in *P. falciparum* on the basis of labeled glycine incorporation experiments (Surolia and Padmanaban, 1992) and a likely δ-aminolevulinic acid (ALA) synthase (ALAS) gene. Curiously, the only apparent enzyme for the next step of the pathway, ∂-aminolevulinate dehydratase (ALAD), appears to have an apicoplast targeting leader (Sato and Wilson, 2002; Van Dooren *et al.*, 2002). This apicoplast ALAD groups with the predominantly Mg^{2+} binding plastid ∂-aminolevulinate dehydratases, rather than with the Zn^{2+} binding mitochondrial equivalents (Sato *et al.*, 2000). At face value this suggests that the mitochondrion synthesizes ALA, and the apicoplast is responsible for the dehydratase step of the pathway. This might explain the physical link between mitochondrion and apicoplast (Waller *et al.*, 2000) if a substrate handover is occurring (see Section V.F.). Remarkably, it has also been claimed that host

ALAD is imported into *Plasmodium* from the host erythrocyte cytoplasm (Bonday *et al.*, 1997; Bonday *et al.*, 2000; Padmanaban and Rangarajan, 2000). If confirmed, this would suggest two sites of ALA processing. Clearly, localization of the enzymes recognized to date, and the characterization of the remaining unidentified enzymes of the heme synthesis pathway are required to clarify this currently confusing issue. This is potentially important for drug therapy as heme synthesis is an established target for herbicides (Prasad and Dailey, 1995), and given *P. falciparum* possesses at least one plant-like heme synthesis enzyme, the elucidation of the full pathway could illuminate further potential drug targets.

VII. Concluding Remarks

The apicoplast is a functional and indispensable organelle of apicomplexan parasites. Although Apicomplexa appear to have abandoned photosynthesis, many of the metabolic functions typically present in plastids like fatty acid, isoprenoid and heme synthesis also occur (at least partially) in the apicoplast.

The plastids found in virtually all apicomplexans studied to date apparently share a common origin and derive from one secondary endosymbiotic event that involved an alga of red algal ancestry as endosymbiont. Taken together, the diverse data in support of such a red algal ancestry (presence of *ycf24/sufB*, genomic and gene-cluster analysis, 23S rRNA, and GAPDH phylogeny) clearly outweigh the apicoplast gene-based data that initially suggested a green algal/euglenoid origin. Furthermore, the evidence indicates that apicomplexans and dinoflagellates – if not all members of the Alveolata and the Chromista (the chromalveolates, see Cavalier-Smith, 1999) – acquired their plastid in the same endosymbiotic event. It is therefore likely that early apicomplexans were photosynthetic and abandoned autotrophy in favor of parasitism. If we accept that apicomplexans and dinoflagellates diverged from a common ancestor already possessing a plastid, an intriguing scenario emerges. Dinoflagellates are marine algae that engage in various interactions with invertebrates. These interactions range from the classic commensal symbioses of zooxanthellae living within the tissues of animals such as corals to more parasitic interactions or even saprophagy in the case of the notorious 'ambush dinoflagellate' *Pfiesteria piscimorta*. The fossil record tells us that dinoflagellates are at least 400 million years old, so the common ancestor of dinoflagellates and apicomplexans must be older. We believe this ancestor was photosynthetic and able to interact intimately with invertebrates (the only animals in existence at this time), usually in the form of symbioses. In this scenario the early apicomplexans would have leant more and more to parasitism and eventually discarded photosynthesis altogether. However, because of the long residence of the plastid, the Apicomplexa had become dependent on some of its anabolic functions. Apparently, keeping a simplified and non-photosynthetic version of their plastid proved easier for these parasites than trying to obtain all the plastid's products from their hosts, and hence the apicoplast persists – a molecular fossil of a former lifestyle.

A detailed model has been proposed for apicoplast division (Striepen *et al.*, 2000) that highlights some fundamental differences between plastid division in apicomplexan

parasites and in plants. While chloroplast division occurs autonomously, division of the apicoplast appears tightly linked to nuclear division, even using parts of the mitotic apparatus. Whether a plastid division ring is truly missing or not (Matsuzaki *et al.*, 2001) is still unclear.

Apicoplast-targeting of nuclear-encoded proteins via the secretory pathway courtesy of a bipartite N-terminal leader sequence has unambiguously been demonstrated and is analogous to that in algae with multiple (more than two) plastid membranes. A thorough understanding of transit peptides and the apicoplast protein import machinery is very useful. Firstly, it will yield knowledge on protein targeting and import mechanisms in plastids acquired through secondary endosymbiosis in general. Apicomplexan parasites will be good models for algae with such 'complex' plastids, particularly *Toxoplasma* which is very amenable to genetic manipulation and experimentation (Roos *et al.*, 1999b). Secondly, components of the import apparatus itself may represent good drug targets, and research efforts in our laboratory to characterize the plastid import machinery have so far identified putative homologues of Toc and Tic components in *P. falciparum* (C. Tonkin, unpublished). Thirdly, the particular characteristics of apicoplast-targeting leader sequences in *P. falciparum* have already led to the development of two bioinformatic tools that are able to predict apicoplast-targeted proteins from malarial genome data (Zuegge *et al.*, 2001) (Foth *et al.*, manuscript in preparation). With the malarial genome sequencing project almost finished, more integrated bioinformatic approaches (Roos *et al.*, 2002) in combination with proteomics performed on isolated organelles will refine and expand our knowledge of the apicoplast proteome.

The prokaryotic nature of its metabolism and its presence throughout the apicomplexan lineage make the apicoplast an excellent drug target. After only a few years of research, a relatively large number of antimalarial compounds with alleged targets in the apicoplast have been identified. If these targets turn out to be viable in therapy, an enormous head start for drug development is already in hand. Since the mode of action and potential mechanisms of resistance for many of these lead compounds is already established in bacterial systems, we can anticipate relatively rapid progress towards utility. Furthermore, we expect apicoplast research to lead to the discovery of new and so far uncharacterized plastid-specific molecular processes that will push the frontiers of plastid biology and plastid evolution. Such new insights will hopefully provide even more key leads for our fight against malaria and other diseases caused by apicomplexan parasites.

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