The cell biology of secondary endosymbiosis – how parasites build, divide and segregate the apicoplast

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Summary
Protozoan parasites of the phylum Apicomplexa harbour a chloroplast-like organelle, the apicoplast. The biosynthetic pathways localized to this organelle are of cyanobacterial origin and therefore offer attractive targets for the development of new drugs for the treatment of malaria and toxoplasmosis. The apicoplast also provides a unique system to study the cell biology of endosymbiosis. This organelle is the product of secondary endosymbiosis, the marriage of an alga and an auxotrophic eukaryote. This origin has led to a fascinating set of novel cellular mechanisms that are clearly distinct from those employed by the plant chloroplast. Here we explore how the apicoplast interacts with its ‘host’ to secure building blocks for its biogenesis and how the organelle is divided and segregated during mitosis. Considerable advances in parasite genetics and genomics have transformed apicomplexans, long considered hard to study, into highly tractable model organisms. We discuss how these resources might be marshalled to develop a detailed mechanistic picture of apicoplast cell biology.

Introduction
Plastids are the cellular home of photosynthesis in eukaryotes and in addition important parts of the cellular metabolism. They are found in plants and a broad range of autotrophic organisms traditionally referred to as algae and somewhat surprisingly in protozoan parasites of the phylum Apicomplexa. Endosymbiosis of a free-living cyanobacterium by a eukaryote is now widely accepted as the evolutionary mechanism that led to this group of organelles. Plastids are semiautonomous and maintain their own (albeit reduced) organellar genome. While the organisms harbouring plastids appear polyphyletic, the organelle itself shows a monophyletic origin. This apparently mixed ancestry is the product of promiscuous horizontal transfer of the organelle. Numerous eukaryotic lines independently captured plastids by secondary endosymbiosis. They engulfed a single celled alga and, through progressive gene transfer to the host nucleus, reduced the endosymbiont to a fully dependent organelle (Stoebe and Maier, 2002).

The plastid of apicomplexans, or apicoplast, is the product of such a secondary endosymbiosis event. The presence of the apicoplast has been demonstrated in all intensely studied apicomplexans with the marked exception of the genus Cryptosporidium (Zhu et al., 2000). Apicomplexa are important human pathogens and claim among their ranks the causative agents of malaria and several AIDS-associated opportunistic infections. It is therefore not surprising that much of the research on this organelle has been geared towards exploiting it as a drug target. Pharmacological and genetic studies have demonstrated that the apicoplast is essential for parasite survival. Genomic (and some experimental) analyses suggest that the apicoplast harbours pathways for the synthesis of fatty acids, isoprenoids and haem (Gardner et al., 2002; Ralph et al., 2004). These pathways are of cyanobacterial origin and differ considerably from equivalent pathways in the human host, making them attractive targets for the development of parasite-specific drugs. Beyond its appeal as a drug target the apicoplast also presents a fascinating and experimentally tractable model to explore the cell biology of endosymbiosis. How is this ménage a trois of two eukaryotes and a prokaryote integrated at the cellular and molecular level? The merger of three previously self-sufficient organisms generates redundancy and choices. Who’s endowment was used to build the new cell and which are the interfaces that bridge the cellular machineries of such divergent phylogenetic origin? Here we will discuss recent advances in our understanding of the mechanisms by which apicomplexans build, divide and segregate their plastids.
Who provides the building blocks for apicoplast biogenesis?

Very few of the estimated 500 apicoplast proteins are actually encoded on the 35 kb organellar genome (Wilson et al., 1996; Foth et al., 2003). As observed for mitochondria and chloroplasts, the apicoplast genome has experienced massive gene transfer to the nuclear genome. This arrangement provides the ‘host’ with tight control over the endosymbiont, yet it also requires a way to route proteins, which are now synthesized outside of the organelle, back into the plastid. In contrast to chloroplasts, which are bounded by two membranes, the apicoplast boasts four membranes, a telltale of its secondary endosymbiotic origin. The outermost membrane is considered a part of the ‘host’s’ endomembrane system. This has important implications for protein targeting. Most apicoplast proteins carry an N-terminal bipartite targeting motif consisting of a classical signal peptide, which mediates cotranslational insertion into the endoplasmic reticulum (ER) and an adjacent transit peptide that is required for routing to the plastid and import into the lumen (Waller et al., 1998; 2000; DeRocher et al., 2000). How apicoplast proteins traffic from the ER to the apicoplast and how they cross the remaining three membranes remains unclear. However, recent work demonstrated that apicoplast protein trafficking is insensitive to the Golgi disrupting agent Brefeldin A and the cis-Golgi localized ERD2 mediated retrieval mechanism (DeRocher et al., 2005; Tonkin et al., 2006). This suggests that trafficking occurs directly from the ER and does not include a sorting step in the Golgi. Models which are currently under consideration include that the outer compartment of the apicoplast is a permanent part of the ER (Fig. 1, route 2), vesicular trafficking from the ER to the apicoplast (route 3), or that apicoplast targeting occurs at a specialized region of the ER that cradles the apicoplast, by either vesicular transport or membrane-spanning tubules or pores (route 1).

Several models have been proposed for how proteins cross the remaining three membranes to the lumen of the organelle (see, e.g. van Dooren et al., 2001). Recently Tomova et al. have produced high-resolution electron tomograms of the apicoplast membranes (Fig. 1C and D, Tomova et al., 2006). The two innermost membranes are tightly apposed while the outer membranes are of more irregular shape. Most interestingly, large regularly distribu-
ulated mass density patches appear to span the two inner as well as the third membrane. This morphology could be consistent with a two-pore model of import (Fig. 1B). If these structures indeed represent protein import complexes as speculated remains to be validated by molecular studies.

In addition to proteins the apicoplast must import many of the small molecule precursors for nucleotide, protein, haem and lipid synthesis (as well as potentially export products of biosynthesis). Yet relatively few transporters have been identified so far (Ralph et al., 2004). A recent study described two apicoplast-targeted putative triose-phosphate transporter (TPT) proteins from *Plasmodium falciparum* and has shed some light on the differential targeting of plastid membrane proteins (Mullin et al., 2006). PfiTPT bears a bipartite leader sequence which is proteolytically removed from the mature protein. From this finding (the candidate processing protease is expected to be a luminal enzyme) and protease protection assays the authors conclude that PfiTPT is most likely localized to the innermost apicoplast membrane. The second transporter PFoTPT lacks a stereotypical apicoplast targeting motif and localizes to the outermost membrane of the organelle. Intriguingly, the protein appears to be unevenly distributed on the apicoplast surface. The apicoplast also has the capability to autonomously synthesize some of the molecules required for it genesis. For example, the synthesis pathway for lipoic acid appears independent from that found in the mitochondrion and dependent on apicoplast type II fatty acid biosynthesis (Wrenger and Muller, 2004; Crawford et al., 2006; Mazumdar et al., 2006). The type II fatty acid synthesis pathway could also provide precursors for some of the lipids required for apicoplast membrane biogenesis. Consistent with this assumption, genetic targeting of this pathway leads to severe defects in apicoplast biogenesis (Mazumdar et al., 2006).

The apicoplast genome remains in prokaryotic hands

The surprising level of effectiveness of several antibiotics (that show tight specificity for prokaryotic targets) against apicomplexan parasites pointed to the fact that the apicoplast might rely on its ancestral cyanobacterial transcription, translation and replication machinery (Clough et al., 1997; 1999; Fichera and Roos, 1997; Camps et al., 2002; Sato and Wilson, 2005). Recently Seow and colleagues described a candidate replicative DNA polymerase for the *P. falciparum* apicoplast (Seow et al., 2005). Interestingly, this gene encodes a helicase, primase, 5′-3′-exonuclease and a prokaryotic Pol-A type DNA polymerase as a single large polypeptide. Once transported into the plastid, this protein is cleaved into at least two smaller functional units (the *Toxoplasma gondii* genome encodes a very similar polypeptide). In both *P. falciparum* and *T. gondii* the genome is present in multiple copies. Surprisingly, however, the topology and mechanism of genome replication appears to differ considerably. The *P. falciparum* apicoplast genome is circular and mainly uses a bidirectional double D-loop replication mechanism (Williamson et al., 2002; Singh et al., 2005). In contrast in the *T. gondii* apicoplast the genome appears to be a linear concatamer of multiple genomes and its replication most likely follows a rolling circle model (Williamson et al., 2001). It is tempting to speculate that the basis for this difference might lie in the different cell and apicoplast division models these two parasites use (see below).

Light microscopic studies in *T. gondii* have suggested that the apicoplast genome is organized into a nucleoid which segregates into two equal portions during apicoplast division (Striepen et al., 2000; Matsuzaki et al., 2001). The molecular basis of faithful apicoplast genome segregation is unknown. Based on the imaging data discussed above a model invoking a role for the centrosome in nucleoid positioning appears reasonable. However, direct experimental support for this model is lacking (this model would require additional proteins within the multiple apicoplast membrane to ‘transmit’ this interaction). The identification of a bacterial type histone-like HU protein presents an attractive candidate molecule potentially orchestrating nucleoid organization (Kobayashi et al., 2002). The HU-protein encoded in the *T. gondii* genome indeed targets to the apicoplast where it colocalizes with its genome and overexpression of this gene results in pronounced missegregation of the apicoplast genome (S. Vaishnava and B. Striepen, unpublished).

Chloroplasts are cyanobacteria – mostly

As plastids cannot form *de novo* they have to be replicated prior to cell division. In chloroplasts this is accomplished using constrictive division rings that form on the inside as well as the outside of the organelle. All chloroplasts studied so far depend largely on their ancestral bacterial division machinery for this purpose (Osteryoung et al., 1998; Miyagishima et al., 2004, Fig. 2). The most conserved element of this machinery is FtsZ, a GTPase structurally related to tubulin and conserved in most eubacteria, archaea and plastid-bearing eukaryotes. FtsZ localizes to the division ring in the chloroplasts of the green plant *Arabidopsis thaliana* and the red alga * Cyanidioschyzon merolae*, and is essential for its function (Stokes et al., 2000; Miyagishima et al., 2004). In bacteria, assembly and positioning of the FtsZ ring is controlled by MinC, D and E. Homologues of MinD and E have been identified in the nuclear genome of *Arabidopsis* and their role in plastid division has been experimentally confirmed.
The putative mitochondrial Dmn1 (our Arc5 queries identify the mitochondrial Dnm1 with low p-score due to conserved dynamin-like sequence of chloroplast division proteins using sequences from genomes of a number of apicomplexans for homologues. One would expect the apicoplast to equally lie in a dramatic shift towards genuinely eukaryotic mechanisms. Even before this organelle was identified as a plastid its proximity to the Golgi and the nucleus had been noticed (e.g. Muller, 1975). In vivo time lapse microscopy in GFP-tagged T. gondii showed that apicoplast division concurred with nuclear division and daughter cell budding (Striepen et al., 2000). During this process the organelle transforms into a tubule and its ends show tight physical association with the centrosomes (Fig. 3A). This hitch to the mitotic spindle ensures faithful segregation of the organelle into the daughter buds. Consequently, pharmacological ablation of spindle microtubules interferes with plastid segregation. How apicoplast fission occurs is less clear and two models have been proposed. The first model is based on electron microscopic observation of apicoplast constriction (Matsuzaki et al., 2001; Colletti et al., 2000; Itoh et al., 2001; Reddy et al., 2002). Chloroplast division also requires Ftn2/ARC6 and Artemis, two inner membrane proteins not found in all eubacteria but restricted to the cyanobacterial lineage (Fulgosi et al., 2002; Vitha et al., 2003). While the luminal division apparatus is clearly in bacterial hands, the cytoplasmatic division ring is an eukaryotic invention. The critical component of this ring appears to be the dynamin-related protein ARC5 (Gao et al., 2003; Miyagishima et al., 2003a). In Arabidopsis, mutations in ARC5 result in chloroplasts that are centrally constricted but fail to complete the division process, indicating that this protein might be required only at later stages of chloroplast division, e.g. to sever already constricted membranes.

**Apicomplexa have lost the conserved chloroplast division machinery**

The secondary endosymbiotic origin of the apicoplast is well established, although there is some controversy about whether the endosymbiont was of red or green algal lineage. However, as both lineages depend on FtsZ for division one would expect the apicoplast to equally employ this conserved machinery. We mined the genomes of a number of apicomplexans for homologues of chloroplast division proteins using sequences from A. thaliana as BLAST queries (ApiDB.org). Figure 2 summarizes the results of these efforts. We did not identify any well-supported homologues for FtsZ, MinD, MinE, ARC5, ARC6 or Artemis (note that FtsZ and ARC5 are highly conserved, see results for C. merolae in Fig. 2 and Miyagishima et al., 2003b).

Apicomplexan genomes encode two dynamin-related proteins. Our Arc5 queries pick up both dynamins with low P-values. However, these dynamins appear more closely related to other members of the dynamin family, such as the dynamin involved in mitochondrial division (Shaw and Nunnari, 2002) as indicated by the considerably stronger P-value when the DMN1 protein is used (see Fig. 2, the second dynamin most likely represents the dynamin involved in vesicle fission). Thus, a clear Arc5 homologue seems to be missing in Apicomplexa. We therefore believe that the FtsZ and ARC5 division machineries, which are conserved among a wide variety of plastid harbouring organisms, are absent in Apicomplexa. It is interesting to note that other organisms harbouring secondary plastids show clear homologues of FtsZ suggesting that the loss of the bacterial division machinery is not an obligatory consequence of secondary endosymbiosis but specific to the phylum Apicomplexa (Fraunholz et al., 1998; Armbrust et al., 2004).

**One ring to rule them all?**

How are apicoplasts divided and segregated in the absence of the conserved machinery? The answer might lie in a dramatic shift towards genuinely eukaryotic mechanisms. The cell biology of secondary endosymbiosis 1383

**Table 1:** Comparative genomic analysis of plastid and mitochondrial division in Apicomplexa. The genomes of Apicomplexa lack genes encoding the conserved chloroplast division machinery. The table shows results of BLAST searches using the ApiDB dataset (ApiDB.org). The right panel shows a simplified model of chloroplast division in A. thaliana. FtsZ forms a luminal, ARC5 a cytoplasmic chloroplast division ring. Min D, E and ARC6 play a role in positioning the division ring while Artemis appears to be involved in the final step of fission and membrane fusion. A short contig with similarity to FtsZ was identified searching ToxoDB, closer inspection however, suggesting a bacterial contamination. Min D, E and ARC6 play a role in positioning the division ring while Artemis appears to be involved in the final step of fission and membrane fusion.1A short contig with similarity to FtsZ was identified searching ToxoDB, closer inspection however, suggesting a bacterial contamination.

<table>
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<th>Gene</th>
<th>A.thaliana</th>
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<th>T.gondii</th>
<th>C.parvum</th>
<th>C.merolae</th>
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(Fulgosi et al., 2003a; Colletti et al., 2000; Itoh et al., 2001; Reddy et al., 2002).
Fig. 3. Centrosome attachment and a constrictive budding ring orchestrate apicoplast division.

A. Dividing T. gondii tachyzoites labelled for the nucleus (histone H2B-RFP), inner membrane complex (IMC3-YFP), apicoplast ($\alpha$-ACP and FNR-RFP), centrosome ($\alpha$-centrin), and MORN1-ring (MORN1-YFP). Reproduced from Striepen and Soldati (2006).

B. Schematic representation of daughter cell budding in T. gondii. The apicoplast is shown in green, nucleus and plastid genome in turquoise, mitotic spindle in lavender, centrosome in red and MORN1 rings in dark-red.

C. Apicoplast morphology in developing intracellular stages of Toxoplasma, Plasmodium, Eimeria and Sarcocystis.

D. A diagram of the three cell cycle and cell division models of Apicomplexa and the apicoplast development associated with each (apicoplast, green; centrosome, red; nucleus, turquoise). The number of meronts has been limited to 8 for simplicity. Note that apicoplast fission concurs with daughter cell budding. Plastid–centrosome association during schizogony is hypothetical and has not been validated experimentally.
Ferguson et al., 2005). These constrictions have been interpreted as plastid division rings. However, as discussed above the genes for the crucial components of these rings appear absent from the apicomplexan genome. A second model has argued that plastid fission might occur through the upward pull of the spindle countered by the downward growth of the daughter cell pellicle (Fig. 2B, Striepen et al., 2000). New support for this idea comes from the discovery of a motile ring at the posterior end of the daughter bud (Gubbels et al., 2006; Hu et al., 2006). The ring identified by the membrane occupation recognition nexus protein MORN1 moves over nucleus and plastid and constricts. The MORN-rings coincide precisely with apicoplast constrictions (see Fig. 2A). The downward movement of the ring is driven by microtubular growth. The constriction might depend on myosin as T. gondii Myosin-C colocalizes with MORN1 at the ring (Delbac et al., 2001; Gubbels et al., 2006). It is important to note that the two fission models are not mutually exclusive. Multiple rings could be at work as seen for the chloroplast. It is, e.g. conceivable that the mitochondrial dynamin like protein might double up and constrict both mitochondrion and apicoplast [however, in P. falciparum the apicoplast clearly divides before the mitochondrion (van Dooren et al., 2005)].

The fission through budding model is consistent with comparative analyses of plastid development in different apicomplexan species. Apicomplexans employ three types of cell division: endodyogeny in which DNA replication is immediately followed by nuclear division and cytokinesis, schizogony where multiple nuclear divisions occur prior to cytokinesis and endopolygeny where multiple rounds of DNA replication result in a polyploid nucleus which fragments and segregates upon budding (see Fig. 3D for a schematic outline). Importantly in all three division modes, plastid fission coincides with daughter cell formation and budding (Striepen et al., 2000; Vaishnava et al., 2005; van Dooren et al., 2005). Laser bleaching experiments in Sarcocystis neurona confirmed that while the parasite is growing the plastid remains a single continuous organelle mirroring the nucleus. Throughout the development and growth of the parasite the plastid maintains tight association with the cell’s multiple centrosomes (Vaishnava et al., 2005). Apicomplexans dividing by schizogony equally show a single plastid that can be highly branched (Fig. 3D). While apicoplast centrosome association has been proposed to occur during budding in P. falciparum, experimental evidence is lacking (van Dooren et al., 2005).

Would the genes involved please step forward
Reverse genetic and in vivo microscopy studies focused on the apicoplast have led to the discovery of a set of fascinating unique mechanisms for organellar biogenesis. Protein targeting occurs through the secretory pathway following a new Golgi-independent route, and apicoplast division employs spindle pole association and a novel cytokinesis ring. However, all models currently suffer from a paucity of molecular data. Spindle pole association, e.g. likely requires the specific interaction of proteins on the surface of the apicoplast with proteins in the centrosome, yet we have only a very sketchy list of the protein composition of both organelles in apicomplexans. The combination of three approaches might help to fill this void. Mining the apicomplexan genomes for candidate genes based on similar function in other organisms followed by experimental verification is an obvious first choice. Work in Trypanosoma brucei, e.g. has shown that Golgi segregation depends on a specific centrin paralogue (He et al., 2004). A systematic genetic test of all apicomplexan centrosins might lead to the centrosomal partner of this interaction. The second approach could exploit the availability of multiple apicomplexan genomes for comparative genomic analysis. Cryptosporidium, the only genus lacking an apicoplast might hold the key here. It is reasonable to hypothesize that proteins required for plastid biogenesis might be conserved in a variety of apicomplexans harbouring apicoplasts yet be missing in Cryptosporidium. Lastly, T. gondii offers the possibility to employ forward genetic screens to identify essential components for plastid biogenesis. Temperature-sensitive mutants are easily obtained in this organism and microscopy-based screens and complementation cloning are feasible (Striepen et al., 2002; Gubbels et al., 2004; White et al., 2005). We now have in place some blueprints and sketches on how to build, divide and segregate the apicoplast. It remains to identify the molecular labourers involved in constructing this fascinating and medically important organelle.

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