Published in final edited form as:

J Eukaryot Microbiol. 2009 ; 56(3): 214–220. doi:10.1111/j.1550-7408.2009.00405.x.

Evolving Insights into Protein Trafficking to the Multiple Compartments of the Apicomplexan Plastid

MARILYN PARSONS, ANURADHA KARNATAKI, and AMY E. DEROCHER

Seattle Biomedical Research Institute, 307 Westlake Ave. N., Seattle, Washington, 98109 USA

Abstract

The apicoplast is a relict plastid found in many medically important apicomplexan parasites, such as *Plasmodium* and *Toxoplasma*. Phylogenetic analysis and the presence of four bounding membranes indicate that the apicoplast arose from a secondary endosymbiosis. Here we review what has been discovered about the complex journey proteins take to reach compartments of the apicoplast. The targeting sequences for luminal proteins are well-defined, but those routing proteins to other compartments are only beginning to be studied. Recent work suggests that the trafficking mechanisms involve a variety of molecules of different phylogenetic origins. We highlight some remaining questions regarding protein trafficking to this divergent organelle.

The double-membraned plastids of most algae and land plants are the result of an ancient endosymbiosis between a photosynthetic bacterium and an early eukaryote. Eons later, an alga set up house in an early progenitor of modern day apicomplexan parasites and its alveolate relatives. The lineage leading to Apicomplexa jettisoned all of the algal cell except for some of its genes and its plastid. The non-photosynthetic apicomplexan plastid is now called the apicoplast and is bounded by four membranes (Kohler et al. 1997; McFadden et al. 1996) (Fig. 1–3). The inner two of these membranes are thought to be derived from the original plastid envelope and hence are of cyanobacterial origin. In contrast, the outer two membranes are likely of eukaryotic origin, possibly derived from the algal plasma membrane and an apicomplexan membrane such as an endosomal membrane or the plasma membrane (Cavalier-Smith 2003). Although the algal nucleus has been lost, its past presence can be deduced from the existence of genes in the apicomplexans that are phylogenetically related to those of alga, including many that encode proteins that typically reside in plastids (Fast et al. 2001; Ralph et al. 2004b). The non-photosynthetic apicoplast is phylogenetically related to the photosynthetic plastid of the alveolate *Chromera velia*, (Moore et al. 2008). Apicomplexa and other alveolates are proposed to belong to a superfamily of protists, the chromalveolates, which includes several other photosynthetic species (Harper and Keeling 2003; van Dooren et al. 2001). Remarkably, some of these, such as the cryptophytes, still have a functional remnant algal nucleus called the nucleomorph between the second and third membrane of the plastid, substantiating the secondary endosymbiosis theory (Douglas et al. 2001; Wastl and Maier 2000).

The apicoplast genome itself has been reduced to just 35 kB. It encodes some genes required for gene expression (such as ribosomal RNAs and tRNAs), plus a few protein coding genes (Wilson et al. 1996). Hence, almost all proteins required for the metabolic pathways of the apicoplast have been transferred to the nucleus. These metabolic steps include reactions involved in biosynthesis of fatty acids (Mazumdar et al. 2006; Waller et al. 1998), isoprenoids (Jomaa et al. 1999), heme (Varadharajan et al. 2004), and iron-sulfur clusters (Seeber 2002).

As expected from the lack of photosynthetic pathways, the apicoplast also lacks the membranous thylakoids where photosynthesis occurs. Nonetheless, the apicoplast is essential for the survival of both *Plasmodium falciparum* and *Toxoplasma gondii* (Dahl et al. 2006; Fichera and Roos 1997). Since the human host lacks this organelle, the apicoplast-localized prokaryotic-like pathways are considered potential targets for development of novel anti-parasitic drugs (Goodman and McFadden 2007; Wiesner and Jomaa 2007; Wiesner and Seeber 2005). Most of the apicomplexan parasites that are important to the health of humans and farm animals [which include *Eimeria* (Cai et al. 2003) and *Theileria* (Gardner et al. 2005) in addition to the parasites mentioned above], contain an apicoplast. An important exception is *Cryptosporidium* (Zhu et al. 2000). The large majority of work on protein targeting to the apicoplast has been conducted in the more well-developed experimental systems afforded by *P. falciparum* and *T. gondii*.

Sequence determinants of trafficking to the apicoplast lumen

The presence and distinct origins of the four membranes of the apicoplast pose a challenging problem to the cell. As the protein-coding genes are nucleus-encoded, apicoplast luminal proteins themselves must find their way across four membranes. Furthermore, other proteins must also reach the different membranes and intermembrane spaces within the apicoplast. Specific protein import machinery must exist at each membrane to facilitate transport of proteins, while allowing others to be retained. It has been hypothesized that the import machinery of each membrane reflects the origin of that membrane (van Dooren et al. 2001).

When the 35 kD DNA was recognized as similar to that of chloroplasts (as opposed to mitochondria), and as residing in a multimembraned compartment, the search began for apicomplexan genes encoding proteins homologous to those of chloroplasts. These studies exploited the nascent genome projects in *T. gondii* (Ajioka et al. 1998; Gajria et al. 2007; Kissinger et al. 2003) and *P. falciparum* (Gardner et al. 2002). Several candidate genes were identified, allowing experiments to identify the determinants required for targeting of those proteins to the apicoplast. Notably these proteins had N-terminal extensions as compared to their bacterial counterparts. The N-terminal extensions commenced with a predicted signal peptide, presumably to route the proteins into the endoplasmic reticulum (ER). When these extensions were fused to the 5' end of the gene-encoding green fluorescent protein (GFP) and expressed in the parasites, the resulting proteins localized to the apicoplast lumen (DeRocher et al. 2000; Waller et al. 1998, 2000; Yung et al. 2001). However, when only the signal peptide region was used, the GFP fusion proteins were secreted. Furthermore, signal peptides from other proteins of the secretory system could be substituted for those of apicoplast proteins without affecting targeting to the organelle (Tonkin et al. 2006b). Thus it appears that the signal peptides of apicoplast proteins function to insert those proteins into the ER. Furthermore, the signal peptide is required for apicoplast targeting, since its deletion led to cytosolic or mitochondrial localization of different proteins (DeRocher et al. 2000; Harb et al. 2004; Waller et al. 2000; Yung et al. 2001). Thus the initial step in targeting of proteins to the apicoplast is fundamentally different from that in targeting to primary plastids, in which proteins are typically synthesized in the cytosol and routed directly into the plastid. On the other hand, this first step, entry into the ER, seems to be a common feature for secondary plastids, whether they are thought to be derived from red alga [e.g. Apicomplexa, dinoflagellates (Sharples et al. 1996) and stramenopiles (heterokonts) (Bhaya and Grossman 1991)] or green alga [e.g. *Euglena* (Sulli et al. 1999; Sulli and Schwartzbach 1995)]. This finding reinforces the concept that the outermost membrane of secondary plastids is related to that of the secretory system. In fact, the secondary plastids of stramenopiles bear ribosomes on their outer surface, indicating that proteins are directly imported across the outer plastid membrane during synthesis (Gibbs 1979). However, ribosomes are not detected on the outer membrane of the apicoplast.

Interestingly, the N-terminal extension present on apicoplast luminal proteins consists of more than just a signal peptide. It is followed by a region of variable length and sequence which is enriched in basic amino acids and reduced for acidic amino acids. This region is similar to sequences which route proteins to the stroma of chloroplasts, dubbed transit peptides, which are also enriched for basic and hydroxylated amino acids (reviewed in (Bruce 2001)). In *P. falciparum*, the AT bias of the nuclear genome means that most of the basic amino acids are lysine and asparagine, whereas in *T. gondii* (which lacks such an AT bias) they are predominantly lysine and arginine (Ralph et al. 2004a). This region is also required for routing proteins to the apicoplast (DeRocher et al. 2000; Waller et al. 2000) and at least one such region, that of *T. gondii* ribosomal protein S9, has been shown to allow import of a reporter protein into pea chloroplasts (DeRocher et al. 2000). In recognition of these conserved characteristics, this portion of the apicoplast bipartite targeting sequence is also called the transit peptide. Mutagenesis experiments on the transit peptide of acyl carrier protein (ACP) have shown that an overall positive charge of the transit peptide, particularly near the N-terminus, is important for proper targeting to the apicoplast in both *P. falciparum* and *T. gondii* (Foth et al. 2003; Tonkin, Roos, and McFadden 2006a). However, the exact location of the basic residues is not important for proper targeting. The ACP transit peptide in *P. falciparum* also encodes a predicted Hsp70 binding site and point mutagenesis in this site prevented targeting to the apicoplast suggesting that Hsp70 acts as a molecular chaperone during protein trafficking to the apicoplast (Foth et al. 2003). The apicomplexan transit peptides lack the hydrophobic stop-transfer sequences often seen on proteins destined for the three-membraned secondary plastids of *Euglena* and dinoflagellates (Patron et al. 2005; Sulli et al. 1999; Sulli and Schwartzbach 1995). The stop-transfer sequences arrest import into the ER, so that only the transit peptide is imported. Hence these proteins traffic as single pass transmembrane proteins. In contrast proteins destined for the apicoplast lumen appear to be fully imported into the ER prior to trafficking.

In most examples studied thus far, the predicted bipartite trafficking sequence is sufficient to route a reporter to the apicoplast lumen. However, in the case of superoxide dismutase 2, this bipartite sequence routes a reporter to the mitochondrion (Brydges and Carruthers 2003). Only when the full-length protein is fused to GFP is the protein also localized to the apicoplast (Pino et al. 2007). Additional proteins, such as the thioredoxin-dependent peroxidase, aconitase, and a pyruvate kinase are also dually localized to the mitochondrion and apicoplast (Pino et al. 2007; Saito et al. 2008). The apicoplast and mitochondrial pyruvate kinase are encoded by the same nuclear gene, but initiate translation from different sites (Saito et al. 2008). In other cases alternative splicing or alternative translation start sites have been ruled out (Pino et al. 2007), suggesting that the same initial translation product can be routed either to the mitochondrion or to the apicoplast via the ER. The proposed model is that the signal sequence of these proteins binds relatively poorly to the ER signal recognition particle, allowing some of the molecules to be fully translated in the cytosol and then imported into the mitochondrion. Conversely, although some apicoplast transit peptides resemble mitochondrial targeting sequences, the presence of a strong signal peptide routes the proteins effectively into the ER, precluding targeting of those proteins to the mitochondrion. This is clearly different from the situation with primary plastids, where organelle-specific targeting is accomplished by the transit peptide itself.

Apicomplexan parasites have several unique secretory organelles including dense granules, micronemes, and rhoptries (see Fig. 1). Apicoplast proteins must be distinguished from proteins targeted to these organelles, and for luminal proteins the transit peptide fulfills that function. It is probable that the transit peptides of apicoplast proteins have distinct functional domains (Foth et al. 2003; Harb et al. 2004), reflecting their required actions at different steps of the protein trafficking process. An extensive study of the *T. gondii* ferredoxin reductase transit peptide indicated that even though several deletions had no discernable effect on apicoplast

targeting, larger deletions from the transit peptide led to accumulation at the periphery of the apicoplast, mislocalization to the rhoptries, or secretion. Like the transit peptides of chloroplasts, the apicomplexan transit peptides are cleaved to yield the mature protein. A protease related to the plastid stromal processing peptidase has been identified in *P. falciparum* and this protease also bears a signal and transit sequence, indicating that it resides in the apicoplast lumen (van Dooren et al. 2002).

The similarities between various apicoplast targeting sequences from *P. falciparum* led to the development of algorithms that predict whether a protein is likely to reside in the apicoplast (Foth et al. 2003; Zuegge et al. 2001). Application of one of these algorithms to the *P. falciparum* genome, coupled with knowledge about chloroplast metabolic pathways, led to the identification of over 500 candidate apicoplast proteins including those participating in the pathways noted earlier (Ralph et al. 2004b). As yet, relatively few have been experimentally verified to reside in the plastid. Furthermore many of the proteins predicted to be targeted to the plastid are of unknown function (i.e. hypothetical), raising the possibility that additional pathways may map to the organelle.

Proteins targeted to the outer compartments of the apicoplast

Very few proteins localized to the apicoplast membranes or intermembrane spaces have been identified thus far. The trafficking of these proteins as compared to luminal proteins is summarized in Table 1 and their localization is depicted in Fig. 2. Interestingly, only two of the identified proteins contain a typical bipartite targeting sequence: One is a predicted transporter of sugar phosphates and related molecules dubbed the *P. falciparum* inner membrane triose phosphate transporter (PfiTPT) (Mullin et al. 2006). This molecule was so-named because of its similarity to translocators that reside in the chloroplast inner membrane and exchange phosphorylated C3, C5, and C6 compounds for inorganic phosphate (although the specificity of PfiTPT has not been demonstrated). PfiTPT, which has multiple transmembrane domains, is processed to remove the transit peptide, indicating that the N-terminus of the molecule was exposed to a peptidase, most likely the stromal processing peptidase mentioned above. This bipartite targeting sequence was able to escort GFP to the apicoplast lumen in *T. gondii* (Karnataki et al. 2007a). Furthermore, PfiTPT is not sensitive to exogenous protease, arguing that the protein is not exposed to the cytosol. The authors propose therefore that this protein is confined to the inner membrane of the apicoplast (Mullin et al. 2006). The other protein is TgTic20 a homolog of a chloroplast inner membrane translocon component which was identified in *T. gondii* (van Dooren et al. 2008). The C-terminus of TgTic20 was demonstrated to be exposed to the lumen of the apicoplast using the split GFP system (Cabantous, Terwilliger, and Waldo 2005). Here, the C-terminal segment of GFP was fused to the C-terminus of TgTic20, and the bulk of GFP was directed to the apicoplast lumen using the apicoplast targeting sequence of ferredoxin reductase. Although neither partial protein alone is fluorescent, if they are in the same compartment they will interact to yield a fluorescent protein. The fluorescent signal obtained indicates that the C-terminus of TgTic20 lies within the apicoplast lumen. Because the signal and transit regions of PfiTPT and TgTic20 can confer localization of a reporter to the apicoplast lumen, these proteins are thought to traffick similarly to luminal proteins, although they are of course embedded in the membrane (Mullin et al. 2006).

Other non-luminal proteins of the apicoplast lack the typical N-terminal bipartite targeting sequence. Two examples are the putative orthologues *P. falciparum* outer membrane triose phosphate transporter PfoTPT (Mullin et al. 2006) and *T. gondii* apicoplast phosphate translocator TgAPT1 (Karnataki et al. 2007a), both of which are related to PfiTPT. Like PfiTPT, the specificity of these translocators has not been determined. Neither protein undergoes processing at the N-terminus. PfoTPT appears to be in the outer apicoplast

membrane based on its sensitivity to exogenous protease following recovery of organelles after hypotonic lysis of the parasites (Mullin et al. 2006). In contrast, TgAPT1 appears to localize to multiple membranes of the *T. gondii* apicoplast, as shown by immunoelectron microscopy, although the close spacing of the membranes makes it difficult to determine whether it resides in all four membranes (Karnataki et al. 2007a). Although the permeability of the individual membranes of the apicoplast has not yet been explored, all of the membranes from which they are putatively derived, with the exception of the outer envelope membrane of the chloroplast, are impermeable to small charged molecules. The predicted metabolic pathways of the apicoplast indicate that several charged molecules must be imported across all four membranes to serve as substrates for luminal enzymes. Hence it is likely that TgAPT1 populates multiple membranes to provide the transport functions that are accomplished by the two related transporters in *P. falciparum*.

We identified another apicoplast integral membrane protein, TgFtsH1, in *T. gondii* and a related FtsH in *P. falciparum* (Karnataki et al. 2007b). FtsH family members are membrane-bound zinc metalloproteases that are found in bacteria, mitochondria and chloroplasts and degrade mis-folded membrane proteins (the abbreviation Fts is derived from the filamentous temperature sensitive phenotype of bacterial mutants). Like TgAPT1 and PfoTPT, TgFtsH1 lacks the typical bipartite targeting sequence; however, unlike those proteins, TgFtsH1 undergoes processing at both the N- and C-termini (AK., unpubl. data), and has only a single transmembrane domain. Immunoelectron microscopy studies of this integral membrane protease show that TgFtsH1 also likely populates multiple membranes. These findings raise the question: how are some molecules targeted to multiple membranes whereas others appear to be restricted to specific membranes?

Recent studies from our laboratory show that a thioredoxin-like protein resides in multiple intermembrane spaces of the apicoplast (DeRocher et al. 2008) (Fig. 4). Thioredoxins are involved in redox homeostasis in multiple cellular compartments and in chloroplasts they have been shown to bind proteins involved in a variety of biochemical pathways including fatty acid, isoprenoid, and heme biosynthesis (Balmer et al. 2003). In the photosynthetic cyanobacterium *Synechocystis*, the major thioredoxin targets appear to be membrane proteins (Mata-Cabana, Florencio, and Lindahl 2007). The *T. gondii* apicoplast thioredoxin (TgATrx1), is in part soluble and in part peripherally associated with membranes (DeRocher et al. 2008). Although TgATrx1 lacks the canonical N-terminal bipartite targeting sequence, it does have a predicted N-terminal signal anchor sequence which could route the protein into the ER. Indeed, the region containing the signal anchor sequence is required for targeting to the apicoplast. Deletion analysis shows that sequences within a 160 aa region downstream of the signal anchor are also required for proper targeting. This region does not closely resemble a transit peptide, so further experimental work will be required to determine the specific motifs that provide for apicoplast localization. Nonetheless, the two regions together, when fused to GFP, route the fusion protein to a donut shaped region around the apicoplast lumen; the same immunofluorescence pattern seen for the native TgATrx1. This pattern is therefore unlike that seen when GFP is fused to the signal and transit peptides of luminal proteins. Hence this targeting sequence is functionally distinct from those of apicoplast luminal proteins.

Although the signal and transit peptide of PfiTPT and TgTic20 are presumably required for localization of the molecule to the plastid and appear to be functionally the same as luminal targeting sequences, the data obtained thus far suggest that the sequences that targets protein to the outer membranes of the apicoplast are totally different. The algorithms designed to identify the apicoplast proteins do not recognize these proteins as being apicoplast-targeted. Motifs that are sufficient for proper trafficking of TgAPT1 and its homologue PfoTPT, as well as TgFtsH1, have not been identified. Both the single transmembrane domain and the peptidase domain of TgFtsH1 are required for proper localization, since their deletion leads to cytosolic

or ER localization respectively (Karnataki et al. 2007b), but there is no evidence that they are sufficient. There are no obvious similarities between the transporters and the protease that enable sequence alignment or suggest a common motif for targeting. This is perhaps not surprising given the very different predicted structures and properties of the molecules.

Proteins associated with apicoplast membranes are present in vesicles

Immunoelectron microscopy experiments with antibodies directed against epitope-tagged TgFtsH1, TgAPT1, and TgATrx1 (expression was driven by the cognate promoters) showed labeling not only of the peripheral compartments of the apicoplast, but also of spherical vesicles (DeRocher et al. 2008; Karnataki et al. 2007a, 2007b). In particular, C-terminally tagged TgATrx1 identifies an abundant set of vesicles on immunoelectron microscopy (Fig. 5). The preponderance of the gold particles marking the locations of TgAPT1 and TgFtsH1 were localized close to membranes in the vesicles, suggesting that are routed as integral membrane proteins (Karnataki et al. 2007a, 2007b). Furthermore TgATrx1, the protein that eventually is localized to intermembrane spaces, is also predominantly adjacent to membranes while residing in the vesicles (DeRocher et al. 2008). This suggests that although TgATrx1 ultimately behaves as a soluble or peripheral membrane protein, in vesicles it is closely associated with the vesicle membrane. In thin sections, the vesicles took up approximately 3% of the surface area of the parasite. Morphologically similar vesicles can be seen in untransfected cells, indicating they are not an artifact of overexpression. In retrospect, the vesicles were readily observed on immunofluorescence analyses as apparent tubules and vesicles extending from the apicoplast at the time of apicoplast elongation. We have suggested that these are apicoplast-specific transport vesicles. However, no immunofluorescence or immunoelectron microscopy evidence has been published that shows the association of luminal proteins with vesicles.

Mechanisms of trafficking: from the ER to apicoplast

As with luminal proteins, the first step in trafficking proteins localized to the outer compartments of the apicoplast appears to be entry into the ER. The data supporting this contention include the fact that certain deletion mutants of TgFtsH1 and TgAPT1 are retained in the ER (Karnataki et al. 2007b) (ADR., unpubl. data). Full-length epitope tagged versions of these proteins also show some ER localization, the extent of which varies during the plastid division cycle in *T. gondii*. Furthermore, as mentioned above, deletion of the sole transmembrane domain of FtsH1 leads to cytosolic localization, indicating that the transmembrane domain likely functions as an internal signal sequence.

Several potential trafficking routes from the ER have been proposed for proteins destined for the apicoplast; these differ in the mechanism of trafficking from the ER and the location of sorting from other proteins of the secretory system. In brief, they include 1) trafficking via a direct connection with the ER, with protein sorting at the apicoplast; 2) vesicular trafficking directly to the Golgi apparatus, followed by sorting; 3) vesicular trafficking directly to the apicoplast, followed by sorting and 4) sorting at the ER, followed by vesicular trafficking to the apicoplast. The first possibility proposes that the outermost apicoplast membrane is distinct yet contiguous with the ER, as it is in the secondary plastids of several alga (Gibbs 1979). In this model, apicoplast proteins would be retained at the apicoplast while other secretory proteins would move on to the Golgi. In Apicomplexa, the lack of ribosomes and the presence of a plastid-specific transporter protein on the outermost membrane of the apicoplast (e.g. PfoTPT) clearly indicate a functional segregation of this membrane from the ER. No physical connections between the ER and the apicoplast have been observed upon electron microscopy, although it is possible these connections are rare and/or transient. The presence of the spherical vesicles bearing TgATrx1 or the apicoplast membrane proteins is harder to reconcile with this model, but it could be that they represent enlarged, specialized regions of the ER. It is also

possible they represent protein being trafficked from the apicoplast to some unidentified hydrolytic compartment. In this model, as well as the third model which also proposes sorting of proteins at the apicoplast, one might predict that COPII vesicles, which transport proteins to the Golgi, would be formed in the vicinity of the apicoplast. Although this has not been studied, COPI, which mediates retrograde trafficking and the retrograde trafficking receptor ERGIC2, are detected at the Golgi and ER, but not at the apicoplast (Hager et al. 1999; Pfluger et al. 2005). This provides some circumstantial evidence that trafficking from the ER to the Golgi bypasses the apicoplast.

The last three models all involve vesicular trafficking from the ER but differ as to where the sorting of apicoplast-targeted proteins from those addressed to other locations in the secretory system. Hence, the vesicles bearing the apicoplast membrane proteins would provide for transport to the apicoplast. A priori, sorting at the level of the Golgi would seem most likely since that is where most sorting of newly synthesized proteins of the secretory system occurs. However, the trafficking of apicoplast luminal proteins is insensitive to the Golgi inhibitor brefeldin A in both *T. gondii* (DeRocher et al. 2005) and *P. falciparum* (Tonkin et al. 2006b). In *T. gondii*, it is also insensitive to low temperature, which blocks trafficking to the Golgi apparatus (DeRocher et al. 2005). Although similar experiments on membrane proteins have not yet been published, these studies argue against sorting in the Golgi apparatus (model 2). One caveat is that all of the studies luminal protein trafficking were performed using heterologous promoters, which might alter the timing of expression, and hence the availability of particular trafficking pathways to the newly synthesized proteins.

In the third model, the vesicles bearing apicoplast membrane proteins are not apicoplast-specific, but rather represent the first step in trafficking of all secretory molecules (Tonkin, Kalanon, and McFadden 2007). The insensitivity of luminal protein trafficking to brefeldin A may argue against this model, as may the observed location of retrograde trafficking molecules described above. However, as yet direct experimental evidence does not distinguish this model from the fourth model where protein sorting occurs in the ER via the formation of apicoplast-specific and Golgi-specific vesicles.

With respect to all of the models invoking vesicular trafficking, it should be noted that vesicles bearing proteins targeted to the lumen or innermost membrane of the apicoplast have not been reported. Furthermore, the tubular/vesicular staining so easily seen for membrane proteins in immunofluorescence experiments is not observed for luminal proteins (although most studies used heterologous promoters). It is also possible that there is more than one way to reach the apicoplast. Nonetheless, if the vesicles bearing membrane proteins are indeed targeting to the apicoplast, these vesicles must bear distinct molecules that allow the vesicles and target membranes to identify one another and fuse. Such molecules could include the small GTP-binding proteins (Rabs), and SNARES, which facilitate membrane fusion. Although it is not certain that similar molecules will be involved in the trafficking of the identified vesicles, it appears to be a good place to start looking.

ER to apicoplast trafficking of membrane proteins is regulated during the cell cycle in *T. gondii*, increasing at the time of apicoplast elongation. Furthermore, we observed a larger proportion of ER-localized TgAPT1 when the protein was expressed from the TgDHFR promoter as opposed to its own promoter, suggesting that timing of expression could be important (the levels of protein expression were somewhat lower than with the TgAPT1 promoter) (Karnataki et al. 2007a). An earlier study in *P. falciparum* using promoters differentially regulated during the erythrocytic cell cycle showed that GFP bearing an apicoplast targeting sequence was secreted in early stages (rings) but localized to the apicoplast in the later stages (trophozoites) (Cheresh et al. 2002). Furthermore, in *P. falciparum*, transcripts encoding apicoplast targeted proteins are coordinately expressed during the

trophozoite stage (Bozdech et al. 2003). Together these data suggest the capacity to transport proteins to the apicoplast is regulated during the plastid division cycle in Apicomplexa. However, these studies address somewhat different steps in trafficking, since the membrane protein TgAPT1 was retained in the ER whereas the luminal protein escaped the ER but was mislocalized. This difference could reflect a difference in species, in how membrane and soluble proteins are handled by the parasites, or in the pathways the individual proteins need to follow. For example, the ability of proteins to unfold or fold could be important in trafficking.

Protein trafficking within apicoplast

Once proteins have passed the first membrane of the apicoplast, they now must cross three membranes to reach the apicoplast lumen. According to the secondary endosymbiosis hypothesis, the two inner membranes would contain homologues to the translocons of the inner and outer membranes of the chloroplast [Tic and Toc respectively, reviewed in (Soll and Schleiff 2004)]. As noted above, a homologue of Tic20 has been identified in *T. gondii* and regulated knockouts of Tic20 show that the protein is required for import of proteins to the lumen of the apicoplast (van Dooren et al. 2008). A candidate Tic22 is also present in the genomes of both *T. gondii* and *P. falciparum*, suggesting the presence of a Tic-related complex in the inner membrane of the apicoplast. The Toc complex includes receptors for the transit peptides which contain a characteristic GTP-binding motif and a trans-membrane pore comprised of the beta-barrel protein Toc75. Thus far no homologues of proteins of the Toc apparatus have been identified from the completed genome sequences of *T. gondii* or *P. falciparum*, leaving open the question of how proteins traverse this membrane. Indeed proteins of the Toc complex remain to be discovered in other secondary plastids (McFadden and van Dooren 2004).

How then do proteins cross the periplastid and plastid outer membranes of the apicoplast? One set of candidates are homologues of the ER-associated degradation (ERAD) pathway, which transports misfolded proteins from the ER back to the cytosol for proteasomal degradation. Chromalveolates, cousins of apicomplexans, have duplicated, diverged copies of much of their ERAD machinery. Several of these duplicate proteins are in the periplastid compartment of the red alga *Phaeodactylum tricornutum* suggesting that they comprise the translocon across the third, periplastid membrane (Sommer et al. 2007). Furthermore, some of these proteins, including Der1 and Cdc48 related proteins, have homologues in apicomplexans where they have been proposed to act as translocons across both the second and third apicoplast membranes (Sommer et al. 2007; Tonkin et al. 2007).

Perspectives

Plastids have been co-opted by other organisms many times during evolution. The apicoplast falls near the end of the continuum from endosymbiosis to organelle to genome-free relict. In the dozen years since the apicoplast was identified, studies conducted in several laboratories have significantly contributed to our understanding of this organelle, which is essential for several human and veterinary pathogens. Deciphering the signals that route proteins to the apicoplast allowed the identification of candidate proteomes for the plastids of *P. falciparum*, and *T. gondii*, and hence potential drug targets. Much work is yet to be done to fully understand trafficking to this organelle. For example, all proteins destined for the apicoplast are first imported into the ER, but how they are directed from the ER to the plastid remains unsolved. Several models have been proposed but what really happens awaits experimental evidence. A transit peptide domain marks proteins destined for the apicoplast lumen but what signals direct membrane proteins to the apicoplast? Characterization of such sequences might lead to bioinformatic identification of proteins involved in metabolic pathways or processes unique to the organelle. Next, how are these signals recognized? We

are now getting glimpses of how luminal proteins are escorted across multiple membranes to their final destination, but still do not understand how proteins are routed to specific membranes. A related question is: to what extent do the four apicoplast membranes differ in protein composition, and, by extension, function? All of the protein transport processes are expected to be essential to the parasite. Some may be shared between apicoplasts and the chloroplasts of chromists, so cross-fertilization will surely continue. Even though we have made excellent progress in furthering our understanding of this important organelle there are still several secrets enclosed within the four membranes of the apicoplast.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH R01 AI50506.

LITERATURE CITED

- Ajioka JW, Boothroyd JC, Brunk BP, Hehl A, Hillier L, Manger ID, Marra M, Overton GC, Roos DS, Wan KL, Waterston R, Sibley LD. Gene discovery by EST sequencing in *Toxoplasma gondii* reveals sequences restricted to the apicomplexa. *Genome Res* 1998;8:18–28. [PubMed: 9445484]
- Balmer Y, Koller A, del Val G, Manieri W, Schurmann P, Buchanan BB. Proteomics gives insight into the regulatory function of chloroplast thioredoxins. *Proc Natl Acad Sci USA* 2003;100:370–375. [PubMed: 12509500]
- Bhaya D, Grossman A. Targeting proteins to diatom plastids involves transport through an endoplasmic reticulum. *Mol Gen Genet* 1991;229:400–404. [PubMed: 1944228]
- Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS Biol* 2003;1:5.
- Bruce BD. The paradox of plastid transit peptides: conservation of function despite divergence in primary structure. *Biochim Biophys Acta* 2001;1541:2–21. [PubMed: 11750659]
- Brydges SD, Carruthers VB. Mutation of an unusual mitochondrial targeting sequence of SODB2 produces multiple targeting fates in *Toxoplasma gondii*. *J Cell Sci* 2003;116:4675–4685. [PubMed: 14576360]
- Cabantous S, Terwilliger TC, Waldo GS. Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat Biotechnol* 2005;23:102–107. [PubMed: 15580262]
- Cai X, Fuller AL, McDougald LR, Zhu G. Apicoplast genome of the coccidian *Eimeria tenella*. *Gene* 2003;321:39–46. [PubMed: 14636990]
- Cavalier-Smith T. Genomic reduction and evolution of novel genetic membranes and protein-targeting machinery in eukaryote-eukaryote chimaeras (meta-algae). *Philos Trans R Soc Lond B Biol Sci* 2003;358:109–134. [PubMed: 12594921]
- Cheresh P, Harrison T, Fujioka H, Haldar K. Targeting the malarial plastid via the parasitophorous vacuole. *J Biol Chem* 2002;277:16265–16277. [PubMed: 11815606]
- Dahl EL, Shock JL, Shenai BR, Gut J, DeRisi JL, Rosenthal PJ. Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*. *Antimicrob Agents Chemother* 2006;50:3124–3131. [PubMed: 16940111]
- DeRocher A, Gilbert B, Feagin JE, Parsons M. Dissection of brefeldin A-sensitive and -insensitive steps in apicoplast protein targeting. *J Cell Sci* 2005;118:565–574. [PubMed: 15657083]
- DeRocher A, Hagen CB, Froehlich JE, Feagin JE, Parsons M. Analysis of targeting sequences demonstrates that trafficking to the *Toxoplasma gondii* plastid branches off the secretory system. *J Cell Sci* 2000;113:3969–3977. [PubMed: 11058084]
- DeRocher AE, Coppens I, Karnataki A, Gilbert LA, Rome ME, Feagin JE, Bradley PJ, Parsons M. A thioredoxin family protein of the apicoplast periphery identifies abundant candidate transport vesicles in *Toxoplasma gondii*. *Eukaryot Cell* 2008;7:1518–1529. [PubMed: 18586952]

- Douglas S, Zauner S, Fraunholz M, Beaton M, Penny S, Deng LT, Wu X, Reith M, Cavalier-Smith T, Maier UG. The highly reduced genome of an enslaved algal nucleus. *Nature* 2001;410:1091–1096. [PubMed: 11323671]
- Fast NM, Kissinger JC, Roos DS, Keeling PJ. Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids. *Mol Biol Evol* 2001;18:418–426. [PubMed: 11230543]
- Fichera ME, Roos DS. A plastid organelle as a drug target in apicomplexan parasites. *Nature* 1997;390:407–409. [PubMed: 9389481]
- Foth BJ, Ralph SA, Tonkin CJ, Struck NS, Fraunholz M, Roos DS, Cowman AF, McFadden GI. Dissecting apicoplast targeting in the malaria parasite *Plasmodium falciparum*. *Science* 2003;299:705–708. [PubMed: 12560551]
- Gajria B, Bahl A, Brestelli J, Dommer J, Fischer S, Gao X, Heiges M, Iodice J, Kissinger JC, Mackey AJ, Pinney DF, Roos DS, Stoeckert CJ Jr, Wang H, Brunk BP. ToxoDB: an integrated *Toxoplasma gondii* database resource. *Nucleic Acids Res* 2007;36:D553–D556. [PubMed: 18003657]
- Gardner MJ, Bishop R, Shah T, de Villiers EP, Carlton JM, Hall N, Ren Q, Paulsen IT, Pain A, Berriman M, Wilson RJ, Sato S, Ralph SA, Mann DJ, Xiong Z, Shallom SJ, Weidman J, Jiang L, Lynn J, Weaver B, Shoaibi A, Domingo AR, Wasawo D, Crabtree J, Wortman JR, Haas B, Angiuoli SV, Creasy TH, Lu C, Suh B, Silva JC, Utterback TR, Feldblyum TV, Perteau M, Allen J, Nierman WC, Taracha EL, Salzberg SL, White OR, Fitzhugh HA, Morzaria S, Venter JC, Fraser CM, Nene V. Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. *Science* 2005;309:134–137. [PubMed: 15994558]
- Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Perteau M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 2002;419:498–511. [PubMed: 12368864]
- Gibbs SP. The route of entry of cytoplasmically synthesized proteins into chloroplasts of algae possessing chloroplast ER. *J Cell Sci* 1979;35:253–266. [PubMed: 422674]
- Goodman CD, McFadden GI. Fatty acid biosynthesis as a drug target in apicomplexan parasites. *Curr Drug Targets* 2007;8:15–30. [PubMed: 17266528]
- Hager KM, Striepen B, Tilney LG, Roos DS. The nuclear envelope serves as an intermediary between the ER and Golgi complex in the intracellular parasite *Toxoplasma gondii*. *J Cell Sci* 1999;112:2631–2638. [PubMed: 10413671]
- Harb OS, Chatterjee B, Fraunholz MJ, Crawford MJ, Nishi M, Roos DS. Multiple functionally redundant signals mediate targeting to the apicoplast in the apicomplexan parasite *Toxoplasma gondii*. *Eukaryot Cell* 2004;3:663–674. [PubMed: 15189987]
- Harper JT, Keeling PJ. Nucleus-encoded, plastid-targeted glyceraldehyde-3-phosphate dehydrogenase (GAPDH) indicates a single origin for chromalveolate plastids. *Mol Biol Evol* 2003;20:1730–1735. [PubMed: 12885964]
- Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK, Soldati D, Beck E. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science* 1999;285:1573–1576. [PubMed: 10477522]
- Karnataki A, DeRocher AE, Coppens I, Feagin JE, Parsons M. A membrane protease is targeted to the relict plastid of *Toxoplasma* via an internal signal sequence. *Traffic* 2007b;8:1543–1553. [PubMed: 17822404]
- Karnataki A, DeRocher A, Coppens I, Nash C, Feagin JE, Parsons M. Cell cycle-regulated vesicular trafficking of *Toxoplasma* APT1, a protein localized to multiple apicoplast membranes. *Mol Microbiol* 2007a;63:1653–1668. [PubMed: 17367386]
- Kissinger JC, Gajria B, Li L, Paulsen IT, Roos DS. ToxoDB: accessing the *Toxoplasma gondii* genome. *Nucleic Acids Res* 2003;31:234–236. [PubMed: 12519989]

- Kohler S, Delwiche CF, Denny PW, Tilney LG, Webster P, Wilson RJ, Palmer JD, Roos DS. A plastid of probable green algal origin in Apicomplexan parasites. *Science* 1997;275:1485–1489. [PubMed: 9045615]
- Mata-Cabana A, Florencio FJ, Lindahl M. Membrane proteins from the cyanobacterium *Synechocystis* sp PCC 6803 interacting with thioredoxin. *Proteomics* 2007;7:3953–3963. [PubMed: 17922517]
- Mazumdar J, Wilson H, Masek K, Hunter A, Striepen B. Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in *Toxoplasma gondii*. *Proc Natl Acad Sci USA* 2006;103:13192–13197. [PubMed: 16920791]
- McFadden GI, van Dooren GG. Evolution: red algal genome affirms a common origin of all plastids. *Curr Biol* 2004;14:R514–R516. [PubMed: 15242632]
- McFadden GI, Reith ME, Munholland J, Lang-Unnasch N. Plastid in human parasites. *Nature* 1996;381:482. [PubMed: 8632819]
- Moore RB, Obornik M, Janouskovec J, Chrudimsky T, Vancova M, Green DH, Wright SW, Davies NW, Bolch CJ, Heimann K, Slapeta J, Hoegh-Guldberg O, Logsdon JM, Carter DA. A photosynthetic alveolate closely related to apicomplexan parasites. *Nature* 2008;451:959–963. [PubMed: 18288187]
- Mullin KA, Lim L, Ralph SA, Spurck TP, Handman E, McFadden GI. Membrane transporters in the relict plastid of malaria parasites. *Proc Natl Acad Sci USA* 2006;103:9572–9577. [PubMed: 16760253]
- Patron NJ, Waller RF, Archibald JM, Keeling PJ. Complex protein targeting to dinoflagellate plastids. *J Mol Biol* 2005;348:1015–1024. [PubMed: 15843030]
- Pflugger SL, Goodson HV, Moran JM, Ruggiero CJ, Ye X, Emmons KM, Hager KM. Receptor for retrograde transport in the apicomplexan parasite *Toxoplasma gondii*. *Eukaryot Cell* 2005;4:432–442. [PubMed: 15701805]
- Pino P, Foth BJ, Kwok LY, Sheiner L, Schepers R, Soldati T, Soldati-Favre D. Dual targeting of antioxidant and metabolic enzymes to the mitochondrion and the apicoplast of *Toxoplasma gondii*. *PLoS Pathog* 2007;3:e115. [PubMed: 17784785]
- Ralph SA, Foth BJ, Hall N, McFadden GI. Evolutionary pressures on apicoplast transit peptides. *Mol Biol Evol* 2004a;21:2183–2194. [PubMed: 15317876]
- Ralph SA, van Dooren GG, Waller RF, Crawford MJ, Fraunholz MJ, Foth BJ, Tonkin CJ, Roos DS, McFadden GI. Tropical infectious diseases: metabolic maps and functions of the *Plasmodium falciparum* apicoplast. *Nat Rev Microbiol* 2004b;2:203–216. [PubMed: 15083156]
- Saito T, Nishi M, Lim MI, Wu B, Maeda T, Hashimoto H, Takeuchi T, Roos DS, Asai T. A novel GDP-dependent pyruvate kinase isozyme from *Toxoplasma gondii* localizes to both the apicoplast and the mitochondrion. *J Biol Chem* 2008;283:14041–14052. [PubMed: 18326043]
- Seeber F. Biogenesis of iron-sulphur clusters in amitochondriate and apicomplexan protists. *Int J Parasitol* 2002;32:1207. [PubMed: 12204220]
- Sharples FP, Wrench PM, Ou K, Hiller RG. Two distinct forms of the peridinin-chlorophyll a-protein from *Amphidinium carterae*. *Biochim Biophys Acta* 1996;1276:117–123. [PubMed: 8816945]
- Soll J, Schleiff E. Protein import into chloroplasts. *Nat Rev Mol Cell Biol* 2004;5:198–208. [PubMed: 14991000]
- Sommer MS, Gould SB, Lehmann P, Gruber A, Przyborski JM, Maier UG. Der1-mediated pre-protein import into the periplastid compartment of chromalveolates? *Mol Biol Evol* 2007;24:918–928. [PubMed: 17244602]
- Sulli C, Schwartzbach SD. The polyprotein precursor to the *Euglena* light-harvesting chlorophyll a/b-binding protein is transported to the Golgi apparatus prior to chloroplast import and polyprotein processing. *J Biol Chem* 1995;270:13084–13090. [PubMed: 7768903]
- Sulli C, Fang Z, Muchhal U, Schwartzbach SD. Topology of *Euglena* chloroplast protein precursors within endoplasmic reticulum to Golgi to chloroplast transport vesicles. *J Biol Chem* 1999;274:457–463. [PubMed: 9867865]
- Tonkin CJ, Kalanon M, McFadden GI. Protein targeting to the malaria parasite plastid. *Traffic* 2007;9:166–175. [PubMed: 17900270]
- Tonkin CJ, Roos DS, McFadden GI. N-terminal positively charged amino acids, but not their exact position, are important for apicoplast transit peptide fidelity in *Toxoplasma gondii*. *Mol Biochem Parasitol* 2006a;150:192–200. [PubMed: 16963133]

- Tonkin CJ, Struck NS, Mullin KA, Stimmler LM, McFadden GI. Evidence for Golgi-independent transport from the early secretory pathway to the plastid in malaria parasites. *Mol Microbiol* 2006b; 61:614–630. [PubMed: 16787449]
- van Dooren GG, Schwartzbach SD, Osafune T, McFadden GI. Translocation of proteins across the multiple membranes of complex plastids. *Biochim Biophys Acta* 2001;1541:34–53. [PubMed: 11750661]
- van Dooren GG, Su V, D’Ombrain MC, McFadden GI. Processing of an apicoplast leader sequence in *Plasmodium falciparum* and the identification of a putative leader cleavage enzyme. *J Biol Chem* 2002;277:23612–23619. [PubMed: 11976331]
- van Dooren GG, Tomova C, Agrawal S, Humbel BM, Striepen B. *Toxoplasma gondii* Tic20 is essential for apicoplast protein import. *Proc Natl Acad Sci USA* 2008;105:13574–13579. [PubMed: 18757752]
- Varadharajan S, Sagar BK, Rangarajan PN, Padmanaban G. Localization of ferrochelatase in *Plasmodium falciparum*. *Biochem J* 2004;384:429–436. [PubMed: 15307818]
- Waller RF, Keeling PJ, Donald RGK, Striepen B, Handman E, Lang-Unnasch N, Cowman AF, Besra GS, Roos DS, McFadden G. Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 1998;95:12352–12357. [PubMed: 9770490]
- Waller RF, Reed MB, Cowman AF, McFadden GI. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. *EMBO J* 2000;19:1794–1802. [PubMed: 10775264]
- Wastl J, Maier UG. Transport of proteins into cryptomonads complex plastids. *J Biol Chem* 2000;275:23194–23198. [PubMed: 10787421]
- Wiesner J, Jomaa H. Isoprenoid biosynthesis of the apicoplast as drug target. *Curr Drug Targets* 2007;8:3–13. [PubMed: 17266527]
- Wiesner J, Seeber F. The plastid-derived organelle of protozoan human parasites as a target of established and emerging drugs. *Expert Opin Ther Targets* 2005;9:23–44. [PubMed: 15757480]
- Wilson RJ, Denny PW, Preiser PR, Rangachari K, Roberts K, Roy A, Whyte A, Strath M, Moore DJ, Moore PW, Williamson DH. Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. *J Mol Biol* 1996;261:155–172. [PubMed: 8757284]
- Yung S, Unnasch TR, Lang-Unnasch N. Analysis of apicoplast targeting and transit peptide processing in *Toxoplasma gondii* by deletional and insertional mutagenesis. *Mol Biochem Parasitol* 2001;118:11–21. [PubMed: 11704269]
- Zhu G, Marchewka MJ, Keithly JS. *Cryptosporidium parvum* appears to lack a plastid genome. *Microbiology* 2000;146:315–321. [PubMed: 10708370]
- Zuegge J, Ralph S, Schmuker M, McFadden GI, Schneider G. Deciphering apicoplast targeting signals - feature extraction from nuclear-encoded precursors of *Plasmodium falciparum* apicoplast proteins. *Gene* 2001;280:19–26. [PubMed: 11738814]

Figure 1–3. *T. gondii* and the apicoplast

1. Transmission EM of a *T. gondii* tachyzoite showing the apicoplast (A), a dense granule (DG), endoplasmic reticulum (ER), Golgi (G), inner membrane complex (IMC), micronemes (Mc), mitochondrion (Mt), nucleus (N) nuclear envelope (NE), plasma membrane (PM), parasitophorous vacuole (PV), and parasitophorous vacuole membrane (PVM). Bar = 500 nm. The inner membrane complex is homologous to the alveolae in other chromalveolates. **2.** A cartoon of the apicoplast pointing out the membranes derived from the plastid (PL inner and PL outer), periplastid membrane, and outermost membrane. The proteins Tic20 and PfiTPT, which reside in the innermost membrane, are shown. Although TgATrx1, TgAPT1 and TgFtsH1 all appear to inhabit multiple compartments, their precise location within the apicoplast is not known; therefore possible locations are indicated by grey arrows. **3.** Enlargement of the apicoplast from panel A. Bar = 200 nm. The apicoplast in this cell is somewhat smaller than is typically seen and may be a transverse section of an elongated apicoplast.

Figure 4, 5. Immunoelectron microscopy of TgATrx1

Cells transfected with TgATrx1 tagged with HA epitope tags at the C-terminus were analyzed with anti-HA monoclonal antibody followed by protein A gold. **4.** Apicoplast showing labeling on multiple membranes. Bar = 100 nm. **5.** TgATrx1 present on apicoplast (a) and abundant vesicles (v). Arrows indicate site where vesicle appears to be fusing with outer membrane of the apicoplast. Bar = 200 nm. This figure is reproduced from (DeRocher et al. 2008) © American Society for Microbiology.

Table 1

	Luminal proteins	Inner membrane proteins	Proteins of outer compartments
Signal sequence	N-terminal	N-terminal	internal
Transit peptide	Yes	Yes	no
Seen in vesicles	Unknown	Unknown	Yes (<i>T. gondii</i>)
Traffics through Golgi	No	Not tested	Not tested
Trafficking modulated during plastid cycle	Yes (<i>P. falciparum</i>)	Not tested	Yes (<i>T. gondii</i>)