VOLUME 9 • NO. 2 • FEBRUARY 2008

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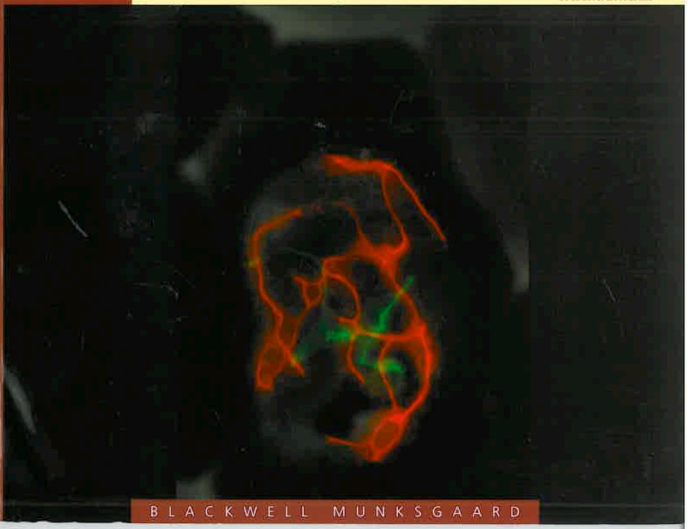

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Traffic 2008; **9:** 166–175
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doi: 10.1111/j.1600-0854.2007.00660.x

Review

Protein Targeting to the Malaria Parasite Plastid

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The relict plastid, or apicoplast, of the malaria parasite *Plasmodium falciparum* is an essential organelle and a promising drug target. Most apicoplast proteins are nuclear encoded and post-translationally targeted into the organelle using a bipartite N-terminal extension, consisting of a typical endomembrane signal peptide and a plant-like transit peptide. Apicoplast protein targeting commences through the parasite's secretory pathway. We review recent experimental evidence suggesting that the apicoplast resides in the mainstream endomembrane system proximal to the Golgi. Further, we explore possible mechanisms for translocation of nuclear-encoded apicoplast proteins across the four bounding membranes. Recent insights into the composition of the transit peptide and how it is cleaved and degraded after use are also examined. Characterization of apicoplast targeting has not only shed light on how this group of parasites mediate intracellular protein trafficking events but also it has helped identify new targets for therapeutics. The distinctive leader sequences of apicoplast proteins make them readily identifiable, allowing assembly of a virtual organelle metabolome from the genome. Such analysis has lead to the identification of several biochemical pathways that are absent from the human host and thus represent novel therapeutic targets for parasitic infection.

Key words: apicoplast, chloroplast, endosymbiosis, ERAD, transit peptide

Received 21 June 2007, revised and accepted for publication 21 September 2007, uncorrected manuscript published online 26 September 2007, published online 13 November 2007

Who would have ever guessed that *Plasmodium falciparum*, the blood-dwelling parasite responsible for the most severe form of malaria, would be the evil cousin of photosynthetic algae? Indeed, almost all parasites belonging to the phylum Apicomplexa, which also includes several other medically and agriculturally important pathogens, contain a non-photosynthetic plastid – or apicoplast (1,2). Parasite resistance to many front-line antimalarials has reinvigorated the hunt for new drugs to fight malaria, and the presence of plant- and prokaryote-derived metabolic pathways within the apicoplast has offered a welcome new line of attack (3).

Ten years on from the identification of the apicoplast, we examine the progress in understanding how this organelle is supplied with proteins by the parasite and the insights that have emerged (1,2). The small, circular genome of the apicoplast encodes only a minor subset of the organelle's proteome, mostly prokaryotic housekeeping genes (2,4,5). Indeed, the apicoplast genome (at only 35 kb) is the smallest known plastid DNA encoding only 30 proteins (2,5). The vast majority of apicoplast protein genes have at some point been transferred to the nuclear genome, and over 500 proteins are predicted to be targeted back to the apicoplast post-translationally (6). The organelle is thus utterly dependent on the nucleocytoplasm. Given that the apicoplast is surrounded by four membranes, how does such a complex targeting process take place? Herein, we review the possible mechanisms employed to translocate proteins across apicoplast membranes. We examine recent evidence suggesting a route for trafficking through the endomembrane system, and we discuss the distinctive N-terminal targeting signals of apicoplast proteins. Furthermore, by understanding the important features of the apicoplast-targeting sequence, it has been possible to predict the organelles proteome and metabolome and we discuss this in context of identifying new targets for therapeutic intervention.

Origin of Apicoplast Membranes

Four membranes surround the apicoplast, and to understand how proteins traffic through these barriers, one must consider their evolutionary origin. All plastids – photosynthetic or otherwise – are derived from endosymbiosis, the engulfment of a photosynthetic organism by a eukaryotic host cell (7). The first (primary) endosymbiosis gave rise to two membrane plastids of land plants, green algae and red algae (8). The inner membranes (IMs) and outer membranes (OMs) of chloroplasts are therefore derived from the two membranes of the engulfed Gram-negative cyanobacterium. However, the apicoplast, like other 'complex' plastids, is derived from another subsequent (secondary) endosymbiosis (9). This secondary engulfment resulted in four bounding membranes around the apicoplast (7,10–12). The origin of these extra membranes in complex plastids remains controversial, but the

general consensus suggests that the outermost membrane derives from the endomembrane system, whereas the subtending membrane [known as the periplastid membrane (PPM)] is the remnant of the secondary endosymbiont's plasma membrane (11).

Pathway of Apicoplast Protein Targeting

Conversion of endosymbionts into enslaved plastids involved massive gene transfer to the host cell nucleus, along with the subsequent targeting of plastid proteins back to the organelle (12,13). Primary plastids utilize an N-terminal transit peptide to facilitate translocation across the two membranes (14). However, complex plastid proteins require an additional targeting domain, a signal peptide, in order to cross the extra membranes. Together, this so-called bipartite leader is necessary and sufficient to mediate translocation across the four apicoplast membranes (15). Furthermore, it has been shown that removal of the transit peptide domain prevents apicoplast import and leads to protein secretion, whereas removal of the signal peptide prevents entry into the endomembrane system and the protein remains cytosolic (16–18). Thus, nuclear-encoded apicoplast proteins traffic through the secretory pathway, and all subsequent translocation into the apicoplast is mediated by the transit peptide. Further, we have recently shown that the signal peptide sequence of apicoplast proteins contains no specific targeting information beyond insertion into the endomembrane system as swapping an apicoplast signal peptide with that of unrelated secretory proteins still mediates correct apicoplast targeting so long as a transit peptide is downstream of the signal peptide (19).

Recently, the specific subcellular localization of the apicoplast has been investigated. Several organisms with complex plastids have an outer plastid membrane that is continuous with the perinuclear endoplasmic reticulum (ER) and studded with ribosomes (20), meaning that these plastids reside within the rough ER lumen and are thus in the mainstream of secretory protein flow. No ribosomes are evident on the outer membrane of the apicoplast and no thorough analysis, such as electron microscopy tomography, has been performed on the apicoplast; so, it is not possible to pinpoint by morphology exactly where within the endomembrane system the apicoplast lies. Fluorescent imaging of parasites using both apicoplast markers and ER-localized markers reveals intimate physical association between the ER and the apicoplast (19). Because apicoplast protein targeting is insensitive to Brefeldin A – a fungal metabolite known to block ER to Golgi retrograde transport in model eukaryotic systems – it seems most likely that the apicoplast is upstream (proximal) of the Golgi (19,21) (Figure 1). Moreover, experiments using a construct containing both a bipartite apicoplast-targeting leader and an ER retention sequence (recognized in the *cis* Golgi) found that apicoplast targeting still occurs,

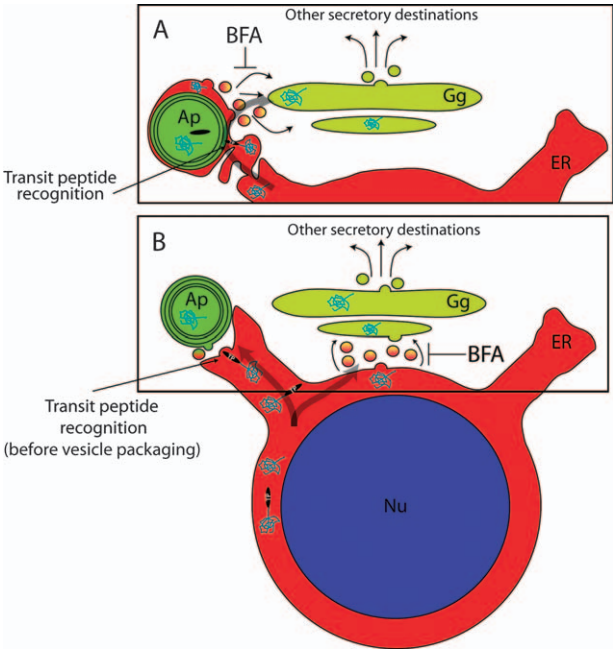

Figure 1: Models for the pathway of apicoplast-targeted proteins. Given the available data (see text), two models can explain the pathway that the nuclear-encoded apicoplast proteins take through the secretory pathway. A) The first model predicts that the apicoplast (Ap) physically sits within the ER. This implies that all secretory proteins wash over the second outermost apicoplast membrane (also called the PPM), and only those with a transit peptide (TP) are recognized and imported. Other secretory proteins continue on and are packaged into vesicle-mediated transport step between ER and Golgi (Gg) (secretory protein flow denoted by large transparent arrow). B) The second model proposes that the apicoplast sits in a terminal endomembrane location. This model requires that there be an apicoplast transit peptide receptor, capable of recognizing all transit peptide-bearing proteins from other secretory proteins and packaging these into vesicles that bud from the ER and fuse with the outer membrane of the apicoplast. This requires a separation of apicoplast and non-apicoplast proteins in the ER (denoted by split large transparent arrow). All other secretory proteins would pass through a separate route to the Golgi. Brefeldin A (BFA) blocks ER to Golgi traffic but does not block apicoplast targeting. Given the two models proposed, the two possibilities where BFA could act are outlined (A and B). Nu, nucleus.

demonstrating that the transit peptide is dominant or is recognized before the ER retention motif (19,21). Together, these experiments imply that the apicoplast is located between the rough ER and the Golgi (Figure 1).

However, several unanswered questions remain. First, does the apicoplast lie within the general smooth ER lumen or is it a specialized side compartment of the endomembrane system? These two possibilities have significant implication for the targeting system. In the former case (Figure 1, model A), all secretory proteins would essentially wash over the PPM and only those proteins bearing a transit peptide would be recognized and imported. Conversely, if the apicoplast is a discrete,

terminal side-branch of the early endomembrane system (Figure 1, model B), a specialized system for trafficking ER-derived vesicles containing apicoplast protein cargo would need to exist. In model A, all proteins destined for secretion through the Golgi will come into contact, at least briefly, with the apicoplast (Figure 1, model A), but in model B, the transit peptide is recognized in the ER prior to subsequent targeting, and only apicoplast proteins come into contact with the organelle (Figure 1, model B). Although it seems counterintuitive that all secretory proteins pass over the apicoplast, this is in fact exactly what happens in related organisms with plastids in the lumen of the rough ER (see above). It is also noteworthy that apicoplast proteins are predicted to make up almost half of all secretory proteins in *P. falciparum*; so, model A is not grossly inefficient. Indeed, model A is more parsimonious because model B requires a novel endomembrane-resident transit peptide receptor to recognize and package apicoplast proteins into vesicles for fusion with the outermost apicoplast membrane (Figure 1, model B). This hypothetical receptor would need to identify the 500 different transit peptides appended to each nuclear-encoded apicoplast protein and also recognize, somehow, apicoplast proteins that do not have a classical bipartite leader (see below). Model B also necessitates the existence of a unique system of vesicle targeting to direct only apicoplast protein-containing vesicles to this subdomain (the apicoplast) of the ER. There is precedence for vesicular shuttling in targeting to some secondary plastids; however, these organelles are post Golgi and only have three membranes, which changes transport architecture significantly (22).

Karnataki et al. observed vesicular transport of a protein to the apicoplast of *Toxoplasma gondii* (another apicomplexan parasite) and concluded that this supported model B (22). However, it should be pointed out that both model A and model B could involve packaging of apicoplast-destined proteins into vesicles (Figure 1). Vesicular traffic is not diagnostic of either model. Model B differs principally in that these vesicles would be vectorally trafficked, whereas in model A, the vesicles would be part of the mainstream endomembrane traffic. Model A also allows for connections of the ER to the outermost apicoplast membrane. If all secretory traffic does indeed pass over the apicoplast, we would expect either a continuous connection between the ER and the apicoplast outer membrane or a substantial number of vesicles arriving at and departing from the apicoplast. Neither has been observed, which is at odds with model A (Figure 1, model A). However, this does not discount that a transient continuity exists between the ER membrane and the outer apicoplast membrane; it may just be difficult to visualize.

The initial characterization of apicoplast targeting identified the bipartite leader as sufficient and necessary for apicoplast targeting (18), but recent characterization of proteins resident in the outer apicoplast membrane has revealed a new targeting system. A putative apicoplast phosphate transporter (termed APT1 in *T. gondii* or *Pf*oTPT in *P. falciparum*) localizes to the outermost apicoplast membrane but has no apparent leader (23,24). A transmembrane domain of *Pf*oTPT/*Tg*APT1 has been proposed to perform double duty as an internal, non-cleaved signal peptide, and the transporter is proposed to traffic in the membrane of apicoplast-destined vesicles (23,24). Traffic of *Pf*oTPT/*Tg*APT1 to the apicoplast could occur by either model A or model B (Figure 1). In model A, some factor would need to retain *Pf*oTPT/*Tg*APT1 in the apicoplast membrane after non-vectorial traffic. In model B, the endomembrane system would need to identify *Pf*oTPT/*Tg*APT1, in the absence of a classical bipartite leader, and target those vesicles to the apicoplast. In any case, working out how a leaderless apicoplast protein targets to the apicoplast is an interesting question and reveals that much remains to be learned about alternative protein trafficking routes, particularly for the little-known apicoplast membrane proteins. Given the lack of typical N-terminal extension, what are the signals needed for correct localization? Does *Pf*oTPT/*Tg*APT1 traffic through the ER or directly insert into the outermost apicoplast membrane? Can *Pf*oTPT/*Tg*APT1 auto insert itself, or does it require a membrane chaperone? Figure 2 outlines these possibilities.

Requirements for Apicoplast Transit Peptide Fidelity

Apicoplast transit peptides are reminiscent of plant chloroplast transit peptides in that they vary enormously in length, have no primary consensus or motif and are only distinguished by the enrichment of hydrophilic and basic amino acids (25). Attempts to define key components of apicoplast transit peptides by serial deletion experiments on model transit peptides have been unable to identify a universal sequence that defines all transit peptides (16,17,26), and the emerging paradigm is that no primary consensus sequence or motif exists. Comparison of the amino acid composition of transit peptides from diverse plastid-containing organisms found that the *P. falciparum* transit peptides have the same bias towards basic and hydrophilic amino acids as other transit peptides (25). However, the particular amino acids preferred are heavily influenced by the AT codon bias of the particular genome (25). For example, *P. falciparum* has an approximately 80% AT 20% GC bias (27) and apicoplast transit peptides are enriched in lysine (K) and asparagine (N), amino acids with codons not requiring G's or C's. Other apicomplexans with more balanced AT content in their DNA have transit peptides with amino acid compositions more similar to that of plant transit peptides (25). Significantly, apicoplast transit peptides differ from plant transit peptides by containing less hydroxylated residues (25). Hydroxylated amino acids have been shown to be phosphorylated and may allow discrimination between chloroplast and mitochondrial transit peptides. Given that apicoplast transit peptides (and complex plastids in general) are recognized

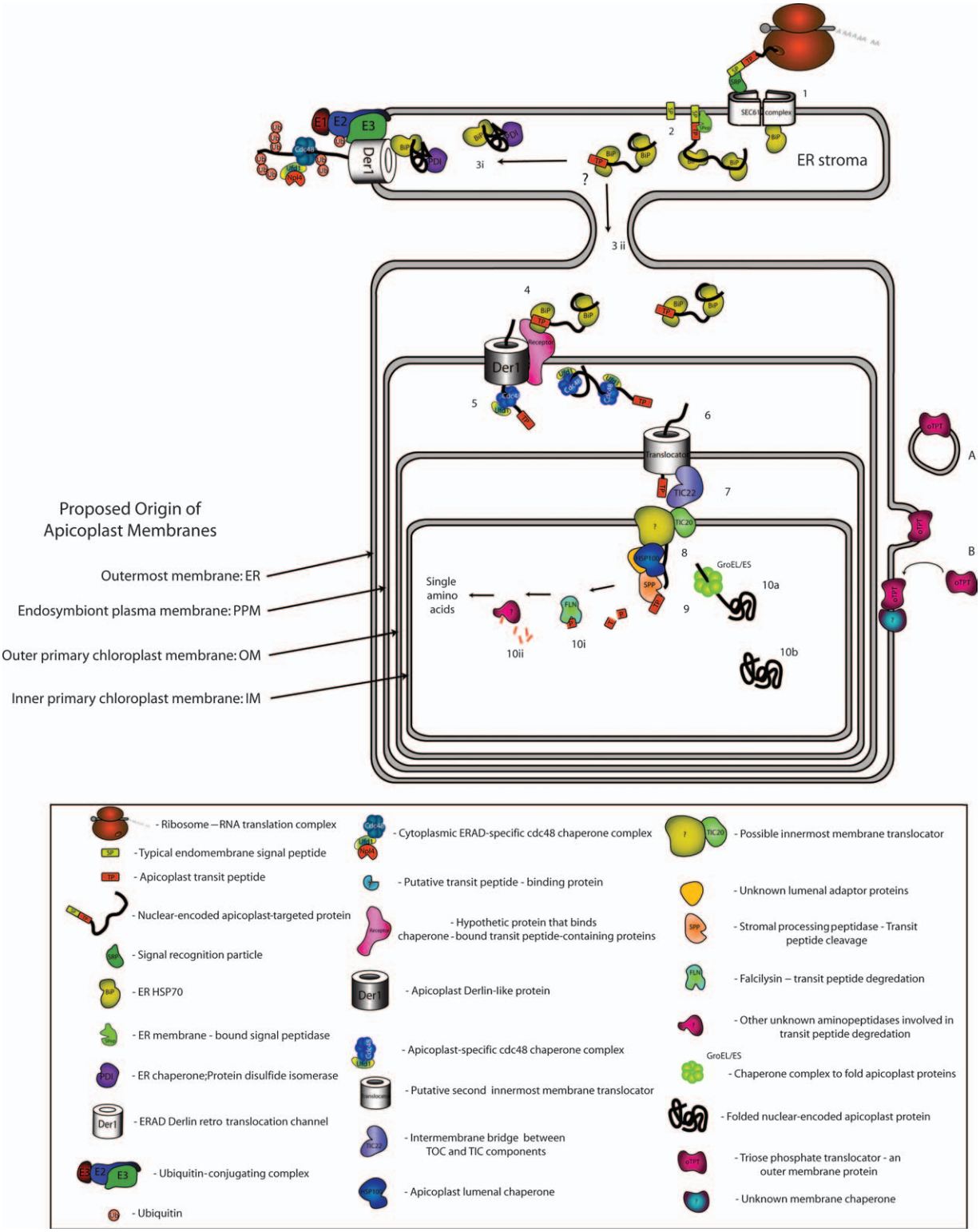

Figure 2: Legend on next page.

in the ER and are therefore spatially separated from the mitochondria, phosphorylation of transit peptides may not be required (25), and apicoplast transit peptides devoid of serines (S) and threonines (T) still can mediate accurate apicoplast targeting (18). Furthermore, the *P. falciparum* genome seems to lack serine and threonine kinases that

could be targeted to the ER, making transit peptide phosphorylation highly unlikely.

Scrutiny of apicoplast transit peptides suggested that the presence of positively-charged residues and depletion of acidic residues may be important features of apicoplast transit peptides (6), and point mutagenesis studies confirmed that a net basic charge, particularly in the N-terminal portion, is a critical for apicoplast transit fidelity of *P. falciparum* (6). Recently, these findings were corroborated and extended in *T. gondii* transit peptides (28). It was shown that positive charge, but more so the absence of negative charge, was important for transit peptide fidelity. By serially removing positive charge in the same *T. gondii* apicoplast transit peptides, it was also deduced that positive charge at the N-terminus was much more important than at the C-terminal portion of the transit peptide (28). Furthermore, if positive-charged residues were re-introduced at the N-terminus – even at different positions – this was sufficient to restore targeting demonstrating that N-terminal positive charge, but not its exact position, is important for apicoplast transit peptide fidelity (28).

Plant transit peptides are predicted to bind Hsp70, and this interaction is important for translocation into plant plastids (29). Using a similar rationale for apicoplast transit peptides, the *Escherichia coli* Dnak (Hsp70)-binding prediction algorithm was applied to predicted apicoplast proteins (see below), and >90% of transit peptides are predicted to bind Hsp70 (6). This suggested that apicoplast targeting might involve a 'transit peptide first' orientation that facilitates recognition and membrane translocation. Interestingly, apicoplast transit peptides also resemble plant transit peptides in that they contain two Hsp70-binding sites, approximately 26 amino acids apart (6). This configuration supposedly facilitates the simultaneous pushing and pulling by molecular chaperones on either side of a membrane as it passes through a translocator. Furthermore, when the predicted Hsp70 high-affinity binding sites were removed by point mutagenesis in a model transit peptide, targeting was severely disrupted (6). This strongly suggests that an Hsp70 interaction with the transit peptide is important for apicoplast targeting.

15-Deoxyspergualin (DSG) is an immunosuppressant with antimalarial activity. Recently, it was shown that DSG in *P. falciparum* has multiple targets within the parasite, including the Hsp70 molecules and the inhibition of polyamine synthesis (30,31). In addition to these targets, it was concluded that the most sensitive target of all is the inhibition of targeting of nuclear-encoded apicoplast proteins (30). How DSG could inhibit apicoplast targeting remains unknown, but an attractive hypothesis is that DSG inhibits chaperone–transit peptide interactions (Hsp70 binding) thus preventing apicoplast targeting. This is the first substance proposed to block apicoplast protein targeting, and further studies of how DSG inhibits this process are warranted.

Traversing Four Apicoplast Membranes

While the protein signal requirements for apicoplast targeting have been extensively investigated, the molecular mechanism of translocation across the apicoplast membranes remains poorly understood. Here, we discuss recent models proposed to explain how two targeting signals mediate passage across four apicoplast membranes.

As discussed previously, the space between the outermost membrane and the PPM is most likely connected with the ER lumen, no matter which model (A or B) is invoked (Figure 1). Thus, apicoplast proteins have effectively crossed the outermost membrane when they enter the ER lumen by cotranslation at the Sec61 complex in the rough ER (Figure 2, step 1). As the signal peptide is removed during cotranslation (Figure 2, step 2), apicoplast proteins retain only the transit peptide for traversing three remaining membranes, the PPM, the OM and the IM (Figure 2).

Figure 2: Apicoplast membrane origin and a theoretical model for translocation of proteins across the apicoplast's four membranes. From the point of translation, apicoplast proteins must transverse four membranes. Given that the apicoplast is topologically within the ER, no matter what model is invoked (Figure 1), translocation across the first membrane most likely occurs by Sec61 complex (steps 1 and 2). The PPM is likely derived from the secondary endosymbionts plasma membrane. The ERAD hypothesis is the most parsimonious yet to describe PPM translocation (see text) (steps 4 and 5). It is unknown how apicoplast proteins are discriminated by the apicoplast ERAD system versus the classical ERAD system (denoted by the question mark; step 3i and 3ii). The PPM is most likely the point of discrimination between apicoplast and other secretory proteins and therefore a transit peptide receptor may be found at this membrane (step 4). The OM is derived from the OM of the primary plastid. No Toc homologues can be found, and no strong leads exist for factors that could mediate transport across the OM (see text) (step 6). The IM is derived from the IM of the primary plastid. Translocation across the IM could be mediated by homologues of the Tic subunits (steps 7 and 8). After translocation across all apicoplast membranes, the transit peptide is likely cleaved off by SPP (see text) (step 9). Proteins can then be folded by endogenous chaperones (step 10a and 10b). Free transit peptides can then be degraded by co-operation between SPP and FLN (and perhaps other proteases) (step 10i and 10ii). The outermost apicoplast membrane protein *Pf*oTPT/*Tg*APT1 does not have an apicoplast leader, and it is unknown how targeting occurs. Two possibilities exist: (i) *Pf*oTPT/*Tg*APT1 inserts into the ER through a nested signal peptide and is delivered to the apicoplast or (ii) *Pf*oTPT/*Tg*APT1 can auto insert directly into the apicoplast membrane through a chaperone-mediated process. For more information on apicoplast membrane translocation, see the main text.

Several models have been proposed for translocation across the PPM, but none has yet been supported by any experimental evidence (10,11,20,32,33). Recently, a new model invoking an adaptation of a plastid-localized, ER-associated degradation (ERAD) translocation system has been proposed to explain PPM translocation (Figure 2, steps 4 and 5). The ERAD model proposes that a relict version of the ERAD translocase from the endosymbiont endomembrane system is now responsible for PPM translocation in plastids of *P. falciparum* as well as related algae known as diatoms and cryptomonads (34).

Classically, ERAD involves the recognition, retrotranslocation and degradation of misfolded ER proteins by cytosolic proteasomes (35–38). Misfolded proteins are somehow identified by the lumenal chaperones Binding Protein (BiP) and protein disulfide isomerase (PDI), enabling discrimination from folding intermediates or correctly folded proteins (39–41) (Figure 2, step 3i). Subsequently, ERAD substrates (misfolded proteins) are returned to the cytosol across the ER membrane through a proteinacious channel, formed by either Sec61 (42) or Der1 (Derlin1 in mammals) (43,44) (Figure 2, step 3i). This retrotranslocation is driven by an AAA (ATPase associated with diverse cellular activity) adenosine triphosphatase (ATPase) motor, utilizing either Cdc48 (p97/VCP in mammals) complexed with its cofactors Ufd1 and Npl4 (45–47) (Figure 2, step 3i) or the 19 S regulatory subunit (or PA700) of the proteasome itself (40,48). Ubiquitination of the ERAD substrate may also occur either before or during retrotranslocation by the cytosolic and membrane-bound factors E1, E2 and E3 (47,49,50) (Figure 2, step 3i). As more data become available, it is becoming clear that different ERAD substrates, with misfolded lesions in differing locations (i.e. in the lumen, at the membrane or in the cytosol), require distinct ERAD pathways, utilizing different components, for removal from the ER (35,51,52). Consequently, many different proteins fulfil redundant roles in ERAD, depending on the particular substrate requiring retrotranslocation.

Recently, Sommer et al. have shown that two versions of the ERAD system occur in organisms with 'complex plastids' of red algal origin, and this second system is apparently located in the plastid compartment (34). Sommer et al. hypothesize that this supernumerary ERAD has been recruited as a PPM translocator, effectively translocating plastid proteins from the lumen of the ER into the plastid by changing the substrates that it translocates (34) (Figure 2, steps 4 and 5). In support of this model, they identified several ERAD paralogues with plastid-targeting bipartite leaders in organisms containing complex plastids, including *P. falciparum* and two other related algal species (34). Significantly, the plastid-localized ERAD system contains fundamental components required for translocation, including a putative translocation pore (Der1) and a translocation motor (Cdc48 and its cofactor, Ufd1) (34) (Figure 2, steps 4 and 5).

Intriguingly, *P. falciparum* and diatoms (which also have a complex plastid) have two paralogues of plastid-localized Der1, although the significance of this is unclear. In comparison with the classical ERAD machinery, the plastid-localized versions appear to be somewhat stripped down – perhaps retaining the minimal components for translocation (Figure 2, steps 4 and 5). For example, the set of enzymes for ubiquitination is incomplete, which makes sense given that plastid-destined proteins would not be anticipated to undergo ubiquitination and degradation by the proteasome. Plastid localization of some of the duplicated ERAD components, including the bipartite leader of one Der1 paralogue in *P. falciparum*, was confirmed by green fluorescent protein fusions (34).

While there is not yet any direct evidence that the plastid-localized ERAD is involved in apicoplast protein translocation, circumstantial evidence favours this model over previous invocations. For example, the direction of protein translocation at the plastid-localized ERAD is consistent with apicoplast import as both ERAD systems function to translocate proteins away from the ER lumen (Figure 2, steps 3i and 4). Further, the plastid ERAD system is present as an almost complete translocation system with receptors (both BiP and PDI in the ER lumen), a potential translocon pore (Der1) and a motor complex (Cdc48/Ufd1), all likely to be localized in the apicoplast (34) (Figure 2, steps 4 and 5). Moreover, the apparent location of ERAD components to complex plastids that lack ER is a compelling argument for their participation in translocation across a plastid membrane. Cryptomonad algae retain the endosymbiont nucleus and their plastid-based ERAD system might still be associated with the nuclear envelope, but diatom plastids and apicoplasts have no ER; so, one has to ask what could ERAD be doing in their complex plastids? While some critical ERAD components such as Npl4 and components of the ubiquitinylation pathway are apparently absent from the plastid (34), these elements may yet be identified in the future or perhaps they are not required for apicoplast protein translocation. It is also noteworthy that the transit peptide of plastid proteins itself forms an ideal ERAD substrate as it is small and soluble, likely associated with chaperones (see above) and possibly intrinsically unfolded (6,25). Identifying the plastid ERAD as a transit peptide receptor and translocator precludes the need to reinvent or relocate novel translocation machinery for the apicoplast PPM.

After crossing the PPM, apicoplast proteins, still retaining a transit peptide, enter the periplastid compartment (between the PPM and the OM) and face the two innermost membranes of the apicoplast (Figure 2, step 5). These two membranes are derived from the cyanobacterial primary endosymbiont (discussed earlier) and are topologically equivalent to the OMs and IMs of primary plastids (including plant chloroplasts) (11,12). Current models predict Toc and Tic translocons, as found in primary (plant) plastids, on the OM and IM, respectively, to facilitate import into the stroma (10–12,33,53).

The OM is likely to contain only core translocation constituents, perhaps even just the translocating pore itself, as the critical function of transit peptide recognition over other secretory proteins has likely been shifted upwards to the PPM (Figure 2, step 4). Consequently, proteins analogous to the Toc receptors (e.g. the guanosine triphosphatases Toc34 and Toc159 or the TPR-containing receptor Toc64) are likely to be absent or instead found at the PPM (Figure 2, step 4).

While there is currently no evidence to confirm or dispute the putative role of a Toc translocon at the OM, extensive bioinformatics searches have so far failed to identify its central component, Toc75. In plants, Toc75 – a β-barrel pore – forms the translocation channel across the OM (54). However, in secondary plastids derived from red algae, Toc75 homologues remain conspicuously absent, despite the availability of several fully sequenced genomes, including *P. falciparum* and *T. gondii* and two diatoms. Thus, alternative scenarios are worth entertaining. We propose that the second Der1 homologue identified by Sommer et al. (34) might be localized in the OM and replace Toc75, however the localization, orientation and particularly the function of this protein remain to be experimentally tested (Figure 2, step 6).

Finally, translocation across the IM is probably facilitated by a translocon homologous to the Tic translocon in plant chloroplasts as orthologues of Tic22 [a putative intermembrane space adaptor (55–57)] (Figure 2, step 7) and Tic20 [a possible translocation channel (58,59)] have been identified in both *T. gondii* and *P. falciparum* (van Dooren GG, University of Georgia, Athens; unpublished data) (Figure 2, step 8). Orthologues of ClpC, an AAA ATPase chaperone implicated as the motor for primary plastid translocation (58,60), have also been predicted to localize to the apicoplast (Figure 2, step 8), with one ClpC homologue retained on the *P. falciparum* apicoplast genome itself (5). Furthermore, there are several other heat shock proteins that have likely apicoplast leaders that could further facilitate apicoplast protein translocation. Although several members of the Tic complex are thus identifiable and likely targeted to the apicoplast IM, one member, the putative pore-forming protein Tic110 (12), is not evident in any apicomplexa begging the question of what its role is and whether Tic20 alone is the pore-forming protein.

Given this new 'ERAD' theory on plastid PPM translocation, further questions are raised regarding plastid targeting. For instance, how do transit peptides escape the classical ERAD but simultaneously become substrates for the plastid-localized ERAD (Figure 2, step 3i versus 3ii)? Put another way, what distinguishes a transit peptide from a misfolded protein? Given the sophistication and flexibility that the classical, ER-localized, ERAD shows towards recognizing its diverse array of substrates, it would not be surprising if the plastid-localized ERAD displays a similar capacity to deal with such complexity.

Processing of the Transit Peptide

After protein translocation through the four apicoplast membranes, the transit peptide has fulfilled its role and is finally removed (Figure 2, step 9). Proteins are then likely folded by resident apicoplast chaperones and rendered active (Figure 2, step 10a and 10b). In plants, the stromal processing peptidase (SPP) is responsible for transit peptide cleavage, and both *P. falciparum* and *T. gondii* have a clear homologue with a characteristic bipartite apicoplast-targeting leader that is capable of directing a reporter protein to the apicoplast (61,62). Indeed, parasites that lack an apicoplast (by means of a segregation defect) cannot cleave transit peptides from nascent nuclear-encoded apicoplast proteins further suggesting that cleavage occurs within the apicoplast (63). Nevertheless, it remains to be formally proven that SPP is responsible for transit peptide cleavage.

In plant chloroplasts, SPP cleaves the transit peptide into two sub fragments but does not participate in further degradation (64) (Figure 2, step 9). Rather, another zinc metallopeptidase protease known as PreP (precursor processing enzyme) is responsible for transit peptide degradation (Figure 2, step 10i). A homologue of PreP exists in Apicomplexa, but it was originally described as a food vacuole enzyme involved in haemoglobin degradation and named falcilysin (65). Recent targeting experiments have shown that falcilysin is targeted to multiple sites, apparently being located in the food vacuole, the apicoplast and the mitochondrion (66). Intriguingly, falcilysin exhibits two different pH optima, each with different substrate preferences (67). Thus, at acidic pH, as occurs in the food vacuole, falcilysin has a preference for haemoglobin-like substrates, but at more neutral pH (as might occur in the apicoplast), it prefers basic substrates and is able to cleave an apicoplast transit peptide *in vitro* (66). Falcilysin may thus have multiple roles in the parasite, but how it is targeted to multiple destinations remains an open question (66,68). Intriguingly, falcilysin is apparently unable to cleave peptides to single amino acid constituents and thus another enzyme(s) might be responsible for this process (Figure 2, step 10ii). There are several candidates that could fulfil this role, or it could be possible that at this point, the general apicoplast protein turnover machinery can take over and complete the process (Figure 2, step 10ii).

Kinetics of Protein Targeting to the Apicoplast

Steady-state analyses of apicoplast proteins on Western blots typically show two molecular mass species. The larger protein is a precursor apicoplast protein with an attached transit peptide, while the smaller protein is the mature, apicoplast-localized protein from which the transit peptide has been removed (21,61). Such a large amount of unprocessed precursor is never seen in proteins targeted to primary plastids, and it is likely that this is a reflection of

the extra time in takes apicoplasts (and other complex plastids) proteins to transverse through the secretory pathway. Pulse–chase experiments indicate that the journey from the site of synthesis in the rough ER to the site of transit peptide removal in the apicoplast stroma takes about 45–60 min in *P. falciparum* or *T. gondii* (21,61), which is consistent with the time required for protein trafficking to other complex plastids (69).

Predicting the Apicoplast Proteome Using Features of the Transit Peptide

Analysis of apicoplast targeting has contributed significantly to the understanding of intracellular trafficking events in malaria and other apicomplexan parasites. Furthermore, analysis of the apicoplast bipartite leader sequence has allowed us to identify a large cohort of proteins (approximately 500) that are predicted to localize to the apicoplast on the basis of having a bipartite N-terminal leader. Apicoplast proteins are extremely attractive drug targets, especially considering that the apicoplast is indispensable (70) and is of prokaryotic origin.

To predict all apicoplast proteins from the completed *P. falciparum* genome, a computer algorithm was created to identify proteins that contained N-termini with a specific acidic:basic ratio and specific amino acid bias (discussed above), and this was used in conjunction with the already available signal peptide prediction tool SignalP (71). This tool, termed PlasmoAP, identified 545 putative apicoplast proteins, which represents approximately 10% of the genome. This set of proteins was then used to construct an apicoplast metabolic map (3), which indicated that the apicoplast performs very much like a 'plant in the dark'. Thus, despite the lack of photosynthesis, the apicoplast retains complete biosynthetic pathways for isoprene subunit synthesis, fatty acid biosynthesis, Fe:S centre formation and a shared heme biosynthetic pathway that are almost identical to those of plant plastids. Apicoplast biosynthetic pathways are attractive drug targets as they are essential and either radically different to the equivalent pathways in the host or absent from the host altogether and susceptible to numerous antibacterials and herbicides (3).

Conclusions

Since it was first identified in 1996 (1), the apicoplast has now assumed an important place in drug development strategies and proffers much hope for new therapeutic intervention of apicomplexan-caused diseases – including malaria. Cell biological experiments are unravelling the complex targeting process that mediates protein translocation across the four apicoplast membranes and how targeting of proteins to the apicoplast occurs courtesy of the bipartite N-terminal leader. The distinctive nature of these leaders has enabled a bioinformatic approach to identify targeted proteins by mining the genome data, providing a welcome avenue to capitalize on this resource to fight the disease.

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