ELSEVIER

Protein targeting to destinations of the secretory pathway in the malaria parasite *Plasmodium falciparum*

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The secretory pathway in the malaria parasite *Plasmodium falciparum* has many unique aspects in terms of protein destinations and trafficking mechanisms. Recently, several exciting insights into protein trafficking within this intracellular parasite have been unveiled: these include signals that are required for targeting of proteins to the red blood cell and the relict plastid (known as the apicoplast); and the elucidation of the pathways of the haemoglobin proteases targeted to the food vacuole. Protein-targeting to the apical organelles in *P. falciparum*, however, is still not very well understood, but available research offers a tantalising glimpse of the system.

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Introduction

Plasmodium falciparum is the causative agent of the most severe form of malaria and infects over 300 million people and kills approximately 2 million people annually. Most of the pathology associated with *P. falciparum* infection occurs during the asexual cycle when this unicellular eukaryotic parasite invades, rapidly grows and divides into multiple daughter cells before finally bursting from the host.

The malaria parasite *P. falciparum* has a range of unique organelles all fed by the protein secretory pathway. Indeed, protein secretory pathways are fundamental to the biology and pathology of *P. falciparum* infection and have great potential as targets for intervention. Like all other eukaryotes, most secretory proteins in *P. falciparum* contain an N–terminal signal peptide for translocation into the ER and additional protein-targeting motifs that provide essential information for subsequent targeting to secretory destinations. There are at least 12 secretory destinations in *P. falciparum*, many of them unique and some of the signals and pathways of proteins travelling to such destinations have recently been revealed. The focus of this review is on recent advances in understanding the organization of the *P. falciparum* secretory pathway, with special emphasis on protein targeting to parasite organelles.

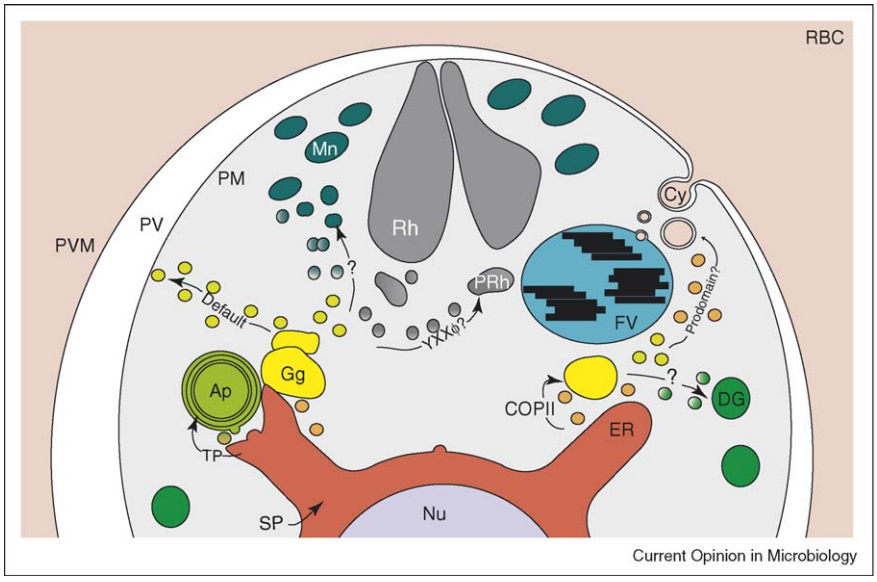
Trafficking to the host red blood cell

During invasion into the erythrocyte, the parasite constructs a parasitophorous vacuole membrane (PVM) that encloses it within a parasitophorous vacuole (PV) and separates it from the host cell cytosol (Figure 1). In order to grow and develop within this protected compartment, the parasite activates a remarkable host cell remodelling process. This converts a simple, terminally differentiated erythrocyte into an ideal host cell that supports the parasite's nutritional needs, and allows adherence of the cell to vascular endothelia in order to avoid detection by the host immune mechanisms (Figure 2). This remodelling requires trafficking of proteins out of the parasite and into the host cell. Recently, advances have been made in the understanding of this process, such as the elucidation of the PEXEL/VTS (plasmodium export element/vacuolar targeting signal) targeting motif that is responsible for translocation proteins across the PVM [1••,2••] and the identification of the first molecule involved in host cell targeting (Figure 2) [3••]. There have been several recently published reviews that comprehensively discuss this aspect of the secretory pathway [4,5]: instead of re-reviewing the literature here, the recent findings have been summarized in Figure 1.

Trafficking to the food vacuole

Rapid growth of the parasite within the host erythrocyte is achieved, in part, by digesting haemoglobin within a lysosomal-like compartment that is referred to as the food vacuole (FV). Haemoglobin proteolysis takes place by a series of parasite-derived proteases [6]. All of the known FV proteases bear a putative N–terminal signal-peptide, signifying that they are transported through the endoplasmic reticulum (ER), and a prodomain that might have a role in targeting from the ER to the FV. Recently, the targeting of two FV-targeted proteases has been studied in detail and they have been shown to traffic to the FV by two different routes.

Figure 1



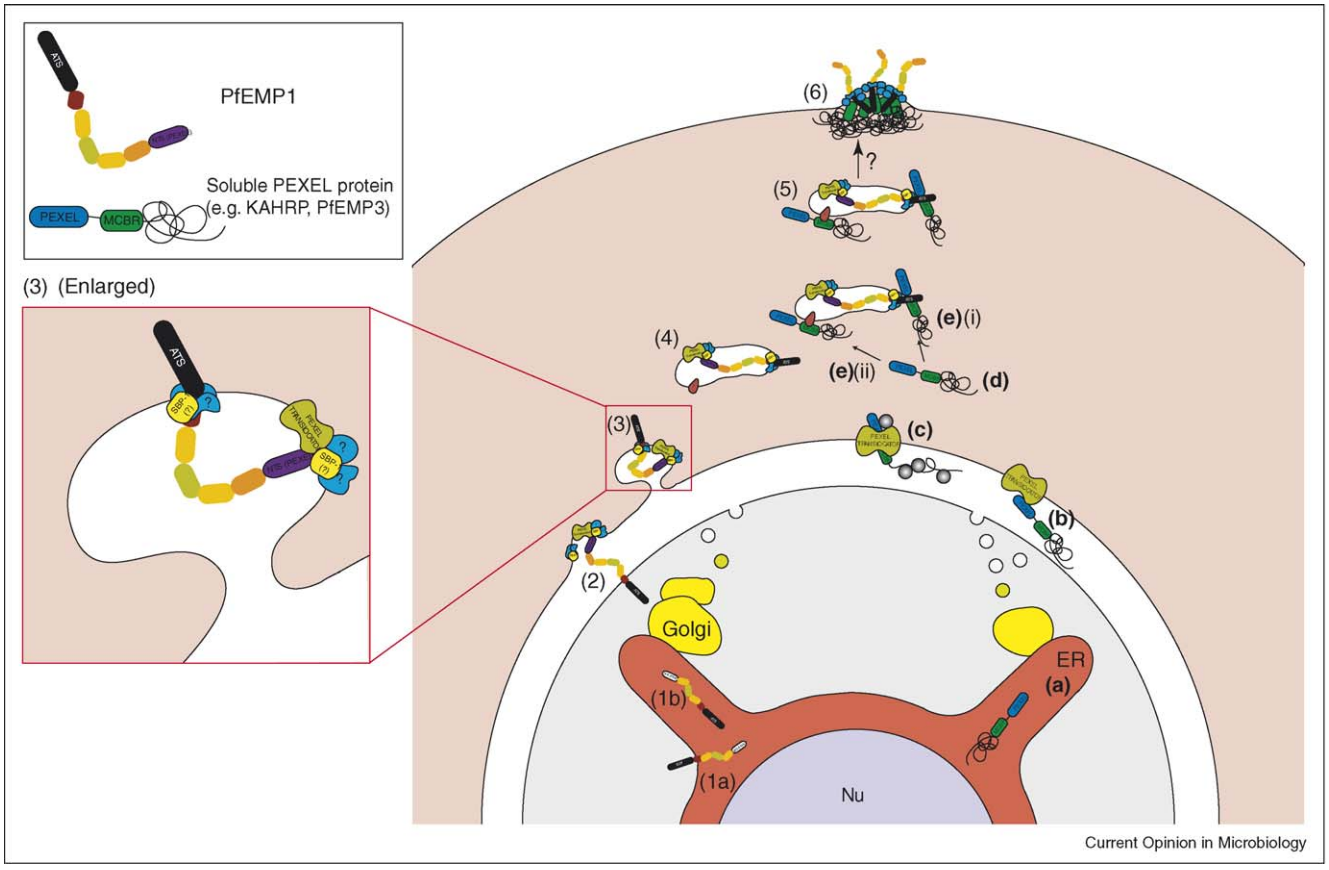
Intracellular secretory transport in *Plasmodium falciparum*. *P. falciparum* (light grey) resides in the PV (white) and is separated from the red blood cell cytoplasm (pink) by the PVM. The parasite ER (red) resembles a simple perinuclear structure (nucleus in blue) with so-called horns protruding from each side. Most secreted proteins require a signal peptide (SP) in order to enter the ER [32•]. The Golgi complex (yellow) is less elaborate than in model systems and seems to be in a simple unstacked conformation [33–34,35•]. The apicoplast (light green) has four membranes, and nuclear-encoded proteins require a SP and transit peptide (TP) for correct targeting to the apicoplast [8]. Protein traffic to the apicoplast occurs through the secretory pathway but it is likely that they do not pass through the Golgi [13•,14•]. Haemoglobin is endocytosed by the cytostome (Cy) and trafficked to the FV (aqua) in vesicles (orange) [10]. FV haemoglobin proteases traffic through the secretory pathway and there is evidence for direct transport to the cytostome (orange vesicles) and through the PV (yellow vesicles) [6,7••]. Three apical organelles involved in red blood cell invasion are also synthesised *de novo* by the secretory pathway. Very little is known about targeting to the rhoptries (Rh; grey), micronemes (Mn; dark green) and dense granules (DG; light green) in *P. falciparum*. In *T. gondii* targeting of transmembrane rhoptry proteins involves a tyrosine-based sorting motif (YXXϕ) in the cytoplasmic tail, through pre-rhoptry compartments (PRh) [27] and this might be true for some *P. falciparum* proteins. Targeting of *T. gondii* transmembrane microneme proteins requires tyrosine-based signals and an acidic patch [22], but this is not absolutely required for *P. falciparum* [23]. Dense granules are the default destination for secretory proteins in *T. gondii* [30], however, this cannot be the case for *P. falciparum* as these organelles are not synthesised until late in the intraerythrocytic cycle. It is presumed that vesicular trafficking delivers proteins to the dense granules (grey vesicles).

The first was a study of the FV-targeted protease plasmepsin II, an aspartic protease involved in the initial steps of haemoglobin degradation [7••]. It was shown that plasmepsin II travels through the ER, as an integral membrane protein, and is initially delivered to the cytostome by an uncharacterised mechanism, probably involving vesicular shuttling, before subsequent localisation to the FV. Upon localisation of plasmepsin II to the FV membrane, the proregion, which contains the only transmembrane domain in the protein, is removed by an uncharacterised protease, termed the 'PM convertase', releasing mature, soluble plasmepsin II into the FV [7••].

More recently, trafficking of another FV protease, dipeptide aminopeptidase 1 (DPAP1) has been shown to use a different pathway to plasmepsin II [6]. DPAP1 does not traffic directly to the cytostome, as is the case for plasmepsin II. Instead DPAP1 accumulates in the PV before moving to the FV [6]. Like plasmepsin II, DPAP1 also has a signal sequence, but it does not have a transmembrane domain. DPAP1 is also processed differently to plasmepsin II, by excision of an internal domain postulated to be between the so-called exclusion domain and the two catalytic domains [6]. It therefore appears that plasmepsin II and DPAP1 take different routes to reach the FV and, given that they have differences in their prodomain structure and solubility during trafficking, it is possible that the prodomain itself is responsible for the targeting route of proteins to the FV (Figure 1). Because reporter proteins with a signal peptide are secreted into the PV and are also subsequently found in the FV, there appears to be no additional sequence information necessary for FV-targeting [8,9]. It will be interesting to determine the role of the prodomain in FV proteases, and perhaps it specifies another route of targeting or increases the efficiency of targeting to the FV.

Digestion of haemoglobin by the parasite first requires the engulfment of portions of host cytoplasm across the PV and the plasma membrane (PM) and into the FV (Figure 1). This process has been morphologically characterised and involves a specialised endocytic-like

Figure 2



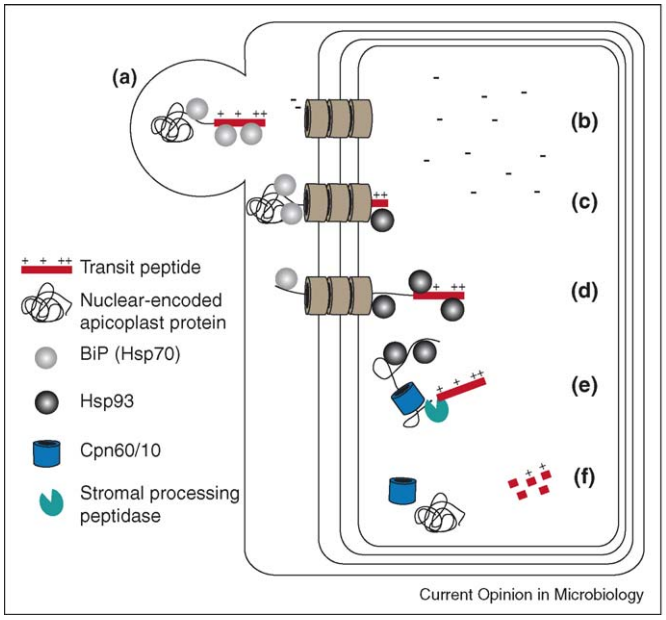
Targeting proteins into and through the red blood cell. There are two routes for red blood cell (pink) targeted proteins **(a–e)** and **(1–6)**. (a) Soluble host targeted proteins contain an N–terminal signal peptide for translocation into the ER (red) and transport to the PV [9]. (b and c) Host-targeted proteins probably associate with chaperones to maintain a translocatable conformation, whereas the PEXEL/VTS sequence mediates transport across the PVM [1••,2••]. The putative PEXEL/VTS translocator is presumably within the PVM, however it has not yet been established where PEXEL/VTS recognition takes place. (d) Upon translocation into the red blood cell cytosol proteins that are destined for the underside of the red blood cell membrane (i.e. KAHRP [knob associated histidine-rich protein] and PfEMP3 [*P. falciparum* erythrocyte membrane protein 3]) associate with parasite-derived membrane-bound structures called Maurer's clefts [9,37]. The Maurer's cleft binding region (or MCBR) of KAHRP and PfEMP3 are unrelated to one another but both mediate transitory binding to the Maurer's clefts by interacting with a membrane-bound protein such as **(e)(i)** PfEMP1 (see below) or **(e)(ii)** an unknown receptor. It is not known how proteins move from the Maurer's clefts to the underside of the red blood cell. (1) The variant surface protein PfEMP1 does not contain a typical signal peptide and it is unclear how this protein is exported to the host cell. PfEMP1 contains a C–terminal transmembrane domain that probably acts as a stop transfer signal for entry into the ER **(1a)**. Transport of PfEMP1 presumably occurs as a transmembrane protein through the secretory pathway [38], although others have demonstrated that PfEMP1 is initially transported as a soluble protein **(1b)** [39]. (2–3) Recent work shows Maurer's clefts originate from the PVM and it likely that PfEMP1 is packaged into Maurer's clefts as they are forming (3 enlarged) [40]. (4) PfEMP1 then traffics to the red blood cell membrane through Maurer's clefts with its C–terminal tail exposed to the red blood cell cytoplasm [38]. A multiple spanning membrane protein SBP1 (spectrin binding protein 1) is necessary for transport of PfEMP1 from the Maurer's clefts to the red cell surface but not other host-targeted parasite proteins [3]. It is possible that SBP1 acts as a ligand for correct PfEMP1 deposition within the Maurer's cleft or that it acts as a receptor for the PfEMP1 PEXEL or another unidentified sequence (3-englarged). (5–6) It is unknown how PfEMP1 gets from the Maurer's clefts to the surface of the red blood cell.

organelle, referred to as the cytostome (Figure 1) [10]. The molecular events mediating this process, however, are completely unknown. The *P. falciparum* genome encodes a suite of homologues that are involved in endocytosis in mammalian and yeast, such as the Rabs, putative SNARE-like molecules and clathrin coat components. Experimental evidence is required to show that these homologues are involved in the acquisition of haemoglobin from the host cell (Figure 1) [11].

Protein trafficking to the apicoplast

P. falciparum contains a relict plastid called the apicoplast that is essential for parasite survival [12]. The apicoplast is surrounded by four membranes and most apicoplast proteins are nuclear-encoded and require a two-part N–terminal extension made up of a signal peptide and a transit peptide (Figure 1) [8]. Earlier work has shown that the signal peptide resembles a classical eukaryotic signal peptide and mediates targeting into the ER

Figure 3



A model for apicoplast targeting. **(a)** ER vesicles arrive at the apicoplast surface carrying a transit peptide-bearing protein. The positively charged transit peptide is held in unfolded conformation by the Hsp70 chaperone, BiP. **(b)** Transit peptide bearing proteins might be brought to the surface by an electrophoretic attraction to a negatively charged translocation complex, which is analogous to the Toc/Tic translocation machinery of plant plastids. **(c)** The positive charge of apicoplast transit peptides is further electrophoretically attracted into the reducing apicoplast lumen. **(d,e)** Hsp93 (ClpC) might also attach to the transit peptide and contribute to the movement of the protein through the membranes as a ratchet that only allows anterograde transport. **(f)** Once inside the apicoplast, the transit peptide is removed by the stromal processing peptidase (SPP). **(g)** The attached protein is folded into an active conformation with help from Cpn60/Cpn10 and the transit peptide is degraded into its amino acid constituents by an unknown protease.

whereas the transit peptide mediates all remaining steps (Figure 1) [8]. Recently, two studies have shed light on the pathway apicoplast proteins take through the secretory system [13•,14•]. It has been shown that is signal peptides from other secreted proteins can mediate apicoplast targeting in combination with a transit peptide, therefore demonstrating that no specific targeting information is found in an apicoplast signal peptide [13•]. It has also been shown that the *P. falciparum* apicoplast has a close association with parasites ER [13•] and that apicoplast-targeting is Brefeldin-A (BFA)-insensitive [13•,14•]. Furthermore, an apicoplast-targeted protein with an incorporated ER retention signal that is recognised by ERD2 in the *cis*-Golgi is still targeted to the apicoplast [13•,14•]; however, when the transit peptide is mutated or deleted, the ER retrieval signal takes over signifying a dominance of transit peptide function over *cis*-Golgi retrieval of the ER retention signal [13•]. Overall, these studies strongly suggest that apicoplast targeted proteins do not traverse the Golgi but rather divert straight from the ER to the apicoplast, in a similar way to some other organisms with multimembrane-bound plastids (Figure 1) [14•,15].

After entry into the secretory pathway, the transit peptide mediates the remaining steps to the apicoplast. Apicoplast transit-peptides, like plant chloroplast transit-peptides, have no consensus sequence, are variable in length and are not predicted to have any secondary structure. Transit peptides are identifiable by a predominance of hydrophilic and basic amino acids, and a corresponding lack of acidic residues [16]. Recently, it was shown that plastid transit-peptides from a wide range of organisms share these common features, although it appears that biases in codon usage influence the actual amino acids used in transit peptides. *P. falciparum*, for instance, is highly AT-rich and therefore uses lysine and asparagine, which can be both coded for by AT-only codons, in its apicoplast transit peptides, whereas other organisms with more balanced codon usages use a wider range of amino acids with equivalent characteristics [16]. Overall, it appears that transit peptides have relatively loose structural constraints, depending primarily on their overall average composition for their function.

What features of apicoplast transit-peptides are physiologically important? To test whether positive charge is essential for transit-peptide function, a series of point mutations were created to perturb the most N–terminal charge in a *P. falciparum* transit peptide [17]. By individually substituting the first two N–terminal lysine residues with alanine, glutamic acid or aspartic acid it was demonstrated that the positive charge of the N–terminal

is crucial for transit-peptide fidelity. More so, apicoplast targeting was altered more dramatically when the first positive charge was changed when compared to when the second was changed [17], signifying its greater importance in transit-peptide function.

Hsp70 chaperones also appear to be important for import of mitochondrial and plastid proteins by unfolding and folding the proteins before and after membrane translocation, respectively. The apicoplast transit peptide contains a region that is predicted to be suitable for Hsp70 chaperone binding, and this putative binding site was crucial for apicoplast transit-peptide function *in vivo* [17]. A physical interaction between an apicoplast transit peptide and Hsp70 has also recently been demonstrated [18]. Overall, it seems apparent that transit peptides are very simple and confer their information through a simple charge profile, and possibly an Hsp70 chaperone binding site.

The identification of important characteristics of apicoplast transit peptides suggests a model of transit-peptide recognition and apicoplast protein import (Figure 3). After entry into the ER, BiP (the resident ER-Hsp70) binds the transit peptide to keep it in an unfolded state. The transit peptide is then be recognised and translocated through the apicoplast membranes by an as yet undefined protein complex that is analogous (or even homologous) to the Toc/Tic (translocon of the outer/inner envelope membrane of chloroplasts) machinery in plants (Figure 3) [19]. Perhaps, a negative-charged patch on a docking protein permits initial interaction with the transit peptide and the reducing environment created by the metabolic processes of the apicoplast provides an electrostatic force that pulls the transit peptide bearing protein through the membranes in a similar manner to the way proteins are translocated into the mitochondria (Figure 3) [20]. Additional mechanical force required for protein import could be provided by other resident chaperones (ClpC) in the apicoplast. The protein could then be folded and the transit peptide removed (Figure 3).

Trafficking to the apical organelles

Approximately 35 hours after invasion, when most of the haemoglobin has been consumed, the parasite initiates simultaneous synthesis of up to 32 daughter merozoites, each of which are capable of invading a new red blood cell. As part of merozoite formation, the apical organelles consisting of the rhoptries, micronemes and dense granules are formed *de novo* by the secretory pathway (Figure 1).

Micronemes are storage organelles for adhesive proteins and release their contents onto the parasite surface upon host cell recognition (Figure 1) [21]. In *T. gondii*, transmembrane micronemal proteins use a tyrosine-based sorting motif and an acidic patch of residues in the C–terminal tail [22]. These features are also found on several *P. falciparum* micronemal proteins. However, in the case of the micronemal protein erythrocyte binding antigen 175 (EBA175), it is clear that neither the tyrosine-based motif nor the acidic residues are required for micronemal targeting [23]. Instead, an unknown signal within the cysteine-rich region directs EBA175 to the micronemes and then unknown features located within the cytoplasmic tail enable EBA175 to function as an invasion ligand [23]. Targeting of soluble proteins to the micronemes in *T. gondii* relies on their interaction with escorter transmembrane molecules, but no such process has been described for any soluble micronemal protein in *P. falciparum* [24].

Rhoptries are large club-shaped organelles that are found at the apex of the polarized merozoite, and these organelles contribute to the invasion process as well as to the synthesis of the PV (Figure 1). Rhoptry-targeting is BFA-sensitive, indicating that rhoptry proteins pass through the ER and Golgi [25,26]. Rhoptry protein targeting in *T. gondii* relies on a tyrosine-based motif, YXXϕ (where X is any amino acid and ϕ is a bulky hydrophobic residue), similar to that found in endosomal-targeted proteins in mammalian and yeast cells [27]. Nothing is known of the requirements for rhoptry targeting in *P. falciparum*, although many of these proteins do contain a C–terminal tyrosine motif. Rhoptry-targeting in *T. gondii* also relies on the packaging of transmembrane proteins into clathrin-coated vesicles by an interaction of the C–terminal tyrosine-based motif with the μ1 subunit of the AP-1 (adaptor protein complex 1) clathrin-coat complex [28]. All homologous components that are necessary for this targeting mechanism are present in *P. falciparum*, but whether they function in rhoptry targeting is unknown [11]. Soluble rhoptry proteins do not contain a tyrosine-based motif and probably rely on interaction with membrane-anchored rhoptry-targeted proteins in order to be correctly located, in a similar mechanism to that used by soluble micronemal proteins. The soluble *P. falciparum* rhoptry-associated protein 2 (RAP2), for instance, relies on an interaction with RAP1 for correct targeting [29]. In turn RAP1 might be bound to RAMA (rhoptry-associated membrane antigen), which is a GPI (glycosyl-phosphatidylinositol) protein located in the rhoptries. How such an entourage of proteins is directed to the rhoptries remains unknown.

The third set of apical organelles are the dense granules, which probably expel their contents into the PV upon entry into the host cell (Figure 1). All proteins destined for the PV in *T. gondii* transit through the dense granules [30]. However, in *P. falciparum* there are many secreted proteins targeted to PV and red blood cells that are expressed even before dense granules are synthesised, implying that there is another route in malaria parasites. The only known requirement for dense granule targeting

in *P. falciparum* is timing of expression. Ring infected erythrocyte antigen (RESA) is normally packaged into dense granules late in the cell cycle and correct targeting was only observed under control of an endogenous promoter, but not under a heterologous promoter [31].

The molecular organization of the secretory pathway

Little is known about the molecular organization of the secretory pathway of *P. falciparum*. The ER resembles a simple perinuclear structure with two protruding 'horns' [32•], whereas the Golgi is rudimentary with a so-called 'unstacked' conformation (Figure 1) [33–34,35•]. Like all eukaryotic cells, *P. falciparum* must rely on a huge supply of molecular machinery to shuffle proteins through membranes and in and out of vesicles in order to target proteins to their correct destination [36]. The genome of *P. falciparum* has many homologues of core secretory proteins such as the Rabs, the ARFs, the COP components and the Sec apparatus [11], but there is very little experimental evidence for the specific role of any of these components.

Protein targeting in *P. falciparum* has been generally understudied and there are many intriguing questions that remain unanswered. Whereas some protein signals have been identified for localisation to organelles such as the apicoplast and export into the host cell, the molecular machinery for these and other membrane translocation events is unknown. The availability of the genome sequence has been important in revealing the conserved trafficking machinery but has not enabled the identification of components for movement of proteins to some of the locations unique to this parasite and its intracellular and host cell compartments. The molecular machinery for targeting to these compartments provides a fascinating avenue for future investigation and potentially might be a useful target for design of novel therapeutics to control this devastating disease of humans.

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