Export of *Plasmodium* Proteins via a Novel Secretory Pathway

M.F. Wiser, H.N. Lanners and R.A. Bafford

The intraerythrocytic location of the malaria parasite necessitates modification of the host cell. These alterations are mediated either directly or indirectly by parasite proteins exported to specific compartments within the host cell. However, little is known about how the parasite specifically targets proteins to locations beyond its plasma membrane. Mark Wiser, Norbert Lanners and Richard Bafford here propose an alternative secretory pathway for the export of parasite proteins into the host erythrocyte. The first step of this pathway is probably an endoplasmic reticulum (ER)-like organelle that is distinct from the normal ER. Possible mechanisms of protein trafficking in the infected erythrocyte are also discussed. The proposed ER-like organelle and alternative secretory pathway raise many questions about the cell biology of protein export and trafficking in Plasmodium*.*

The malaria parasite, like other Apicomplexa, spends much of its life as an intracellular parasite. During its intraerythrocytic stage, the parasite extensively modifies the cytoplasm and plasma membrane of the host erythrocyte. Well-known ultrastructural alterations include the electron-dense knobs on *Plasmodium falciparum*-infected erythrocytes and caveola–vesicle complexes on *P. vivax*-infected erythrocytes. In addition, membranous clefts are found within the host cell cytoplasm and numerous *Plasmodium* antigens are associated with poorly defined inclusions, which are probably localized to these intraerythrocytic membranes. Parasite-induced intraerythrocytic membranes have been variously described as Maurer's clefts, the parasitophorous duct[1], and the tubovesicular membrane (TVM) network[2]. The exact relationships between these various membrane structures are not known, but for simplicity we will refer to them collectively as the intraerythrocytic membranes (IEMs). Parasite proteins are targeted differentially to the host membrane and to these various intraerythrocytic compartments[3], thus raising questions as to how the parasite is able to target proteins to distinct locations beyond its own plasma membrane. Recently, we have described an ER-like compartment that appears to be an early step in this extraparasite transport process[4].

A novel ER-like organelle in *Plasmodium*

Most models for the export of *Plasmodium* proteins to the host cell usually have the exported proteins initially routed to the parasite plasma membrane via the classic secretory pathway[5,6]. This classic pathway consists of the ER and Golgi, which are just beginning to be characterized in *Plasmodium* (Box 1). However, recent data suggest that many *Plasmodium* proteins destined for export are not processed by the ER and Golgi, but are exported via an alternative secretory pathway[4]. This proposition is based on the observation that brefeldin A (BFA) leads to the accumulation of exported proteins in a compartment morphologically distinct from the ER. Although, BFA blocks the movement of proteins from the ER to the Golgi and leads to their accumulation in the ER[7], this novel compartment is not the ER. Localization studies using antibodies against BiP, an ER marker, reveal that the *Plasmodium* ER is a diffuse network of vesicles in the parasite cytoplasm[8,9]. In addition, BFA treatment leads to the accumulation of a parasite plasma membrane protein in these diffuse cytoplasmic vesicles characteristic of the ER[4]. Exported proteins, however, do not exhibit this diffuse pattern of cytoplasmic fluorescence after BFA treatment. Immunofluorescence studies (Fig. 1) and immunoelectron microscopy[4] indicate that this novel BFA-induced compartment is located close to the periphery of the parasite.

Interestingly, two ER-type Ca^{2+}-ATPases have been described in *P. falciparum*. Antibodies against one of these, designated PfATPase4, recognize a compartment at the parasite periphery[10] that exhibits a similar immunofluorescence pattern to the BFA-induced compartment. The observation that *Plasmodium* has two ER-type Ca^{2+}-ATPases and the accumulation of exported proteins into a compartment distinct from the ER suggest that the parasite has two distinct ER-like organelles. Furthermore, a second ER-like organelle suggests that the parasite has two parallel secretory pathways. One of these secretory pathways would be analogous to the classic ER and Golgi and would function in the targeting and sorting of proteins destined for the parasite plasma membrane and intraparasite organelles. The other pathway presumably specializes in the export of

Box 1. The Classic Secretory Pathway in *Plasmodium*

Little is known about the secretory pathway in *Plasmodium*. Morphological studies describe the endoplasmic reticulum (ER) as a loose network of vesicles[42] and the Golgi as small coated vesicle-like structures[43,44] instead of the classic stacks. The parasite's ER and Golgi appear to be more developed in the later stages of the replicative cycle[45]. Some protein components of the intracellular secretory pathway have been identified in *Plasmodium*. These include: ER-type Ca^{2+}-ATPases[10,46]; Sec61α, a component of the translocation pore in the ER[47]; BiP[8], a chaperone in the lumen of the ER; reticulocalbin[48], an ER Ca^{2+}-binding protein; and ERD2 (Ref. 9), a protein resident in the *cis*-Golgi, which functions to return proteins to the ER. In addition, several Ras-like monomeric GTP-binding proteins and accessory proteins have been identified in *P. falciparum*[45]. It has been argued that the malaria parasite lacks a 'classic' Golgi[49]. However, the presence of a parasite rab6 homolog[50], which functions in intra-Golgi transport of the medial and *trans*-Golgi, and its segregation from ERD2 argues for the presence of a Golgi[51].

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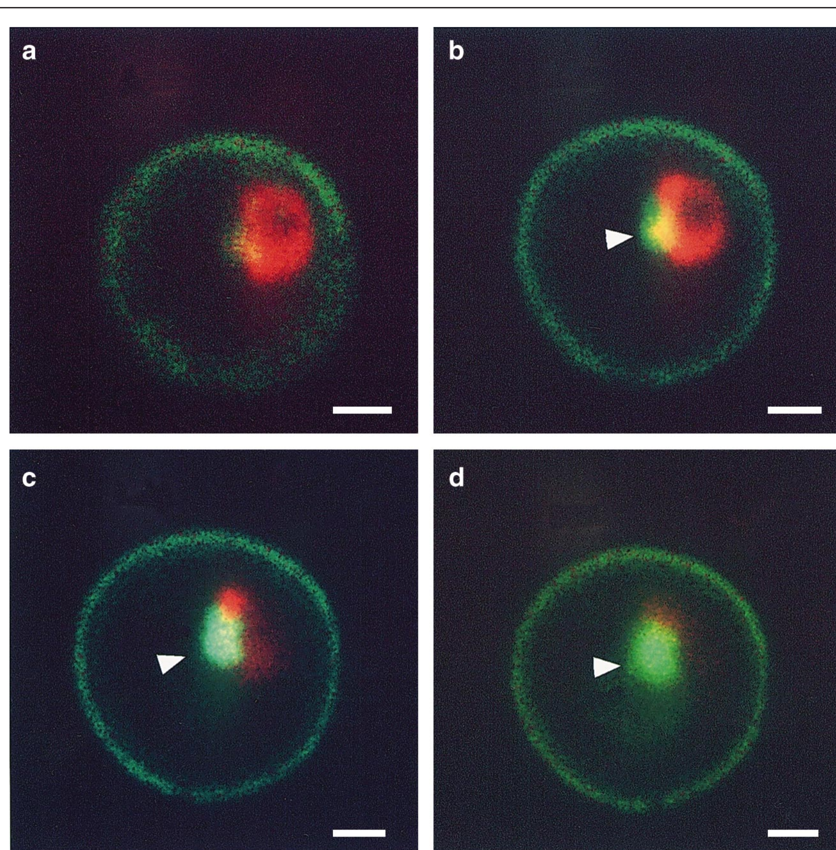

Fig. 1. Immunofluorescent confocal microscopy demonstrating the secondary endoplasmic reticulum of Apicomplexa (sERA). Four consecutive 0.5 μm optical planes are shown (a–d) of a *Plasmodium berghei*-infected erythrocyte treated with brefeldin A (BFA) and examined by confocal immunofluorescence microscopy with monoclonal antibody 16.3. Monoclonal antibody 16.3 recognizes a 65 kDa parasite protein associated with the erythrocyte membrane[28]. Treatment with BFA results in the accumulation of exported *Plasmodium* proteins (green) into a compartment at the parasite periphery. Counterstaining nucleic acids with ethidium bromide (red) reveals little overlap with the BFA-induced compartment. In these particular sections the sERA wraps around the parasite periphery and appears as a flattened disk on the top side of the parasite in this orientation (d). Exported proteins do not accumulate in this compartment in the absence of BFA[4]. Scale bars = 1 μm.

proteins into the host cell. *Cryptosporidium* might also have two ER-type Ca^{2+}-ATPases[11]. Localization studies with antibodies raised against a *Cryptosporidium* Ca^{2+}-ATPase reveal two distinct localization patterns. One is a diffuse perinuclear pattern, presumably the ER, and the other pattern is discrete vesicles near the parasite periphery at the apical end. This coincidence of an ER-type Ca^{2+}-ATPase localized at the parasite periphery in two different parasites suggests that this novel organelle may be a general feature of Apicomplexa. A second ER-like organelle has not been described in any other eukaryote and raises questions about its origin. This novel organelle and the alternative secretory pathway could possibly be related to the other unique organelles found in the Apicomplexa (Box 2). We propose the name secondary ER of Apicomplexa, or sERA.

In our model of extraparasite transport (Fig. 2) the sERA is the first step of an alternative secretory pathway that operates in parallel with the classic protein transport pathway. This alternative pathway is established immediately after merozoite invasion and functions throughout the erythrocytic stage. The rapid export of proteins from the parasite[12] suggests that processing through multiple compartments is not likely. Presumably, mRNA of exported *Plasmodium* proteins is either translated at the sERA or proteins are post-translationally imported into the sERA. Many exported *Plasmodium* proteins have typical eukaryotic signal sequences and some of these are capable of being translocated across microsomal membranes *in vitro*[13]. It is not clear how mRNA and/or proteins are differentially targeted to either the sERA or the ER. In addition, proteins can be targeted to the sERA via dense granules. For example, Ag-3008 (Ref. 14) is synthesized in the previous merogonic cycle as a dense granule protein and transits through the sERA on its way towards its final destination of the PVM[4]. Dense granules are secretory vesicles of Apicomplexa and are probably the output of the classic secretory pathway[15].

Exported proteins presumably move into the parasitophorous vacuole (PV) after transit through the sERA, as has been demonstrated for the transit of glycophorin-binding protein to the IEM[16]. The juxtaposition of the sERA with the parasite plasma membrane[4] is also consistent with the PV being the next step in extraparasite transport. However, the mechanisms by which proteins move from the sERA into the PV are not known. The BFA block implies that transfer vesicles[7] and G proteins are involved. BFA inhibits the guanine nucleotide exchange on a Ras-like G protein known as ADP-ribosylation factor (ARF)[17] and multiple ARF homologues have been identified in *P. falciparum*[18,19]. The recent report that small transfer vesicles fuse into vesicular-tubular clusters that resemble the *cis*-Golgi[20] inspires speculation that similar mechanisms might also operate in the export of *Plasmodium* proteins.

Transport of proteins to their final destinations

Proteins destined for different locations within the infected erythrocyte are simultaneously found in the sERA after BFA treatment[4]. Therefore, sorting into the different intraerythrocytic compartments occurs after the sERA. Some sorting might occur at the level of the PV, as proteins destined for the PV could simply be retained and those destined for the PVM could be incorporated into the membrane. Interestingly, sphingomyelin synthetase, a Golgi marker, is associated with the PVM/TVM[21] and a monoclonal antibody against an intraerythrocytic cleft protein recognizes a *cis*-Golgi protein of mammals[22], leading to speculations that the TVM might be involved in Golgi functions such as intracellular protein transport and sorting[2]. However, recent data indicate that the TVM does not play a role in extraparasite transport, but might be involved in the acquisition of nutrients[23]. Furthermore, studies with fluorescent lipid probes indicate that there is little, if

Box 2. Origins of the sERA: Dual Roles for the Apical Organelles?

An additional unanswered question about the secondary ER of Apicomplexa (sERA) and the alternative secretory pathway concerns its origin. Membranes originate from pre-existing membranes and do not arise *de novo*. One possibility is that the apical organelles play some role in the establishment of the sERA. For example, the release of rhoptries or micronemes during merozoite invasion could establish domains on the parasite membrane serving as a foundation for the sERA. A single sERA located adjacent to the parasite plasma membrane is consistent with such a phenomenon. Interestingly, vesicles containing an ER-type Ca^{2+}-ATPase are located at the apical end of *Cryptosporidium* sporozoites[11]. The association of some rhoptry proteins with the parasitophorous vacuolar membrane (PVM)[52] indicates that rhoptry components are important for PVM formation and therefore could also be involved in extraparasite transport. The observation that Ag-3008 (Ref. 15) is transferred from the dense granules to the PVM via the sERA immediately after merozoite invasion[4] establishes a link between the alternative secretory pathway and specialized organelles of the Apicomplexa. Similarly, the formation of membranous projections from the PVM as a result of dense granule release[53] and the possible movement of ring-infected erythrocyte surface antigen (RESA) from the dense granules to the erythrocyte membrane via the intraerythrocytic membranes (IEMs)[35] are consistent with a role for dense granules in the establishment of the alternative secretory pathway.

Fig. 2. Proposed model for secretory processes in *Plasmodium*. Major components of the two parallel secretory pathways are shown schematically. Proteins destined for export are routed through a unique endoplasmic reticulum (ER)-like organelle located at the parasite periphery, either co- or post-translationally. Some proteins associated with dense granules (DG) are also routed through the secondary ER of Apicomplexa (sERA). Sorting into different erythrocytic compartments probably occurs at the level of the parasitophorous vacuole (PV) or its membrane. The mechanism of sorting is not known but probably does not involve vesicular trafficking between the PV membrane (PVM) or intraerythrocytic membranes (IEMs) and the erythrocyte membrane. Some exported proteins could be released directly into the erythrocyte cytoplasm and then bind when they reach their specific destinations such as the IEMs or the erythrocyte membrane. The trafficking of proteins to their final or intermediate destinations might involve molecular chaperones (Chp).

any, vesicular movement between the PVM and the erythrocyte membrane[24,25]. Nonetheless, IEMs appear to play some role in extraparasite transport. For example, ultrastructural studies reveal electron-dense material associated with Maurer's clefts, implying that this electron-dense material is a component of the knobs on route to the erythrocyte membrane[26]. It has also been suggested that Ag-Pf332 is transferred from intraerythrocytic clefts to the erythrocyte membrane by a mechanism not involving membrane fusion[27].

Immunofluorescence studies reveal that Pc(em)93, a 93 kDa *P. chabaudi* protein associated with the erythrocyte membrane[28], is occasionally found associated with vesicle-like structures adjacent to the parasite during the period of Pc(em)93 synthesis[29]. The paucity of these structures is consistent with the rapid transport of Pc(em)93 to the erythrocyte membrane[12]. Pc(em)93 is not seen to be associated with structures that extend to the erythrocyte membrane and we propose that Pc(em)93 is released directly into the host cytoplasm from these vesicle-like structures that are adjacent to the parasite[29]. Interestingly, Pc(em)93 and similar proteins from *P. berghei* bind specifically to the cytoplasmic face of the erythrocyte membrane[30]. Several *P. falciparum* proteins, including the knob-associated protein[31], ring-infected erythrocyte surface antigen (RESA)[32] and mature parasite-infected erythrocyte surface antigen (MESA)[33], also bind specifically to proteins located on the cytoplasmic face of the erythrocyte membrane. This affinity that some *Plasmodium* proteins have for the cytoplasmic face of the erythrocyte membrane might be part of their targeting mechanism. However, the knob-associated protein[34], as well as RESA[35], Ag-Pf332 (Refs 27,36) and PfEMP-1 (Ref. 37) initially associate with the IEMs before being found at their final destination of the erythrocyte membrane. It is not clear whether this intermediate step in the transport to the host membrane involves proteins moving along the IEMs or whether proteins are released into the erythrocyte cytoplasm and then bind to the cytoplasmic face of the IEMs. These two possibilities are not mutually exclusive and both mechanisms could be operational; different proteins might utilize different mechanisms.

Accessory proteins, such as molecular chaperones, could also participate in the targeting of parasite proteins to the erythrocyte membrane or other intraerythrocytic locations. Interestingly, antibodies raised against a *P. berghei* co-chaperone crossreact with HSP70 (Ref. 38) and a protein(s) in the cytoplasm of infected erythrocytes[39]. Exported chaperones could participate in the targeting of parasite proteins to their final destinations and the assembly of protein complexes, such as knobs, on the host erythrocyte membrane. An analogous situation has been described for transport of proteins to the outer membrane of bacteria. For example, the surface pili of bacteria are assembled on the outer bacterial membrane from several different proteins[40]. Chaperones found in the periplasmic space sequester the protein subunits, thus controlling their ordered translocation across the outer membrane and preventing their premature assembly into pili.

Conclusions

Much still needs to be learned about how the malaria parasite alters its host cell. A major question still to be answered concerns the targeting of exported proteins to the sERA and the alternative secretory pathway. The ability to target transfected genes correctly in *Plasmodium*[41] demonstrates that it will be possible to determine the signal sequences specific for the sERA empirically. Other future research includes the isolation and biochemical characterization of the sERA and the determination of the fate of exported proteins when they leave the sERA. Additional research into secretory processes in *Plasmodium* and other Apicomplexa will probably reveal more unique cell biology in this fascinating phylum.

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Techniques

Single-cell *in vivo* Measurements of Ion Concentrations within the Intracellular Parasite *Plasmodium falciparum*

S. Wünsch, P. Horrocks, M. Gekle and M. Lanzer

Rapid progress has been made in the study of intracellular ion activities of eukaryotic cells through the recent combination of high-resolution microscopy with fluorimetric ion-specific probes. This technique allows a specific ion concentration within a single living cell to be monitored on-line with high temporal and spatial resolution. In this report, Stefan Wünsch, Paul Horrocks, Michael Gekle and Michael Lanzer evaluate the application of single-cell fluorimetry to the study of transport processes in Plasmodium falciparum.

Protozoan parasites impose a severe burden on the public health of many developing nations, accounting for a significant proportion of the morbidity and mortality associated with infectious diseases worldwide. This, together with the rapidly increasing incidence of drug resistance, underscores the need to identify novel targets for rational drug design programs, an endeavor complicated by the evolutionary relatedness of the eukaryotic protozoa and their vertebrate hosts. Valuable clues to the identification of novel drug targets can be gleaned from the mode of action of current antiparasite drugs. Recent work shows that some of the more effective antiparasite drugs specifically target or interact with parasite transport mechanisms: for example, the antimalarial drug chloroquine interacts with the *Plasmodium falciparum* Na^+–H^+ ion exchanger, facilitating its own uptake through stimulation of this protein[1–3]; another example is the anthelmintic drug ivermectin, which opens up Cl^- channels, thereby disrupting Cl^- ion homeostasis[4,5]. Other drugs that utilize intrinsic transport mechanisms for their uptake are methotrexate, which is taken up by a high-affinity folate transporter in *Leishmania major*[6], and melarsan, which is taken up by a high-affinity adenosine transporter in trypanosomes[7]. The fact that many drugs interact specifically with parasite transport processes indicates that the proteins mediating them are sufficiently different from their host homologues. This is probably a reflection of the parasitic lifestyle, where parasites have adapted these transport processes in order to survive and exploit the very different environments they encounter during their often complex life cycles.

Exploring the potential of parasite transporters as novel drug targets requires a better understanding of the parasite's physiology in order to identify essential transport processes and the proteins mediating them. Until recently, physiological techniques, such as patch clamping, did not readily lend themselves to the investigation of microorganisms or more complex systems, such as intracellular protozoan parasites. However, recent advances in physiological techniques are now providing us with the tools necessary to investigate transport mechanisms within parasites. Here we describe the application of ratio imaging, in combination with high-resolution microscopy, to measure Na^+–H^+ exchange in *P. falciparum* at the single-cell level, although this technique is applicable to any transport process involving the exchange of ions.

Single-cell ratio imaging of ion fluxes

Ratio imaging relies upon the use of ion-specific fluorochromes, a wide range of which is commercially available[8]. Most fluorochromes are available as a membrane-permeable precursor; this is added to the cell of interest

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