

An Alternate Secretory Pathway in *Plasmodium*: More Questions than Answers.

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ABSTRACT. The malaria parasite extensively modifies the host erythrocyte. Many of these modifications are mediated by proteins that are exported from the parasite and targeted to specific locations within the infected erythrocyte. However, little is known about how the parasite targets proteins to specific locations beyond its own plasma membrane. Treatment of infected erythrocytes with brefeldin-A results in the accumulation of many exported *Plasmodium* proteins into a compartment that is distinct from the endoplasmic reticulum. Proteins destined for the host erythrocyte membrane, the parasitophorous vacuole, or inclusions within the erythrocyte cytoplasm accumulate in this novel compartment and co-localization studies indicate that there is a single compartment per parasite. Exported proteins only accumulate in this novel compartment if brefeldin-A treatment is concurrent with their synthesis. This novel compartment is probably a membrane-bound organelle located at the parasite periphery and may be the first step in an alternate secretory pathway that specializes in the export of proteins into the host cell. Such an alternate secretory pathway raises questions about how exported proteins are differentially targeted to this novel organelle versus the endoplasmic reticulum and the fate of exported proteins after this novel organelle.

hemoglobin, as well as a polymerization mechanism to deal with the toxicity of the free heme generated by this digestion (Francis et al 1997). In addition, new permeability pathways have been described in the infected erythrocyte (this volume: Kirk, 1999). The malaria parasite also extensively modifies the cytoplasm and plasma membrane of the host erythrocyte during its intraerythrocytic stage. The electron-dense protuberances on the surface of *Plasmodium falciparum*-infected erythrocytes and caveola-vesicle complexes on *P. vivax*-infected erythrocytes are well known ultrastructural changes in the host erythrocyte (Aikawa 1977). Several distinct parasite induced compartments are also found in the cytoplasm of *Plasmodium*-infected erythrocytes (Gormley et al 1992, Stenzel & Kara 1989). In addition, numerous parasite antigens are localized to ill-defined inclusions within the erythrocyte cytoplasm. Ultrastructural studies suggest that these antigens are associated with the parasite-induced intraerythrocytic membranes (Hinterberg et al 1994, Martinez et al 1998). These intraerythrocytic membranes are probably extensions of the parasitophorous vacuole membrane (PVM). However, these antigens are not associated with the entire PVM, but probably associated with distinct domains of the PVM extensions as illustrated by co-localization studies (Haldar 1998).

Intracellular parasites experience many barriers in their exploitation of host cells. In addition to problems associated with entering host cells, parasites must also survive in the confines of another cell. This intracellular survival involves both impeding host cell defenses and carrying out metabolic processes. Many intracellular parasites have evolved unique metabolic features to compensate for their rather esoteric environments. For example, the intraerythrocytic malaria parasite has evolved several proteases for the specific digestion of

Many of these erythrocyte modifications are probably related to the metabolic demands and acquisition of nutrients by the parasite. Other alterations, such as the *P. falciparum* knobs, play more specialized roles in parasite survival (Crabb et al 1997). Regardless of their functions, parasite proteins exported into the host erythrocyte directly or indirectly mediate these host modifications. Furthermore, exported parasite proteins are specifically targeted to distinct locations within the infected erythrocyte. This trafficking of proteins to locations beyond the parasite plasma membrane raises

many intriguing questions about *Plasmodium* cell biology.

An alternate secretory pathway

It is generally assumed that proteins destined for export into the host erythrocyte are first routed to the parasite plasma membrane via the classical secretory pathway which consists of the endoplasmic reticulum (ER) and Golgi apparatus (Halder 1998, Lingelbach 1997). However, studies using brefeldin A (BFA) as an inhibitor of secretory pathway suggest that the ER and Golgi are not directly involved (Wiser et al 1999, Wiser et al 1997). BFA is a fungal metabolite that blocks the movement of proteins from the ER to the Golgi and leads to an accumulation of exported proteins in the ER (Klausner et al 1992). Treatment of infected erythrocytes with BFA leads to the accumulation of several distinct exported *Plasmodium* proteins (Table 1) in a compartment at the parasite periphery which is morphologically distinct from the ER (Figure 1). In contrast, the merozoite surface protein-1 (MSP1), a parasite membrane protein, accumulates in a compartment morphologically similar to the ER following BFA treatment (Wiser et al 1997). The exported proteins are never observed in a compartment with the morphological characteristics of the ER. Furthermore, the rapid export of proteins from the parasite (Wiser & Lanners 1992) indicates that processing of exported proteins through multiple compartments is unlikely. Therefore, this novel BFA-induced compartment is analogous to the ER since exported proteins are first found there instead of the ER, and because BFA inhibits the trafficking out of this compartment.

Interestingly, two ER-type Ca^{2+} -ATPases, designated PfATPase4 and PfATPase6, have been described in *P. falciparum*. Antibodies against PfATPase4 recognize a

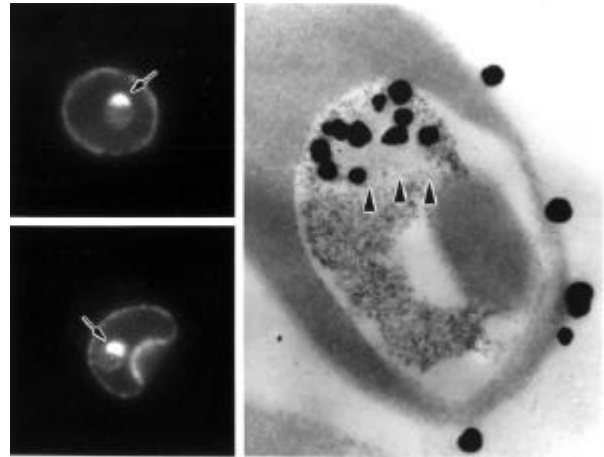


FIGURE 1. Microscopic characteristics of sERA. *P. chabaudi*-infected erythrocytes were treated with BFA and analyzed by immunofluorescence (left) and pre-embedding immunoelectron microscopy (right) with mAb-13.5 against Pc(em)93 as described in Wiser et al (1997). Treatment with BFA results in the accumulation of Pc(em)93 in a compartment at the parasite periphery (arrows). This compartment is clearly distinct from the ER as defined by BiP, a molecular chaperone of the ER (Kumar et al 1991). A demarcation between the parasite cytoplasm and the BFA-induced compartment is observed at the ultrastructural level (arrowheads) suggesting that the sERA is membrane bound.

compartment at the parasite periphery that exhibits a similar immunofluorescence pattern as the BFA-induced compartment (Dyer et al 1996). The observation that *Plasmodium* has two ER-type Ca^{2+} -ATPases and the accumulation of exported proteins into a compartment distinct from the ER suggests that the parasite has two distinct ER-like organelles. *Cryptosporidium* may also have two ER-type Ca^{2+} -ATPases (Zhu & Keithly 1997). Localization studies with antibodies raised against a

Cryptosporidium Ca^{2+} -ATPase reveal two distinct localization patterns. One pattern is a diffuse perinuclear localization, 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 this novel ER-like organelle may be a general feature of apicomplexa. Therefore, we propose to call this novel compartment the secondary ER of

TABLE 1. Exported *Plasmodium* proteins localized to the parasite periphery following brefeldin A treatment

Protein	Final destination	Reference
Pc(em)93	Erythrocyte membrane	Wiser et al (1997)
Pb(em)65	Erythrocyte membrane	Wiser et al (1997)
Ag-Pf332	Erythrocyte membrane	Hinterberg et al (1994)
MESA	Erythrocyte membrane	Mattei (1999)
Pb(ec)31	Inclusions in erythrocyte cytoplasm	Wiser et al (1997)
Pb(ec)13	Inclusions in erythrocyte cytoplasm	Wiser et al (1997)
Pc(ec)19	Inclusions in erythrocyte cytoplasm	Wiser et al (1997)
GBP	Inclusions in erythrocyte cytoplasm	Benting et al (1994)
HRP-II	Inclusions in erythrocyte cytoplasm	Mattei (1999)
Pfsar1p ^a	Inclusions in erythrocyte cytoplasm	Albano et al (1999)
Ag-3008 ^b	Parasitophorous vacuole membrane	Wiser et al (1997)

^aPfsar1p is localized to both inclusions in the erythrocyte cytoplasm and the parasite periphery in the presence or absence of BFA treatment. ^bAg-3008, a *P. chabaudi* exp-1 homologue, is initially synthesized during the schizont stage and localized to dense granules (Favaloro et al 1993) before its transport to the PVM.

apicomplexa, or sERA.

A second ER-like organelle suggests that the parasite has two parallel secretory pathways. One of these secretory pathways is analogous to the classical ER and Golgi and functions in the trafficking of proteins destined for the parasite plasma membrane and intraparasite organelles. The other pathway presumably specializes in the export of proteins into the host cell. Such a duplication of the secretory pathway is thus far unprecedented in other eukaryotic organisms. However, peroxisomes are believed to be derived from the ER without passing through the Golgi and are capable of translocating proteins from the cytoplasm (Titovenko & Tachubinski 1998). Other alternate secretory pathways have been described. For example, the export of mating factor- α in *Saccharomyces cerevisiae* is mediated by a transporter in the ATP-binding cassette (ABC) family (Kuchler et al 1989). In addition, protein secretion which is insensitive to pharmacological agents that perturb the ER and Golgi and that is independent of ABC-transporters has been described (Cleves et al 1996). However, such alternate secretory pathways are distinct from the alternate pathway proposed for *Plasmodium* in that the *Plasmodium* alternate pathway is sensitive to BFA, whereas these other alternate pathways are insensitive to BFA. However, a BFA-insensitive pathway is proposed for the export of the knob associated histidine-rich protein-1 (KAHRP-1) from *P. falciparum* (this volume: Mattei, 1999).

Analysis of sERA by density gradient centrifugation

Preliminary experiments examining the feasibility of isolating the sERA by density gradient centrifugation were carried out. A lysate was prepared from BFA-treated parasites and subjected to centrifugation on a discontinuous Percoll gradient. The material accumulating at the interfaces was examined by immunoblotting with monoclonal antibodies (mAb) against different subcellular compartments. The lack of a definitive marker for the sERA was circumvented by analyzing an exported protein, Pb(ec)31, which had accumulated in the sERA as a result of BFA treatment (Table 1). Isolation of the parasites before the density gradient centrifugation removes the Pb(ec)31 already exported into the host cytoplasm. Following centrifugation, the majority of Pb(ec)31 is found at the interface between the 30% and 45% Percoll layers (Figure 2). As expected, no Pb(ec)31 was recovered from control gradients of parasite lysates prepared without prior BFA treatment (not shown). Pb(em)65,

another exported protein accumulating in sERA following BFA treatment (Table 1), is also enriched at the 30/45% interface (not shown).

The gradient fractions were also analyzed with a mAb against MSP-1 and a mAb against a parasite cytosolic protein called Pbpp58 (Wiser et al 1996). MSP1 is found at the 10/20% and 20/30% interfaces (Figure

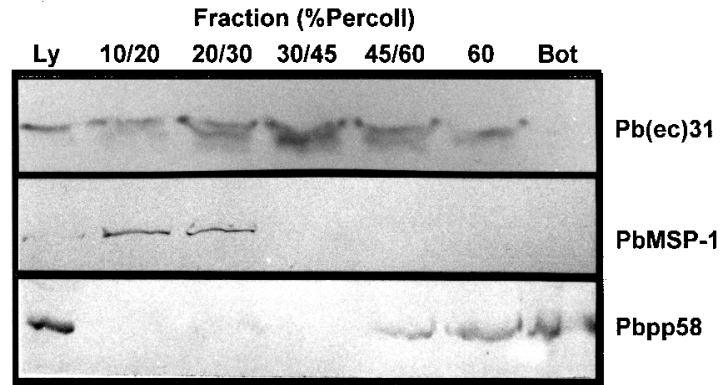


FIGURE 2. Analysis of the sERA by density gradient centrifugation. *P. berghei*-infected erythrocytes were treated with BFA as described in Wiser et al (1997) followed by parasite isolation on Percoll gradients as described in Wiser & Schweiger (1985). Isolated parasites were resuspended in 60% Percoll and lysed with a French press at 1000 psi. The lysate was then underlaid on a discontinuous Percoll gradient consisting of 3-4 ml layers of 10%, 20%, 30% and 45% Percoll and subjected to centrifugation as described in Grab et al (1987). Material at the interfaces (% Percoll indicated above lanes) was collected by centrifugation at 100,000xg and analyzed by immunoblotting with mAb-I2.6, mAb-F4.4 or mAb-b5 which recognize Pb(ec)31, PbMSP-1 and Pbpp58, respectively. The sample in lane 60 contained intact parasites detected as a visible brown band between the 45/60% interface and the bottom (Bot) of the gradient. (Ly = lysate)

2) indicating that separation of subcellular compartments is occurring. The majority of Pbpp58 is found at the bottom of the gradient. This is expected for a soluble cytosolic protein since the parasites were lysed in 60% Percoll and the sample was underlaid on the gradient. The small amount of Pbpp58 in the lighter fractions is probably due to unlysed parasites. Also notable is the complete lack of Pb(ec)31 at the bottom of the gradient. This result indicates that Pb(ec)31 is membrane bound, and therefore the sERA is a membrane bound organelle as suggested by the electron microscopy studies (Figure 1). More work is clearly needed on the biochemical and morphological characterization of this novel organelle.

Targeting signals for the sERA?

The proposition of two parallel secretory pathways implies that signals within exported proteins will differentially target them for the sERA instead of the ER. However, comparison of the N-terminal sequences from *P. chabaudi* proteins localized to different

subcellular compartments did not reveal any distinct features of proteins exported into the host erythrocyte (Table 2). All of the proteins exhibit a canonical signal sequence (von Heijne 1985), consisting of 1-3 positive amino acids in the first seven residues, followed by a hydrophobic core of 7-13 residues, and a cleavage region of 5-7 residues which are generally uncharged but somewhat polar. Many *P. falciparum* proteins that are exported from the parasite also have N-termini that

Subsequent trafficking and sorting after sERA

Another major question in the extraparasite transport process concerns the fate of proteins after the sERA and their trafficking to intraerythrocytic locations. Co-localization of proteins destined for different intraerythrocytic sites indicates that sorting occurs subsequent to the sERA (Wiser et al 1997). Glycophorin-binding protein (GBP) traverses the parasitophorous

TABLE 2. Canonical signal sequences from selected *Plasmodium chabaudi* proteins

<i>Protein</i>	<i>Location</i>	<i>Sequence</i> ^a	<i>Acc. No.</i>
MSP-1	Parasite Membrane	MKA <u>I</u> GLLFSFVFFAIYCK S ◆E	M34947
AMA-1	Rhoptries	MKEI <u>YYIVILCSLYLINL</u> GNCSEG/T	M25248
Ag-3008	Dense Granules, PVM ^b	MKVP <u>KVSAIFLVFLLSVCF</u> LPSP/LSC/D	L19784
Pc(em)93	Erythrocyte Membrane	MNKGYIK <u>TIFII</u> LSLVAYTSNKA/H	U80896
PcEMP-1	Erythrocyte Membrane	MKAISL GLISSIIFSIVLAKNSSG/S	M95789

^aFeatures of signal sequences for *P. chabaudi* proteins obtained from GenBank are highlighted. Positive residues in first seven are in bold, the hydrophobic core is double underlined, and a polar cleavage region is underlined. The / denotes potential cleavage sites and the ◆ denotes a confirmed cleavage site (O'Dea et al 1995). ^bAg-3008, a *P. chabaudi* exp-1 homologue, is initially synthesized during the schizont stage and localized to dense granules (Favaloro et al 1993) before its transport to the PVM in the early ring stage (Wiser et al 1997).

resemble signal sequences, and some of these can be translocated across mammalian microsomal membranes in vitro (Lingelbach 1993). Some exported proteins, however, do not have typical signal sequences and are not translocated across microsomes. Many of the proteins lacking the canonical N-terminal sequence do have a stretch of hydrophobic amino acids within the first 100 residues which could serve as a signal sequence (Foley & Tilley 1998). The nature of the signal sequence for the sERA will probably not be revealed by analysis of gene sequences and will likely require an empirical approach involving gene transfection.

Little is known about proteolytic processing associated with the removal of signal sequences in *Plasmodium*. Proteolytic processing of rhoptry proteins is inhibited by BFA (Howard & Schmidt 1995, Ogun & Holder 1994). BFA treatment, however, did not affect the sizes of Pc(em)93, Pb(em)65 and Pb(ec)31 (R.A. Bafford & M.F. Wiser, unpublished work 1996). Attempts to sequence the N-terminus of Pc(em)93 revealed that it is blocked (M.F. Wiser, unpublished work 1994). N-termini are usually acetylated in the cytoplasm and removal of signal sequences results in exported proteins not having block N-termini (Bradshaw 1989). The blocked N-terminus of Pc(em)93 is consistent with a lack of proteolytic processing associated with its export. On the other hand the blocked N-terminus inspires speculation that the targeting signals may be post-translational modifications to the exported proteins. Some analysis of the nature of the block is warranted.

vacuole en route to its final destination in the erythrocyte cytoplasm (Ansong et al 1996). This observation and the location of the sERA near the parasite periphery suggest that exported proteins move from the sERA into the parasitophorous vacuole. Some sorting could occur at the level of the parasitophorous vacuole. For example, parasitophorous vacuole proteins could simply be retained and those destined for the PVM could be incorporated into the membrane. A *Toxoplasma* protein has been demonstrated to integrate into the PVM following secretion of a soluble form (Ossorio et al 1994). Proteins associated with domains or the intraerythrocytic membranes could move along membrane extensions of the PVM. However, studies with fluorescent lipid probes indicate that there is little, if any, vesicular traffic between the PVM and the erythrocyte membrane (Haldar & Uyetake 1992, Pouvelle et al 1994). Therefore, it is unlikely that transport to the erythrocyte membrane involves vesicles derived from the PVM or fusion of the PVM with the erythrocyte membrane.

Pc(em)93 is occasionally associated with vesicle-like structures adjacent to the parasite during the early ring stage at a time coincident with its synthesis (Lanners et al 1999). The paucity of these structures is consistent with the rapid transport of Pc(em)93 to the erythrocyte membrane (Wiser & Lanners 1992). These structures do not extend to the erythrocyte membrane and are only observed to be juxtaposed to the parasite. Aggregates of RESA, a *P. falciparum* exported protein, that are closely associated with the parasitophorous

vacuole in newly invaded erythrocytes have also been described (Culvenor et al 1991). In addition, an ATP-binding cassette protein from *P. falciparum* has been localized to electron-dense areas at the parasite periphery and to convolutions of the PVM (Bozdech et al 1998). The relations between these various structures projecting from the PVM are not known. One possibility is that these PVM associated structures are involved in the release of parasite proteins into the host erythrocyte cytoplasm. A direct export of proteins into the host cytoplasm is consistent with the lack of vesicular traffic between the PVM and erythrocyte membrane. Interestingly, several parasite proteins that

anisms, proteins still need to be transferred to the erythrocyte membrane. It has been suggested that Ag-Pf332 is directly transferred from intraerythrocytic clefts to the erythrocyte membrane by a mechanism not involving membrane fusion (Hinterberg et al 1994). Such a direct transfer would favor a model in which proteins are released into the erythrocyte cytoplasm and then associate with the cytoplasmic face of the intraerythrocytic membranes.

Accessory proteins, such as molecular chaperones, could also participate in the targeting of parasite proteins to the intraerythrocytic membranes and/or erythrocyte membrane. Furthermore, molecular chaperones could participate in the assembly of supra-

molecular complexes, such as knobs, or the insertion of parasite proteins into the erythrocyte membrane (eg., PfEMP-1). An analogous situation has been described for the transport of proteins to the outer membrane of bacteria. For example, surface pili of bacteria are assembled on the outer bacterial membrane from several different proteins (Hultgren et al 1993). Chaperones in the periplasmic space

sequester the protein subunits, thus controlling their ordered insertion into the outer membrane and preventing the premature assembly of pili. Indirect evidence for the existence of parasite chaperones in the erythrocyte cytoplasm has been reported (Wiser et al 1996).

Summary

The mechanisms of transport and targeting of parasite proteins to specific locations beyond its own plasma membrane is an intriguing aspect of *Plasmodium* cell biology. Much still needs to be learned about trafficking in the malaria parasite as exemplified by the numerous question marks in our working model for extraparasite transport (Figure 3). A key feature of this model is a proposed ER-like organelle which functions as an early compartment for many exported *Plasmodium* proteins. Questions regarding the biochemical characteristics of the sERA, how proteins are targeted to the sERA, and the fate of proteins after the sERA remain to be answered.

TABLE 3. Binding of *Plasmodium* proteins to erythrocyte membranes and associations with erythrocyte cytoplasmic inclusions

<i>Protein</i>	<i>IOV</i>	<i>Reference</i>	<i>IEM</i>	<i>Reference</i>
Pb(em)65, Pc(em)93	+	Wiser et al (1990)	*	Lanners et al (1999)
KAHRP-1	+	Kilejian et al (1991)	+	Taylor et al (1987)
RESA	+	Foley et al (1991)	*	Culvenor et al (1991)
MESA	+	Lustigman et al (1990)	n.d.	
Ag-Pf332	n.d.		+	Hinterberg et al (1994)
PfEMP-1	n.d.		+	Crabb et al (1997)

Plusses (+) in the relevant columns denote *Plasmodium* proteins that bind to inside-out vesicles (IOV) in vitro and/or that exhibit an immunofluorescence pattern consistent with intraerythrocytic inclusions. These inclusions are presumably associated with intraerythrocytic membranes (IEM) and represent an intermediate step in their transport to the erythrocyte membrane. *Immediately after merozoite invasions Pc(em)93 is transiently associated with vesicle-like structures which are adjacent to the parasite and RESA is transiently observed in aggregates closely associated with the parasitophorous vacuole. However, neither exhibits an inclusion-type immunofluorescence pattern. (n.d. = not determined)

are associated with the erythrocyte membrane specifically bind to the cytoplasmic face of the erythrocyte membrane (Table 3). This affinity of some *Plasmodium* proteins for the erythrocyte membrane may be a central component of the targeting mechanism.

Many of the erythrocyte membrane associated proteins are localized to inclusions in the erythrocyte cytoplasm before becoming associated with the host membrane (Table 3). Similarly, ultrastructural studies reveal electron-dense material associated with Maurer's clefts, implying that knob components are routed to the host membrane via intraerythrocytic membranes (Aikawa et al 1986). These observations suggest that proteins could move through or along the intraerythrocytic membranes en route to the erythrocyte membrane. Alternatively, proteins could be released directly into the erythrocyte cytoplasm and then become transiently associated with the cytoplasmic face of the intraerythrocytic membranes. These two mechanisms are not mutually exclusive and different parasite proteins could utilize different mechanisms. Regardless of the mech-

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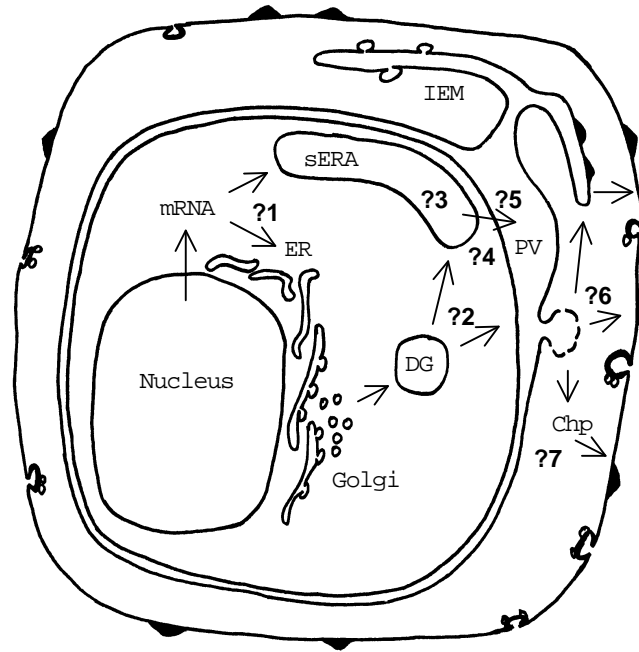


FIGURE 3. Schematic representation of current working model for extraparasite transport. The major subcellular components and possible pathways for the movement of exported proteins are shown. Many issues still need to be resolved about secretory processes in *Plasmodium*. Potential questions are denoted. 1) What are the differential targeting signals for the two ER-like organelles? 2) What are the relationships between components of the classical secretory pathway, such as the dense granules (DG), and the alternate secretory pathway? 3) What are the biochemical characteristics of sERA? 4) How are proteins moved from the sERA to the parasitophorous vacuole? 5) How do the PVM and the intraerythrocytic membranes (IEM) participate in the trafficking of exported proteins? 6) How are proteins released from the parasitophorous vacuole (PV) and then targeted to the erythrocyte membrane or other intraerythrocytic compartments? 7) Do molecular chaperones participate in the trafficking?

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