Biosynthesis, export and processing of a 45 kDa protein detected in membrane clefts of erythrocytes infected with *Plasmodium falciparum*

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During its asexual life cycle, the human malaria parasite *Plasmodium falciparum* exports numerous proteins beyond its surface to its host erythrocyte. We have studied the biosynthesis, processing and export of a 45 kDa parasite protein resident in membrane clefts in the erythrocyte cytoplasm. Our results indicate that this cleft protein is made as a single tightly membrane-bound 45 kDa polypeptide in ring- and trophozoite-infected erythrocytes (0–36 h in the life cycle). Using ring/trophozoite parasites released from erythrocytes, the 45 kDa protein is shown to be efficiently transported to the cell surface. This export is specifically blocked by the drug brefeldin A, and at 15 and 20 °C. These results indicate that transport blocks seen in the Golgi of mammalian cells are conserved in *P. falciparum*. Further, the newly synthesized 45 kDa protein passes through parasite Golgi compartments before its export to clefts in the erythrocyte. In mid-to-late-ring-infected erythrocytes, a fraction of the newly synthesized 45 kDa protein is processed to a second membrane-bound phosphorylated 47 kDa protein. The $t_{\frac{1}{2}}$ of this processing step is about 4 h, suggesting that it occurs subsequent to protein export from the parasite. Evidence is presented that, in later trophozoite stages (24–36 h), the exported 45 and 47 kDa proteins are partially converted into soluble molecules in the intraerythrocytic space. Taken together, the results indicate that the lower eukaryote *P. falciparum* modulates a classical secretory pathway to support membrane export beyond its plasma membrane to clefts in the erythrocyte. Subsequent to export, phosphorylation and/or conversion into a soluble form may regulate the interactions of the 45 kDa protein with the clefts during parasite development.

INTRODUCTION

Plasmodium falciparum is a protozoan which causes the most virulent form of human malaria. Its asexual life cycle is entirely responsible for the symptoms of the disease and begins with the invasion of an erythrocyte by a merozoite. The resulting intraerythrocytic parasite develops within a parasitophorous vacuole through morphologically distinct ring, trophozoite and schizont stages. The uninfected erythrocyte has no intracellular organelles and lacks the machinery for *de novo* lipid and protein biosynthesis (Gronowicz et al., 1984; Chasis et al., 1989). In contrast, the parasite actively synthesizes and exports protein and lipids beyond its plasma membrane to develop its parasitophorous vacuolar membrane, a complex of tubovesicular structures in the cytoplasm of the erythrocyte and the erythrocyte membrane (Barnwell, 1990; Vial et al., 1990; Haldar, 1992; Elmendorf and Haldar, 1993a). However, very little is known about the routes of membrane-protein export through the parasite.

The major pathway for the export of protein and membranes in eukaryotic cells is the secretory pathway (Rothman and Orci, 1992). Proteins are recruited into the endoplasmic reticulum (ER) by a signal-mediated process, exported through the Golgi and released at the plasma membrane. Distinct compartments of this pathway display characteristic activities of protein modification and transport, and these have been essential in defining stepwise transport through the secretory pathway. In addition to classical secretory release, higher eukaryotic cells and yeast can also export proteins (such as interleukin 1β and a factor) directly from the cytoplasm across the plasma membrane (Kuchler et al., 1989; Rubartelli et al., 1990). There is evidence for a classical secretory pathway, comprised of an ER–Golgi complex in *Plasmodium* [reviewed in Elmendorf and Haldar (1993a)]. The Golgi has at least two compartments, one containing a parasite homologue of ERD2 (a receptor for protein retention in the ER) and the other sphingomyelin synthase (Elmendorf and Haldar, 1993b). Constitutive secretion of soluble lumenal proteins through *P. falciparum* is rapid [5 min from the onset of translation (Crary and Haldar, 1992)], suggesting that the parasite has a simple secretory apparatus relative to that of higher eukaryotes. *Plasmodium* is unable to carry out N-linked glycosylation (Dieckman-Schuppert et al., 1992), a major protein modification of eukaryotic secretion, but is capable of glycosylphosphatidylinositol (GPI) addition (Haldar et al., 1985; Schwartz et al., 1986). Other protein-processing activities of parasite secretion remain undefined. The drug brefeldin A reorganizes the parasite ERD2 Golgi compartment back to the ER (Elmendorf and Haldar, 1993b) and blocks secretory release of soluble lumenal proteins from the parasite (Crary and Haldar, 1992; Elmendorf et al., 1992). We also have evidence for a brefeldin-insensitive pathway of export, suggesting that protein export in *Plasmodium* can occur by pathways independent of classical secretion (Elmendorf et al., 1992). Consistently, a number of proteins lacking canonical N-terminal signal sequences appear to be exported by the parasite (Lingelbach, 1993), and it has been proposed that multiple pathways exist for protein export from the parasite (Elmendorf and Haldar, 1993a; Gormley et al., 1992).

By transmission electron microscopy, tubovesicular structures

Abbreviations used: EM, erythrocyte membrane; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; HRP, histidine-rich protein; PMSF, phenylmethanesulphonyl fluoride; TVM, tubovesicular membrane; DTT, dithiothreitol.

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in the cytoplasm of the erythrocyte appear as 'clefts' and large loops, clearly separated from each other, the parasitophorous vacuolar membrane and the erythrocyte membrane (EM) respectively (Barnwell, 1990). However, using scanning electron and laser confocal microscopy, the intraerythrocytic structures appear as a tubovesicular membrane (TVM) network, connected to the parasitophorous vacuolar surface and extending to the EM (Elford and Ferguson, 1993; Elmendorf and Haldar, 1994). This network can be prominently labelled with fluorescent lipids. In earlier studies, lipid-labelled cells were perfused with diaminobenzidine, irradiated in a fluorescence microscope and processed for electron microscopy. In the resulting thin sections, prominent, black, photo-oxidation products of polymerized diaminobenzidine were seen in the clefts and loops, confirming that they were in fact domains of the TVM network, but appeared as isolated structures in transverse or longitudinal thin sections (Haldar et al., 1991). The clefts and loops contain parasite proteins and the export of these proteins is thought to underlie their development in the erythrocyte (Simmons et al., 1987; Kara et al., 1988; Hui and Siddiqui, 1988; Etzion and Perkins, 1989; Stanley et al., 1989; Li et al., 1991). Although the parasite may engage in classical secretion as well as additional pathways of protein export, it remains unclear (i) which pathway drives export of membrane proteins from the parasite and what are its component transport steps, (ii) whether exported proteins are recycled back to the parasite, (iii) what types of post-translational protein modifications are effected during membrane trafficking between the parasite and tubovesicular structures and (iv) how these processes are regulated during asexual parasite development.

As a first step to understanding these processes we have investigated the biosynthetic export and processing of a 45 kDa protein which is a major antigenic resident of the clefts. We find that the protein is synthesized as a tightly membrane-bound form. It is exported by the classical secretory pathway and undergoes conserved steps of membrane transport through the Golgi during ring and trophozoite stages. The exported protein, its phosphorylated and soluble products accumulate in the erythrocyte and are not recycled back to the parasite. The implications of these data are discussed with respect to mechanisms of parasite membrane export and stage-specific modifications of the 45 kDa cleft protein in the erythrocyte.

EXPERIMENTAL

Materials

RPMI 1640 was from Gibco. BSA, pepstatin A, leupeptin, iodoacetamide, phenylmethanesulphonyl fluoride (PMSF), gentamicin, hypoxanthine and cycloheximide were purchased from Sigma. A^+ human serum was from Gemini Bio Products. Percoll was from Pharmacia. [^{32}P]P_i was obtained from Amersham, and [^{35}S]Tran label was from ICN. The stainless-steel ball homogenizer (internal bore diameter, 0.635 cm; ball diameter, 0.6335 cm) was custom built at the Biochemistry Shop, Stanford University. The steel balls were obtained from Industrial Techtonics. All other reagents were of superior analytical grade.

Culture of *P. falciparum* FCR-3/A_2

The clonal line of *P. falciparum* FCR-3/A_2 was cultured *in vitro* by a modification (Haldar et al., 1985) of the method of Trager and Jensen (1976). The basic culture medium contained RPMI 1640, 25 mM Hepes, pH 7.4, 11 mM glucose, 92 μM hypoxanthine, 0.18% $NaHCO_3$ and 25 μg/ml gentamicin, and was supplemented with 10% A^+ human serum. The parasites were grown in A^+ erythrocytes at 2.5–5% haematocrit at parasitaemias of 2–30%. Parasite cultures were synchronized by separation of the early and late stages over Percoll gradients and the subsequent separate reincubation of these stages in culture.

Pulse–chase studies

Pulse-labelling experiments were performed with synchronized cultures of infected erythrocytes from the early ring (0–12 h after invasion) to schizont (> 40 h after invasion) stages. *P. falciparum*-infected erythrocytes of the appropriate stage were resuspended in methionine-free RPMI 1640 at a density of 2.5×10^8 parasites/ml and metabolically labelled with 300 μCi/ml [^{35}S]Tran label for 60 s at 37 °C. Cycloheximide (100 μg/ml) was immediately added to block additional protein synthesis. The cells were then washed twice in RPMI 1640 containing methionine at 0–2 °C and stored as a frozen pellet at −20 °C. Where indicated, the pulse-labelled cells were chased in RPMI 1640 containing methionine at 37 °C for 1, 4 or 8 h. At the end of chase period the cells were washed twice in RPMI 1640 and stored at −20 °C.

Extraction of cells for immunoprecipitation

Cells were lysed on ice for 10 min in 5 vol. of 10 mM Tris/HCl (pH 7.4), containing 10 μg/ml pepstatin A, 50 μg/ml leupeptin, 5 mM iodoacetamide and 1 mM PMSF (lysis buffer). Lysates were centrifuged at 100000 ***g*** for 30 min. The supernatant was adjusted to 20 mM Tris/HCl (pH 7.4), 20 mM EDTA, 0.5% Triton X-100, 1 mg/ml BSA and 400 mM NaCl and subjected to immunoprecipitation (Li et al., 1991). The membrane pellet was resuspended in PBS containing 1% Triton X-100, 1% sodium deoxycholate and 0.5% SDS. The extract was clarified by centrifugation at 100000 ***g*** and subjected to immunoprecipitation as described above. Where indicated the membrane fractions were also extracted with (i) 1 M NaCl, (ii) 100 mM Na_2CO_3, pH 11 or (iii) hypotonic lysis buffer containing 1% Triton X-100. Subsequent to these treatments, the membranes were pelleted at 100000 ***g*** for 30 min at 4 °C. Supernatants and pellets were subjected to immunoprecipitation. Immunoprecipitated complexes were analysed by SDS/PAGE (Laemmli, 1970) and fluorography.

Isolation of released *P. falciparum* parasites and the EM/TVM fraction

Parasites were released from their erythrocyte host cells as described previously (Elmendorf et al., 1992). Erythrocytes infected with rings and trophozoites at 15–30% parasitaemia were washed once, resuspended at 6% haematocrit in homogenization buffer [65 mM sucrose, 15 mM EDTA (potassium salt), 85 mM Hepes, pH 7.4, 0.3 mM dithiothreitol (DTT), 1 mg/ml BSA] at 20 °C, and subjected to homogenization with a stainless-steel ball homogenizer until release of parasites exceeded 90% (10–15 passages). The homogenate was diluted 10-fold into buffer A [95 mM potassium acetate, 15 mM EDTA (sodium salt), 6.5 mM sucrose, 20 mM Hepes, pH 7.4, 0.3 mM DTT, 1 mg/ml BSA, 8 mg/ml glucose] and centrifuged twice at 2000 ***g*** for 10 min at 0 °C. The cell-free supernatant was stored for further use in the isolation of the EM/TVM fraction. The cell pellets were resuspended in 4 vol. of buffer A containing a vitamin/amino acid supplement (Gibco) and layered on to a 10%/40% Percoll gradient in buffer A. The gradient was centrifuged at 2000 ***g*** for 10 min at 0 °C. The 10%/40% interface containing the released parasites was collected and washed three times.

The EM/TVM fraction was isolated from the cell-free homogenate on a sucrose cushion in buffer A [27 ml of homogenate/10 ml sucrose cushion in Beckman Ultra Clear Tubes (2.54 cm × 8.89 cm)] and centrifuged for 100000 *g* for 60 min at 2 °C in a Beckman SW 28 rotor. The membrane interfaces were pooled and diluted into buffer A and stored at −70 °C.

Biosynthesis and export of the 45 kDa protein in released parasites

Released parasites were radiolabelled in 50 μCi/ml [^{35}S]Tran label at a density of 1×10^7–5×10^7 per ml of buffer B [95 mM potassium acetate, 10 mM sucrose, 1 mM EDTA (sodium salt), 5 mM $MgCl_2$, 30 mM Hepes, pH 7.4, 0.3 mM DTT, 8 mg/ml glucose and 1 × vitamin/amino acid mixture from a methionine-free RPMI 1640 Select Amine Kit] for 60 min at 37 °C. Where indicated, brefeldin A was added before the addition of radiolabel. For the temperature blocks, incubation with the radiolabel was carried out at 15 or 20 °C for 60 min. Incubation mixtures were subjected to centrifugation at 2000 *g* for 10 min, and the resultant cell pellet was washed three times in buffer A without BSA and containing 5 mg/ml L-methionine. The supernatant was subjected to a second step of centrifugation (30 min; 100000 *g*; TLA 100.2 rotor; 2 °C) to separate high-speed pellet and supernatant fractions. Proteins in the pellet and supernatant fractions were subjected to immunoprecipitation with monoclonal antibody LWLI or MOPC 104E as described previously (Li et al., 1991; Elmendorf et al., 1992) and analysed by SDS/PAGE (Laemmli, 1970) and fluorography.

[^{32}P]P_i incorporation and phosphoamino acid analysis

Infected erythrocytes were washed free of serum in RPMI 1640 and then resuspended in phosphate-free RPMI 1640, supplemented with 25 mM Hepes, 10% human serum that had been dialysed extensively against 150 mM NaCl and 200 μCi/ml $NaH_2{}^{32}PO_4$. Parasites were labelled for 10 h from the mid-late ring (12–24 h) to trophozoite (~ 30 h) stage. Extracts were prepared and subjected to immunoprecipitation with the monoclonal antibody LWLI as described by Li et al. (1991) and the samples were analysed by SDS/PAGE and autoradiography. Phosphorylated protein bands were excised from gels and partially hydrolysed in 5.7 M HCl for 1 h at 60 °C. Phosphoamino acids were separated on t.l.c. plates by electrophoresis in two dimensions (Boyle et al., 1991).

RESULTS

Detection of the newly synthesized 45 kDa cleft protein by the monoclonal antibody LWLI and its stage-specific expression in infected erythrocytes

We have previously described a monoclonal antibody LWLI which bound to membrane clefts (also referred to as intra-erythrocytic cisternae or Maurer's clefts) in the infected-erythrocyte cytoplasm (Li et al., 1991). The antibody recognized three proteins in schizont-infected cells. However, the dominant antigen, a parasite-encoded protein of 45 kDa, was the only LWLI-reactive protein found in the early ring stages, ~ 6 h in the sexual cycle (Li et al., 1991). As shown in Figure 1, indirect immunofluorescence assays in 6 h rings indicate a punctate fluorescence pattern in the erythrocyte, which is characteristic of LWLI binding in these cells. Thus it is reasonable to conclude that the 45 kDa protein is delivered to the clefts, and provides a suitable marker to study (i) the biosynthetic processing of a cleft protein and its regulation by the life cycle and (ii) routes of protein export from the vacuolar parasite.

To study the biosynthesis of the 45 kDa protein, early-ring-stage parasites (0–12 h) were pulse-labelled with [^{35}S]Tran label for 60 s and subjected to immunoprecipitation with either LWLI or a control antibody, MOPC 104E. As shown in Figure 2, lane 2, a newly synthesized 45 kDa protein was specifically recognized

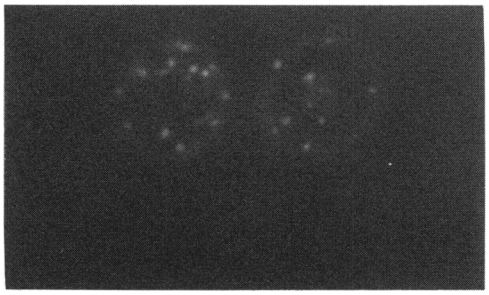

Figure 1 Indirect immunofluorescence of 0–6 h-ring-infected erythrocytes with LWLI

Blood smears of early rings were fixed with acetone and probed with LWLI and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody as described previously (Li et al., 1991) and examined in the fluorescence microscope under filter settings for fluorescein. Outlines of the erythrocyte and the ring parasite (seen more clearly on the cell on the right) were stained with Evans Blue dye.

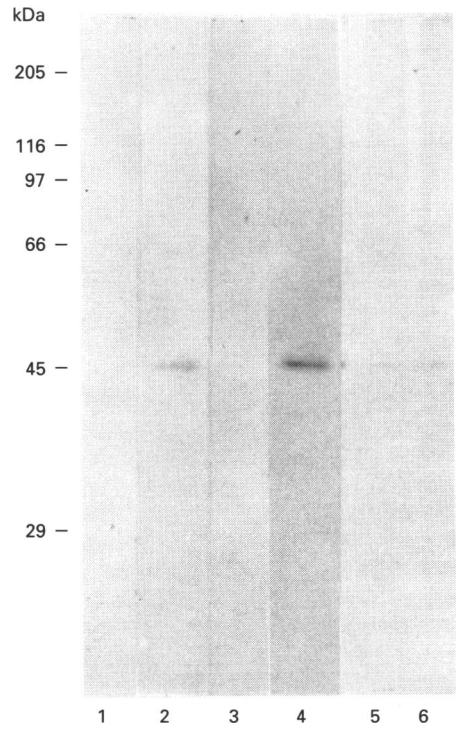

Figure 2 Stage-specific synthesis of the 45 kDa protein

Parasitized erythrocytes at the appropriate stage were pulsed with [^{35}S]Tran label for 60 s and subsequently subjected to immunoprecipitation with either the control antibody MOPC (odd-numbered lanes) or LWLI (even-numbered lanes). Lanes 1 and 2, early rings (0–12 h); lanes 3 and 4, mid-late rings (12–24 h); lanes 5 and 6, schizonts > 40 h. Molecular-mass markers used were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

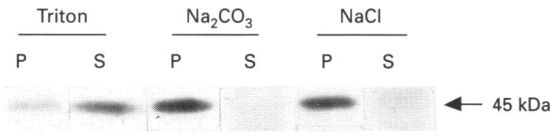

Figure 3 Demonstration that newly synthesized 45 kDa protein is membrane associated

Mid-late-ring-infected erythrocytes (12–24 h) were pulse-labelled with [^{35}S]Tran label for 60 s and lysed in hypotonic buffer. The membranes were collected by centrifugation and subsequently extracted in 1% Triton X-100, 0.1 M Na_2CO_3 or 1 M NaCl and subjected to centrifugation (see the Experimental section for details). The resulting supernatant (S) and pellets (P) were subjected to immunoprecipitation with LWLI.

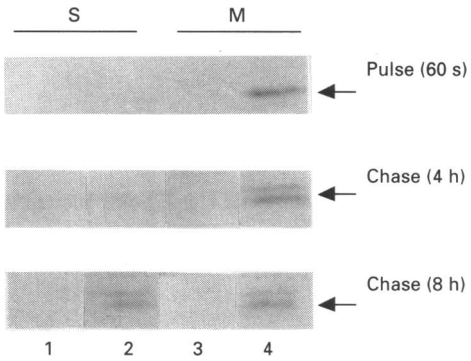

Figure 4 Biosynthetic processing of the 45 kDa protein

Mid-late rings (12–24 h) were pulse-labelled with [^{35}S]Tran label and then chased for 4 h and 8 h in RPMI 1640 medium containing methionine. At 0, 4 and 8 h of chase, the cells were lysed in hypotonic buffer and separated by centrifugation into soluble (S) and membrane (M) fractions, which were subjected to immunoprecipitation by LWLI (lanes 2 and 4) or MOPC 104E (lanes 1 and 3). The arrow indicates the 45 kDa protein.

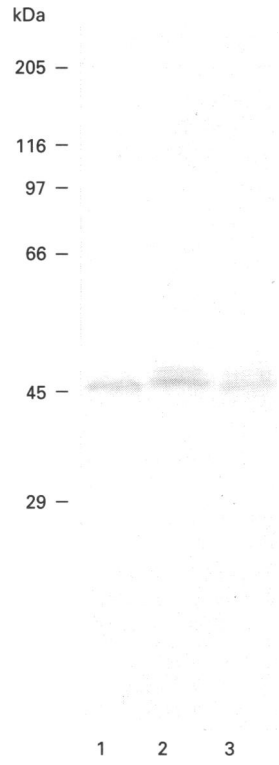

Figure 5 Accumulation of the 45 and 47 kDa proteins from ring to schizont stages

Infected erythrocytes at the indicated stages were solubilized in SDS/PAGE sample buffer, electrophoresed, transferred to nitrocellulose and probed with LWLI in a Western blot. Lane 1, early rings (0–12 h); lane 2, mid-late rings (12–24 h); lane 3, schizonts (> 40 h). Molecular-mass markers were as in Figure 2.

by LWLI. The control monoclonal antibody, MOPC 104E, which recognizes α-1–4-linked glucose did not recognize any parasite proteins (lane 1). Cycloheximide was added immediately after the pulse to block any lag in radiolabel incorporation and hence the protein detected in Figure 2, lane 2, indicates polypeptide synthesized within a minute of translation. Independent studies in our laboratory have indicated that export of newly synthesized proteins from ring-stage parasites requires a minimum of 4–5 min from the onset of translation (Crary and Haldar, 1992). Hence the radiolabelled protein recognized by LWLI in Figure 2, lane 2, lies within the parasite. The micrographs in Figure 1 indicate that the antibody recognizes protein exported to the erythrocyte. Thus it appears that LWLI can detect the newly synthesized 45 kDa protein within the parasite as well as its exported form/s in the erythrocyte. Similarly, the newly synthesized 45 kDa protein can also be immunoprecipitated from pulse-labelled mid to late rings (12–24 h; Figure 2, lane 4). In contrast, no radiolabelled proteins were specifically recognized in pulse-labelled schizonts (36–44 h; Figure 2, lane 6). Our results therefore indicate that the cleft protein is actively synthesized during the first 24 h of intraerythrocytic development as a single 45 kDa polypeptide: no precursors (of higher or lower molecular mass) were detectable in the fluorograms despite the prolonged exposure of the gels to film (not shown). Protein synthesis is detected at the trophozoite stage (not shown) but decreases at schizogony, indicating its stage-specific nature in the infected erythrocyte.

Association of the newly synthesized 45 kDa protein with membrane

Our previous studies by immunoelectron microscopy indicated that the 45 kDa protein was found associated with flattened lamellar membranes of the erythrocyte cytoplasm (Li et al., 1991). To examine the membrane association of the newly synthesized protein, pulse-labelled ring- and trophozoite-infected cells were lysed in a hypotonic buffer. Membrane and soluble components were separated by high-speed centrifugation and the membrane pellets were subjected to a variety of chemical treatments. As the maximum synthesis of the 45 kDa protein was detected in mid to late rings, the experiments shown were conducted at these stages of growth. After hypotonic lysis, the newly synthesized 45 kDa protein was detected entirely in the membrane fraction from where it could be efficiently extracted in 1% Triton (Figure 3). It could not, however, be released from the membranes by 0.1 M Na_2CO_3, pH 11.5, or 1 M NaCl. Hence the newly synthesized 45 kDa protein is tightly associated with membranes and likely to be an integral membrane protein in the parasite. (The absence of radiolabelled protein from the supernatant after hypotonic lysis is not shown for these experiments but a representative result is shown in Figure 4.)

Stage-specific post-translational processing of the 45 kDa protein

We next examined the biosynthetic processing of the newly synthesized 45 kDa protein. Pulse-labelled mid/late-ring-infected erythrocytes were chased for 4 h and subjected to immuno-

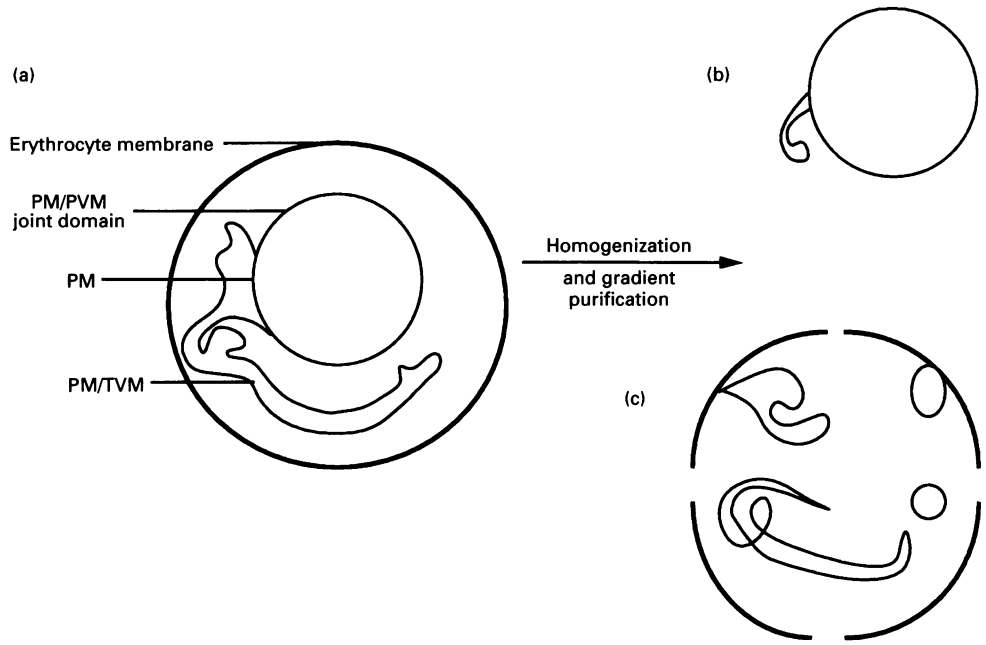

Figure 6 Fractionation of *P. falciparum*-infected erythrocytes

(**a**) Infected erythrocyte; (**b**) released parasite with parasitophorous vacuolar membrane (PVM); (**c**) EM and TVM. The TVM is found associated with the EM and also free in this fraction. PM, parasite plasma membrane.

precipitation with LWLI. As shown in Figure 4 (lane 4), after a 4 h chase, a 47 kDa membrane-bound protein was clearly detected along with the 45 kDa molecule. One-dimensional *Staphylococcus aureus* V8 protease maps of the labelled 45 kDa and 47 kDa proteins were indistinguishable from each other and the characteristic fragment pattern previously described for the 45 kDa molecule (Li et al., 1991; results not shown). Thus the 47 kDa species is not a coprecipitated protein but a processed form of the 45 kDa protein. The 47 kDa protein was not detected within a 60 min chase period (not shown), suggesting that the *in vivo* processing of the 45 to 47 kDa form is very slow. When the chase period was extended to 8 h, the 45 and 47 kDa species were detected in both membrane and supernatant fractions, with virtually no loss of radiolabel in the total immunoprecipitated protein pool (Figure 4, lanes 2 and 4; chase 8 h). The results again confirm that the proteins are stable but are further processed to soluble forms. After an 8 h chase the parasite population is well into the trophozoite stage, suggesting that the conversion into the soluble proteins may be specific to this stage. These results strongly suggest that the appearance of the soluble forms of the cleft proteins is stage-specific and is not detected earlier than 24–36 h in the asexual cycle.

The high stability of the 45 kDa and 47 kDa proteins suggests that they should accumulate in infected erythrocytes during the asexual cycle. We therefore compared the total levels of these proteins at different stages of parasite growth. As shown in the Western blot in Figure 5, early-ring-stage parasites contained the 45 kDa protein alone (lane 1), whereas both the 45 and the 47 kDa species were present in mid to late ring stages (Figures 5, lane 2). We had previously reported that LWLI recognizes a second distinct parasite protein of 50 kDa protein, present in late rings (Li et al., 1991). It is, however, a much weaker reaction than that observed with the dominant 45 kDa/47 kDa antigens and therefore not detected with the amounts of parasite material used in Figure 5. The detection of the 47 kDa protein in mid to late rings by Western blots confirms our studies on the biosynthesis of the 47 kDa protein at this stage of growth. Given the low turnover of both proteins in infected erythrocytes, it is likely that proteins synthesized in late rings accumulate during schizogony. Yet the amounts of the 45 and 47 kDa proteins at the schizont stages are comparable, possibly a little reduced compared with that in the mid-late rings (Figure 5, lane 3 compared with lane 2). This strongly suggests that the synthesis of the 45 kDa protein is severely depressed or possibly shut off at schizogony, consistent with our inability to detect new synthesis of the 45 kDa protein at the schizont stages (see Figure 2, lane 6). In summary, our studies on the biosynthesis and processing of the 45 kDa protein in infected erythrocytes indicate that the molecule is actively synthesized in ring and trophozoite stages, 0–36 h after invasion. In the mid-late rings the newly synthesized membrane-bound protein is slowly converted into a 47 kDa polypeptide. The soluble forms of the 45 and 47 kDa molecules appear at the trophozoite stage. Thus synthesis, as well as two separate processing steps of the protein, are regulated in a stage-specific manner. They overlap but do not display the identical temporal distribution suggesting that synthesis and each processing step need to be separated in parasite development.

Distribution of the 45 kDa protein in ring- and trophozoite-infected erythrocytes

In order to determine the distribution of the 47 and 45 kDa proteins in late-ring-infected cells, we relied on a method of mechanical homogenization that disrupts infected erythrocytes to release (i) intact malaria parasites and (ii) a membrane fraction enriched in EM and TVM enriched in clefts (Elmendorf et al., 1992; Elmendorf and Haldar, 1994; for reference, see Figure 6). Cell equivalents of purified released parasites and the EM/TVM fraction were probed in Western blots with antibodies against PfERD2 (a marker for Golgi membranes within the

Table 1 Distribution and percentage recovery of the 45/47 kDa proteins and other parasite markers between released parasites and the EM/TVM

For PfERD2, merozoite surface protein 1 (MSP1), the 45 kDa and 47 kDa proteins, cell equivalents of fractions were probed in Western blots with the appropriate antibody and subjected to densitometric analysis. The average of three PfERD2, six MSP1 and three 45 kDa, 47 kDa determinations are shown. The numbers have been rounded off to the closest whole number. PfERD2 and MSP1 were never detected in a soluble fraction. The 47 kDa species was never detected in association with released parasites.

	Percentage of infected erythrocytes			
Fraction	PfERD2 (Golgi)	MSP1 (plasma membrane)	45 kDa	47 kDa
(A) Infected erythocytes	100	100	100	100
(B) Released parasites	90	75	2	–
(C) EM/TVM	5	17	49	51
(D) Erythrocyte cytosol	–	–	36	41
(E) Percentage recovery (B)–(D)	95	92	87	92

parasite) as well as LWLI in the Western Blots. The presence of the proteins in the different fractions was determined by densitometric analysis of Western blots (Towbin et al., 1979). As shown in Table 1, the EM/TVM fraction contained 49% of the 45 kDa protein, and 36% was detected as a soluble protein in an erythrocyte cytosolic fraction generated on homogenization of infected erythrocytes. Approx. 2% of the 45 kDa protein was detected in released parasites. The 47 kDa protein was found in the exported EM/TVM fraction and in the cytosolic fraction, but could not be detected in the released parasite fraction. In contrast, PfERD2 is quantitatively retained in the released parasite fraction. Approx. 5% of PfERD2 was detected in the EM/TVM fraction indicating that these membranes are not significantly contaminated with internal parasite membranes. Similarly 75% of the merozoite surface protein 1 (a marker for the parasite plasma membrane) was detected in the released parasites, with 17% in the EM/TVM fraction. This again confirms a good separation between the EM/TVM fraction and the released parasite membranes (including the parasite plasma membrane). The presence of the 45 and the 47 kDa proteins in the cytosolic fraction is consistent with their biosynthetic processing to soluble forms, discussed above. These results strongly support the suggestion that by the late-ring/trophozoite-infected stages all forms of both the 45 and 47 kDa proteins reside quantitatively beyond the parasite plasma membrane. Hence the formation of the soluble species is not likely to result from endocytosis of the exported membrane-bound forms and subsequent digestion in a compartment within the parasite.

Biosynthesis and export of the 45 kDa protein in late-ring/trophozoite-stage parasites released from EM

The results of the preceding section indicate that, at steady state, the 45 kDa protein (and its products) accumulate beyond the parasite. Hence the newly synthesized protein made within the parasite must be quantitatively exported. To study this process of export we investigated the synthesis, transport and processing of the 45 kDa protein in parasites released from their EMs. The released parasites were incubated with [^{35}S]Tran label in working buffer B (see the Experimental section), and allowed to synthesize proteins for 60 min as previously described (Elmendorf et al., 1992). The cells were separated from the incubation medium by centrifugation at 2000 *g*. When the cell-associated proteins were

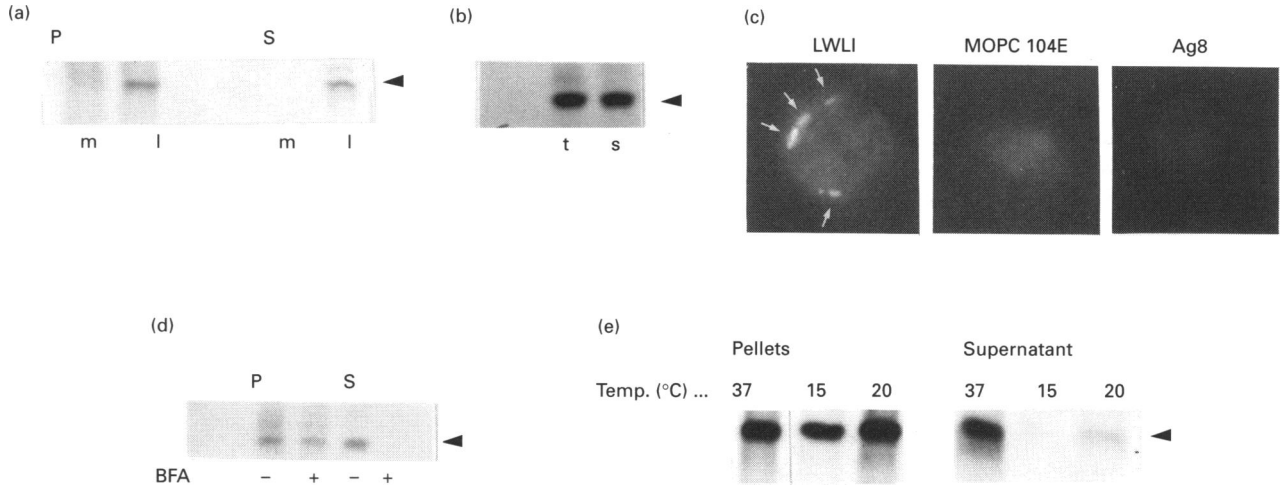

Figure 7 Biosynthesis and secretory export of the 45 kDa protein from released rings and trophozoites

(**a**) Released parasites were incubated with [^{35}S]Tran label for 60 min at 37 °C, and the cells were collected by centrifugation at 2000 *g* (P) and subjected to immunoprecipitation by LWLI (l) or MOPC (m). The supernatant from the low-speed spin was subjected to further centrifugation at 100000 *g*. The 45 kDa protein was detected in the supernatant fraction (S) of this high-speed spin. The pellet of the high-speed spin contained no detectable signal (not shown). The arrowhead indicates the 45 kDa protein. The faint upper band in (l) in the S fraction is not reliably detected in the immunoprecipitates. (**b**) Released parasites metabolically labelled as in (**a**) were extracted in detergent and subjected to immunoprecipitation (t). Alternatively, an equal amount of the intact radiolabelled cells was incubated with LWLI. Excess antibody was removed by washing the cells three times in buffer A. The cells were subsequently extracted in detergent and subjected to immunoprecipitation by adding goat anti-mouse second antibody-conjugated Sepharose (s). The arrowhead indicates the 45 kDa protein. (**c**) Released parasites were incubated with LWLI, or the control antibodies, MOPC 104E, Ag8 (parent hybridoma cell line). Excess antibody was removed by washing cells in buffer A. The cells were subsequently incubated with FITC-labelled goat anti-mouse antibodies. The unbound antibodies were removed by washing and cells were viewed by fluorescence microscopy under filter settings for fluorescein. (**d**) Released parasites were incubated with 5 μg/ml brefeldin A, or 'mock' treated, then subsequently labelled and analysed as in (**a**). The arrowhead indicates the 45 kDa protein. (**e**) Released parasites wee incubated with radiolabel at the indicated temperatures for 60 min and then processed as described in (**a**). The arrowhead indicates the 45 kDa protein.

solubilized in detergent and incubated with the relevant antibodies, a newly synthesized 45 kDa protein was immunoprecipitated by LWLI but not by the control antibody MOPC 104E (Figure 7a). A soluble form of 45 kDa protein was also specifically detected in the extracellular medium, as expected from its detection in trophozoites in pulse–chase studies. Neither the cell-associated nor supernatant forms of the 47 kDa protein were detected. (The faint upper band immunoprecipitated by LWLI in the supernatant in Figure 7(a) is not reproducibly detected in the immunoprecipitates.) This is consistent with the slow appearance of the 47 kDa protein over 4 h and our inability to detect it after 60 min in infected erythrocytes.

To determine how much of the newly synthesized cell-associated protein was at the surface of the released parasites, intact radiolabelled parasites were incubated with LWLI. The excess unbound antibody was removed by washing, the cells were subsequently solubilized and extracts were incubated with the second antibody (Figure 7b, lane 5). In a parallel incubation, the radiolabelled cells were first solubilized with detergent, and the total protein in these extracts was solubilized with LWLI (Figure 7b, lane t). Comparing these two lanes, the newly synthesized protein associated with the parasites was quantitatively detectable on the surface. Indirect immunofluorescence assays shown in Figure 7(c) indicate areas of LWLI binding on the surface of live intact free parasites. Neither MOPC, the control IgM, nor ascites from Ag8 produced a specific fluorescence pattern in cells. It remains unclear whether the punctate pattern (indicated by arrows) is due to antibody-induced clustering of the 45 kDa protein or reflects specific concentration of the protein in membrane domains subsequent to protein export to the parasite surface. All of the cell-associated 45 kDa protein is tightly associated with membrane and could not be dissociated by treatment with Na_2CO_3 and/or urea (not shown). These results indicate that, within 60 min, detectable levels of the 45 kDa protein are synthesized and efficiently exported to the surface of released parasites. This export occurs in a simple well-defined buffer system, ostensibly lacking a defined extracellular signal or erythrocyte cytosol, and therefore appears to be constitutive in nature. A portion of the exported protein is processed to its soluble form, as seen in trophozoite-infected erythrocytes.

Our pulse–chase studies in infected erythrocytes indicate that the soluble 45 kDa protein is a processed form of its membrane-bound precursor. Detection of the protein in the supernatant of the released-parasite incubation mixture and in the erythrocyte cytosolic fraction suggests that it is formed at the surface of the parasite. Therefore it may be used as a convenient marker to assay for the export of the 45 kDa protein from the parasites. We have shown that the drug brefeldin A, which reorganizes the parasite ERD2 Golgi compartment back to the ER, inhibits protein export from malarial parasites. As shown in Figure 7(d), brefeldin A inhibits the appearance of the 45 kDa protein in the supernatant. In contrast, substantial synthesis of the protein is detected in the cell pellet in the presence and absence of the drug. This strongly supports the idea that the biosynthetic export of the 45 kDa protein occurs by a classical pathway of secretion, via the ER–Golgi complex of the parasite. Two additional blocks in secretory transport have been detected at 15 and 20 °C in *cis*- and *trans*-Golgi networks respectively in mammalian cells. As shown in Figure 7(e), the appearance of the soluble form of the 45 kDa protein in the supernatant is inhibited when the parasites are incubated at 15 and 20 °C, although the block at 20 °C is sometimes partial. In contrast, substantial levels of protein are seen in association with the cell pellets at these temperatures, consistent with specific temperature blocks in protein export through a classical secretory pathway.

Post translational modification of the 45 kDa protein by phosphorylation

Our results indicate that the 45 kDa protein is processed to a 47 kDa molecule in infected erythrocytes. However, this processing is not detected in early rings and begins only at the mid-late ring stage. As the mid to late ring stages were also the maximal times of protein synthesis, we investigated post-translational modifications of the 45 kDa protein at these stages of growth. In order to determine whether the 45 kDa protein was phosphorylated, infected erythrocytes were incubated with [^{32}P]-P_i for 2 h, subjected to immunoprecipitation by LWLI or MOPC and analysed by SDS/PAGE and fluorography. As shown in Figure 8, lanes 1 and 2, a radiolabelled band of 45 kDa was specifically detected in the mid-late ring stages. Incorporation of phosphate into the protein was not detected at schizogony. However, if mid to late rings are pulse-labelled with [^{32}P]P_i and allowed to mature for 20 h into schizogony, the label remains stably associated with the protein (not shown). This suggests that a fraction of the protein is stably phosphorylated and does not undergo multiple rounds of dephosphorylation and rephosphorylation. The phosphorylated protein migrates a little above the 45 kDa marker (Figure 8) with the 47 kDa molecule (not shown), consistent with the appearance of the higher-molecular-mass form in mid to late rings. Phosphoamino acid analysis (Figure 9)

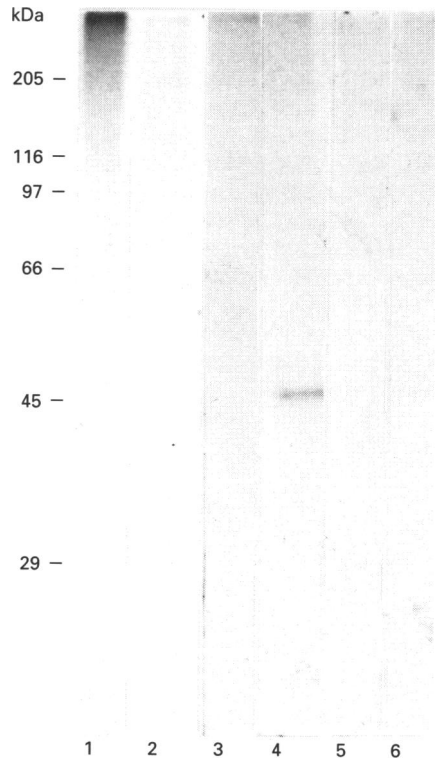

Figure 8 Phosphorylation of the 45 kDa cleft protein occurs in mid-late-ring-infected erythrocytes

Infected erythrocytes at the indicated stages were incubated with [^{32}P]P_i as described and subjected to immunoprecipitation with MOPC (odd lanes) or LWLI (even lanes). Lanes 1 and 2, early rings (0–12 h); lanes 3 and 4, mid to late rings (12–24 h); lanes 5 and 6, schizonts > 40 h. Molecular-mass markers were as in Figure 2.

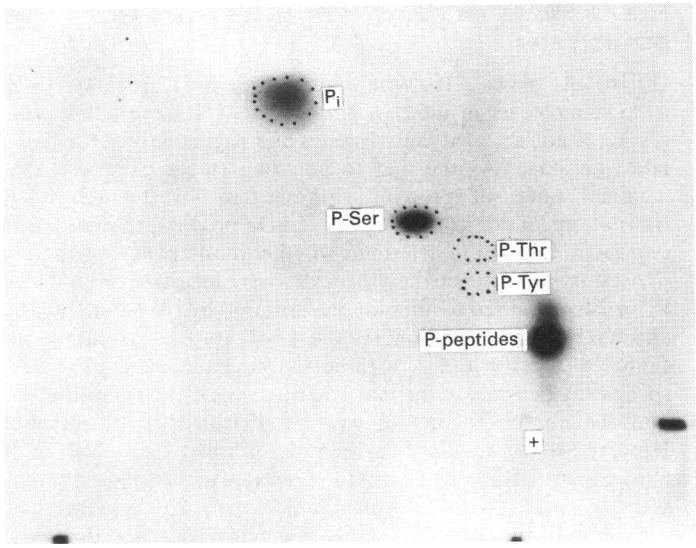

Figure 9 Phosphoamino acid analysis

The phosphorylated protein band was subjected to partial hydrolysis and separated on cellulose t.l.c. plates as described in the Experimental section. The expected migrations of phosphorylated tyrosine, serine and threonine (P-Tyr, P-Ser and P-Thr) are as indicated.

indicates that phosphorylation occurs at a serine residue, suggesting that it is catalysed by a serine/threonine kinase.

As judged by the lack of incorporation of radiolabelled glucosamine and sulphate (not shown), the 45 kDa cleft protein did not contain major post-translational modifications of the secretory pathway such as N-linked glycans, GPI anchors, *O*-glycans and tyrosine sulphation. As the 45 kDa protein is first exported and subsequently processed to the 47 kDa species, it is reasonable to presume that phosphorylation occurs beyond the parasite plasma membrane and not within the plasmodial secretory pathway. Consistent with this hypothesis, we were unable to detect phosphorylation of the newly synthesized 45 kDa protein in late rings released from their EMs incubated with [^{32}P]-P_i for 2 h (not shown). Regulation of the relevant serine/threonine kinase in the phosphorylation of the 45 kDa protein is not understood.

DISCUSSION

In a *Plasmodium*-infected erythrocyte the parasitophorous vacuolar membrane separates the parasite from the cytoplasm of the erythrocyte. The parasite exports proteins past its plasma and vacuolar membranes to clefts in the erythrocyte. In this report we have demonstrated the biosynthesis, transport and processing of a 45 kDa marker protein of the cytoplasmic membrane clefts. We developed and used a monoclonal antibody LWLI to detect the newly synthesized and exported forms of this protein in infected erythrocytes. Our previous studies indicated that the protein was detected in ring-, trophozoite- and schizont-infected erythrocytes. In this study we find that new synthesis of the protein is detected only in the ring and trophozoite stages. The precise onset of synthesis between 0 and 6 h rings cannot be defined because of the limitations in culturing *Plasmodium*. However, 6 h rings clearly make the protein, indicating that synthesis occurs early in ring development. Trophozoites at 24–36 h also synthesize the protein, while schizonts of 40 h and later do not. The shut-off time of protein synthesis in schizonts is not precisely known, but is expected to lie somewhere between 30 and 40 h of development, i.e. at the onset of schizogony. Pulse–chase studies indicate that the 45 kDa protein is a very stable molecule. Hence, although it is not synthesized at schizogony, protein made in rings and trophozoites persists at the later stages of schizont development.

Like the 45 kDa cleft protein, several additional parasite proteins have been shown to be exported past the parasite plasma membrane (to either the vacuolar surface or the erythrocyte). The histidine-rich protein I (HRPI) is a parasite protein which is detected in 'knobs' on the cytoplasmic face of the EM (Taylor et al., 1987). HRPII (Howard et al., 1986; Gormley et al., 1992) and glycophorin-binding protein 130 (Perkins, 1988) are parasite proteins delivered to the erythrocyte cytoplasm, and EM protein 2 (EM2) is detected in association with the EM (Howard et al., 1987). The Exp1 protein has been reported in the vacuolar membrane and membrane loops of erythrocyte cytoplasm (Simmons et al., 1987). As shown by immunolocalization studies and Western-blot analysis, all of these proteins are synthesized and exported by ring- and trophozoite-stage parasites. Biosynthetic studies have only been carried out with the 45 kDa cleft protein (as reported in the present paper) and HRPI (Vernot Hernandez and Heidrich, 1984). In both cases the proteins are synthesized and exported early in the life cycle and then accumulate (in the absence of new protein synthesis) in the erythrocyte at schizogony. In contrast, proteins of the parasite plasma membrane and the vacuolar space are synthesized and exported only at schizogony [reviewed by Haldar and Holder (1993)], suggesting a dichotomy between the regulation of synthesis and export of proteins required to modify the erythrocyte and tubovesicular structures and those required for the growth of the parasite plasma membrane and the parasitophorous vacuole.

The kinetics of export of the 45 kDa protein (which are of the order of minutes) are much more rapid than those reported for the formation of the 47 kDa protein, suggesting that the 45 kDa protein is first exported and then processed to its 47 kDa form (Figure 10a). The precise kinetics of export have not been measured because limitations of the experimental system preclude such rapid transport measurements for the 45 kDa protein. We also find that the 45 kDa protein undergoes phosphorylation to a 47 kDa protein subsequent to its export from the parasite. Phosphorylation occurs in a stage-specific manner suggesting a mechanism of activating/modifying the function of the exported protein at a desired point in the cell cycle. Several parasite proteins exported to the erythrocyte are phosphorylated, but their stage-specific regulation has not been investigated (Coppel et al., 1988; Wiser and Lanners, 1992). Both the 45 and 47 kDa proteins are converted into soluble forms at the trophozoite stage. The apparent molecular sizes of these soluble proteins are indistinguishable from those of their membrane-bound precursors, suggesting that solubilization is due to cleavage of an unidentified lipid or a short polypeptide transmembrane anchor from each precursor protein. The conversion into soluble forms also suggests additional stage-specific mechanisms for regulating membrane functions of the cleft protein subsequent to its export into the erythrocyte (summarized in Figure 10a). There is no evidence that the 45 kDa protein or its processed 47 kDa protein are recycled back to the parasite at least up to the trophozoite stage.

We have previously shown that released parasites can display elements of both classical and alternative pathways of protein export (Elmendorf et al., 1992). We now show that the export of the membrane-bound 45 kDa protein is blocked by the drug, brefeldin A, which reorganizes the PfERD2 compartment back

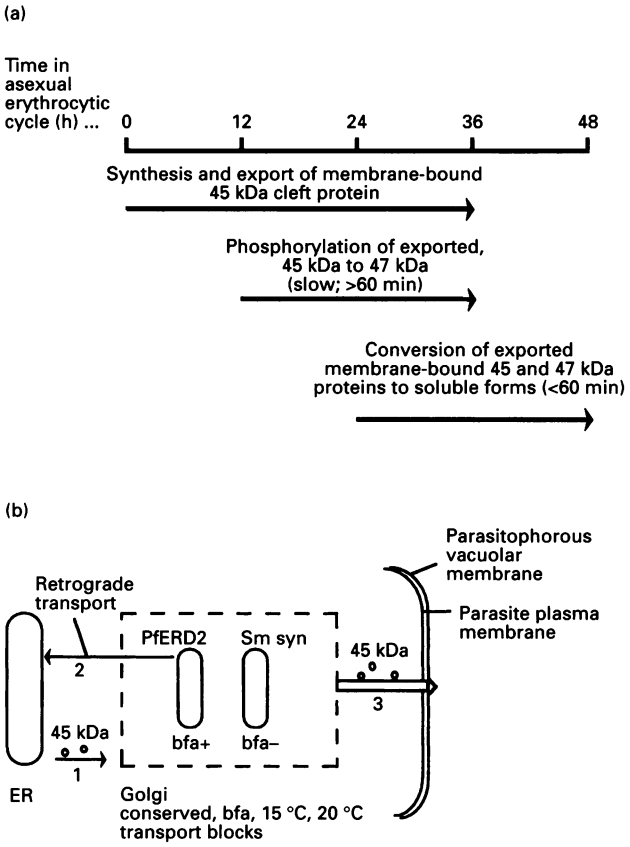

Figure 10 (a) Summary of biosynthesis, export and processing of the 45 kDa cleft protein during the asexual intraerythrocytic cycle and (b) secretory export of the 45 kDa protein in rings and trophozoites of *P. falciparum*

In (**b**), steps 1 and 3 are probable steps of vesicular transport, although this remains unstudied. Step 2 is expected to be retrograde transport from the Golgi to the ER. Sm syn, sphingomyelin synthase; bfa+, reorganized to ER by brefeldin A; bfa−, not reorganized by brefeldin A; open circles, vesicles; arrows indicate directions of transport.

to the ER. Hence the 45 kDa protein is likely to pass through this Golgi compartment. We also detect specific blocks in the export of the 45 kDa protein at 15 and 20 °C. These temperature blocks have been respectively ascribed to the intermediate compartment/*cis*-Golgi network and the *trans*-Golgi/*trans*-Golgi network in mammalian cells [reviewed in Elmendorf and Haldar (1993a)]. Their presence in *Plasmodium* indicates that, although the parasite has a simple secretory pathway (Crary and Haldar, 1992), it contains conserved steps of membrane transport in the Golgi of ring- and trophozoite-stage parasites (as shown in Figure 10b). The 45 kDa protein is exported via these conserved steps of transport through the Golgi and eventually targeted beyond the parasite plasma membrane to the clefts. In contrast, secretion in higher vertebrae cells delivers membrane up to the plasma membrane alone.

The Golgi is the major site for sorting of proteins and lipids in the secretory pathway. Our present studies indicate that distinct blocks in membrane-protein transport through the Golgi are conserved in *Plasmodium*. However, our studies on the organization of the Golgi complex of the parasite indicate that ERD2 and sphingomyelin synthase, localized in the same compartment (the *cis*-Golgi) in mammalian cells, are separated in *Plasmodium*. Furthermore, the perinuclear Golgi compartment containing sphingomyelin synthase in the parasite is insensitive to brefeldin A, indicating that its dynamics are distinct from that in mammalian cells (Elmendorf and Haldar, 1993b). A fraction of the sphingomyelin synthase is exported past the parasite plasma membrane to a network of TVMs (Elmendorf and Haldar, 1994), of which the clefts are a part (Haldar et al., 1991). As indicated above, the 45 kDa protein is expected to cross the ERD2 site. It is possible that subsequent transport to the perinuclear sphingomyelin synthase compartment regulates export beyond the parasite plasma membrane to the vacuolar–tubovesicular membranes. Further experimentation is required to define the molecular determinants underlying the formation and targeting of Golgi-derived transport vesicles to and beyond the parasite plasma membrane.

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