Trafficking of Malarial Proteins to the Host Cell Cytoplasm and Erythrocyte Surface Membrane Involves Multiple Pathways

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Abstract. During the asexual stage of malaria infection, the intracellular parasite exports membranes into the erythrocyte cytoplasm and lipids and proteins to the host cell membrane, essentially "transforming" the erythrocyte. To investigate lipid and protein trafficking pathways within *Plasmodium falciparum*-infected erythrocytes, synchronous cultures are temporally analyzed by confocal fluorescence imaging microscopy for the production, location and morphology of exported membranes (vesicles) and parasite proteins. Highly mobile vesicles are observed as early as 4 h postinvasion in the erythrocyte cytoplasm of infected erythrocytes incubated in vitro with C_6-NBD-labeled phospholipids. These vesicles are most prevalent in the trophozoite stage. An immunofluorescence technique is developed to simultaneously determine the morphology and distribution of the fluorescent membranes and a number of parasite proteins within a single parasitized erythrocyte. Parasite proteins are visualized with FITC- or Texas red-labeled monoclonal antibodies. Double-label immunofluorescence reveals that of the five parasite antigens examined, only one was predominantly associated with membranes in the erythrocyte cytoplasm. Two other parasite antigens localized only in part to these vesicles, with the majority of the exported antigens present in lipid-free aggregates in the host cell cytoplasm. Another parasite antigen transported into the erythrocyte cytoplasm is localized exclusively in lipid-free aggregates. A parasite plasma membrane (PPM) and/or parasitophorous vacuolar membrane (PVM) antigen which is not exported always colocalizes with fluorescent lipids in the PPM/PVM. Visualization of two parasite proteins simultaneously using FITC- and Texas red-labeled 2° antibodies reveals that some parasite proteins are constitutively transported in the same vesicles, whereas other are segregated before export. Of the four exported antigens, only one appears to cross the barriers of the PPM and PVM through membrane-mediated events, whereas the others are exported across the PPM/PVM to the host cell cytoplasm and surface membrane through lipid (vesicle)-independent pathways.

THE invasion of erythrocytes by *Plasmodium falciparum* parasites elicits major changes in the structure, composition, antigenicity, and function of the host cell membrane. These modifications produce significant changes in the biological properties of the host erythrocyte. One change of patho-physiological importance is that parasitized erythrocytes (infected red blood cells, IRBC)[1] become adherent to receptors on vascular endothelium. As a result, IRBC sequester within postcapillary venules, causing vessel occlusion and the pathologic condition of cerebral malaria (2, 3, 9). It is likely that this affliction is mediated by the presence of malarial proteins in the erythrocyte membrane (4, 24, 32). During infection, the parasite actively imports obligate nutrients from the serum and exports lipids, proteins and membranes to the erythrocyte cytoplasm and the erythrocyte membrane (10, 24, 26, 43). Previous investigations have shown that the phospholipid, fatty acid, and cholesterol contents of the erythrocyte membrane and its structure are extensively modified during asexual infection (26, 35, 36, 47, 51). Earlier experiments from this laboratory have suggested that host cell lipid modifications are probably caused by the action of parasite phospholipases and acyl transferases and/or the transfer of lipids from the parasite to the erythrocyte membrane (26).

Parasite synthesized proteins are found in various intracellular compartments throughout the IRBC. These include parasite subcellular organelles, the parasite plasma membrane (PPM), the parasitophorous vacuolar membrane

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1. *Abbreviations used in this paper*: C_6-NBD-PC, 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine; C_6NBD-PE, 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylethanolamine; CFIM, confocal fluorescence imaging microscopy; IRBC, infected red blood cell; PLP, periodate-lysine-paraformaldehyde; PPM, parasite plasma membrane; PVM, parasitophorous vacuolar membrane; RBCM, red blood cell membrane; RT, room temperature.

(PVM), membranous and nonmembrane bound aggregates in the host cytoplasm, and the erythrocyte membrane (reference 1 and reviewed in 7, 10, 23, 24, 29). At least three different types of membrane structures have been found within the erythrocyte cytoplasm of IRBC that are not detected in uninfected cells. These structures have been described as long and slender unit membrane "clefts" (Maurer's clefts), circular unit membranes with electron dense contents, and vesicles with multiple membranous whorls. Electron dense material (probably protein-rich aggregates) has been found associated with these membranes, or clumped in nonmembrane bound aggregates (1, 7, 10, 23, 24, 46). All of these structures detected within the host cytoplasm may be active in the trafficking of proteins and membranes between the parasite and the host plasma membrane. It has been proposed that trafficking systems similar to those characterized for other eukaryotic cells may be used to shuttle proteins and membranes between the parasite and the host cell plasma membrane (15, 21, 22, 44, 45). In the host-parasite system, however, the trafficking of proteins and membranes would appear more complicated. Newly synthesized proteins must traverse the parasite Golgi complex, cross the PPM, PVM and the host cytoplasm in order to reach the erythrocyte membrane. The mechanisms and potential signals involved in assisting parasite proteins to traverse these membranes and direct them to the host plasma membrane are unknown. The regulation of these transport pathways must be under parasite control because mature erythrocytes have limited lipid synthetic or metabolic capabilities, lack cytoplasmic membranes or organelles, and do not have the machinery to synthesize and process proteins.

A number of investigations have described the subcellular location of malarial proteins within IRBC. In each case, specific MAbs that react with the antigen of interest by immunoprecipitation and/or Western blotting analysis have been used for immunoelectron microscopy studies to reveal aspects of subcellular protein compartmentation and trafficking. Some biochemical and immunological characteristics of the various parasite antigens we have investigated are summarized in Table I and described below.

The very large (M_r ~300 kD) malaria protein designated PfEMP2 (22) (also called MESA, [13]) has been localized to parasite membranes, electron-dense spheres budding off the PVM, to unit membranes within the erythrocyte cytosol (not Maurer's clefts), and to the cytoplasmic face of the erythrocyte membrane under electron dense protrusions that have been coined "knobs" (13, 22, 25). PfHRP2 is a soluble, histidine-rich protein that is exported from the asexual malaria parasite to the erythrocyte cytoplasm and released into the culture supernatant. PfHRP2 in the host erythrocyte cytoplasm is distributed in packets that do not appear to be membrane-bound. These packets of PfHRP2 are found as well underlying the erythrocyte membrane. The PfHRP2 sequence indicates a potential NH_2-terminal signal peptide region typical of secretory proteins and no hydrophobic transmembrane domains (10, 21, 24, 52). The distribution of PfHRP1, also called the knob-associated histidine-rich protein (28), in IRBC has been previously investigated. It is not surface exposed but associated with knobs at the erythrocyte membrane. PfHRP1 has been localized to electron dense spheres and large membrane whorls in the erythrocyte cytoplasm, to the parasite plasma membrane, the PVM and under the surface membrane at knobs (31, 41, 46). An antigenically cross-reactive group of malarial proteins has been identified with human MAb's 33G2 (49, 50) and 41E11 (20). MAbs 33G2 and 41E11 have been shown to react on fixed immunofluorescence assays with the malaria parasite, with undefined structures in the host erythrocyte cytoplasm, and the IRBC surface membrane (20, 49, 50). Not all malarial antigens exported from the parasite move to the host erythrocyte cytoplasm or IRBC outer membrane (12). For example, antigens defined by the IgG murine MAb 677-1 are located at the parasite PPM and PVM (D. Taylor, personal communication), but not external to the PVM.

In this investigation, we begin to elucidate specific trafficking pathways for different malarial proteins in malaria-infected erythrocytes. We describe the kinetics of production, cellular distribution and morphological appearance of membranes and proteins exported from the intracellular parasite to the erythrocyte cytoplasm and IRBC surface membrane. Protocols were devised to simultaneously observe fluorescent-labeled membranes and proteins by CFIM, and their production and distribution was analyzed as a function of parasite developmental stage. Our results offer evidence for (*a*) direct association of exported parasite proteins with membranous structures in the erythrocyte cytoplasm; (*b*) transport of parasite proteins through the erythrocyte cytoplasm by vesicle-independent pathways; (*c*) constitutive export of parasite proteins and (*d*) segregation of parasite proteins before export to the erythrocyte membrane.

Table I. Characteristics of Parasite Antigens

Antigen	Mol Wt (kD)	MAb used to visualize antigen	Antibody class and origin	Exported to host cytoplasm and RBC plasma membrane	Reference
PfHRP1	90	89/E8	IgG_{2a}, mouse	yes	46
PfHRP2	72	1D6, 2G12	IgG, mouse	yes	40
PfEMP2	~300	4H9.1	IgM, mouse	yes	22
Antigens reactive with MAb 41E11	>1,000, 320, 175, 160, and 138	41E11	IgM, rat	yes	20
PPM/PVM Antigens	43	677-1	IgG_1, mouse	no	D. W. Taylor, unpublished results

Materials and Methods

Cell Culture

Clone 5 from the FCR-3 Gambian strain of *P. falciparum* was grown as described previously (16,48). Briefly, IRBC were thawed from liquid N_2 using the three-step Fenwall solution protocol (12% NaCl, pH 6.0; 1.6 NaCl, pH 5.5; 0.9% NaCl + 0.2% dextrose, pH 5.0). Infected blood was maintained at 5% hematocrit in petri dishes, changing the media daily using RPMI 1640 culture media containing 24 mM $NaHCO_3$, 25 mM Hepes, 21.1 mM dextrose, 2 mM glutathione, 0.44 mM hypoxanthine and 66 mg/L gentamycin. Culture media was supplemented with 10% human serum (heat inactivated). The knob-positive (K^+) phenotype was maintained in vitro by treating IRBC weekly with RPMI medium containing 1–2% gelatin (38). Cultures were synchronized by selecting for ring stage parasites using 5% sorbitol treatments (30).

Stage Analysis

To observe vesicle and protein production as a function of parasite development, highly synchronous cultures were maintained. Cultures were subjected to several days of multiple 5% sorbitol treatments to continuously maintain newly infected ring stage parasites within several hours of the initial invasion (30). At the late trophozoite or early schizont stage, knobby IRBC were enriched with 1% gelatin. A 5% sorbitol treatment was performed on IRBC the following day within 3–4 h of the initial invasion to retain only the early ring stage. At the late schizont stage, cells were then floated at 25% hematocrit on a 65% percoll gradient, centrifuged at 3,000 rpm and the enriched band of IRBC collected, washed and returned to culture overnight. Giemsa smears of the reinvading parasites were taken until released merozoites and newly formed rings were detected. A 5% sorbitol treatment was performed on the cells immediately and the surviving rings collected and put back in culture. IRBC treated by these methods were synchronous to within 3–4 h.

Fluorescent Lipid Labeling of IRBC

Synchronous IRBC were labeled with the fluorescent lipid 1-acyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl] phosphatidylethanolamine, C6-NBD-PE; or with 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-amino]caproyl] phosphatidylcholine, C6-NBD-PC (Avanti Polar Lipids, Inc., Birmingham, AL). 5×10^7 cells were incubated with 1.33–2.66 μM C6-NBD-PE or PC (diluted in PBS) for 60 min at 37°C. The labeled cell suspension was centrifuged at 1,500 rpm, the supernatant removed, and the cells resuspended in 1 ml of PBS. The cell suspension was placed in an open Dvorak-Stotler culture chamber (Nicholson Precision Instruments Inc., Gaithersburg, MD) and viewed by laser scanning confocal microscopy.

Confocal Fluorescence Imaging Microscopy

Confocal microscopy was performed with a Bio-Rad MRC-600 system (Bio-Rad Laboratories, Cambridge, MA) interfaced to an Olympus IMT-2 inverted microscope using an air cooled argon laser for excitation. Under the conditions of most experiments, x-y resolution was ~0.2 μm and x-z resolution was ~0.8 μm.

Immunofluorescence

Samples of synchronous cultures were taken at different time points during the 48 h life cycle and processed for indirect immunofluorescence by two different methods. Using the first method, smears of IRBC were made on glass slides, allowed to dry, fixed for 2 min with 4° or −20°C acetone and dried again. Slides were washed with PBS and nonspecific antibody binding blocked with 1% BSA or 5% human serum in PBS for 30 min. Primary antibody dilutions (1:100 or 1:200) made in the same blocking solution were added for 60 min at room temperature (RT), washed, and the secondary antibody (FITC or Texas red-labeled goat anti-mouse IgG or IgM [Fisher Biotech, Inc., Philadelphia, PA]) was incubated with the cells for 30 min at RT. After the final wash, coverslips were mounted on the cells using 90% glycerol in PBS. The primary MAb's against parasite proteins utilized in these experiments are described in Table I.

The second method used a similar labeling protocol, but with different fixation. Coverslips were boiled in 0.1 N HCl for 10 min, washed with H_2O and ethanol and allowed to dry. The coverslips were coated with a thin (1.5 μL) film of the bioadhesive Cell Tak (Collaborative Research, Bedford, MA) using a bent pipette, dried and washed with ethanol for 5 min and then 2 times with deionized H_2O for 3 min each. Dried Cell Tak coverslips were stored for several days at 4°C. 5×10^7 cells were suspended in 1 ml of PBS, 30 μL was added to Cell Tak coated coverslips, and the cells were allowed to settle and adhere for 30–60 min at 37°C. Excess PBS was blotted from the coverslips and the IRBC were fixed overnight (12–24 h) in freshly made periodate-lysine-paraformaldehyde (PLP). PLP fixative consists of 2% paraformaldehyde in 37 mM sodium phosphate buffer (equimolar concentrations of Na_2HPO_4 + NaH_2PO_4, pH 7.4), 10 mM sodium m-periodate, and 74.5 mM DL-Lysine-HCL (Sigma Chemical Co., St. Louis, MO) (37). This hypo-osmotic buffer helps permeabilize the erythrocyte membrane, allowing antibody penetration, without perturbing membrane morphology. The combination of periodate and lysine is necessary to create cross-linked aldehyde groups, which are then stabilized by the paraformaldehyde (37). After fixation, cells were quenched three times with 0.5 mg/ml $NaBH_4$ and washed three times in PBS. IRBC stained for PfHRP1 could only be fixed for 1–3 h using the PLP fixative. Nonspecific binding was inhibited by diluting antibodies in PBS containing 5% human serum. All antibody dilutions were added directly on top of the adherent cells, taking care never to allow the cells to dry during the labeling procedure. Fixed cells on Cell Tak coverslips were washed between incubations by dipping three times in PBS for 20 s each. Finally, coverslips were inverted and mounted onto slides using 90% glycerol.

Colocalization of Parasite Proteins or Proteins and Membranes within Infected Erythrocytes

Double Labeling of Parasite Proteins. Using synchronous cultures and PLP fixation, parasite proteins were detected simultaneously by labeling with two different fluorophores and using the dual channel detection mode of the confocal microscope system to collect the green and red emissions simultaneously. By observing labeling patterns, the production and location of different parasite proteins was determined as a function of stage, and compared directly within the same cell. The green signal arising from proteins labeled with FITC-2° antibodies was collected first, stored and then irreversibly destroyed by continuous exposure to high intensity laser light (for simplicity, we define this as photobleaching). The signal from the Texas red-labeled proteins was then collected in the red channel and stored. In this way, potential misinterpretation of the data due to bleeding of FITC fluorescence into the red channel was eliminated. The Bio-Rad MRC 600 software allows the stored green and red signals to be pseudo-colored and merged pixel by pixel so that the protein labeling patterns can be simultaneously displayed for each infected cell.

Parasite Proteins and Fluorescent Membranes. Similar double-label imaging was performed on synchronous cultures as described above, but instead proteins and membranes were detected simultaneously. PLP fixation on Cell Tak coverslips was found to be imperative for this experiment in order to retain membrane morphology, preserve antigenicity and allow antibody penetration. Cells were allowed to adhere to coverslips, fixed and labeled with 1° and 2° antibodies and extensively washed to remove serum. The cells were then incubated with 2.66 μM C_6-NBD-PE or PC diluted in PBS for 2 h, washed in PBS and mounted on slides using 90% glycerol. C_6-NBD-PE or PC-labeled phospholipids were imaged first, stored on the computer, and the NBD-fluorescence irreversibly destroyed with continuous laser scanning. The Texas red signal from the labeled proteins was collected in the red channel and the image stored. Images of the same cell(s) were computer merged and the protein and lipid fluorescent patterns compared.

Results

CFIM Analysis of IRBC Labeling with C_6-NBD-PC

IRBC were labeled in vitro with the fluorescent lipid C_6-NBD-PC for 30–60 min at 37°C, washed, resuspended in PBS and viewed by CFIM. C_6-NBD-PC was internalized into living IRBC, labeling the host plasma membrane, intracellular parasite, and mobile vesicles within the erythrocyte cytoplasm (Fig. 1). This labeling pattern was obtained for infected cells incubated with a wide range of concentrations of NBD-PC (1.33–5.0 μM). Grellier et al. (17) observed similar labeling patterns in erythrocytes infected with *P. falciparum* labeled at 37°C. We have previously shown that labeling of the parasite and membranes in the erythrocyte cytoplasm oc-

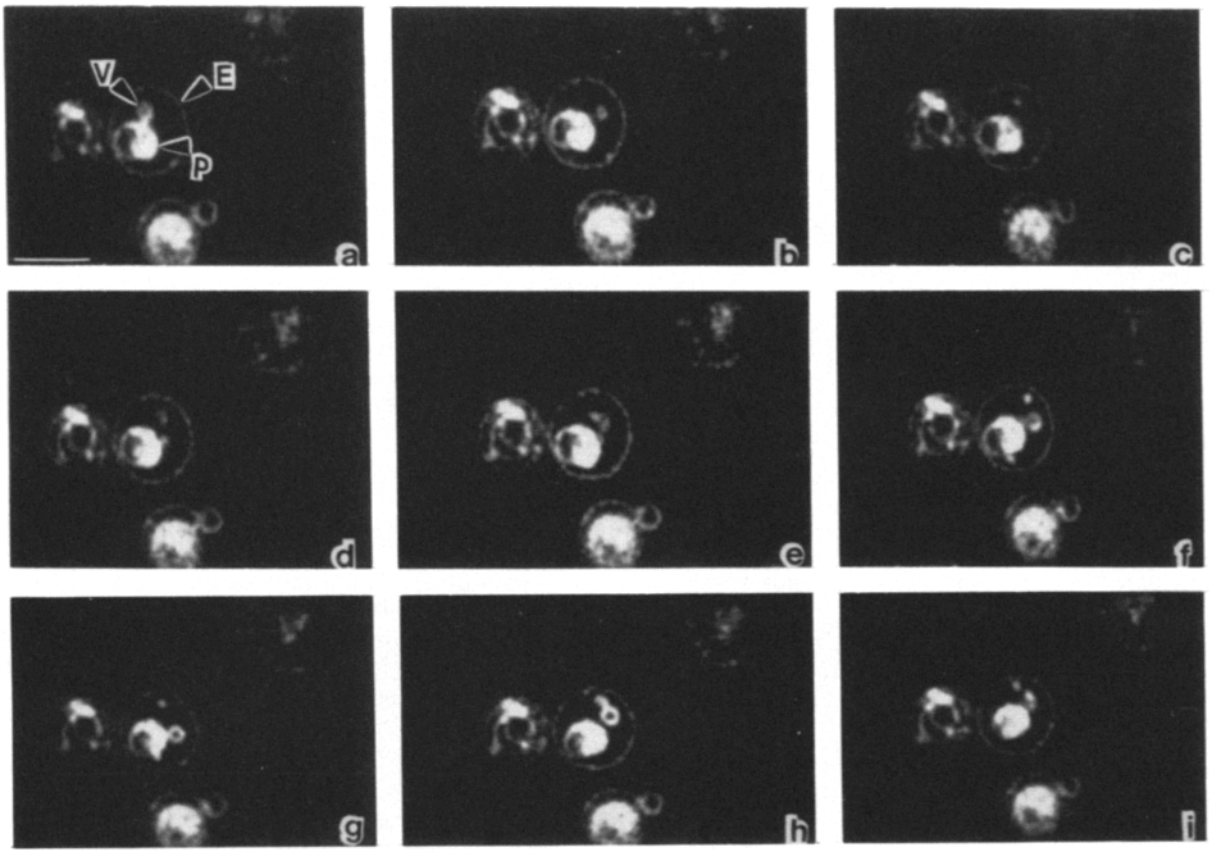

Figure 1. Mobile vesicles in the host cytoplasm of *Plasmodium falciparum* infected erythrocytes. Trophozoite-stage IRBC were suspended in PBS, incubated in the presence of 1.33 μM C_6-NBD-PC at 37°C for 1 h and washed to remove unincorporated label. Live cells were then placed in an open Dvorak-Stotler chamber and viewed by CFIM, using an excitation wavelength of 488 nm. Sequential images (*a–i*) of the same cell in the same focal plane were recorded every 0.75 s. Under conditions of the experiment the x-y resolution was ~0.2 μm and the x-z resolution ~0.8 μm. (*a*) The parasite (*P*), erythrocyte membrane (*E*) and cytoplasmic vesicles (*V*) labeled with NBD-PC are marked in the cell of interest. The image series depicts vesicles that appear to break off the PVM and move throughout the erythrocyte cytosol. Note that the parasite does not change position within the IRBC. Scale bar = 7 μm.

curs through nonendocytic pathways and does not depend on the class of NBD-labeled phospholipid (39). In contrast to an earlier study by Haldar et al. (18), we find that internalization of NBD-PC is not energy dependent. It seems that NBD-PC gains access to internal parasite membranes by a combination of (*a*) transbilayer movement across the erythrocyte membrane followed by monomer diffusion and (*b*) passive diffusion through the parasitophorous duct (39). Based on these results, it is reasonable to assume that the NBD-lipids label all membranes within the IRBC.

Fig. 1 depicts a temporal series of images collected every 0.75 s showing mobile NBD lipid-labeled vesicles of ~0.5 μm. These structures appear to move by Brownian motion within the host cytoplasm. Uninfected cells from the same cultures, or noncultured erythrocytes, did not contain any internal structures when labeled under identical conditions. Labeling with C_6-NBD-PE gave similar results, with highly mobile, fluorescent structures detected in the host cytoplasm. It has been previously documented by immunoelectron microscopy, that parasite proteins are often located in membranous structures within the host cytoplasm of *P. falciparum* infected erythrocytes (1, 15, 22, 24, 44, 45, 46). We propose that the dynamic structures detected by C_6-NBD-PC and PE may be responsible for trafficking of parasite proteins to the host cell membrane. The vesicles that we observe are quite large (0.2–0.8 μM) compared to Golgi-derived vesicles in eukaryotic cells (~0.05 μm). Vesicles of those dimensions are well below the size resolution (~0.2 μm) of the light microscope.

Temporal Analysis of the Distribution of C_6-NBD-PC and C_6-NBD-PE in IRBC

The erythrocytic life cycle of the malaria parasite *P. falciparum* lasts for ~46–48 h (Fig. 2). After merozoite entry into the erythrocyte, the parasite develops a vacuole and becomes a ring form, shaped like a signet ring, with the nucleus as the seal. This stage lasts ~18 h. The ring grows by increasing its cytoplasm and feeding on the erythrocyte cytoplasm by ingesting hemoglobin, which is digested in food vacuoles. The presence of electron-dense, black pigment in the vacuoles is characteristic of trophozoite-stage parasites. This stage persists for 18–30 h. During this time, the parasite is the most metabolically active (14). During the following 16–18 h, the schizont stage, the nucleus divides 3 to 5 times to form 8–32 nuclei. At this stage, the parasite occupies most

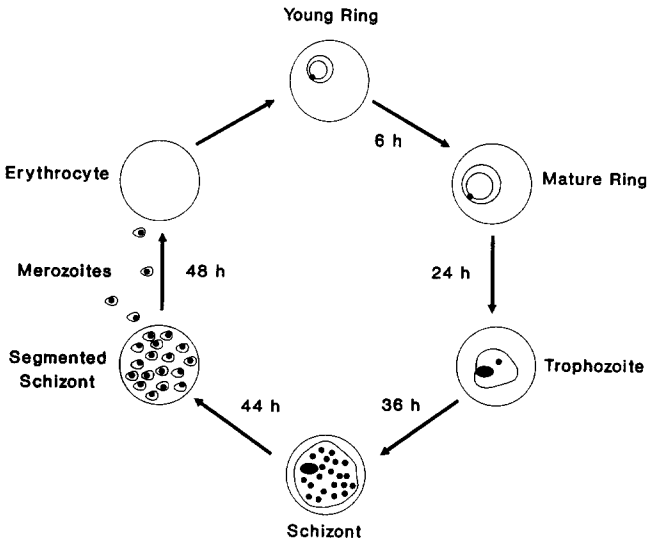

Figure 2. Schematic of the erythrocytic life cycle of *Plasmodium falciparum.* Infection commences with the invasion of erythrocytes by merozoites. The erythrocytic life cycle lasts for ~46–48 h. The small filled circles represent the parasite nucleus; the large filled circles represent the food vacuoles where degraded hemoglobin is stored. A description of the cogent features of each stage is provided in the Results section.

of the erythrocyte and the pigment concentrates in a single mass. During the final 2 h of the life cycle, the merozoites containing a single nucleus bud off from the schizont, leaving behind a pigment-containing residual body. The erythrocyte membrane disintegrates, releasing infectious merozoites into the bloodstream.

In vitro incubation of synchronous IRBC with the fluorescent lipids C_6-NBD-PE or PC revealed the presence of highly mobile vesicular structures (~0.5 μm) in the erythrocyte cytoplasm as early as 4 h postinvasion (Fig. 3 *a*). In addition, the fluorescent lipids heavily labeled the parasite and the erythrocyte membrane.

By the late ring stage, ~50% of IRBC contained cytoplasmic vesicles and/or budding PPM/PVM protrusions (Fig. 3 *b*, Table II). At the trophozoite stage, between 18 and 30 h postinvasion, all IRBC contain various sized membranes in the erythrocyte cytoplasm (0.2–0.8 μm) and/or membranous protrusions extending from the PVM (Fig. 3 *c*, Table II). Some of the protrusions from the PPM/PVM appeared to extend to the erythrocyte membrane. Similar structures have also been observed in IRBC by Grellier et al. (17) and Haldar and Uyetake (19). In the latter study, it was suggested that membrane protrusions from the PVM/PPM might be involved in exporting parasite lipids to intraerythrocytic compartments. However, no evidence for transport of parasite lipids to the erythrocyte surface membrane was presented by

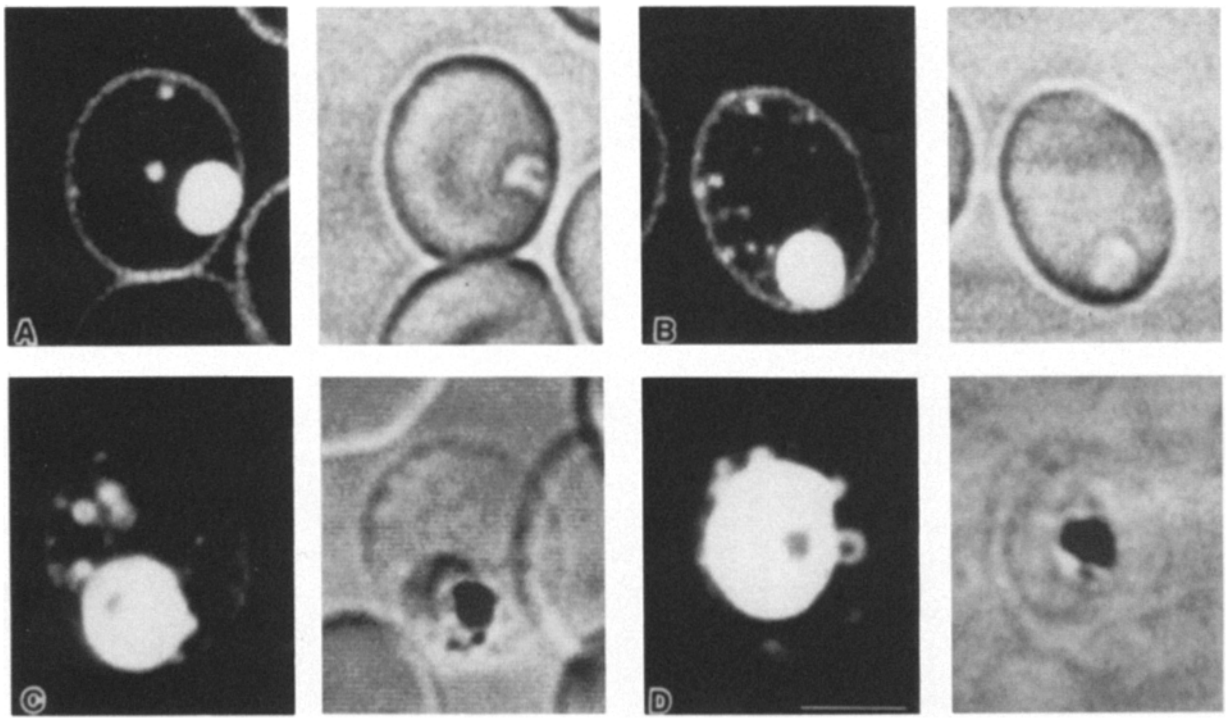

Figure 3. Membrane export as a function of parasite developmental stage. IRBC were suspended in PBS (5×10^7 cells/ml), labeled with 2.6 μM C_6-NBD-PC for 1 h at 37°C, and washed to remove unincorporated label. The labeled, living cells were suspended in PBS and viewed by CFIM. Each pair of images shows the fluorescent labeling and the corresponding transmitted light image. Only infected cells contained internal labeled membranes. (*A*) Early ring stage, 4–8 h post invasion; (*B*) late ring stage, 12–18 h post invasion; (*C*) mid-trophozoite stage, 20–30 h post invasion; (*D*) late schizont stage, 36–40 h post invasion. The images were collected and processed for maximum resolution and contrast of the vesicles in the erythrocyte cytoplasm. Therefore, the fluorescence in the various compartments is not quantitative. Scale bar = 3.4 μm.

Table II. Fluorescent Membrane Labeling of IRBC

Time (h)*	% IRBC with labeled parasite protrusions and cytoplasmic membranes‡
4–8	50.0 ± 4
12–14	52.3 ± 3
24–26	90.3 ± 6
40–46	36.3 ± 5

* Times are hours post-invasion; ‡Percentages (± SD) are the result of four separate experiments, with 100 IRBC counted for each time point.

Haldar and Uyetake (19). Uninfected erythrocytes did not contain any intracellular membranes, as would be expected for mature erythrocytes. As the parasite continued to mature and enlarge within the erythrocyte during the schizont stage, the number of free vesicles in the cytoplasm decreased dramatically, (Fig. 3 *d*, Table II) until virtually no vesicles were detected between 46–48 h. A summary of membrane production as a function of developmental stage and labeling patterns within IRBC is presented in Table III.

Temporal Analysis of Parasite Protein Production and Distribution

Samples from highly synchronous cultures were fixed with the PLP method and stained for parasite proteins using the immunofluorescence techniques outlined in Methods. Control samples were prepared with each set to insure that fluorescent secondary antibodies did not bind nonspecifically to the cells, and that they did not nonspecifically cross-react with the primary antibodies. The results discussed below and depicted in Fig. 4 are from samples fixed by the PLP method. Similar results were obtained for IRBC fixed with acetone. A summary of protein production as a function of developmental stage and the labeling patterns within IRBC is presented in Table III.

Using CFIM analysis to detect the presence of individual malarial protein antigens, parasite protein synthesis was detected as early as 4 h postinvasion. At the early ring stage (4–8 h), PfEMP2 was confined to the parasite (Fig. 4 *a*), whereas PfHRP2 was detected both at the parasite and spread diffusely across the host cytoplasm in structures smaller than 0.5 μm (Fig. 4 *d*). These results are in good agreement with an earlier study describing PfHRP2, wherein this protein was localized by cryothin-section immunoelectron microscopy to the parasite cytoplasm and "packets" in the erythrocyte cytoplasm (21). By contrast, MAb 41E11 labeled only the parasite and erythrocyte membranes (Fig. 4 *b*).

The antigens associated with the PPM/PVM recognized by MAb 677-1, and PfHRP1 were not detectable until the late ring stage (12–18 h postinvasion). PfHRP1 was in a diffuse pattern throughout the host cytoplasm and at the host plasma membrane (Fig. 4 *c*), while the PPM/PVM protein(s) were only detected around the parasite (Fig. 4 *e*). During this time, several parasite proteins were detected at locations beyond the parasite. PfHRP1 and PfHRP2 were seen in the

Table III. Protein and Membrane Labeling as a Function of Parasite Developmental Stage

Parasite protein or membrane	Time (h)	Internal parasite structures	Parasite PPM/PVM	Diffuse label across RBC cytoplasm	Aggregates in erythrocyte cytoplasm	RBCM
C6-NBD labeled membranes	4–8	++	++	−	+	++
	12–18	++	++	−	++	++
	20–30	++	++	−	+++	++
	36–40	+++	+++	−	−	+++
PfEMP2 (MAb 4H9.1 or 8B7.4)	4–8	+	−	−	−	−
	12–18	+	−	+	+	+
	20–30	++	−	+	+++	++
	36–40	++	−·	−	+	+++
PfHRP1 (MAb 89/E8)	4–8	−	−	−	−	−
	12–18	+	−	+	−	+
	20–30	+	−	++	−	++
	36–40	+	−	+	−	+++
PfHRP2 (MAb 1D6 and 2G12)	4–8	+	−	+	−	+
	12–18	+	−	++	±	+
	20–30	+	−	+++	±	++
	36–40	+	−	+	−	+++
PPM/PVM protein (MAb 677-1)	4–8	−	+	−	−	−
	12–18	−	++	−	−	−
	20–30	−	+++	−	−	−
	36–40	−	+++	−	−	−
Antigens reactive with MAb 41E11	4–8	++	+	−	−	++
	12–18	+	++	+	−	++
	20–30	+	+	−	+++	±
	36–40	+	−	−	±	+

Grading system for chart: (−) feature not detected; (±) <5% of IRBC contain labeled feature; (+) >70% of IRBC contain dimly labeled feature; (++) >70% of IRBC contain brightly labeled feature; (+++) 90% of IRBC contain brightly labeled feature.

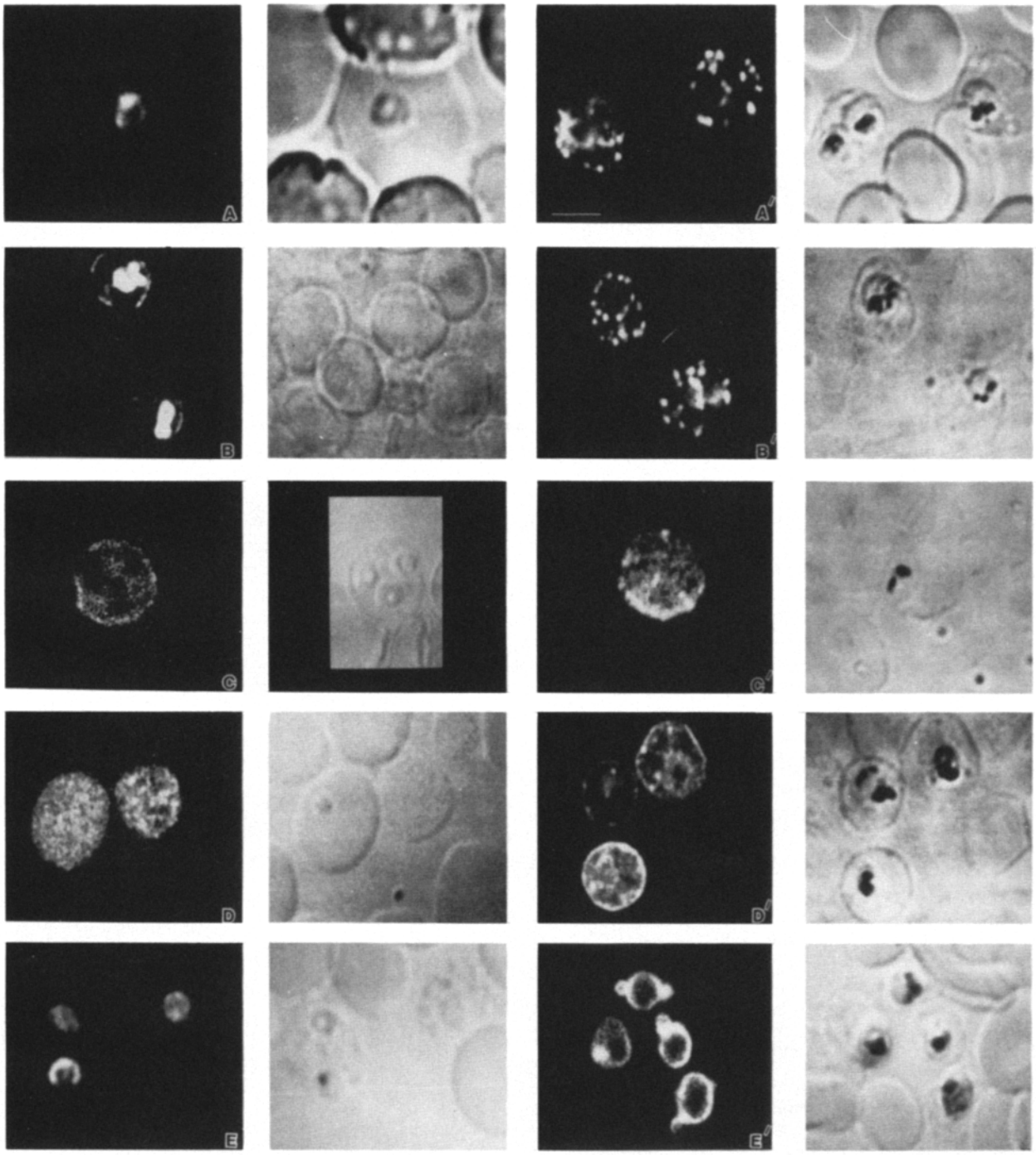

Figure 4. Production of parasite proteins as a function of developmental stage. Cultures were synchronized, fixed with the PLP method and stained with MAbs to parasite proteins. Images *A–E* show fluorescently labeled IRBC in the early to late (4–18 h post-invasion) ring stage, with the corresponding transmitted light image. The second set of paired images (*A′–E′*) depict IRBC in the midtrophozoite stage, between 20–30 h post invasion, with the corresponding transmitted image. (*A* and *A′*) MAb 4H9.1; (*B* and *B′*) MAb 41E11; (*C* and *C′*) MAb 89/E8; (*D* and *D′*) MAb 1D6; (*E* and *E′*) MAb 677-1. The fluorescence intensity is not quantitative because it was computer enhanced for maximal brightness. Scale bar = 4.5 μm.

parasite and in the host cytoplasm, often as a diffuse labeling pattern, with the cytoplasm also occasionally containing PfHRP2 in larger (≤0.5 μm) aggregates (data not shown). MAb 41E11 labeled the parasite and erythrocyte membrane, with an increased amount of diffuse fluorescence in the host cytoplasm, while PfEMP2 was detected at the parasite and in small aggregates (0.2–0.5 μm) within the host cytoplasm (data not shown). In Fig. 3 it can be seen that slightly larger (and more abundant) C_6-NBD phospholipid labeled vesicles were also detected at the late ring stage.

At the midtrophozoite stage (20–30 h post invasion), ~0.5 μm, punctate structures within the host cytoplasm were la-

beled with MAb 4H9.1 (Fig. 4 *a'*) and MAb 41E11 (Fig. 4 *b'*). In an earlier study, PfEMP2 was shown by cryothin-section immunoelectron microscopy to be associated with the PVM, occasionally in unit membrane whorls up to 1 μm in diameter, but for the most part in 0.2–0.6 μm electron dense spheres in the host cell cytoplasm that occasionally abutted the erythrocyte membrane (22). PfHRP1 (Fig. 4 *c'*) and PfHRP2 (Fig. 4 *d'*) were detected in smaller (0.2–0.5 μm) stipled-structures that gave an overall diffuse pattern across the cytoplasm. Some larger aggregates containing PfHRP2 in the host cytoplasm were also occasionally observed.

Referring to Fig. 3 and Table II, this is the same time period that the highest frequency of C_6-NBD phospholipid-labeled membranous vesicles and PVM protrusions were detected. The similarities in labeling patterns within the IRBC for both membranes and some of the parasite proteins suggest that vesicles in the erythrocyte cytoplasm are involved in trafficking between the parasite and the erythrocyte membrane. The PPM/PVM antigen is not exported from the parasite during this period, although it appears to be associated with structures that protrude from the PPM/PVM 20 h postinvasion (Fig. 4 *e'*).

As the parasite enlarges within the erythrocyte during the schizont stage (between 36–40 h postinvasion), the number of fluorescent membrane and protein aggregates detected in the erythrocyte cytoplasm decreased significantly (data not shown). By contrast, labeling of the erythrocyte membrane was more abundant for PfEMP2, PfHRP1, PfHRP2 and the antigens reactive with MAb 41E11, which were often in large (~0.5–1.0 μm) patchy areas. The PPM/PVM associated protein was still restricted to the parasite, with little or no membrane extensions labeled. Similarly, C_6-NBD-PE or PC labeled vesicles were detected less frequently at this stage, as the parasite begins to completely occupy the host cytoplasm (data not shown). The accumulation of a large amount of labeled parasite proteins at the erythrocyte membrane and the loss of vesicle-type structures in the host cytoplasm may indicate a decrease in the trafficking of membranes and proteins from the parasite to the host plasma membrane at this stage.

Colocalization of Membranous Vesicles and Parasite Proteins

To determine if the vesicular structures labeled with C_6-NBD-PE or PC colocalized with proteins exported from the parasite, a method was developed to simultaneously observe membranes and proteins within the same IRBC. By adhering cells to Cell-Tak coated coverslips and fixing with PLP, the erythrocyte membrane was sufficiently permeabilized to allow antibody penetration and the membranes could be stained with C_6-NBD-PE or PC. Cells stained with both fluorescent lipids and antibodies were visualized by CFIM.

Experiments were performed to insure that the membrane labeling pattern detected with PLP-fixed cells was similar to that obtained with live cells, i.e., to check that fixation did not alter membrane morphology in IRBC. Cells adhered to Cell-Tak coated coverslips were incubated with C_6-NBD-PC for 2 h, washed in PBS, the cover slip mounted in a Dvorak chamber, and the cells viewed by CFIM. Cells on the same coverslip were then fixed with PLP overnight, gently washed so as not to dislodge any cells, and the same field of cells in the same focal plane reexamined. We observed that the cells shifted position slightly overnight and after x-y realignment (which often resulted in unacceptably low levels of NBD fluorescence after the repeated exposures of laser light), the vesicles were not at the same positions as seen in the living cells (data not shown). This was not unexpected, since it was shown in Fig. 1 that the NBD-lipid labeled vesicles in the host cell cytoplasm were extremely dynamic. In addition, PLP is a light fixative and consequently requires some time for full effectiveness. As a result of these difficulties an alternative approach was developed.

We examined the distribution of C_6-NBD-PC in hundreds of living IRBC, counting the number of erythrocyte cytoplasmic vesicles/cell, estimating their size and measuring the fluorescence in the erythrocyte membrane, in each erythrocyte cytoplasmic vesicle and in the parasite compartment (data not shown). Average values were calculated for each compartment. Similar measurements were made for IRBC that were labeled as live cells with C_6-NBD-PC and then fixed overnight with PLP as described above. The cells flattened slightly as a result of PLP fixation by ~0.3 to 0.5 μm (~8–10% of the thickness of live cells). A small reduction in erythrocyte membrane fluorescence (5–10%) was noticed in PLP-fixed IRBC. In contrast, no significant decrease in parasite or vesicle fluorescence was detected as a consequence of fixation. The average number of C_6-NBD-PC-labeled vesicles/IRBC was the same (3) in live and fixed

Table IV. Colocalization Between Parasite Proteins and Membranes Labeled with NBD-PC or NBD-PE

Protein	Time (h)	Colocalizes* with parasite ppm/pvm	Colocalizes with membranes in host cytoplasm	Colocalizes at erythrocyte membrane
PfEMP2	4–8	+	−	−
	12–18	+	−	−
	20–30	++	±	+
	36–40	++	±	++
PfHRP1	4–8	−	−	−
	12–18	+	−	+
	20–30	±	−	++
	36–40	±	−	++
PfHRP2	4–8	+	−	+
	12–18	+	±	+
	20–30	±	±	++
	36–40	±	−	++
PVM/PPM Protein	4–8	+	−	−
	12–18	++	−	−
	20–30	++	−	−
	36–40	++	−	−
MAb 41E11 reactive antigens	4–8	++	−	++
	12–18	++	+	+
	20–30	+	++	+
	36–40	±	±	++

* Colocalization is defined as the fluorescence arising from the NBD-phospholipids and Texas red-labeled 2° antibodies being localized to exactly the same structures in the same cell. Grading system for chart: (−) protein not colocalized with membranes labeled with NBD-PC or PE; (±) 10% of IRBC contain the same structures labeled with NBD-phospholipid and Texas red antibody; (+) >60% of IRBC contain the same structures labeled with NBD-phospholipid and Texas red antibody; (++) >90% of IRBC contain the same structures labeled with NBD-phospholipid and Texas red antibody.

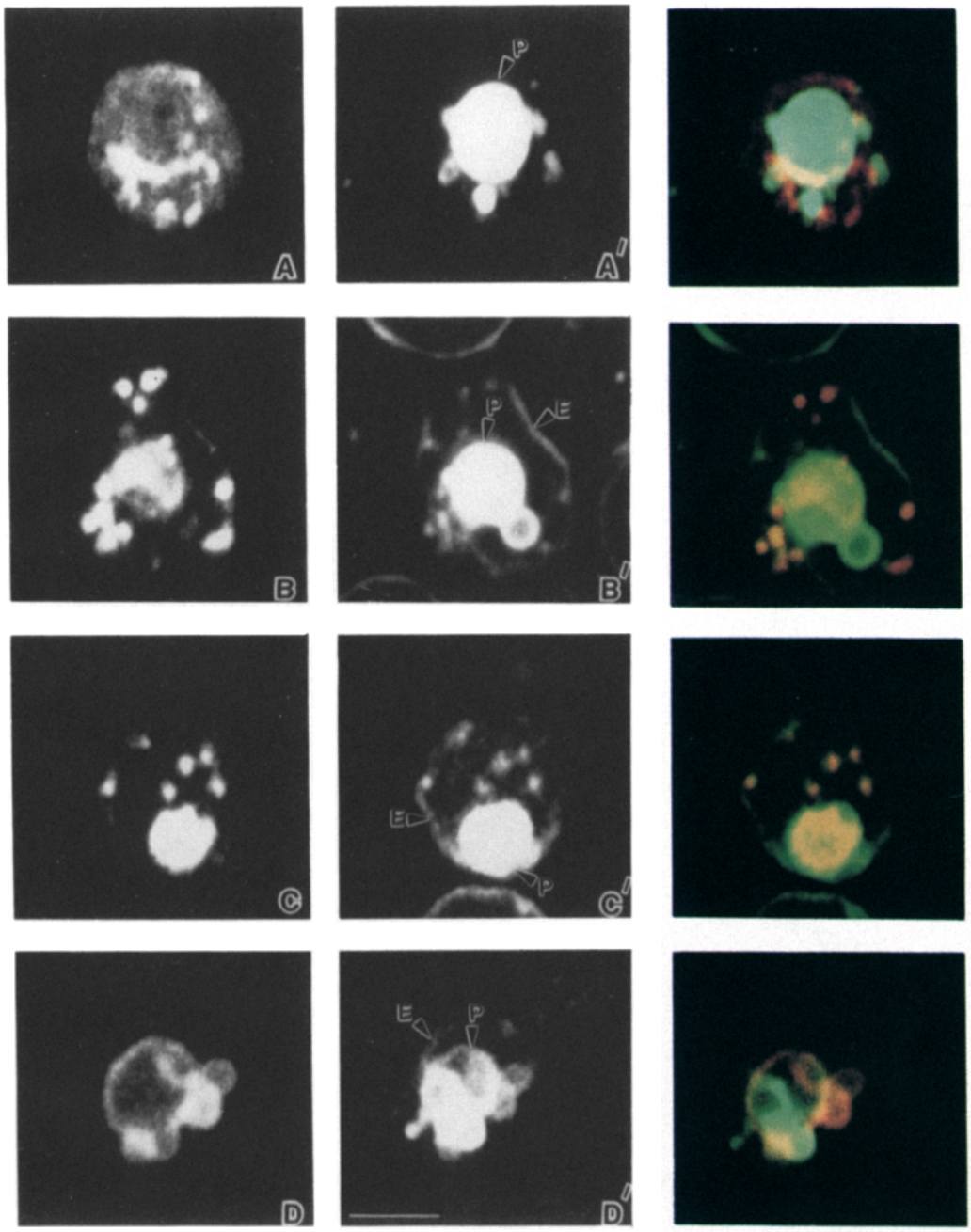

Figure 5. Colocalization of parasite proteins with vesicles in the host cytoplasm. Synchronous trophozoite stage IRBC (20–30 h) were fixed by the PLP method, stained with Texas Red labeled MAbs, then labeled with 2.6 μM C_6-NBD-PC and viewed by CFIM. Images of NBD fluorescence were imaged and stored, the NBD fluorescence irreversibly destroyed by photobleaching and the fluorescence from the Texas red labeled MAb's in the same focal plane of identical cells visualized and stored. (*A*) PfHRP2; (*B*) PfEMP2; (*C*) antigens reactive with MAb 41E11; (*D*) PPM/PVM antigens reactive with MAb 677-1; (*A'–D'*) are the images of the identical cells labeled with NBD-PC. The third column depicts computer merged color images showing both colocalized and nonoverlapping structures: areas containing only NBD-lipid fluorescence are green; Texas red labeled proteins are red and areas where overlap (colocalization) occurs are orange-yellow. (*P*) = parasite, (*E*) = erythrocyte membrane, scale bar = 3.8 μm.

samples as was their size (0.2–0.8 μm). These controls demonstrate that fixation with PLP does not alter the morphology of IRBC (including vesicles/membranes in the cytoplasm) or deplete intracellular fluorescence. Furthermore, when 90% glycerol and a coverslip were added to PLP-fixed, NBD-lipid labeled samples, the fluorescence intensity and labeling patterns were unchanged (data not shown).

To insure that the Texas red-antibody signal for the parasite proteins had minimal signal overlap contribution from C_6-NBD-PE or PC, the images for fluorescent NBD lipid-labeled membranes were collected first. After storage of the image, the NBD phospholipid fluorescence was destroyed by continuous exposure to high intensity laser light. The Texas Red protein signal for the same cells was then collected and

stored. By this method, the position of the cells is not changed and identical focal planes are examined. The C_6-NBD-PC/PE and Texas Red images were then computer merged using MRC-600 software and analyzed for areas of lipid and protein colocalization.

At the early to late ring stage, no vesicle colocalization was detected between parasite proteins and C_6-NBD-PE or PC-labeled cytoplasmic membranes, even those that were detected beyond the parasite such as PfHRP2 and the antigens labeled by MAb 41E11 (refer to Table IV for a temporal summary of protein and NBD-labeled vesicle colocalization). Colocalization between parasite proteins and fluorescent membrane-vesicles was highest at the trophozoite stage, which corresponded with the time of the highest protein synthesis rate (14), and when the highest percentage of IRBC contained C_6-NBD-lipid labeled vesicle structures.

Two of the parasite proteins, PfHRP1 (data not shown) and PfHRP2 (Fig. 5 *a*), generally failed to colocalize with NBD-lipid labeled vesicles or parasite PPM/PVM protrusions. PfHRP1 and PfHRP2 were diffusely distributed throughout the erythrocyte. On occasion, some focal points of protein expression (PfHRP2) in the cytoplasm of the erythrocyte would colocalize with NBD-labeled vesicles; this was mostly seen in the trophozoite stage between 20–30 h postinvasion (data not shown).

Although the parasite protein PfEMP2 was seen abundantly in punctate structures throughout the host cytoplasm, these protein aggregates usually did not colocalize to C_6-NBD-PC/PE labeled membranous vesicles. Occasionally during the trophozoite stage (20–30 h), some protein areas would overlap with NBD-membrane vesicles (see Fig. 5 *b*), but often there was more PfEMP2 protein-aggregate areas than NBD-lipid vesicles. At the schizont stage (36–40 h), few cytoplasmic vesicles were detected due to the large parasite occupying the entire erythrocyte (data not shown). At this late stage, PfEMP2 was also mostly detected in small aggregates that abutted the erythrocyte membrane and contained C_6-NBD-PE or PC. Colocalization was also observed at the PPM/PVM.

Antigens reactive with MAb 41E11 consistently colocalized with NBD-PE or PC-labeled vesicular structures in the erythrocyte cytoplasm, especially in the trophozoite stage (20–30 h post invasion) (Fig. 5 *c*). During the early ring stage (4–8 h), MAb 41E11 labeling was restricted to the parasite and in focal patches around the erythrocyte membrane; NBD-lipids also exhibited this fluorescent pattern (data not shown). It must be noted that some vesicular-structures were also detected in the erythrocyte cytoplasm that did not contain the antigens defined by MAb 41E11.

Throughout the parasite life cycle, the PPM/PVM antigens recognized by MAb 677-1 did not colocalize with NBD-PE or PC cytoplasmic vesicles. Often though, membranous extensions from the parasite (which were never detected as free structures in the cytoplasm) labeled with MAb 677-1 also had an identical NBD-lipid labeling pattern (Fig. 5 *d*). These structures are probably protrusions of the PVM and/or PPM.

The detection of lipids and proteins colocalized to identical vesicular structures within the same cells is strong evidence for membrane-associated protein trafficking between the parasite and the host plasma membrane. In addition to the colocalized regions of proteins and membranes, lipid-free, protein-rich areas and membranes devoid of the five parasite antigens we investigated, were also observed in the erythrocyte cytoplasm. These results suggest a multitude of trafficking pathways within the infected erythrocyte.

Colocalization of Parasite Proteins

The results of the previous experiments indicated that individual parasite proteins may be segregated and trafficked to the erythrocyte plasma membrane collectively within the same vesicular structure, or along separate routes. To further elucidate these pathways, synchronous IRBC fixed via the PLP method were subjected to double-label immunofluorescence at different time points during the intraerythrocytic life cycle. Dual channel CFIM was performed on double-labeled IRBC so that the distribution of two different proteins could be detected simultaneously within the same cell. Images for the proteins labeled with FITC-labeled antibodies were collected first, stored, and the FITC signal was subsequently destroyed by photobleaching. An image of the same focal plane was then collected for the proteins labeled with Texas red antibodies and merged with the FITC image for the same cell (see Table V for a summary of protein colocalization).

In IRBC double-labeled with MAb 4H9.1 and an anti-glycophorin antibody, 10F7MN (M. R. van Schravendijk, personal communication), PfEMP2 was detected submembranously, while 10F7MN labeled the surface of IRBC (data not shown). This is in agreement with previous reports that showed that PfEMP2 moves from the parasite to the erythrocyte membrane, but remains submembranous, mostly localized under knobs (22, 25). PfEMP2 was often detected surrounding the parasite, and sometimes overlapped the PPM/PVM region labeled by MAb 677-1. However, any PfEMP2-containing aggregates in the erythrocyte cytoplasm did not contain antigen(s) recognized by MAb 677-1, which remained confined to the parasite PPM/PVM (Fig. 6 *a*).

The histidine-rich protein, PfHRP2, which is a soluble protein secreted from intact IRBC, does not colocalize well with any of the other proteins investigated here. PfHRP2 is mostly detected as a diffuse labeling pattern throughout the host cytoplasm, with an occasional aggregate colocalizing to a vesicular-dot region identified by MAb 41E11 or MAb 4H9.1 (Fig. 6 *b*). Similar results were found for PfHRP1, in that its diffuse staining pattern across the erythrocyte cyto-

Table V. Colocalization of Parasite Proteins in the Erythrocyte Cytoplasm

Proteins	PfEMP2	PfHRP1	PfHRP2	PPM/PVM Antigen	MAb 41E11 Antigens
PfEMP2	NA	−	±	−	+
PfHRP1	−	NA	±	−	−
PfHRP2	±	±	NA	−	±
PPM/PVM Antigen	−	−	−	NA	−
MAb 41E11 Antigens	+	−	±	−	NA

Grading system for colocalization of proteins within the same structures in the same cell. NA, not applicable; (−) proteins in erythrocyte cytoplasm do not colocalize; (±) <10% of IRBC contain structures in the erythrocyte cytoplasm in which different parasite proteins colocalize; (+) >70% of IRBC contain structures in the erythrocyte cytoplasm in which different parasite proteins colocalize.

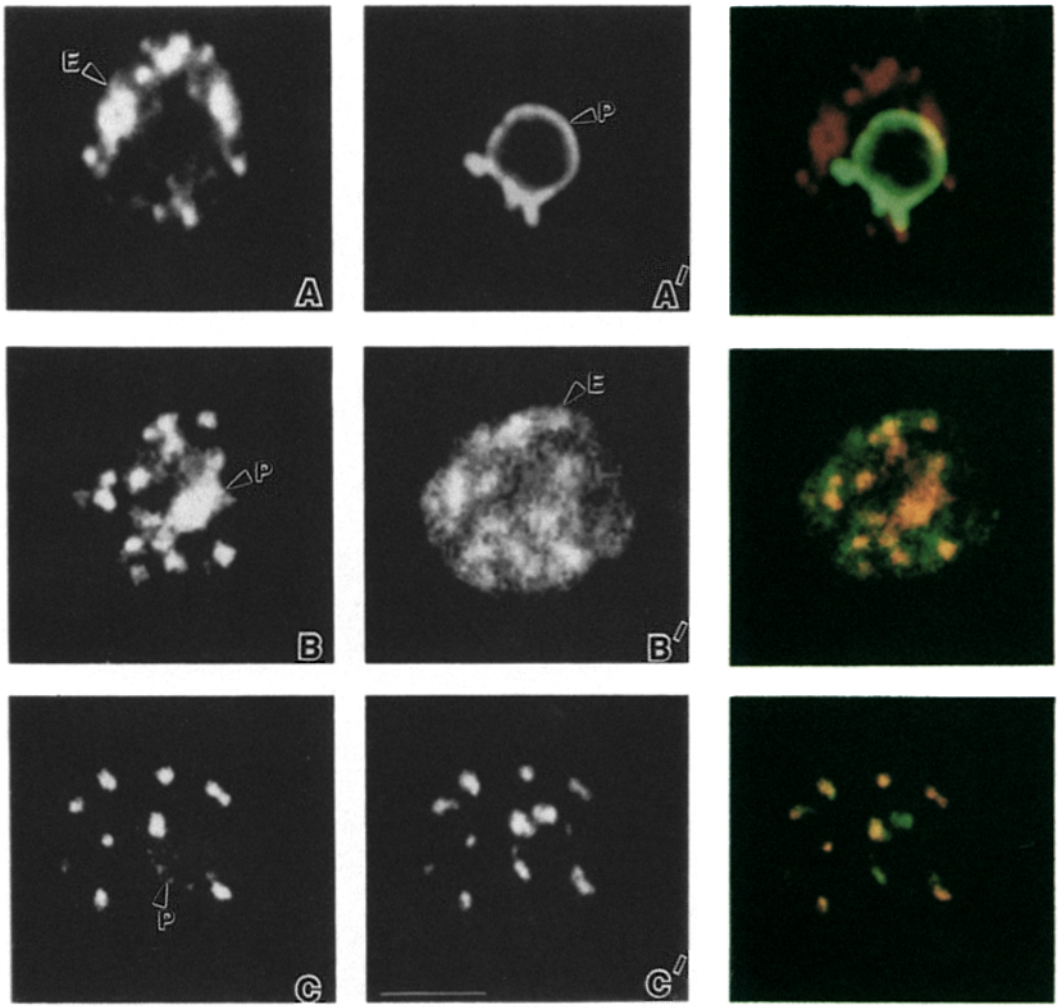

Figure 6. Colocalization of parasite proteins by double-label immunofluorescence. Trophozoite stage IRBC (20–30 h) were fixed by the PLP method and stained with two different MAb's against parasite proteins. The cells were viewed by CFIM, first imaging and storing the image of the FITC labeled antigens, photobleaching the FITC signal by continuous laser scanning, then imaging and storing the Texas red labeled antigens. By computer merging of the images, areas of protein colocalization could be distinguished from areas where only individual antigens were localized. (*A*) anti-PfEMP2 (Texas red), (*A'*) PPM/PVM antigens reactive with MAb 677-1 (FITC); (*B*) anti-PfEMP2 (Texas red), (*B'*) anti-PfHRP2 (FITC); (*C*) anti-PfEMP2 (Texas Red), (*C'*) antigens reactive with MAb 41E11 (FITC). The third column depicts computer merged color images of the corresponding IRBC, showing areas of protein colocalization and nonoverlapping areas. Colocalized area are orange/yellow, whereas nonoverlapping regions are either green (FITC) or red (Texas red). Scale bar = 4.2 μm.

plasm was very different from the cytoplasmic aggregates seen with MAb 4H9.1 and MAb 41E11 (data not shown). The proteins defined by these MAbs did not colocalize with PfHRP1.

The cytoplasmic punctate areas labeled by PfEMP2 colocalized well with the parasite antigens identified by MAb 41E11. Initially at the early ring stage, MAb 41E11 labeled both the parasite and the erythrocyte membranes, while PfEMP2 was confined to the parasite. By the late ring stage, both the antigens labeled by MAb 41E11 and PfEMP2 were detected in cytoplasmic aggregate areas, and the two proteins often colocalized to the same structures (data not shown). By the midtrophozoite stage, protein aggregate labeling significantly overlapped between the two proteins (Fig. 6 *c*). By immunoblot analysis the proteins identified by MAb 41E11 do not cross-react with PfEMP2, demonstrating that the MAb's are detecting separate antigens (20). As the parasite enters the schizont stage, PfEMP2 and the antigens reactive with MAb 41E11 were detected strongly at the erythrocyte membrane (data not shown). Colocalization of these proteins in the same structures in the erythrocyte cytoplasm suggests that they are transported by the same pathways to the host plasma membrane.

Taken together, these results strongly suggest that parasite proteins are segregated at the PPM or PVM before export into the erythrocyte cytoplasm. Transport of the parasite antigens recognized by MAb 41E11 appears to be vesicle mediated. PfEMP2 and PfHPR2 were occasionally observed in vesicles in the erythrocyte cytoplasm. However, for the most part, PfHRP1, PfHPR2, and PfEMP2 are transported through the erythrocyte cytoplasm in lipid-free protein aggregates. Instances were also observed where multiple parasite pro-

teins were transported in the same structures (PfEMP2, antigen(s) recognized by MAb 41E11, PfHRP2) or alone (PfHRP1). Colocalization of proteins to the same cytoplasmic vesicles may indicate a common transport pathway for a particular subset of proteins (PfEMP2 and the antigens labeled by MAB 41E11), while discrete labeling patterns obtained for PfHRP1 and PfHRP2 suggests diverse carrier systems for trafficking of other parasite proteins.

Discussion

It is now well established that a number of parasite proteins are exported to the host cell cytoplasm, host cell membrane and serum during asexual parasite development. There is little information, however, on the pathways by which parasite antigens are exported from the intracellular parasite to these destinations. Using confocal fluorescence microscopy, we observed a population of highly mobile vesicles in the host cell cytoplasm of IRBC labeled with C_6-NBD-PC or C_6-NBD-PE in vitro. A fixation method was developed which allowed us to determine whether these cytoplasmic vesicles were involved in trafficking of parasite proteins to the erythrocyte membrane. Using double-label confocal fluorescence microscopy, we established that the antigens recognized by MAb 41E11 (D260, Ag 332, and Pf155/RESA), and occasionally PfEMP2 and PfHRP2, were found to be associated with membranes in the host cell cytoplasm. By simultaneously visualizing two parasite proteins within the same IRBC using FITC and Texas red labeled 2° antibodies, we determined that PfHRP1, and >90% of PfEMP2 and PfHRP2, were distributed throughout the host cell in lipid-free aggregates. The sizes of these aggregates were variable, with PfHRP1 present in uniformly small structures (<0.2 μm diameter) and PfHRP2 in a mixture of large (~0.5 μm) and small (<0.2 μm) structures. The PPM/PVM antigen(s) recognized by MAb 677-1 were not exported from the parasite throughout the entire life cycle. These antigen(s) were often present in membranous protrusions extending from the PVM, that did not appear to contain the other parasite proteins that were analyzed. These results indicate that protein and membrane transport from the parasite to the host cell membrane is a complex process, involving multiple trafficking pathways and the segregation of chemically diverse proteins into discrete carrier pathways. A schematic summarizing protein and membrane trafficking pathways in IRBC is shown in Fig. 7.

The proteins analyzed in these experiments were chosen because they had been previously characterized immunochemically and their distribution within IRBC at some stages of parasite development delineated by immunofluorescence and cryothin-section electron microscopy using specific monoclonal antibodies. The diverse labeling patterns observed for different antigens demonstrates that individual proteins leave the parasite at specific times by distinct routes during the malaria life cycle, with segregation of protein subgroups into different trafficking pathways occurring at the level of the parasite and/or PVM. For instance, PfHRP2 is detected within the host cytoplasm immediately after invasion, whereas PfEMP2 and the PPM/PVM protein(s) are confined to the parasite at the early ring stage. As the infection develops to the late ring/early trophozoite stage,

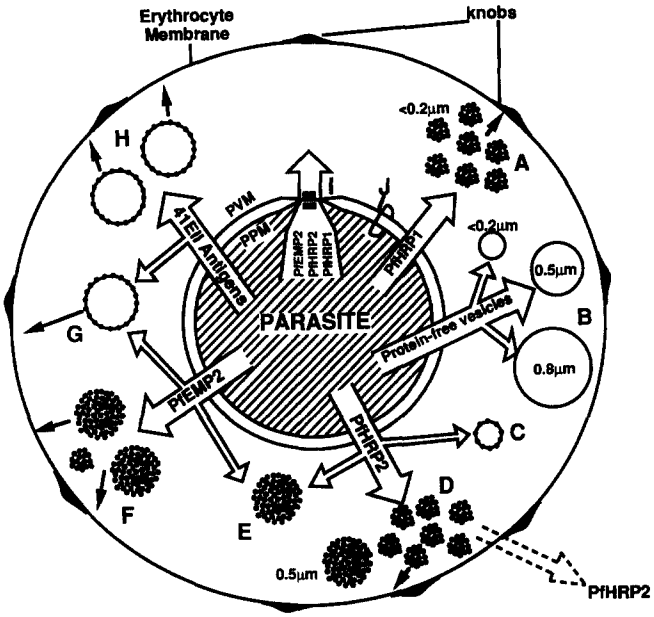

Figure 7. Schematic of membrane and protein trafficking pathways in IRBC. Malaria proteins are exported from the parasite to the erythrocyte cytoplasm and surface membrane through different pathways. (*A*) PfHRP1 traverses the PPM/PVM and is transported in lipid-free packets (<0.2-μm diam) across the host cytosol, eventually reaching knobs; (*B*) C6-NBD-PC/PE labeled membranes (*vesicles*) are exported to the erythrocyte cytoplasm which do not contain PfHRP1, PfHRP2, PfEMP2 or antigens recognized by MAb 677-1 and MAb 41E11; (*C*) and (*D*) PfHRP2 is transported across the PPM/PVM and into the erythrocyte cytosol mostly in 0.2–0.5-μm lipid-free packets, and occasionally in NBD-lipid labeled membranes (0.2 μm). Some PfHRP2 is transported across the erythrocyte membrane and into the culture medium (*dashed arrow*); (*E*) On occasion, PfHRP2 and PfEMP2 colocalize to the same 0.5-μm packets; (*F*) PfEMP2 is transported into the host cytosol, mostly in lipid-free packets of varying size (0.2–0.5 μm); (*G*) PfEMP2 and MAb 41E11 antigens are transported from the parasite in the same vesicles; (*H*) transport of antigens recognized by MAb 41E11 into the host cytosol mostly in ~0.5 μm, NBD-lipid labeled vesicles; (*I*) PfEMP2, PfHRP1, and PfHRP2 may cross the PPM and PVM via putative "contact sites" (areas where the PPM and PVM are closely apposed) with protein chaperones (■) assisting protein transport across these membranes and through the host cytosol; (*J*) PPM/PVM antigens recognized by MAb 677-1 are not exported into host cytosol.

PfEMP2 and the antigens labeled by MAb 41E11 are detected as discrete punctate areas within the cytoplasm, indicative of their release from the parasite. This pattern of punctate labeling becomes more widespread during the trophozoite stage. PfHRP2 and PfHRP1 are secreted from the parasite in a different pathway, wherein the proteins are arranged in small particles giving rise to a more diffuse labeling pattern. Eventually, the host cell membrane becomes enriched at the end of the schizont stage with all but the PPM/PVM antigen(s), with a corresponding decrease in protein-associated areas and membrane-vesicles in the erythrocyte cytoplasm, and a lack of colocalization between them. This may signify the end of the majority of transport between the parasite and host membrane.

Double-labeling experiments showed little colocalization

of PfHRP1 and PfHRP2 with the other proteins, which also indicates segregation of distinct proteins into separate trafficking routes external to the malaria parasite. On the other hand, colocalization of proteins such as PfEMP2 and the antigens recognized by MAb 41E11 indicates transport of other proteins beyond the PPM. Not all of the parasite proteins investigated here are trafficked through the host cytoplasm; the protein(s) identified by MAb 677-1 is confined to the PPM/PVM region. This antigen must either contain a sequence which causes it to be retained in the PPM/PVM or lack a sequence necessary for export into the erythrocyte cytoplasm.

It is an important question to address whether the lipid and protein labeling patterns we visualized in IRBC fixed with the PLP method are representative of the living cell. From our previous investigations with living IRBC, we have shown that the NBD-labeled phospholipids label the host cell membrane, membranes in the erythrocyte cytosol and parasite membranes through nonendocytic processes (39). We did not detect any differences in the labeling patterns when comparing IRBC labeled in vitro with C_6-NBD-PC or PE to those obtained with IRBC fixed with PLP and then labeled. All membranes in the IRBC appear to be equally accessible to labeling by NBD-phospholipids. In addition, the results we obtained by CFIM for PfEMP2, PfHRP2 and PfHRP1 using PLP-fixed IRBC were very similar to those obtained previously using cryothin-section immunoelectron microscopy and the same monoclonal antibodies (21, 22, 46).

One common, yet perplexing, feature that arose from our analysis of protein distribution in IRBC is the seemingly enormous amount of parasite proteins in the erythrocyte cytoplasm. The majority of PfHRP1 and the soluble, secreted protein PfHRP2 within IRBC appears to be localized in the host cytoplasm or associated with the erythrocyte membrane. This export from the parasite is not transient because pulse radiolabeling studies with ^{3}H-histidine indicate new synthesis of both PfHRP1 and PfHRP2 throughout the entire trophozoite stage and early schizogony (R. Howard, unpublished results). Why are these proteins exported in such high quantities? It seems unlikely that such export over many hours is required to produce the morphologic changes, such as knob formation, accompanying asexual parasite development. We now know that the intracellular parasite, but not the host erythrocyte membrane, is capable of fluid-phase endocytosis (Pouvelle, B. and T. F. Taraschi, unpublished results). Thus, a situation exists wherein the parasite is actively exporting lipids, proteins and membranes, yet the physical size of the IRBC does not change. We suggest that a possible explanation for the impressive scale of malaria protein export is that vesicles containing various parasite antigens are continually being released from the IRBC into the serum. Indeed, Lenstra et al. (33) reported that vesicles containing PfHRP1 bud from the host cell membrane of IRBC and can be isolated from in vitro cultures of *P. falciparum* infected erythrocytes. Howard et al. (21) have also determined that PfHRP2 is secreted continuously from intact infected cells, accumulating in culture supernatant from the early ring stage. Malarial proteins, both soluble and membrane bound, might fulfill numerous roles in plasma, including elicitation of immune responses of advantage to the parasite or diversion of antibody or cell-mediated arms of effector immune responses that would otherwise destroy IRBC.

Comparison of lipid and protein trafficking pathways in this host-parasite system has revealed a number of peculiarities compared to other eukaryotic cells. The highly mobile vesicular structures in the erythrocyte cytoplasm that bear parasite protein antigens recognized by MAb 41E11 are quite large (0.2–0.8 μm) compared to typical exocytic vesicles (0.05 μm). These vesicles were observed to move by a random, Brownian motion. Because vesicle motion was not significantly slowed by lowering the temperature to 4°C or depleting cellular ATP (T. F. Taraschi, unpublished results), these vesicles appear not to be directed along a microtubular or filamentous network. In contrast to vesicle-associated antigens defined by MAb 41E11, the antigens identified by PfHRP1, PfHRP2 and PfEMP2 were generally present in lipid-free protein aggregates in the host cell cytoplasm. The mode of transport of these proteins across the PVM and host cell cytoplasm is unknown but presumably quite different to that of membrane vesicle-associated malarial proteins. Protein chaperones, such as the 70-kD heat shock proteins (27), may facilitate export of PfHRP1, PfHRP2, and PfEMP2 across the PVM and erythrocyte cytosol (Fig. 7). Exported parasite proteins may cross the PPM and PVM through structures analogous to contact sites in mitochondria (42). In fact, transmission electron micrographs of IRBC reveal areas of close membrane apposition and "crossing-over" points between the PPM and PVM that may correspond to such contact sites (23, 24, and references therein). Once the unfolded malarial proteins pass through contact sites at the PPM/PVM interface, they could be bound by chaperones and escorted to their target destinations. On the basis of the observation that the bulk of PfHRP1 is in the host cytoplasm, it has already been suggested (24) that PfHRP1 may act as a chaperone or transport protein for other exported parasite proteins. However, the PfHRP1 sequence does not bear much resemblance to molecular chaperones or chaperonins that have been characterized to date (5, 8, 11, 34).

CFIM provides the opportunity to observe dynamic processes in living cells. By using membrane labels that define specific compartments, it should be possible to determine if the vesicles in the host cytoplasm that carry parasite proteins originate from the parasite or the PVM. Models of membrane traffic have been proposed that incorporate both possibilities (23, 24). Such information is crucial to determine the mechanism(s) whereby newly synthesized parasite proteins traverse the PPM and PVM. We also need to describe how proteins such as PfHRP1, PfHRP2, PfEMP2 are specifically routed for incorporation with the IRBC surface membrane. Definitive answers to the questions raised by our work can be expected once it becomes possible to transfect malaria parasites with mutant malarial genes and the methods used here applied to studying the routing of mutant gene products.

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References

1. Aikawa, M., S. Uni, A. T. Andrutis, and R. J. Howard. 1986. Membrane associated electron dense material of the asexual stages of *Plasmodium falciparum*: evidence for movement from the intracellular parasite to the erythrocyte membrane. *Am. J. Trop. Med. Hyg.* 35:30–36.
2. Aikawa, M. 1988. Human cerebral malaria. *Am. J. Trop. Med. Hyg.* 39:3–10.
3. Aikawa, M., M. Iseki, J. W. Barnwell, D. W. Taylor, M. M. Oo, and R. J. Howard. 1990. The pathology of human cerebral malaria. *Am. J. Trop. Med. Hyg.* 43:30–37.
4. Aley, S. B., J. A. Sherwood, and R. J. Howard. 1984. Knob-positive and Knob-negative *Plasmodium falciparum* differ in expression of strain specific malarial antigen on the surface of infected erythrocytes. *J. Exp. Med.* 160:1585–1590.
5. Ardeshir, F., J. E. Flint, Y. Matsumoto, M. Aikawa, R. T. Reese, and H. Stanley. 1987. cDNA sequence encoding a *Plasmodium falciparum* protein associated with knobs and localization of the protein to electron-dense regions in membranes of infected erythrocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1421–1427.
6. Atkinson, C. T., M. Aikawa, G. Perry, T. Fujino, V. Bennett, E. A. Davidson, and R. J. Howard. 1987. Ultrastructural localization of erythrocyte cytoskeleton and integral membrane proteins in *Plasmodium falciparum* infected erythrocytes. *Eur. J. Cell Biol.* 45:192–199.
7. Atkinson, C. T., and M. Aikawa. 1990. Ultrastructure of malaria infected erythrocytes. *Blood Cells.* 16:351–368.
8. Bardwell, J. C. A., and E. A. Craig. 1984. Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. *Proc. Natl. Acad. Sci. USA* 81:848–852.
9. Barnwell, J. W., A. S. Asch, R. L. Nachman, M. Yamaya, M. Aikawa, and P. Ingravallo. 1989. A human 88-kd membrane glycoprotein (CD36) functions in vitro as a receptor for cytoadherence ligand on *Plasmodium falciparum*-infected erythrocytes. *J. Clin. Invest.* 84:765–772.
10. Barnwell, J. W. 1990. Vesicle mediated transport of membrane and proteins in malaria infected red blood cells. *Blood Cells.* 16:379–395.
11. Bianco, A. E., J. M. Favaloro, T. R. Burkot, J. G. Culvenor, P. E. Crewther, G. V. Brown, R. F. Anders, R. L. Coppel, and D. J. Kemp. 1986. A repetitive antigen of *Plasmodium falciparum* that is homologous to heat shock protein 70 of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* 83:8713–8717.
12. Coppel, R. L., A. F. Cowman, K. R. Lingelbach, G. V. Brown, R. B. Saint, D. J. Kemp, and R. F. Anders. 1983. Isolate-specific S-antigen of *Plasmodium falciparum* contains a repeated sequence of eleven amino acids. *Nature (Lond.).* 306:751–756.
13. Coppel, R. L., S. Lustigman, L. Murray, and R. F. Anders. 1988. MESA is a *Plasmodium falciparum* phosphoprotein associated with the erythrocyte membrane skeleton. *Mol. Biochem. Parasitol.* 31:223–232.
14. de Rojas, M. O., and M. Wasserman. 1985. Temporal Relationships on macromolecular synthesis during the asexual cell cycle of *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* 79:792–796.
15. Etzion, Z., and M. E. Perkins. 1989. Localization of parasite encoded protein to erythrocyte cytoplasmic vesicles of *Plasmodium falciparum* infected cells. *Eur. J. Cell Biol.* 48:174–179.
16. Green, T. J., G. Gadsden, T. Seed, R. Jacobs, M. Morhardt, and R. Brackett. 1985. Cloning and characterization of *Plasmodium falciparum* FCR-3/FMG Strain. *Am. J. Trop. Med. Hyg.* 34:24–30.
17. Grellier, P., D. Rigomier, V. Clavey, J. C. Fruchart, and J. Schrevel. 1991. Lipid traffic between high density lipoproteins and *Plasmodium falciparum*-infected red blood cells. *J. Cell Biol.* 112:267–277.
18. Haldar, K., A. F. de Amorim, and G. A. M. Cross. 1989. Transport of fluorescent phospholipid analogues from the erythrocyte membrane to the parasite in *Plasmodium falciparum*-infected cells. *J. Cell Biol.* 108: 2183–2192.
19. Haldar, K., and L. Uyetake. 1992. The movement of fluorescent endocytic tracers in *P. falciparum*-infected erythrocytes. *Mol. Biochem. Parasitol.* 50:161–177.
20. Handunnetti, S., B. L. Pasloske, M. R. van Schravendijk, J. C. Aguiar, T. F. Taraschi, J. A. Gormley, and R. J. Howard. 1992. The characterization of two monoclonal antibodies which react with high molecular weight antigens of asexual *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 54:231–246.
21. Howard, R. J., S. Uni, M. Aikawa, S. B. Aley, J. H. Leech, A. M. Lew, T. E. Wellems, J. Rener, and D. W. Taylor. 1986. Secretion of a malarial his-rich protein (PfHRP2) from *Plasmodium falciparum*-infected erythrocytes. *J. Cell Biol.* 103:1296–1277.
22. Howard, R. J., J. A. Lyon, S. Uni, A. J. Saul, S. B. Aley, F. Klotz, L. J. Panton, J. A. Sherwood, K. Marsh, M. Aikawa, and E. P. Rock. 1987. Transport of an Mr ~300,000 *Plasmodium falciparum* protein (PfEMP2) from the intraerythrocytic asexual parasite to the cytoplasmic face of the host cell membrane. *J. Cell Biol.* 104:1269–1280.
23. Howard, R. J., S. Uni, J. A. Lyon, D. W. Taylor, W. Daniel, and M. Aikawa. 1987. Export of *Plasmodium falciparum* proteins to the host erythrocyte membrane: special problems of protein trafficking and topogenesis. *In* Host-Parasite Cellular and Molecular Interactions in Protozoal Infections. K. P. Chang and D. Snary, editors. Springer-Verlag, Berlin/Heidelberg. 281–296.
24. Howard, R. J. 1988. *Plasmodium falciparum* proteins at the host erythrocyte membrane: biological and immunological significance and novel parasite organelles which deliver them to the cell surface. *In* The Biology of Parasitism. Alan R. Liss, Inc., 111–145.
25. Howard, R. J., J. W. Barnwell, E. P. Rock, J. Neequaye, D. Ofori-Adjei, W. L. Maloy, J. A. Lyon, and A. Saul. 1988. Two approximately 300-kd *P. falciparum* proteins at the surface membrane of infected erythrocytes. *Mol. Biochem. Parasitol.* 27:207–224.
26. Hsiao, L., R. J. Howard, M. Aikawa, and T. F. Taraschi. 1991. Modification of host cell membrane composition by the intra-erythrocytic human malaria parasite *Plasmodium falciparum*. *Biochem. J.* 274:121–132.
27. Kang, P., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner. 1990. Requirement for hsp 70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature (Lond.).* 348:137–143.
28. Kilejian, A. 1979. Characterization of a protein correlated with the production of knob-like protrusions on membranes of erythrocytes infected with *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA.* 76:4650–4653.
29. Knapp, B., E. Hundt, and K. R. Lingelbach. 1991. Structure and possible function of *Plasmodium falciparum* proteins exported to the erythrocyte membrane. *Parasitol. Res.* 77:277–282.
30. Lambros, C., and J. P. Vanderberg. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65:418–420.
31. Leech, J. H., J. W. Barnwell, M. Aikawa, L. H. Miller, and R. J. Howard. 1984. *Plasmodium falciparum* malaria: association of knobs on the surface of infected erythrocytes with a histidine-rich protein and the erythrocyte skeleton. *J. Cell Biol.* 98:1256–1264.
32. Leech, J. H., J. W. Barnwell, L. H. Miller, and R. J. Howard. 1984. Identification of a strain specific malarial antigen exposed on the surface of *Plasmodium falciparum* infected erythrocytes. *J. Exp. Med.* 159: 1567–1575.
33. Lenstra, R., A. Samso, B. Andrieu, J. Le Bras, and F. Gailbert. 1988. Viruslike particles containing knob-associated histidine-rich protein are secreted into the culture medium of *Plasmodium falciparum* in vitro cultures. *Biochem. Biophys. Res. Commun.* 151:749–757.
34. Lindquist, S. 1986. The heat-shock response. *Annu. Rev. Biochem.* 55: 1151–1191.
35. Maguire, P. A., and I. W. Sherman. 1990. Phospholipid composition, cholesterol content and cholesterol exchange in infected erythrocytes. *Mol. Biochem. Parasitol.* 38:105–112.
36. Maguire, P. A., J. Prudhomme, and I. W. Sherman. 1991. Alterations in erythrocyte membrane phospholipid organization due to intracellular growth of the human malaria parasite, *Plasmodium falciparum*. *Parasitology.* 102:179–186.
37. McLean, I. W., and P. L. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077–1083.
38. Pasvol, G. 1978. Separation of viable schizont-infected cells of *Plasmodium falciparum* from human blood. *Ann. Trop. Med. Hyg.* 72:1–2.
39. Pouvelle, B., R. Spiegel, L. Hsiao, R. J. Howard, R. L. Morris, A. P. Thomas, and T. F. Taraschi. 1991. Direct access to serum macromolecules by intraerythrocytic malaria parasites. *Nature (Lond.).* 353: 73–75.
40. Rock, E. P., K. Marsh, A. J. Saul, T. E. Wellems, D. W. Taylor, W. L. Maloy, and R. J. Howard. 1987. Comparative analysis of the *Plasmodium falciparum* histidine-rich proteins HRP-I, HRP-II, and HRP-III in malaria parasites of diverse origin. *Parasitology.* 95:209–227.
41. Rock, E. P., A. J. Saul, D. W. Taylor, J. H. Leech, J. A. Sherwood, and R. J. Howard. 1988. Expression of the histidine-rich protein PfHRP1 by knob-positive *Plasmodium falciparum* is not sufficient for cytoadherence of infected erythrocytes. *Infect. Immun.* 56:3301–3304.
42. Schleyer, M., and W. Neupert. 1985. Transport of proteins into mitochondria: translocation intermediates spanning contact sites between outer and inner membranes. *Cell.* 43:339–350.
43. Sherman, I. W. 1979. Biochemistry of *Plasmodium* (malarial parasites) *Microbiol. Rev.* 43:453–495.
44. Stanley, H. A., S. G. Langreth, and R. T. Reese. 1989. *Plasmodium falciparum* antigens associated with membrane structures in the host erythrocyte cytoplasm. *Mol. Biochem. Parasitol.* 36:139–150.
45. Stenzel, D. J. and U. A. K. Kara. 1989. Sorting of malarial antigens into vesicular compartments within the host cell cytoplasm as demonstrated by immuno electron microscopy. *Eur. J. Cell Biol.* 49:311–318.
46. Taylor, D. W., M. Parra, G. B. Chapman, M. E. Stearns, J. Rener, M. Aikawa, S. Uni, L. J. Panton, S. B. Aley, and R. J. Howard. 1987. *Plasmodium falciparum* histidine rich protein PfHRP1: its subcellular localization to the host erythrocyte membrane under knobs using a monoclonal antibody. *Mol. Biochem. Parasitol.* 25:165–174.
47. Taraschi, T. F., A. Parashar, M. Hooks, and H. Rubin. 1986. Perturbation of red cell membrane structure during intracellular maturation of *P. fal-*

ciparum. Science (Wash. DC). 232:102–104.
48. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science (Wash. DC).* 193:673–675.
49. Udomsangpetch, R., M. Aikawa, K. Berzins, M. Wahlgren, and P. Perlmann. 1989. Cytoadherence of knobless *Plasmodium falciparum*-infected erythrocytes and its inhibition by a human monoclonal antibody. *Nature (Lond.).* 338:763–765.
50. Udomsangpetch, R., J. Carlsson, B. Wahlin, G. Holmquist, L. S. Ozaki, A. Scherf, D. Mattei, O. Mercereau-Puijalon, S. Uni, M. Aikawa, and P. Perlmann. 1989. Reactivity of the human monoclonal antibody 33G2 with repeated sequences of three distinct *Plasmodium falciparum* antigens. *J. Immunol.* 142:3620–3626.
51. Vial, H. J., M. J. Thuet, J. L. Broussal, and J. R. Philippot. 1982. Phospholipid biosynthesis by *Plasmodium knowlesi*-infected erythrocytes: the incorporation of phospholipid precursors and the identification of previously undetected metabolic pathways. *J. Parasitol.* 68:379–391.
52. Wellems, T. E., and R. J. Howard. 1986. Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA.* 83:6065–6069.