# Transport of an $M_r$ ~300,000 *Plasmodium falciparum* Protein (Pf EMP 2) from the Intraerythrocytic Asexual Parasite to the Cytoplasmic Face of the Host Cell Membrane

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***Abstract.*** The profound changes in the morphology, antigenicity, and functional properties of the host erythrocyte membrane induced by intraerythrocytic parasites of the human malaria *Plasmodium falciparum* are poorly understood at the molecular level. We have used mouse mAbs to identify a very large malarial protein ($M_r$ ~300,000) that is exported from the parasite and deposited on the cytoplasmic face of the erythrocyte membrane. This protein is denoted *P. falciparum* erythrocyte membrane protein 2 (Pf EMP 2). The mAbs did not react with the surface of intact infected erythrocytes, nor was Pf EMP 2 accessible to exogenous proteases or lactoperoxidase-catalyzed radioiodination of intact cells. The mAbs also had no effect on in vitro cytoadherence of infected cells to the C32 amelanotic melanoma cell line. These properties distinguish Pf EMP 2 from Pf EMP 1, the cell surface malarial protein of similar size that is associated with the cytoadherent property of *P. falciparum*–infected erythrocytes. The mAbs did not react with Pf EMP 1. In one strain of parasite there was a significant difference in relative mobility of the $^{125}$I-surface-labeled Pf EMP 1 and the biosynthetically labeled Pf EMP 2, further distinguishing these proteins. By cryo–thin-section immunoelectron microscopy we identified organelles involved in the transit of Pf EMP through the erythrocyte cytoplasm to the internal face of the erythrocyte membrane where the protein is associated with electron-dense material under knobs. These results show that the intraerythrocytic malaria parasite has evolved a novel system for transporting malarial proteins beyond its own plasma membrane, through a vacuolar membrane and the host erythrocyte cytoplasm to the erythrocyte membrane, where they become membrane bound and presumably alter the properties of this membrane to the parasite's advantage.

ASEXUAL malaria parasites markedly alter the properties of the host erythrocyte within which they grow, differentiate, and multiply. The erythrocyte membrane, which separates the parasite from the external environment, is altered in several ways including its shape, antigenicity, and functional properties (7, 8, 25). We presume that some of these alterations reflect the requirements of this obligate intracellular parasite to modify the relatively limited transport and enzymatic properties of the erythrocyte membrane to suit its own metabolic requirements. Furthermore, the host cell membrane is an interface at which the parasite and immune system interact since malarial protein antigens are expressed at the cell surface and are recognized by antibodies (2, 12, 19). There is little information on the molecular basis for these alterations and the pathways by which malarial antigens are exported from the intracellular parasite to the host cell membrane. Earlier we suggested a hypothetical model for transport and topological orientation of such antigens involving membrane vesicles in the erythrocyte cytoplasm (7).

Here we use mAbs to identify a previously undescribed protein of the human parasite *Plasmodium falciparum* that is associated with the erythrocyte membrane. This protein is *P. falciparum* erythrocyte membrane protein 2 (Pf EMP 2),[1] denoted as such to distinguish it from another protein on the surface of infected erythrocytes (Pf EMP 1) (20) thought to be responsible for attachment of infected erythrocytes to capillary endothelium in vivo (21), and thrombospondin (24) and endothelial cells (30) in vitro. The results of immunofluorescence, immunoelectron microscopy, cell-surface radioiodination, and protease treatment suggest that Pf EMP

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1. *Abbreviations used in this paper*: Pf EMP 1 and 2, *Plasmodium falciparum* erythrocyte membrane proteins 1 and 2; STI, soybean trypsin inhibitor.

2 is attached to the erythrocyte membrane, but not exposed at the infected cell's outer surface. With cryo-thin-section immunoelectron microscopy, we explored how this malarial protein traverses the membranes and erythrocyte cytoplasm, separating the parasite from the erythrocyte surface membrane. The results have general implications on the extent to which parasitization of the host cell has created a new cell with properties that lack in the individual components.

## Materials and Methods

### Parasites and In Vitro Culture

The Malayan Camp (6) and St. Lucia (4) strains of *P. falciparum*, both knob-positive ($K^+$) parasites, were obtained from *Aotus* monkeys and cryopreserved (19). Synchronous populations of ring-stage parasites (10–30% parasitemia) were obtained after thawing and were cultured in vitro up to the late schizont stage by standard procedures (19).

### Biosynthetic Radiolabeling

Infected erythrocytes were cultured with 100 μCi/ml of various $^3$H- or $^{35}$S-labeled amino acids to biosynthetically label malarial proteins (12, 19). Uninfected erythrocytes do not incorporate label into protein under these conditions.

### Monoclonal Antibodies

Mouse mAbs were obtained by fusing .653 myeloma cells (14) with spleen cells from mice (15) immunized with schizont antigens. mAb Pf9.4H9.1 was from a DBA/2J mouse primed and boosted with immune complexes from $1 \times 10^6$ parasites (23). mAb Pfl2.8B7.4 was from a BALB/cJ mouse immunized with glutaraldehyde-fixed parasites and immune complexes. The immunization regimen for this mouse will be described elsewhere (23a). Several mAbs appeared to react with the erythrocyte membrane on indirect immunofluorescence screening of air-dried, methanol-fixed thin films of infected blood. These were cloned by limiting dilution in vitro, and ascites were prepared. mAb Pfl2.8B7.4, denoted mAb 8B7.4, was an $IgG_1(k)$ and mAb PF9.4H9.1, denoted mAb 4H9.1, was an IgM. Both mAbs were purified using protein A–Sepharose. mAb 8B7.4 was also purified as follows: The crude globulin fraction of mouse ascites was isolated by precipitation with 18% sodium sulfate (wt/vol), followed by precipitation with 14% sodium sulfate (wt/vol). The precipitate was dialyzed against 0.01 M Tris-HCl buffer, pH 8.0, and was applied to an ion-exchange column of DEAE cellulose (DE-52; Whatman Inc., Clifton, NJ) equilibrated with the same buffer. Fractions containing IgG were eluted with a linear salt gradient from 0.01 M Tris-HCl to 0.3 M NaCl-0.01 M Tris-HCl, pH 8.0 (17).

### Indirect Immunofluorescence

Indirect immunofluorescence was performed using ascites or protein A–purified antibody on fixed or unfixed erythrocytes. Immunofluorescence with methanol- or acetone-fixed smears of infected blood was performed as described previously (12). For immunofluorescence detection of bound mAb with unfixed erythrocytes we used either fluorescein-conjugated rabbit anti-mouse IgG (or IgM as appropriate), or goat anti-mouse Ig followed by fluorescein-conjugated rabbit anti-goat IgG. Immunofluorescence was also performed with very mature infected cells that were cultured with protease inhibitors to prevent cell rupture (22).

### Protease Treatments

Methanol-fixed slides of infected blood were overlaid with various concentrations of trypsin (type XIII; Sigma Chemical Co., St. Louis, MO) (0, 0.1, 1, 10 μg/ml) or 10 μg/ml trypsin plus 30 μg/ml soybean trypsin inhibitor (STI) in PBS and incubated 5 min at 23°C. The slides were quickly washed, incubated 5 min in PBS plus 30 μg/ml STI, washed again, and processed directly for immunofluorescence.

Intact infected erythrocytes that contained a synchronous population of late trophozoites were resuspended at $1 \times 10^9$ cells/ml in RPMI-1640 medium containing various levels of trypsin (0–1.0 mg/ml) (type XIII; Sigma Chemical Co.) or chymotrypsin (type VII; Sigma Chemical Co.) (0–10 μg/ml) and incubated 5 min at 23°C. Reaction was terminated by addition of STI (30 μg/ml) or L-1-tosylamide-2-phenylethyl chloromethyl ketone (0.2 mm), respectively, and washing the cells four times in RPMI-1640 medium.

### Immunoelectron Microscopy

To label surface antigens, intact unfixed infected erythrocytes were washed in RPMI-1640 and resuspended at $1 \times 10^9$ cells/ml in medium containing 0.5% wt/vol BSA and various concentrations of protein A–purified antibody (0, 0.10, and 1.0 μg/ml). After incubation for 30 min at 37°C, the cells were washed three times in medium plus BSA and incubated under the same conditions with a 1:30 dilution of rabbit IgG anti-mouse IgG or IgM (Cappel Laboratories, Malvern, PA), washed again, and incubated with gold-conjugated protein A (EY Laboratories, San Mateo, CA). This was followed by washing and fixation in 1% glutaraldehyde, then by standard methods of transmission electron microscopy (1).

Alternatively, to visualize antibody binding to the cytoplasmic face of the host erythrocyte membrane, infected erythrocytes were lysed by treatment for 2 min at 23°C with 0.1% wt/vol saponin in RPMI-1640 medium (a 1:2 vol/vol ratio of packed cells and saponin solution), washed three times in RPMI-1640 plus 0.5% BSA, and processed for immunoelectron microscopy with the mAb as for the intact cells (centrifugation of saponin-treated cells was for 1 min on an Eppendorf centrifuge).

### Cryo-thin-section Immunoelectron Microscopy

Infected blood was lightly fixed with formaldehyde/glutaraldehyde and frozen sections prepared (27). The sections were incubated with mAb (1:6,000–1:8,000 dilutions of ascites), washed, and incubated with protein A–gold. In some experiments an additional step of incubation with rabbit anti-mouse IgG or IgM was included, but this had no effect on the results. Parallel control incubations were performed without mAb or with irrelevant mAbs (1A1D5, IgM; 2D2C5, $IgG_1$).

### Cell-surface Radioiodination

Intact infected erythrocytes were purified on Percoll gradients containing sorbitol (2) and cell surface proteins $^{125}$I-labeled by the lactoperoxidase method (11). The cells were then fractionated again (on a 60%/40% Percoll step-gradient) to remove broken cells (11).

### Detergent Extraction

Infected cells were extracted sequentially with 1% Triton X-100 in PBS and 2% SDS in PBS for immunoprecipitation or immunoblotting, or with 1% Triton X-100 in PBS (9, 10) and SDS sample buffer (18) for examination of Coomassie Blue–stained proteins.

### Immunoprecipitation

Triton X-100 extracts were used directly for immunoprecipitation. SDS extracts were diluted with addition of Triton X-100 to 0.2% SDS, 1.5% Triton X-100 (9, 10). Immune complexes were purified by adsorption to protein A–Sepharose (10). Bound antigens were recovered by treatment of the washed beads with SDS sample buffer (containing 5% wt/vol SDS) at 100°C for 5 min (18).

### SDS PAGE and Radioactivity Analysis

SDS PAGE was performed on 1.5-mm thick slab gels (5–10% gradients of acrylamide) with the Laemmli (18) system of discontinuous buffers. $^3$H- or $^{35}$S-labeled proteins were detected by fluorography (3) after impregnation of gels with EN$^3$HANCE (New England Nuclear, Boston, MA). $^{125}$I-labeled proteins were detected by autoradiography (26).

For precise comparison of the $M_r$ of $^3$H-labeled antigens immunoprecipitated by mAbs and $^{125}$I-labeled antigens immunoprecipitated by antisera that block cytoadherence, samples were subjected to electrophoresis in multiple adjacent lanes on sets of identical acrylamide gradient gels poured with a Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Electrophoresis was continued for an additional 60 min at 24 mA/gel after the dye marker migrated off the end. $^3$H- and $^{125}$I-tracks were processed separately for fluorography/autoradiography and the dried gels realigned precisely using reference points stabbed with an ink pen.

The relative $M_r$ of Coomassie Blue–stained proteins in the SDS extracts of Triton X-100–insoluble material from Malayan Camp and St. Lucia

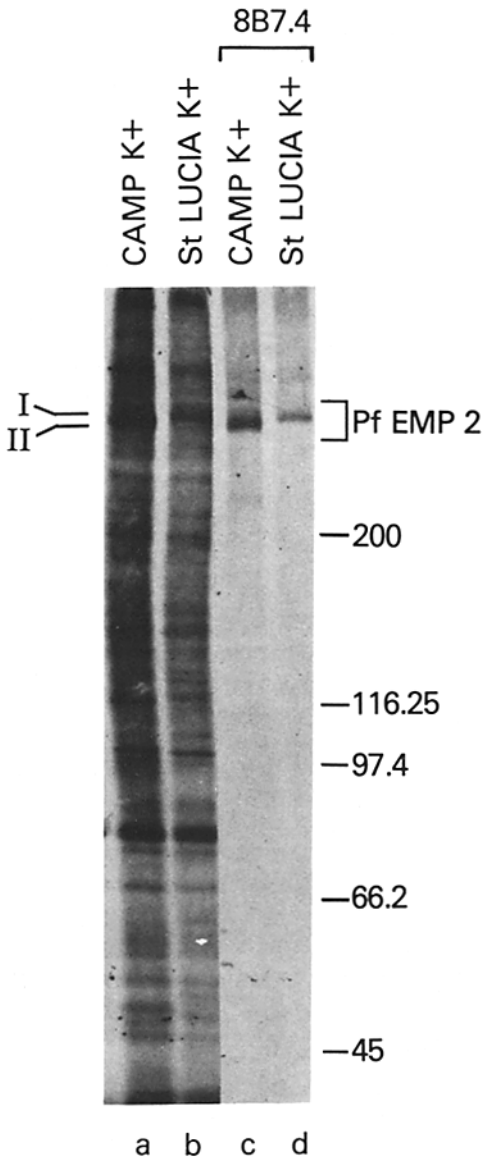


*Figure 1.* Immunoprecipitation of Pf EMP 2 using mAb 8B7.4. Infected erythrocytes of the Malayan Camp and St. Lucia strains in *Aotus* RBC were biosynthetically radiolabeled by culture from the ring to late-trophozoite stage in the presence of [$^3$H]isoleucine, extracted in Triton X-100, and the insoluble material extracted in 2% SDS. The detergent extracts were incubated with mAb, immune complexes purified using protein A-Sepharose, and analyzed by SDS PAGE and fluorography. Lanes *a–d*, SDS extract only; lanes *a* and *b*, SDS extracts before immunoprecipitation; lanes *c* and *d*, immunoprecipitation with mAb 8B7.4. Control IgG and IgM mAbs did not immunoprecipitate any proteins from SDS extracts. The relative mobilities of the specifically immunoprecipitated proteins from St. Lucia and Malayan Camp-strain parasites are denoted I and II, respectively.

strains were examined statistically. The relative mobilities of bands at $M_r$ ~300,000 and the spectrin 1.1 band were determined by lasar scanning densitometry (model 2202 UltroScan Lasar Densitometer; LKB, Bromma, Sweden) and analysis of variance performed with results from multiple gel lanes. The difference in mobility (arbitrary units) for Pf EMP 2 of St. Lucia and Malayan Camp parasites (mean values of 605.0 and 642.5, respectively) was significantly greater than the difference in mobilities of the corresponding spectrin 1.1 band (mean values of 773.0 and 779.5) at the 0.001 level.

### *In Vitro Cytoadherence*

In vitro cytoadherence of infected erythrocytes containing late-trophozoites was performed as described elsewhere (30) using formalin-fixed C32 amelanotic melanoma cells (28). The published protocol for testing antibody blockade was used (29), except that in our experiments, the concentration of antibody in the preincubation with infected erythrocytes was not changed during the incubation with melanoma cells.

### *Antisera Specific for Pf EMP 2*

Pf EMP 2 was purified by immunoadsorption and a rabbit antiserum prepared as follows: protein A-purified mAb 8B7.4 was coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's directions. $5 \times 10^9$ infected erythrocytes were extracted in 2 ml of 1% wt/vol Triton X-100 plus 10 μg/ml DNAase in PBS (10 min at 0°C with vortexing), and the insoluble material was recovered after centrifugation (Eppendorf centrifuge, 5 min, 4°C) and extracted with 500 μl of 2% wt/vol SDS in PBS. Extraction was facilitated by passage of the insoluble material through a 25-gauge needle. After centrifugation (Eppendorf centrifuge, 5 min, room temperature), the supernatant was passed over a 2-ml AGI18 ion-exchange column (BioRad Laboratories, Richmond, CA) to remove excess SDS. The sample was diluted by the addition of 4.5 ml of 2% wt/vol Triton X-100 in PBS and loaded onto a 1-ml column of 8B7.4-coupled Sepharose. The column was washed with 10 ml of PBS plus 1% wt/vol Triton X-100 and the beads divided into 250-μl portions for storage at −90°C. A rabbit was immunized with 250 μl of the Sepharose-8B7.4-immobilized antigen beads in Freund's complete adjuvant and boosted 4 wk later with the same amount of beads in Freund's incomplete adjuvant.

A mouse antiserum against part of the Pf EMP 2 protein was produced by immunization with a fusion protein consisting of a chimera of β-galactosidase and an 80 amino acid *P. falciparum*-encoded sequence that reacts with mAb 8B7.4. Selection of the λgt11 genomic clone expressing this fusion protein and the sequence of the malarial DNA will be described elsewhere (Saul, A., manuscript in preparation).

## *Results*

### *Immunochemical Identification of the Antigen Recognized by mAbs 8B7.4 and 4H9.1*

mAb 8B7.4 and mAb 4H9.1 gave an immunofluorescence reaction with infected erythrocytes that suggested localization of the appropriate antigen(s) to the infected cell's outer membrane. Neither mAb reacted with uninfected erythrocytes. The antigen recognized by these mAbs was identified by immunoprecipitation of biosynthetically radiolabeled proteins using protein A–Sepharose (Fig. 1) and by solid-phase adsorption to antibody coated on plastic (not shown). No malarial proteins were specifically immunoprecipitated from the Triton X-100 extract. In contrast, mAbs 8B7.4 and 4H9.1 both immunoprecipitated a single $M_r$ ~300,000 malarial protein from SDS extracts (Fig. 1). The immunoprecipitated antigen was biosynthetically radiolabeled by several amino acids, including $^3$H-labeled histidine, leucine, or isoleucine and $^{35}$S-labeled methionine. Western blotting with these mAbs also identified a single $M_r$ ~300,000 band in SDS extracts (not shown). The mobility of the reactive antigen differed in the two *Aotus* monkey-adapted parasites shown in Fig. 1 and also in a variety of culture-adapted strains or clones of *P. falciparum* (not shown). When uninfected RBCs were purified from the radiolabeled infected blood using a density gradient and extracted and immunoprecipitated in parallel, no radiolabeled bands were immunoprecipitated, nor were any bands detected by Western blotting. Thus, the antigen identified by mAbs 8B7.4 and 4H9.1 is a product of malarial protein synthesis. With each parasite, the immunoprecipitated antigen co-migrated with the major radiolabeled

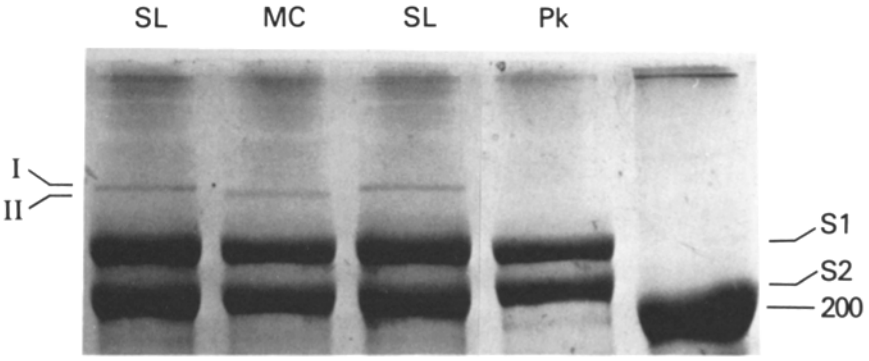


*Figure 2.* Identification of Pf EMP 2 as a Coomassie Blue-stained protein in the Triton X-100-insoluble fraction from purified infected erythrocytes. Infected cells (>88% parasitemia, late trophozoites) were purified from *Aotus*-infected blood containing St. Lucia or Malayan Camp strain *P. falciparum* or rhesus monkey blood infected with clone Pk1(A+) of *P. knowlesi* (Pk). After extraction with Triton X-100, the insoluble material was extracted with SDS and analyzed by SDS PAGE and Coomassie Blue staining. The figure shows bands with relative mobility of I and II for St. Lucia and Malayan Camp parasites, respectively, together with the spectrin doublets of *Aotus* and rhesus erythrocytes (S1 and S2). The myosin marker at 200,000 D is indicated. The relative mobilities of bands I and II as measured by scanning laser densitometry (arbitrary units) were significantly different: I, 642.5; II, 605.0 (SE 38.5, $P \ll 0.001$; see Materials and Methods).

band of the SDS extract of Triton X-100-insoluble material (Fig. 1). In some experiments longer fluorographic exposure of immunoprecipitation gels revealed several discrete minor bands that migrated faster than the major antigen band. Such bands are probably partial proteolytic breakdown products of the $M_r$ ~300,000 antigen.

The estimation of the $M_r$ of this antigen ($M_r$ ~300,000) will contain large errors since this protein migrates more slowly than the highest $M_r$ marker (myosin, $M_r$ ~200,000) used to form a standard curve. The antigen also migrates more slowly than the spectrin doublet ($M_r$ ~215,000 and 240,000). The size of the antigen that reacts with mAbs 8B7.4 and 4H9.1 is comparable to that of PF EMP 1, and both antigens exhibit size diversity in different isolates. Below we show that the mAbs do not react with Pf EMP 1. Accordingly, we designate the protein that reacts with mAbs 8B7.4 and 4H9.1 as Pf EMP 2. The relative mobility on SDS PAGE of these very large antigens will be described with Roman numerals (increasing mobility as the numerals increase). In Fig. 1 the immunoprecipitated antigen for Malayan Camp strain parasites is identified at relative mobility II to indicate that its mobility is slightly greater than the immunoprecipitated antigen of St. Lucia strain parasites, identified at relative mobility I.

With each parasite strain a Coomassie Blue-stained band was identified at the same relative mobility as the antigen immunoprecipitated by the mAbs (Fig. 2). This band was not extracted from purified infected erythrocytes by Triton X-100. There was significantly less protein in this band than in the spectrin bands derived from the host erythrocyte membrane (S1 and S2, Fig. 2). The Coomassie Blue-stained bands were absent from extracts of uninfected erythrocytes or purified infected erythrocytes from the simian malaria *P. knowlesi* (Fig. 2, Pk). The two mAbs also failed to react with *P. knowlesi* on immunofluorescence and immunoprecipitation analysis.

### *Stage Specificity of Parasite Reactivity with mAbs*

Cryopreserved *Aotus* blood containing ring-stage parasites was thawed and cultured as a synchronous parasite population to identify the stages at which the antigen recognized by mAbs 8B7.4 and 4H9.1 is expressed. mAbs 8B7.4 and 4H9.1 reacted strongly on indirect immunofluorescence with acetone- or methanol-fixed infected erythrocytes containing late trophozoites. Fig. 3 shows that the patterns for the Malayan Camp and St. Lucia strains of $K^+$ parasites were indistinguishable with mAb 8B7.4. Uninfected erythrocytes did not fluoresce. Fluorescence was not seen with ring stage parasites but was first seen in early trophozoites as relatively weak focal patches within the parasite. With mid-trophozoites and the appearance of refractile malaria pigment

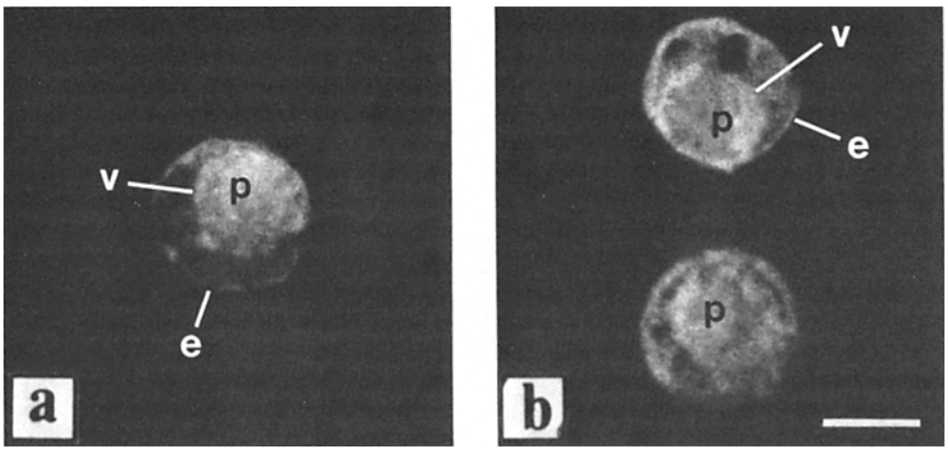


*Figure 3.* Subcellular localization of Pf EMP 2 by indirect immunofluorescence. *Aotus* blood containing Malayan Camp strain (*a*) or St. Lucia strain (*b*) parasites at the late trophozoite stage was smeared, fixed with acetone, and distribution of Pf EMP 2 examined by indirect immunofluorescence using mAb 8B7.4. The host erythrocyte surface membrane is labeled *e*; the plasma membrane of the parasite and/or vacuolar membrane and the parasite cytoplasm are labeled *v* and *p*, respectively. Bar, 5 μm.

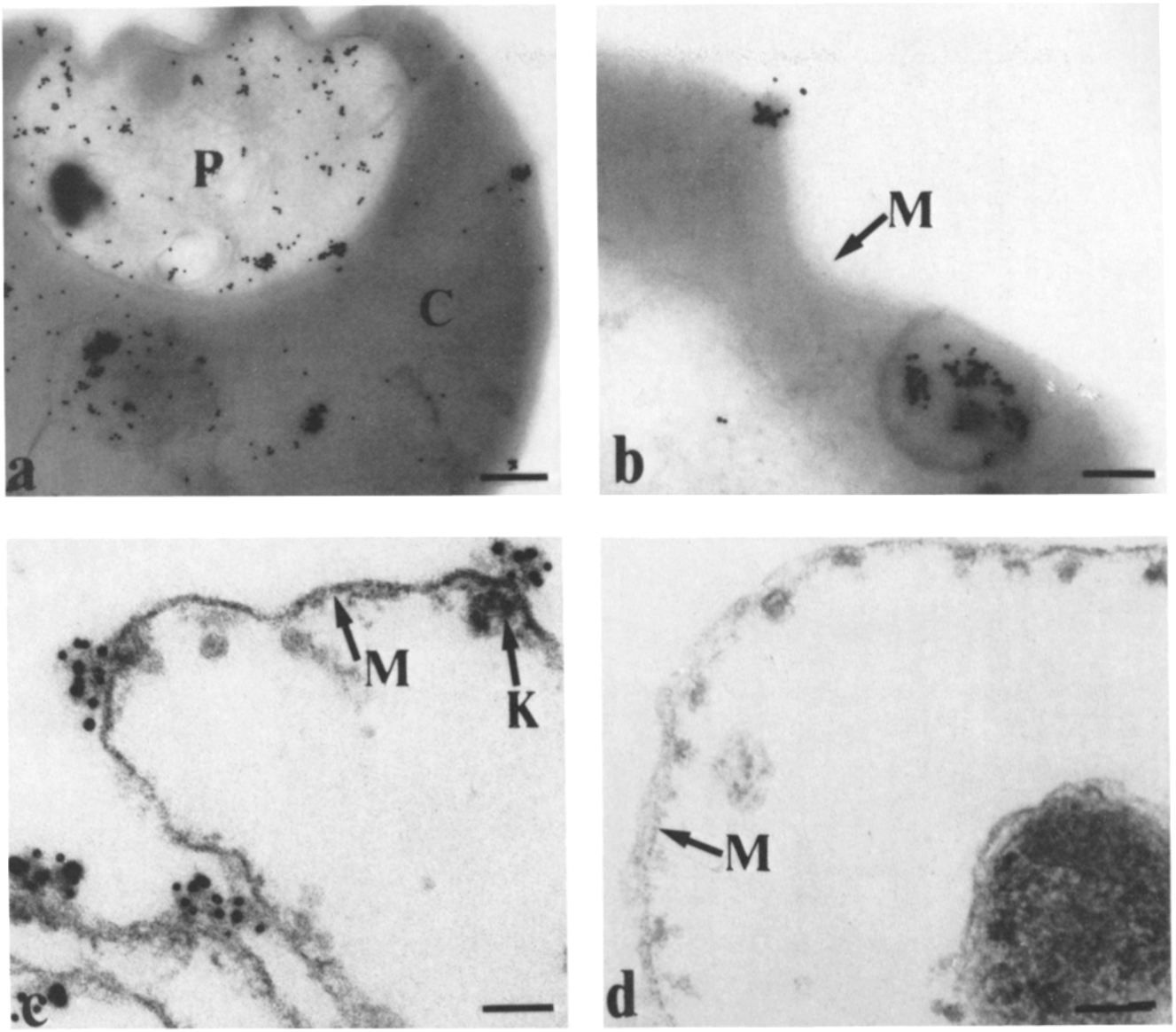


*Figure 4.* Subcellular localization of Pf EMP 2 using mAb 8B7.4 and cryo–thin-section immunoelectron microscopy (*a* and *b*) or immunoelectron microscopy of saponin-lysed infected erythrocytes (*c* and *d*). (*a*) *P*, parasite; *C*, erythrocyte cytoplasm. Bar, 1 μm. (*b*) *M*, infected erythrocyte surface membrane. Bar, 0.5 μm. (*c*) *M*, infected erythrocyte surface membrane, cytoplasmic face identified by position of submembranous electron-dense material and orientation of knob (*K*). Bar, 0.1 μm. (*d*) Control showing results after treatment with control IgG mAb (2D2C5). Bar, 0.5 μm.

within the parasite, the fluorescence became more intense and widely dispersed in small grains that appeared to be in the RBC cytoplasm. At the late-trophozoite stage fluorescence was distributed over the entire infected erythrocyte (Fig. 3). From comparison of this fluorescence pattern with those for other mAbs that react with the parasite plasma membrane, we could discern three subcellular areas of mAb reactivity: the infected erythrocyte membrane was positive with a very fine granular pattern over its entire area; the cytoplasm of the host erythrocyte was positive with roughly spherical focal patches; the parasite and/or the vacuolar membrane that ensheaths the parasite was also positive (Fig. 3). As the parasites matured into schizonts the peripheral fluorescence associated with the erythrocyte membrane increased in intensity and remained up to the time of infected cell rupture and release of new merozoites.

Western blotting also revealed that Pf EMP 2 appears in early trophozoites and accumulates continuously through to at least the late-schizont stage. The relative mobility of Pf EMP 2 did not alter during these steps of parasite differentiation and asexual division.

### *mAbs 8B7.4 and 4H9.1 Recognize Different Epitopes*

A survey of indirect immunofluorescence reactivity of laboratory strains of *P. falciparum* and six isolates collected and analyzed directly from patients in The Gambia, West Africa, revealed that the two mAbs differed in their patterns of reactivity (not shown). mAb 8B7.4 reacted with all samples tested, whereas mAb 4H9.1 failed to react with five of the Gambian isolates. With St. Lucia strain parasites in *Aotus* erythrocytes, mAb 4H9.1 also reacted very weakly on immu-

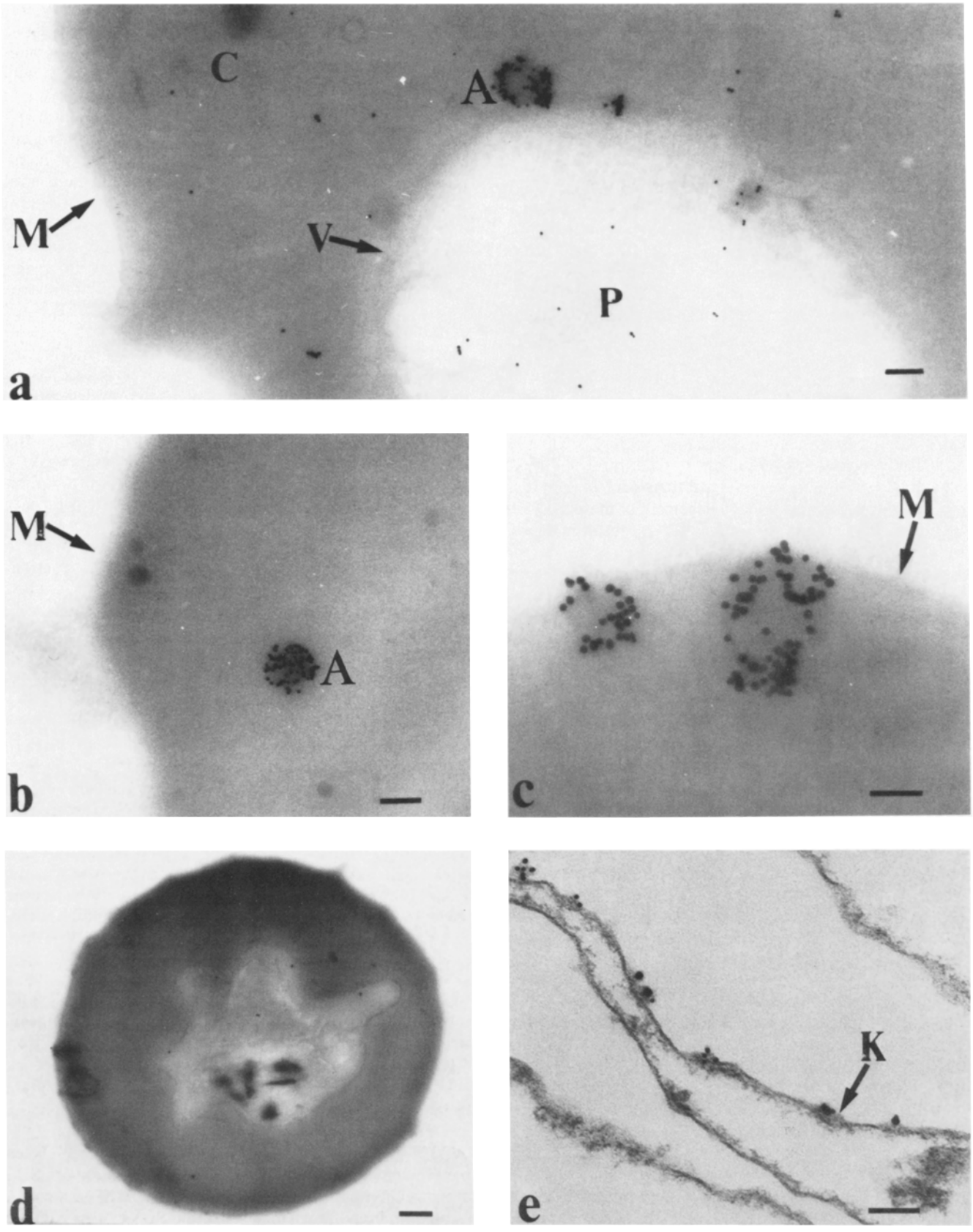


*Figure 5.* Subcellular localization of Pf EMP 2 using IgM mAb 4H9.1 and cryo–thin-section–immunoelectron microscopy (*a–d*) or immunoelectron microscopy of saponin-lysed infected cells (*e*). (*a*) *P*, parasite; *C*, erythrocyte cytoplasm; *V*, parasitophorous vacuole membrane; *M*, infected erythrocyte surface membrane; *A*, electron-dense clumps associated with Pf EMP 2 antigen. Bar, 0.5 μm. (*b*) Bar, 0.5 μm. (*c*) Bar, 0.25 μm. (*d*) Control showing results after treatment with a control IgM mAb (1A1D5). Bar, 1 μm. (*e*) Submembranous area of knob (*K*) identified by concavity of surface membrane and electron-dense material. Bar, 0.25 μm.

nofluorescence, Western blotting, or immunoprecipitation of [$^3$H]leucine-labeled proteins. In contrast, with Malayan Camp parasites, its reactivity was indistinguishable from mAb 8B7.4.

### *Localization of Pf EMP 2 by Immunoelectron Microscopy*

Immunoelectron microscopy was performed with two methods designed to localize an intracellular antigen.

In one approach, the infected erythrocytes were lightly fixed in formaldehyde/glutaraldehyde, frozen, thin-sectioned, and the sections incubated sequentially with mAb, secondary antibodies, and protein A–gold. Fig. 4, *a* and *b* shows the results of this cryo–thin-section immunoelectron microscopy for mAb 8B7.4, whereas Fig. 5, *a–d* shows the comparable data for mAb 4H9.1. The results for the two mAbs are described together since they gave identical patterns of protein A–gold deposition.

Incubation of the thin sections with either mAb led to deposition of gold particles on infected erythrocytes containing late trophozoites, but not on adjacent uninfected erythrocytes. Identical results were obtained with the following combinations: mAb 8B7.4/protein A–gold; mAb 8B7.4/rabbit anti–mouse IgG/protein A–gold; mAb 4H9.1/rabbit anti–mouse IgM/protein A–gold. Replacement of these mAbs by control mAbs of the same subclass did not result in deposition of gold particles (e.g., Fig. 5 *d*).

Gold particles were specifically deposited in the interior of the intraerythrocytic parasite (Fig. 4 *a*). It was not possible to identify whether the antigen was associated with parasite cytoplasm or membranes. Gold particles were also located at several sites external to the malaria parasite but within the infected cell. Electron-dense clumps containing the antigen recognized by these mAbs were seen "budding off" the parasite (*A* in Fig. 5 *a*). These clumps contained >40 individual gold particles on the thin section and measured roughly 200–600 nm in diameter. The location and size of these clumps are similar to those of unit membrane vesicles seen budding off the parasitophorous vacuole membrane by transmission electron microscopy (1). Some of these electron-dense clumps decorated with gold particles were clearly associated with a peripheral unit membrane, while others showed no obvious membrane. The electron-dense clump and gold particles seen in Fig. 5 *b* are of the latter type. In this case the clump is in the cytoplasm of the erythrocyte, distant from the parasite and parasitophorous vacuole membrane. The clumps of Pf EMP 2 were also seen abutting the infected erythrocyte surface membrane (Figs. 4 *b* and 5 *c*). The example in Fig. 4 *b* is interesting in that ~0.2-μm diameter electron-dense material is decorated with protein A–gold, and within a unit membrane vesicle of ~1-μm diam that appears to be fusing with the surface membrane. Gold particles were occasionally seen at and under knobs on the infected erythrocyte surface. An example is shown in Fig. 4 *b*. However, the majority of knobs seen in cryo–thin-sections were not labeled with gold particles. The surface membrane of the infected erythrocyte in between obvious knob protrusions was never labeled. Thus, Pf EMP 2 was located predominantly in the parasite cytoplasm and in 200–600-nm diam clumps between the parasitophorous vacuole membrane and infected erythrocyte surface membrane.

The second approach for localization of intracellular antigen used the detergent saponin to lyse the infected erythrocyte surface membrane, thereby rendering the cytoplasmic face of this membrane accessible to the mAbs. Saponin-treated infected erythrocytes were incubated in series with mAb, secondary antibody, and protein A–gold and fixed in glutaraldehyde for conventional transmission electron microscopy. Inclusion of mAb 8B7.4 or 4H9.1 in the first incubation led to deposition of protein A–gold on these membrane preparations (Figs. 4 *c* and 5 *c*), whereas the control incubations with irrelevant mAbs were uniformly negative (e.g., Fig. 4 *d*). Because the topological relationships of different membranes are disrupted with saponin treatment and centrifugation, it is not possible to identify any membranes with certainty other than the infected erythrocyte surface membrane and parasite plasma membrane. The infected erythrocyte surface membrane and its orientation with respect to external and cytoplasmic faces can be defined by the orientation of knobs. The cytoplasmic face of the outer membrane is identified by the submembrane electron-dense material of 75–100-nm diam and convexity of membrane over this material (see the arrows labeled *K* in Figs. 4 *c* and 5 *d*). With saponin-treated infected cells, protein A–gold was deposited on surface membrane–associated electron-dense material. In the example of Fig. 4 *c* with mAb 8B7.4, the electron-dense material is on the external face of the surface membrane at knobs. Fig. 5 *e* shows an example with mAb 4H9.1 in which the protein A–gold is bound to submembraneous electron-dense material, again at knobs. Both types of deposition (external and cytoplasmic face of the surface membrane) were seen with these mAbs in different sections. Protein A–gold was not deposited on either face of the surface membrane in the areas between knobs where electron-dense material is absent.

### *Pf EMP 2 Cannot Be Demonstrated on Intact P-RBC Using Antibodies or Proteases*

Neither mAb 8B7.4 nor 4H9.1 reacted with intact, unfixed infected cells in an indirect immunofluorescence test. Furthermore, neither the polyclonal rabbit serum raised against the whole Pf EMP 2 molecule, nor the mouse antisera raised against the fusion protein encoding an epitope reactive with mAb 8B7.4 reacted (1:50 dilution). Both of these sera were strongly reactive with air-dried, methanol-fixed infected cells. It is known from the differential reactivity of these sera with a series of fusion proteins that their predominant specificity is not that of mAb 8B7.4 (Saul, A., unpublished data). When immunoelectron microscopy was performed with intact infected erythrocytes, no gold particles were found attached to the cell surface after treatment with mAbs 8B7.4 or 4H9.1 and gold-conjugated protein A.

Pf EMP 2 was inaccessible to trypsin and chymotrypsin in intact infected cells. At levels of 0–1.0 mg/ml of trypsin or 0–0.1 mg/ml chymotrypsin, treatment for 5 min at 23°C had no effect on the SDS PAGE patterns of radiolabeled malarial proteins in either the Triton X-100 extract or SDS extract of Triton X-100–insoluble material (not shown). In particular, immunoprecipitation of the SDS extract with the mAbs showed that these enzymes had no effect on the amount of Pf EMP 2 recovered with mAbs 8B7.4 or 4H9.1, or any effect on the relative mobility of this antigen (Fig. 6). Both of these proteases completely degraded Pf EMP 2

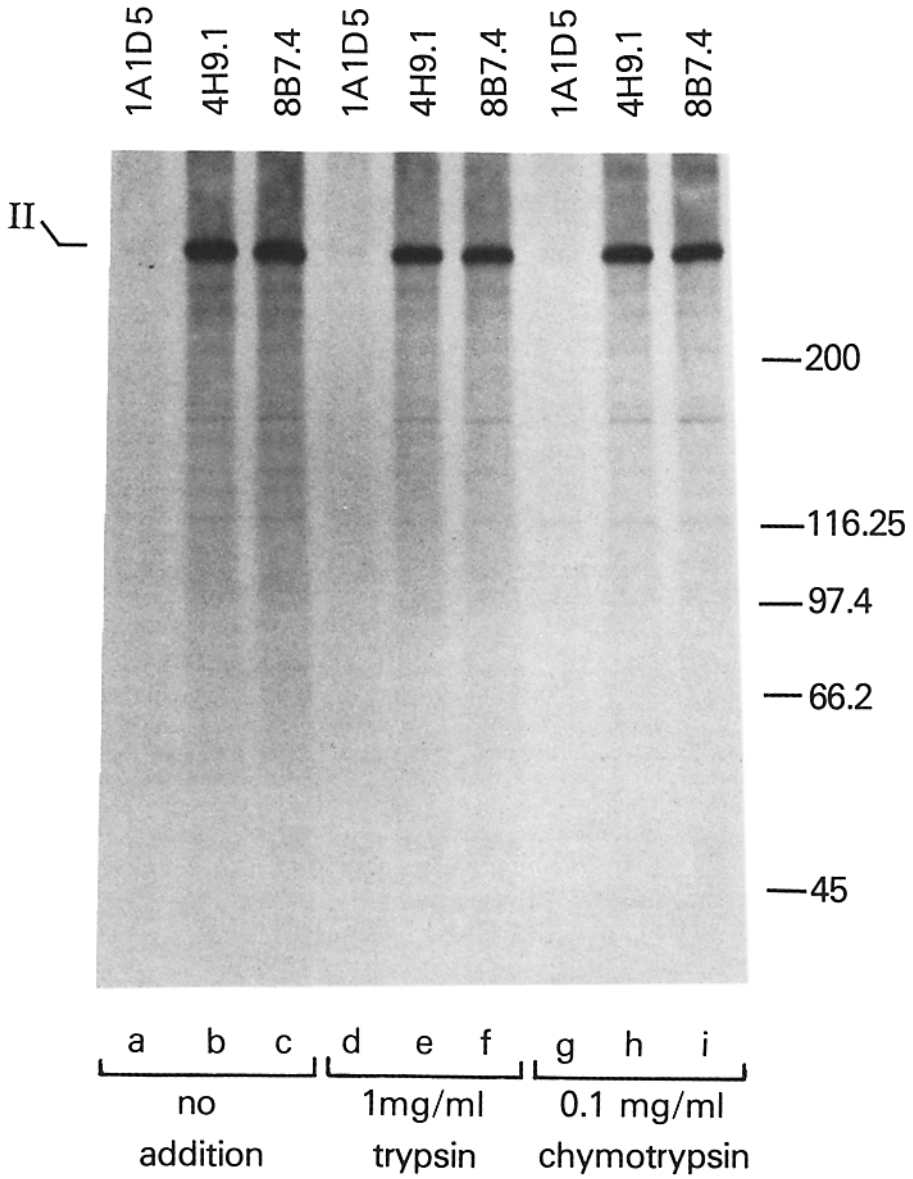


*Figure 6.* Failure of exogenous proteases to cleave Pf EMP 2 with intact infected erythrocytes. Malayan Camp strain parasites were biosynthetically radiolabeled by uptake of [$^3$H]isoleucine, intact infected cells purified on a sorbitol-Percoll density gradient and treated with various levels of trypsin or α-chymotrypsin. After protease inactivation the cells were washed and sequentially extracted with Triton X-100 and SDS. Immunoprecipitation of the SDS extract was performed with control IgM mAb (1A1D5), mAb 4H9.1, or mAb 8B7.4. At the maximum levels of enzymes tested (shown here) there was no detectable loss of immunoprecipitable Pf EMP 2.

bound to mAb 8B7.4 on protein A–Sepharose and also abolished the immunofluorescence reactivity of air-dried, methanol-fixed cells.

Pretreatment of the cells with trypsin (0.1–10 μg/ml trypsin, 5 min, 23°C) did not uncover cryptic epitopes recognized by the two mAbs, as judged by subsequent immunofluorescence assays on intact cells. The trypsin-treated cells were, however, strongly positive after air-drying and methanol fixation. With very mature P-RBC stabilized by the addition of protease inhibitors (22), Pf EMP 2 remained inaccessible to mAbs 8B7.4 or 4H9.1 until loss of hemoglobin indicated rupture of the erythrocyte membrane. Cells that had lost hemoglobin were strongly fluorescence-positive at the RBC membrane.

### *Pf EMP 2 Is Not $^{125}$I-Surface-labeled under Conditions That Label the Surface Antigen Pf EMP 1*

We examined whether Pf EMP 2, as defined by mAbs 4H9.1 and 8B7.4, was the same as Pf EMP 1, the cell-surface malarial protein of similar size associated with the acquired cytoadherence property of *P. falciparum*–infected erythrocytes (20). St. Lucia and Malayan Camp parasites were thawed simultaneously, part of each culture biosynthetically radiolabeled to allow identification of the antigen recognized by the mAbs, and the remainder used for $^{125}$I-surface labeling of purified infected erythrocytes at the trophozoite stage.

Pf EMP 1 was identified as follows (data not shown): this protein was $^{125}$I-surface labeled with $K^+$ but not $K^-$ parasites (2); it was recovered from the 2% SDS extract of Triton X-100–insoluble material but absent from the Triton X-100 extracts (20); Pf EMP 1 was of mobility III with St. Lucia parasites and mobility II with Malayan Camp parasites (20).

The biosynthetically labeled antigen defined by mAbs 8B7.4 and 4H9.1 (Pf EMP 2) had the same relative mobility as $^{125}$I-labeled Pf EMP 1 with Malayan Camp strain (relative mobility II) (not shown). In contrast, the mAbs immunoprecipitated $^3$H-labeled Pf EMP 2 of relative mobility I from St. Lucia parasites that was significantly lower in relative mobility than the $^{125}$I-labeled Pf EMP 1 band of relative mobility III (Fig. 7). The metabolically labeled antigens im-

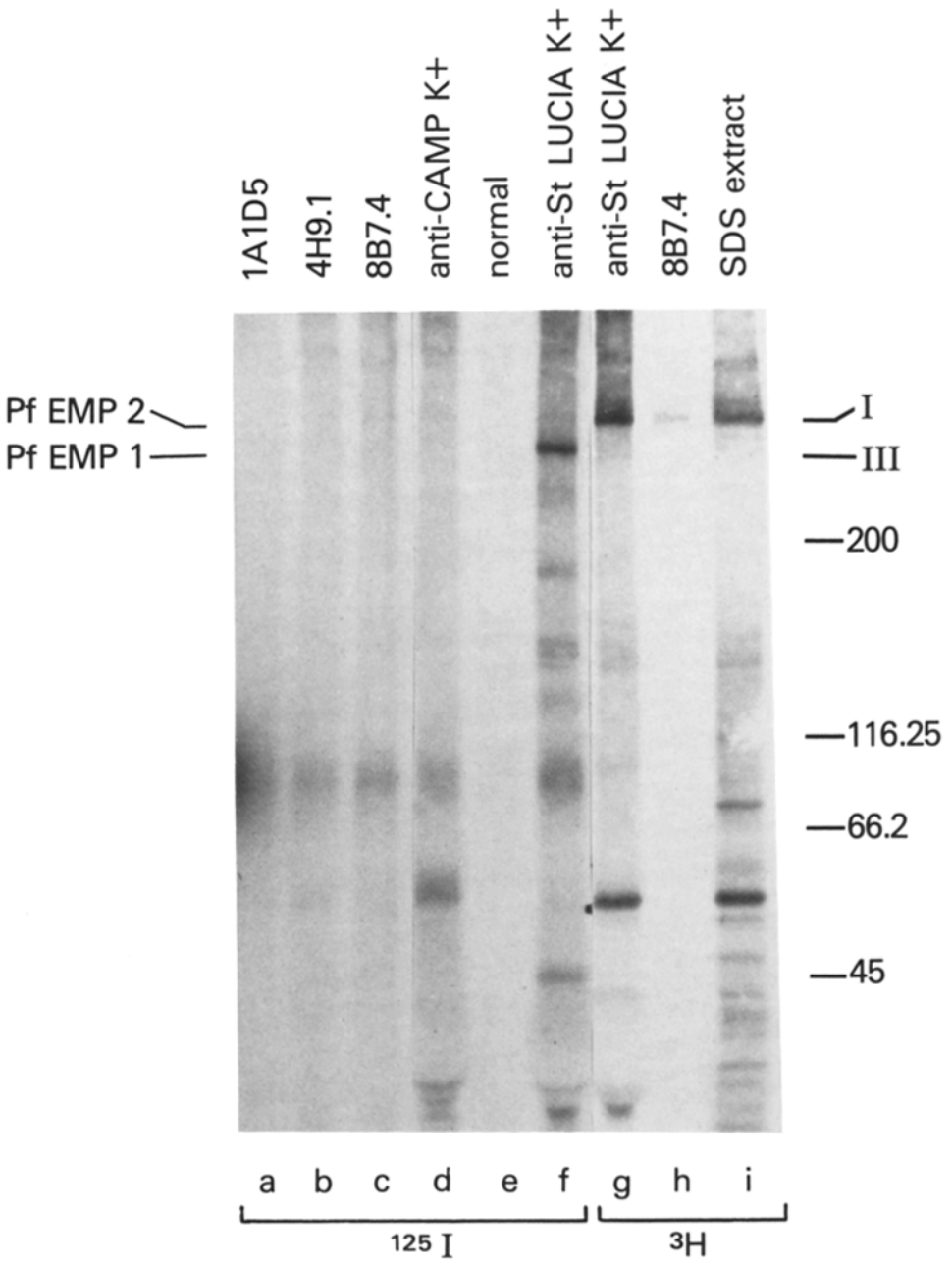


*Figure 7.* Comparison of biosynthetically radiolabeled Pf EMP 2 and cell-surface $^{125}$I-labeled Pf EMP 1 from St. Lucia parasites. SDS extracts were immunoprecipitated with control IgM mAb (1AID5); mAbs 4H9.1 or 8B7.4; an *Aotus* antiserum, which blocked in vitro cytoadherence of Malayan Camp infected cells but not St. Lucia infected cells (anti–Camp $K^+$); normal *Aotus* antiserum; or an *Aotus* antiserum which blocked in vitro cytoadherence of St. Lucia but not Malayan Camp infected cells (anti–St. Lucia $K^+$), and subjected to electrophoresis adjacent to the nonimmunoprecipitated [$^3$H]isoleucine-labeled SDS extract (lane *i*). Pf EMP 2 (relative mobility I), as defined by immunoprecipitation with mAbs 8B7.4 or 4H9.1, was $^3$H-labeled but incorporated very little $^{125}$I-radioactivity. Under the same conditions, Pf EMP 1 (relative mobility III) was strongly $^{125}$I-labeled and immunoprecipitated only by antiserum that can block in vitro cytoadherence. Pf EMP 1 was unreactive with mAbs.

munoprecipitated by mAbs co-migrated in both strains with the most heavily labeled malarial proteins in the SDS extracts of Triton X-100–insoluble material (relative mobilities II and I in lanes *a* and *b*, respectively, Fig. 1).

Immunoprecipitation of $^{125}$I- and $^3$H-labeled antigens was also performed with *Aotus* antisera known to specifically block cytoadherence of St. Lucia or Malayan Camp infected erythrocytes but not both strains (Fig. 7). Since the relative mobility of $^{125}$I-surface labeled Pf EMP 1 and biosynthetically labeled Pf EMP 2 were identical with Malayan Camp parasites, the most informative experiment is shown with St. Lucia parasites. mAb 8B7.4 immunoprecipitated a $^3$H-labeled molecule of relative mobility I (Fig. 7, lane *h*), but only immunoprecipitated a very low level of $^{125}$I-radioactivity at this position (Fig. 7, lane *c*). *Aotus* antiserum, which blocked in vitro cytoadherence of St. Lucia but not Malayan Camp parasites, immunoprecipitated a strongly labeled $^{125}$I-band at relative mobility III (lane *f*), whereas the *Aotus* antiserum capable of blocking Malayan Camp but not St. Lucia strain did not react with this band (lane *d*). The band at relative mobility III is therefore Pf EMP 1, the cytoadherence protein. mAbs 4H9.1 and 8B7.4 failed to immunoprecipitate any $^{125}$I-radioactivity at relative mobility III (lanes *b* and *c*), consequently they do not react with the Pf EMP 1 surface protein.

It is of interest to note that the hyperimmune *Aotus* antiserum specific for cytoadherence blockade of St. Lucia but not Malayan Camp parasites immunoprecipitated a biosynthetically labeled antigen with the same relative mobility as the Pf EMP 2 molecule immunoprecipitated by mAb 8B7.4 (relative mobility I; Fig. 7, lane *g*). There was a very faint band of $^3$H-radioactivity at relative mobility III in this gel lane

corresponding to the Pf EMP 1 protein. This result agrees with our previous observation that the amount of biosynthetically labeled Pf EMP 1 protein in the SDS extract of Triton X-100–insoluble protein is extremely small (20).

The immunoprecipitation results with Malayan Camp strain were quantitatively identical to those in Fig. 7 except that in this case the $^{125}$I- and $^{3}$H-bands co-migrated at relative mobility II. In this case also the mAbs immunoprecipitated a very small amount of $^{125}$I-radioactivity compared with the *Aotus* antiserum capable of blocking cytoadherence (not shown).

These results show that Pf EMP 2, as defined by mAbs 8B7.9 and 4H9.1, is different from Pf EMP 1.

### *Antibodies Specific for Pf EMP 2 Fail to Block Cytoadherence*

The cytoadherence of Malayan Camp strain-infected erythrocytes to C32 amelanotic melanoma cells was tested in the presence of the following antibodies: ion-exchange purified mAb 8B7.4 (100 μg/ml); mAb 8B7.4 or mAb 4H9.1 ascites (1:10 dilution); rabbit antiserum to Pf EMP 2 (1:10 dilution); mouse antiserum to a fusion protein that reacts with mAb 8B7.4 (1:10 dilution). These antibodies had no inhibitory effects, whereas antiserum from an *Aotus* monkey infected with Malayan Camp parasites blocked cytoadherence by 50% at 1:50 dilution. We conclude that the epitopes on Pf EMP 2 that are recognized by these antibodies do not appear to be involved in the cytoadherence properties of infected erythrocytes.

## *Discussion*

Our previous studies on the malarial proteins that contribute to the altered erythrocyte membrane properties of *P. falciparum*-infected cells identified a surface-exposed malarial protein of $M_r$ ~300,000, denoted Pf EMP 1, that could be linked to the capacity of infected erythrocytes to cytoadhere to endothelial cells (20). In this paper we have used two mAbs, 8B7.4 and 4H9.1, to identify another malarial protein, denoted Pf EMP 2, that shares the size ($M_r$ ~300,000) and host cell membrane association of Pf EMP 1, but differs from the latter molecule in its topological arrangement in the membrane. Pf EMP 2 is submembranous, located in the host cell cytoplasm and under knob protrusions of the host erythrocyte membrane. There is no evidence for a direct role of Pf EMP 2 in the cytoadherence property of infected erythrocytes.

Pf EMP 2 exhibits size diversity in different *P. falciparum* isolates in the range $M_r$ ~250,000–350,000. With the St. Lucia and Malayan Camp strains of *P. falciparum*, Pf EMP 2 had relative mobilities of I and II, respectively, whereas Pf EMP 1 had relative mobilities of III and II. Since these two proteins co-migrate with Malayan Camp parasites, the critical experiments to distinguish these proteins were performed with the St. Lucia strain. We have also used the mAbs to examine whether knobless variants of *P. falciparum* (i.e., $K^-$), which do not cytoadhere, also synthesize Pf EMP 2. All $K^-$ parasites examined to date, whether derived from *Aotus* monkey infections or long-term, culture-adapted parasites, express Pf EMP 2. The relative mobility of Pf EMP 2 was identical in $K^-$ and $K^+$ parasites derived from the same isolate (Howard, R., unpublished data).

The stage specificity of synthesis of Pf EMP 2 during the asexual cycle was similar to that of the knob protrusions at the host erythrocyte membrane, expression of Pf EMP 1 at the cell surface, and synthesis of another malarial protein (the histidine-rich protein, PfHRP 1) that we have also localized to the submembranous material at knobs (reviewed in reference 8). These changes in the host cell membrane are not evident with ring-stage parasites. Pf EMP 2 was synthesized first at the early trophozoite stage and localized to the intracellular parasite. As the trophozoites matured, immunofluorescence reactivity with the mAbs spread to small granules throughout host cell cytoplasm, and later, to the entire erythrocyte membrane. By the late-trophozoite/early-schizont stage all infected cells expressed Pf EMP 2 over the erythrocyte membrane.

Cryo–thin-section immunoelectron microscopy identified Pf EMP 2 within the malaria parasite, consistent with its synthesis as a malarial protein, as well as external to the intracellular parasite in the erythrocyte cytoplasm. The likely path of intracellular movement of Pf EMP 2 can be suggested from the facts that as a malarial protein it must originate within the parasite and that it is finally deposited at the host cell membrane. Dense aggregates of protein A–gold were evident as roughly circular electron-dense bodies that appear to bud from the parasitophorous vacuole membrane, which ensheaths the parasite. Beyond the parasitophorous vacuole membrane, in the cytoplasm of the host erythrocyte, Pf EMP 2 did not appear to be associated with unit membrane vesicles or membrane clefts (also called "Maurers clefts" in the malaria-infected erythrocyte). Occasionally, unit membrane whorls up to 1-μm diam surrounded an aggregate of Pf EMP 2, but the protein was generally associated with electron-dense material in putative spheres of 200–600-nm diam. These electron-dense aggregates of Pf EMP 2 were also seen abutting the surface membrane of infected erythrocytes. Dense aggregates of protein A–gold were only infrequently seen at knob protrusions by cryo–thin-section immunoelectron microscopy. Gold particles were never observed at the surface membrane in between knobs. The occasional identification of Pf EMP 2 under the surface membrane knobs by the cryo–thin-section technique indicates that this site must represent a significant proportion of the total Pf EMP 2, the knobs being much smaller (roughly 100-nm diam) and more numerous than the cytoplasmic spheres (200–600-nm diam). Immunoelectron microscopy of saponin-treated cells containing mature trophozoites and schizonts confirmed the localization of Pf EMP 2 at knobs. After saponin treatment, mAb 8B7.4 and 4H9.1 reacted with the electron-dense material under most, if not all knobs, but did not bind to the host cell membrane between knobs. In some experiments the electron-dense material under knobs was on the convex side of the knobs (i.e., on the external face of the surface membrane) and was also strongly reactive. Since nonsaponin-treated infected cells are nonreactive with the mAb and this extracellular electron-dense material was not seen by immunoelectron microscopy with untreated cells, we conclude that saponin can disrupt the knob structure to release electron-dense material from below the knob. The electron-dense spherical accretions of Pf EMP 2 in the host erythrocyte cytoplasm (200–600-nm diam) are presumably the precursors of the electron-dense material beneath knobs at the surface membrane (75–100-nm diam).

Parallel immunoelectron microscopy studies with an mAb specific for one of the *P. falciparum* histidine-rich proteins, Pf HRP 1 (or, "knob-associated histidine-rich protein"; 16, 19), also localized this molecule to the submembrane electron dense material at knobs (Taylor, D. W., M. Parra, G. B. Chapman, M. E. Stearns, J. Rener, M. Aikawa, S. Uni, S. B. Aley, L. J. Panton, and R. J. Howard, manuscript submitted for publication). Our combined evidence implicates both Pf EMP 2 and Pf HRP 1 in the submembrane structural and/or functional properties of knobs, but in neither case is there evidence for a direct role in the cell surface property of cytoadherence.

Pf EMP 2, defined by its reaction with mAbs 8B7.4 and 4H9.1, was clearly distinguished from Pf EMP 1 by several criteria other than the different relative mobilities of these proteins in St. Lucia parasites.

Pf EMP 2 was immunoprecipitated as a biosynthetically labeled protein by mAbs 8B7.4 and 4H9.1, whereas Pf EMP 1 was not immunoprecipitated as a surface $^{125}$I-labeled or biosynthetically labeled protein by either of these antibodies. The faster mobility of Pf EMP 1 versus Pf EMP 2 for St. Lucia parasites and difference in mAb reactivity are compatible with the possibility that Pf EMP 2 is an intracellular precursor of the surface-exposed Pf EMP 1 protein and that proteolytic cleavage coincidentally removes the epitopes defined by mAbs 8B7.4 and 4H9.1. If this possibility was also applicable to Malayan Camp parasites, in which the relative mobilities of Pf EMP 1 and Pf EMP 2 are indistinguishable, proteolytic cleavage would have to remove a relatively small peptide bearing the epitopes of both mAbs.

With St. Lucia parasites Pf EMP 2 was shown to be much more abundant than Pf EMP 1. Pf EMP 2 was a major biosynthetically labeled malarial protein and could be identified by Coomassie Blue staining in the Triton X-100–insoluble material. In contrast, at the mobility defined by $^{125}$I-labeled Pf EMP 1, very little if any biosynthetically incorporated radioactivity was identified in nonimmunoprecipitated or immunoprecipitated detergent extracts and there was no Coomassie Blue staining. This difference in abundance has been confirmed for five other *P. falciparum* isolates in which the mobilities of $^{125}$I-labeled Pf EMP 1 and biosynthetically labeled Pf EMP 2 are different (Howard, R., manuscript in preparation).

The dispositions of Pf EMP 1 and Pf EMP 2 in the host erythrocyte membrane were different. Pf EMP 1 is exposed on the surface of intact, unfixed infected cells (2, 20), whereas our evidence indicates that most, perhaps all, Pf EMP 2 is under the erythrocyte membrane, either in the erythrocyte cytoplasm or under the knobs in association with electron-dense material. A variety of specific antibody reagents were used to probe the accessibility of Pf EMP 2 at the surface of intact infected erythrocytes: mAbs 8B7.4 and 4H9.1; a rabbit antiserum raised against purified Pf EMP 2; and a mouse antiserum raised against a β-galactosidase fusion protein expressing a Pf EMP 2 epitope. None of these antibodies reacted at the cell surface, whereas the same reagents gave very strong immunofluorescence reactions (in a pattern suggesting the infected cell outer membrane) when the cells were air-dried and fixed in acetone or methanol. None of these antibodies affected cytoadherence in the in vitro assay, whereas antisera that bind to Pf EMP 1 at the cell surface do block cytoadherence (20, 29).

Pf EMP 2 was accessible to the mAbs with very mature infected erythrocytes containing the late segmenter stage of the parasite that had undergone obvious membrane rupture. Unfixed infected cells that had lost the host erythrocyte cytoplasm, as seen by light microscopy, were strongly fluorescence positive over the entire host cell membrane, whereas cells that had retained the host cytoplasm were negative. Even at the terminal stages of intraerythrocytic growth, Pf EMP 2 is not exposed at the surface of intact cells.

The susceptibilities of Pf EMP 1 and Pf EMP 2 to exogenous proteases were also different. $^{125}$I-surface labeled Pf EMP 1 is completely cleaved by addition of 0.1–1 μg/ml trypsin to intact infected cells and incubation for 5 min at 23°C. These conditions also ablate cytoadherence (20). Treatment of intact infected cells with up to 1 mg/ml exogenous trypsin or up to 0.1 mg/ml α-chymotrypsin had no effect on the immunoreactivity or mobility of Pf EMP 2 as measured by indirect immunofluorescence and immunoprecipitation. A small proportion of Pf EMP 2 may have been protease-cleaved; however, no faster mobility bands were observed after prolonged overexposure of these gels.

The results of $^{125}$I-protein labeling using the lactoperoxidase method also indicated that at most only a very small proportion of Pf EMP 2 is exposed at the cell surface. Immunoprecipitation of St. Lucia parasites with mAb 8B7.4 generally failed to yield a $^{125}$I-labeled radioactive band at relative mobility I or III. In some experiments a very low level of $^{125}$I-radioactivity was immunoprecipitated by mAb 8B7.4 at the mobility corresponding to Pf EMP 2; however, in each case $^{125}$I-radioactivity was also incorporated into spectrin, indicative of some permeation of the labeling probe. With all samples, including those from which $^{125}$I-label could not be immunoprecipitated by mAb 8B7.4, we were able to immunoprecipitate $^{125}$I-labeled Pf EMP 1.

One novel aspect of these results is the concept that an obligate intracellular parasite has engineered a system for transporting a protein through the cytoplasm of its host cell for that protein to associate with membranes beyond its plasma membrane. Since the host cell, as a mature, non-nucleated erythrocyte is devoid of protein-synthetic machinery and the machinery for membrane biogenesis and protein trafficking, all changes in the properties and composition of the host cell cytoplasm and surface membrane must ultimately be under control of the parasite. Some malarial proteins such as the S-antigen (5) are exported from the parasite and accumulate in the parasitophorous vacuole, which ensheaths the intracellular parasite. For these proteins there is no need to postulate special mechanisms for passage through the parasite plasma membrane other than those involved in secretion of soluble proteins from typical eukaryotic cells. In contrast, passage of Pf EMP 2 through the topological barrier of the parasitophorous vacuole membrane as well as the host cell cytoplasm implies that other extra-parasitic mechanisms for protein transport are involved. We have also identified an extra-parasitic route of protein secretion for a soluble histidine-rich protein, Pf HRP 2, that is released from intact infected cells (13); however, we do not know whether Pf HRP 2 destined for secretion, and Pf EMP 2 destined for insertion into the host membrane are exported in association or separately. The extra-parasitic machinery involved in export of these proteins represents the extraordinary extent to which the intracellular malaria parasite has supplemented the properties

of the host erythrocyte to create a parasitized cell with entirely new biological properties.

The authors are grateful to Dr. D. Alling for statistical analysis and Dr. L. Miller for helpful discussions during this study (both from National Institute of Allergy and Infectious Diseases, National Institutes of Health). We also thank the staff and patients of the Medical Research Council Laboratories, Fajara, The Gambia, for provision of *P. falciparum* isolates, and Wilma Davis and Brenda Martin for skillful assistance in manuscript preparation.

Edwin Rock was supported by the Howard Hughes Medical Institute. Lindsey Panton was supported by a grant from the World Health Organization (WHO)-Special Programme for Research and Training in Tropical Diseases (TDR). Shigehiko Uni and Masamichi Aikawa were supported by the United Nations Development Programme/World Bank/WHO Special Programme for Research in Tropical Diseases, a U.S. Public Health Service research grant (AI-10645), and the U.S. Army Research and Development Command (DAMD 17-79-C-9029).

Received for publication 7 October 1986, and in revised form 9 January 1987.

*Note Added in Proof.* A recent study (Coppel, R. L., J. G. Culvenor, A. E. Bianco, P. E. Crewther, H-D Stahl, G. V. Brown, R. F. Anders, and D. J. Kemp, 1986, *Mol. Biochem. Parasitol.*, 20:265-277) described a $M_r$ ~250,000 heterogeneous *P. falciparum* protein (denoted MESA) associated with the surface membrane of infected erythrocytes, using human antibodies affinity purified on extracts from a cDNA clone expressing a partial gene sequence. It was suggested that this cDNA clone corresponded to part of Pf EMP 1. We have prepared rabbit antisera raised against a hexapeptide repeat encoded by the MESA sequence (GESKET) and have shown that these sera do not react with $^{125}$I-surface labeled Pf EMP 1, but react with Pf EMP 2 as defined here by mAbs 8B7.4 and 4H9.1 and rabbit antiserum to purified Pf EMP 2 (Howard, R. J., W. L. Maloy, J. A. Lyon, and A. Saul, manuscript submitted for publication).

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