

# Secretion of a Malarial Histidine-rich Protein (Pf HRP II) from *Plasmodium falciparum*-infected Erythrocytes

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**Abstract.** *Plasmodium falciparum*-infected erythrocytes (IRBCs) synthesize several histidine-rich proteins (HRPs) that accumulate high levels of [<sup>3</sup>H]histidine but very low levels of amino acids such as [<sup>3</sup>H]isoleucine or [<sup>35</sup>S]methionine. We prepared a monoclonal antibody which reacts specifically with one of these HRPs (Pf HRP II) and studied the location and synthesis of this protein during the parasite's intracellular growth. With the knob-positive Malayan Camp strain of *P. falciparum*, the monoclonal antibody identified a multiplet of protein bands with major species at  $M_r$  72,000 and 69,000. Pf HRP II synthesis began with immature parasites (rings) and continued through the trophozoite stage. The  $M_r$  72,000 band of Pf HRP II, but not the

faster moving bands of the multiplet, was recovered as a water-soluble protein from the culture supernatant of intact IRBCs. Approximately 50% of the total [<sup>3</sup>H]histidine radioactivity incorporated into the  $M_r$  72,000 band was extracellular between 2 and 24 h of culture. Immunofluorescence and cryothin-section immunoelectron microscopy localized Pf HRP II to several cell compartments including the parasite cytoplasm, as concentrated "packets" in the host erythrocyte cytoplasm and at the IRBC membrane. Our results provide evidence for an intracellular route of transport for a secreted malarial protein from the parasite through several membranes and the host cell cytoplasm.

THE avian malaria *Plasmodium lophurae* and the major pathogenic human malaria *P. falciparum* synthesize proteins of extraordinary histidine content called histidine-rich proteins (HRPs).<sup>1</sup> Asexual blood stage *P. lophurae* parasites synthesize an HRP (P1 HRP) containing 72 mol % histidine according to amino acid analysis (8) and sequencing of genomic DNA (17). Despite the fact that in duckling infections it can account for 10% of the total parasite dry weight (8) its function is unknown. At least three HRPs are made by blood stage *P. falciparum*, denoted here as Pf HRP I, II, and III in order of discovery. Pf HRP I is associated phenotypically with expression of knob-like protrusions on the surface membrane of infected erythrocytes (IRBCs) (9). Pf HRP I appears to be associated with the IRBC membrane (23), particularly the cytoskeleton (13). Pf

HRP II, described in detail in this report, is expressed by both knob-positive (K<sup>+</sup>) and knob-negative (K<sup>-</sup>) IRBCs (13), unlike Pf HRP I which is only expressed by K<sup>+</sup> IRBC (5, 9, 24). Sequencing of genomic DNA has shown that Pf HRP II contains 35% histidine, as well as relatively high contents of alanine and aspartate (40 and 12%, respectively) (25). Pf HRP III has been recently identified by sequencing a cDNA clone and shown to contain 30% histidine and 29% alanine (19).

Since the roles that these HRPs play in the complex interaction of blood-stage malaria parasite and vertebrate host are unknown, and the structure-function relationship of proteins with such high contents of imidazole groups are still a matter for conjecture, we have prepared monoclonal antibodies (mAb's) to dissect some of their properties. This report describes the synthesis and subcellular location of Pf HRP II using one of these antibodies (mAb 87). We show that although a multiplet of bands react with mAb 87 in IRBCs, only one of these bands is secreted into the culture supernatant. By immunoelectron microscopy, we could identify Pf HRP II in several compartments including the host erythrocyte cytoplasm. Our results provide direct evidence for intra-

1. *Abbreviations used in this paper:* [<sup>3</sup>H]-HIS, [<sup>3</sup>H]histidine; HRP, histidine-rich protein; [<sup>3</sup>H]-ILEU, [<sup>3</sup>H]isoleucine; IRBC, infected erythrocyte; K<sup>+</sup>, knobby; K<sup>-</sup>, knobless; mAb, monoclonal antibody; Pf HRP I, *Plasmodium falciparum* histidine-rich protein I synthesized only by K<sup>+</sup> parasites; Pf HRP II, *Plasmodium falciparum* histidine-rich protein II synthesized by K<sup>+</sup> and K<sup>-</sup> parasites and reactive with mAb 87; RBC, erythrocyte.

cellular trafficking of malarial proteins within IRBCs and have several implications for the extent to which the intracellular parasite can not only alter the properties of the host erythrocyte (RBC), but affect the host through secretion of soluble proteins.

## Materials and Methods

### Culture and Metabolic Labeling

Cryopreserved IRBCs of the uncloned K<sup>+</sup> Malayan Camp strain of *P. falciparum*, derived from *Aotus* monkeys (13), were thawed (15) and fractionated on a Percoll gradient containing sorbitol (1) to remove any leukocytes and mature IRBCs. The purified ring-IRBCs (5–30% parasitemia) were washed twice in RPMI 1640 and resuspended in culture medium (22). Metabolic labeling with 100  $\mu$ Ci/ml of [<sup>3</sup>H]histidine ([<sup>3</sup>H]-HIS) or [<sup>3</sup>H]isoleucine ([<sup>3</sup>H]-ILEU) was performed as described earlier (13) with 2–3  $\times$  10<sup>7</sup> IRBCs/ml and hematocrits of 2–6%.

### Collection of Supernatants and Cells from Culture

Cultures were harvested by low-speed centrifugation (300 g, 5 min); the supernatant was collected and centrifuged again (18,000 g, 30 min at 4°C). A portion of the second supernatant was mixed with an equal volume of 2X SDS sample buffer for SDS PAGE analysis. The remaining supernatant was stored at –90°C. A third centrifugation of the supernatant (110,000 g, 40 min at 23°C) had no effect on the SDS PAGE pattern of soluble radiolabeled proteins.

The pelleted cells from low-speed centrifugation were washed once in RPMI, resuspended in PBS (phosphate-buffered saline; 0.15 M NaCl, 20 mM sodium phosphate, pH 7.3) at 5  $\times$  10<sup>8</sup> RBCs/ml, 50  $\mu$ l removed for SDS PAGE analysis; and the remaining cells were extracted with detergents.

### Detergent Extraction of IRBCs

IRBCs were resuspended in PBS (5  $\times$  10<sup>8</sup> RBC/ml) and a mixture of protease inhibitors added with vortexing to the following final concentrations: 1 mM leupeptin, 1 mM chymostatin, 1 mM pepstatin, 2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride (PMSF) (all from Sigma Chemical Co., St. Louis, MO). An equal volume of 2% Triton X-100 in PBS was added, the sample vortexed, and incubated at 0°C for 30 min. After centrifugation (18,000 g, 30 min) the supernatant (i.e., Triton X-100 extract) was removed. A 50- $\mu$ l portion of supernatant was mixed with 50  $\mu$ l of 2X SDS sample buffer for SDS PAGE. The remaining Triton X-100 extract was stored at –90°C. The pellet (i.e., Triton X-100-insoluble material) was washed once with 1% Triton X-100 in PBS (18,000 g, 30 min) and extracted by vortexing for 10–30 min at 23°C with 2% SDS in PBS (in a volume equivalent to 10<sup>9</sup> RBC/ml). Centrifugation (18,000 g, 30 min, 23°C) fractionated this sample into 2% SDS-soluble material (i.e., supernatant) and an insoluble pellet. A portion of the supernatant was mixed with an equal volume of 2X SDS sample buffer for SDS PAGE analysis and the remainder stored at –90°C.

### Production of Monoclonal Antibodies and Antiserum

BALB/c mice were immunized intraperitoneally with 1  $\times$  10<sup>7</sup> purified IRBCs of Malayan Camp strain, K<sup>+</sup> *P. falciparum* emulsified in complete Freund's adjuvant. Immunization was repeated at 2 and 7 wk using IRBCs in incomplete Freund's adjuvant and finally at 14 wk without adjuvant. 3 d later, spleen cells from the immunized mouse were fused with myeloma cell line P3-NSI/1-Ag 4-1 (11) according to the method of Galfré et al. (4). Cells were plated directly into 660 microtiter wells and cultured (18) such that 2 wk later, 1 or 2 hybrids were observed in all wells. Culture supernatants were collected and screened by radioimmunoassay using intact normal *Aotus* RBC or K<sup>+</sup> or K<sup>-</sup> infected *Aotus* RBC coated onto plastic microtiter wells and <sup>125</sup>I-goat anti-mouse immunoglobulin. 90% of the wells contained mAb's against K<sup>+</sup> and/or K<sup>-</sup> IRBC but not normal *Aotus* RBCs. These cells were expanded and cryopreserved. Secondary screening was performed by immunoprecipitation and SDS PAGE with supernatants from expanded cultures that had been concentrated roughly 10-fold by centrifugation with Amicon Centriflo Membrane Cones. Cells producing mAb's that immunoprecipitated a protein labeled with [<sup>3</sup>H]-HIS but not with [<sup>3</sup>H]-ILEU were passaged into pristane-primed mice for ascites production. In this way we identified mAb 87 which immunoprecipitated Pf HRP II (see

Results). Ouchterlony analysis of mAb 87 from culture supernatants and immunoelectrophoresis of ascites showed that this antibody was homogeneous and of the IgM ( $\mu$ k) isotype.

Rabbit antiserum against the *P. lophurae* HRP was prepared by immunization with 0.3 mg of protein (purified according to the original protocol [8]) emulsified in incomplete Freund's adjuvant. Serum was collected 1 mo after three intramuscular injections at intervals of 6 wk and 6 mo.

### Immunofluorescence and Immunoelectron Microscopy

Immunofluorescence was performed with a 1:100 dilution of mAb 87 ascites (or control IgM mAb 1A1D5) and fluorescein-conjugated goat anti-mouse Ig (Cappel Laboratories, Cochranville, PA) using thin smears of infected blood fixed for 1 s in 100% acetone at 23°C. For immunoelectron microscopy, infected blood was mixed with 1.0% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min at 4°C. Specimens were embedded in 30% bovine serum albumin (BSA) cross-linked with 0.5% glutaraldehyde in phosphate buffer for 10 min, then incubated in 1.3 M sucrose in phosphate buffer for 3 h on a rotator and frozen in liquid nitrogen. Thin sections were cut on a Sorvall MT5000 Ultramicrotome at –91.5°C with an FS1000 Cryo Sectioning Accessory, picked up on a freezing drop of 2.3 M sucrose in phosphate buffer, and transferred onto ionized carbon-coated formvar grids, according to the methods of Tokuyasu (20). After washing in PBS, pH 8.6, containing 0.01 M glycine (PBS-G), the sections were incubated with 2% gelatin in PBS-G for 10 min, washed with PBS-G, and incubated overnight at 23°C on a drop of monoclonal antibody solution diluted with PBS-1% BSA. The specimens were washed with PBS-1% BSA, incubated 2 h at 23°C on a drop of 20-nm gold particles bound to protein A (Polysciences Inc., Warrington, PA), washed with PBS-G, and fixed with 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Staining was performed using 0.5% osmium tetroxide in phosphate buffer, pH 7.4; 2% uranyl acetate in 0.15 M oxalic acid, pH 7.0; and 3% aqueous uranyl acetate. The sections were embedded in a mixture of 2% methylcellulose and 2% polyethylene glycol (*M*, 1,540) in distilled water (4:3:3; vol/vol/vol). To stain membranes, 3  $\mu$ l of 0.8% aqueous uranyl acetate was included per ml of embedding medium. The dried specimens were examined in a JEOL 100CX electron microscope.

### Immunoprecipitation and Immunoblotting

Supernatants from culture and Triton X-100 extracts of washed IRBCs were used directly for immunoprecipitation. SDS extracts were diluted with Triton X-100 solution before addition of antibody (6). Immunoprecipitation was performed by standard methods (6) using 100  $\mu$ l–1.0 ml of antigen solution and 20  $\mu$ l of rabbit antiserum, 200–400  $\mu$ l of hybridoma supernatant, and 3–5  $\mu$ l of mouse ascites. Antigen-antibody complexes were purified using Protein A-Sepharose as solid-phase adsorbent (6). In some experiments, 3  $\mu$ l of rabbit antiserum to mouse IgM (generously provided by Dr. R. Asofsky, National Institute of Allergy and Infectious Diseases) was incubated with the primary complexes of mAb 87 for 2 h at 23°C, however, since mAb 87 binds directly to Protein A this did not affect the results.

Immunoblotting was performed with washed IRBCs or culture supernatants solubilized in 1X SDS sample buffer (21). For electrophoretic transfer to nitrocellulose (21) (30 V for 16 h at 4°C) we used 0.5 M glycine, 50 mM Tris base in 20% vol/vol methanol. After quenching in PBS plus 0.3% wt/vol Tween 20 and 0.3% wt/vol bovine serum albumin, sample strips were incubated 1–2 h at 23°C in a 1:100 dilution of ascites in PBS plus 0.05% wt/vol Tween 20. Bound mAb 87 was detected by incubation with rabbit anti-mouse IgM serum (1:1,000 dilution, 1 h at 23°C) and <sup>125</sup>I-Protein A (1 h at 23°C). The nitrocellulose was dried onto Gelbond (FMC Corporation, Marine Colloids Div., Rockland, ME) for autoradiography.

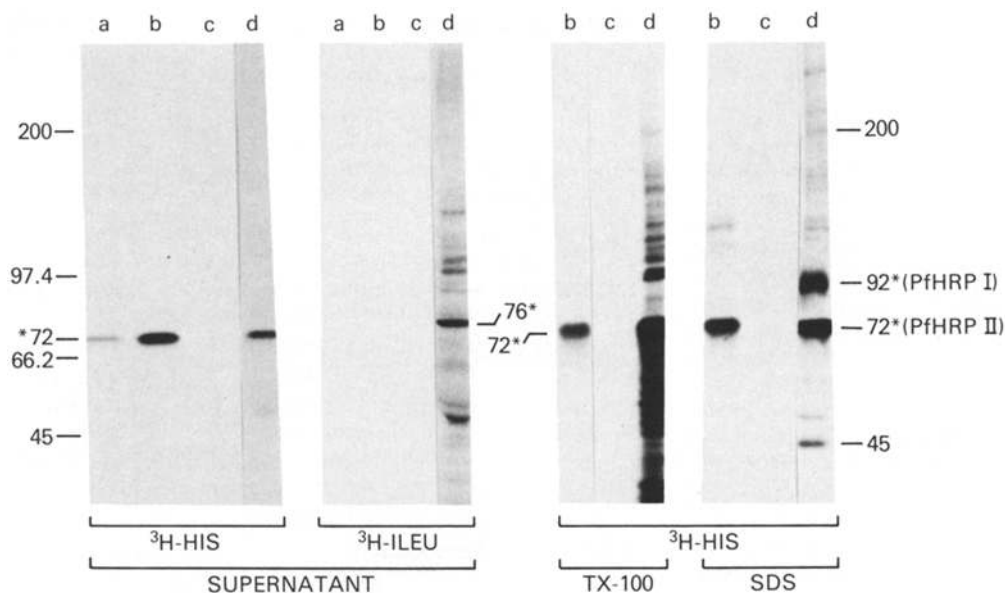
### SDS PAGE and Radioactivity Analysis

SDS PAGE was performed on 1.5-mm-thick gels of 5–15% acrylamide gradients using the Laemmli buffer system (12). The distribution of [<sup>3</sup>H]radioactivity was determined by fluorography (2). Radioactivity in individual protein bands on dried gels was determined after solubilization with Protosol (New England Nuclear, Boston, MA) as described earlier (7).

## Results

### mAb 87 Reacts Specifically with Pf HRP II

Pf HRP II has been described for K<sup>+</sup> Malayan Camp strain



**Figure 1.** Identification of the antigen recognized by mAb 87 by immunoprecipitation of biosynthetically radiolabeled culture supernatant (*SUPERNATANT*), and sequential Triton X-100 and SDS extracts of washed IRBC (*TX-100* and *SDS*, respectively). Lanes *d* show the  $^3\text{H}$ -HIS or  $^3\text{H}$ -ILEU-labeled proteins in each sample before immunoprecipitation. Lanes *b* and *c* show results for mAb 87 and a control IgM mAb (1A1D5), respectively. For the supernatant, lanes *a* show results of immunoprecipitation with rabbit antiserum to the *P. lophurae* HRP. Pf HRP II is identified with an asterisk at  $M_r$  72,000. Pf HRP I is also labeled with an asterisk at  $M_r$  92,000. The  $^3\text{H}$ -ILEU labeled band at  $M_r$  76,000 in the culture supernatant did not co-migrate with Pf HRP II. Molecular mass standards ( $\times 10^{-3}$ ) are indicated with dashes.

*P. falciparum* as a cell-associated  $M_r$  72,000 protein strongly labeled by biosynthetic uptake of  $^3\text{H}$ -HIS but not with  $^3\text{H}$ -ILEU or  $^{35}\text{S}$ methionine (13). Pf HRP II was also expressed by a  $\text{K}^-$  variant of the Malayan Camp strain. In contrast, the  $M_r$  92,000 knob-associated HRP (Pf HRP I) of the same strain was expressed by the  $\text{K}^+$  parasites but not by the  $\text{K}^-$  variant (13). These two HRPs are identified in the extreme right lane of Fig. 1, which shows the SDS PAGE pattern of  $^3\text{H}$ -HIS-labeled proteins of  $\text{K}^+$  parasites extracted with SDS. It should be noted that uptake of  $^3\text{H}$ -amino acids into protein by IRBCs implies biosynthetic incorporation by the malaria parasite since in control cultures of uninfected erythrocytes none of the proteins described here are radiolabeled.

mAb 87 specifically immunoprecipitated an  $M_r$  72,000  $^3\text{H}$ -HIS-labeled band from sequential Triton X-100 and SDS extracts of washed IRBCs (lanes *b*, *TX-100* and *SDS*, Fig. 1). Immunoprecipitation with a control IgM mAb (1A1D5) is shown for comparison (lanes *c*, *TX-100* and *SDS*, Fig. 1). Four pieces of evidence indicate that the antigen immunoprecipitated from IRBCs by mAb 87 is Pf HRP II. (a) The immunoprecipitated antigen has the same  $M_r$  as the  $^3\text{H}$ -HIS-labeled protein in both detergent extracts (compare lanes *b* and *d*, Fig. 1). The protein in these cell extracts characteristically migrates as a broad band with a sharp trailing edge at  $M_r$  72,000 and diffuse leading edge extending to  $M_r$   $\sim$ 60,000. mAb 87 immunoprecipitated an antigen band with the same  $M_r$  range and appearance. (b) The protein immunoprecipitated by mAb 87 is strongly labeled by  $^3\text{H}$ -HIS uptake but not at all with  $^3\text{H}$ -ILEU,  $^3\text{H}$ leucine, or  $^{35}\text{S}$ methionine. (c) mAb 87 also immunoprecipitates an antigen with the same properties as described in points *a* and *b* from the  $\text{K}^-$  variant of Malayan Camp parasites. Thus, expression of the antigen recognized by mAb 87 is not related

to knob-phenotype. mAb 87 also immunoprecipitated an HRP (with slightly different  $M_r$ ) from two other pairs of  $\text{K}^+$  and  $\text{K}^-$  parasites (Howard, R., manuscript in preparation). (d) The antigen immunoprecipitated from IRBCs by mAb 87 co-migrates with a protein immunoprecipitated by antiserum to the *P. lophurae* HRP (not shown). Such rabbit antisera have been shown to cross-react with both Pf HRP I and Pf HRP II of *P. falciparum* (10).

Immunoblotting with mAb 87 (Fig. 2) confirmed the reactivity of this antibody with Pf HRP II. mAb 87 failed to react with proteins of uninfected *Aotus* RBCs transferred to nitrocellulose, indicating that the antibody is specific for an IRBC antigen. The antibody reacted with a multiplet of bands from  $\text{K}^+$  IRBC, the slowest moving component at  $M_r$  72,000, with faster components, or a smear, extending to  $M_r$   $\sim$ 50,000. Fig. 2 shows four discrete bands at  $M_r$  72,000, 63,000, 58,000 and 49,000. The same pattern of mAb 87 reactivity was observed with immunoblots of the  $\text{K}^-$  variant of the same strain (Howard, R., manuscript in preparation).

#### Release of Pf HRP II into Culture Supernatant

Pf HRP II was identified in supernatants of cultured IRBCs by immunoprecipitation (Fig. 1) and immunoblotting using mAb 87. Fig. 1 shows a single sharp band at  $M_r$  72,000 immunoprecipitated from culture supernatants. This band was strongly labeled after biosynthetic labeling with  $^3\text{H}$ -HIS, but was not labeled with  $^3\text{H}$ -ILEU (compare lanes *b*, *SUPERNATANT*, Fig. 1),  $^3\text{H}$ leucine, or  $^{35}\text{S}$ methionine. The extracellular antigen immunoprecipitated by mAb 87 co-migrated with an antigen immunoprecipitated by rabbit antiserum to the *P. lophurae* HRP (lanes *a*, *SUPERNATANT*, Fig. 1), confirming its identity as an HRP. Identical results were obtained with the  $\text{K}^-$  variant (not shown). Immunoblotting

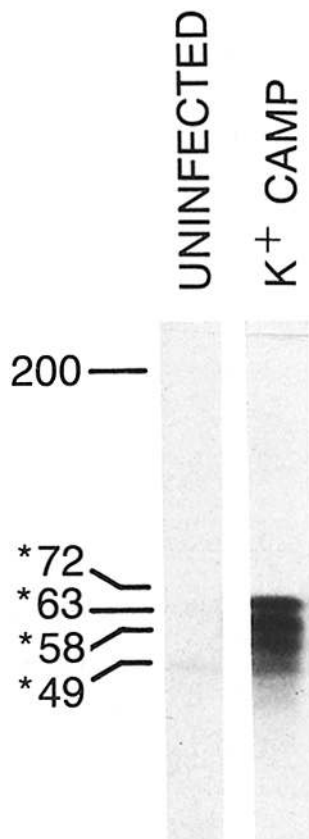


Figure 2. Comparison of the reactivity of mAb 87 with uninfected and *P. falciparum*-infected *Aotus* RBCs, washed uninfected RBCs, or blood containing 30% late trophozoite-infected RBCs. Cells were solubilized in 5% SDS sample buffer, fractionated by SDS PAGE, and electrophoretically transferred to nitrocellulose. After incubation with mAb 87 and rabbit anti-mouse IgM, immune complexes were identified by reactivity with <sup>125</sup>I-Protein A and autoradiography. Antigens discussed in the text are identified by an asterisk and their molecular mass ( $\times 10^{-3}$ ).

of culture supernatants with mAb 87 also identified a single sharp band of antigen reactivity at  $M_r$  72,000. This supernatant antigen corresponded in apparent  $M_r$  to the trailing edge of the Pf HRP II band associated with washed IRBCs. Thus, mAb 87 reacts with a multiplet of Pf HRP II in IRBCs ( $M_r$  72,000–50,000) and a single antigenically related band of  $M_r$  72,000 in culture supernatants.

### Kinetics of HRP Synthesis and Release of Pf HRP II into Supernatants

A kinetic experiment was performed to examine the relationships between the extracellular Pf HRP II, the intracellular Pf HRP II, and the pool of Pf HRP I that previous studies have associated with intracellular or membrane fractions of IRBCs. It was also of interest to determine whether Pf HRP II was released from intact IRBCs before the time of cell rupture and parasite reinvasion. Purified IRBCs containing a synchronous population of immature ring-stage parasites were cultured directly with [<sup>3</sup>H]-HIS and samples of culture supernatant and cells collected at various times and electrophoresed. The results of SDS PAGE and fluorography for the washed cells and the 24 h supernatant are shown in Fig. 3. HRPs were excised from the gels and uptake of [<sup>3</sup>H]-HIS radioactivity into individual bands was measured (Fig. 4).

During the first 16 h of in vitro culture with [<sup>3</sup>H]-HIS, as ring stages developed into trophozoites, a doublet of proteins,  $M_r$  72,000 and 69,000, were the quantitatively major [<sup>3</sup>H]-HIS labeled proteins associated with washed IRBCs. From parallel cultures with [<sup>3</sup>H]-ILEU in which these bands were not radiolabeled we could identify both of these proteins as HRPs. This  $M_r$  72,000 HRP corresponds in  $M_r$  with slowest moving Pf HRP II antigen reactive with mAb 87 by both immunoprecipitation (Fig. 1) and immunoblotting (Fig. 2). The  $M_r$  69,000 HRP was particularly well resolved from the  $M_r$  72,000 band in Fig. 3. It corresponds to part of the faster moving zone of Pf HRP II ( $M_r$  50,000–72,000) seen in other experiments (see Figs. 1 and 2).

Although ring-stage malaria parasites are relatively inactive in protein synthesis (16), it was notable that synthesis of the  $M_r$  72,000 and  $M_r$  69,000 HRP occurred in the first 2 h of culture. The  $M_r$  69,000 band did not continue to accumulate additional radioactivity after 12 h. In contrast, radioactivity accumulated continuously up to 20–24 h in the  $M_r$  72,000 protein. The  $M_r$  72,000 protein was found in the culture supernatant and in the washed IRBCs, while the  $M_r$

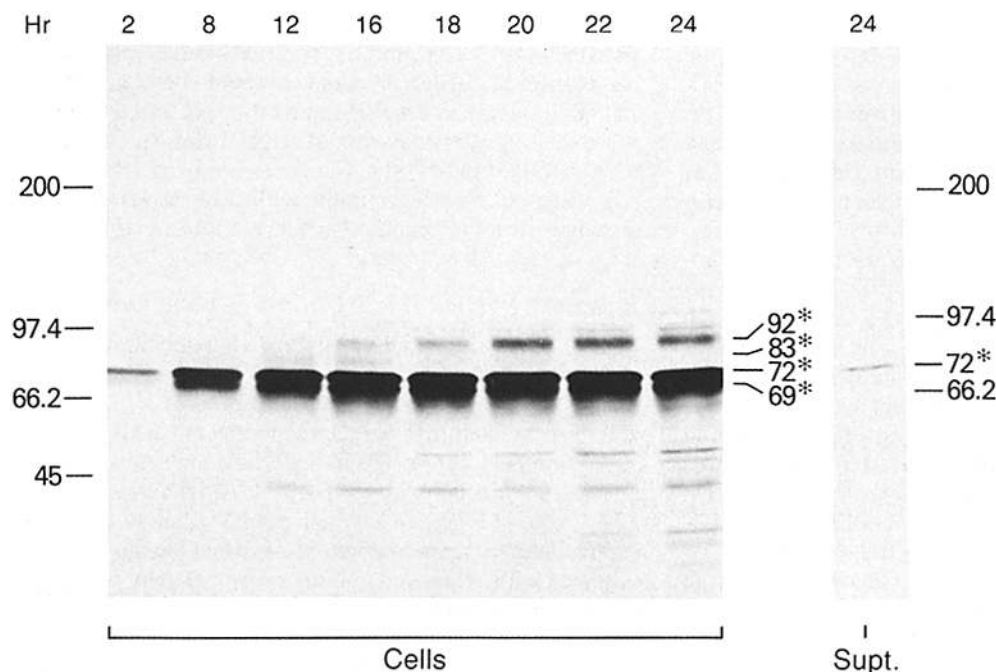
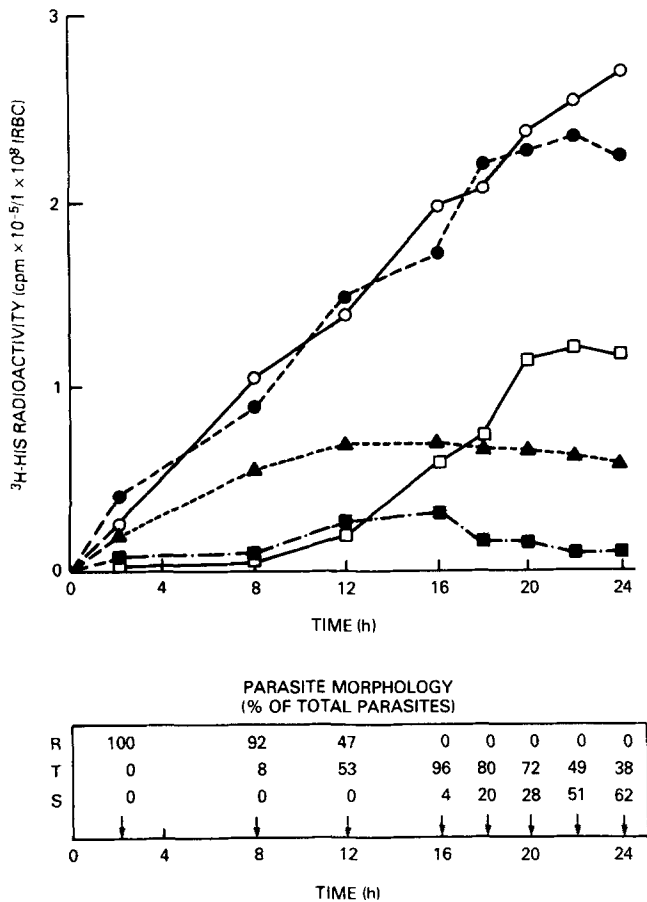


Figure 3. Kinetics of uptake of [<sup>3</sup>H] radioactivity from [<sup>3</sup>H]-HIS into HRP of K<sup>+</sup> Malayan Camp strain *P. falciparum* (35% parasitemia) analyzed by SDS PAGE and fluorography. Parasites were cultured continuously with [<sup>3</sup>H]-HIS from the ring-stage to late-trophozoites and sampled at different times (shown above each gel lane). The washed infected blood (Cells) and culture supernatant after high speed centrifugation (Supt.) were analyzed separately. Proteins discussed in the text are labeled with an asterisk. Molecular mass standards ( $\times 10^{-3}$ ) are indicated by dashes.



**Figure 4.** Quantitative analysis of the kinetics of labeling HRP during synchronous parasite growth. After fluorography of the gel shown in Fig. 3, sections of the gel were excised and their [ $^3\text{H}$ ] radioactivity counted. (○)  $M_r$  72,000 HRP in culture supernatant; (●)  $M_r$  72,000 HRP in IRBC; (▲)  $M_r$  69,000 HRP in IRBC; (□)  $M_r$  92,000 HRP in IRBC; (■)  $M_r$  83,000 HRP in IRBC. At the times indicated by arrows the parasite morphology is shown as the percentage of total parasite as rings (R), trophozoites (T), or schizonts (S). Similar results were obtained in another experiment with a different cryopreservate.

69,000 protein was only recovered from the washed IRBCs (Fig. 3). Approximately half the total radioactivity in the  $M_r$  72,000 protein was found in the supernatant at any time (Fig. 4). This protein was the dominant [ $^3\text{H}$ ]-HIS-labeled protein in the culture supernatant such that at the film exposure time shown in Fig. 3 it was the only component detected (see also Fig. 1, lane d, *SUPERNATANT*).

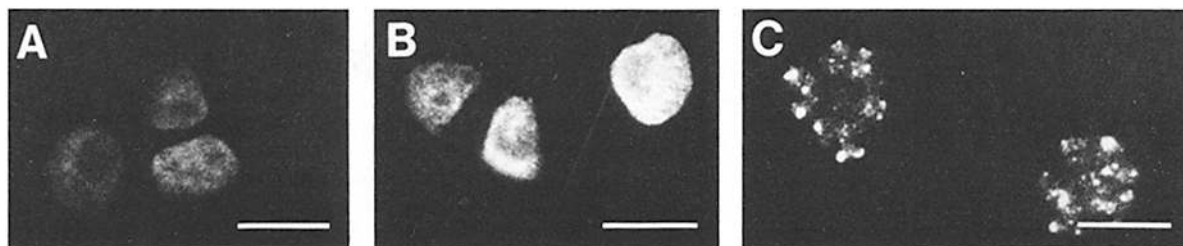
The knob-associated HRP of Malayan Camp strain parasites (Pf HRP I) first incorporated [ $^3\text{H}$ ]-HIS between 8 and 12 h of culture, steadily accumulated more radioactivity up to 20 h of labeling and then failed to accumulate additional radioactivity (Figs. 3 and 4). Another [ $^3\text{H}$ ]-HIS-labeled protein at  $M_r$  83,000 incorporated low level radioactivity at 2 and 8 h, increased to maximal uptake of 8–12 h, and contained less radioactivity thereafter (Figs. 3 and 4). In other experiments we have established that this protein is antigenically cross-reactive with Pf HRP I (Leech, J., unpublished results). The flow of [ $^3\text{H}$ ]-HIS radioactivity through this  $M_r$  83,000 protein and its antigenic relatedness to Pf HRP I are compatible with the hypothesis that this molecule is a precursor for Pf HRP I. An analogous relationship was proposed for an  $M_r$  75,000 HRP of FCR-3/Gambia strain  $K^+$  parasites and the  $M_r$  80,000–85,000 Pf HRP I of this parasite (10).

### Immunofluorescence and Immunoelectron Microscopy with mAb 87

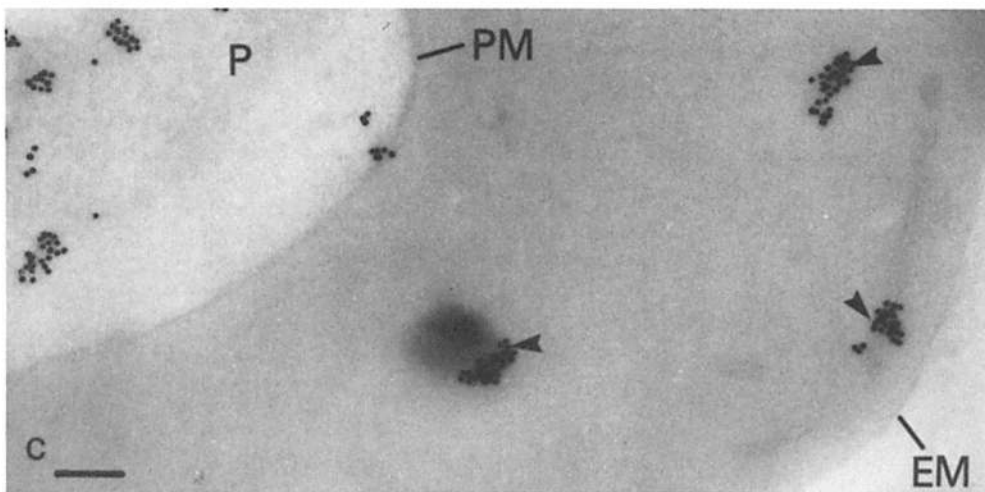
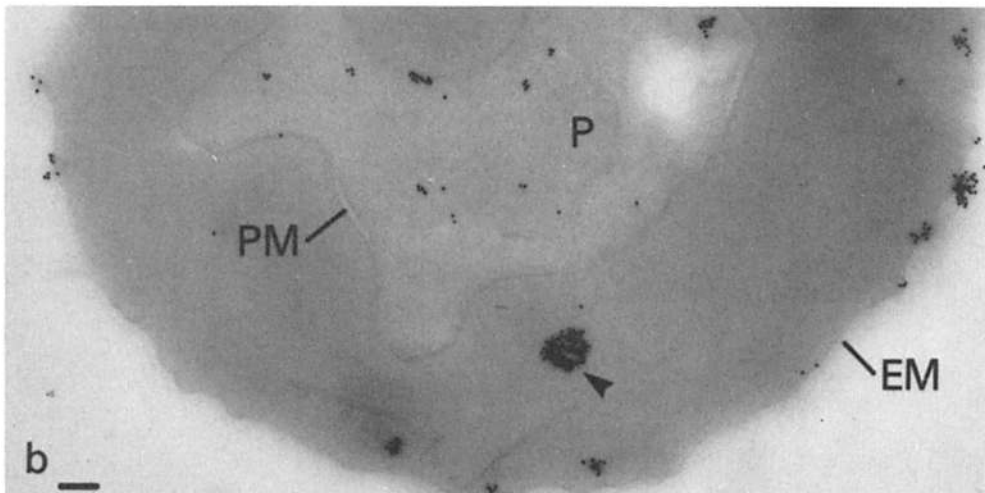
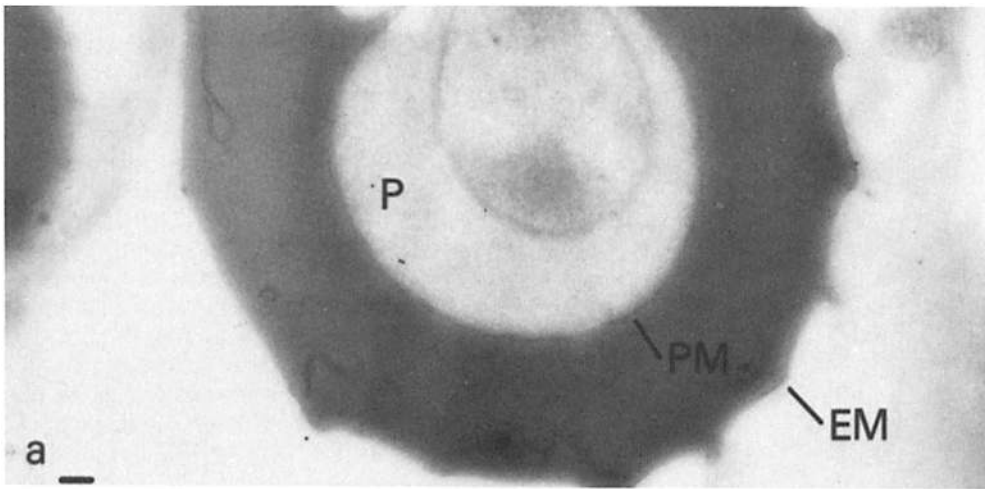
The immunofluorescence results with mAb 87 and acetone-fixed IRBCs of  $K^+$  Malayan Camp Strain parasites are shown in Fig. 5. Identical results were obtained with  $K^-$  parasites of the same strain. mAb 87 reacted in a diffuse granular pattern over the entire IRBC containing ring, trophozoite, or early schizont stages. This implies the presence of the antigen recognized by mAb 87 in the host cell cytoplasm. Often, less fluorescence was seen over part of the IRBC that appeared to correspond to the location of the malaria parasite (Fig. 5, a and b). The fluorescence intensity increased as the parasite matured (compare Figs. 5, a and b). With mature IRBC in which individual merozoites had segmented, the immunofluorescence pattern was quite different. A pronounced granular pattern was observed at this stage with the antigen concentrated in very small areas localized to individual parasites. Uninfected RBCs were always negative by immunofluorescence.

These results are consistent with the identification of the antigen recognized by mAb 87 (Pf HRP II) as a malarial protein that is synthesized and present throughout the asexual growth cycle. The presence of strong immunofluorescence in the host cell cytoplasm is also consistent with the release of Pf HRP II from intact IRBCs and the fact that this antigen must somehow traverse the host cell cytoplasm to be released as a soluble protein.

The subcellular distribution of Pf HRP II was examined in greater detail by cryothin-section immunoelectron microscopy using mAb 87 (Fig. 6). The location of bound mAb 87



**Figure 5.** Indirect immunofluorescent pattern of reactivity of mAb 87 with  $K^+$  Malayan Camp (A and B) or  $K^-$  Malayan Camp (C) *P. falciparum* (in *Aotus* RBC). The parasites were young trophozoites in A, late trophozoites and schizonts in B, and segmenters in C. The immunofluorescence patterns for  $K^+$  and  $K^-$  parasites were identical when compared at the same parasite stage. No fluorescence was observed in controls omitting primary antibody or using a control mAb as primary antibody. Bar, 10  $\mu\text{m}$ .



**Figure 6.** Immunoelectron microscopy of mature trophozoites of K<sup>+</sup> Malayan Camp *P. falciparum* with (a) a control IgM mAb (1A1D5) or (b and c) mAb 87. In a, the ascites was diluted to 1:400, and in b and c, the ascites was diluted to 1:6,000 for reaction with the frozen section. Addition of secondary antibody (rabbit anti-mouse Ig) prior to Protein A-gold had no effect on the results (a and c show the results without secondary antibody). Identical results to a were obtained if primary antibody was omitted and Protein A-gold added either directly or after incubation with secondary antibody. P, parasite; PM, parasite plasma membrane; EM, erythrocyte plasma membrane. Dense packets of Protein A-gold particles reacting with the cytoplasm of the host erythrocyte are indicated by arrows in b and c. Bars, 0.5  $\mu$ m.

was identified by the pattern of Protein A-conjugated colloidal gold particles. Gold particles were associated with IRBCs of all stages of parasite maturity but not with uninfected RBCs. They occurred as clusters within the cytoplasm of the intracellular malaria parasite, as very dense clusters or "packets" within the cytoplasm of the host cell, and as peripheral clusters at and just exterior to the IRBC surface membrane (Figs. 6, b and c). The dense "packets" of gold

particles in the host cell cytoplasm were often but not invariably associated with a localized deposit of electron-dense material (e.g., Fig. 6 c). These "packets" of gold particles were occasionally seen at the unit membrane vesicles (or "Maurer's clefts") in the host cell cytoplasm, but more often these clefts were entirely devoid of gold particles (e.g., Fig. 6 c). The gold clusters associated with the periphery of the IRBCs were generally immediately under the IRBC mem-

brane (Fig. 6 b), suggestive of an intracellular location for Pf HRP II just before release. The peripheral clusters occasionally appeared to be extracellular but it is impossible to exclude the possibility that the RBCs membrane was sectioned tangentially at these points. In some sections, the peripheral clusters were located at knobs (e.g., Fig. 6 b), but there were many sections in which no knob protrusions of the IRBC membrane bound colloidal gold.

The patterns of reactivity for Protein A-gold were shown to reflect specific binding of mAb 87. In the absence of mAb 87 or with a control monoclonal as primary antibody, very few gold particles were associated with IRBCs (Fig. 6 a). When particles did appear in these control sections they were present as single, or at most, two particles together, unlike the clusters of up to ~80 particles seen with mAb 87.

The reactivity of mAb 87 within the malaria parasite confirms the identification of Pf HRP II as a malarial protein. It was noted that in any section of trophozoites probed with mAb 87 there were less gold particles in the parasite than in the host cell cytoplasm, consistent with the immunofluorescence data and the release of >40% of Pf HRP II into culture supernatants. The presence of gold particles in "packets" in the host cell cytoplasm and at the IRBC periphery is also consistent with the release of the Pf HRP II antigen from intact IRBCs.

## Discussion

This report has demonstrated that a *P. falciparum* HRP, Pf HRP II, is synthesized by the intraerythrocytic asexual parasite and exported through the erythrocyte cytoplasm and surface membrane to accumulate in the extracellular culture supernatant. An IgM mouse monoclonal antibody (mAb 87) was shown to specifically react with Pf HRP II, thereby allowing us to study both the molecular form of this antigen by immunoprecipitation and immunoblotting and the subcellular location of Pf HRP II by immunofluorescence and cryothin-section immunoelectron microscopy.

Previous reports distinguished Pf HRP II from the two other HRP of *P. falciparum* according to its expression by both  $K^+$  and  $K^-$  parasites (10, 13) (unlike Pf HRP I which is only expressed by  $K^+$  parasites [5, 9, 13, 24]) and its  $M_r$  on SDS PAGE (Pf HRP III or SHARP is considerably smaller,  $M_r$  ~21,000 [19]). mAb 87 further distinguishes Pf HRP II since this antibody did not react with the other HRPs by immunoprecipitation or immunoblotting (Figs. 1 and 2).

Several lines of evidence show that Pf HRP II as identified here with mAb 87 is in fact "histidine rich." First, immunoprecipitation of biosynthetically radiolabeled malarial proteins with mAb 87 showed that Pf HRP II was labeled by uptake of [ $^3$ H]-HIS but not by uptake of [ $^3$ H]-ILEU, [ $^3$ H]leucine, or [ $^{35}$ S]methionine (e.g., Fig. 1). This result has been obtained with several isolates of  $K^+$  or  $K^-$  parasites cultured in *Aotus* or human erythrocytes (not shown). The analogous protein ( $M_r$  65,000) of FCR-3/Gambia strain parasites was also found to incorporate [ $^3$ H]-HIS but no detectable labeled proline, serine, or lysine (10). Second, mAb 87 has been used to screen a  $\lambda$ GT 11 genomic expression library of *P. falciparum* and identified a fusion protein with a peptide sequence corresponding to part of the gene encoding Pf HRP II (25). The deduced protein sequence from sequencing genomic DNA contains 35% histidine and nu-

merous tandem repeats of Ala-His-His-Ala-Ala-Asp or Ala-His-His (25). Third, Pf HRP II (as immunoprecipitated by mAb 87) co-migrated with a [ $^3$ H]-HIS-labeled antigen immunoprecipitated by rabbit antiserum against the *P. lophurae* HRP (Fig. 1). Such rabbit antisera, raised against a protein containing 72 mol % histidine, appear to immunoprecipitate several HRPs from *P. falciparum* (reference 10, and Howard, R., unpublished data), presumably through immunocross-reactivity with sequences of contiguous histidine residues.

The recovery of Pf HRP II from culture supernatants also distinguishes this HRP from Pf HRP I (comparable information is not yet available for Pf HRP III). Pf HRP I is only solubilized from IRBCs by treatment with 1-2% SDS (reference 13, and see Fig. 1) or by freeze-thaw of parasites and suspension in pH 8.5 buffer containing 2% Triton X-100 and 5 mM EDTA (10). In contrast, Pf HRP II was identified as a water-soluble extracellular protein that could not be pelleted by centrifugation (110,000 g, 40 min), as well as a component of washed IRBCs (Fig. 1).

Extracellular Pf HRP II could arise by artifactual lysis of immature IRBCs, by lysis of mature IRBCs at the time of parasite liberation and reinvasion of new RBCs, or by a specific process of release from intact IRBCs containing living parasites. The following results demonstrate that Pf HRP II is released from RBCs containing growing parasites. Pf HRP II was recovered from 2 and 8 h culture supernatants of ring-IRBCs that had been purified before in vitro culture in such a way that mature parasitized IRBCs were removed. The ring-infected RBCs are much less likely to lyse artifactually in vitro than mature IRBCs. Furthermore, immunofluorescence and immunoelectron microscopy localization studies with acetone or paraformaldehyde/glutaraldehyde-fixed IRBCs, respectively, identified Pf HRP II in the cytoplasm of the IRBCs (Figs. 5 and 6). In particular, the electron microscopy studies identified "packets" of Pf HRP II en route to the RBC membrane and under this membrane in cells with morphologically intact, normal parasites. Thus, Pf HRP II is exported from the parasite to the culture supernatant as a normal metabolic process.

The kinetics of synthesis of Pf HRP II during the asexual growth cycle also distinguish Pf HRP II from Pf HRP I. Pf HRP II incorporated [ $^3$ H]-HIS radioactivity within 2 h when immature parasites (ring-stages) were cultured, whereas Pf HRP I was not labeled until between 6 and 10 h of culture (Figs. 3 and 4). An earlier study showed that Pf HRP I was first synthesized 9 h after merozoite reinvasion and correlated its synthesis and recovery from purified erythrocyte membranes with the time of appearance of knobs at the erythrocyte membrane (23). It should be noted that not only is synthesis of Pf HRP II separated kinetically from knob synthesis, but that Pf HRP II is synthesized by both  $K^+$  and  $K^-$  *P. falciparum* (10, 13). Our kinetic studies also identified an HRP of  $M_r$  83,000 that appears to be a kinetic precursor of the  $M_r$  92,000 Pf HRP I molecule (Figs. 3 and 4). Similar results were obtained with the FCR-3/Gambia strain of parasites wherein an  $M_r$  72,000 precursor of an  $M_r$  85,000 Pf HRP I was identified (10), although a separate set of experiments failed to identify a precursor to Pf HRP I (23).

One of the most intriguing properties of Pf HRP II is the identification of a multiplet of bands reactive with mAb 87 with washed IRBCs ( $M_r$  ~50,000-72,000), yet recovery of only one of these bands ( $M_r$  72,000) in the extracellular

medium (Fig. 1). This property is shared by at least five other isolates of *P. falciparum*: only the slowest moving band associated with IRBCs is exported from the cells (Howard, R., unpublished results). We do not know the structural basis for the differences in apparent  $M_r$  on SDS PAGE for the multiple Pf HRP II bands found within IRBCs. These cell-associated forms of Pf HRP II appear to be different to the extracellular Pf HRP II, since although hypotonic lysis of IRBC releases some of these bands into the supernatant, the bulk of radioactivity in the Pf HRP II multiplet requires Triton X-100 or SDS to effect solubilization (Fig. 1). Since the apparent  $M_r$  of the extracellular Pf HRP II is higher than that of some of the cell-associated bands, proteolysis alone is probably not the mechanism for generation of the water-soluble Pf HRP II exported from the cell.

Our studies on the subcellular localization of Pf HRP II visualized export of this protein from the parasites via the host cell cytoplasm, to the extracellular medium. They also raise several fascinating questions that await additional experiments. Indirect immunofluorescence of acetone-fixed IRBCs with mAb 87 localized Pf HRP II within the asexual parasite (rings, trophozoites, and schizonts) and also within the cytoplasm of the host RBC (Fig. 5). This was confirmed more precisely by cryothin-section immunoelectron microscopy using mAb 87 and Protein A-gold (Fig. 6). Gold particles were evident as very dense clusters or "packets" in the RBC cytoplasm, some of which were associated with electron-dense material of unknown composition, while other clusters were associated with the unit membrane clefts (Maurer's clefts). Many gold particles were detected directly under the RBC membrane. The presence of dense "packets" of Pf HRP II in the host RBC cytoplasm and under the RBC membrane is consistent with the recovery of Pf HRP II in the culture supernatant and the implication that Pf HRP II must traverse the host cytoplasm. These results provide the first detailed evidence for the subcellular route of export of a malarial antigen from the intraerythrocytic parasite to (and in this case, through) the host erythrocyte membrane. It will be of great interest to determine whether other malarial antigens, especially membrane-associated proteins destined for expression on the surface of the IRBC (14), move independently of Pf HRP II through the RBC cytoplasm. We would predict that separate subcellular routes and mechanisms of antigen trafficking must exist in malaria-infected cells for water-soluble and membrane-associated antigens. Additional studies are also required to explain how Pf HRP II traverses the two membrane barriers that appear to separate the intraerythrocytic parasite from the extracellular medium (i.e., the parasitophorous vacuole membrane sheathing the parasite and the host RBC membrane). From our immunoelectron microscopic results, there is no obvious morphological explanation for movement of the dense "packets" of Pf HRP II through these topological barriers.

The function of Pf HRP II for the malaria parasite has not yet been explored. Since at any time during the asexual cycle roughly 50% of this protein was extracellular (Fig. 4), it is likely that it has some special function in the extracellular medium. In vivo, this would correspond to the blood plasma. By radioimmunoassay, we have identified an antigen in malarious plasma or sera which reacts with mAb 87 (Taylor, D., unpublished data). An assay for circulating Pf HRP II may be useful for diagnosis of low levels of blood parasites.

One speculative role of HRP in plasma during malaria stems from in vitro studies demonstrating an inhibitory effect of L-histidine on neutrophil-mediated intracellular killing of *P. falciparum* (3). Neutrophils can mediate intracellular killing of asexual *P. falciparum* parasites via the release of free radicals (3). The capacity of L-histidine to react with free radicals and thereby neutralize their effect on malaria parasites could be fulfilled in vivo by the high histidine content of Pf HRP II released into the plasma.

To conclude, using an mAb that reacts specifically with Pf HRP II, we have shown this protein to be very different to Pf HRP I, or the knob-associated HRP of *P. falciparum*, in that it is exported from living infected cells into the extracellular medium. This study has identified a subcellular route of trafficking of Pf HRP II from the parasite, through the host cell cytoplasm, to the plasma, where we presume this unusual protein mediates a function for the intracellular malaria parasite. Numerous questions remain to be addressed concerning not only its function, but the structural basis for its size heterogeneity within the cell and its precise mechanism of export.

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*Note Added in Proof:* Using a rabbit antiserum raised against a pentapeptide repeat of Pf HRP III (25) we have identified this HRP as a quantitatively minor biosynthetically labeled protein of  $M_r$  45,000 with Malayan Camp *P. falciparum*. mAb 87 cross-reacts weakly with Pf HRP III on immunoprecipitation. A rabbit antiserum raised against the hexapeptide repeat of Pf HRP II (25) does not cross-react with Pf HRP III (or Pf HRP I). The results of immunofluorescence and cryothin-section immunoelectron microscopy with the rabbit antiserum to Pf HRP II are identical to those reported here for mAb 87.

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