Expression of a *Plasmodium falciparum* Histidine-rich Protein II (HRPII) Reveals Sorting of Soluble Proteins in the Periphery of the Host Erythrocyte and Disrupts Transport to the Malarial Food Vacuole*

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The heme polymer hemozoin is produced in the food vacuole (fv) of the parasite after hemoglobin proteolysis and is the target of the drug chloroquine. A candidate heme polymerase, the histidine-rich protein II (HRPII), is proposed to be delivered to the fv by ingestion of the infected-red cell cytoplasm. Here we show that 97% of endogenous *Plasmodium falciparum* (Pf) HRPII (PfHRPII) is secreted as soluble protein in the periphery of the red cell and avoids endocytosis by the parasite, and 3% remains membrane-bound within the parasite. Transfected cells release 90% of a soluble transgene PfHRPII-ynmc into the red cell periphery and contain 10% membrane-bound within the parasite. Yet these cells show a minor reduction in hemozoin production and IC$_{50}$ for chloroquine. They also show decreased transport of resident fv enzyme PfPlasmepsin I, the endoplasmic reticulum (ER) marker PfBiP, and parasite-associated HRPII to fvs. Instead, all three proteins accumulate in the ER, although there is no defect in protein export from the parasite. The data suggest that novel mechanisms of sorting (i) soluble antigens like HRPII in the red cell cytoplasm and (ii) fv-bound membrane complexes in the ER regulate parasite digestive processes.

*Plasmodium falciparum* is a protozoan parasite that causes the most virulent of human malarias (1, 2). During the blood stages of infection, it invades and develops within a parasitophorous vacuolar membrane (PVM) in a red cell (3). The parasite exports numerous proteins to the cytoplasm and membrane of the red cell (4). Association with the host skeleton or insertion across its membrane is thought to be required to keep the protein in the periphery of the red cell. Soluble exported proteins are expected to be ingested with hemoglobin and delivered to the fv within the parasite (3, 5). The parasite ingests and degrades ~80% of the hemoglobin in the red cell (3, 5). Ingestion occurs via a specialized organelle called the cytostome, which is a double membrane invagination of the parasite plasma membrane (PPM) and PVM (see Fig. 1). The cytostome pinches off to form a large, double membrane vesicle containing hemoglobin that fuses with the fv where hemoglobin is degraded (Fig. 1). The released, toxic-free heme is detoxified by polymerization to hemozoin (6, 7). Heme polymerization is also likely to be the main target of chloroquine, of which the emergence of drug resistance is a major problem in controlling malaria (8–10).

Histidine-rich proteins have been shown to function as heme polymerases *in vitro*, and the best characterized of these proteins is *P. falciparum* HRPII (or PfHRPII) (11–13). This protein has been detected in the red cell as well as the fv, leading to the suggestion that it is ingested from the red cell by the cytostome along with hemoglobin and subsequently delivered to the fv. Early studies also suggest that resident fv proteases synthesized in a membrane-bound pro-form are transported to the PVM and then retrieved into the newly developing cytostome, which ingests hemoglobin (11, 14). This has rendered attractive a general model that both membrane and lumenal components of food vacuole may be delivered to the PVM and the red cell from where they are internalized into the fv (5).

This is because (as indicated earlier) soluble proteins in the red cell cytoplasm are not expected to be segregated. Yet whether cytosolic antigens in the red cell can avoid ingestion has never been evaluated. In mammalian cells and yeast, lumenal, resident proteases are thought to be exported from the ER and sorted in the Golgi for transport to the lysosomes and fv, respectively (15, 16), but to what extent this model serves in lower eukaryotic pathogens like the malaria parasite is not known.

The function of HRPII in heme polymerization has been difficult to evaluate in intact, infected red cells. Although the heme polymerase activity (which is ascribed to the histidine-rich protein) emission in-lens scanning electron microscopy; BiP, luminal binding protein.
Transport of HRPII in Plasmodium-infected Red Cells

Immunolocalization Assays—Infected red cells were attached to poly-l-lysine-coated cover slips (20). Where indicated, the cells were permeabilized in 0.01% saponin to release red cell contents. Intact or permeabilized cells were subsequently fixed with 1% paraformaldehyde for 10 min and permeabilized with 0.05% saponin for 20 min. The cells were incubated with 0.2% Triton X-100 for 30 min followed by a second incubation with primary antibodies and secondary antibodies (diluted with PBS containing 0.01% saponin). Isolated vesicles were allowed to air dry and then were permeabilized with saponin and probed with primary and secondary antibodies. Epifluorescence images (30 optical sections of 0.2-mm thickness) were collected using a DeltaVision microscope with a 100× oil immersion objective (NA 1.4) attached to a cooled charge-coupled device (CCD) camera. A series of single optical sections were obtained, and the images were deconvolved on a SGI work station by using SoftWoRx Version 2.5.

Preparation of Lysates for SDS-PAGE and Western Blots—Saponin permeabilization was carried out by mixing infected erythrocytes with 10 volumes of 0.01% saponin followed by incubating at 4 °C for 10 min (this selectively lyses erythrocyte membrane, tubovesicular membrane (TVN), PVM but is expected to leave intact the parasite plasma membrane). The saponin lysates were subjected to centrifugation at 2,200 × g for 10 min, and the supernatant (enriched in the erythrocyte cytosolic content) was collected. The pellet containing isolated parasites was washed three times with cold PBS. Supernatant and pellet fractions were incubated for 30 min with glutamate dehydrogenase activity (by measuring the change in A500 as a result of the conversion of NADPH to NADP+ as described by Vander Jagt et al. (23)) to determine whether the parasite plasma membrane had ruptured or parasites contaminated the supernatant fraction. Hypotonic lysates were prepared by mixing infected cells with 10 volumes of water followed by vigorous vortexing in the presence of protease inhibitors. The lysis was subjected to centrifugation at 100,000 × g, the supernatant and pellet fractions were collected. The samples obtained after saponin treatment or hypotonic lysis were solubilized in 5 volumes of 1× SDS-PAGE sample buffer, boiled for 3–5 min, separated by SDS-PAGE, and transferred to nitrocellulose filters. The filters were incubated with 5% nonfat dry milk for 30 min and subsequently incubated with either anti-HRPII antibody (1:1000) or anti-C-myc antibody (1:500) for 1 h at room temperature. They were then washed with Tris-buffered saline containing 0.05% Tween 20 and incubated with peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature. The bands were identified with ECL solutions as per the manufacturer’s protocol.

Quantitative Measurement of Hemozoin Content—To release hemozoin from parasites, infected cells were first lysed with 0.01% saponin for 30 min at room temperature to release parasites from red cell ghosts. The parasites were washed 3 times with PBS, resuspended in 2.5% SDS in PBS, and subjected to centrifugation at 20,000 × g for 1 h. The supernatant was discarded, and the insoluble pellet was washed again in 2.5% SDS in PBS and then dissolved in 20 mM NaOH. The hemozoin content was measured by determining the absorbance at 400 nm and using a standard curve generated with heme.

Food Vacuole Isolation—Food vacuoles were isolated as described by the method of Goldberg et al. (31). Briefly, cells were harvested by centrifugation, washed 3× with PBS, resuspended in 5 volumes of 5% sorbitol, and incubated at room temperature for 20 min. The lysate was centrifuged at 600 × g for 7 min. The resulting supernatant was centrifuged at 2200 × g for 10 min and the pellet was treated with saponin and processed as described by Goldberg et al. (31). The resulting lysate was layered on top of a Percoll gradient. Isolated vacuoles were collected from the bottom fraction. The enzyme plasmepsin I was used as a marker for purification. Isolated food vacuole fraction showed 10-fold enrichment with a yield of 30%.

Biosynthetic Labeling of the Parasite and Analysis of Metabolically Labeled Exported Proteins—Red cells (1×107) infected with either 3D7 or E8 (15–20% parasitemia) were washed twice with methionine-free RPMI and incubated in 10 ml of PBS containing 4.5 mg/ml glucose and 500 μCi of [35S]methionine-[35S]cysteine at 37°C for 2 h. The cells were collected by centrifugation and washed 3 times with PBS at 4°C. The cells were lysed with 0.01% saponin at room temperature for 10 min and subjected to centrifugation at 2200 × g for 10 min. The supernatant (red cell cytosol) was removed and processed for SDS-PAGE. The gel was dried and exposed to film to analyze the pattern of exported proteins in the different lines.

Flow Cytometry—1×106 cells from E8 or 3D7 cultures were washed in PBS and fixed in 1% formaldehyde in PBS for 15 min. Excess aldehyde was removed, and the cells were incubated with anti-HRPII antibody for 30 min followed by FITC-conjugated secondary antibody for 30 min. The parasite nuclei were stained with rich sequences of this protein and other histidine-rich proteins and peptides has been demonstrated in vitro (11, 13), a laboratory cell line with a natural deletion in HRPII and HRPIII can still produce hemozoin (11). Thus, additional histidine-rich proteins may contribute to hemozoin production. Our understanding has been further limited by the fact that although there is evidence that PHRPII is delivered to the infected red cell cytosol and the fv as well as the extracellular medium, a detailed characterization quantitating the distribution of the protein and its transport properties has not been undertaken. Analysis of the primary structure of HRPII using Signal P suggests an N-terminal signal sequence and predicted cleavage site. However, the values for both are low, and this raises further questions of whether the protein is efficiently recruited into the ER-Golgi secretory pathway in its transport to diverse cellular destinations.

In this study, we have transfected P. falciparum-infected red cells to express PHRPII tagged with a C-terminal c-Myc epitope under continuous selection. Using high resolution microscopy and biochemical cell fractionation assays, we have characterized the distribution of the native protein and its Myc-tagged counterpart in the parasite and the red cell to understand their functional consequences. The specific questions we asked were the following. (i) Are PHRPII and PHRPIImyc exported to the red cell and subsequently endocytosed into the parasite or released across the red cell membrane? (ii) Are PHRPII and PHRPIImyc detected within the parasite and the fV? (iii) Are these proteins recruited into the secretory pathway, and do cells expressing HRPIImyc show perturbations in secretory organization, protein export to the red cell and/or the fV? (v) Do the transformed cells show changes in heme polymerization and/or sensitivity to chloroquine?

MATERIALS AND METHODS

Culturing of Parasites and Drug Treatments—RPML medium 1640 and A+ human serum were obtained from Invitrogen and Gemini Biological Products (Calabasas, CA), respectively. Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences. FITC- or rhodamine-conjugated secondary antibodies were from Cappel, ICN. Monoclonal mouse anti-c-Myc antibodies, 9E10, were purchased from Sigma. Antibodies to PHRP II (HG12, 87) were obtained from Dr. David Ward. Antibodies to plasmepsin I were generated in the Goldberg laboratory (17). Antibodies to PfBiP and PfERD2 were developed in the Haldar laboratory. P. falciparum strains of 3D7, ACPGF, and E8 were cultured in vitro by a modification of the method of Trager and Jensen (18) and Haldar et al. (19). To examine stage-specific events (from 6 to 42 h), parasites were synchronized to 4 h after transfection, pyrimethamine (Sigma) was added to the culture media and maintained at an osmolarity of 0.31 KV and 950 microfarads. Forty-eight h after transfection, pyrimethamine (Sigma) was added to the culture media and maintained at a concentration of 100 ng/ml for 2 days. The concentration of drug was subsequently reduced to 25 ng/ml and maintained at 25 ng/ml. Parasites were cloned by serial dilution to isolate pyrimethamine-resistant HRPII expressing P. falciparum E8 clone.
propidium iodide (10 μl/ml). Where indicated, after fixation the cells were permeabilized by treatment with 0.05% saponin for 20 min and subsequently processed for antibody binding. Samples were analyzed on a BD PharMingen FACSscan (FACSCALIBUR). Data was analyzed with CELLQuest software, and fluorescence histograms and dot plots were displayed on a four-decade logarithmic scale.

RESULTS

Characterization of the Expression and Distribution of Endogenous PfHRPII in the Parasite and the Red Cell during Asexual Development—Plasmodium parasites develop within the parasitophorous vacuole (white) that resides in the erythrocyte. The PVM is connected to a tubovesicular membrane (TVN) network of nutrient transport. Hemoglobin (light gray) is delivered to the parasite by the formation of a double-walled cytostome. Active ingestion of hemoglobin and hemozoin (hz) production is maximal at the late ring and trophozoite stages and is completed by schizogony. Other soluble proteins (including parasite antigens exported to the red cell), indicated by black dots, are also expected to be delivered to the parasite by this pathway. N represents the nucleus. PPM, parasite plasma membrane.

FIG. 1. Asexual life cycle of P. falciparum in red cells. Infection of the red cell is initiated by the extracellular merozoite, which then develops through morphologically distinct ring, trophozoite, and schizont stages. Plasmodium parasites develop within the parasitophorous vacuole (white) that resides in the erythrocyte. The PVM is connected to a tubovesicular membrane (TVN) network of nutrient transport. Hemoglobin (light gray) is delivered to the parasite by the formation of a double-walled cytostome. Active ingestion of hemoglobin and hemozoin (hz) production is maximal at the late ring and trophozoite stages and is completed by schizogony. Other soluble proteins (including parasite antigens exported to the red cell), indicated by black dots, are also expected to be delivered to the parasite by this pathway. N represents the nucleus. PPM, parasite plasma membrane.
chase times and quantitative immunoprecipitations were not provided. This makes it difficult to evaluate how much of the export detected was due to partial cell lysis. Pulse-chase studies are difficult to undertake with PfHRPII because the protein is methionine-poor and, thus, cannot be metabolically labeled with [35S]methionine with any efficiency.

Expression of a Transgene PfHRPIImyc during Asexual Development—To further examine the relationship between the exported and parasite-associated pools of protein, we expressed in trans-PfHRPII tagged at its C terminus with Myc (see Fig. 3A) under the cam promoter using the pHCI plasmid to establish the transformed E8 line (see “Materials and Methods”). Our unpublished studies suggested that cam is active at the trophozoite stage (data not shown). Western blots (Fig. 3B) show that the expression of Myc-tagged HRPII protein in transformed E8 cells is detectable in 18-h rings. Low levels of HRPIImyc are also expressed in 12-h rings and can be detected in single cells by immunofluorescence assays (Fig. 3A, ii) or by loading 2 × 10^6 parasite equivalents of the supernatant (S) and parasite pellet (P) fractions were prepared for SDS-PAGE and Western blots (see “Materials and Methods”) and probed for PfHRPII (i), assayed for glutamate dehydrogenase (GDA) activity (ii), or probed for PfBiP (see “Materials and Methods”) (iii). E, densitometric analyses of Western blots indicating HRPII protein detected in saponin supernatant (i) or pellets (ii) at different times of parasite growth.

Fig. 2. Expression and distribution of PfHRPII in non-transformed 3D7-infected red cells. A, ring-infected (0–12 h) and trophozoite-infected (24–36 h) erythrocytes were fixed, permeabilized, and probed with anti-HRPII antibody followed by FITC-conjugated secondary antibody in indirect immunofluorescence assays, stained with Hoechst dye to visualize parasite nucleus, and imaged by DeltaVision (see “Materials and Methods”). Fluorescence from only the FITC channel is shown. The scale bar is in μm. N indicates the position of parasite nucleus. B, 2 × 10^6-infected red cells (as determined by independent cell counts) were removed at the indicated times of growth from synchronized cultures and subjected to Western blotting using anti-HRPII antibody. C, i, Northern blot of RNA (isolated with TRIzol per the manufacturer’s instruction) from synchronized parasites harvested at the indicated times and hybridized with a HRPII (1 kilobase (kb)) probe. ii, the corresponding RNA gel loaded with 5 μg of indicated RNAs and molecular size markers. D, trophozoite-infected red cells were lysed with 0.01% saponin. 2 × 10^6 parasite equivalents of the supernatant (S) and parasite pellet (P) fractions were prepared for SDS-PAGE and Western blots (see “Materials and Methods”) and probed for PfHRPII (i), assayed for glutamate dehydrogenase (GDA) activity (ii), or probed for PfBiP (see “Materials and Methods”) (iii).
this are unclear. However, because high levels of trans gene expression may be harmful to cells, subsequent analyses of transfected cells was restricted to 33-h trophozoites.

A comparison of total HRPII protein in parent 3D7 and transformed E8 lines during intraerythrocytic development undertaken in Fig. 4A (where samples were run on the same gels and processed simultaneously) shows that rings may contain lower levels of the transgene product. However, as the parasites develop into trophozoites (up to 33 h), there is 3–4-fold more total HRPII protein in transformed cells; schizonts (>36 h) contain even higher levels. Transformed trophozoites release 3–4-fold more HRPII protein in the saponin supernatant (Fig. 4B). This closely reflects elevated levels of protein expression in these cells and suggests that, like its endogenous counterpart, PHRP11mec is primarily exported out of the parasite into red cell cytoplasm and remains there, even when 80% of the hemoglobin is ingested by the parasite.

The release of HRPII and HRPIImec into saponin-sensitive supernatant fractions suggested that they are soluble proteins in the erythrocytic milieu. However, saponin can intercalate with lipids like cholesterol and may, therefore, affect some forms of protein-membrane association. Thus, to determine what fraction of HRPII in 3D7 and E8 cells are soluble, we subjected cells to hypotonic lysis followed by centrifugation at 100,000 × g. As shown (Fig. 4C), 90 and 80% of HRPII appears to be soluble in the 3D7 and E8 cells, respectively. That 97 and 90% are, respectively, exported argues that the bulk of exported PHRP11 protein in both cell lines is soluble. However, a low fraction 10–20% may have a peripheral association with membranes. In addition, examination of consecutive optical sections through the depth of the infected red cells shows that most of the exported protein is detected in sections at the periphery of the red cell (Fig. 4D). In contrast in intermediate sections, the signal is not prominent in the red cell. Thus, it is possible that HRPII and HRPIImec may not freely diffuse through the red cell cytoplasm but may concentrate primarily at the periphery of the red cell and, thus, avoid ingestion along with hemoglobin.

**PHRP11 and PHRP11mec Are Not Transported across the Trophozoite-infected Red Cell Surface**—As indicated earlier, one previous study has proposed that at the trophozoite stage, HRPII is secreted across the red cell membrane into the extracellular medium (26). Our present data (Figs. 2B and 3B) suggest that although HRPII protein is not quantitatively re-
leased from 3D7 or E8 cells, it localizes to the periphery of the red cells; thus, some fraction of the protein could be on the infected red cell surface. To address this we examined both parent and transformed lines for HRPII associated with the infected cell surface by flow cytometry (Fig. 4E). This is a highly sensitive method for protein determination on single cells as well as in a population. Infected erythrocytes can be distinguished from their uninfected counterparts by staining for parasite DNA, and as shown in the insets in Fig. 4E, panels I and II, a peak (i) and intermediate shoulder (ii) followed by a second peak (iii) correspond to separation of uninfected, ring-infected, and trophozoite-/schizont-infected (late stage parasites) cells. In non-permeabilized samples (panels I and II), the bulk of the late parasites (quadrants a and b) and ring-infected cells (quadrants c and d) stain for HRPII at similar levels as uninfected cells (quadrants e and f). Inset, histogram showing the distribution of uninfected and infected red cells.

However, for 99% of the red cell population infected with either late stage 3D7 or E8, no fluorescence was seen at the surface. In contrast, infected red cells permeabilized with saponin show high levels of PHHRPII staining (panels III and IV). The numbers of uninfected red cells in panel III and IV are disproportionately low because despite fixation, saponin-permeabilized uninfected red cells are not quantitatively recovered after centrifugation at 3000 × g (see “Materials and Methods”). Nonetheless panels III and IV demonstrate that the anti-HRPII antibody used in panels I-II indeed recognizes HRPII protein in this assay. Thus, the failure to detect quantitative cell surface expression in panels I and II suggests that neither HRPII nor HRPIImyc are exported to the infected red cell surface (the 3-fold increase in total PHHRPII levels in E8 cells compared with 3D7 cells are obscured by the log scale (conventionally) used in flow cytometry measurements shown in Fig. 4E).

Both HRPII and HRPIImyc Are Recruited into the Secretory Pathway; Elevation of Total HRPII Protein Levels within Transfected Parasites Has No Effect on Protein Export from the Parasite—To confirm that HRPII is recruited to the secretory pathway we examined the distribution of protein in cells treated with the drug brefeldin A (BFA) that blocks secretory
export from cells. An unexpected feature of rings of *P. falciparum* is that extended treatments for 24 h or longer are entirely reversible, in that washing BFA out completely restores parasite protein export (27). This unusual feature enables examination of the effects of this secretory block on long term protein accumulation as rings progress to the trophozoite stage. As shown, in Fig. 5A, in the presence of BFA, all cell-associated HRPIIs detected in both E8 and 3D7 reside within regions that stain with the ER marker PfBiP. This suggests that both endogenous and transgenic proteins are recruited to the ER; that they accumulate in a small region of PfBiP stain in the presence of BFA is consistent with previous results that the drug probably restricts secretory proteins to a region of the ER. Removal of BFA by washing completely restored HRPII and HRPIImyc export (data not shown).

As previously shown (in Fig. 4B) transgenic parasites con-
tain ~10-fold higher levels of PfHRPII within the parasite compared with non-transformed cells. Because the trans gene product is recruited into the secretory pathway, elevation of protein within the parasite could in principle affect secretory functions of the organism. However, as shown in Fig. 5B, accumulation of the trans gene product had no significant effect on the export of metabolically labeled parasite proteins as measured by SDS-PAGE or acid-insoluble counts (not shown). This suggests that PfHRPII* expression does not alter general biosynthetic protein export from the parasite. Furthermore, ultrastructural studies by electron microscopy and indirect immunofluorescence assays (not shown) confirm that in E8 cells there are no detectable changes in secretory membrane organelles, including secreted compartments such as clefts and loops induced in the red cells as well as the ER-Golgi, parasite plasma membrane, and PVM, which have been implicated in protein export pathways. Thus, although it is recruited into the secretory pathway and found at higher levels within the parasite, trans-expressed PfHRPII* has no prominent effect on ultrastructural organization of the parasite or bulk protein export to the red cell.

Effects of PfHRPII* Expression on Hemozoin Production

and Chloroquine IC_{50}—Because PfHRPII is proposed to be a heme polymerase, we investigated whether hemozoin levels as well as chloroquine sensitivity were altered in transfected cells. In 33-h parasites there is ~26% reduction in hemozoin in the transformed E8 line compared with parent 3D7 cells. To control for transfection we also examined a second transfected line expressing a secretory transgene ACPGFP. This gene is not histidine-rich nor is it delivered to the fV. Rather, as shown by Waller (29) and our work (30), it is targeted to another secretory organelle called the apicoplast. The ACPGFP cells show a 30% reduction in growth (measured by incorporation of hypoxanthine into DNA), a 10% reduction in hemozoin, and a 17% reduction in chloroquine sensitivity. This suggests that trans gene expression contributes to a decrease in cell growth (probably due to the cost of carrying the resistance plasmid), which in turn reduces hemozoin production. E8 cells also show a 30% reduction in growth (as measured by hypoxanthine incorporation), which is expected since they express a transgene. However, there is a 26% reduction in hemozoin and a 30% reduction in chloroquine sensitivity. Because hypoxanthine incorporated by E8 and ACPGFP cells is comparable, a 16% reduction in hemozoin and a 13% reduction of chloroquine sensitivity of E8.
Effects of PfHRPIImyc Expression on Association of Secretory Markers in Isolated fv—The observed reduction in hemozoin production in E8 cells led us to investigate the association of HRPII and HRPIImyc with isolated fv. A biochemical analysis with isolated fv was undertaken to facilitate quantitative estimation of marker association with the vacuoles. These fv preparations have been previously characterized to show that they are enriched in resident proteases but relatively depleted in other cellular proteinases (31, 33). They are isolated from trophozoite-stage (24–36 h) parasites, because this is the time of active hemozoin production. The resident fv protein plasmepsin I (global) was used as the marker for purification (24).

Shown in the Western blot in Fig. 5D is the distribution of HRPII and fv proteins detected in parasites and fv isolated from ACPGFP and E8 cells. To provide a quantitative estimation, we loaded equal amounts (10 μg) of protein from (i) isolated parasites and (ii) isolated vacuoles. Densitometric analysis indicates that both E8 and ACPGFP cells produce comparable levels of plasmepsin I and BiP. Yet there was a 10-fold enrichment of plasmepsin I in isolated vacuoles from ACPGFP. Because the same amount of protein was loaded in each lane, this 10-fold reduction cannot be due to a lower number of fs or in the E8 sample. By indirect immunofluorescence, isolated fs from E8 parasites can be stained with antibodies to plasmepsin I (the marker for fv purification) and c-Myc but not the Golgi marker PfERD2 (Fig. 5E). Vacuoles from the ACPGFP line fail to show Myc expression. These data show that the vacuole preparation is indeed enriched for isolated fv; moreover, these vacuoles contain HRPII and HRPIImyc. However, the total amount of HRPII signal associated with E8 vacuoles is comparable with that seen for ACPGFP vacuoles even though the latter parasites have 10-fold less total HRPII protein than E8 cells. In addition, although E8 parasites express comparable levels of plasmepsin I relative to ACPGFP cells, the amount of plasmepsin I seen in vacuoles isolated from E8 parasites is much reduced. One explanation is that the efficiency of transporting both PfHRPII and plasmepsin I to the fv is reduced in E8 cells.

As shown in Fig. 5D, in addition to being enriched for food vacuole enzymes like plasmepsin I, isolated vacuoles from ACPGFP cells also concentrate the ER marker PfBiP. This suggests that PfBiP may be transported to the fv. Alternatively its presence may reflect a contamination of ER membranes in fv preparations. However, if it were a contaminant, comparable levels of BiP would be expected to associate with fv isolated from E8 and ACPGFP cells, since the difference in total levels of BiP between these parasites is less than 2-fold. Instead BiP is markedly decreased in fv isolated from E8 cells. It is important to note that BiP is not released into the red cell cytosol (see Figs. 2 and 3), suggesting that BiP does not “leak” out throughout the secretory pathway. Moreover, as indicated earlier, the Golgi marker PfERD2 is not found in isolated fv (see Fig. 5E and data not shown). Thus, the association of PfBiP with isolated fv is not expected to be due to contamination by secretory membranes but could reflect an ER to fv transport pathway that is disrupted in E8 cells.

To determine the major sites of secretory accumulation of HRPII and plasmepsin I within E8 parasites, we permeabilized infected red cells to release HRPII from the cytosol and then examined the distribution of these proteins relative to other secretory markers. High resolution-digitized fluorescence microscopy (Fig. 6A) show that HRPII and plasmepsin I show substantial colocalization with PfBiP in E8 cells but not markers of the Golgi (ERD2) or the parasite plasma membrane (not shown), consistent with the idea that HRPII and plasmepsin I are blocked in the ER in E8 cells and could be substrates in an ER to fv pathway. Upon hypotonic lysis and centrifugation, all of the HRPII protein in E8 parasites is found in the pellet fraction, suggesting that both ER and fv forms associate with membranes (Fig. 6B). HRPII in ACPGFP parasites is also in the pellet fraction (Fig. 6B), suggesting that membrane association within the parasite is not a consequence of HRPII transgene expression. Its membrane association and, likely, ER location suggests that PfHRPII in E8 parasites is probably not derived by endocytic uptake of soluble PfHRPII delivered to the red cell.

**DISCUSSION**

Our studies suggest that both PfHRPII and PfHRPIImyc are stable proteins that are exported to the red cell and the fv. 3-4 Fold elevation of PfHRPIImyc expression results in a cor-

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**FIG. 7. Model of HRPII transport to erythrocyte cytosol and fv.** Newly synthesized HRPII is recruited into the ER and either exported as soluble protein (97%) or retained in a membrane-associated (3%) form. Soluble HRPII sorts in the periphery of the red cell and is not ingested into the parasite with hemoglobin. The membrane-associated HRPII is transported directly to fv from the ER together with other parasite proteins such as plasmepsin I and PfBiP. The tubovesicular network that extends from the parasitophorous vacuole to the red cell is not shown. **PV** is parasitophorous vacuole.
responding increase in total HRPII release into the red cells but no increase of HRPII protein in the fv. This strongly argues against HRPII undergoing bulk endocytosis with hemoglobin. Moreover most of the protein detected in the E8 parasites shows secretory accumulation in the ER, and it is unlikely that this is a consequence of endocytosis. This raises the question of how HRPII exported to the red cell is excluded from endocytic uptake during the ingestion of hemoglobin. It is possible that hemoglobin exists as a dense semi-crystalline state in the red cells that excludes parasite proteins (such as HRPII and HRPIImyc) in the periphery of the red cells. Alternatively, HRPII may actively segregate. The reason for the punctuate staining of PHRPPII and PHRPPIImyc is not clear but could underlie protein-protein interactions that contribute to their exclusion from hemoglobin. Although the precise mechanisms are not known, our data provide the first evidence that an antigen released into the red cell can “sort” away from hemoglobin, concentrate in the periphery of the red cell, and need not undergo bulk digestive uptake into the fv (see Fig. 7, a model). Thus membrane-bound intermediates may not be required to deliver soluble antigens to the edge of the red cell. This is in marked contrast to the idea that soluble proteins exist in an unsorted mixture in the cytoplasm of a mammalian cell and suggests that cytosolic protein-protein interactions in the absence of membrane may have important implications for eukaryotic cellular transport pathways in general and digestive uptake in Plasmodium in particular.

A C-terminal c-Myc epitope does not significantly interfere with recruitment of HRPII into the secretory pathway, its export from the parasite, or its residence in the red cell. Further significant extracellular release of PHRPPII or PHRPPIImyc does not occur during the first 36 h of intraerythrocytic development. BFA treatment appears to result in protein accumulation in a region of the ER. The trans gene is maximally expressed and exported at the trophozoite and schizont stages, whereas the endogenous protein is exported at the ring stage. This suggests that unlike transport to apical organelles (32), protein secretion to the red cell is not temporally regulated. This argues against a “secondary ER” that has been proposed to exist as a specialized compartment in Plasmodium dedicated to ring stage export to the erythrocyte (33).

Both HRPII and HRPIImyc are also transported to the fv. However, because the bulk of both proteins is delivered to the red cell and remains there, it is reasonable to presume that transport of HRPII to the fv is not due to the presence of a specific targeting signal on these proteins. Rather, transport there may depend on interactions with other proteins; a functional consequence of these interactions may underlie reduced transport of plasmspepin I and BiP to the fv in E8 cells. That BiP along with plasmspepin I is enriched in isolated fvs, suggests that there may be an ER to fv transport pathway. Because BiP is also not expected to contain peptidic signals for transport to the fv, it is likely to be delivered as a complex with other (signal bearing) proteins (such as plasmspepin I), consistent with its function as a chaperone. One possibility is that in addition to its resident-targeting signals, plasmspepin I may complex with BiP for optimal transport to the fv. If newly synthesized HRPII associates with plasmspepin I-BiP complex, it could piggyback into the fv (see Fig. 7). HRPIImyc is delivered to the fv and affects BiP association with the fv, suggesting that it too may use a biosynthetic ER-fv pathway. Biosynthetic pathways of transport to digestive organelles that are independent of endocytic routes have been demonstrated in eukaryotes (34). However proteins are targeted from the Golgi to the yeast vacuole or mammalian lysosomes (35). Transport from the ER to the fv may reflect an unstacked Golgi (decreasing the need for step-wise transport through this organelle) during ring and trophozoite stages of Plasmodia (36) and argues that in primitive eukaryotes the ER may combine both biosynthetic and sorting functions.

How is PHRPPII/HRPIImyc shunted off to the fv pathway differentiated from that exported from the parasite? The fv and secretory forms of the proteins within the parasite are membrane-associated, whereas the exported forms are largely soluble. Because the endogenous HRPII within the parasite is also membrane-associated, the higher level of HRPII associated with E8 parasites is not catalyzed by c-Myc per se but could be due to higher levels of trans gene expression. Treatment with EDTA releases HRPII and HRPIImyc from the membrane (not shown), suggesting that it may require divalent cations such as calcium that are elevated in the ER. In E8 cells, elevated levels of newly synthesized, membrane-bound PHRPPIImyc in the ER may compete BiP away from membrane-associated plasmspepin I, leading to a reduced efficiency of fv transport for all three components.

Recent studies estimate that 1.2 μg PHRPPII in the fv would be sufficient to catalyze heme polymerization in the fv (13). Our present studies showing that a maximum of 3% of endogenous PHRPPII is retained in the parasite would suggest that micromolar concentrations of the protein are probably not achieved in the fv. Furthermore, it is known that loss of HRPII and HRPPIII does not prevent heme polymerization in cells (11). Our data that a vast majority of endogenous HRPII and a trans gene are exported to the red cell cytoplasm and remain there suggest that a major function of this protein is likely to be at its principal site of residence, in the periphery of the red cell or in its released form after host cell rupture. That soluble HRPII can sort away from hemoglobin and that secretory, membrane-bound HRPII can disrupt protein transport to the fv may reflect protein-sorting properties of PHRPPII that underlie novel mechanisms of protein targeting in the red cell and food vacuole of this major human pathogen. Our data also suggest a direct ER to fv transport pathway and a sorting function for the ER in a eukaryotic cell.

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