Plasmodium falciparum Malaria: Association of Knobs on the Surface of Infected Erythrocytes with a Histidine-rich Protein and the Erythrocyte Skeleton

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ABSTRACT *Plasmodium falciparum*-infected erythrocytes (RBC) develop surface protrusions (knobs) which consist of electron-dense submembrane cups and the overlying RBC plasma membrane. Knobs mediate cytoadherence to endothelial cells. Falciparum variants exist that lack knobs. Using knobby (K+) and knobless (K−) variants of two strains of *P. falciparum*, we confirmed Kilejian's original observation that a histidine-rich protein occurred in K+ parasites but not K− variants (Kilejian, A., 1979, *Proc. Natl. Acad. Sci. USA*, 76:4650–4653; and Kilejian, A., 1980, *J. Exp. Med.*, 151:1534–1538). Two additional histidine-rich proteins of lower molecular weight were synthesized by K+ and K− variants of both strains. We used differential detergent extraction and thin-section electron microscopy to investigate the subcellular location of the histidine-rich protein unique to K+ parasites. Triton X-100, Zwittergent 314, cholic acid, CHAPS, and Triton X-100/0.6 M KCl failed to extract the unique histidine-rich protein. The residues insoluble in these detergents contained the unique histidine-rich protein and electron-dense cups. The protein was extracted by 1% SDS and by 1% Triton X-100/9 M urea. The electron-dense cups were missing from the insoluble residues of these detergents. The electron-dense cups and the unique histidine-rich protein appeared to be associated with the RBC skeleton, particularly RBC protein bands 1, 2, 4.1, and 5. We propose that the unique histidine-rich protein binds to the RBC skeleton to form the electron-dense cup. The electron-dense cup produces knobs by forming focal protrusions of the RBC membrane. These protrusions are the specific points of attachment between infected RBC and endothelium.

The human malaria parasite *Plasmodium falciparum* has a 48-h asexual life cycle within the host's erythrocytes (RBC).[1] The intracellular parasite develops from a small ring form into a trophozoite occupying one-half to two-thirds of the RBC. It then divides asexually to produce a schizont, which ruptures the RBC at the end of the 48-h cycle and releases daughter merozoites that immediately invade RBC to reinitiate the cycle. In falciparum malaria only RBC containing rings circulate in peripheral blood (1). RBC containing trophozoites and schizonts sequester by adhering to endothelium of postcapillary venules of several tissues, predominantly heart and skeletal muscle (2). Sequestration of the more mature forms of the parasite prevents their circulation through the spleen, a major site of parasite destruction in malaria (3). Sequestered parasites may also obstruct blood flow, as seen in cerebral malaria.

Attachment of RBC containing trophozoites and schizonts to endothelial cells is mediated by parasite-dependent alterations in the RBC membrane. Infected RBC (IRBC) develop focal surface protrusions (knobs) that have a diameter of 100 nm (4). By transmission electron microscopy the knob is seen

[1] *Abbreviations used in this paper:* CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; HRP, histidine-rich proteins; IRBC, infected RBC; K+, knobby; K−, knobless; RBC, erythrocytes.

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to consist of a submembrane, electron-dense, cup-shaped structure and a protrusion of the RBC membrane that follows the contour of the electron dense cup (5). Kilejian compared knobby (K+) and knobless (K−) IRBC of a Gambian strain of *P. falciparum* (FCR3) and identified a protein with apparent molecular weight (M_r) 80,000 that was unique to K+ IRBC (6). This protein was metabolically labeled preferentially by [^{3}H]proline and [^{3}H]histidine (6, 7). Hadley et al. recently observed a similar histidine-rich protein (HRP) in K+ but not K− IRBC of another strain of *P. falciparum* (Malayan Camp) (8).

In this report we used differential detergent extraction and thin-section electron microscopy to show that the knob-associated HRP had the same solubility characteristics as the electron-dense, cup-shaped component of knobs. Subcellular fractions that contained electron-dense cups also contained the unique knob-associated HRP. These fractions lacked most intracellular parasite material, as judged by electron microscopy, and were depleted of other parasite proteins. Both the electron-dense cups and the unique HRP were closely associated with the RBC skeleton. Our results suggest that the parasite HRP binds to the RBC skeleton to form the structural basis of knobs.

MATERIALS AND METHODS

Materials: Glycerolyte solution for cryopreservation of infected blood was from Fenwal Laboratories (Deerfield, IL). 12% NaCl, 1.6% NaCl, and 0.9% NaCl plus 0.2% glucose used for thawing infected blood were also obtained from Fenwal. RPMI-1640 was purchased from Gibco Laboratories, Inc. (Grand Island, NY), HEPES and Triton X-100 from Sigma Chemical Co. (St. Louis, MO), and horse serum from M. A. Bioproducts (Walkersville, MD). Culture medium 90% deficient in histidine was prepared using the Select-Amine kit (Gibco Laboratories, Inc.) supplemented with 10% (vol/vol) normal RPMI. L-[2, 5-^{3}H]Histidine (40–60 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. Zwittergent 314, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), SDS, and cholic acid were from Calbiochem-Behring Corp., La Jolla, CA. Electrophoresis sample buffer (1 ×) contained 10% glycerol, 5% SDS, 5% 2-mercaptoethanol, .001% bromphenol blue, and 6.25 ml of 1 M Tris buffer, pH 6.8, per 100 ml (9). 2 × sample buffer contained twice the concentration of each reagent.

Parasites: All experiments were performed using uncloned K+ and K− IRBC of the Malayan Camp and St. Lucia strains of *P. falciparum*. Malayan Camp K+ parasites have been maintained in our laboratory by passage in *Aotus* monkeys. Malayan Camp K− parasites were kindly provided by Dr. David Haynes, Walter Reed Army Institute of Research. These parasites had originally been passaged in *Aotus* monkeys and subsequently cultured in vitro for >1 y. The examination of Camp IRBC by transmission electron microscopy showed that the IRBC were K−, as previously described in another strain of *P. falciparum* following long-term cultivation (10). The IRBC remained K− when repassaged into splenectomized monkeys, which served as the source of parasites for our experiments. St. Lucia parasites were provided by Dr. William Collins, Center for Disease Control, Atlanta, GA, where they had been maintained in splenectomized *Aotus* monkeys. In our laboratory the parasites were passaged into splenectomized and intact monkeys. During a chronic, recrudescent infection in a splenectomized *Aotus* monkey, the parasites gradually changed from a mixture of K+ and K− phenotypes to a pure K− phenotype as judged by transmission electron microscopy. The parasites passaged in intact monkeys remained K+. Infected blood from monkeys was cryopreserved at 10–50% parasitemia (11). Experiments comparing K+ and K− parasites were performed with isolates having similar parasitemias. In some experiments K+ parasites obtained from splenectomized monkeys were used to exclude the possibility that the biochemical differences observed between K+ and K− parasites were due to the presence or absence of a spleen in the host. Electron microscopy was used to determine whether samples of infected blood were K+ or K−.

Culture and Metabolic Labeling: Cryopreserved IRBC were thawed (11) and cultured (12) as previously described. RBC containing trophozoites and schizonts were lysed during thawing, yielding parasitemias of 8–30% with a predominance of ring forms. Immediately after thawing, blood was suspended in RPMI-1640 containing 10% of the normal concentration of histidine and supplemented with 10% human AB serum or horse serum, 30 mM HEPES, 0.27% sodium bicarbonate, 10 μg/liter hypoxanthine (Sigma), 2 mg/ml D-glucose, and 50 μg/ml gentamicin (Schering Corp., Kenilworth, NJ). [^{3}H]Histidine was added to 25–50 μCi/ml. Cultures were gassed with 3% O_2, 6% CO_2, and 91% N_2 and maintained in 75-cm^2 flasks (Corning Glass Works, Corning, NY) at 1% hematocrit. The cells were harvested after 20–24 h when the majority of parasites were trophozoites.

Binding to Melanoma Cells: A line of amelanotic melanoma cells has been shown to specifically bind RBC infected with K+ *P. falciparum* parasites (13). This binding reaction appears indistinguishable from the binding of K+ IRBC to human umbilical cord endothelial cells (14) and can therefore be used as an assay of the functional (i.e., binding) capacity of knobs on the IRBC surface.

Cryopreserved IRBC were thawed and cultured in vitro for 20–24 h to the trophozoite stage. 0.5 ml of a suspension of IRBC at 1–2% hematocrit was layered over melanoma cells in Lux 5218 8-well plates (Miles Laboratories, Elkhart, IN) and incubated 90 min at 37°C. The plates were gently rocked by hand every 15 min. Nonadherent cells were removed by washing and adherent RBC were fixed in absolute methanol for Giemsa staining. The number of IRBC bound to 100 melanoma cells was determined by light microscopy and the number of bound cells per melanoma cell was calculated.

Hypotonic Lysis of IRBC: IRBC were harvested, washed twice in RPMI 1640, and lysed in 80 vol of ice cold 20 mM sodium phosphate, pH 7.4, and ghosts were pelleted by centrifugation at 13,000 *g* for 10 min. The supernatant was removed and the pelleted ghosts were solubilized at a ratio of 250 μl of 1 × electrophoresis sample buffer per 1.25 × 10^8 RBC.

Detergent Extraction of IRBC: IRBC were metabolically labeled, washed, and hypotonically lysed as described above. Pelleted ghosts of 1.25 × 10^8 RBC were vortexed and extracted in 250 μl of the following detergents: 1% Triton X-100 in 150 mM NaCl, 20 mM phosphate, pH 7.3 (PBS); 2% Triton X-100 in 0.6 M KCl; 6 mM Zwittergent 314, 10 mM CHAPS, 2% cholic acid, or 1% SDS, all in PBS, pH 7.3. Samples were incubated 1 h on ice, except for the 1% SDS sample which was incubated at 22°C, and layered onto 500 μl of 20% sucrose in PBS in a 1.5 ml Eppendorf tube. After centrifugation (5 min at 13,000 *g*) the supernatant above the sucrose cushion was removed and added to an equal volume of 2 × electrophoresis sample buffer. The cushion of sucrose was aspirated, the pellet was resuspended in the same detergent solution, and this detergent-insoluble material was washed by centrifugation (13,000 *g* for 5 min) before solubilization in 250 μl of 1 × electrophoresis sample buffer. Complete solubilization required heating at 60°C for 30 min with frequent vortexing and disruption of the pellet by gentle aspiration through a 23-gauge needle. Samples were stored at −70°C for ≦48 h before analysis by SDS PAGE.

SDS PAGE: Samples were boiled for 5 min immediately prior to SDS PAGE. When K+ and K− parasites were compared, samples prepared from equal numbers of IRBC were applied to the gels. The discontinuous buffer system of Laemmli (9) was used with a 5–10% acrylamide gradient. The distribution of proteins was determined by Coomassie Blue staining (15) and the distribution of radioactivity by fluorography (16) following impregnation of the gel with ENHANCE (New England Nuclear, Boston, MA).

Electron Microscopy: Samples for electron microscopy were fixed in 2% glutaraldehyde, 0.05 M phosphate, and 4% sucrose, pH 7.4. Pellets of detergent-insoluble material were prepared for electron microscopy by resuspension in the same detergent solution and layering this over 500 μl of the fixation solution in an Eppendorf tube. After centrifugation (13,000 *g* for 5 min) the entire liquid phase was aspirated and the pelleted material was resuspended in fresh fixation solution. Samples were postfixed in 1% osmium tetroxide in Millonig's phosphate buffer, dehydrated in ethanol, and embedded in Spurr. Thin sections were stained with uranyl acetate followed by lead citrate and examined with a JEOL 100 Cx electron microscope.

Quantitative Analysis of Protein Radioactivity: After fluorography of dried gels, lanes of interest were excised and cut into 1- or 2-mm slices. These were rehydrated in 1 ml distilled water for 1 h, 1 ml of Protosol (New England Nuclear) was added, and the sample was kept at 23° for 24 h, then 30 min at 45°C. 20 μl of Aquasol (New England Nuclear) was added and liquid scintillation counting was performed after decay of chemiluminescence.

Densitometry: Densitometry of Coomassie Blue-stained gels or fluorographs was performed on a Transidyne 2955 scanning densitometer (Transidyne General Corporation, Ann Arbor, MI). The area and percent of total area contained in specific peaks was determined with a Transidyne 2967 computing integrator.

RESULTS

Binding of IRBC to Melanoma Cells

K+ IRBC of both Malayan Camp and St. Lucia strains were cultured in vitro to the trophozoite stage and their

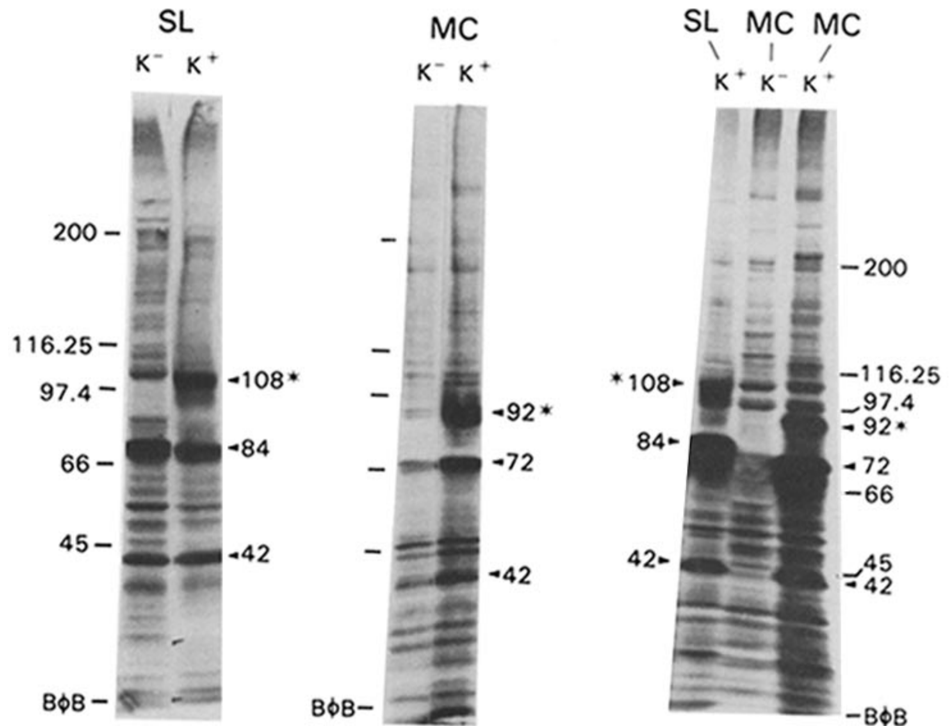

FIGURE 1 Electrophoretic analysis and fluorography of *P. falciparum* proteins labeled with [^{3}H]histidine during in vitro growth from rings to trophozoites. Knobless (*K−*) and knobby (*K+*) variants of St. Lucia (*SL*) and Malayan Camp (*MC*) parasites were compared. IRBC were hypotonically lysed in 20 mM phosphate, pH 7.4, and centrifuged, and the ghosts were solubilized in electrophoresis sample buffer. Samples prepared from equal numbers of K− or K+ IRBC were applied to each lane and electrophoresed in a 5–10% polyacrylamide gel. Molecular weight standards (× 10^{-3}) and the bromphenol blue (*BϕB*) dye front are shown by dashes. The three major [^{3}H]histidine-labeled proteins (HRP) are marked by arrowheads. The HRP unique to K+ parasites is indicated by an asterisk. For the three lanes on the right side of the figure, IRBC were solubilized in 1 × electrophoresis sample buffer without prior hypotonic lysis and were electrophoresed in adjacent lanes. Parasitemias: SL K− (13%); SL K+ (15%); MC K− (23%); MC K+ (18%).

capacity to bind in vitro to melanoma cells was tested. An average of 10–50 IRBC bound per melanoma cell in five separate experiments with each strain. K− IRBC of both strains cultured to the same stage did not bind to melanoma cells (0 IRBC per melanoma cell).

Comparison of [^{3}H]Histidine-labeled Proteins of Hypotonically Lysed and Intact K+ and K− IRBC

Biosynthetically labeled K+ IRBC of both Malayan Camp and St. Lucia strains contained three major histidine-labeled proteins (HRP).[2] With St. Lucia parasites the major HRP had M_r of 108,000, 84,000, and 42,000 whereas with Malayan Camp parasites the major HRP had M_r of 92,000, 72,000 and 42,000 (Fig. 1). In both strains only one HRP was uniquely associated with K+ IRBC: the M_r 108,000 HRP from St. Lucia and the M_r 92,000 HRP from Malayan Camp. The two lower M_r HRP were labeled with approximately equal intensity in K+ and K− St. Lucia IRBC but appeared to contain more radioactivity in K+ compared with K− Malayan Camp IRBC. The results were reproducible in four independent experiments with St. Lucia parasites and in 10 independent experiments with Malayan Camp parasites. The M_r of the knob-associated HRP were constant in experiments employing parasites from different *Aotus* monkeys.

[2] We have referred to these three proteins as histidine-rich proteins because they are preferentially and intensely labeled with [^{3}H]histidine in biosynthetic labeling experiments. They therefore appear to contain more histidine residues than other malarial proteins. However, their histidine content has not been directly analyzed.

Detergent Effects on the Knob-associated HRP of Malayan Camp Parasites

Hypotonically lysed or intact K+ IRBC were incubated with detergents and centrifuged to obtain a soluble extract and an insoluble residue. The residue was then solubilized in 1 × electrophoresis sample buffer and the [^{3}H]histidine-labeled proteins of the extract and residue were compared by SDS PAGE and fluorography (Fig. 2). Triton X-100, Zwittergent 314, CHAPS, cholic acid, and Triton X-100 in 0.6 M KCl did not solubilize the M_r 92,000 HRP, but did solubilize the majority of other biosynthetically labeled proteins. These detergents also extracted the majority of proteins labeled by [^{3}H]isoleucine (not shown). Densitometry of the fluorograph showed that after these detergent extractions, the M_r 92,000 protein accounted for 70–75% of the [^{3}H]histidine-labeled proteins in the insoluble material, compared with 10–12% in hypotonically lysed IRBC. The enrichment of the M_r 92,000 malarial protein, compared with other radiolabeled malarial proteins, was therefore approximately sevenfold in the detergent-insoluble material as compared with hypotonically lysed IRBC. In contrast, 1% SDS completely solubilized the M_r 92,000 HRP. Urea (9 M) plus 1% Triton X-100 solubilized 90% of this protein as judged by quantitative analysis of radioactivity. When [^{3}H]histidine-labeled IRBC of K+ and K− parasites were treated in parallel with 1% Triton X-100 in PBS or 2% Triton X-100 in 0.6 M KCl and detergent-insoluble material was examined by SDS PAGE (Fig. 3), all of the M_r 92,000 HRP and some of M_r 72,000 HRP of K+ IRBC remained in the residue. There were no [^{3}H]histidine-labeled proteins enriched in the insoluble residues of K− IRBC. The M_r 108,000 HRP of K+ St. Lucia parasites had the same solubility characteristics as the M_r 92,000 HRP of

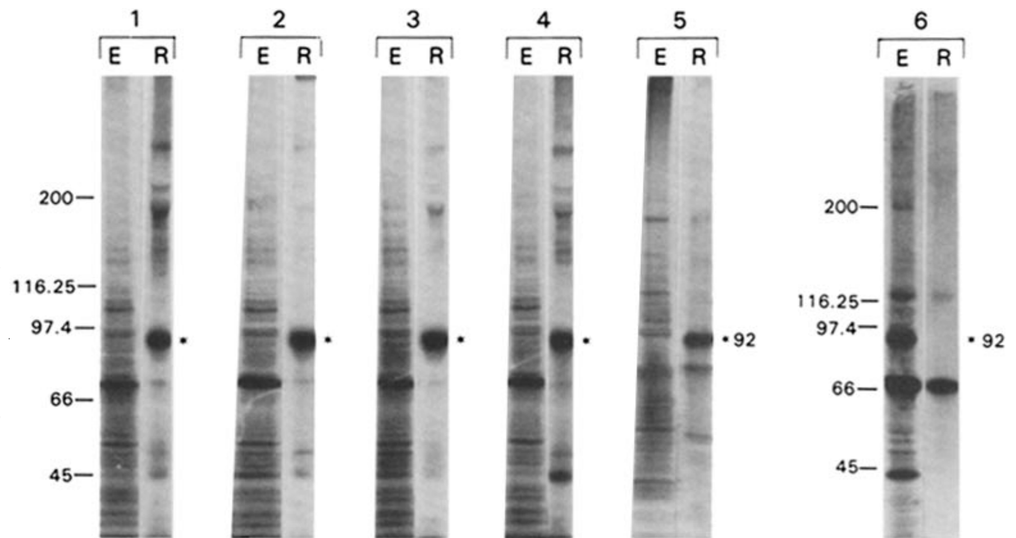

FIGURE 2 [^{3}H]Histidine-labeled proteins in soluble extracts (*E*) and insoluble residues (*R*) of knobby (K+) IRBC of the Malayan Camp strain of *P. falciparum* after metabolic labeling. 30% of the RBC contained trophozoites. Extractions were performed with six detergent solutions: 1% Triton X-100 (*1*), 6 mM Zwittergent 314 (*2*), 10 mM CHAPS (*3*), 2% cholic acid (*4*), 2% Triton X-100 in 0.6 M KCl (*5*), and 1% SDS (*6*). The extracts and residues shown for each detergent were prepared from the same sample of IRBC. The amount of sample loaded and the exposure time of the fluorograph were adjusted so that lanes *E* and *R* reflect similar proportions of extract and residue for each detergent solution. M_r standards (× 10^{-3}) are marked by dashes. The knob-associated M_r 92,000 protein is marked by an asterisk.

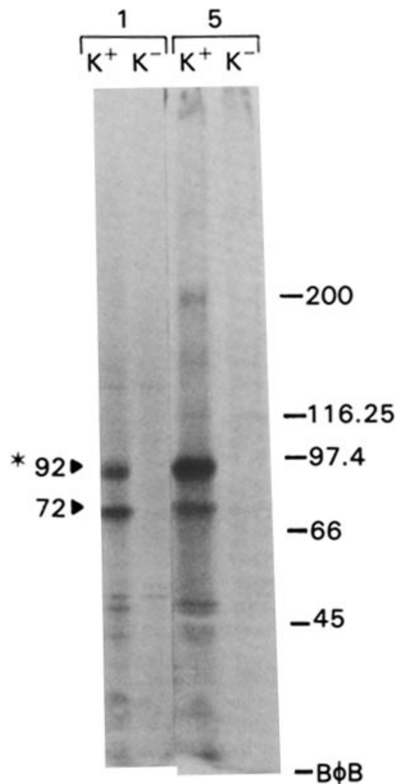

FIGURE 3 Comparison of [^{3}H]histidine-labeled proteins contained in the insoluble residues of knobby (*K+*) (9% parasitemia) and knobless (*K−*) (8% parasitemia) IRBC (Malayan Camp strain) after extraction with 1% Triton X-100 (*1*) or 2% Triton X-100 in 0.6 M KCl (*5*). M_r standards (× 10^{-3}) and the dye front are indicated by dashes. The M_r 92,000 and 72,000 proteins are marked by arrowheads and the knob-associated M_r 92,000 protein is marked by an asterisk.

K+ Malayan Camp parasites, i.e., it was insoluble in 1% Triton X-100 and 6 mM Zwittergent 314 and soluble in 1% SDS (not shown).

Association of the M_r 92,000 HRP with RBC Proteins

Detergent extracts and residues of RBC infected with Malayan Camp parasites were electrophoresed and examined by Coomassie Blue staining (Figs. 4 and 5). Proteins of the *Aotus* RBC membrane could be identified since they had M_r almost identical to those of human RBC membrane proteins (15) and similar solubility properties (17, 18). The M_r 92,000 HRP was identified by its electrophoretic mobility and by its presence in K+ but not K− parasites (Fig. 5). In contrast, other Coomassie Blue-stained proteins were shared by both K+ and K− parasites (Fig. 5). Residues from extraction of K+ IRBC with Triton X-100, CHAPS, Zwittergent 314, and cholic acid contained the M_r 92,000 protein, several proteins characteristic of the RBC skeleton, and variable amounts of band 3 (Fig. 4 and Table I). The M_r 92,000 protein was detected as a prominent Coomassie Blue-stained band comparable in amount to bands 4.2 and 5, even though only 30% of the RBC in this experiment were parasitized. Inspection of the Coomassie Blue-stained gel indicated that the M_r 92,000 protein was the predominant parasite protein in the insoluble residues. Extracts and residues of K+ and K− IRBC incubated in 1% Triton X-100 or 2% Triton X-100 in 0.6 M KCl were compared (Fig. 5). Bands 2.1, 3, and 4.2 were completely extracted by 2% Triton X-100 in 0.6 M KCl from both K+ and K− IRBC, yielding residues that contained RBC skeletal proteins 1, 2, 4.1, and 5. Only residues of K+ IRBC contained the M_r 92,000 protein, which again was detected as a Coomassie Blue-stained band even though only 10% of the RBC were parasitized.

Detergent Effects on the Electron-dense Cups

The residue after detergent extraction was examined by transmission electron microscopy. Treatment of hypotonically lysed K+ IRBC with 1% Triton X-100 produced insoluble residues containing RBC skeletons together with some parasite-derived material (Fig. 6*a*). Compared with intact IRBC (Fig. 6*a*, inset), the characteristic lipid bilayer structure

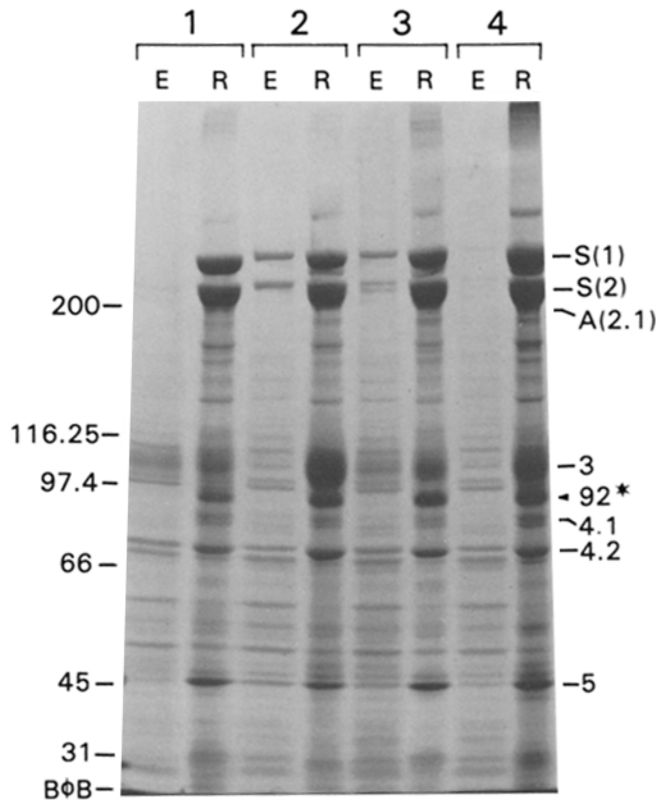

FIGURE 4 Association of the M_r 92,000 protein with RBC membrane proteins. Coomassie Blue-stained extracts (*E*) and insoluble residues (*R*) of K+ Malayan Camp (30% parasitemia) IRBC were compared after extraction with 1% Triton X-100 (*1*), 10 mM CHAPS (*2*), 6 mM Zwittergent 314 (*3*), and 2% cholic acid (*4*). M_r standards (× 10^{-3}) are marked by dashes on the left. RBC membrane proteins spectrin, *S(1)* and *S(2)*, ankyrin *A(2.1)*, bands *3*, *4.1*, *4.2*, and *5* (dashes) and the M_r 92,000 knob-associated protein (arrowhead and asterisk) are marked on the right.

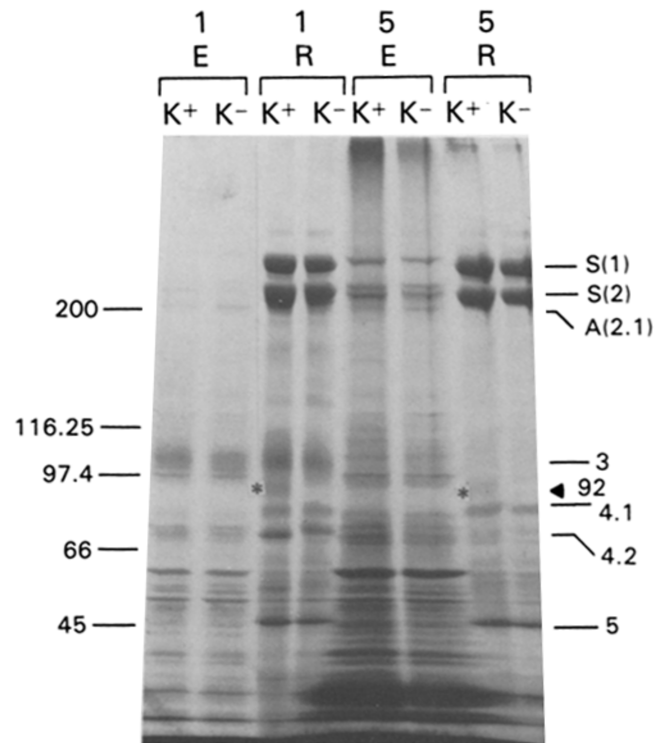

FIGURE 5 Comparison of extracts (*E*) and insoluble residues (*R*) of knobby (*K+*) (10% parasitemia) and knobless (*K–*) (11% parasitemia) Malayan Camp IRBC after extraction with 1% Triton X-100 (*1*) or 2% Triton X-100 in 0.6 mM KCl (*5*). Residues from equal numbers of K+ and K– IRBC at equivalent stages of parasite maturity were electrophoresed. M_r standards (× 10^{-3}) are indicated by dashes on the left. RBC membrane proteins spectrin, *S(1)* and *S(2)*, ankyrin *A(2.1)*, bands *3*, *4.1*, *4.2*, and *5* (dashes), and the M_r 92,000 knob-associated protein (arrowhead) are marked on the right. The broad Coomassie Blue-stained band at the top of the gel in 2% Triton X-100/0.6 M KCl extracts of K+ and K– IRBC is probably aggregated band 3 (19).

TABLE I

*Composition of Insoluble Residues after Extraction with Different Detergents**

Detergent	Electron-dense cups	M_r 92,000 Histidine-labeled protein	RBC protein bands: 1 and 2 (Spectrin)	2.1 (Ankyrin)	3	4.1	4.2	5 (Actin)
1% Triton X-100	+[‡]	+	+	+	+[§]	+	+	+
10 mM CHAPS	+	+	+	+	+	+	+	+
6 mM Zwittergent 314	+	+	+	+	+[§]	+	+	+
2% Cholic acid	+	+	+	+	+	+	+	+
2% Triton X-100/0.6 M KCl	+	+	+	–	–	+	–	+
1% SDS	–	–	–	–	–	–	–	–
1% Triton X-100/9 M urea	–	–	–	–	–	–	–	–

* IRBC were incubated in detergent and centrifuged (5 min, Eppendorf centrifuge) on a 20% sucrose cushion, and duplicate insoluble residues were examined by transmission electron microscopy and by SDS PAGE after solubilization in electrophoresis sample buffer (see Materials and Methods). The presence of electron-dense cups was assessed by electron microscopy. The M_r 92,000 HRP was identified by fluorography of [^{3}H]histidine-labeled proteins after SDS PAGE. RBC proteins were identified by Coomassie Blue-staining.

‡ + Present in detergent-insoluble residues.
– Absent from detergent-insoluble residues.

§ Band 3 was partially extracted by 1% Triton X-100 and by 6 mM Zwittergent 314.

of the RBC membrane was not observed (Fig. 6, *a* and *b*). The electron-dense structure of the knob was uniformly associated with the skeletal shell. Treatment of hypotonically lysed K– IRBC with 1% Triton X-100 produced residues containing RBC skeletons of identical appearance to those from K+ IRBC, except that they did not contain the electron-dense cups (Fig. 6*c*). Extraction with cholic acid (Fig. 7*a*), Zwittergent 314 (Fig. 7*b*), or CHAPS (data not shown) yielded

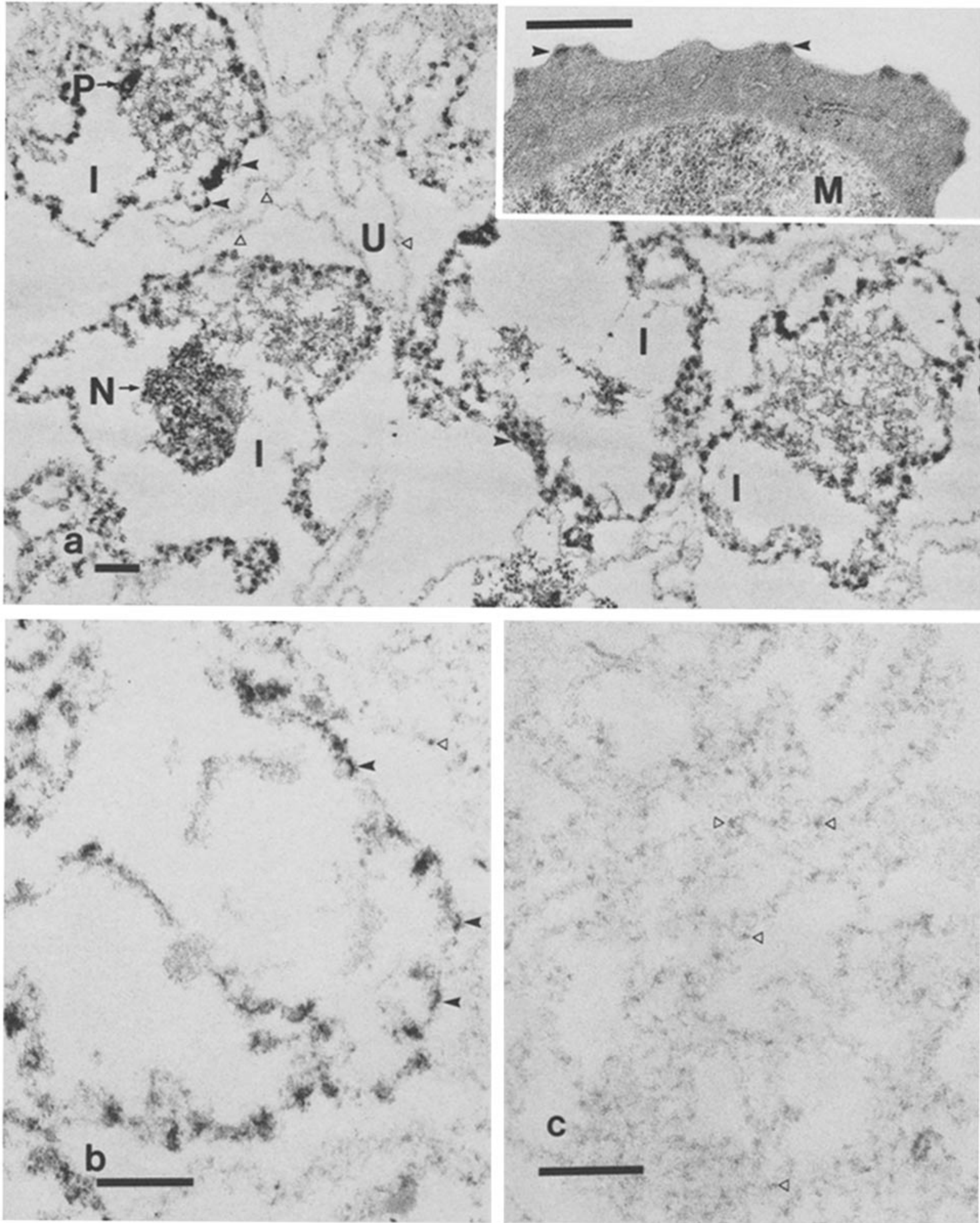

FIGURE 6 Electron micrographs of detergent-insoluble residues of RBC infected by K+ or K− Malayan Camp parasites after hypotonic lysis and extraction in various detergents. (*a*) K+ IRBC extracted with 1% Triton X-100. The residue contains RBC skeletons of both infected (*I*) and uninfected (*U*) RBC, malarial pigment (*P*), and some parasite material (*N*). Cup-shaped electron-dense structures (solid arrowhead) are embedded in the skeleton of IRBC. Smaller dot-like particles (open arrowhead) are embedded in the skeletons of both infected and uninfected RBC. Bar, 0.5 μM. × 15,000. *Inset*: Intact K+ IRBC for comparison. Intracellular parasite is indicated (*M*). Parasitemia 30%. Bar, 0.5 μM. × 35,000. (*b*) Higher magnification of *a*. Lipid bilayer structure of the RBC membrane has been extracted. Bar, 0.5 μM. × 32,000. (*c*) K^- IRBC extracted with 1% Triton X-100. The residue contains RBC skeletons but no electron-dense cups. The small dot-like particles are apparent. Parasitemia 23%. Bar, 0.5 μM. × 35,000.

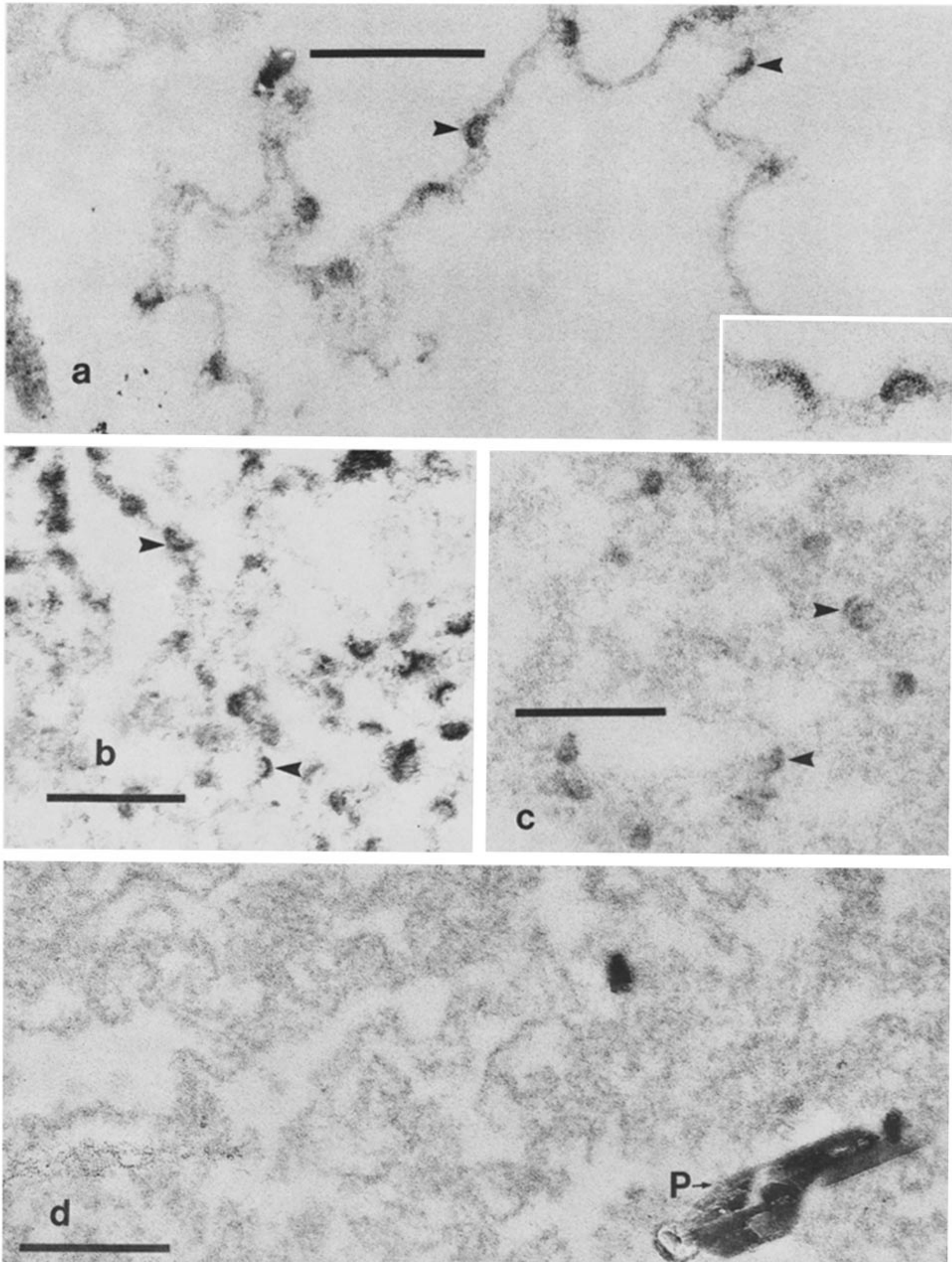

FIGURE 7 Detergent-insoluble residues after hypotonic lysis and extraction of K+ IRBC in various detergents. Parasitemia: 30% (*a*, *b*, and *d*); 10% (*c*). (*a*) 2% cholic acid. Electron-dense cups (arrowhead) oriented convex outward produce focal protrusions of the RBC skeleton. Bar, 0.5 μM. × 60,000. *Inset:* × 88,000. (*b*) 6 mM Zwittergent 314. Bar 0.5 μm. × 47,000. (*c*) 2% Triton X-100 in 0.6 M KCl. Cells were not hypotonically lysed before extraction. Bar, 0.5 μM. × 52,000. (*d*) 1% SDS. The residue contains pigment (*P*) and amorphous membranous material. Electron-dense cups are not observed. Bar, 0.5 μM. × 52,000.

residues that were depleted of most parasite material but that contained the electron-dense cups embedded in fibrillar material. After extraction with cholic acid, the residue contained electron-dense structures (convex outwards) that appeared to produce focal-outward protrusions of the RBC skeleton (Fig. 7*a*, inset). Extraction with 2% Triton X-100 in 0.6 M KCl also yielded residues containing electron-dense cups in a dispersed fibrillar matrix (Fig. 7*c*). In this case, however, the matrix was very amorphous and lacked the linear continuity of RBC skeletons seen with other detergent extractions. Residues of hypotonically lysed K+ IRBC extracted with 1% SDS contained pigment, scattered parasite nuclei, and some amorphous membranous material, but no electron-dense cups (Fig. 7*d*). Similarly, residues after extraction with 9 M urea in 1% Triton X-100 contained no electron-dense cups (not shown).

Detergent Effects on HRP Shared by K+ and K− IRBC

IRBC of both Malayan Camp and St. Lucia strains contained two major HRP that were shared by K+ and K− IRBC. In both strains these HRP had lower M_r than the knob-associated HRP (72,000 and 42,000 with Malayan Camp; 84,000 and 42,000 with St. Lucia). The M_r 72,000 HRP was present in both extracts and residues of K+ Malayan Camp IRBC after extraction with Triton X-100, Zwittergent 314, CHAPS, cholic acid, 2% Triton X-100 in 0.6 mM KCl, or SDS (Fig. 2). The M_r 42,000 HRP could be identified only in the residue after extraction with 2% cholic acid (Fig. 2, lane *4*) and in the extract after extraction with 1% SDS (Fig. 2, lane *6*).

DISCUSSION

Plasmodium falciparum is unique among human malaria parasites in its capacity to sequester by binding to endothelial cells. Sequestration by RBC containing trophozoites and schizonts may contribute to the virulence of falciparum malaria by allowing the parasite to avoid spleen-dependent immune mechanisms and by obstructing blood flow. Knobs on the surface of IRBC appear to be necessary for binding to endothelial cells. The evidence for this is as follows. First, all parasites that sequester (*P. falciparum*, *P. fragile*, and *P. coatneyi*) have knobs (4, 20–22). Second, when falciparum parasites lose knobs, they no longer sequester in vivo or in vitro (reference 11 and J. Barnwell and J. Leech, unpublished observations). IRBC of the K+ phenotype of the two strains studied here were shown to bind to melanoma cells in vitro and to be sequestered in vivo. In contrast, IRBC of the K− phenotype neither bound in vitro nor sequestered. Third, sequestration and cytoadherence occur as the parasite matures from the ring to trophozoite stage and are associated with the appearance of knobs (13). Fourth, the knob is the point of apposition to endothelium (5, 13, 14, 20).

Kilejian's original observation of a HRP unique to K+ IRBC of the FCR3 strain (6, 7) was recently confirmed by Hadley et al. (8). This recent study (8) also identified an HRP unique to K+ IRBC of the Malayan Camp strain. In this report we examined K+ and K− IRBC of a third *P. falciparum* strain (St. Lucia) and showed again that K+ IRBC synthesized an HRP that K− IRBC of the same strain did not synthesize. This observation strengthens the association of a unique HRP with K+ IRBC and indicates that synthesis of a knob-associated HRP is probably a general phenomenon of K+ *P. falciparum*. Failure to synthesize an analogous protein appears to be a general feature of K− *P. falciparum* and is not due to coincidental, unrelated variation of the K− parasite. In additional studies, the M_r 92,000 HRP was not detected with K− Malayan Camp parasites by immunoprecipitation or immunoblotting analyses using immune serum from humans and *Aotus* monkeys or a monospecific rabbit serum against the M_r 92,000 HRP (J. H. Leech, unpublished observations). These studies strongly suggest that K− parasites fail to synthesize this protein.

The M_r of the knob-associated HRP of the Malayan Camp and FCR3 strains was 92,000;[3] the M_r of the comparable molecule in the St. Lucia strain was 108,000. Despite these differences in M_r these two proteins appear to be analogous for the following reasons. (*a*) Each protein is unique to K+ parasites. (*b*) The proteins have identical solubility characteristics. (*c*) A monospecific rabbit serum raised against the M_r 92,000 HRP of K+ Malayan Camp parasites reacts specifically with the M_r 108,000 HRP of K+ St. Lucia parasites as determined by immunoblotting and immunoprecipitation (J. H. Leech, unpublished observations). The significance of the variation in M_r of the knob-associated HRP is not known. However, since K+ IRBC of both strains bound to endothelial cells and amelanotic melanoma cells and since the knobs of these parasitized cells were structurally indistinguishable, the variation in M_r of the knob-associated HRP does not appear to affect these shared properties.

Two HRP with lower molecular weights than the knob-associated HRP were observed in both K+ and K− IRBC of the Malayan Camp and St. Lucia strains. Although there appeared to be increased labeling of these proteins in K+ compared with K− IRBC of the Malayan Camp strain, approximately equal amounts of radioactivity accumulated in these proteins in K+ and K− IRBC of the St. Lucia strain. Neither Kilejian (6) nor Hadley et al. (8) observed the lower molecular weight HRP in the FCR3 strain. The apparent differences in expression of the lower M_r HRP among K+ and K− IRBC of the three strains may be related to details of parasite treatment after their isolation from infected humans. Parasites studied immediately after isolation from an infected human synthesized three proteins that were intensely labeled by [^{3}H]histidine (J. H. Leech, unpublished observation). The K+ and K− FCR3 parasites and K− Malayan Camp parasites had been maintained for long periods in continuous culture. The other three parasites had been maintained by passage in monkeys with limited time in vitro. It is possible, therefore, that decreased expression of the lower M_r HRP may result from continuous in vitro culture. Whatever the function of these proteins, they are not unique to K+ parasites nor do they appear to be synthesized in different amounts by K+ and K− IRBC of the St. Lucia strain.

Previous ultrastructural studies of *P. falciparum* showed that the electron-dense cups were below the plasma membrane of the IRBC (5). In this study we have shown that the electron-dense cups remained in a fuzzy fibrillar network after detergent extraction of the lipid bilayer of the plasma membrane. We conclude that this fibrillar network represents the RBC skeleton, based on the M_r of its constituent proteins and on

[3] The M_r of this protein was reported by Hadley et al. (8) to be 89,000. We have recalculated this M_r based on the information that the molecular weight of phosphorylase β is 97,400 rather than 92,500 (23).

its morphological appearance compared with similarly prepared skeletons of normal human RBC (17, 18, 24). The electron-dense cup remained associated with the RBC skeleton after extraction with neutral and zwitterionic detergents, suggesting that it is bound to the skeleton.

Comparison of ultrastructural studies with the protein content of extracted cell residues (Table I) showed that detergent residues containing the electron-dense cups included the M_r 92,000 HRP. Detergent residues that did not contain cups had little or no M_r 92,000 HRP. The residues insoluble in cholic acid, Zwittergent 314, and 2% Triton X-100 in 0.6 M KCl contained the electron-dense cups and appeared to be largely depleted of other parasite material (Fig. 7 *a–c*). The M_r 92,000 HRP was a predominant parasite protein in these residues as judged by fluorography of SDS polyacrylamide gels of malarial proteins metabolically labeled by [^{3}H]histidine or [^{3}H]isoleucine and by Coomassie Blue staining (Figs. 2–5). Thus, there was a correlation in the extracted cell residues between the presence of the electron-dense cups and the presence of the M_r 92,000 HRP. In addition, the cups and the protein co-purify, suggesting that the M_r 92,000 HRP is a constitutent of the cups.

Evidence for an association between the electron-dense cups, the RBC skeleton, and the M_r 92,000 HRP is also summarized in Table I. Insoluble residues which consisted of cups bound to RBC skeleton contained the M_r 92,000 HRP and several proteins typical of the RBC skeleton. Variable amounts of band 3, the major transmembrane protein of the RBC membrane (24), were also present in these residues. The electron-dense cups and the M_r 92,000 HRP do not appear to be tightly linked to either band 3 or the lipid bilayer of the RBC membrane. When IRBC were extracted with 2% Triton X-100 in 0.6 M KCl, the insoluble residue contained electron-dense cups associated with a fibrillar matrix similar to the RBC skeleton (Fig. 7 *c*). However, band 3 was completely solubilized together with skeletal proteins 2.1 (ankyrin) and 4.2 (Fig. 5). Similar concentrations of Triton X-100 have been shown to extract >95% of RBC membrane phospholipid from human RBC (24). Taken together, the ultrastructural and biochemical data indicate that the cups and the M_r 92,000 HRP are anchored to the RBC skeleton. These experiments do not exclude the possibility that the M_r 92,000 HRP interacts with or penetrates the RBC membrane. They do suggest, however, that the cup and the M_r 92,000 HRP are tightly bound to the RBC skeleton, perhaps by interaction with skeletal proteins 1, 2, 4.1, or 5.

The functional unit of sequestration, the knob, consists of an electron-dense, submembrane cup and the overlying plasma membrane that attaches specifically to endothelium. We propose the following model for the structure of the knob. Since the M_r 92,000 HRP of Malayan Camp parasites is unique to K+ IRBC and is associated with electron-dense cups and the RBC skeleton in residues after detergent extraction (Table I), this protein may bind to the RBC skeleton to form the structural basis of the cup. The cup produces a focal protrusion of the skeleton which pushes out the RBC membrane, forming a knob. Whether the HRP crosses the bilayer structure of the membrane and is directly involved in cytoadherence is unknown. Since antibody blocks binding to endothelium in a strain-specific manner (25), we suspect that adherence is mediated by a molecule synthesized by the parasite. Thus, the knob may be analogous to coated pits of mammalian cells (26). The knob-specific HRP, like clathrin, may determine the shape of the membrane. Another molecule, a postulated cytoadherence factor, could extend across the bilayer to the external surface where it mediates binding to endothelium.

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