

HOMOLOGY BETWEEN A HISTIDINE-RICH PROTEIN FROM
PLASMODIUM LOPHURAE AND A PROTEIN
ASSOCIATED WITH THE KNOB-LIKE
PROTRUSIONS ON MEMBRANES OF ERYTHROCYTES
INFECTED WITH *PLASMODIUM FALCIPARUM**

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Intraerythrocytic stages of *Plasmodium lophuræ* contain prominent cytoplasmic granules. The main chemical constituent of isolated granules was found to be a rather peculiar protein with five major amino acids: 73% histidine, 7.5% proline, 7% alanine, 6% glutamic acid, and 2.1% aspartic acid (1, 2). Indirect experimental evidence indicated that polar organelles of merozoites may also contain a histidine-rich protein (3). Because the contents of polar organelles have been implicated to participate in infection of erythrocytes by merozoites, it seemed reasonable to test the purified protein as a vaccine. This antigen protected ducklings against infection with *P. lophuræ* (4).

Although erythrocytic stages of mammalian species of malaria do not have cytoplasmic granules similar to those of *P. lophuræ*, polar organelles are a common feature of all malaria merozoites. Therefore, the presence of a histidine-rich protein in *P. falciparum* was investigated. A dual-labeling experiment with histidine (which comprises 70% of the protein) and serine (which is lacking) indicated the presence of a 55,000-mol wt protein that had a greater histidine:serine ratio than all other labeled proteins (5). This was considered as evidence for the presence of a histidine-rich protein in *P. falciparum*. In the course of recent experiments on metabolically labeled constituents of *P. falciparum*, a striking difference in extent of incorporation of various labeled amino acids was observed in an 80,000-mol wt protein. This protein had been previously shown to be correlated with the knob-like protrusions that appear on membranes of erythrocytes infected with *P. falciparum* (6).

This communication is a report on the similarity of the amino acid composition of the histidine-rich protein (HRP) of *P. lophuræ* and the knob protein (KP) of *P. falciparum*.

Materials and Methods

P. falciparum (FCR-3/Gambia) was cultured in 100-mm Petri dishes in a candle jar (7). Parasitized erythrocytes were labeled metabolically by adding one of the following amino acids to culture medium: L-[2,3,4,5-³H]proline (0.5 μ Ci/ml); L-[3-³H]histidine (10 μ Ci/ml); [U-¹⁴C]protein hydrolysate (0.5 μ Ci/ml); L-[³⁵S]methionine (0.5 μ Ci/ml); or L-[4,5-³H]leucine (10 μ Ci/ml).

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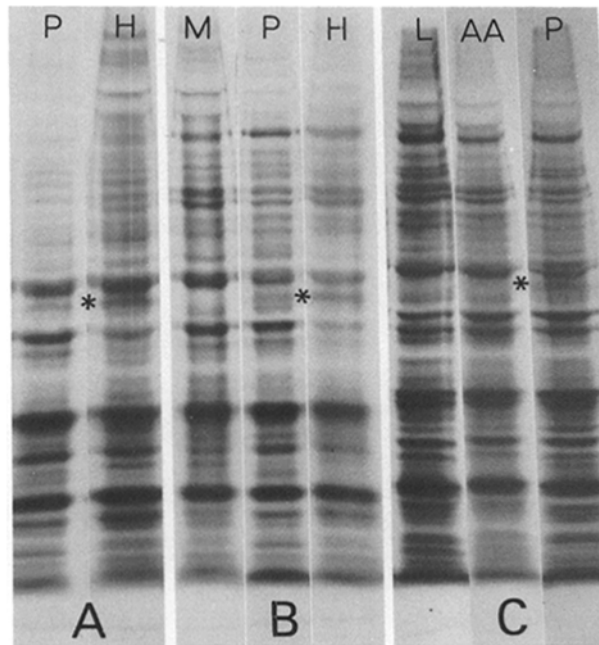


FIG. 1. Fluorogram of metabolically labeled *P. falciparum* analyzed by polyacrylamide gel electrophoresis. A, B, and C are from three independent experiments. P, proline; H, histidine; M, methionine; L, leucine; AA, protein hydrolysate. Asterisk marks the KP.

Labeled substrate was added when the majority of parasites were in the ring stage, and the cultures were collected 20 h later. Samples were prepared and analyzed by polyacrylamide gel electrophoresis as described previously (6). Radioactive proteins were visualized by fluorography (8).

Rabbits were immunized with HRP by three intraperitoneal injections of 1 mg of antigen each at 20-d intervals. For the first injection, the antigen was emulsified with 1 ml of Freund's complete adjuvant. Antiserum was collected 10 d after the last injection.

Reactivity of anti-HRP with knobs was tested by immunoelectron microscopy as described previously (9).

Results and Discussion

In a previous study, KP had been metabolically labeled with radioactive proline (6). The rationale for selecting this particular amino acid was simply for practical reasons; because the culture medium does not contain proline, dilution of labeled substrate could be avoided. When parasites were labeled with a mixture of amino acids derived from a protein hydrolysate, KP showed a striking reduction of radioactivity in relation to all other major proteins. Inspection of the composition of the protein hydrolysate (CFB.104, Amersham Corp., Arlington Heights, Ill.) showed the presence of all major amino acids except for histidine and methionine. The poor incorporation of mixed amino acids suggested possible similarity of KP to HRP. In several independent experiments, relative incorporation of selected amino acids in KP was tested. The results of three experiments are shown in Fig. 1. Similar to HRP, KP incorporates more histidine than proline, no detectable levels of methionine or leucine, and very little from an amino acid mixture that lacks histidine. In experiment B, the lane labeled with histidine was intentionally underexposed to film to illustrate the

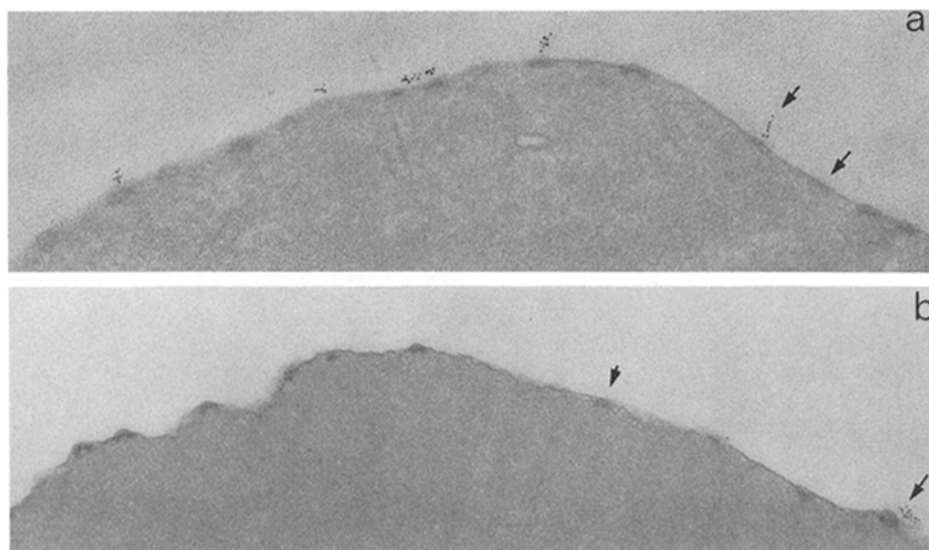


FIG. 2. Human erythrocytes infected with *P. falciparum* were incubated in a 1:2 dilution of anti-HRP prepared in rabbits (a) or a 1:2 dilution of normal rabbit serum (b), and subsequently treated with ferritin-labeled anti-rabbit IgG. Arrows indicate knobs on the membrane of host erythrocyte. $\times 62,500$.

difference from proline; similarly, samples labeled with methionine and leucine were overexposed to show the lack of labeled KP. Inspection of fluorograms from several experiments did not show a similar peculiarity of amino acid incorporation in any other major protein of *P. falciparum*.

The results of immunoelectron microscopy suggested possible immunological cross-reactivity between HRP and KP. At a 1:4 dilution of anti-HRP, only a few knobs on infected erythrocytes showed ferritin label and control samples showed no apparent binding. At a 1:2 dilution of anti-HRP, the labeling of knobs was significantly increased but a few ferritin grains were also observed in control samples (Fig. 2b). Fig. 2a illustrates the average amounts of binding observed with a 1:2 dilution of antiserum; some erythrocytes showed more label and others less. Considering the limitations of the method used, these results have to be interpreted with reservations. More quantitative methods are required for conclusive evidence on the immunological cross-reactivity. Unfortunately, because of the high charge of HRP and its insolubility at physiological pH, it has not been possible to apply standard immunological procedures for quantitation of antibody titers.

Even though HRP has a 37,500 mol wt, on sodium dodecyl sulfate-polyacrylamide gels it migrates as a 45,000- to 50,000-mol wt protein. This might be a result of peculiar binding of the detergent to this unusual protein. KP shows a mobility of $\sim 80,000$ mol wt. Though the data on the amino acid incorporation leave no doubt on homology of KP and HRP, the extent of similarity between these two proteins will await purification and amino acid analysis.

In view of the data, some of the hypotheses based on past experiments have to be reevaluated. The 55,000-mol wt protein reported from *P. falciparum* (5) is apparently a protein that has relatively more histidine than serine. As is apparent from the data in Fig. 1, comparison of incorporation of any two single amino acids shows some

relative differences in minor constituents. Furthermore, the experiment that was carried out soon after the discovery of the culture system was based on asynchronous cultures, growing at low levels of parasitemia. With hindsight, it is apparent that the crude samples that were analyzed could not have had detectable levels of labeled KP. The hypothesis that HRP might be a constituent of polar organelles of merozoites was based on incorporation of high levels of histidine in these organelles as well as the reactivity of purified HRP with membranes (3). It is interesting that KP is also reactive with membranes because the knobs have been shown to adhere to endothelial cells (10). Recent studies on stage-specific proteins of *P. falciparum* indicate that KP is not a major constituent in a merozoite-enriched preparation, though the possibility of minor amounts cannot be ruled out (11). Therefore, the possible participation of HRP in merozoite entry will remain a hypothesis until proven or disproven conclusively.

The knobs were found to be antigenically different from adjacent areas of erythrocyte membrane devoid of them (9). The presence of anti-knob antibody in serum of experimentally immunized monkeys was recently shown by immunoelectron microscopy (12). To date, all successful experimental vaccinations with a particular antigen in one species of malaria have been shown to be applicable to homologous antigens from other species. Because HRP was already shown to be a functional antigen for vaccination of ducklings, the homology of KP and HRP strengthens the hypothesis proposed in a previous study (9) that KP has the potential for use as a defined malaria vaccine.

Summary

The incorporation of several radioactive amino acids into the knob protein of *Plasmodium falciparum* was compared. Histidine showed better incorporation than proline. A protein hydrolysate, which had all major amino acids except histidine and methionine, showed relatively poor incorporation as compared with proline, and no labeling could be detected with methionine or leucine. These results strongly suggest that the amino acid composition of the knob protein has the same peculiarities as that of a histidine-rich protein characterized from *P. lophurae*. Immunoelectron microscopy suggested possible immunological cross-reactivity between these two proteins.

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