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Characterization of a protein correlated with the production of knob-like protrusions on membranes of erythrocytes infected with *Plasmodium falciparum*

(malaria)

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ABSTRACT **Membranes of erythrocytes infected with *Plasmodium falciparum* develop protrusions called "knobs." These protrusions are not apparent on erythrocytes infected with young parasites (rings) but develop with the growth of parasites to the trophozoite and schizont stages. The nature and origin of knobs were characterized by comparing the stage-specific proteins of two culture lines of *P. falciparum*, K+ and K−. K+ parasites produce knobs; K− parasites do not. Erythrocytes infected with both types of parasites were labeled metabolically and samples were analyzed by electrophoresis in sodium dodecyl sulfate/polyacrylamide gels. There were no apparent differences in Coomassie blue-stained or radioactive components of rings of K+ and K− parasites. However, erythrocytes infected with K+ trophozoites or schizonts showed a major labeled protein, with an apparent molecular weight of ≈80,000, that was not present in any developmental stage of K− parasites or in K+ rings. A fraction enriched in membranes from erythrocytes infected with K+ trophozoites showed enrichment of this protein. The results indicate that this protein, synthesized by the parasites, is correlated with the formation of knobs on the host cell membrane. Two additional labeled components were identified. These appeared with the development of schizonts in both K+ and K− parasites and are therefore stage-dependent and not correlated with knobs.**

Membranes of erythrocytes infected with *Plasmodium falciparum* develop morphological alterations that were first described as "knob-like protrusions" (1). These protrusions are not apparent on erythrocytes that are infected with ring stages; they develop with the growth of parasites. In infected hosts, erythrocytes parasitized with multinucleate stages become sequestered and are not seen in the peripheral circulation. The knobs have been shown to be the sites of adhesion between sequestered erythrocytes and venous endothelial cells (2).

A previous study (3) demonstrated that these protrusions were antigenically different from adjacent areas devoid of them. In an attempt to determine whether knob constituents were antigens of parasite origin that were incorporated into the host cell membrane, membrane fractions enriched in knobs were isolated from metabolically labeled infected erythrocytes and compared with fractions devoid of knobs. An additional labeled protein, with an apparent molecular weight of 70,000–80,000, was identified in samples enriched with knobs (4). From these results it could not be concluded with certainty whether this protein was a knob constituent or some other protein associated with membranes. Recently, it was noted that in cultures of an African strain of *P. falciparum* (FCR-3/Gambia) that had been maintained in flow vials (5) for >2 years by W. Trager some parasites failed to produce knobs (6). We were able to select for these parasites and obtain a culture line that showed mainly (about 99%) parasitized erythrocytes with smooth membranes (A. Kilejian and W. Trager, unpublished data). For purposes of discussion, the parent strain will be referred to as K+ and the derived culture line that does not produce knobs, as K−.

This communication is a report on the comparison of stage-specific proteins of K+ and K− parasites. The results show that a metabolically labeled protein, of ≈80,000 molecular weight, that had been noted in a previous study (4) is correlated with the production of knobs. Two additional components that are synthesized during the development of merozoites have been identified.

MATERIALS AND METHODS

Parasites. *P. falciparum* (FCR-3/Gambia) was cultured in 100-mm petri dishes in a candle jar (7). Cultures of K+ and K− parasites were synchronized by treatment with sorbitol (8).

Labeling of Stage-Specific Proteins. The developmental stages selected for comparison were: (*i*) uninucleate parasites that were predominantly in ring form; (*ii*) trophozoites that showed at least two nuclei but were not close to segmentation; and (*iii*) mature schizonts. L-[2,3,4,5-^{3}H]Proline (Amersham) was added to the culture medium [2.5 μCi (1 Ci = 3.7×10^{10} becquerels)/ml] of K+ and K− parasites that were developing synchronously at 18–20% parasitemia. The different stages were labeled in sequence—i.e., as one stage was collected, labeling of the next stage was initiated. Because parasites appear as rings for 14–16 hr, it was not possible to determine the precise age of parasites for the initiation of labeling. The proper time for collection of each sample was determined from stained smears made at intervals of 5–6 hr. The incubation time in labeled substrate for rings, trophozoites, and schizonts of K+ parasites was 12, 16, and 13 hr, respectively; for K− parasites it was 8, 13, and 11 hr.

Preparation and Analysis of Samples. Erythrocytes collected from culture were pelleted by centrifugation (10 min, 900 × *g*, 20°C). After one washing with 0.14 M NaCl/10 mM phosphate, pH 8/5 mM glucose/1 mM $CaCl_2$, the pellets (0.5 ml) were lysed by suspending them in 40 ml of 20 mM phosphate, pH 8/0.5 mM phenylmethylsulfonyl fluoride. The suspensions were centrifuged immediately (10 min, 1200 × *g*, 20°C). After the supernatant and a buffy layer of normal erythrocyte ghosts were removed, 1 ml of 20 mM phosphate pH 8 buffer was added and the suspension was transferred to a microfuge tube. Samples were centrifuged for 1 min (Beckman, Microfuge B) and the pellets were kept at −80°C until analyzed. To prepare a membrane-enriched fraction, 2 ml of erythrocytes infected with K+ trophozoites (20% parasitemia) was suspended in 10 ml of 0.1% saponin (Eastman) in the washing buffer (see above). After 5 min at room temperature,

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

30 ml of the buffered saline was added and the hemolyzed samples were centrifuged (10 min, 900 × *g*, 4°C). The pellet was suspended in 10 ml of ice-cold 0.1 M phosphate buffer at pH 8 and forced twice through a syringe fitted with a 26-gauge needle. Parasites were pelleted (10 min, 500 × *g*, 4°C) and membrane fragments from the supernatant were collected by centrifugation (60 min, 130,000 × *g*, Beckman rotor 50.1, 4°C).

Sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis was performed in 1.5-mm slab gels with a discontinuous buffer system (9). A stacking gel with 4% acrylamide and a separating gel of either 7.5% or an exponential gradient of 5–15% was used. Gels were stained with Coomassie brilliant blue. To be able to correlate radioactivity with specific stained bands and to avoid variations due to different degrees of stretching of gels during slicing, adjacent lanes of stained samples that were to be compared were aligned along graph paper and sliced into 2 mm slices simultaneously. Subsequently, the sliced gels were cut perpendicularly in 1-cm widths. For measurement of radioactivity, gel slices were first solubilized by incubating in 0.3 ml of $H_2O_2/HClO_4$, 2:1 (vol/vol), for 4 hr at 60°C.

RESULTS

Preliminary experiments designed to determine solubility properties of knob constituents indicated that repeated washes of infected erythrocytes with isosmotic buffers that were free of protein and divalent cations caused considerable membrane-blebbing and loss of knobs. In addition, extensive exposure to low ionic strength buffers (5–10 mM, pH 8) seemed to extract some component of the knobs as judged by electron microscopy. To avoid any loss of knob constituents in this study, the exposure of parasitized erythrocytes to buffers was reduced to a minimum.

The electrophoretic patterns of erythrocytes infected with different developmental stages of K+ and K− parasites are shown in Fig. 1. For ease of description, selected components are labeled alphabetically. Any component associated with knobs would be predicted to be absent in all developmental states of K− parasites, present in multinucleate forms of K+ parasites, and absent or decreased in K+ rings (because knobs are not apparent at this stage of development).

All these criteria were met by one stained band (band d in Fig. 1) with an apparent molecular weight around 80,000. This protein was present only in trophozoites and schizonts of K+ samples. Examination of the staining patterns for stage-specific proteins showed two minor components that were limited to schizonts of both lines of parasites (Fig. 1, bands a and b).

From the stained proteins it was not possible to distinguish between host and parasite components. However, because mature erythrocytes do not synthesize proteins, all radioactive proteins from the metabolically labeled samples had to originate from the parasites. The distribution of radioactivity along sliced gels is shown in Figs. 2 and 3. Rings of K+ and K− parasites showed almost an identical pattern of labeled proteins (Fig. 2 *left*). Similar to stained samples, K+ trophozoites showed a prominent labeled component (Fig. 2 *center*, peak d) that was totally absent in K− trophozoites and perhaps present only in trace amounts of K+ rings (Fig. 2 *left*, note shoulder on peak c). Despite the presence of a distinct stained component d in K+ schizonts, there was very little measurable comigrating radioactivity (Fig. 2 *right*, peak d). The most likely explanation is that synthesis of this constituent is mainly at the trophozoite stage and very little was synthesized during segmentation when labeled substrate was present. The relative increase of radioactivity in peaks a and b in schizonts (Fig. 2 *right*) paralleled

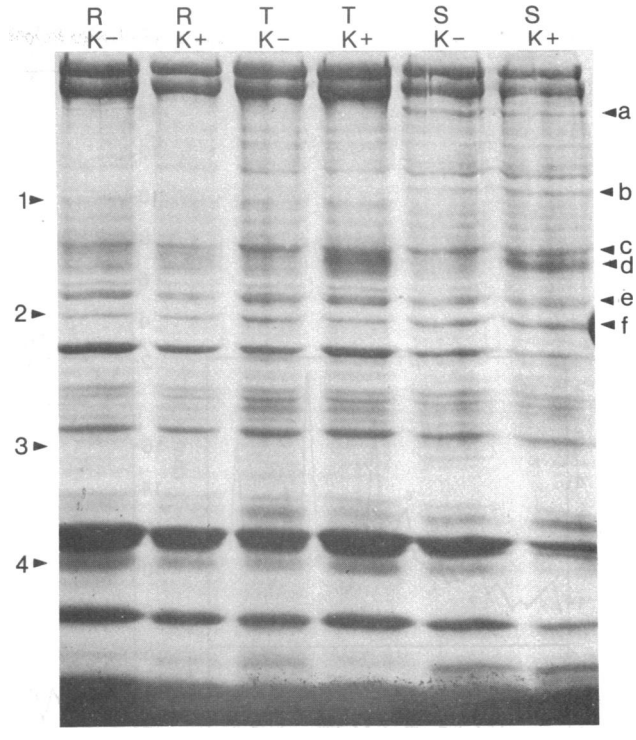

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of erythrocytes infected with different developmental stages of K+ and K− *P. falciparum*. R, ring; T, trophozoite; S, schizont. Numbers at the left indicate molecular weight marker proteins: 1, β-galactosidase, 130,000; 2, bovine serum albumin, 68,000; 3, ovalbumin, 45,000; 4, chymotrypsinogen 25,000. Letters show selected components discussed in text.

the appearance of the corresponding stained components (Fig. 1). From the limited information of this study, it is not possible to conclude whether peaks a and b represent merozoite or membrane components. Another consistent stage-related difference between trophozoites and schizonts of both lines of parasites was a change in relative proportions of peaks e and f (compare Fig. 2 *center* and *right*). Preliminary studies indicate that peak f may be a glycoprotein associated with the plasma or parasitophorous vacuole membrane of the parasite; it can be labeled with radioactive glucosamine and has been purified by concanavalin A affinity chromatography (unpublished data).

The distribution of radioactivity of a membrane-enriched sample that was prepared from erythrocytes infected with K+ trophozoites is shown in Fig. 3. The enrichment of peak d relative to other major labeled peaks seen in trophozoite samples further supports the correlation of this component with knobs.

DISCUSSION

Comparison of stage-specific proteins from metabolically labeled K+ and K− *P. falciparum* has shown clearly that a component of parasite origin identified in a previous study (4) is indeed correlated with production of knobs. Because erythrocyte membranes free of membrane contaminants from parasites have not been analyzed, it could be argued that the identified component could indirectly mediate the formation of knobs rather than be a constituent of it. This remote possibility could be ruled out only by production of specific antibody because it has not been possible to prepare host membranes free of parasite contaminants by the standard procedures used for isolation of membranes. As *P. falciparum* develops, fragments

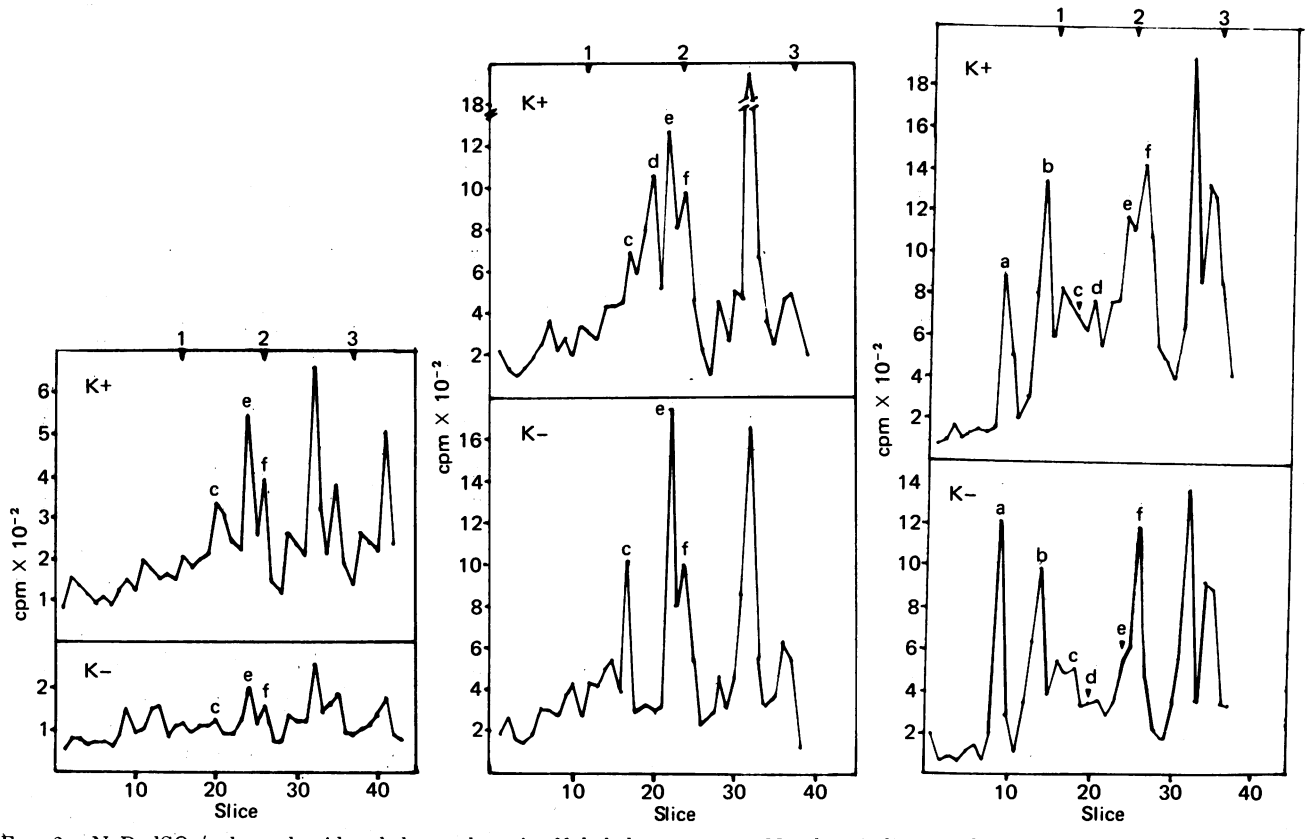

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of labeled components. Numbers indicate mobility of molecular weight markers as in Fig. 1. Letters correspond to stained bands shown in Fig. 1. (*Left*) Ring stages of K+ and K− parasites. (*Center*) Trophozoite stages of K+ and K− parasites. (*Right*) K+ and K− schizonts.

of parasitophorous vacuole membrane (described as Maurer's clefts by light microscopy) are released into the host cell cytoplasm; therefore, regardless of method of homogenization, release of host cell membrane is accompanied by simultaneous release of membranes of parasite origin. The presence of these membrane structures in the host cytoplasm makes them a logical vehicle for the insertion of parasite metabolites into the host erythrocyte membrane.

Differential solubility properties of major polypeptides of human erythrocyte ghosts have been well documented (10, 11). The results of this study suggest that standard conditions (12) used in preparation of hemoglobin-free ghosts result in considerable losses of some components from membranes of parasitized cells. The inclusion of divalent cations in wash media and minimal exposure of infected cells to buffers of low ionic strength has considerably increased the recovery of the knob component in analyzed samples. It is possible that the brief period of manipulation of the test material has also helped reduce the possibility of enzymatic degradation.

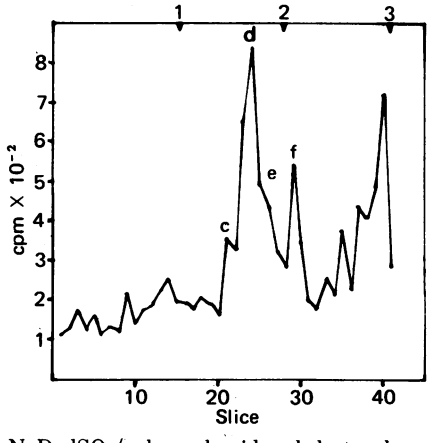

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of membrane-enriched fraction from K+ trophozoites. Letters and numbers as in Fig. 2.

With the exception of components a, b, and d, which are stage-related and therefore of parasite origin, it is not possible to distinguish overlapping components of host and parasite in stained gel bands. Even though the samples are not suited for precise comparison with polypeptides of membranes of normal erythrocytes, one striking change merits mention. There is a drastic reduction of component 3, which has been shown to constitute 25% of stainable proteins of erythrocyte ghosts and which in stained gels occupies a broad molecular weight region extending from 88,000 to 105,000 (11). In infected samples, its leading edge corresponds to radiolabeled band c; it is not possible to distinguish between the contributions of host and parasite to the stained band c (Fig. 1). Preliminary studies indicate that reduction in component 3 may be in part due to exposure of erythrocytes to metabolites of parasites that are released from schizonts into the culture medium, along with the release of merozoites.

Antigenic modifications of infected host erythrocyte membranes have been shown in infections with other species of malaria parasites, irrespective of presence or absence of morphological modifications (13–16). In none of these instances has the use of surface antigens in eliciting protective immunity been tested. However, selective destruction of infected erythrocytes has been correlated with the immune state of the host (17). Because knobs play a role in sequestration of *P. falciparum*,

they may be important for the development or survival of parasites within the host. Now that a knob constituent is identified, its possible use in immunization can be tested experimentally.

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