Membrane-associated Phosphoproteins in *Plasmodium berghei*-infected Murine Erythrocytes

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ABSTRACT Normal and *Plasmodium berghei* (NYU-2 strain)-infected murine erythrocytes display substantially different patterns of plasma membrane phosphoproteins phosphorylation. Intact erythrocytes (normal and parasite infected) incubated with $^{32}P_i$ and isolated washed erythrocyte plasma membranes incubated with γ-^{32}P-ATP were analyzed for phosphoproteins by SDS PAGE and autoradiography. Two new phosphoproteins of molecular weight 45,000 (pp45) and 68,000 (pp68), which are absent in normal erythrocyte membranes, are associated with the membranes of infected erythrocytes subjected to both intact-cell and isolated-membrane phosphorylation conditions. Two-dimensional gel electrophoresis indicates that pp45 and pp68 are of parasite origin. Partial or complete proteolytic digestion reveals that pp45 is phosphorylated at similar amino acid residues both in intact cells and in isolated membranes. The pp45 phosphoprotein can be detected at as low as 3% parasitemia and its phosphorylation is not affected by 10 μM cAMP, 1 mM Ca^{2+}, or 5 mM EGTA. Extraction of isolated washed plasma membranes with 0.5% Triton X-100 or 0.1 M NaOH indicates that pp45 is detergent insoluble and only partially extractable with NaOH, suggesting that pp45 is closely associated with the host erythrocyte plasma membrane.

Malaria is one of the most thoroughly studied intracellular parasites, yet relatively little is known of the metabolic and regulatory interactions between the parasite and host cell. Many changes in the structure and function of host erythrocyte membranes are known to occur after malarial infection (1, 2). These aberrations include increased permeability to glucose (3), increased amino acid transport (4), increased Na^+ content and permeability (5), increased Ca^{2+} content and influx (6), increased osmotic fragility (7), and the appearance of new polypeptides (8–10).

Protein phosphorylation, a regulatory mechanism of primary physiological importance, is catalyzed by protein kinases (11). Specific erythrocyte membrane proteins are known to undergo reversible phosphorylation (12). Although no definitive role for the phosphorylation of these proteins has been described, abnormalties in erythrocyte membrane protein phosphorylation have been shown to occur in pathological conditions such as sickle cell anemia (13), hereditary spherocytosis (14), hereditary stomatocytosis (15), and muscular dystrophies (16). In this paper we report striking qualitative differences in the phosphoproteins associated with the plasma membranes from normal and *Plasmodium berghei*-infected murine erythrocytes.

MATERIALS AND METHODS

Malaria Infection and Parasite Isolation: *P. berghei* (NYU-2 strain) was maintained in Swiss white mice as previously described (17). Blood was obtained by axillary incision of anesthetized mice and aspiration into heparin. Unless otherwise noted, 70–85% of the erythrocytes from infected animals were parasitized. Blood was filtered through powdered cellulose (Whatman CF-11) to deplete it of white cells and platelets (18). Normal and infected erythrocytes were lysed in 20 mM phosphate buffer, pH 7.6, at 4°C, and intact parasites were removed by centrifugation at 1,000 *g*. Parasites were washed three times in isotonic-alkaline KCl (19). Host cell membranes (normal and parasitized) were isolated from the remaining 1,000 *g* supernatant by centrifugation at 15,000 *g* and washed in 20 mM phosphate buffer, pH 7.6, three times. Microscopic examination of the isolated membranes indicated some parasite trapping by the ghosts. Assays of glutamate dehydrogenase (a parasite enzyme not present in normal erythrocytes) associated with the isolated erythrocyte membranes indicated that parasite protein contamination in all cases was <10%. Protein was assayed by the method of Lowry et al. (20) using bovine serum albumin (BSA) as a standard.

Protein Phosphorylation of Intact Cells and Isolated-Membranes: Normal and infected erythrocytes were isolated as described above and washed once in phosphate-free minimum essential medium (MEM) (Flow Laboratories, Inc., McLean, VA). Protein phosphorylation occurring within intact erythrocytes was ascertained by incubating the cells at 37°C for 1 h in phosphate-free MEM plus 1 mCi/ml $^{32}PO_4$ (New England Nuclear, Boston, MA). Parasites and erythrocyte membranes were isolated, washed as described above, and solubilized in gel electrophoresis sample buffer.

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Alternatively, isolated erythrocyte membranes (1 mg/ml) prepared as described above were incubated in a final volume of 0.1 ml containing 50 mM 2-(*N*-morpholino) ethane sulfonic acid (MES), pH 6.0; 10 mM $MgCl_2$; 1 μM γ-^{32}P-ATP (20–50 Ci/mmole) (21) and various other additives as indicated for 2 min at 30°C. The reaction was terminated by the addition of 0.1 ml of double-strength gel electrophoresis sample buffer

Electrophoresis: Phosphoproteins (20 μg of total protein per lane) were analyzed by SDS PAGE followed by autoradiography. The discontinuous SDS PAGE system as described by Laemmli (22) was used with 0.75-mm slabs of 9% acrylamide.

Electrophoresis was performed at 20 mA per gel for 3–4 h. Gels were fixed in 50% methanol/10% acetic acid, stained by the method of Holbrook and Leaver (23) in Coomassie Blue G-250 and perchloric acid, and destained in 5% methanol/7% acetic acid. Phosphoproteins were detected by autoradiography of dried gels using Cronex-4 x-ray film and Cronex Lighting Plus intensifying screens (24). Two-dimensional gel electrophoresis was performed as described by O'Farrell (25) using 8–15% polyacrylamide gradient gels for the second dimension.

Selective Extraction of Membrane Proteins: Normal and infected host erythrocyte membranes isolated and phosphorylated as described above (except for terminating the reaction by washing the membranes once) were extracted with either 0.1 M NaOH or 0.5% Triton X-100 in 20 mM phosphate buffer, pH 7.6, for 30 min at 0°C as described by Steck and Yu (26, 27). The soluble and insoluble fractions were separated by centrifugation at 27,000 *g* for 30 min. Before centrifugation, an aliquot was removed as an unextracted control. The pellet was resuspended in the extraction buffer to the same volume as before centrifugation.

Two-dimensional Peptide Mapping: Following SDS PAGE and autoradiography, pp45 was excised from the gel and eluted into 0.05 M NH_4HCO_3, pH 8.3, 0.1% SDS at 37°C for 12–16 h. The eluate was filtered through glass wool, and 30 mg of carrier BSA was added. Trichloroacetic acid (TCA) was added to bring the final concentration to 20% TCA and the mixture allowed to precipitate for 16 h at 0°C. The precipitate was collected by centrifugation, dissolved in 0.2 ml of 0.1 M NaOH, and reprecipitated with 20% TCA at 0°C for ~1 h. The precipitate was collected again by centrifugation, then dissolved in 0.2 ml of 1 M NH_4OH, and reprecipitated in 20% TCA for 1 h at 0°C. The precipitate collected by centrifugation was then oxidized in 0.1 ml of performic acid (19 vol formic acid, 1 vol 30% H_2O_2) for 1 h at 0°C followed by lyophilization twice from 10 vol of H_2O. The protein was then dissolved in 0.1 ml of freshly prepared 0.05 M NH_4HCO_3, pH 8.3, and 5 μg of *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, IN) was added, followed by incubation at 37°C for 1 h. The pH was then checked and adjusted with HCl, and an additional 5 μg of protease was added followed by incubation for 5 additional h. The sample was lyophilized twice from 10 vol of H_2O. The peptides were then dissolved in 0.1 M NH_4OH and spotted onto thin-layer cellulose sheets (Polygram cel 300, 20 × 20 cm, Macherey-Nagel and Co.). The peptides were first separated by ascending chromotography in 2° butanol, *n*-propanol, isoamyl alcohol, pyridine, H_2O (1:1:1:3:3). The sheet was dried at room temperature and subjected to electrophoresis at 1,800 V for ~10 min in pyridine, acetic acid, H_2O (100:3:879), pH 6.5. The sheets were dried and then autoradiographed with Cronex 4 x-ray film and Lightning plus intensifying screens.

RESULTS

P. berghei-infected murine erythrocyte membranes have several new proteins and phosphoproteins as detected by two-dimensional gel electrophoresis followed by protein staining and autoradiography (Fig. 1). The most prominent new phosphoproteins associated within the host cell membranes detected by $^{32}P_i$ incorporation have molecular weights of 45,000 (pp45) and 68,000 (pp68) and pIs of 3.8, as determined by a preparative isoelectric focusing (data not shown). Two-dimensional gel electrophoresis indicates that pp45 and pp68 are of parasite origin since protein-staining bands corresponding with these phosphoproteins can be detected only in membranes from infected cells. The appearance of these new phosphoproteins is not likely the result of contamination by the parasites since these phosphoproteins are only minor components in the isolated parasites, which could be due to host membrane contamination of the parasites. In addition, the staining intensity of pp45 and pp68 is greater in the host membranes than in the isolated parasites (Fig. 1, *c* and *e*).

The isolated parasites also have a 45,000-dalton phosphoprotein of a different pI (~5.3) than pp45 found associated with the host membrane. Whether or not there is a relationship between these two proteins cannot be determined at this time. However, the two proteins are phosphorylated differently when analyzed by partial proteolysis.

The possibility that these new phosphoproteins are proteolytic fragments of spectrin has been ruled out. Fig. 2 shows partial proteolytic digestions with papain of spectrin (200), pp68, and pp45. As can be seen, all three are clearly unique. The same conclusions are reached if either the *S. aureus* V8 protease or chymotrypsin is substituted for papain.

The degree of phosphorylation of pp45 and pp68 increases with degree of parasitemia (Fig. 3). We believe that this is due to an increase in the substrates (pp45 and pp68) rather than the protein kinase activity since there is also enhanced staining intensity of pp45 and pp68 on two-dimensional gels as parasitemia progresses (data not shown). The decrease in spectrin phosphorylation (Fig. 3) is not a consistent observation. However, the decrease in the phosphorylation of the 50,000-dalton phosphoprotein is reproducible.

Phosphorylation of pp45 and pp68 is not affected by 10 μM cAMP, 1 mM Ca^{2+}, or 5 mM EGTA (not shown). The phosphorylation of pp45 and pp68 has a pH optimum of 6.0 and is unaffected by NaCl up to 0.2 M NaCl. However, at concentrations >0.2 M NaCl, phosphorylation of all phosphoproteins is markedly inhibited.

The same pattern of protein phosphorylation is found by either labeling intact cells with $^{32}P_i$ and then isolating membranes, or by isolating membranes and then labeling with γ-^{32}P-ATP (Fig. 4), indicating that the protein kinase responsible for these reactions remains associated with the erythrocyte membrane during isolation. Furthermore, partial proteolytic digestion of pp45 by the method of Cleveland et al. (28) (Fig. 5) reveals that pp45 labeled by either method (intact cell or isolated membrane) is phosphorylated at similar amino acid residues. Complete proteolytic digestion with *S. aureus* V8 protease followed by two-dimensional peptide mapping also demonstrates that the same sites on pp45 are phosphorylated by labeling intact cells with $^{32}P_i$ or isolated membranes with γ-^{32}P-ATP (Fig. 6), indicating that the phosphorylation is probably physiologically significant (29). However, there is one minor difference in the two-dimensional peptide maps. Two major phosphopeptides are generated during phosphorylation of isolated membranes with γ-^{32}P-ATP, whereas only one major phosphopeptide occurs following phosphorylation of intact cells with $^{32}P_i$. The upper peptide seen in Fig. 6*b* is present only as a very minor spot in Fig. 6*a*. Further digestion of pp45 with either trypsin or papain results in the same peptide pattern, suggesting that the V8 protease digestion is complete and that there is only one (or possibly two) major site(s) of phosphorylation on pp45. Both serine and threonine residues are phosphorylated on pp45, with serine being >95% of the total phosphoamino acids (not shown).

Further evidence that pp45 is associated with the host erythrocyte membrane is the selective solubility of pp45 with Triton X-100 and 0.1 M NaOH (Fig. 7). Neither pp45 nor pp68 is soluble in Triton X-100, suggesting that neither is an integral membrane protein (27). However, 0.1 M NaOH completely extracts pp68 and spectrin but only partially extracts pp45. Repeated extraction with 0.1 M NaOH does not solubilize any more of the membrane-bound pp45, indicating a very strong association between pp45 and the host cell membrane. In addition, extraction with 5 mM β-mercapto-

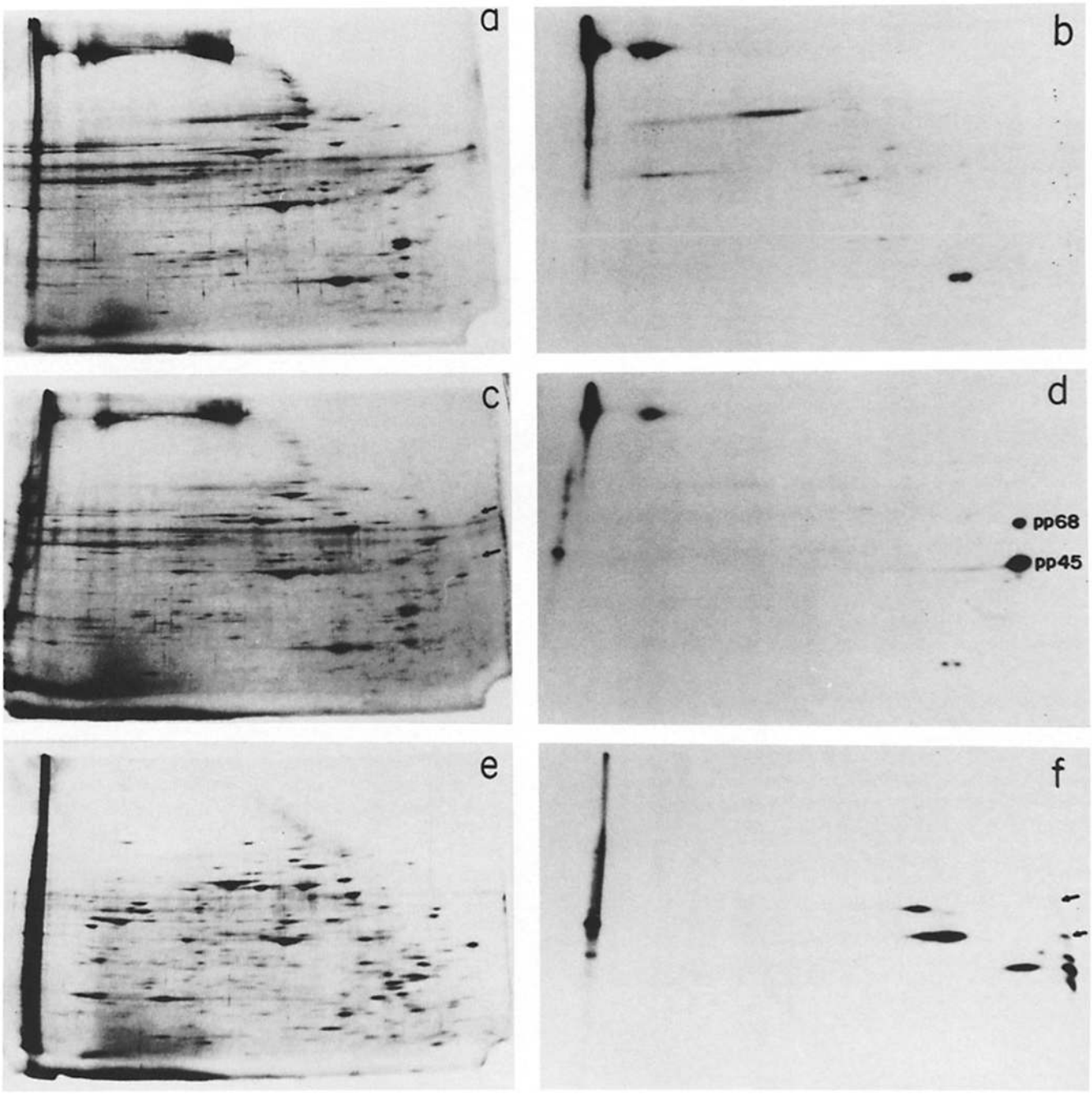

FIGURE 1 Two-dimensional gel electrophoresis of membranes from normal and *P. berghei*-infected erythrocytes and isolated parasites. Membranes and parasites were isolated as described in Materials and Methods. Isolated membranes and parasites (sonicated after isolation) were phosphorylated as described in Materials and Methods, except that the reaction was stopped by adding 10 vol of acetone (−20°). The precipitate was collected by centrifugation, then lyophilized, resolubilized in O'Farrell lysis buffer (25), and 250 μg of protein of each sample was subjected to two-dimensional gel electrophoresis as described (25). Coomassie Blue-stained gels are shown on the left and the corresponding autoradiographs are shown at the right of membranes from normal erythrocytes (*a* and *b*), host membranes from infected erythrocytes (*c* and *d*) and isolated parasites (*e* and *f*). The positions of pp68 and pp45 are indicated in (*d*), and the arrows in (*c*) indicate the staining proteins corresponding to pp68 and pp45, and the arrows in (*f*) indicate pp45 and pp68 in the isolated parasites.

ethanol in 0.1 M NaOH does not result in any additional solubilization of pp45, indicating that disulfide bonds are not involved with the membrane association. Examination of Coomassie Blue-stained proteins indicates that the extraction is complete since all the spectrin and actin become solubilized, yet all of band 3 remains insoluble. This same result was observed whether membranes from intact cells labeled with $^{32}P_i$ or isolated membranes labeled with γ-^{32}P-ATP were used. Both the NaOH-soluble and -insoluble forms of pp45 are phosphorylated similarly when examined by partial proteolysis or complete proteolysis followed by two-dimensional peptide mapping (data not shown).

DISCUSSION

P. berghei infection substantially alters the host erythrocyte plasma membrane and, as shown in this study, causes the appearance of new membrane-associated phosphoproteins. The most prominent of these have molecular weights of

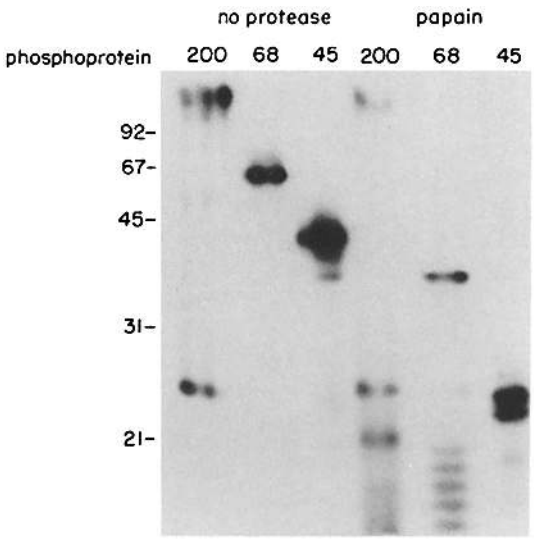

FIGURE 2 Partial proteolytic digestion of spectrin, pp68, and pp45. Spectrin (200), pp68 (68), and pp45 (45) were excised from gels after autoradiography and analyzed by partial proteolysis as described by Cleveland et al. (28). The excised gel slices of the phosphoproteins were loaded onto a 15% polyacryamide gel and overlayed with either no protease or 1 μg of papain as indicated. Digestion was then allowed to proceed during electrophoresis. (Numbers at left, × 10^{-3}).

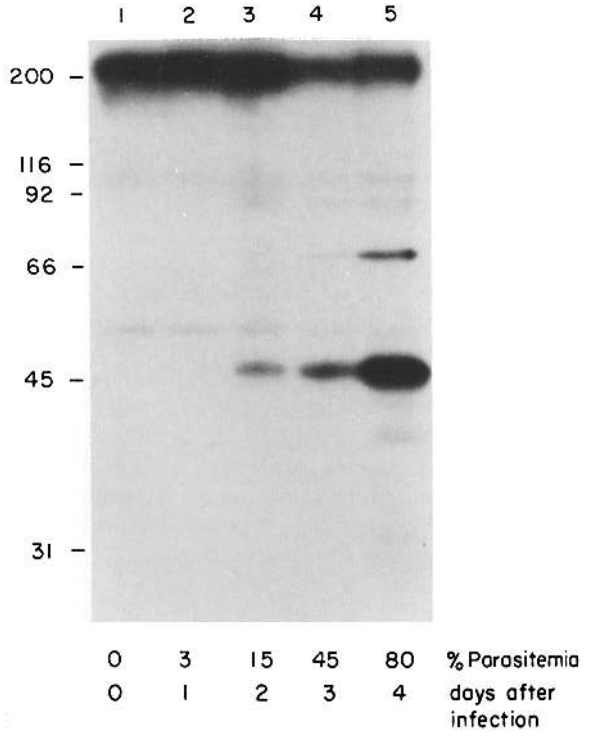

FIGURE 3 Appearance of new phosphoproteins during the course of infection. Blood was obtained on successive days after inoculation. Membranes were isolated and phosphorylated as described in Materials and Methods. Autoradiograph of SDS PAGE is shown with the days after infection and the percent parasitemia indicated. (Numbers at left, × 10^{-3}.)

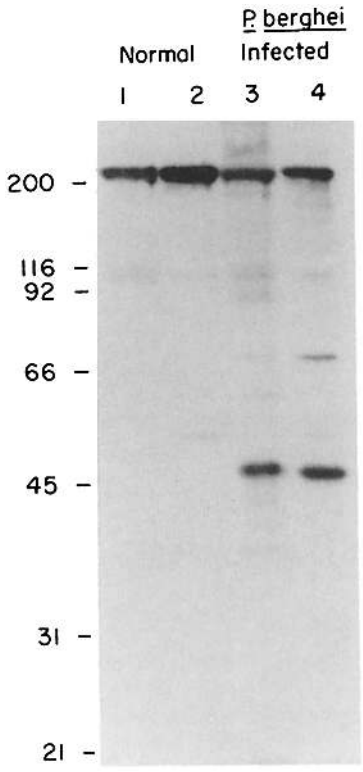

FIGURE 4 Comparison of host cell membrane phosphorylation in intact cells and isolated membranes. Intact cells were incubated in PO_4-free MEM plus 1 mCi/ml $^{32}PO_4$ (lanes *1* and *3*) or isolated membranes were incubated with γ-^{32}P-ATP (lanes *2* and *4*) as described in Materials and Methods. (Numbers at left, × 10^{-3}.)

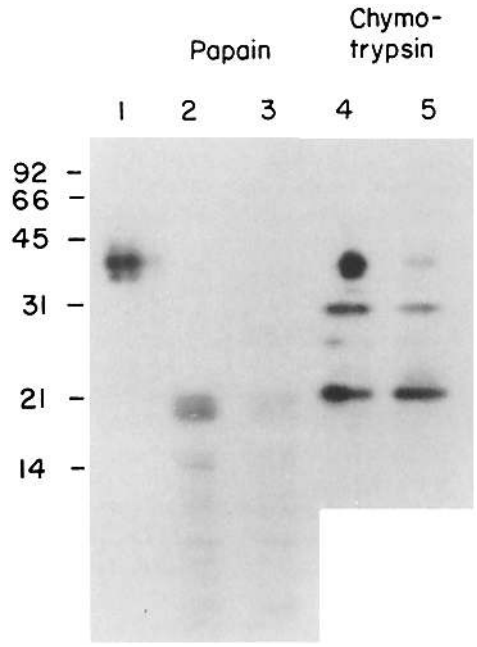

FIGURE 5 Partial proteolytic digestion of pp45. The 45,000-dalton phosphoprotein was excised from gels after autoradiography of either intact cell or isolated membrane labeled samples and partially digested as described in Fig. 2 with 1 μg of either papain or chymotrypsin as indicated. In lane *1* no protease was added, and lanes *2* and *4* are pp45 from isolated membrane labeled samples, while lanes *3* and *5* are pp45 from intact cell labeled samples. (Numbers at left, × 10^{-3}.)

45,000 (pp45) and 68,000 (pp68). These readily phosphorylated proteins most likely originate from the parasite since proteins corresponding to pp45 and pp68 can be detected by Coomassie Blue staining on two-dimensional gels of membranes from infected erythrocytes but not of membranes from normal erythrocytes. Furthermore, the intensity of pp45 and pp68 protein staining increases in proportion to the percentage of infected erythrocytes (data not shown), suggesting that these phosphoproteins are, indeed, synthesized by the parasite.

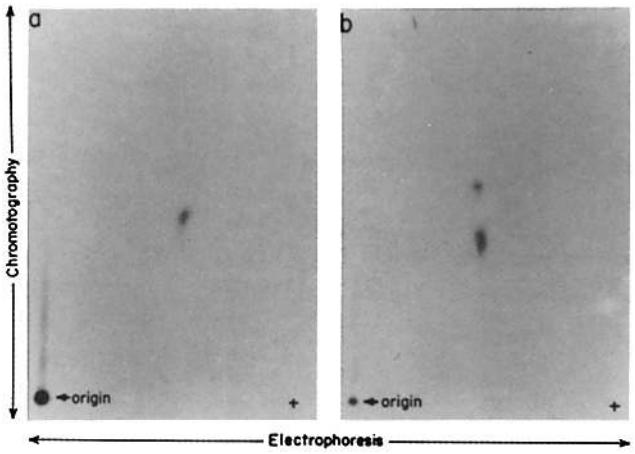

FIGURE 6 Two-dimensional peptide mapping of pp45. After autoradiography, pp45 from either intact cell (*a*) or isolated membrane (*b*) labeled samples were excised from gels, eluted, digested, and chromatographed as described in Materials and Methods. The major phosphopeptide *a* and the lower major phosphopeptide in *b* are equivalent.

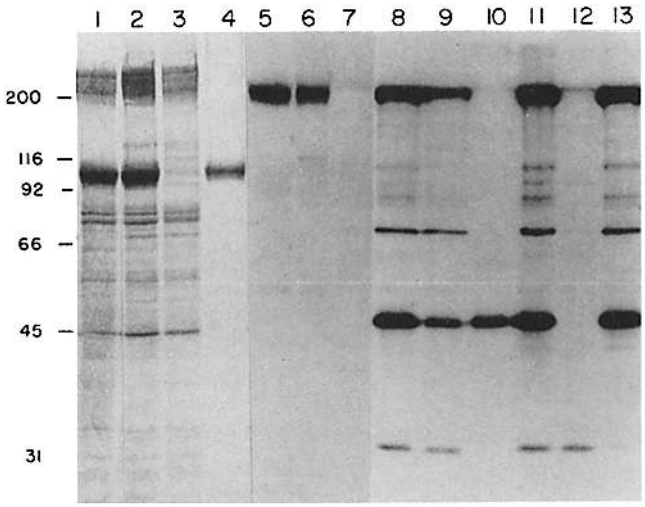

FIGURE 7 Selective solubility of host membrane-associated proteins. Membranes were isolated and phosphorylated as described in Materials and Methods. After phosphorylation the membranes were washed once in 20 mM phosphate buffer, pH 7.6. The membranes were then resuspended in the appropriate extraction buffer at a concentration of 1 mg/ml and extracted as described in Materials and Methods. Lanes *1–4* are Coomassie Blue-stained proteins showing unextracted membranes from normal (*1*) and infected (*2*) cells along with the NaOH-soluble (*3*) and -insoluble (*4*) fractions from host membranes of infected cells. Lanes *5–7* are the autoradiographs from NaOH extractions of normal red cell membranes showing unextracted (*5*), soluble (*6*), and insoluble (*7*) fractions. Lanes *8–10* are the autoradiographs from NaOH extractions of host membranes from infected cells showing unextracted (*8*), soluble (*9*), and insoluble (*10*) fractions. Lanes *11–13* are the autoradiographs from Triton X-100 extractions of host membranes from infected cells showing unextracted (*11*), soluble (*12*), and insoluble (*13*) fraction. (Numbers at left, × 1,000.)

In addition, we have data (not shown) indicating that membranes from *P. chabaudi*-parasitized murine erythrocytes also have new phosphoproteins but of different molecular weights than the phosphoproteins from *P. berghei*-infected cells. This indicates that the novel membrane-associated phosphoproteins from murine erythrocytes infected with *Plasmodium* (ssp.) are not likely to be host derived. In addition, pp45 and pp68 are not proteolytic products of spectrin as shown by partial proteolysis. Furthermore, there is no decrease in a normal erythrocyte protein large enough to account for the appearance of pp45 and pp68.

Although pp45 is associated with host erythrocyte membranes, the mechanism of its interaction with the host cell is not precisely known. We find that pp45 is not extractable with 0.5% Triton X-100 but is partially extractable with either 0.1 mM EDTA or 0.1 M NaOH, suggesting that pp45 may exist in two membrane-associated forms. The low pI (3.8) of pp45 indicates that this protein contains many acidic residues. In this repect, it is interesting that digestion of pp45 with *S. aureus* V8 protease, which cleaves specifically at glutamic acid residues (30), results in marked degradation of the protein (data not shown). The above observations indicate that, like the erythrocyte membrane protein spectrin, pp45 may be rich in glutamate (31).

In proportion to the amount of Coomassie Blue-stainable protein present, pp45 appears to be more heavily phosphorylated than any of the normal murine erythrocyte membrane proteins. The origin of the protein kinase(s) responsible for this phosphorylation has not yet been determined, although it must be at least partially membrane associated to explain the phosphorylation of pp45 in isolated membranes. Although the phosphorylation of pp45 in isolated membranes is cAMP independent, the Walsh cAMP-dependent protein kinase inhibitor protein (32) inhibits it phosphorylation by 30–50% (not shown), suggesting that pp45 may be phosphorylated by the cAMP-dependent protein kinase. The reason why no cAMP dependence is seen may be that the cAMP-dependent protein kinase is already activated by the elevated levels of cAMP known to occur in *P. berghei*-infected erythrocytes (33).

A 42,000-dalton phosphoprotein has previously been reported to be associated with membranes from *P. berghei*-infected murine erythrocytes (34). However, these earlier investigators suggested that the phosphoprotein was red cell actin on the basis of molecular weight, extractability with 0.1 mM EDTA, and nonextractability with 1% Triton X-100. We believe that this protein and pp45 are the same due to the similarities we find in the two; however, we have shown that pp45 is clearly distinguishable from erythrocyte actin by two-dimensional gel electrophoresis. In addition, a 44,000-dalton protein with a pI of 3.9 has previously been reported from the surface membrane of *P. berghei* sporozoites (an exo-erythrocytic form of the parasite) (35). Monoclonal antibodies directed against this protein do not cross-react with other stages of malarial parasites (36). However, the chemical similarity between this protein and pp45 does suggest that the two may be related.

Several investigators have reported new proteins in the 130,000- to 210,000-dalton range associated with the host membrane during malaria infection (8–10, 37). We also find new Coomassie Blue-stainable bands in this molecular weight range associated with the host membrane from infected cells (e.g., Fig. 7). Whether all of these reported novel proteins are the same or similar remains to be determined. However, it is clear that new proteins of similar molecular weight are consistently associated with host membranes during malarial infections from various species.

The possible functions of the above plasmodial proteins

associated with the host erythrocytes membrane remain unknown, although in one case a possible structural role for such a protein has been postulated. Kilejian (37) has reported that the appearance of "knobs" on the surface of erythrocytes infected with *P. falciparum* is due to an 80,000-dalton parasite protein associated with the host cell membrane. The functional roles of the phosphoproteins which we have described here await elucidation.

In summary, at least two novel phosphoproteins, pp45 and pp68, are associated with the membranes from *P. berghei*-infected murine erythrocytes. We have studied pp45 in greater detail and find that it is specifically associated with the host cell membrane. This novel phosphoprotein and its phosphorylation may be physiologically significant for the following reasons: (*a*) pp45 remains preferentially associated with the host erythrocyte membrane during fractionation of parasite-infected erythrocytes (*b*); pp45 is strongly associated with the host erythrocyte membrane as exemplified by its resistance to extractions with detergents and NaOH; and (*c*) pp45 is phosphorylated both in intact parasitized cells and in isolated host membranes in an identical manner. The association of pp45 with the host plasma membrane and its phosphorylation may play a role in the alterations of erythrocyte transport, permeability, or membrane structure that accompany parasite infection.

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