Phosphorylation of Protein 4.1 in Plasmodium falciparum-Infected Human Red Blood Cells

By Athar H. Chishti, George J. Maalouf, Shirin Marfatia, Jiri Palek, W. Wang, Derek Fisher, and Shih-Chun Liu

The composition of the erythrocyte plasma membrane is extensively modified during the intracellular growth of the malaria parasite Plasmodium falciparum. It has been previously shown that an 80-kD phosphoprotein is associated with the plasma membrane of human red blood cells (RBCs) infected with trophozoite/schizont stage malaria parasites. However, the identity of this 80-kD phosphoprotein is controversial. One line of evidence suggests that this protein is a phosphorylated form of RBC protein 4.1 and that it forms a tight complex with the mature parasite-infected erythrocyte surface antigen. In contrast, evidence from another group indicates that the 80-kD protein is derived from the intracellular malaria parasite. To resolve whether the 80-kD protein is indeed RBC protein 4.1, we made use of RBCs obtained from a patient with homozygous 4.1(−) negative hereditary elliptocytosis. RBCs from this patient are completely devoid of protein 4.1. We report here that this lack of protein 4.1 is correlated with the absence of phosphorylation of the 80-kD protein in parasite-infected RBCs, a finding that provides conclusive evidence that the 80-kD phosphoprotein is indeed protein 4.1. In addition, we also identify and partially characterize a casein kinase that phosphorylates protein 4.1 in P falciparum-infected human RBCs. Based on these results, we suggest that the maturation of malaria parasites in human RBCs is accompanied by the phosphorylation of protein 4.1. This phosphorylation of RBC protein 4.1 may provide a mechanism by which the intracellular malaria parasite alters the mechanical properties of the host plasma membrane and modulates parasite growth and survival in vivo.

The Malaria parasite Plasmodium falciparum undergoes three distinct morphologic changes during its 48-hour life cycle inside human red blood cells (RBCs). After invasion into RBCs, the parasites mature to the ring stage. The subsequent growth to the trophozoite stage is associated with marked morphologic and biochemical changes in the host plasma membrane. Further maturation of trophozoite-stage parasites to the schizont stage leads to the loss of RBC biconcave shape and a dramatic reduction in cell deformability. Although the molecular basis for these changes is not yet known, it has been proposed that specific parasite-induced alterations in the host plasma membrane may be necessary for the growth and maturation of the intracellular malaria parasites.

In normal human RBCs, all major membrane proteins including spectrin, ankyrin, adducin, band 3, protein 4.1, protein 4.2, and dematin serve as substrates for a multitude of protein kinases. More importantly, the phosphorylation of protein 4.1, dematin, and band 3 has been shown to downregulate their binary and ternary interactions in vitro. Therefore, it has been proposed that one of the mechanisms by which the intracellular malaria parasite can modify the host plasma membrane may involve the selective phosphorylation of RBC membrane proteins. Indeed, several RBC membrane-associated proteins are known to undergo changes in phosphorylation on maturation of the malaria parasite. More specifically, a major phosphoryl protein of approximately 80 kD was selectively phosphorylated at the trophozoite/schizont stage. Based on the lack of reactivity of the 80-kD phosphoprotein to various antibodies raised against RBC membrane proteins including protein 4.1, it has been suggested that the 80-kD protein is derived from the intracellular parasite. In contrast, the 80-kD phosphoprotein that coimmunoprecipitated with mature parasite-infected erythrocyte surface antigen (MESA) produced one-dimensional peptide maps that were similar to those from protein 4.1 raising the possibility that this 80-kD protein is a phosphorylated form of protein 4.1.

In this study we examine the phosphorylation of the 80-kD protein in RBCs that were infected with the malaria parasite P falciparum and we identify the 80-kD phosphorylated protein as host protein 4.1. This evidence is based on the absence of phosphorylation of this protein in RBCs obtained from a patient with homozygous hereditary elliptocytosis and a complete deficiency of protein 4.1. In addition, we report the partial characterization of a casein kinase that specifically phosphorylates protein 4.1 in infected RBCs. Based on these results, we propose that the selective phosphorylation of protein 4.1 may provide a mechanism by which the intracellular malaria parasite modifies the host RBC membrane in vivo.

Materials and Methods

Protein 4.1-deficient RBCs. The patient (DW), of African-American origin, suffers from homozygous 4.1(−) hereditary elliptocytosis. After splenectomy as a child, the patient is asymptomatic and not receiving any transfusions. The patient’s RBCs are completely deficient of all isoforms of protein 4.1 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

Parasite culture. A “knobby” P falciparum line 7901 (Palo Alto, Uganda strain) was used in the majority of experiments. In some experiments, the A-2(K') strain was used. The A-2(K') strain is a clonal derivative of the nonclonal Gambian FcR-3 line (provided by Dr J. Ravetch, Sloan-Kettering Cancer Center, NY). The in vitro parasite culture medium contained fresh A+ human erythrocytes, RPMI 1640, glucose, gentamycin sulfate, HEPES, NaHCO₃, L-glutamin, and 5% human serum. The parasites were grown in RPMI 1640 supplemented with 0.5% glucose, 0.01% gentamycin sulfate, 10 mM HEPES (pH 7.3), and 30 mM NaHCO₃ (pH 7.3) under a 2% CO₂ atmosphere.

Address reprint requests to A.H. Chishti, PhD, ACH4, St Elizabeth's Hospital, Tufts University School of Medicine, Boston; the Department of Biochemistry and Molecular Biology and The Rowlatt Institute for Science, Harvard University, Cambridge, MA; and St Jude Children's Research Hospital, Memphis, TN.

Submitted June 24, 1993; accepted January 10, 1994.

Supported by the National Institutes of Health Grant No. HL37462.

From the Department of Biomedical Research, St Elizabeth’s Hospital, Tufts University School of Medicine, Boston; the Department of Biochemistry and Molecular Biology and The Rowlatt Institute for Science, Harvard University, Cambridge, MA; and St Jude Children’s Research Hospital, Memphis, TN.
tamine, pyruvic acid, and 15% A" human serum as described by Trager and Jensen.11 The parasites were grown up to 6% parasitemia at 37°C under 5% CO2, 1% O2, and 94% N2. Ring-stage parasites were synchronized in 5% sorbitol.12 Trophozoite and schizont-stage infected RBCs were then selected for the "knobby" phenotype using the gelatin flotation method.13

Isolation of parasite-infected RBC membranes. Trophozoite/schizont-infected RBCs at 5% parasitemia were enriched to greater than 95% by centrifugation through sorbitol-Percoll (Sigma, St Louis, MO) gradients.14 Infected RBCs were hypotonically lysed in 5 mmol/L sodium phosphate pH 8.0, 0.5 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1.0 μg/mL leupeptin and the lysates were centrifuged at 15,000g. The pelleted material had two distinct layers: a tight gray zone of parasite material at the bottom of the tube, and a less dense white layer of RBC membranes above the gray zone. The membrane fraction was removed and recentrifuged to remove any residual parasite material. This membrane fraction, which is referred to as "infected ghosts" was used in the phosphorylation assays.

Metabolic labeling of infected RBCs. For the 32P-labeling of trophozoite/schizont-infected RBCs, intact parasite infected RBCs were washed in phosphate- and glutamine-free Minimum Essential Medium (Flow Laboratories, McLean, VA). The culture medium was further supplemented with 25 mmol/L HEPES, 10 μg/mL gentamycin sulfate, and 15% human A" serum. The human A" serum was dialyzed against sterile normal saline. Cells were labeled for 4 hours at 37°C in a culture medium to which 2.0 mCi/mL of 32P-orthophosphate was added. After incubation, the cells were washed with the culture medium and the RBC membranes were prepared as described above. Metabolic labeling of the infected RBC was performed in methionine-free RPMI 1640 (Select-amine kit, GIBCO Laboratories, Grand Island, NY) containing 100 μCi/mL of 35S-methionine (1,140 Ci/mmol; ICN, Irvine, CA). Radiolabeled RBCs were then washed once with the methionine-free culture medium, and the RBC membranes were prepared as described above.

Phosphorylation assay. Phosphorylation of infected ghosts was performed in a reaction mixture containing 50 mmol/L Tris-HCl pH 7.5, 8.0 mmol/L MgCl₂, 1.0 mmol/L dithiothreitol (DTT), 50 μmol/L adenosine triphosphate (ATP), and 200 μCi carrier-free [γ-32P]ATP. After incubation at 30°C for 10 minutes, the radiolabeled infected ghosts were separated using 10% to 18% gradient acrylamide gels and visualized by silver staining and autoradiography. Two-dimensional peptide mapping of the 125I-labeled 80-kD protein and the 32P-labeled 80-kD protein was performed as described.16

Purification of parasite casein kinase. Trophozoite/schizont-infected RBCs were metabolically labeled with 35S-methionine as described above. The labeled parasite-infected RBCs were incubated with 0.1% saponin for 10 minutes at 37°C to make the RBC membranes permeable. Saponin-treated parasite lysates were centrifuged at 10,000g, and the supernatant containing RBC membrane fragments was removed. Intact parasites were washed twice with the saponin solution and finally with phosphate-buffered saline. 35S-labeled intact parasites that were essentially free of RBC membranes were hypotonically lysed in 10 vol of cold distilled water. Parasite lysates were then centrifuged at 50,000g for 30 minutes and the supernatant collected. This supernatant, referred to as the "cytosolic extract," was then loaded on a casein-Sepharose column (0.5 x 5 cm) that was equilibrated with 20 mmol/L Tris-HCL, pH 7.5, and 1.0 mmol/L DTT. After extensively washing the column, the bound proteins were eluted using a 0 to 0.5 mmol/L KC1 gradient (100 mL). Column fractions were assayed for both radioactivity and casein kinase activity. Affinity-purified casein kinase fractions were pooled and further purified on a diethyl aminoethyl (DEAE)-Sephacel column (Pharmacia, Piscataway, NJ). The bound enzyme was eluted with a 0 to 0.5 mol/L KC1 gradient. In both purification steps, the peak of casein kinase activity overlapped that of the [35S] radioactivity.

RESULTS

Selective phosphorylation of the 80-kD protein. To compare the phosphorylation of membrane proteins in uninfected and parasite-infected human RBCs, phosphorylation assays were performed using both intact RBCs and isolated RBC membranes (Fig 1). When in vitro phosphorylation assays were performed on the RBC membranes isolated from uninfected RBCs, no appreciable phosphorylation signal was detected in the region where protein 4.1 migrates on SDS-gels (Fig 1, lane 8). In contrast, phosphorylation of infected ghosts produced selective phosphorylation of the 80-kD protein (Fig 1, lane 10, arrowhead). A comparison of in vitro protein phosphorylations at similar spectrin content also indicated an enhancement in the phosphorylation of band 3 in trophozoite-infected RBCs (60% increase compared with uninfected cells, Fig 1: lanes 8 and 10).

The 80-kD protein was again selectively phosphorylated when intact RBCs containing trophozoite/schizont-stage parasites were metabolically phosphorylated in the presence of 32PO4 (Fig 1, lane 6, arrowhead). However, the enhanced phosphorylation of band 3, as detected in infected ghosts, was not observed in intact trophozoite-infected RBCs. Similarly, when intact RBCs containing ring-stage malaria parasites were metabolically phosphorylated, no phosphorylation of the 80-kD protein was observed (Fig 1, lane 4). In fact, ring-infected RBCs reproducibly show a 90% decrease in the labeling of β-spectrin compared with the same number of uninfected cells (Fig 1, lanes 2 and 4). This result is consistent with a previous report demonstrating dephosphorylation of the major erythrocyte membrane proteins on merozoite invasion.8

Phosphorylation of the 80-kD protein in normal and 4.1(-) RBCs. To obtain further insight into the origins of the 80-kD protein and its relationship to the phosphorylated
form of RBC protein 4.1, metabolic phosphorylations were performed in both normal and protein 4.1(−) RBCs that were infected with malaria parasites at the trophozoite/schizont stage (Fig 2). These RBCs do not contain protein 4.1 or any of its isoforms as determined by the Western blot analysis. As shown in Fig 2, no phosphorylation of the 80-kD phosphoprotein was observed in RBC membranes isolated from protein 4.1(−) RBCs that were infected with malaria parasites. It should be noted that this absence of phosphorylation of the 80-kD protein is not simply caused by the reduced invasion of 4.1(−) RBCs by malaria parasites. The evidence in support of this conclusion is provided by the fact that although 4.1(−) RBCs have reduced parasitemia, both normal and 4.1(−) RBCs that contained mature parasites were concentrated by centrifugation through Percoll and an equal number of cells were used in the phosphorylation assays. Moreover, the parasites in 4.1(−) RBCs appeared morphologically normal and could be propagated when cultured with normal RBCs (not shown). These results strongly suggest that the 80-kD phosphoprotein in mature parasite-infected RBCs is host membrane protein 4.1 and that it is not derived from the intracellular parasite.

Structural similarity between the 80-kD phosphoprotein and protein 4.1. Because the mobility of the 80-kD phosphoprotein on SDS-polyacrylamide gels is identical to that of protein 4.1 (Fig 1), we examined structural similarities by one- and two-dimensional peptide mapping. As shown in Fig 3, the mobilities of three major peptides derived from the cyanogen bromide cleavage of the 80-kD phosphoprotein were similar to those of peptides derived from purified protein 4.1 (Fig 3, compare lanes 1 and 2). The three radiolabeled peptides derived from the 32P-labeled 80-kD protein were also contained within the silver-stained peptide map of protein 4.1 (Fig 3, compare lane 3 with lane 1). In addition, the phosphopeptide maps of the 80-kD protein phosphorylated in either isolated RBC membranes or in intact RBCs were identical (Fig 3, lanes 4 and 5) indicating that similar sites were phosphorylated under these conditions. The structural relationship between protein 4.1 and the 80-kD phosphoprotein was further examined by two-dimensional peptide mapping analysis. Although the 125I-protein 4.1 peptide map shared some spots with the phosphopeptide map of 32P-labeled 80-kD protein, there were many spots that were unique to both peptide maps (not shown). This result is consistent with the fact that the phosphorylated peptides exhibited altered mobilities during charge-based separation.

Phosphorylation of the 80-kD phosphoprotein. To elucidate the basis of hyper-phosphorylation of the 80-kD protein in mature parasite-infected RBCs, we first compared the total protein kinase activity in both uninfected and infected RBCs (Fig 4). The RBCs were lysed in the hypotonic buffers, and ruptured cells were separated into cytosol and membrane fractions by high-speed centrifugation. The protein kinase activity of the cytosol and membrane fractions was measured using casein as the substrate. The casein kinase activity was 20-fold higher in trophozoite/schizont-infected red cells (Fig 4, lanes 5 through 8) compared with uninfected RBCs (Fig 4, lanes 1 through 4). Both the cytosol and membrane fractions of the infected RBCs exhibited elevated casein kinase activity. A similar increase in total protein kinase activity was also observed using phosvitin as an exogenous substrate. In contrast, basic protein substrates such as histones and protamine were not phosphorylated by this protein kinase (data not shown).

In addition to the increase in protein kinase activity, the increase in phosphorylation of the 80-kD protein may be a consequence of the increased phosphate turnover caused by the activation of protein phosphatases. To examine this possibility, membranes from parasite-infected RBCs were incubated under conditions that promoted dephosphorylation in vitro. RBC membranes that contained the 32P-labeled 80-kD protein were incubated with either the membrane or the
endo within the linear range of phosphorylation kinetics. The membrane-associated protein kinase exclusively phosphorated serine residues of the 80-kD protein, and both ATP and guanosine triphosphate (GTP) could be used as phosphate donors in the reaction (not shown). Phosphorylation of the 80-kD protein was strongly inhibited by NaCl and KCl and 50% inhibition was observed at approximately 100 mmol/L salt concentration (not shown). The phosphorylation was not affected by calcium, calmodulin, EGTA, or trifluoperazine and so excluded participation of the calcium/calmodulin protein kinases (Table 1). In addition, the protein kinase activity was not affected by cyclic adenosine monophosphate (cAMP) and staurosporine (up to 36 nmol/L), suggesting that neither cAMP-dependent protein kinase nor protein kinase C was directly responsible for the phosphorylation of 80-kD protein (Table 1). Staurosporine at 36 nmol/L is known to inhibit cAMP kinase and protein kinase C in vitro. In contrast, phosphorylation of the 80-kD protein was inhibited by N-ethylmaleimide and 2,3-diphosphoglycerate (Table 1). Heparin inhibited the in vitro

cytosol fractions derived from unlabeled mature parasite-infected RBCs. No loss of radioactivity from the 32P-labeled 80-kD protein was observed in the presence of magnesium, which is known to promote dephosphorylation of the RBC membrane proteins (data not shown), suggesting that the observed hyper-phosphorylation of the 80-kD protein is likely caused by the increased protein kinase activity in parasite-infected RBCs.

Effect of the protein kinase inhibitors. To identify and characterize the protein kinase activity that catalyzed phosphorylation of the 80-kD protein, we first determined the kinetics of phosphorylation. All subsequent assays were performed within the linear range of phosphorylation kinetics. The membrane-associated protein kinase exclusively phosphorated serine residues of the 80-kD protein, and both ATP and guanosine triphosphate (GTP) could be used as phosphate donors in the reaction (not shown). Phosphorylation of the 80-kD protein was strongly inhibited by NaCl and KCl and 50% inhibition was observed at approximately 100 mmol/L salt concentration (not shown). The phosphorylation was not affected by calcium, calmodulin, EGTA, or trifluoperazine and so excluded participation of the calcium/calmodulin protein kinases (Table 1). In addition, the protein kinase activity was not affected by cyclic adenosine monophosphate (cAMP) and staurosporine (up to 36 nmol/L), suggesting that neither cAMP-dependent protein kinase nor protein kinase C was directly responsible for the phosphorylation of 80-kD protein (Table 1). Staurosporine at 36 nmol/L is known to inhibit cAMP kinase and protein kinase C in vitro. In contrast, phosphorylation of the 80-kD protein was inhibited by N-ethylmaleimide and 2,3-diphosphoglycerate (Table 1). Heparin inhibited the in vitro
phosphorylation of the 80-kD protein in a dose-dependent manner with a 50% inhibition at 1.0 μg/mL (Table 1). Finally, the identity of the protein kinase was established using CKI-7, a specific inhibitor of the casein kinases (Table 1). CKI-7, an N-(2-aminoethyl)-5-chloro-isoquinoline-8-sulfonamide, is known to inhibit casein kinase I and casein kinase II with IC₅₀ values of 9.5 and 195 μmol/L, respectively.¹¹ The IC₅₀ value of CKI-7 for the inhibition of phosphorylation of the 80-kD protein was 30 μmol/L (Table 1). These results strongly suggest that the phosphorylation of the 80-kD protein was catalyzed by a casein kinase in trophozoite/schizont-infected RBCs.

Purification and characterization of the casein kinase. As shown in Fig 4, the casein kinase activity was significantly higher in trophozoite/schizont-infected cells than in uninfected RBCs. To determine the origin of this casein kinase activity, trophozoite/schizont-infected RBCs were metabolically labeled in the presence of [³⁵S]-methionine. The mature parasites were then isolated using selective lysis in the presence of saponin.²² The cytosolic casein kinase fraction was then obtained by hypotonic lysis of purified parasites, and further purified by affinity chromatography on the immobilized casein. As shown in Fig 5A, the casein kinase activity peak completely overlapped the [³⁵S] radioactivity peak, strongly suggesting that the casein kinase was metabolically labeled and therefore derived from the malaria parasite. The affinity-purified casein kinase was further purified using anion-exchange chromatography (Fig 5B). Again, the casein kinase activity and the [³⁵S] radioactivity peaks overlapped. The properties of the purified casein kinase were similar to those of the membrane-bound casein kinase that phosphorylated the 80-kD protein in trophozoite/schizont-infected RBCs (data not shown). These observations suggest that these two enzyme activities, which show similar biochemical properties, may be the same and may be derived from the intracellular parasite. However, further experiments are necessary to prove the hypothesis that the parasite-derived casein kinase is translocated to the host membrane in infected RBCs.

**DISCUSSION**

The overall aim of this study was to identify an 80-kD phosphoprotein that is prominently phosphorylated at the trophozoite/schizont stage of parasite development, and to characterize the protein kinase that mediates this phosphorylation. As shown in Fig 1, phosphorylation of the 80-kD protein was dependent on the stage of parasite development. Such developmentally regulated phosphorylation of the 80-kD protein is consistent with previously reported phosphorylation events in *P. falciparum*-infected human RBCs.³⁰ However, there are two conflicting views with respect to the identity of the 80-kD phosphoprotein. One group⁴ suggested that the 80-kD phosphoprotein was derived from the intracellular malaria parasite, based on the observation that antibodies specific to protein 4.1 failed to immunoprecipitate [³⁵S]-labeled 80-kD protein from infected RBCs. In contrast, others concluded that the 80-kD protein is a phosphorylated form of RBC protein 4.1, as suggested by (1) the absence of metabolic radiolabeling of 80-kD protein in infected RBCs in the presence of [³⁵S]-methionine or a mixture of 16 radiolabeled amino acids, and (2) the similarities of one-dimensional peptide maps of the 80-kD protein and protein 4.1.

Our results confirm the observation of Lustigman et al.⁹ to identify an 80-kD phosphoprotein at the trophozoite/schizont stage of parasite development, and to characterize the protein kinase that mediates this phosphorylation. As shown in Fig 1, phosphorylation of the 80-kD protein was dependent on the stage of parasite development. Such developmentally regulated phosphorylation of the 80-kD protein is consistent with previously reported phosphorylation events in *P. falciparum*-infected human RBCs. However, there are two conflicting views with respect to the identity of the 80-kD phosphoprotein. One group suggested that the 80-kD phosphoprotein was derived from the intracellular malaria parasite, based on the observation that antibodies specific to protein 4.1 failed to immunoprecipitate [³⁵S]-labeled 80-kD protein from infected RBCs. In contrast, others concluded that the 80-kD protein is a phosphorylated form of RBC protein 4.1, as suggested by (1) the absence of metabolic radiolabeling of 80-kD protein in infected RBCs in the presence of [³⁵S]-methionine or a mixture of 16 radiolabeled amino acids, and (2) the similarities of one-dimensional peptide maps of the 80-kD protein and protein 4.1.

Our results confirm the observation of Lustigman et al.⁹ to identify an 80-kD phosphoprotein at the trophozoite/schizont stage of parasite development, and to characterize the protein kinase that mediates this phosphorylation. As shown in Fig 1, phosphorylation of the 80-kD protein was dependent on the stage of parasite development. Such developmentally regulated phosphorylation of the 80-kD protein is consistent with previously reported phosphorylation events in *P. falciparum*-infected human RBCs. However, there are two conflicting views with respect to the identity of the 80-kD phosphoprotein. One group suggested that the 80-kD phosphoprotein was derived from the intracellular malaria parasite, based on the observation that antibodies specific to protein 4.1 failed to immunoprecipitate [³⁵S]-labeled 80-kD protein from infected RBCs. In contrast, others concluded that the 80-kD protein is a phosphorylated form of RBC protein 4.1, as suggested by (1) the absence of metabolic radiolabeling of 80-kD protein in infected RBCs in the presence of [³⁵S]-methionine or a mixture of 16 radiolabeled amino acids, and (2) the similarities of one-dimensional peptide maps of the 80-kD protein and protein 4.1.

Our results confirm the observation of Lustigman et al.⁹ to identify an 80-kD phosphoprotein at the trophozoite/schizont stage of parasite development, and to characterize the protein kinase that mediates this phosphorylation. As shown in Fig 1, phosphorylation of the 80-kD protein was dependent on the stage of parasite development. Such developmentally regulated phosphorylation of the 80-kD protein is consistent with previously reported phosphorylation events in *P. falciparum*-infected human RBCs. However, there are two conflicting views with respect to the identity of the 80-kD phosphoprotein. One group suggested that the 80-kD phosphoprotein was derived from the intracellular malaria parasite, based on the observation that antibodies specific to protein 4.1 failed to immunoprecipitate [³⁵S]-labeled 80-kD protein from infected RBCs. In contrast, others concluded that the 80-kD protein is a phosphorylated form of RBC protein 4.1, as suggested by (1) the absence of metabolic radiolabeling of 80-kD protein in infected RBCs in the presence of [³⁵S]-methionine or a mixture of 16 radiolabeled amino acids, and (2) the similarities of one-dimensional peptide maps of the 80-kD protein and protein 4.1.

---

**Table 1. Inhibition of Phosphorylation of Erythrocyte Membrane-Associated 80-kD Protein**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>Inhibition of Kinase Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>16 μmol/L</td>
<td>None</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>16 μg/mL</td>
<td>None</td>
</tr>
<tr>
<td>EGTA</td>
<td>5 mmol/L</td>
<td>None</td>
</tr>
<tr>
<td>Trifluoroperazine</td>
<td>25-160 μmol/L</td>
<td>None</td>
</tr>
<tr>
<td>cAMP</td>
<td>60-300 μmol/L</td>
<td>None</td>
</tr>
<tr>
<td>Stauroporine</td>
<td>4-36 nmol/L</td>
<td>None</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>8 mmol/L</td>
<td>75</td>
</tr>
<tr>
<td>2,3-Diphosphoglycerate</td>
<td>5 mmol/L</td>
<td>50</td>
</tr>
<tr>
<td>Heparin</td>
<td>1 μg/mL</td>
<td>50</td>
</tr>
<tr>
<td>CKI-7*</td>
<td>10 μmol/L</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>30 μmol/L</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>100 μmol/L</td>
<td>70</td>
</tr>
</tbody>
</table>

* CKI-7, N-(2-aminoethyl)-5-chloro-isoquinoline-8-sulfonamide.
and show that the 80-kD protein is indeed protein 4.1 based on the following observations: (1) The elliptocytic RBCs, which completely lack all isoforms of protein 4.1, fail to show any phosphorylation of the 80-kD protein on parasite development (Fig 2). (2) Both the \[^{32}P\]-labeled 80-kD protein and RBC protein 4.1 have similar molecular weights (Fig 1), one-dimensional peptide maps (Fig 3), and membrane association properties (Fig 4). In contrast, the two-dimensional peptide maps of \[^{32}P\]-protein 4.1 and the \[^{35}S\]-80-kD protein were distinctly different and shared only three spots (not shown). This apparently contradictory result is consistent with the reported altered migration of phosphorylated peptides during charge separation. On account of this fact the two-dimensional peptide maps cannot be used to establish the identity of these two proteins.

Our second task was to characterize the protein kinase involved in the phosphorylation of the 80-kD protein in parasite-infected RBCs. A type 1 casein kinase has been previously characterized in both \(P. \)berghei and \(P. \)falciparum. More recently, phosphorylation of the parasite proteins was correlated with parasite invasion and development in infected RBCs; however, the corresponding protein kinases were not identified. The data presented here describe the properties of the casein kinase that phosphorylates the 80-kD protein in \(P. \)falciparum-infected human RBCs. The properties of the casein kinase in infected RBCs resemble those of casein kinase II with respect to substrate specificity and enzyme inhibition by specific inhibitors (Table 1), whereas the elution of enzyme from the ion-exchange matrix resembles that of casein kinase I (Fig 5B). Because the biochemical characteristics of the casein kinase reported here appear to share the features of both type I and type II casein kinases, primary structure determination will be required to unambiguously establish its classification.

Human RBCs contain several active casein kinases. It appears unlikely that the casein kinase activity detected in the infected RBCs represents an activated form of an RBC casein kinase because the properties of the casein kinase reported here are distinct from those of the known casein kinases in RBCs. Boivin and Galand have purified a casein kinase from the cytosol of normal human RBCs that was stimulated by monovalent salts and inhibited by calcium. These properties are different from the casein kinase we have characterized in infected RBCs. A cAMP-kinase was previously characterized from normal RBC cytosol, which phosphorylated casein, spectrin, and protein 4.1. This casein kinase was not inhibited by 2,3-diphosphoglycerate, a property different from the casein kinase reported in this manuscript. Similarly, Clari and Moret have characterized a casein kinase from human RBC cytosol that phosphorylated spectrin and protein 4.1. This enzyme was not inhibited by either NaCl or 2,3-diphosphoglycerate.

Our data also show that the casein kinase isolated from parasite-infected RBCs can be metabolically labeled (Fig 5), and this further supports the hypothesis that the enzyme is of parasite origin. Although the functional significance of these observations is presently unknown, the phosphorylation of protein 4.1 is known to dramatically reduce both its spectrin-actin and membrane-binding properties. The selective phosphorylation of RBC membrane proteins by the intracellular malaria parasite may influence events such as the loss of cell shape and deformability, reorganization of the membrane skeleton and membrane permeability, membrane rupture during merozoite release, and the modification of RBC surface antigens. Whether the parasite induced phosphorylation of protein 4.1 brings about any such effects in intact RBCs remains to be investigated.

ACKNOWLEDGMENT

We thank Linda Oak for valuable technical assistance with the culture of malaria parasites.

REFERENCES

3. Howard RJ: Malarial proteins at the membrane of Plasmodium falciparum-infected erythrocytes and their involvement in cytoadherence to endothelial cells. Prog Allergy 41:98, 1988
Phosphorylation of protein 4.1 in Plasmodium falciparum-infected human red blood cells

AH Chishti, GJ Maalouf, S Marfatia, J Palek, W Wang, D Fisher and SC Liu