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A Plasmodium Protein Kinase That Is Developmentally Regulated, Stimulated by Spermine, and Inhibited by Quercetin

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Plasmodium berghei–infected murine red cells possess protein kinase activity that is associated with the isolated parasites. Schizonts contain significantly higher levels of this protein kinase than the more immature forms, suggesting a relationship between this enzyme activity and parasite development. Partially purified protein kinase has a K_m for ATP of ~30 μM, whereas the K_m for GTP is ~300 μM and the substrate preference is phosvitin > casein > > histone > protamine. The Mg^{2+} optimum is 10–20 mM, and the protein kinase activity is stimulated by the polyamines spermine and spermidine. The flavone, quercetin, inhibits the protein kinase activity in a competitive manner with respect to ATP (Ki ~3 μM), and P chabaudi also has a very similarly regulated protein kinase. Protein kinases from both species are very similar to the type I casein kinase.

Key words: protein kinase, Plasmodium berghei, Plasmodium chabaudi, malaria, polyamine stimulation, quercetin inhibition

Protein kinases are a class of enzymes which catalyze the transfer of the gamma phosphate from nucleotide triphosphates (primarily ATP) to a variety of protein substrates. This reversible post-translational event is a ubiquitous mechanism for regulation of a large and diverse number of cellular events [1,2]. In addition to a variety of animal sources, protein kinases are reported in protozoa [3], fungi [4], plants [5], and bacteria [6]. However, very little is known concerning parasite-associated protein kinases except for the viruses [7]. Protein kinases have previously been described in the Trypanosomes, a group of parasitic protozoa [8,9]. This paper describes a protein kinase from the malarial parasites Plasmodium berghei and P chabaudi.

METHODS

Materials

Phosvitin and 2-N-morpholino ethanesulfonic acid (MES) were obtained from Calbiochem-Behring. Casein, histone, polyamines, dithiothreitol (DTT), phospho-

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serine, phosphothreonine, ninhydrin, and ATP were obtained from Sigma. Percoll was obtained from Pharmacia and Hypaque-M, 75% from Winthrop. DEAE-cellulose (DE52) and powdered cellulose (CF-11) were obtained from Whatman. Ultrapure HCl was obtained from Pierce, and thin-layer cellulose sheets from Brinkman. Phosphotyrosine was the gift of Dr M.S. Collett, University of Minnesota, and γ-^{32}P-ATP was synthesized by the method of Johnson and Walseth [10]. All other reagents were at least reagent grade.

Malaria Infection and Parasite Isolation

P berghei (NYU-2 strain) and P chabaudi (PC-F clone, obtained from Dr M. Hommel, Harvard Medical School) were maintained in Swiss white mice as previously described [11]. Blood was obtained by axillary incision of anesthetized mice and aspiration into heparin. Blood was filtered through powdered cellulose to deplete it of white cells and platelets [12]. It is important to note that no detectable elevation of reticulocytes was observed during the infection. Infected red blood cells (>80% parasitemias) were lysed and parasites isolated as previously described [13]. Isolated parasites were lysed by sonication followed by centrifugation at 100,000g for 30 min.

Percoll-Hypaque Density-Gradient Centrifugation

Schizont and late trophozoite-infected cells were separated from early trophozoite-infected and uninfected cells by a modification of the procedure of Vettore et al [14]. The Percoll-Hypaque solution of density 1.095 g/ml was prepared by mixing 35 ml Percoll, 12 ml Hypaque-M, 25 ml 0.9% NaCl, and 28 ml H_2O. Washed, infected red blood cells depleted of platelets and white cells (0.5–1.0 ml packed cells) were layered onto 10 ml of Percoll-Hypaque solution and centrifuged at 25,000g for 10 min in a fixed-angle rotor. The cells were divided into light and heavy fractions with cells of intermediate densities included with the light fraction. All cells were washed three times in 0.9% NaCl to remove the Percoll-Hypaque solution before lysis and isolation of parasites as described above.

DEAE-Cellulose Chromatography

The 100,000g supernatants from the lysed parasites were chromatographed on DEAE-cellulose as described [15] except that 7–20 mg of protein was loaded onto each column. The peak fraction of protein kinase activity was pooled, dispensed into aliquots, and stored at −70°C. The enzyme is stable for at least 1 month with no loss of activity. Because of the limited amount of material available for any single isolation, different batches of enzyme with slightly varying specific activities were used throughout the study.

Assays

Protein kinase was assayed by the method previously described [16], except that the pH was 6.1, and 150 μg of either casein or phosvitin, 0.5 mM DTT, and 10 mM $MgCl_2$ were used. Protein was measured by the method of Lowry et al [17] using BSA as a standard.

Phosphoamino Acid Analysis

Casein and phosvitin (50 μg) were phosphorylated in 0.15 ml containing 50 mM MES, pH 6.1, 10 mM $MgCl_2$, 1 μM γ-^{32}P-ATP (20 Ci/mmole) for 2 min at 30°C.

The reaction was terminated by precipitation with 2 ml of cold 20% trichloroacetic acid (TCA). The precipitate was collected by centrifugation, dissolved in 0.2 ml 1 M NH_4OH, and reprecipitated with 2 ml of cold 20% TCA. After centrifugation, the pellet was suspended in 50 μl of ultrapure 6 N HCl, sealed into a melting point capillary tube, and hydrolyzed for 1.5 hr at 110°C. The hydrolysate was dried and dissolved in 10 μl of phosphoamino acid standards containing 5 mM each of phosphoserine, phosphothreonine, and phosphotyrosine. Five microliters was then spotted on a thin-layer cellulose sheet and electrophoresed at 1,700 V in a Savant tank as previously described [18] using pyridine, acetic acid, H_2O (1:10:189), pH 3.5. Standard phosphoamino acids were stained with ninhydrin, and radiolabeled phosphoamino acids were detected by autoradiography.

RESULTS

Crude lysates of P berghei–infected murine erythrocytes contain a 10- to 20-fold higher level of casein kinase than uninfected erythrocytes (Table I). This auxiliary P berghei protein kinase (PbPK) activity is associated exclusively with the isolated parasites, the majority occurring within the 100,000g supernatant of lysed parasites. Chromatography of crude parasite lysates on DEAE-cellulose resulted in a peak of activity eluting between 0.04 and 0.18 M NaCl. Further purification or initial purification by other means resulted in total loss of activity. However, PbPK does not absorb to CM-cellulose and can subsequently be chromatographed on DEAE-cellulose, yielding an identical enzyme as purified on DEAE-cellulose alone. Isolation of PbPK by either method typically results in a 4- to 7-fold purification.

Infected blood cells separated on Percoll-Hypaque as described in Methods yields a light fraction, consisting of schizont- and late trophozoite-infected cells, and a heavy fraction, consisting of ring stage and early trophozoite-infected and uninfected cells [19]. Parasites isolated from cells of the light fraction have significantly higher levels of protein kinase activity per milligram protein than the parasites isolated from cells of the heavy fraction (Table II). This increase in PbPK activity is also observed after chromatography of the 100,000g supernatants on parallel DEAE-cellulose columns and in the specific activity of the pooled PbPK peaks (data not shown).

Also shown in Table II is the substrate preference of PbPK from crude lysates. Phosvitin incorporates approximately 25% more phosphate than casein on a per-milligram basis; histone and protamine are only phosphorylated slightly.

Substrate and cofactor requirements of PbPK were further characterized using the DEAE partially purified enzyme. The protein kinase prefers ATP (K_m ~30 μM) over GTP (K_m ~300 μM). Cyclic AMP, cGMP, diacylglycerol, or calmodulin—

TABLE I. Casein Kinase Activity From Hypotonic Lysates of Normal and Infected Red Cells and Isolated Parasites

Crude lysate	Specific activity[a]
Normal RBCs	8.9 ± 2.8
Infected RBCs	190 ± 21
Isolated parasites	510 ± 14

[a]pmoles P_i incorporated per min per mg lysate protein ± SD (n = 3).

TABLE II. Comparison of Parasite Protein Kinase Activity From Mature (light fraction) and Immature (heavy fraction) Parasites Using Various Exogenous Protein Kinase Substrates*

Substrate	Enzyme source	
	Light fraction	Heavy fraction
None	28.5 ± 1.4	nd[a]
Phosvitin	1520 ± 97	76.1 ± 9.2
Casein	1240 ± 20	45.4 ± 1.3
Histone	93.5 ± 2.8	nd
Protamine	56.5 ± 6.6	nd

*Infected blood was separated into light and heavy fractions on Percoll-Hypaque density gradients, then the respective parasites were isolated. Shown is the protein kinase activity with various exogenous substrates of the 100,000g supernatants of lysed parasites expressed as pmoles P_i incorporated per minute mg parasite protein ± SD (n = 3).
[a]No detectable activity.

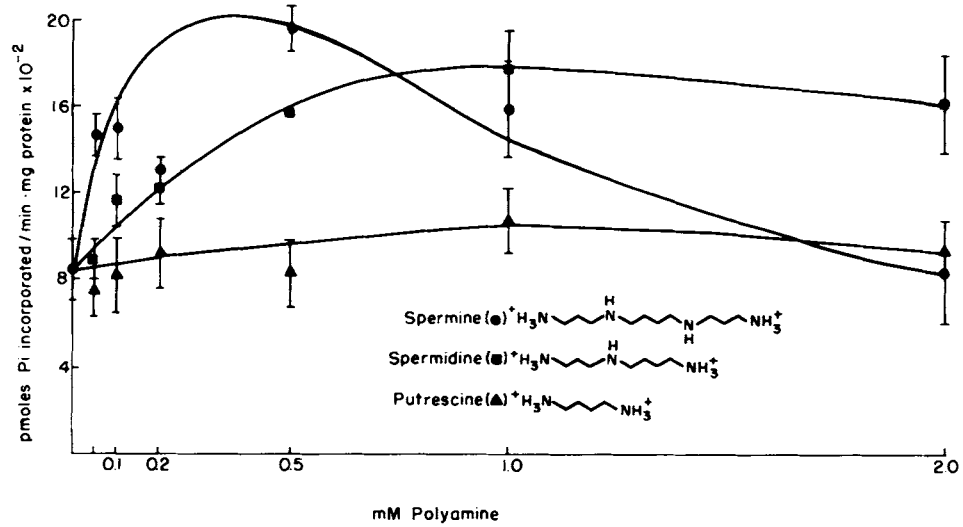

Fig. 1. Polyamine stimulation of PbPK. PbPK was partially purified as described. Protein kinase activity was measured using phosvitin as substrate and in the presence of various concentrations of polyamines as indicated.

known regulators of other protein kinases—have no effect on PbPK, whereas Ca^{2+} slightly inhibits in the presence of Mg^{2+}. However, spermine and spermidine stimulate PbPK 2- to 4-fold, whereas putrescine has little, if any, effect (Fig. 1).

As shown in Figure 2 the stimulation by spermine is dependent on Mg^{2+} concentration. At lower Mg^{2+} concentrations the degree of stimulation by 0.5 mM spermine is greater than at higher Mg^{2+} stimulations (Fig. 2a). Alternatively, spermine may simply lower the Mg^{2+} optimum. This effect seems to be primarily due to Mg^{2+} changing the spermine optimum, and not from Mg^{2+} changing the maximum protein kinase activity (Fig. 2b). However, spermine, even up to 50 mM, cannot replace the absolute divalent cation requirement. Substitution of Mg^{2+} with other

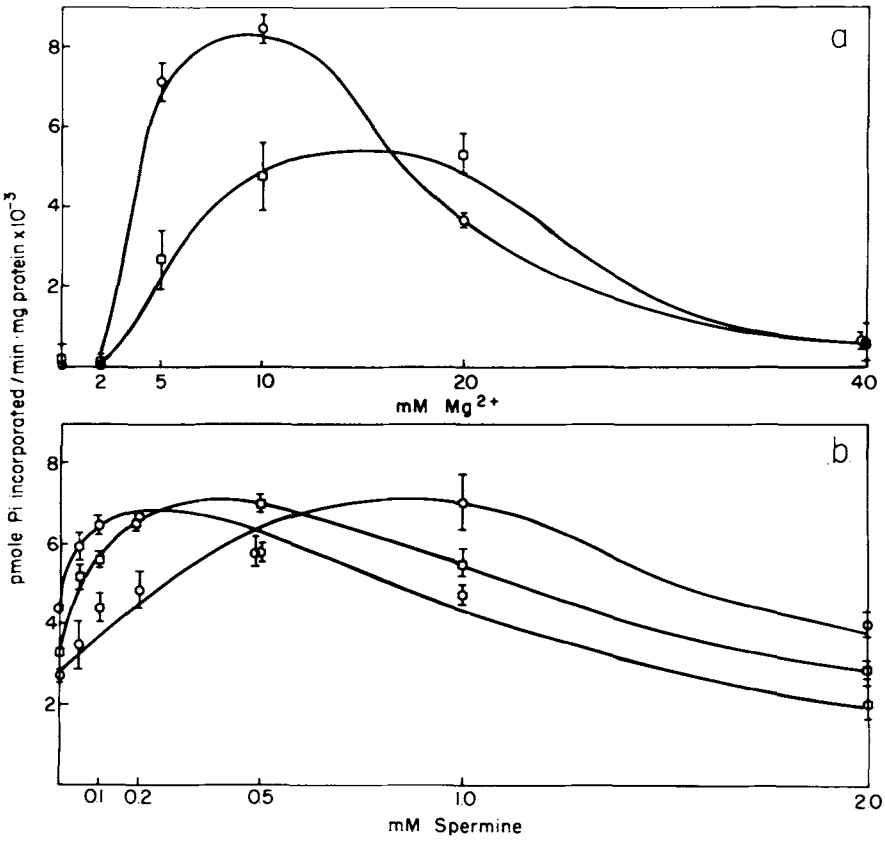

Fig. 2. Effects of Mg^{2+} and spermine on PbPK. PbPK was purified as described. a) The Mg^{2+} optimum in the absence (□) and presence (○) of 0.5 mM spermine. b) The spermine optimum in the presence of 5 mM (○), 10 mM (□), and 15 mM (⬡) Mg^{2+}.

divalent cations, such as Mn^{2+}, Co^{2+}, and Ca^{2+}, results in 10-fold lower activity (data not shown).

Kinetic analysis of the effects of spermine and Mg^{2+} reveals that the two modulate the reaction by slightly different mechanisms. The major effect of spermine is to increase the V_{max} approximately 2-fold with only a slight lowering of the K_m for ATP (Fig. 3a). With respect to casein and phosvitin, spermine does not change the K_m and increases the V_{max} (data not shown). On the other hand, Mg^{2+} results in both a small decrease in the K_m and a slight increase in the V_{max} with respect to ATP (Fig. 3b).

Another effector of PbPK is the flavone, quercetin. Quercetin inhibits the protein kinase activity in a manner competitive with ATP as indicated by only a change in the apparent K_m associated with an increase in quercetin concentration (Fig. 4). The K_i of quercetin is 3.2 μM while the K_m for ATP in this instance is 31 μM. Cochet *et al* [20] also find quercetin to be a competitive inhibitor of the G-type casein kinase from bovine adrenocortical tissue.

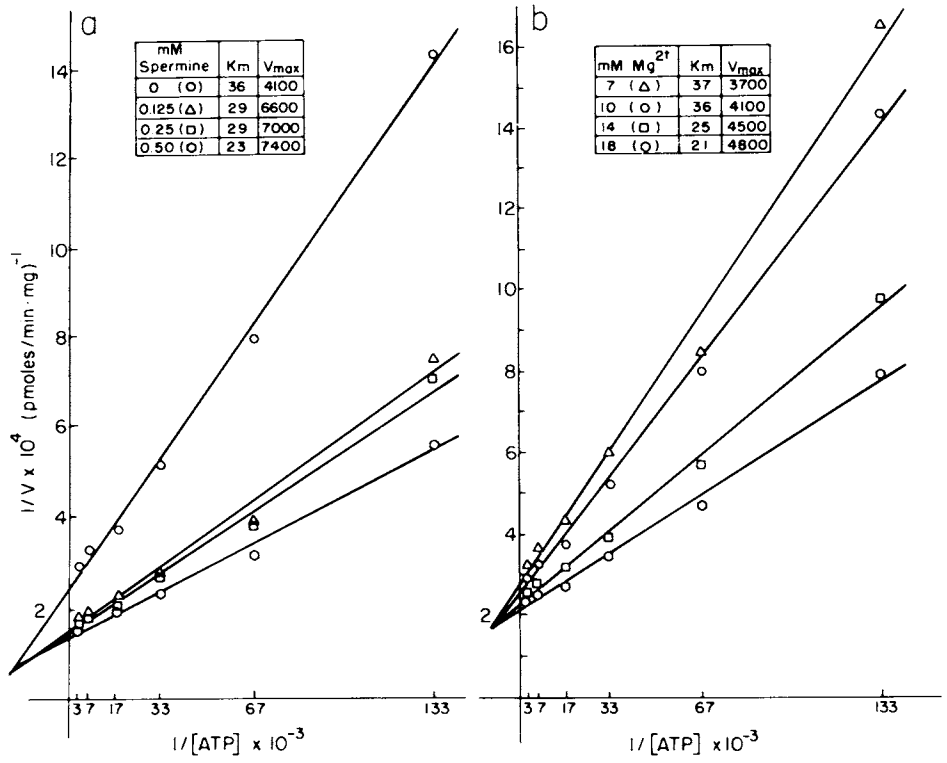

Fig. 3. Double-reciprocal analysis of spermine and Mg^{2+} effects. PbPK was purified and assayed as described. a) The effects of spermine in the presence of 10 mM Mg^{2+}; b) the effects of various Mg^{2+} concentrations, as indicated. The slopes and intercepts were calculated by linear regression analysis. The calculated K_m's and V_{max}'s for the various conditions are shown in the insets.

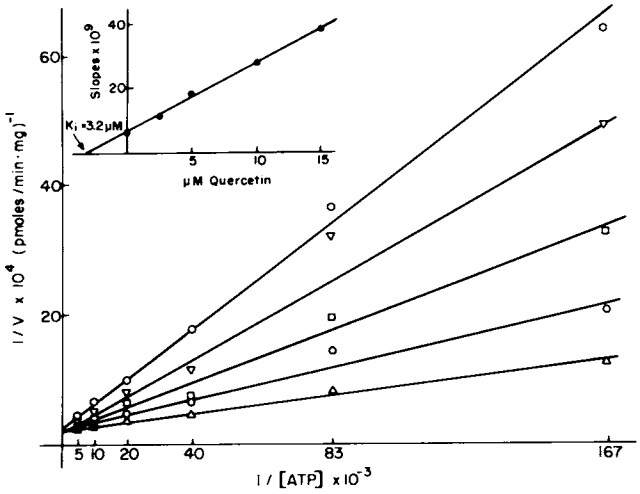

Fig. 4. Quercetin inhibition. PbPK was purified and assayed as described in the presence of various concentrations of quercetin: 0 μM (△), 2.5 μM (○), 5 μM (□), 10 μM (▽) and 15 μM (◇). The inset shows a replot of the slopes versus the quercetin concentration.

Spermine stimulation and quercetin inhibition of PbPK are independent of one another, in that the same degree of quercetin inhibition is seen at any spermine concentration and the same degree of spermine stimulation is seen at any quercetin concentration. In addition, a very similar protein kinase is associated with P chabaudi (PcPK), which is also stimulated by spermine and inhibited by quercetin (data not shown). Moreover, the protein kinases isolated from the heavy and light fractions of parasite-infected cells separated on Percoll-Hypaque are both stimulated by spermine and inhibited by quercetin (data not shown).

Casein is phosphorylated almost exclusively at serine residues by both PbPK and PcPK, whereas phosvitin is phosphorylated predominantly at serine residues with some phosphorylation at threonine and at a unique amino acid residue by both protein kinases (Fig. 5). The nature or identity of the unique amino acid residue is unknown at this time; however, no major phosphoprotein of the parasite is phosphorylated at a similar residue (data not shown), suggesting that it is probably not physiologically significant. In addition, the intrinsic erythrocyte membrane protein kinase, a type I casein kinase [32], also phosphorylates phosvitin at this same unique amino acid

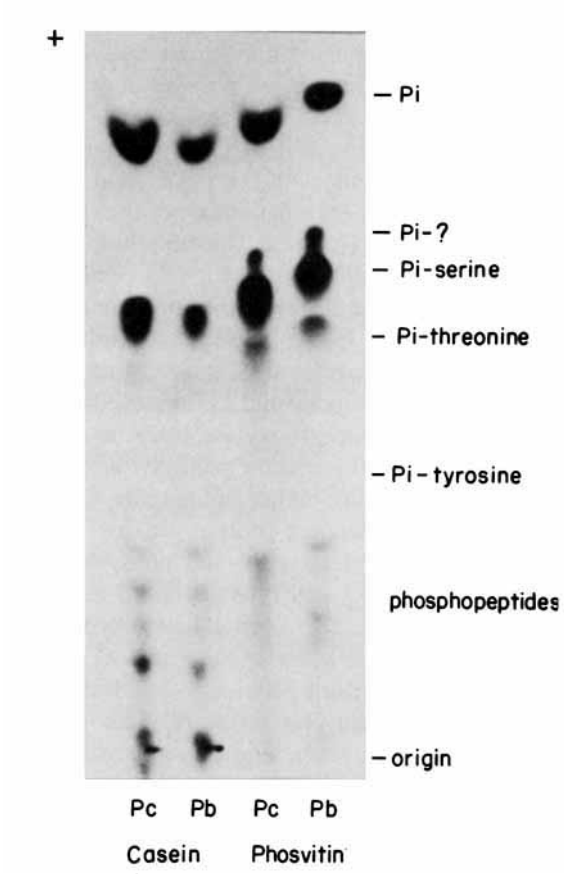

Fig. 5. Phosphoamino acid analysis. Phosphoamino acids were analyzed as described in Methods. Shown is an autoradiograph of partially hydrolyzed casein and phosvitin phosphorylated by either PcPK or PbPK as indicated.

residue (data not shown). Spermine has no effect on the pattern of phosphoamino acid phosphorylation in either casein or phosvitin. Again, both of the protein kinases from P berghei and P chabaudi appear identical in terms of phosphoamino acid preference in respect to the phosphorylation of exogenous substrates.

DISCUSSION

Our results clearly show that one or more cAMP-independent protein kinases are associated with the malarial parasites P berghei and P chabaudi. This protein kinase activity is highest with the acidic substrates, phosvitin and casein, rather than basic substrates, such as histone and protamine. Furthermore, the specific activity of lysates containing the plasmodial protein kinase increases as the parasite matures from a trophozoite (ingestion of host cytoplasm and parasite growth) to a schizont, (nuclear division and differentiation of cytoplasmic organelles) [21]. Presumably, protein phosphorylation-dephosphorylation events could be involved in coordinating and regulating the pleiotropic events of parasite differentiation and proliferation. Indeed, protein kinases and protein phosphorylation are known to be involved in cell proliferation events in other species [22,23]. In this respect, it is interesting that polyamines, which are also known to be involved in cell differentiation and proliferation [24], stimulate the plasmodial protein kinase activity. However, owing to the impure nature of the protein kinase, it is not possible at this time to conclude the exact regulatory mechanisms of the plasmodial protein kinase.

The evidence we have presented by no means constitutes proof that spermine or spermidine are physiological effectors of the plasmodial protein kinase, but we feel that it is plausible. The spermine stimulation is not due to spermine substituting for Mg^{2+} since 1) Mg^{2+} is absolutely required for protein kinase activity; 2) putrescine, in contrast to spermine, has little effect (Fig. 1); and 3) changes in the Mg^{2+} concentration seem only to change the spermine optimum and not the maximal activity (Fig. 2). Furthermore, spermine causes an increase in the V_{max} with little effect on the K_m (Fig. 3), as would be expected for regulators of protein kinases, since little regulation of protein kinase activity would be achieved by affecting the affinity of ATP [2]. In addition, ornithine decarboxylase (ODC), the rate-limiting enzyme of polyamine synthesis, increases in specific activity as the parasite matures [25], suggesting that polyamine levels are higher in the more mature parasites.

A number of cAMP-independent protein kinases are classified as casein (or phosvitin) kinases since they prefer casein and phosvitin over histone. Two basic types have been described: type I and type II, based on the order of elution from DEAE-cellulose [32]. They have also been called A-type and G-type, respectively, since type I readily distinguishes between ATP and GTP, and type II uses either nucleotide with aproximately the same efficiency. In addition, the type I casein kinase phosphorylates casein predominantly as serine residues whereas type II phosphorylates casein equally well at both serine and threonine residues. Also shown in this report is that the plasmodial protein kinase and another type I casein kinase, the erythrocyte membrane protein kinase, phosphorylate phosvitin as a unique and unidentified phosphoamino acid. In respect to elution from DEAE-cellulose, nucleotide preference, and phosphoamino acid preference, the plasmodial protein kinase is similar to the type I isozyme. However, Cochet et al [20] found that quercetin selectively inhibits the G-type casein kinase and not the A-type casein kinase from

bovine adrenocortical tissue. This indicates that the plasmodial protein kinase, although very similar, may not be exactly analogous to the mammalian type I casein kinase.

Several polyamine-dependent protein phosphorylations have been previously reported. Murray et al [29] reported stimulation of cAMP- independent and inhibition of cAMP-dependent protein kinases from mouse epidermis. However, their cAMP-independent protein kinase elute from DEAE-cellulose at greater than 0.25 M NaCl, and the optimum for spermine stimulation was 10-fold higher than the optimum for stimulation of the plasmodial protein kinase. Atmar and Kuehn [30] have reported that ODC is phosphorylated by a polyamine-dependent protein kinase; but this protein kinase exhibited low activity when casein was used as the substrate, and the plasmodial protein kinase does not phosphorylate ODC (data not shown). In addition, a protein kinase from hepatoma cells is stimulated by spermine with casein as substrate but not stimulated by spermine with phosvitin as a substrate [31]. Spermine was found in this case to lower the K_m for casein. The plasmodial protein kinase shows the same degree of stimulation by spermine with either casein of phosvitin as substrates, and spermine was not found to affect the K_m of either.

Proteins from parasitic organisms that are unique from analogous host proteins may serve as targets for chemotherapeutic agents [26]. In this regard, the protein kinase from the malarial parasite may be sufficiently different from host protein kinases that specific inhibitory drugs might be designed. In this respect we have shown that quercetin inhibits the plasmodial protein kinase. Quercetin has been previously shown to selectively inhibit certain protein kinases from Ehrlich ascites tumor cells [27], and, in addition, has been shown to inhibit the growth and proliferation of various transformed cells in vitro [28].

In summary, this paper characterizes a cAMP-independent protein kinase from the malarial parasites P berghei and P chabaudi. This enzyme appears to be involved in the maturation of the parasites, and is stimulated by polyamines and inhibited by quercetin. In addition, the enzyme was found to be similar to other previously described protein kinases, but not identical to any of them. Future work will be concerned with the elucidation of the role this protein plays in malarial physiology and its potential as a therapeutic target.

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