PfPKB, a Protein Kinase B-like Enzyme from Plasmodium falciparum

II. IDENTIFICATION OF CALCIUM/CALMODULIN AS ITS UPSTREAM ACTIVATOR AND DISSECTION OF A NOVEL SIGNALING PATHWAY*

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Intracellular cell signaling cascades of protozoan parasite Plasmodium falciparum are not clearly understood. We have reported previously (Kumar, A., Vaid, A., Syin, C., and Sharma, P. (2004) J. Biol. Chem. 279, 24255–24264) the identification and characterization of a protein kinase B-like enzyme in P. falciparum (PfPKB). PfPKB lacks the phosphoinositide-interacting pleckstrin homology domain present in mammalian protein kinase B. Therefore, the mechanism of PfPKB regulation was expected to be different from that of the host and had remained unknown. We have identified calmodulin (CaM) as the regulator of PfPKB activity. A CaM binding domain was mapped in the N-terminal region of PfPKB. CaM, in a calcium-dependent manner, interacts with this domain and activates PfPKB. CaM associates with PfPKB in the parasite and regulates its activity. Furthermore phospholipase C acts as an upstream regulator of this cascade as it facilitates the release of calcium from intracellular stores. This is one of the first multicomponent signaling pathways to be dissected in the malaria parasite.

Plasmodium falciparum is responsible for most cases of human malaria worldwide. This parasite invades both hepatocytes as well as erythrocytes in human host, but it is the erythrocytic phase of its life cycle that causes severe pathogenesis of malaria. After invading erythrocytes, the parasite undergoes well defined developmental changes inside the erythrocyte host. The parasite adopts a ringlike morphology and acquires necessary nutrients from the host during the trophozoite stages. Subsequently nuclear division gives rise to multinucleated schizont containing ~24 merozoites. These merozoites when released after schizont rupture invade fresh erythrocytes to start another cycle of asexual development. Although it is known that Plasmodium can utilize host G-protein signaling (2) and alters phosphorylation of erythrocyte cytoskeletal proteins during infection (3), parasite signaling pathways have remained largely uncharacterized. Given the importance of cell signaling cascades in proliferation and differentiation of eukaryotic cells, dissection of signal transduction mechanisms may provide useful insights about the development of this protozoan parasite. Plasmodium genome analysis revealed that there are close to 65 protein kinases, major mediators of cell signaling, in P. falciparum (4, 5). Apart from a few of these kinases (6–10), the function and mechanism of regulation and identity of cellular targets of most of these enzymes is largely unknown.

We recently identified a protein kinase B-like enzyme in P. falciparum (PfPKB)3. Despite sharing significant sequence homology (~70%) with the catalytic domain of PKB, PfPKB lacks a pleckstrin homology (PH) domain present at the N terminus of the mammalian enzyme. The N-terminal region (NTR) of PfPKB is inhibitory as its deletion results in PfPKB catalytic activation (1). The NTR does not exhibit similarity with any other protein in the non-redundant protein data base. PKB binds phosphoinositides via the PH domain, which is crucial for its membrane translocation and catalytic activation. Whereas PKB is activated by phosphoinositide-dependent kinase 1-mediated phosphorylation at Thr-308 in its activation loop (11), autophosphorylation of PfPKB at its analogous Ser-271 residue results in its activation (1). PfPKB is expressed mainly in the schizont/merozoite stages of P. falciparum. Using a pharmacological inhibitor, we had proposed a role of PfPKB during schizont-to-ring transition of the parasite (1). Despite this information, it had remained unknown how PfPKB is regulated in P. falciparum. In the present study we identified calmodulin as the upstream regulator of PfPKB activity in vitro and in vivo. These findings resulted in identification of a novel signaling pathway in the malaria parasite.

EXPERIMENTAL PROCEDURES

Reagents—pGEX4T1-PfPKB and pET-NTR plasmids used for protein expression and anti-PfPKB rabbit antisera used in these studies have been described earlier (1). Site-directed mutagenesis was carried out using the QuikChange site-di-

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3 The abbreviations used are: PfPKB, protein kinase B-like enzyme in P. falciparum; CaM, calmodulin; ΔPfPKB, deletion of PfPKB lacking the NTR; CBD, calmodulin binding domain; NTR, N-terminal region; PKB, mammalian protein kinase B; PLC, phospholipase C; PfPKB-IP, PfPKB immunoprecipitate; PI3K, phosphatidylinositol 3-kinase; scr, scrambled; PH, pleckstrin homology; GST, glutathione S-transferase; BAPTA-AM, 1,2-bis(2-amino-phenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxyethyl) ester.
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Site-directed Mutagenesis and Recombinant Protein Expression—For expression of PfPKB as a GST fusion protein, pGEX4T1-PfPKB plasmid construct was used (1). Deletion mutant ΔCBD and S271A mutants were generated by using the above mentioned PfPKB construct and the QuikChange site-directed mutagenesis kit (Stratagene). Recombinant proteins were expressed and purified as described earlier (1). Protein concentration was estimated by performing densitometry of SDS-PAGE gels using NIH Image software.

Immunoblotting and Immunoprecipitation—Parasites were released from infected erythrocytes by 0.05% (w/v) saponin treatment. Cell-free protein extracts from specific parasite stages were prepared by suspending parasite pellets in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 20 μM sodium fluoride, 20 μM β-glycerophosphate, 100 μM sodium orthovanadate, and 1X Complete protease inhibitor mixture (Roche Applied Science) using a syringe and a needle. In most experiments 1 mM CaCl₂ was also included in the buffer, and 1 mM EGTA was added to perform experiments in calcium-free buffer. Lysates were cleared by centrifugation at 14,000 × g for 30 min. PfPKB was immunoprecipitated from the schizont or the merozoite lysates using anti-PfPKB antisera (1). 50–100 μl of immunoprecipitated PfPKB were expressed and purified as described above. The phosphorimage shows that PfPKB (lane 2) and not S271A (lane 4) was autophosphorylated in the presence of Ca²⁺/CaM. Unlike PfPKB, S271A mutant failed to exhibit CaM-mediated activation (E). Fold activation was calculated by comparing the ability of PfPKB or S271A to phosphate crosstide in the presence or absence of CaM (see “Experimental Procedures”). Error bars reflect S.E. from more than three experimental determinations.

Parasite Culture—P. falciparum strain 3D7 was cultured at 37°C in RPMI 1640 medium using either 10% AB⁺ human serum or 0.5% Albumax II (Invitrogen) (complete medium). Cultures were gassed with 7% CO₂, 5% O₂, and 88% N₂, and synchronization of the parasites in culture was achieved by sorbitol treatment (1, 12). Sorbitol synchronization yielded parasites in ring form; these rings matured to trophozoites 30–36 h later. After nuclear division schizonts containing merozoites were observed. Ruptured schizonts with emerging merozoites were seen after ~44 h; this was followed by formation of fresh rings as a result of red blood cell invasion. Typically pharmacological inhibitors were incubated with schizonts for 15–60 min (~3% parasitemia) at 5% hematocrit.

Figure 1. CaM activates PfPKB in a calcium-dependent manner. A, schematic representation of PfPKB domain architecture. Autophosphorylation of Ser-271 in PfPKB catalytic domain (C.D.) can result in its activation, whereas its NTR (red) prevents its autophosphorylation as well as catalytic activation (1). The blue bar indicates the presence of a putative N-terminal signal peptide in PfPKB. Recombinant GST-PfPKB (1.4 μM) was preincubated with various concentrations of CaM and 100 μM CaCl₂, and kinase assays were performed using a small peptide, crosstide, as substrate. B, CaM activates PfPKB in a calcium-dependent manner. GST-PfPKB was incubated in the presence or absence of 5 μM CaM and 100 μM CaCl₂, and kinase assays were assayed as described in B, D and E. CaM regulates PfPKB activity in a calcium-dependent manner. GST-PfPKB was incubated in the presence or absence of 5 μM CaM in a buffer containing either 20 μM CaCl₂ or 1 mM EGTA (0 mM CaCl₂), and PfPKB activity was assayed as described in B, D and E. CaM promotes autophosphorylation of PfPKB, which is responsible for its activation. Equal amounts of GST-PfPKB or its S271A mutant were incubated with (lanes 2 and 4) or without (lanes 1 and 3) 5 μM CaM and 100 μM CaCl₂, in the absence (D) or presence of crosstide (E), and kinase assays were performed as described above. The phosphorimage shows that PfPKB (lane 2) and not S271A (lane 4) was autophosphorylated in the presence of Ca²⁺/CaM. Unlike PfPKB, S271A mutant failed to exhibit CaM-mediated activation (E). Fold activation was calculated by comparing the ability of PfPKB or S271A to phosphate crosstide in the presence or absence of CaM (see “Experimental Procedures”). Error bars reflect S.E. from more than three experimental determinations.

rected mutagenesis kit (Stratagene). The peptides crosstide (GRPRTSFFAEG), calmodulin (CaM) binding domain (CBD) peptide (IGKKRLNSMSLYERKKRI), and scrambled (scr) peptide (MKLSGKRYNSRLKEIRSRIK) were custom synthesized by Peptron. scr peptide has an amino acid composition similar to CBD, but their arrangement has been scrambled. U73322, U73122, and W7 were purchased from Calbiochem. Anti-CaM monoclonal antibody against Dictyostelium discoideum CaM and purified bovine CaM were also obtained from Calbiochem. Unless indicated, all other fine chemicals were purchased from Sigma.

_protein extracts from specific para- site stages were prepared by sus- pending parasite pellets in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 20 μM sodium fluoride, 20 μM β-glycerophosphate, 100 μM sodium orthovanadate, and 1X Complete protease inhibitor mixture (Roche Applied Science) using a syringe and a needle. In most experiments 1 mM CaCl₂ was also included in the buffer, and 1 mM EGTA was added to perform experiments in calcium-free buffer. Lysates were cleared by centrifugation at 14,000 × g for 30 min. PfPKB was immunoprecipitated from the schizont or the merozoite lysates using anti-PfPKB antisera (1). 50–100 μg of lystate were incubated with the antisera at 4°C overnight on an end-to-end shaker. Subsequently antigen-antibody complexes were incubated with 50 μl of protein A/G-Sepharose for 6 h with shaking at 4°C. Resin was washed with the lysis buffer several times and was finally resuspended in 50 μl of 1X kinase assay buffer. After separation of parasite lysters or PfPKB-IP on SDS-PAGE gels, Western blotting was performed as described previously (1).

Assay of Kinase Activity—GUST fusion proteins of PfPKB (or its variants) or 10 μl of immunoprecipitated PfPKB were assayed in a buffer containing 50 mM Tris, pH 7.5, 10 mM magnesium chloride, 1 mM dithiothreitol, and 100 μM [γ-32P]ATP (6000 Ci/mmol) using a small peptide substrate (“crosstide”) or histone H₁AS as the phosphate acceptor substrate. Reactions were terminated by spotting the reaction mixture on P81 phosphocellulose paper (Whatmann), and phosphate incorporation was measured by scintillation counting of the P81 paper. When histone was used as the substrate, reactions were stopped by boiling the reaction mixture in SDS-PAGE loading buffer. After electrophoresis, phosphate incorporation in histone was visualized by using a Fuji FLA5000 phos-
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Calmodulin Activates PFPKB Activity in a Calcium-dependent Manner—We have previously reported that PFPKB shares significant homology with the catalytic domain of PKB. However, it lacks the N-terminal PH domain present in PKB (Fig. 1A). When NTR of PFPKB is deleted it results in its catalytic complex only in the presence of calcium. E. CBD peptide prevents activation of PFPKB by CaM. GST–PFPKB was incubated with 5 μM CaM, which had been preincubated with the indicated concentration of CBD, scr-CBD peptides, water, and kinase assays were performed using 1 mM crosstide as substrate as described under "Experimental Procedures." Activity of CaM-PFPKB in the absence of CBD peptides was considered as 100%. Data are presented as mean ± S.E. of three independent experiments. C.D., catalytic domain.

RESULTS

Phorimaging system. For typical CaM activation experiments, recombinant proteins were incubated with purified bovine CaM in the presence or absence of CaCl₂ 15 min prior to addition of the phosphoacceptor substrate (100 μM cross- tide or 5 μg of histone) and ATP. Unless indicated, the concentration of CaM and CaCl₂ used in kinase assays was 5 and 100 μM, respectively. For experiments described in Fig. 1B, Ca²⁺/CaM was preincubated with GST-PFPKB 1 h and ATP prior to the addition of crosstide. 1 unit of PFPKB activity is equivalent to 1 μmol of phosphate/min/mg. All experiments were done at least three times.

CaM-Peptide Interaction—CaM-peptide interaction experiments (Fig. 2D) were performed as described earlier (13) with a few modifications. Briefly CaM and peptides were incubated in a buffer containing 25 mM Tris·HCl, pH 7.4, 192 mM glycerol for 1 h at room temperature in the presence (1 mM CaCl₂) or absence (2 mM EGTA) of calcium. The mixture was resolved by 15% native PAGE at constant current of 25 mA.
The maximal activation of PfPKB only in the absence of CaM (Fig. 1), a well-established PKB substrate (15) (Fig. 1). Recombinant PfPKB resulted in a dose-dependent increase in peptide phosphorylation with CaM from other eukaryotes (14). Incubation of CaM with almost all eukaryotes including major calcium-binding protein ubiquitously expressed in and is well conserved across species. P. falciparum homologue of CaM (GenBank accession number X56950) shares ~97% homology with CaM from other eukaryotes (14). Incubation of CaM with recombinant PfPKB resulted in a dose-dependent increase in its activity as judged by its ability to phosphorylate a small peptide, crosstide, a well-established PKB substrate (15) (Fig. 1B).

The maximal activation of 1.4 µM PfPKB was achieved at ~2 µM CaM with a K_{CaM} of ~0.5 µM. Importantly, PfPKB activation by CaM was dependent on calcium (Fig. 1C). Because it has been shown previously that PfPKB activation is dependent on autophosphorylation of Ser-271 in its activation loop (1), it was worth exploring the role of phosphorylation of this site in CaM-mediated PfPKB activation. Calcium/CaM catalyzed autophosphorylation of PfPKB (Fig. 1D), and mutation of its Ser-271 to Ala (S271A) resulted in almost complete loss of its autophosphorylation and concomitant attenuation of its catalytic activity (Fig. 1E). Collectively, these data suggest that Ca^{2+}/CaM activates PfPKB by promoting its autophosphorylation at Ser-271 (Fig. 1, B–E).

Identification of a CBD in PfPKB—CaM interacts with segments of proteins that form amphipathic α-helices and are rich in basic and hydrophobic amino acids (16). On examination of NTR of PfPKB, a 21-amino acid motif possessing a putative Ca^{2+}/CaM binding site was identified (Fig. 2). To test whether this motif is the CBD of PfPKB, a deletion mutant of PfPKB lacking these 21 amino acids (ΔCBD) was created. Unlike PfPKB, CaM failed to activate this mutant (Fig. 2B). Direct binding of PfPKB and ΔCBD was tested by using CaM immobilized on agarose. Whereas PfPKB exhibited significant binding to CaM-agarose, ΔCBD mutant failed to interact with CaM (Fig. 2C), suggesting that this 21-amino acid stretch is the only CaM binding site in PfPKB. As expected, PfPKB did not show binding to CaM in the absence of calcium (Fig. 2C, inset). CaM-CBD interaction was further validated by using a synthetic peptide corresponding to a pseudosubstrate motif in the CBD, which spans the pseudosubstrate motif, causes a conformational change resulting in the dissociation of NTR from the catalytic cleft thereby facilitating the autophosphorylation of Ser-271 of the activation loop. These events result in the catalytic activation of PfPKB. Phos-S271, phosphorylated Ser-271.

A pseudosubstrate motif is present in the CBD. A, ΔPfPKB, an N terminus-deleted version of PfPKB lacking the first 98 residues that is active independently of CaM, was incubated in a kinase assay mixture with or without CaM peptide. Histone was used as the phosphate acceptor substrate. Inhibition of histone phosphorylation was accompanied by a simultaneous increase in CBD peptide phosphorylation. B, ΔPfPKB was incubated in a kinase assay mixture, and 50 µM CBD or CBD-598A peptide was used as phosphate acceptor substrate. The phosphorylation of these peptides was measured as described for crosstide in Fig. 1. The average from two determinations done at the same time is shown, and error bars indicate S.E. C, 4 µM His6-NTR (6xHis-NTR) was preincubated with buffer alone or with 4 µM CaM and 100 µM CaCl2 before addition to the kinase assay mixture containing ΔPfPKB. Kinase activity was determined as described above, and the activity in the absence of NTR was considered as 100%. D, 2.5 µM His6-NTR was either preincubated with buffer alone (lane 1) or with 100 µM CaCl2 and 2.5 µM CaM (lane 2) before addition of 2.5 µM GST-ΔPfPKB. Glutathione-Sepharose beads were used to pull down the complex of GST-ΔPfPKB and His6-NTR. After washing, the proteins bound to the beads were analyzed by SDS-PAGE. Coomassie staining of the gels revealed the presence of NTR bound to PfPKB only in the absence of CaM (lane 1). In the presence of Ca^{2+}/CaM, no NTR-PfPKB interaction was observed (lane 2). Lane 3 is the input His6-NTR used for the experiment. E, a model for PfPKB activation by Ca^{2+}/CaM. PfPKB is locked in an inactive state as a pseudosubstrate region in the NTR occupies its catalytic cleft. Ca^{2+}/CaM binding to CBD, which spans the pseudosubstrate motif, causes a conformational change resulting in the dissociation of NTR from the catalytic cleft thereby facilitating the autophosphorylation of Ser-271 of the activation loop. These events result in the catalytic activation of PfPKB. Phos-S271, phosphorylated Ser-271.
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FIGURE 4. CaM regulates PFKPB activity in P. falciparum cultures. A, PFKPB and CaM associate in P. falciparum. PFKPB was immunoprecipitated from schizonts (both panels) or red blood cell (right panel) lysates, and immunoprecipitate IP was electrophoresed on an SDS-PAGE gel followed by Western blot analysis using anti-CaM antibody. Detection of CaM in the immunoprecipitate of PFKPB suggested that these proteins interact in the parasite (lane 2, both panels). Mock immunoprecipitation experiments were performed by using preimmune antisera (left panel, lane 1) or red blood cell lysate (right panel, lane 1). B, CaM inhibitor W7 inhibits PFKPB activation in P. falciparum cultures. Schizont-rich P. falciparum cultures were treated with 50 μM W7. Subsequently PFKPB was immunoprecipitated from parasite protein lysates, and PFKPB-IP-associated activity was assayed. Significant loss in PFKPB activity was observed upon W7 treatment. Addition of 0.1 mM CaCl₂ and 5 μM CaM to PFKPB-IP from W7-treated parasites resulted in a significant recovery of PFKPB activity. The results are presented as mean ± S.E. of three independent experiments; the activity of PFKPB in Me₅SO (DMSO)-treated parasites was considered as 100%. C, PFKPB was immunoprecipitated from schizonts in a buffer containing either 1 mM CaCl₂ or EGTA, and immunoblotting was performed to detect the associated CaM. A representative of three independent experiments is shown in the figure. H, heavy.

The CBD sequence. CBD peptide exhibited CaM binding as it caused a mobility shift of CaM on a native PAGE gel in the presence of calcium. When calcium was excluded from the reaction mixture CBD did not interact with CaM as it failed to exhibit a shift in its electrophoretic mobility (Fig. 2D). CBD peptide prevented PFKPB activation in a dose-dependent manner when incubated with CaM (Fig. 2E) indicating that this peptide competes with the CBD of PFKPB for CaM.

Data presented in Fig. 1 and our previous studies (1) indicate that the NTR is inhibitory for PFKPB in the absence of CaM binding. It is possible that the NTR either keeps PFKPB in an inactive state either by masking its catalytic cleft and/or by physically interacting with its active site. To investigate this, ΔPFKPB, a deletion mutant of PFKPB that lacks most of the NTR and first nine residues of CBD and is constitutively active (1), was used. Incubation of ΔPFKPB with CBD peptide inhibited its ability to phosphorylate histone. Interestingly this was accompanied by simultaneous phosphorylation of the CBD peptide (Fig. 3A). These observations indicated that the CBD peptide can also interact with PFKPB active site. A similar inhibition in phosphorylation of crostide by ΔPFKPB was observed (data not shown). Upon close examination of CBD sequence, an RXRXS type motif was found embedded in the CBD (Fig. 2A) that closely resembles a putative substrate motif for AGC family kinases like PFKPB (17) and is not present in ΔPFKPB. It is possible that this motif may act as a pseudosubstrate and thus have affinity for PFKPB active site. Replacement of the Ser (Ser-98 of PFKPB) in the RXRXS motif to Ala resulted in a loss of phosphoconformation by CBD/NTR as the RXRXS pseudosubstrate motif present in this domain occupies the catalytic cleft of the kinase. Ca²⁺/CaM binding to CBD induces a conformational change that causes the release of NTR from the catalytic site resulting in PFKPB autophosphorylation and its catalytic activation (Fig. 3E).

Regulation of PFKPB by CaM in P. falciparum—Whereas PFKPB is specifically expressed in schizonts/merozoites (1), CaM is present in all intraerythrocytic stages (18). To determine whether PFKPB interacts with CaM in the parasite, PFKPB was immunoprecipitated from schizonts followed by Western blotting for CaM. CaM was co-immunoprecipitated with PFKPB indicating that these proteins associate in the parasite. In contrast, mock immunoprecipitation experiments performed with either preimmune antisera or red blood cell lysates did not show the presence of CaM (Fig. 4A). The ability of CaM to activate PFKPB in P. falciparum was tested by using W7, a specific CaM inhibitor, which has been used previously to demonstrate its role in erythrocyte invasion (19, 20). Incubation of either schizonts or free merozoites (data not shown) with W7 resulted in a significant loss of PFKPB activity indicating that CaM is a PFKPB regulator in vivo. This was further established when addition of purified CaM to the PFKPB-IP from W7-treated parasites led to a significant recovery of PFKPB activity (Fig. 4B). Collectively these observations establish that CaM is a regulator of PFKPB in P. falciparum. Moreover when PFKPB was immunoprecipitated in the absence of calcium, a significant loss of CaM binding resulted (Fig. 4C), suggesting
that calcium is necessary for CaM-PfPKB interaction in the parasite.

**Phospholipase C-mediated Calcium Release Regulates CaM-PfPKB Interaction**—Because experiments described above (Figs. 1 and 4C) suggest that the activation of PfPKB by CaM is dependent on calcium, it was important to investigate the mechanism via which parasitic calcium regulates PfPKB. Intra-cellular calcium levels of the parasite are tightly regulated in *Plasmodium*, and inhibitors of phospholipase C (PLC) (21), which block inositol 1,4,5-trisphosphate formation, prevent release of free calcium from intracellular parasite stores (22, 23). To investigate the role of PLC in PfPKB activation, schizonts were incubated with either U73122, a specific inhibitor of PLC, or its less potent analogue U73322. U73122 treatment resulted in a significant attenuation of PfPKB activity. In contrast, U73322 only caused a marginal effect (Fig. 5A). The loss of PfPKB activity was accompanied by a reduction in amount of CaM co-immunoprecipitated with PfPKB observed only in U73122-treated parasite without altering levels of PfPKB and CaM in whole parasite lysates (bottom panels). A representative of three independent experiments is shown. H, heavy.

**DISCUSSION**

Based on the sequence homology we had identified PfPKB as a PKB-like kinase in *P. falciparum*. The PI3K-PKB pathway is a major player in a wide variety of cellular processes in mammalian cells (24, 25). *Plasmodium* possesses only one PI3K homologue and PfPKB, a protein kinase B-like enzyme in *P. falciparum* (1). PfPKB shares several common features with the catalytic domain of PKB such as the regulatory motif in the T-loop that has a regulatory Ser-271, which is in a location similar to Thr-308 of PKB (1). PKB is regulated by interaction of 3’-phosphorylated phosphoinositides with its N-terminal PH domain (26) and phosphorylation of PKB at Thr-308 by phosphoinositide-dependent kinase 1. In contrast, PfPKB is activated by autophosphorylation of Ser-271. The NTR of PfPKB does not have a PH or any other modular domain, but it prevents PfPKB activation (1). Because NTR failed to exhibit any similarity with proteins in the non-redundant data base, it was difficult to postulate its mode of regulation. Biochemical studies performed with PfPKB indicated that it is directly regulated by Ca$^{2+}$/CaM. Whereas PfPKB is expressed mainly in the schizont/merozoite stages, our recent findings suggest that PfPI3K homologue in *P. falciparum* may be present mainly during trophozoite stages; this also fits well with a phosphoinositide-independent mechanism of PfPKB regulation.

CaM interacts with segments on proteins that are rich in basic and hydrophobic residues and have the propensity of forming α-helices. Typically hydrophobic residues in CBDs of target proteins are critical for anchoring them to hydrophobic

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4. P. Sharma, unpublished results.

5. A. Vaid and P. Sharma, manuscript in preparation.

6. A. Vaid and P. Sharma, unpublished results.
FIGURE 7. A novel signaling pathway in P. falciparum. Studies described in this report suggest that CaM activates PfPKB in a calcium-dependent manner by interacting with a CBD in its N terminus (Figs. 1 and 2). Phospholipase C, the enzyme involved in generation of inositol 1,4,5-trisphosphate (I(1,4,5)P3), acts as an upstream regulator of this pathway (Fig. 5) as inositol 1,4,5-trisphosphate may facilitate the release of calcium needed for PfPKB activation. IP(4,5)P2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol.

CaMKD-dependent protein kinases, and more strikingly a protein kinase C-like enzyme seems to be absent (4). Given its versatility in eukaryotic signaling, lack of a protein kinase C-like enzyme in Plasmodium was indeed surprising. Protein kinase C belongs to the same AGC class of kinases as cAMP-dependent protein kinase and PKB, which share significant homology in their catalytic domain region. For instance, PKB and protein kinase C have ~67% similarity in their catalytic domains. In addition to the similarity between PfPKB and PKB, it is important to note that PfPKB also shares reasonable homology (~64%) with mammalian protein kinase C (1). We used this information to identify a protein kinase C inhibitor, Go6983, as an inhibitor of PfPKB. When added to schizont stage cultures, this inhibitor blocked ring formation suggesting that PfPKB may be involved in schizont-to-ring transition (1); no other parasitic stage was affected by this compound. Schizont/merozoite-specific expression (1) may allow PfPKB to play a role in early/late stages of parasite life cycle.

Release of intracellular calcium is critical for various parasitic functions (29). Importantly it appears to be indispensable for successful erythrocyte invasion (30, 31). CaM is known to localize at strategic locations in merozoites and control invasion (19, 20). However, the lack of identity of Ca2+/CaM targets leaves a gap in understanding their role in the parasite life cycle. We have shown that CaM interacts and regulates PfPKB in response to upstream events in the schizont. Therefore, it is possible that PfPKB may be one of the major targets via which CaM may control important parasitic processes like invasion. Identification of downstream targets of PfPKB will help further in unraveling the function of this pathway.

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