Role of Ca\(^{2+}\)/Calmodulin-PfPKB Signaling Pathway in Erythrocyte Invasion by *Plasmodium falciparum*

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Molecular mechanisms by which signaling pathways operate in the malaria parasite and control its development are promiscuous. Recently, we reported the identification of a signaling pathway in *Plasmodium falciparum*, which involves activation of protein kinase B-like enzyme (PfPKB) by calcium/calmodulin (Vaid, A., and Sharma, P. (2006) J. Biol. Chem. 281, 27126–27133). Studies carried out to elucidate the function of this pathway suggested that it may be important for erythrocyte invasion. Blocking the function of the upstream activators of this pathway, calmodulin and phospholipase C, resulted in impaired invasion. To evaluate if this signaling cascade controls invasion by regulating PfPKB, inhibitors against this kinase were developed. PfPKB inhibitors dramatically reduced the ability of the parasite to invade erythrocytes. Furthermore, we demonstrate that PfPKB associates with actin-myosin motor and phospho-rylates PfGAP45 (glideosome-associated protein 45), one of the important components of the motor complex, which may help explain its role in erythrocyte invasion.

Almost 500 million cases of malaria are reported each year, and 1–3 million of these cases result in human death. Apicomplexan parasite *Plasmodium*, which is responsible for malaria, has a complex life cycle. It develops inside both human and insect hosts, but its development inside human erythrocytes is the major cause of malarial pathology. Upon invasion of the erythrocyte, the parasite resides inside the parasite vacuole and undergoes various developmental changes. Subsequent to nuclear division, several merozoites are generated, which upon release invade fresh erythrocytes resulting in higher parasitemia. It is clear that erythrocyte invasion is a key step for the parasite to infect the human host and therefore is a key target for intervention.

Erythrocyte invasion is a multistep process wherein interaction between the merozoite and erythrocyte is followed by reorientation of the merozoite, which results in the formation of a tight junction between the merozoite apical end and the erythrocyte membrane (2). Although reasonable information is available about the receptor-ligand interactions involved in recognition and formation of the junction, parasite events that cause the initiation of invasion process are poorly understood. It has been reported that parasite calcium levels need to be optimal for successful invasion (3, 4). Therefore, it is reasonable to speculate that calcium-dependent signaling pathways may be critical in controlling signaling events.

Sequencing of the *Plasmodium* genome and subsequent *in silico* work has indicated the presence of several putative signaling proteins in the parasite (5–8). However, the information about parasite signaling networks is very limited.

Mammalian protein kinase B (PKB)\(^3\) is regulated by phosphoinositides as they regulate its cellular localization and activation by interacting with a pleckstrin homology domain present at its N terminus. PKB is activated upon phosphorylation by PDK1 (9). In contrast, PfPKB, which is a PKB-like enzyme in *P. falciparum*, is regulated via a PDK1 and Phosphatidylinositol 3-kinase-independent mechanism (1, 10). Recently, we described a signaling pathway in *Plasmodium falciparum*, which involved the activation of PfPKB by calcium/calmodulin (CaM). Calmodulin interacts with the N-terminal region (NTR) of PfPKB and promotes its autophosphorylation-dependent activation. The intracellular calcium necessary for the activation of this pathway was mobilized from intra-parasitic stores by phospholipase C (1). Despite this information, the function of this pathway had remained unexplored. In this study, we demonstrate that CaM and intracellular calcium are important for invasion, and phospholipase C was found to play an important role in this process as it mediated intracellular calcium release needed for invasion. Because inhibition of PfPKB impaired invasion, we conclude that this calcium/calmodulin signaling pathway is crucial for erythrocyte invasion. In addition, we provide evidence that PfPKB interacts with actin-myosin motor complex and phosphorylates PfGAP45 (glideosome-associated protein 45).

**EXPERIMENTAL PROCEDURES**

Reagents—PfPKB-pGEX4T1 plasmid used for protein expression and anti-PfPKB rabbit antisera used in these studies have been described earlier (10). The following peptides: cross-

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\(^1\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. I–V.

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\(^3\) The abbreviations used are: PKB, mammalian protein kinase B; CaM, calmodulin; CBD, calmodulin binding domain; PfGAP45, *P. falciparum* homologue of Glideosome-Associated Protein 45; NTR, N-terminal region; PfPKB, protein kinase B-like enzyme in *P. falciparum*; SPF1, PfPKB deletion of PfPKB lacking the NTR that is catalytically active; PLC, phospholipase C; IP, immunoprecipitate; IMC, inner membrane complex; scr, scrambled; FITC, fluorescein isothiocyanate; PMTIP, *P. falciparum* homologue of myosin tail interacting protein.
PfPKB Signaling Pathway in Malarial Invasion

FIGURE 1. A, calmodulin is involved in erythrocyte invasion. Parasite cultures were synchronized and schizont stage parasites were treated either with MeSO (DMSO) or 50 μM W7. Blood smears were made after the indicated time to assess the ring formation. B, phospholipase C controls invasion by regulating intracellular calcium levels. Schizonts isolated from synchronized parasites were treated in culture with MeSO alone, 30 mM U73122 or U73322, the combination of U73122 and 10 μM ionomycin, or 25 μM 1,2- bist(aminophenoxo)ethane-N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM). Thin blood smears of the culture were prepared after the indicated time, and the number of rings formed after invasion was counted. Data in both panels indicate that the percentage of ring-infected parasites is representative of at least three experiments. Error bars reflect S.E. of replicates of a single experiment.

Cell Culture, Synchronization, Preparation of Free Merozoites, Inhibitor Treatment, and Invasion Assays—P. falciparum strain 3D7 was cultured at 37°C in RPMI 1640 medium using either 10% AB+ human serum (11) 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 (12).

RBC-free merozoites were prepared by using a previously published method with slight modifications (13, 14). Briefly, sorbitol synchronized parasites at ring stages were collected and washed with a buffer containing 10 mM Tris, pH 7.6, 150 mM NaCl, 10 mM glucose, 1 mM CaCl2 and incubated at 10% hematocrit in the same buffer with 1 mg/ml trypsin for 60 min at 37°C. After washing with complete medium, parasites were resuspended immediately in complete medium and were incubated with fresh erythrocytes for invasion. Smears were stained with Giemsa and examined microscopically and were typically found free of either erythrocytes or intracellular parasitic stages.

To determine the effect of inhibitors on PfPKB activity, inhibitors were added to either schizonts or merozoites in culture. Subsequently, immunoprecipitation was performed, and the IP was used for kinase assays (described below). For typical invasion assays, parasite cultures were synchronized using sorbitol as described above, and peptides and pharmacological inhibitors were added to mid-late schizonts (~3% parasitemia) and 5% hematocrit, which was maintained using fresh erythrocytes. The formation of rings was analyzed periodically after every 2 h. For experiments with free merozoites, 1–5 × 106 merozoites were incubated with peptides or inhibitors for ~10 min in complete medium prior to the addition of fresh erythrocytes at 5% hematocrit in culture medium. New ring stage infection was scored after 3 h by microscopic examination of Giemsa-stained blood smears. At least 1000 erythrocytes from several different fields were counted for each experiment.

Recombinant Protein Expression and Generation of Antisera—For expression of ΔPfPKB as a GST fusion protein, the pGEX4T1-ΔPfPKB plasmid construct was used, as described previously (10). For expression of PfGAP45 and PfMTIP, corresponding cDNAs were amplified and cloned in PET28a and PQE30-UA vectors, respectively, and expressed as His6-tagged proteins in BL21(DE3)-RIL Escherichia coli strain and purified using nickel-nitriilotriacetic acid affinity chromatography. These recombinant proteins were used to raise antiserum in rabbits or mice using standard procedures. PfMTIP was also expressed as a GST fusion protein. For this purpose, PfMTIP was cloned in pGEX4T3 vector and expressed in BL21-RIL strain as described previously (15). Recombinant proteins were quantified and normalized by performing densitometry of SDS-polyacrylamide gels.

Metabolic Labeling of Parasites—Tightly synchronized parasite cultures (~6–8% parasitemia) were washed at least three times either with phosphate or methionine-deficient RPMI 1640 medium (HyClone). Subsequently, parasites were cultured in media supplemented with 10% human serum. CBD peptide or pharmacological inhibitors were added to schizonts, which corresponded to ~42–44 h post-invasion parasites. Inorganic 32P radionuclide in the form of [32P]orthophosphoric acid (2 mCi/ml) (16) or [35S]Met/Cys (0.3 mCi/ml) (17, 18) was added to cultures and incubated for 2 and 4 h, respectively.

tide (GRPRRTSSFAEG), CBD peptide (IGKKRLRNSLSYEKRRIR), scr-peptide (MKLSGKRYNRSRLKEIRSRK), and CBD-(1–15) (IGKKRLRNSLSYLERS) were custom-synthesized by Peptron, South Korea. Scr peptides have similar amino acid composition as CBD, but their arrangement has been scrambled. U73322, U73122, and W7 were purchased from Calbiochem. A443654 and A739985.3 were provided by Dr. Vincent Giranda, Abbott.

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Peptide inhibitors attenuate PfPKB activity in parasites. A, domain structure of PfPKB. The sequence of the N-terminal CBD present in the N-terminal region and sequences of peptides used in this work are shown; an RRXXS pseudosubstrate region present in the CBD is also highlighted. B, CBD and CBD-(1–15) peptide inhibit activity of recombinant \( \Delta \)PfPKB. Recombinant \( \Delta \)PfPKB was incubated in a kinase assay buffer with phosphor acceptor substrate crosstide (1 mM) in presence or absence of 50 \( \mu \)M CBD and CBD-(1–15) peptides. Phosphate incorporation in crosstide was determined as described under “Experimental Procedures.” C, CBD and CBD-(1–15) peptides inhibit PfPKB in P. falciparum. Parasites were incubated with the indicated amount of 50 \( \mu \)M CBD, CBD-(1–15), or 100 \( \mu \)M scr-CBD peptide. Subsequently, the activity of PfPKB immunoprecipitated from parasite lysates was assayed using 1 mM crosstide as substrate. D, localization of the CBD peptide in P. falciparum merozoites. CBD-peptide conjugated with FITC at its N terminus was added to merozoites. FITC-associated fluorescence (green) was observed inside the merozoite at the apical end. Data representative of more than three independent experiments are shown in all panels.

Subsequently, parasite lysates were prepared, and immunoprecipitation was performed as described below. The IP was resuspended in SDS-PAGE loading buffer and electrophoresed, and labeled proteins were detected using a Fuji FLA5000 scanner.

Western Blotting and Immunoprecipitation—Parasites were released from infected erythrocytes by 0.05% (w/v) saponin treatment. Cell-free protein extracts were prepared by homogenizing parasite pellets in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 20 \( \mu \)M sodium fluoride, 20 \( \mu \)M \( \beta \)-glycerophosphate, 100 \( \mu \)M sodium orthovanadate, and 1X Complete Protease inhibitor mixture (Roche Applied Science). Particulate material was removed by centrifugation at 14,000 \( \times \) g for 30 min. PfPKB, PfGAP45, and PfMTIP were immunoprecipitated from the schizont or merozoite lysates using appropriate antisera as described previously (1). For co-immunoprecipitation experiments, IP was electrophoresed on SDS-PAGE followed by Western blotting (1).

Immunofluorescence and FITC-Peptide Localization— Immunofluorescence was performed on thin blood smears of parasite cultures. Thin blood smears were first fixed with cold methanol and blocked with 3% bovine serum albumin (prepared in phosphate-buffered saline) before incubation with primary antibody. After washing with blocking buffer, samples were incubated with secondary antibodies labeled with FITC/Texas red or AlexaFluor488/594. For experiments performed with FITC-labeled peptides, CBD peptides were added to schizont stage parasite cultures. After 2 h, thin smears were processed for immunofluorescence for MSP-1 as described above. Slides were viewed using an Olympus fluorescence microscope. For indirect immunofluorescence assays described in Fig. 6A, confocal microscopy was performed using an Olympus FV1000 confocal microscope. The single z-stack shown was processed using Olympus Fluoview 1.6 software. Images were processed using Image ProPlus or Adobe Photoshop software.

Assay of Kinase Activity—Catalytic activity of GST-\( \Delta \)PfPKB or 10 \( \mu \)l of immunoprecipitated PfPKB were assayed as described previously (1) in a buffer containing 50 mM Tris, pH 7.5, 10 mM magnesium chloride, 1 mM dithiothreitol, and 100 \( \mu \)M (\( \gamma \)-\( ^{32} \)P)ATP (6000 Ci/mmol) using a small peptide substrate “crosstide” (100 \( \mu \)M, unless otherwise indicated) or other recombinant proteins like PfGAP45. Reactions were stopped by spotting the assay mixture on P11 phosphocellulose paper, and phosphate incorporation was measured by scintillation counting of the P11 paper. When recombinant proteins were used as substrate, reactions were stopped by boiling the mixtures in SDS-PAGE loading buffer (1, 10). After electrophoresis, phosphate incorporation in substrate proteins was visualized by using a Fuji FLA5000 scanner. For PfPKB inhibition assays, pharmacological or peptide inhibitors were preincubated with \( \Delta \)PfPKB 15 min prior to the addition of phosphoacceptor substrate and ATP.

RESULTS

Phospholipase C and Calmodulin Inhibitors Block Erythrocyte Invasion—PfPKB is regulated by CaM by promoting its autophosphorylation in a calcium-dependent manner in vitro and in parasites. The calcium necessary for PfPKB activation by CaM is dependent on the activation of phospholipase C (PLC) (1). Therefore, the PfPKB pathway is regulated by CaM and phospholipase C-mediated calcium release (1). Specific inhibitors of both CaM (19) and PLC (20, 21) have been used successfully to block the activity of these proteins in Plasmodium. In this study, we used these inhibitors to evaluate the function of CaM and PLC in invasion.
When the CaM inhibitor W7 was added to schizonts, it blocked the formation of rings suggesting that CaM plays a role in invasion (Fig. 1A), which is consistent with results published previously (19). The role of PLC was determined by using its analogue U73322 or Me2SO treatment of schizonts, addition of U73322, which was used as a negative control. In comparison with U73322 or Me2SO treatment of schizonts, addition of U73122 inhibited new ring formation significantly suggesting that phospholipase C may be important for invasion. To explore if PLC controls invasion by regulating calcium release, U73122 was used in combination with ionomycin, a calcium ionophore. In this case, inhibition of invasion was significantly protected suggesting that PLC may control this process by regulating intracellular calcium release (Fig. 1B). Similar results were obtained when free merozoites were used for invasion assays (data not shown). Previously, we have demonstrated that CaM and PLC inhibitors block PfPKB activity. When U73122 was used in the presence of ionomycin, PfPKB activity was restored suggesting that PLC-mediated calcium release is critical for PfPKB to function (1). Therefore, it was reasonable to speculate that the modulation of PfPKB activity caused because of the inhibition of CaM and PLC may contribute to impaired invasion.

**Calmodulin-dependent Activation of PfPKB May Be Important for Erythrocyte Invasion; Design and Use of Peptide Inhibitors of PfPKB—CaM and PLC appear to play a role in invasion (Fig. 1), and because they act as upstream regulators of PfPKB (1), the contribution of PfPKB in this process was worth investigating. Two different strategies were used to inhibit PfPKB as follows: 1) a pharmacological inhibitor competitive against ATP, and 2) peptide inhibitors, which target either PfPKB catalytic cleft and/or compete with CaM for PfPKB.

Previous biochemical studies (1) had suggested that a calmodulin binding domain (CBD) exists in the NTR of PfPKB (Fig. 2A). In addition, a pseudo-substrate RXRXS motif is also embedded in the CBD, which probably holds PfPKB in an inactive state by interacting with its catalytic site. This was confirmed when a 21-amino acid peptide corresponding to the CBD motif could interact with CaM and prevent the activation of PfPKB in vitro (1). This peptide can also compete directly with the catalytic site of PfPKB (Fig. 2B) as it inhibited the activity of ΔPfPKB, a variant of PfPKB that lacks the CBD and the NTR of PfPKB and therefore is active independent of Ca2+/CaM (1, 10). This "two-pronged" ability of the CBD peptide to inhibit PfPKB activity made it a putative tool for studying PfPKB function. Because this peptide may alter the function of other CaM-binding proteins, it was also important to generate a version of this peptide that could inhibit PfPKB exclusively by interacting with its active site without interacting with CaM. For this purpose, a truncated peptide, which lacked the last six amino acids of CBD, was synthesized. This peptide retained the pseudosubstrate RXRXS motif (Fig. 2A). Even though the CBD-(1–15) peptide failed to interact with CaM (supplemental Fig. 1A), it effectively inhibited the activity of ΔPfPKB (Fig. 2B). Therefore, CBD-(1–15) does not interact with CaM and works exclusively as a pseudosubstrate inhibitor of PfPKB, which interacts with the catalytic domain.

These peptide inhibitors were added to parasites followed by immunoprecipitation of PfPKB, and the kinase activity associated with PfPKB-IP was assayed. Both CBD and CBD-(1–15) peptides inhibited PfPKB activity, and the control scrambled version of peptides were ineffective (Fig. 2C). To demonstrate if these peptides enter the parasite, FITC-labeled CBD-(1–15) (Fig. 2D) and CBD (supplemental Fig. 1B) were added to parasite cultures. The peptide-associated fluorescence was observed inside most schizonts/merozoites, which indicated that these peptides might work intracellularly. Like PfPKB, peptides also concentrated at the apical end of the parasite as observed for PfPKB (supplemental Fig. 1B), and peptide associated fluorescence was largely absent from uninfected erythrocytes (data not shown). In contrast, FITC-labeled scr-CBD peptide, which does not inhibit and interact with PfPKB (Fig. 2B, supplemental Fig. 1D), was not observed inside the parasite.

These peptides were subsequently used to probe PfPKB function. When CBD and CBD-(1–15) peptide were added to se-
menter/mature schizonts, a marked decrease in the formation of fresh rings was observed (Fig. 3A, left panel). Because there was no significant change in the number of schizonts (Fig. 3A, right panel) or their morphology, it is reasonable to infer that the decreased ring formation may be due to altered invasion and not due to defects on egress and maturation. Furthermore, invasion assays were also performed with free merozoites isolated from *P. falciparum* cultures. These merozoites when added to erythrocytes in culture resulted in the formation of rings, which was indicative of successful invasion (supplemental Fig. II). Although scr-CBD peptide-treated merozoites efficiently invaded erythrocytes, both CBD and CBD-(1–15) caused a significant reduction in the formation of rings indicating that invasion was significantly impaired (Fig. 3B). To rule out the effect of these peptides on other parasitic stages, changes in response to these peptides on the entire life cycle were monitored. The growth of parasites was normal until ~46 h, and there were no significant changes in morphology of intraerythrocytic stages. Subsequent formation of rings in control cultures was indicative of the initiation of the next cycle. The peptide inhibitor-treated cultures had significantly less number of rings (supplemental Fig. IIIA), which was most likely due to impaired invasion as observed in above-described experiments (Fig. 3).

**A443654 Is an Effective Inhibitor of PfPKB Which Blocks Invasion**—Until recently, there were no commercial inhibitors available for PKB-like enzymes from any organism. A443654 was developed as a specific inhibitor of mammalian PKB/AKT enzymes, which is competitive against ATP (22). Given the sequence the high similarity between PfPKB and PKB/AKT in the catalytic domain (10), A443654 was chosen as a possible candidate to inhibit PfPKB. A443654 successfully inhibited the activity of recombinant PfPKB in vitro with reasonable IC_{50} values (~200 nM) (Fig. 4A). Importantly, A443654 treatment of schizonts resulted in a significant reduction of parasite PfPKB activity (Fig. 4B). To explore the function of PfPKB, A443654 was added to the ring stage parasites, and their growth was monitored at regular intervals. There was no change in parasitemia until ~46 h, and at this point, a sudden decrease in rings formed in the next round of the life cycle (supplemental Fig. IIIIB) was observed, which could be a result of impaired invasion. To explore this more specifically, invasion assays were performed using schizonts as well as free merozoites as described above for peptide inhibitors (Fig. 3). When schizonts were incubated with the inhibitor, no significant change in their number or morphology was observed. However, the number of rings formed subsequent to invasion was effectively reduced, IC_{50} ~ 250 nM (Fig. 5A). Similar impairment of invasion was observed when free merozoites were used for assays indicating that A443654 blocks invasion (Fig. 5B). The inactive analogue of A443654 failed to cause any significant changes to PfPKB activity or invasion of the parasites in control experiments (supplemental Fig. IV). These data corroborate well with results obtained with peptide inhibitors and collectively highlight the importance of PfPKB activity in invasion of erythrocytes.

**PfPKB Interacts with PfGAP45 and Actin-Myosin Motor Complex**—It has been demonstrated recently that the actin-myosin motor present in merozoites is important for the invasion of the host by *Toxoplasma* (23, 24), *Plasmodium* sporozoites (25), and merozoites (17, 18, 26). GAP45 and GAP50, which are part of the inner membrane complex (IMC), are responsible for anchoring the myosin A-myosin tail interacting protein complex with parasite cytoskeleton in sporozoites as well as merozoites (15, 17, 26). Even though it is realized that the proper orientation and the assembly of this motor may be a prerequisite for successful invasion (2, 17, 18, 26), the actual mechanism via which this motor functions is not yet clear. PfPKB (supplemental Fig. IIB) (10) and its regulator CaM (19, 27) localize at the apical end of merozoites, which probably facilitates their participation in invasion. Given the apical localization of PfPKB and proteins of the motor complex and their importance in invasion, the possibility of their interaction was tested.

Immunofluorescence studies revealed reasonable overlap in localization between PfGAP45 and PfPKB, which was concentrated more in the region where PfGAP45 expression was maximal (Fig. 6A). Partial co-localization between these proteins may be due to the proposed dynamic nature of the motor complex (17). Interaction between PfPKB and PfGAP45 was tested by using GST pulldown assays. Like PfMTIP, which has been suggested to interact with PfGAP45 (15, 18), PfPKB also exhibited binding to PfGAP45 (Fig. 6B).
The interaction of PfPKB with PfGAP45 and actin-myosin motor was further evaluated by performing immunoprecipitation experiments from \textsuperscript{35}S-labeled parasite proteins. SDS-PAGE of PfGAP45-IP and PfMTIP-IP exhibited a profile highly similar to that published in recent reports (17, 18); a doublet of PfGAP45 in addition to bands for PfMyoA (~94 kDa), PfGAP50 (~42 kDa), and PfMTIP (~27 kDa) was observed (Fig. 6C, lanes 2 and 3). A band corresponding to an unidentified ~50-kDa protein was also noted in previous studies (18), which fits well with the expected size of PfPKB. Western blotting using anti-PfPKB antisera performed on PfGAP45-IP confirmed the presence of PfPKB in GAP45-IP (Fig. 6C, right panel). When PfPKB was immunoprecipitated, PfGAP45 and the other above-mentioned motor proteins were also pulled down (Fig. 6C, lane 1), and the protein profile was reasonably similar to that observed for PfMTIP-IP and PfGAP45-IP (lanes 2 and 3). Control antisera prepared from pre-immune bleeds did not precipitate any of these proteins (supplemental Fig. V). These data indicate that PfPKB associates with the motor protein complex, and it is likely that its affinity toward PfGAP45 results in this interaction.

\textit{PfGAP45 is a Substrate of PfPKB—} Given the results presented in Fig. 6, it was worth testing if PfPKB phosphorylates PfGAP45. To assess this, recombinant PfGAP45 was incubated with constitutively active ΔPfPKB. PfGAP45 turned out to be reasonable substrate of PfPKB (Fig. 7A). To establish if PfGAP45 is a target of PfPKB in the parasite, schizonts were incubated with CBD-(1–15) inhibitor or scrCBD-(1–15) control peptide and labeled with \textsuperscript{32}Porthophosphate. Subsequently, PfGAP45 was immunoprecipitated, and the IP was electrophoresed. A band corresponding to PfGAP45 was labeled in control cultures indicating that it is phosphorylated in the parasite (Fig. 7B, lane 2). When
parasites were treated with the CBD-(1–15) peptide (Fig. 7B, lane 1), a significant reduction in PfGAP45 phosphorylation was observed. Expectedly, PfPKB autophosphorylation was also lowered significantly in the presence of CBD-(1–15) inhibitor (Fig. 7B, lane 1). Similar observations were made when A443654 was used to inhibit PfPKB activity (data not shown). These data strongly suggest that PfGAP45 may be a PfPKB target in *P. falciparum*. Because GAP45 is an important component of the motor complex, which is important for linking the motor with IMC, it is possible that phosphorylation by PfPKB may be important for proper functioning of the motor, which may help explain the role of PfPKB in invasion.

Based on the present findings, we extend the previously published model (1) for the PfPKB pathway in *P. falciparum* (Fig. 8); PLC-mediated calcium release results in the activation of PfPKB by Ca²⁺/CaM, which is important for invasion. The targeting of PfGAP45 by PfPKB may be important for proper functioning of the actin-myosin motor, which may be crucial for successful erythrocyte invasion.

**DISCUSSION**

Although the importance of calcium (3, 28, 29) as well as calmodulin (19, 27) has been implicated in erythrocyte invasion by *Plasmodium*, the identity of signaling pathways and underlying mechanisms has remained largely unknown. This study demonstrates that PLC-mediated control of calcium release is important for merozoite invasion. Results reported here as well as previous studies indicate that CaM (19) may be involved in invasion, and localization of CaM at the apical end of merozoites may facilitate this process (19, 27). PfPKB is one of the very few CaM targets to be identified in *P. falciparum* (1), which corroborates well with its proposed role in invasion. The role of

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**FIGURE 7.** **PfPKB phosphorylates PfGAP45 in *P. falciparum.** A, recombinant GST–ΔPfPKB was incubated with Histagged PfGAP45 in a kinase assay mix, and the reaction mixture was separated on a SDS-polyacrylamide gel followed by autoradiography. PfGAP45 was phosphorylated by PfPKB. B, PfPKB schizonts were either treated with 25 μM CBD-(1–15) or 100 μM scr-CBD-(1–15) peptides followed by labeling with [³²P]orthophosphate. PfGAP45 was immunoprecipitated, and the IP was electrophoresed and analyzed by autoradiography. Inhibition in PfPKB auto-phosphorylation as well as PfGAP45 phosphorylation was observed. Lower panel shows a PfGAP45 Western blot performed on parasite lysates from the same experiment.

**FIGURE 8.** **A model for the role of PfPKB pathway in *P. falciparum.** Calcium release from intracellular stores is facilitated by PfPLC, which results in the activation of PfPKB by Ca²⁺/CaM (1). In this study, we demonstrate that PLC controls invasion by regulating calcium release from intracellular stores and also engages CaM, one of the major calcium sensors, in this process (Fig. 1). PfPKB is the downstream target of Ca²⁺/CaM by which invasion may be regulated (Figs. 3 and 5). PfGAP45 is an important component of the glideosome (15, 17, 18), and its phosphorylation by PfPKB (Figs. 6 and 7) may be important for proper functioning of the actin-myosin motor.
PfPKB, which is a downstream target of this pathway, was elucidated by using two novel inhibitors. A443654, a recently developed indazole-pyridine series compound, is probably the most effective and specific inhibitor of PKB/AKT. This ATP competitive inhibitor (22, 30) turned out to be reasonably effective against PfPKB (Fig. 4). The crystal structure of this compound in complex with human PKBβ was solved recently (31). Sequence comparison suggested that several key residues of PKBβ (Glu-230, Phe-439, Lys-181, Glu-236, and Phe-163) that are implicated in binding with this inhibitor (31) are conserved in PfPKB (Glu-192, Phe-401, Lys-143, Glu-198, and Tyr-125), which most likely results in reasonable efficacy for PfPKB.

An independent approach based on biochemical studies was used to develop peptide inhibitors against PfPKB. Peptide inhibitors have served as potent tools for functional studies of kinases like cAMP-dependent protein kinase, protein kinase C, c-Jun N-terminal kinase (JNK), cyclin-dependent kinase, protein kinase AKT, cGMP-dependent protein kinase, and extracellular signal-regulated kinase (ERK) (32–35) as these inhibitors can target the substrate-binding cleft or sites other than the ATP binding pocket. The NTR of PfPKB keeps it inactive by interacting with the catalytic site. CaM binding to the CBD, which is present in the NTR, promotes autophosphorylation and stimulation of PfPKB activity (1). An RXRXS pseudosubstrate motif within the CBD is most likely responsible for keeping PfPKB inhibited. This information was used to successfully design CBD and CBD-(1–15) peptides as PfPKB inhibitors.

Clearly, use of this inhibitor in parasites was aided by the ability of these peptides to enter parasites. It has been demonstrated that peptides of up to ~2400 Da size can easily enter the malaria parasite (36), although the mechanism of transport is not clear. Arginine-rich peptides are known to be extremely cell-permeable and have also been used for cellular delivery of peptide inhibitors of kinases (35). It is noteworthy that CBD peptides have several arginines, which may possibly facilitate their entry in cells.

A443654 as well as inhibitor peptides prevented the formation of rings without significantly altering other parasite stages. Further confirmation of these results was provided when PfPKB inhibitors blocked invasion in assays performed with free merozoites. Collectively, these observations indicate that inhibition of PfPKB will aid in the development of novel antimalarial strategies.

Recently, several reports have implicated the actin-myosin motor in sporozoite and merozoite invasion (26, 26, 37, 38). GAP45 along with GAP50 is responsible for anchoring the actin-myosin motor complex to the IMC (17, 26). Although in-depth understanding of how this motor operates is largely unclear, the complex seems to be transient and dynamic in nature (17). GAP45 is myristoylated as well as palmitoylated (18), which may be crucial for its targeting to the glideosome. Although not demonstrated, the influence of phosphorylation in the function of motor proteins, including GAP45, has been speculated (15). Calcium is considered important for the regulation of motility and invasion by apicomplexan parasites (29, 39–41). Therefore, phosphorylation of motor complex proteins by calcium-dependent signaling pathways may be an important step in invasion. Here, we demonstrate that PfPKB phosphorylates PGAP45 in parasites, and immunoprecipitation experiments revealed that PfPKB is associated with the motor complex. Given the importance of the motor complex in invasion, it is possible that PfPKB-mediated phosphorylation of PGAP45 may regulate functioning of the actin-myosin motor.

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