Identification of physiological substrates of Plasmodium falciparum PfPK5, a CDK-like kinase

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IDENTIFICATIN OF PHYSIOLOGICAL SUBSTRATES OF PLASMODIUM FALCIPARUM PFK5, A CDK-LIKE KINASE

by

CATHERINE SULLENBERGER

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Molecular and Microbiology in the College of Biomedical Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Chair: Dr. Debopam Chakrabarti
Abstract

Malaria is one of the most devastating infectious diseases causing 1-3 million fatalities a year. The majority of these cases occur amongst children in developing countries. Malarial strains in these areas are exhibiting increasing resistance to canonical treatments proving the importance of new drug targets for anti-malarials. Identification of new drug targets is dependent upon a better understanding of the molecular biology of the parasitic agent of malaria, *Plasmodium*. The regulation of *Plasmodium*’s complex life cycle is still not well understood. Elucidation of signaling pathways involved in *Plasmodium* cell cycle regulation will provide insights into how the parasite thrives in human cells. A subset of kinases, referred to as cyclin-dependent kinases (CDKs), are crucial regulators of eukaryotic cell cycle progression. *In silico* studies show high homology between mammalian CDK’s and a group of CDK-like *Plasmodium* kinases including PfPK5 (*Plasmodium falciparum* protein kinase 5). *Plasmodium* homologues to CDK regulators, cyclins, have also been identified. Understanding the role of PfPK5 in cell cycle regulation would require analysis of subcellular localization and cell cycle-dependent expression. Immunofluorescence assays demonstrate that PfPK5 is localized in the nucleus. PfPK5’s expression profile, as determined by western blotting, shows highest expression in the schizont stage, the stage when the atypical multiple nucleated form of the parasite is observed. Possible PfPK5 interacting partners were detected by performing an anti-PfPK5 immunoprecipitation assay. Additionally, a hemagglutinin (HA)-tagged PfPK5 construct was made to increase the sensitivity of immunoprecipitation assay and identification of PfPK5
interacting partners. The characterization of PfPK5 and its interacting partners may prove useful in identification of novel drug targets in the future.
Acknowledgements

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Introduction

In 2008 alone the Center for Disease Control reported up to 311 million cases of malaria worldwide. 1,003,000 of these cases resulted in death, most of the fatalities being amongst young children in developing countries. 90 countries are considered malarious putting 36% of the total world population at risk with almost half of the countries being located in sub-Saharan Africa (19). The disease is contracted from mosquitoes and as such resistance to pesticides like DDT in malaria-endemic countries (10) has greatly hindered alleviation of the disease. This threat is being intensified by the fact that Plasmodium strains have been developing drug resistance to canonical anti-malarials. Thus the need for new anti-malarials is of vital importance (4).

Malaria: A Devastating Tropical Disease

Malaria has been considered one of the most important tropical diseases (19). Currently there is no effective vaccine (13). The threat of malaria continues to grow as traditional treatments are becoming obsolete with the emergence of drug resistant parasites. Drug resistance has occurred rapidly over the past few decades. Quinine is a natural antimalarial that has been used since the 1600s (23) but is rare and thus expensive making it far from an optimal treatment for underdeveloped countries. A synthetic equivalent, chloroquine, was created to circumvent this problem in the mid 1940s (23). Although drug resistance to quinine was not observed until 1910 (23) chloroquine resistance emerged as early as 1957 (23). Sulfadoxine-pyrimethamine drugs were introduced as a way to treat malaria in 1967 but resistance was reported in that same year (23). Sulfadoxine-pyrimethamine resistance is especially detrimental due to the fact that it
is one of the only affordable drug treatments in Africa (23). Another common treatment, mefloquine, was introduced in the late 70s and after only 5 years mefloquine resistance was reported (23). Inadequate drug supplies, improper dosing regimens and the high price of more effective anti-malarials continue to aggravate the problem of drug resistance in developing countries (3). From 1925 to 1996 1,223 new drugs were designed and of these only 3 were anti-malarials (10). As such malaria has resurfaced in countries where it had been previously eradicated such as Iraq and Turkey (19).

Initial symptoms of malaria are flu-like including high fever, headache and muscle pain. High fever associated with malaria subsides and reoccurs about every 48 hours. Other complications linked to malaria are renal failure, pulmonary edema, cardiovascular impairment and anemia (22).

The Parasitic Agent of Malaria

The causative agent of malaria is the protozoan parasite *Plasmodium*. The *Plasmodium* species are apicomplexans. Common to the apicomplexan phylum are apical organelles that reside in the anterior end of the organism’s invasive form (16). These proteins form an apical complex which is involved in the invasion of host cells (16). There are four parasitic species that cause malaria in humans: *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium falciparum* with *P. falciparum* and *P. vivax* being the more widely distributed and encountered species. *P. falciparum*, the most lethal of the species, is responsible for approximately 95% of malaria related deaths (11). Virulence factors that contribute to the potency of *P. falciparum* infection include the ability of infected red blood cells to adhere to
endothelial cells causing agglutination of non-infected red blood cells in blood vessels thus disrupting blood flow (rosetting) (17). In vitro tests have demonstrated that TNFα, a pro-inflammatory cytokine, may have a role in the pathogenicity of *P. falciparum*. In vitro studies have also shown that TNFα has the ability to modulate ICAM-1, an endothelial cell involved in rosetting (17). Patients infected with malaria exhibit raised TNFα concentrations (17). Antigenic variation is a common evasion mechanism and has been demonstrated in *P. falciparum* (2). Studies report that the antigenic variation of *P. falciparum* erythrocyte membrane protein 1 (mbp1) resulted in different expression patterns on the surfaces of infected red blood cells (2). Exponential increase in parasitemia from schizont to merozoite and the transitory extracellular exposure of merozoites are likely to aid in evasion from host immune cells in most *Plasmodium* species.

**The Non-Canonical Life Cycle of Plasmodium**

The *Plasmodium* life cycle is atypical and complex. It has adapted to survive in vertebrate and invertebrate hosts (16). In mosquitoes *Plasmodium* demonstrates a sexual life cycle in the midgut of the *Anopheles* female vector. However, upon invasion of the host it arrests cell cycle until it has successfully penetrated host hepatocytes where the parasite undergoes asexual replication, egression of hepatocytes and invasion of new red blood cells (RBCs) (14).

On average 15 parasitic sporozoites are transferred from mosquito saliva to a human host (18). Upon entry into the blood stream they travel to the liver and invade hepatocytes where they undergo exo-erythrocytic schizogony forming multiple merozoites, which have the ability to
invade erythrocytes. Once inside the erythrocyte the parasite enters a unique asexual, intra-erythrocytic life cycle. The first phase of the asexual life cycle is referred to as the ring stage. Ring stage parasites take up a minimal volume of the red blood cell. They feed on hemoglobin and increase in size until they reach the trophozoite stage. Transition from ring to trophozoite is also marked by the development of a purple pigmentation, the result of hemozoin crystal formation from the toxic heme byproduct of hemoglobin metabolism. The trophozoite stage is followed by the schizont stage, which occupies almost the entire RBC. Most DNA replication occurs during this stage. Multiple rounds of DNA replication without cytokinesis result in a multinucleated schizont. The RBC eventually lyses releasing a variable number of segmenters (7). Segmenters that do not quickly re-infect new RBCs die or arrest their cycle for differentiation into gametocytes. Gametocytes represent a reproductive sexual stage. After ingestion by the female mosquito (male feed on plant nectar) gametocytes undergo gametogenesis in the mid-gut of the insect. Gametogenesis involves the fusion of 2 gametocytes to a zygote, which differentiates into an ookinet. The ookinete crosses the peritrophic membrane and midgut epithelium to become immobilized at the epithelium basal lamina where it develops into an oocyst (14). From the oocyst thousands of sporozoites are formed (14) which travel to the salivary glands to await re-infection. This complex life cycle can be visualized in figure 1. 
Erythrocytic-shizogony has no clear correlation to the traditional cell cycle progression demonstrated by eukaryotes (7). Traditionally cells progress through four cell cycle phases: G1, S, G2, and M. Gap phases (G1 and G2) serve as checkpoints for progression into DNA synthesis (S) and mitosis/meiosis (M). Entry into the S phase from G1 checks that all DNA has been replicated, that damaged DNA has been repaired, and that environmental conditions favor synthesis (20). Once the synthesis has been initiated it will go to completion (20). Entry into M from G2 checks that replication has been completed, damaged DNA has been repaired and that the cell is large enough to accommodate cell division. A mitotic checkpoint ensures sister chromatids attach to spindles on opposite polar ends. If checkpoint requirements are not met the cell cycle is arrested to ensure fidelity of genetic transfer (20). Interestingly, during the trophozoite and schizont stages *Plasmodium* exhibits multiple rounds of synthesis and mitosis without gap phases.

Although the intra-erythrocytic asexual life cycle of *P. falciparum* has no clear correlation to the typical life cycle phases of eukaryotes, *P. falciparum* homologues to cell cycle
regulators, cyclin dependent kinases (CDKs), have been identified as well as their regulating subunits, cyclins (6). This evidence suggests that though the cell cycle progression diverges in *Plasmodium*, they may undergo similar regulating mechanisms observed in traditional eukaryotes such as regulation by CDKs and cyclins. Understanding these mechanisms would help elucidate the signaling pathways involved in *Plasmodium*

**Cell Cycle Regulation**

Signaling pathways are regulated and propagated by various protein kinases and phosphatases. There are seven clusters of protein kinases associated with eukaryotes: 1. CK1 (casein kinases) 2. CMGC (cyclin dependent kinases), MAPK (mitogen activated protein kinase), GSK3 (glycogen synthase kinase), CLKs (CDK-like) 3. TKL (tyrosine kinase-like) 4. AGC (PKA, PKG and PKC kinases) 5. CAMK (calcium/calmodulin dependent kinases) 6. STE (MAPK regulators) 7. TyrK (tyrosine kinases) (5). The *Plasmodium* kinome however lacks TyrK and STE families. In fact it has been determined that the *P. falciparum* genome contains only 65 protein kinases (21), in comparison to the human genome which encodes 500 protein kinase genes.

Cell cycle signaling pathways in eukaryotes are regulated by CDKs. The regulating unit of CDKs, referred to as cyclins, determine CDK activity. CDKs exhibit steady state expression while cyclins are degraded and synthesized in a cyclical manner. In typical eukaryotes CDK activity is low during the G1 phase due to inhibition of cyclin synthesis and high levels of cyclin degradation (20). During the transition from G1 to S, cyclin synthesis is induced and cyclin degradation is inhibited facilitating an increase in CDK activity (20). CDK activity remains high
during S, G2 and M phases (20). Following the completion of mitosis, Cdc20 (cell division cycle protein 20) and Cdh1 (APC activating protein) (members of the anaphase-promoting complex APC) label cyclins for degradation which reduces CDK activity and brings the cell cycle back to the G1 phase (20). CDKs show preference for cyclin binding partners (6). Thus the appropriate CDK can be activated in its respective cell cycle phase. Generally CDKs also require ATP binding and activation by cyclin activating kinases (CAKs) in addition to cyclin binding (5).

PfCDKs exhibit 40-60% homology with eukaryotic kinase domains of CDKs (6) suggesting similar functions. There are however large extensions or insertions in these conserved sequences, which may be indicative of unique regulatory features.

**Plasmodium falciparum Protein Kinase 5**

PfPK5 is a proline-directed Ser/Thr CDK-like kinase that was isolated using sequences of mammalian CDK2 and CDK1 (14). PfPK5 has been shown to have several sequence characteristics and functionalities that suggest similar roles and regulation to CDKs. PfPK5 is the closest homologue to metazoan CDKs demonstrating roughly 58% identity with CDK2 (11). PfPK5 shows strong structural similarities with CDK2 especially in the cyclin binding domain. CDK2 contains a PSTAIRE sequence involved in cyclin binding and *in silico* studies show that PfPK5 has a conserved PSTAIRE-like sequence (PSTTIRE) (6). Homology between amino acid sequences in the catalytic ATP binding domain in eukaryotic CDKs and PfPK5 has also been identified (11). PfPK5 also demonstrates 60% sequence homology with CDK1 and CDK5 (6) though it is structurally more similar to CDK2.
In addition to sequence similarity, PfPK5 shows similar activation and inhibition activity with CDKs. Activation of CDKs requires phosphorylation of a conserved threonine residue. In mammals this residue is Thr-169 (7). The analogous residue of the Thr-169 residue in PfPK5 is Thr-158 (9). It was demonstrated that mutagenesis of Thr-158 to glutamic acid (mimics phosphorylated Thr) enhanced PfPK5 activity while replacement with valine (cannot be phosphorylated) prevented PfPK5 from exhibiting activity. PfPK5 was also shown to respond to CDK 2 inhibitors (11).

While PfPK5 shares several characteristics with its CDK homologues, it also demonstrates many unique characteristics. PfPK5 shows promiscuity in cyclin binding partners and has also been shown to be activated by non-cyclin vertebrate activators such as RINGO from Xenopus (12). Activation by proteins such as RINGO, which share no sequence homology to cyclins, suggests that the P. falciparum genome may code for cryptic cell cycle regulators which cannot be identified based solely on database searches (14). Not only does PfPK5 have a promiscuous nature but it demonstrates low-level phosphorylation in the absence of cyclins, though cyclins significantly enhance activity (12). Therefore cyclins may not have as great a role in the regulation of PfPK5. Determination of regulatory mechanisms involved in the PfPK5 signaling pathway has therefore provoked much interest. The differences observed in CDKs and PfPK5 suggest that proteins unique to the parasite associate with and regulate PfPK5. If identified, these proteins could serve as novel drug targets.
Characterization of PfPK5

The expression profile of Cdc2, a yeast CDK homologue of PfPK5, and its cyclin-like regulator, Cdc13, show similar cyclical expression both demonstrating peak expression in the M phase of the cell cycle (8). These are the expected results for a CDK and its cyclin subunit; expression of the cyclin will oscillate in accordance with cell cycle progression and because CDK activity is dependent upon cyclin binding, CDK expression should mirror cyclin expression. To determine if PfPK5 exhibits this type of expression profile, stage-specific western blots were performed against PfPK5 and PfCyc3. The data gathered from this experiment will determine if PfPK5 and PfCyc3 are possible interacting partners. This will also indicate which stage exhibits the highest PfPK5 expression, suggesting when the kinase is active and proposing the cell cycle phase PfPK5 is involved in regulating.

Immunofluorescence assays were also conducted to determine if PfPK5 demonstrates nuclear localization as would be required if PfPK5 is involved in cell cycle regulation. The assays will also be used to determine if PfPK5 co-localizes with PfCyclins1-3. Proteins involved in the same pathway often exhibit co-localization, which aids in substrate channeling and efficiency. Co-localization would support PfPK5 interaction with PfCyclins.

Identification of the entire complement of positive and negative PfPK5 regulators and each of their functions is necessary in order to understand the Plasmodium life cycle (6). To identify possible interacting partners a PfPK5 immunoprecipitation was conducted.
An HA-tagged (hemagglutinin) PfPK5 construct was created for *Plasmodium* transfection for use in further characterization. An HA-tag immunoprecipitation will validate the data generated by the PfPK5 immunoprecipitation.

Here we have further characterized PfPK5’s role in cell cycle regulation through immunofluorescence assays and determination of its expression profile. Furthermore, we have identified potential interacting partners of PfPK5 by performing an immunoprecipitation. The generation of a PfPK5-HA construct will also prove useful in future work aimed at uncovering the role PfPK5 plays in cell cycle regulation, as well as identifying previously unidentified PfPK5 interacting partners and validating preliminary data gathered here.
Methods

Culturing *Plasmodium falciparum*

Cultures were maintained in RPMI 1640 media supplemented with 5% albumax at a 4% hematocrit in a 5% CO₂ humidified incubator at 37°C. Fresh media was added daily. Cultures were split as needed to maintain 3-5% parasitemia (as determined my geimsa staining and microscopy). For use in experiments, cultures were brought up to 10-20% parasitemia before being harvested (see below for harvesting protocol).

**Synchronization of Malarial Culture**

Cultures were resuspended and centrifuged at 2000rpm, room temperature, for 5min. The pellet was resuspended in 5X the pellet volume of 5% sorbitol. The culture was incubated with sorbitol for 15min at 37°C. The culture was centrifuged as before and the pellet was washed twice in 25mL RPMI 1640. The pellet was resuspended in its original volume of culture and plated back in a non-treated 100cm by 20cm culture dish.

**Immunofluorescence Assays**

Asynchronous malarial culture (*P. falciparum 3D7*) was resuspended and centrifuged at 1500rpm, 4°C for 3min (2ml/IFA). The pellet was resuspended in PBS (1ml/1ml culture) and centrifuged at the same conditions as before. The pellet was resuspended in fixing solution (4% glutaraldehyde, .0075% paraformaldehyde) and incubated at 37°C for 30min. After incubation the cells were centrifuged at 1850rpm, 4°C for 3min and the pellet rinsed in PBS. Cells were
made permeable by treatment with 0.1% triton-X100 (1ml/1ml culture) for 10min at room temperature. Cells were centrifuged as before. The pellet was resuspended in 125mM glycine and incubated for 10min at room temperature to reduce remaining aldehydes. Then the cells were centrifuged again as before.

The pellet was then resuspended in 3% BSA for blocking at room temperature for 1 hour. Cells were centrifuged as before and resuspended in PBS. 1ml per IFA was aliquoted into a microcentrifuge tube. Primary antibodies were added and incubated at 4°C overnight with the following dilutions: rabbit αPfPK5 1:2000, chicken αPfCyc1-3 1:5000, goat αPfPK5 1:1000. Cells were centrifuged at 2000rpm, 4°C for 2 min. The pellet was rinsed 3 times in PBS for 5min and centrifuged as before following each wash. Secondary antibody was added and incubated for two hours at room temperature with the following dilutions: αrabbit-AlexaFlur™555 1:1000, αchicken-AlexaFlur™488 1:1000, αgoat-AlexaFlur™488 1:1000. Cells were centrifuged as before. Again the pellet was washed 3 times with PBS as before. Cells were centrifuged as before and 4μl were added to prepared cover slips (see below) and incubated for 30min at room temperature. Fixed cover slips were mounted on a microscope slide over 2μl of mounting solution.

**Preparation of Cover Slips**

Cover slips were rinsed in 70% ethanol followed by a PBS rinse. 200μl of poly-L lysine were added to the cover slip and incubated for 30min. The poly-L lysine was then removed and 200μl PBS was added.
**Western Blots**

*Time Points*

*P. falciparum* 3D7 culture was resuspended and treated with 0.05% saponin. Cells were centrifuged at 4000rpm, 4°C for 10min. The pellet was rinsed twice in PBS and centrifuged as before. Parasite lysate was obtained from the pellet by treatment with 8M urea. Cell cultures were harvested every 8 hours for 48 hours. 100μg of each time point was run on an SDS-PAGE gel and transferred to a PVDF membrane by semi-dry transfer at 15V for 45min. The membrane was blocked in 5% milk-PBS-t for 45 min. Primary antibody was added and incubated overnight at 4°C with the following dilutions: rabbit αPfPK5 1:5000, chicken αPfcyc3- 1:2500. The membrane was washed in 5% milk-PBS-t 3 times for 10min. Secondary antibody was then added and incubated at room temperature for 2 hours at the following dilutions: αrabbit-HRP 1:10000, αchicken-HRP 1:10000. Following incubation the membrane was washed 3 times in PBS-t and once in PBS. Finally, the membrane was developed by chemiluminescence using the Immun-Star Western Kit from Biorad.

**PfPK5-HA Expression**

PfPK5-HA and *P. falciparum* 3D7 parasite pellets were obtained as above. Parasite lysate was obtained by treatment with M-per cell lysis buffer (1μl lysis buffer/1mg pellet incubated on ice for 30min while vortexing every 5min, centrifuged down and the supernatant collected). 100μg of each lysate was run on an SDS-PAGE gel and transferred overnight at 10V. Incubations and washes were performed the same as above (see time points). Primary antibody
dilutions were as follows: goat αHA 1:1000, rabbit αPfPK5 1:1000. Secondary antibody
dilutions were as follows: donkey αgoat-HRP 1:2000, αrabbit-HRP 1:2000.

**Anti-PfPK5 Immunoprecipitation**

20μl of control resin and 20μl of A/G plus resin were added to separate spin columns and
centrifuged at 1000rpm, 4°C for 1min and the flow-through discarded. Columns were washed
with coupling buffer. 10μg of anti-PfPK5 antibodies were diluted with dH₂O and coupling
buffer and added to each column for a 1 hour incubation at room temperature. Columns were
centrifuged as before and washed twice with coupling buffer. Columns were then incubated
with coupling buffer, 2.5mM DSS and dH₂O for 1 hour to crosslink antibody to beads. The
columns were washed twice with elution buffer and twice with immunoprecipitation lysis/wash
buffer. 1mg of *P. falciparum* 3D7 lysate was added to the control beads and the anti-PfPK5
crosslinked beads and incubated at 4°C overnight. Columns were centrifuged as before and
washed 3 times with immunoprecipitation lysis/wash buffer and once with conditioning buffer.
The beads were then resuspended in gel loading buffer and prepared for SDS-PAGE analysis and
silver staining.

*Silver Staining*

The SDS gel was rinsed briefly with ultrapure water and fixed overnight in fixative
solution (40% ethanol, 10% acetic acid). Fixative solution was decanted and the gel was washed
in 30% ethanol for 10min. Ethanol was decanted and the gel was incubated for 10 min in
sensitizing solution (0.02% sodium thiosulfate). Sensitizing solution was decanted and the gel
was washed in 30% ethanol as before. The gel was then rinsed in ultrapure water for 10min.
The gel was stained by a 15min incubation in staining solution (0.1% silver nitrate). Stainer was decanted and the gel was rinsed for 30 sec in ultrapure water. The gel was developed by incubation in the developer solution (2% sodium carbonate, 0.04% formalin) for ~25min (until bands of desired intensity are observed). 10ml of stopper solution (5% glacial acetic acid) was added to gel in developing solution and incubated 10min to stop developing. Developer and stopper were decanted and gel was rinsed in ultrapure water for 10min.

**PfPK5-HA Cloning**

PfPK5-HA, flanked by XhoI and AvrII cut sites, was generated via reverse transcriptase PCR. PCR settings were as follows: 42°C-30”; 95°C-30”; [95°C-30”; 46°C-30”; 68°C-6”]X5; [95°C-30”; 52°C-30”; 68°C-6”]X35; 68°C. The PCR product was purified (Promega PCR Clean-Up System) and ligated into pGEMT easy vector (Promega pGEMT Easy Vector System) and transformed into E. coli XL10 Gold cells. Accuracy of the nucleic acid sequence was confirmed by dideoxy sequencing. PfPK5-HA-pGEMT was digested with XhoI and AvrII overnight at 37°C. The digest was heat inactivated at 80°C and run on an agarose gel. PfPK5-HA (insert) was extracted from the gel. pDC2-1600-MAHRP was digested in the same manner, treated with phosphatase for 20 min at 65°C, run on an agarose gel and the pDC2-1600 (vector backbone) was extracted. The insert and vector were ligated with quick ligase and transformed into E. coli XL10 ultra-competent cells. These clones were also sequenced confirmed by dideoxy sequencing as well.
**Plasmodium Transfection**

2.5 mL of ring-stage *P. falciparum* integrase culture was resuspended and centrifuged at 1500rpm, 25°C for 3min. The pellet was resuspended and washed with cytomix (1ml/1ml culture) and centrifuged as before. The pellet was resuspended in 300μl cytomix. 100ug of PfPK5-HA and 25ug of pINT in cytomix were added to the resuspended pellet. The cells were iced for 30sec and electroporated. The transfection was plated in a 6 well plate with 5ml RPMI 1640 media and 280μl of blood. After 4 hours the transfection was treated with drug media (RPMI 1640/G-418/Blasticidin/WR 99210).

**Genomic DNA Extraction of PfPK5-HA**

Parasite pellet was obtained as described under Western Blot. Genomic DNA extraction was performed on the pellet. The pellet was first washed in 5mL cell wash buffer (50mM Tris pH7.5, 100mM NaCl), centrifuged and the supernatant discarded. The pellet was resuspended in 1mL cell wash buffer. 250mM EDTA, 250μl 10% SDS and 25μl RNase (10mg/mL) were added and incubated at 37°C for 1 hour. 250μl of 10mg/mL proteinase K was added and incubated at 60°C for 2 hours. After incubation, an equal volume of phenol-chloroform-isoamyl alcohol was added to the lysate and centrifuged at 7000g, 10 min, at room temperature. The aqueous layer was removed and kept. An equal volume of chloroform-isoamyl alcohol was added, incubated at room temperature for 5min and centrifuged as before. The aqueous layer was again removed and kept. The aqueous phase volume was diluted with 10mL TE buffer followed by addition of 1mL 3M sodium acetate and 22mL of 100% ethanol. Genomic DNA was precipitated, removed and
washed in 5mL 70% ethanol. The ethanol was removed and the pellet air dried for 45min. 200μl of TE buffer was added to the pellet and then stored overnight at 4°C.

**PCR Screen of PfPK5-HA Transfection**

3 PCR reactions were set up with digested PfPK5-HA genomic DNA (Nco1 at 37°C for 4 hours) as template DNA and the following primers: 1) F:5’ of CG6 gene and R:3’BSD gene 2) F:5’ of PfPK5 gene and R:3’ of CG6 gene 3) F:5’ of CG6 gene and R:3’CG6 gene. PCR conditions were as follows: 95°C-2min’; [95°C-30”; 45°C-30”; 72°C-6min]X5; [95°C 30”; 50°C-30”; 72°C-6min]X35; 72°C 10min. PCR products were run on an agarose gel for visualization.

**Southern Blot of PfPK5-HA Transfection**

*Generation of membrane*

PfPK5-HA genomic DNA (same as used in the PCR Screen) and 3D7 genomic DNA (obtained as above) was digested at 37°C overnight with XmaI and EcoRV. Undigested and digested samples were run on an agarose gel. The gel was rinsed in denaturing solution for 15min twice. The gel was then rinsed briefly in water and then in neutralizing solution for 45min. A nitrocellulose membrane was wet with water and then 20X SSC. Next the gel was transferred to the membrane overnight via capillary action. Lanes on the membrane were marked with a soft lead pencil. The membrane was then soaked in 5X SSC for 5min and placed on a wet paper towel for UV crosslinking to immobilize DNA. The membrane was dried at room temperature between two Whatman 3mm papers.

*Pre-hybridization*
The membrane was placed in a hybridization bottle, DNA side up. 20mL of pre-hybridization buffer (10% NaPPi, 10%SDS, 100XDenhalt’s Solution, 20XSSC) and 200μl of 1mg/mL sonicated salmon sperm DNA were added to the membrane and incubated in the hybridization oven at 60°C for 2 hours.

Radioactive label of probe

(Strategene Prime-it II kit): 25ng of template DNA (PfPK5 sequence being labeled), 10μl of random oligonucleotide primers and water were added up to 34μl in a microcentrifuge tube. The reaction was boiled for 5min and briefly centrifuged at room temperature. 10μl 5X dATP primer buffer, 5μl of labeled nucleotide ([α-32P]dATP at 3000Ci/mmol), and 1μl Exo(-) Klenow enzymes were added to the reaction tube and incubated at 37°C for 10min. 2μl of stop mix were added to reaction tube following the 10min incubation.

Hybridization

The denatured probe was added to membrane/pre-hybridization solution and incubated overnight at 60°C in the hybridization oven. The membrane was then washed in 50mL wash buffer (2X SSC, 10%SDS, 10%NaPPi and water) for 5min in the hybridization oven. This solution was decanted and the wash was repeated. The membrane was next washed in 60mL wash buffer 2 (0.2X SSC, 10%SDS, 10%NaPPi and water) for 15min in the hybridization oven. This solution was decanted and the wash repeated twice. Bands on the membrane were finally visualized with autoradiography at -80°C for 2 days.
Results

PfPK5 Demonstrates Nuclear Localization and Co-localization with PfCyclins

A protein’s role in cellular biology dictates its subcellular localization. Proteins involved in nuclear processes thus localize to the nucleus. To determine if PfPK5 localizes to the nucleus, supporting its suggested role in cell cycle regulation, immunofluorescence assays (IFAs) have been performed. IFAs use fluorophore-tagged antibodies and confocal microscopy to determine a protein’s localization. Here anti-histone antibody was used as a nuclear marker. First, infected red blood cells are fixed with formaldehyde to preserve the parasite’s intra-erythrocytic location as well as endogenous localization of parasitic proteins. The erythrocyte and parasite membranes were made permeable by treatment with triton-X100 so that antibodies would have access to parasitic proteins. After incubation with primary antibodies (αPfPK5 and αhistone) the cells were washed and incubated with the appropriate fluorophore-tagged antibodies. The cells were washed again and fixed to microscope slides for visualization via confocal microscopy. Results in figure 2 show PfPK5 antibodies co-localize with histone antibodies.
Figure 2 - PfPK5 vs histone IFA results show nuclear localization of PfPK5. PfPK5 is visualized in green and histone in red. Overlay images merge PfPK5 and histone images and superimpose them on DIC images of the cell. Yellow spots indicate overlap of red (histone) and green (PfPK5). DIC: differential interference contrast.

To determine if PfPk5 demonstrates co-localization with *P. falciparum* cyclins, thus indicating potential cyclin-mediated regulation of PfPK5, additional IFAs were performed using antibodies against identified PfCyclin homologues. Results in figures 3-5 show that PfPK5 co-localizes with PfCyclin1 (fig 3), PfCyclin2 (fig 4) and especially with PfCyclin3 (fig 5). PfCyc1 and PfCyc2 show partial recruitment to PfPK5 but both also localize in the cytoplasm where PfPK5 is absent. PfCyc3 however shows tight co-localization throughout the intra-erythrocytic stages.
Figure 3 - PfPK5 vs PfCyclin1 IFA results show partial recruitment of PfCyclin1 (green) by PfPK5 (red). Merged images represent localization of both proteins with yellow representing overlap of green (PfCyclin1) and red (PfPK5). Overlay images superimpose merged images on DIC images. DIC: differential interference contrast.
Figure 4 - PfPK5 vs PfCyc2 IFA results show partial recruitment of PfCyc2 (green) by PfPK5 (red). Merged images represent localization of both proteins with yellow representing overlap of green (PfCyclin1) and red (PfPK5). Overlay images overlay merged images on DIC images. DIC: differential interference contrast.
Figure 5 - PfPK5 vs PfCyc3 IFA results show recruitment of PfCyc3 (green) by PfPK5 (red) throughout the intra-erythrocytic stages with top row representing early infection (late ring/early trophozoite) and the last row representing late infection (late trophozoite/early schizont). Merged images represent localization of both proteins with yellow representing the overlap of green (PfCyc3) and red (PfPK5). Overlay images superimpose merged images on DIC images. DIC: differential interference contrast.

**Expression Profile of PfPK5 and PfCyc3 Indicate Peak Expression during Late Schizont Stages**

To determine when PfPK5 is most actively expressed, stage-specific western blots were performed. Stage-specific western blots were also generated against PfCyc3 since localization studies and previous data suggested possible interactions between PfPK5 and PfCyc3. Culture was harvested every 8 hours throughout the parasite’s 48 hour life cycle. The cell-free extracts were resolved by SDS-PAGE gel electrophoresis. PfPK5 migrates through the gel as expected as a 33kDa protein. However PfCyc3, a 27kDa protein, migrates through the SDS gel as a
52kDa protein. Results in figures 6-8 show cyclical expression of PfPK5 and PfCyc3. Both also demonstrate a trend of increasing levels of expression from early infection to late infection.

PfPK5 and PfCyc3 expression peaks in the late schizont stage. Densitometry (fig 8A-8B) confirms the qualitative trend of increasing cyclical expression demonstrated by band intensity in the western blots.

Figure 6 - Stage-specific anti-PfPK5 western blot. The band at 33kDa is PfPK5. Segmenter/Early Ring: re-infecting parasites and up to 8 hours post infection. Late ring: up to 16 hours post infection. Early trophozoite: up to 24 post infection. Late trophozoite: up to 32 hours post infection. Early schizont: up to 40 hours post infection. Late schizont: up to 48 hours post infection.

Figure 7 - Stage-specific anti-PfCyc3 western blot demonstrates PfCyc3 expression profile. The band at 52kD is PfCyc3. Stages are represented as in PfPK5 western in fig 6.
Figure 8 - Figure 8A and 8B. Densitometry of PfPK5 and PfCyclin3 stage-specific expression quantitatively show a trend of increasing expression from early infection to late infection. PfPK5 and PfCyc3 expression peaks during the late schizont stage. Stages are represented as in figures 6 and 7.

**Anti-PfPK5 Immunoprecipitation Two Possible PfPK5 Interacting Partners**

In order to pull-down possible PfPK5 interacting partners, anti-PfPK5 antibodies were crosslinked to agarose beads through their interactions between Protein A/G which is immobilized on the resin. Asynchronous lysate was incubated with the crosslinked resin overnight and the beads were washed, resuspended in gel loading buffer, centrifuged down and the supernatant run on an SDS-PAGE gel. Control resin lacked the A/G protein and was thus not able to crosslink with antibodies. Control resin was also incubated with asynchronous parasite lysate, washed and loaded on the gel. The SDS gel was visualized by silver staining as can be seen in figure 9. Bands at 150kDa, 100kDa, 70kDa and approximately 30kDa represent possible
PfPK5 interacting partners. Bands visualized at 50kDa and 25kDa may be attributed to the heavy and light chains of the antibodies. Mass spectrometry will be used to identify these proteins and further characterization of the putative interacting partners will determine the type of interaction, if any, the proteins share with PfPK5.

Figure 9 - Visualization of SDS gel after silver staining reveals possible PfPk5 interacting partners. Control: Agarose beads lacking A/G protein conjugation.
FT: Flow-through after primary antibody incubation
Red arrow: Bands at 150kDa, 100kDa, 70kDa and ~30kDa represent potential interacting partner of PfPK5

**Generation of PfPK5-HA Construct**

Reverse transcriptase PCR was used to generate an HA-tagged PfPK5 with flanking AvrII and Xho1 cut sites from a *P. falciparum* mRNA library. PfPK5 is 867bp. The HA tag and the enzyme cut sites make recombinant PfPK5 a 906bp gene. Figure 10 shows the agarose gel of
the unpurified PCR product with a strong band at approximately 900bp. The recombinant PfPK5-HA gene was ligated into a pGEMT Easy vector (3015bp) and transformed into *E.coli* XL10 Ultra-competent cells. Ligation colonies were miniprepped and screened by digestion with XhoI and AvrII. The digested DNA was run on an agarose gel revealing bands at approximately 900bp and 3000bp as seen in figure 11. The clones were also confirmed by dideoxy sequencing.

To facilitate ligation of PfPK5-HA into a pDC2-1600-GFP-MAHRP (8349bp) construct for malarial transfection the construct was digested with AvrII and XhoI which removed the GFP-MAHRP cassette. The digest was run on an agarose gel (fig 12B) and the pDC2-1600 (6822bp) vector backbone extracted. The PfPK5-HA-pGEMT was also digested with AvrII and XhoI, run on an agarose gel (fig 12A) and the PfPK5-HA insert was extracted. The gel extracts were purified and ligated with quick ligase. Colonies were cloned into *E. coli* XL10-gold ultra-competent cells and screened via an XhoI and AvrII double digest. Screening results can be seen in figure 13. Two clones show bands at 900bp and 7000bp. These bands were sequence confirmed.
Figure 14 - Agarose gel of double digest of PfPK5-HA-pGEMT with AvrII and Xho1 yields GFP-MAHRP and pDC2-1600 at 7000bp
PfPK5-HA Transfection Shows Expression of the HA

The transfection efficiency of *P. falciparum* is extremely low. Episomal transfections are difficult to propagate through the entire life cycle because they can be lost during mitotic segregation (15). In order to overcome this deficiency, a transfection method mediated by *Mycobacterium Bxb1* integrase was developed. This integration method will facilitate homologous crossover of the gene with a specific site on the parasitic chromosome. The crossover event occurs between an attP site on the vector and an attB site on the chromosome and is performed by mycobacteriophage *Bxb1* integrase. *P. falciparum* cell lines were generated with the attB crossover sites (15). The attB vector that carries the gene of interest contains a blasticidin (BSD) marker for selection. The vector that expresses the integrase enzyme (pINT vector) has a neomycin marker and is transiently co-transfected with the integrase construct. The co-transfection allows for larger gene sequences to be inserted into the integrase vector and allows for more efficient recombination (15). Once the chromosomal crossover has occurred the parasite will no longer need antibiotic treatment as the unneeded vector will be lost.

Following the transfection of synchronized malarial culture western blotting was used to determine if PfPK5-HA was being expressed. Western blotting against PfPK5 shows that PfPK5 (33kDa) has higher expression in the transfected culture as opposed to the untransfected malarial culture (fig 14). Western blotting against the HA tag shows that HA is only present in the transfected culture (fig 15). The HA band is visualized at approximately 33kDa as well.
Figure 15 - Anti-PfPK5 western blot shows that PfPK5 (red arrow at 33kDa) is more highly expressed in the PfPK5-HA transfected line (PfPK5). 3D7: untransfected *P. falciparum* culture.
Figure 16 - Anti-HA western blot shows HA tag expression at ~33kDa (red arrow) only in the PfPK-HA transfected culture (PfPK5). 3D7: untransfected *P. falciparum* culture

**PCR Screening Demonstrates PfPK5-HA Was Not Integrated into the Parasite CG6 Locus**

A genomic DNA extraction was used to isolate nuclear DNA from the PfPK5-HA transfected line to screen for integration via PCR. PfPK5-HA-pDC2-1600 integration would disrupt the CG6 gene of *P. falciparum* 3D7, a nonessential gene. Three PCR reactions were set up. One reaction used a forward primer that recognizes the parasite chromosome (5’ end of the CG6 gene) and a reverse primer that recognizes the construct (3’ end of the BSD gene used as a selective marker). Another reaction uses a forward primer that recognizes the construct (5’ end of the PfPK5 gene) and a reverse primer that recognizes the parasite (3’ end of the CG6 gene). The last reaction was set up using the 5’ and 3’ end of the CG6 gene (chromosomal recognition). The unpurified PCR products were run on an agarose gel as can be seen in figure 16. The
reactions that used primers against the construct and the parasite chromosome show no bands while the reaction that used chromosomal primers show a small band around 300bp.

**PfPK5-HA Does Not Co-localize with Endogenous PfPK5**

IFAs against endogenous PfPK5 and PfPK5-HA were performed to determine if the expressed PfPK5-HA was localizing with endogenous PfPK5. Figure 17 demonstrates that PfPK5-HA localizes to the cytosol and does not exhibit the nuclear localization of endogenous PfPK5.

![Figure 17 Agarose results from screening of PfPK5-HA-pDC2-1600 chromosomal integration by PCR.](image)

Lane 1: Primers 1 and 2
Lane 2: Primers 3 and 4
Lane 3: Primers 1 and 4

- Primer 1: 5' of CG6 gene (gene interrupted)
- Primer 2: 3' of BSD gene
- Primer 3: 5' of PfPK5 gene
- Primer 4: 3' of CG6 gene

![Figure 18 - PfPK5-HA vs PfPK5 IFA results show that recombinant PfPK5 (green) did not co-localize with endogenous PfPK5 (red). The nucleus is stained blue with DAPI. PfPK5-HA localization is shown in green and PfPK5 in red.](image)
Discussion

PfPK5 demonstrates predominantly nuclear localization (fig 2). This data reinforces PfPK5’s suggested role in nuclear processes. PfPK5 also showed co-localization with PfCyclins (fig 3-5). The tightest co-localization was observed between PfCyc3 and PfPK5 suggesting that PfCyc3 may interact with PfPK5. The expression profile of PfPK5 (fig 6- and 8A) demonstrates cyclical expression throughout the intra-erythrocytic life cycle of P. falciparum increasing from early infection to late infection. PfPK5 expression peaks during the late schizont stage which is the stage responsible for mitotic events, indicating that PfPK5 may be involved in this nuclear process. PfCyclin3 demonstrates the same cyclical expression pattern also showing peak expression levels during the schizont stage (fig 7 and 8B). Based on their corresponding expression patterns there may be a correlation between the up-regulation of PfCyc3 and PfPK5 during the schizont stage. This is seen in most CDK/cyclin relationships in which oscillating levels of cyclins activate steady-state levels of the CDK. Thus, co-localization and expression studies suggest that PfCyc3 may be involved in the regulation of the PfPK5 signaling pathway and that the PfPK5 signaling pathway may be involved in Plasmodium’s divergent mitotic processes. These results support our previous published work which shows that the RNA polymerase C-terminal domain phosphorylation by PfPK5 was significantly stimulated by recombinant PfCyc3 (Merckx et al, 2003, JBC).

PfCyc3 was visualized at 52kDa after migration through an SDS gel. Interestingly PfCyc3 is a 27kDa protein. This may be due to post-translational modification such as
ubiquitination. Cyclins are synthesized and degraded in a cyclical pattern that aids in the progression of the cell cycle, thus cyclins are commonly ubiquitinated.

The anti-PfPK5 immunoprecipitation revealed 4 bands in the PfPK5 elution upon analysis by SDS-PAGE and silver staining. Red arrows in figure 8 at 150kDa, 100kDa, 70kDa and ~30kDa represent potential PfPK5 interactors. Mass spectrometry will be used to identify these proteins. The potential of these proteins to serve as PfPK5 regulators or substrates will be deduced via bioinformatic analysis. Further localization and expression studies will determine if the indicated proteins are physically able to interact with PfPK5. In vitro kinase assays will validate if the putative interacting partners are substrates of PfPK5. Proteins that demonstrate interaction but do not show phosphorylation by PfPK5 may be regulators of the PfPK5 pathway. There are also distinct bands at ~50kDa and ~25kDa. These are the relative sizes of the heavy and light chains of antibodies and these bands may therefore be attributed to PfPK5 antibody that came loose from the resin when the beads were boiled (see methods). Determination of PfPK5 interacting partners will aid in illumination of the pathways responsible for Plasmodium’s untraditional cell cycle. Plasmodium proteins that associate with a crucial cell cycle regulator such as PfPK5 may prove to be novel drug targets. Further characterization of PfPK5’s interacting partners will also provide more insight into the structure PfPK5 assumes when binding to these proteins and therefore help guide rational drug design.

The recombinant PfPK5 gene is about 906bp. The PCR product in figure 10 resulted in a band around 900bp indicating successful generation of the HA-tagged PfPK5. After ligation into the pGEMT Easy vector digestion with AvrII and Xho1 (cut sites flanking recombinant PfPK5)
yields bands around 3000bp and 900bp. The pGEMT Easy vector is 3015bp and PfPK5-HA is 916bp so bands at 3000 and 900 indicate a successful ligation which was confirmed by DNA sequencing. Digestion of the PfPK5-HA-pGEMT construct was performed again to extract the PfPK5-HA insert for ligation into the pDC2-1600 vector (fig 12A). The vector was prepared by digesting pDC2-1600-GFP-MAHRP with XhoI and AvrII as well (fig 12B). The GFP-MAHRP cassette was left behind and the vector backbone extracted (~6822bp). Purified vector and insert extracts were ligated and clones screened again with XhoI and AvrII (fig 13). Lanes 3 and 4 show bands around 900bp and 7000bp that indicate the PfPK5-HA gene and the pDC2-1600 vector respectively. The other lanes represent clones that did not take up a successfully ligated construct. The construct was used for transfection after sequence confirmation by dideoxy sequencing.

Western blotting showed expression of the HA-tag in the transfected line only (fig 15). The HA tag is on the C-terminus of PfPK5 therefore expression of HA would require expression of PfPK5. It was also shown that PfPK5 was more highly expressed in the transfected line (fig 14) as would be expected with the introduction of a duplicate PfPK5 gene. Both the HA and PfPK5 bands are visualized at ~33kDa indicating that the H-tag was not cleaved from PfPK5 post-translationally or otherwise altered after expression. Western blotting thus confirms expression of PfPK5-HA.

However, PCR results show that while expressed, PfPK5-HA was not integrated into the parasite’s endogenous CG6 gene (fig 16). In the first PCR reaction (fig 16 lane 1) a forward primer that recognizes the chromosome (5’of CG6 gene) and a reverse primer that recognizes the
construct (3’ of BSD gene) were utilized. The absence of a band indicates that primers were not able to bind the same strand of DNA, an indication that integration at the CG6 site did not occur. The second PCR reaction (lane2) also used a primer recognizing the chromosome (3’ of CG6) and one recognizing the construct (5’ of PfPK5). Again elongation and amplification were not observed. The final PCR reaction (lane 3) contained the 3’ and 5’ end of the CG6 gene, which would be disrupted upon integration of the construct. This PCR reaction resulted in a small band around 300bp, indicative of amplification of the CG6 gene. Had the construct been integrated a much larger PCR product would have been visualized as the entire construct (~8kb) is integrated into the CG6 loci of the chromosome. In addition to the absence of PCR products, IFAs also show that PfPK5-HA does not co-localize with endogenous PfPK5 (fig 17). Since the recombinant PfPK5 was expressed it can be suggested that the transfection was not integrated, an episomal transfection occurred, or that it was integrated at a different site on the chromosome. Its localization to a subcellular compartment other than the nucleus suggests that if the gene was integrated part of the gene sequence was altered or lost. As a result the recombinant PfPK5 did not localize with its endogenous substrates and would therefore be unable to interact with them. A southern blot was also performed to confirm PCR results and determine if the recombinant PfPK5 was integrated elsewhere in the chromosome. This data has yet to be analyzed (being developed).

**Significance of Work**

The information gained from this experiment shows PfPK5 is nuclear and it can be predicted that the signaling pathway it propagates is likely involved in DNA replication or
division. Peak expression of PfPK5 during the late schizont stage hints at the later. PfCyc3 and PfPK5 coordinated expression levels and co-localization strongly suggests that they are interacting partners. Also several potential interacting partners have been isolated and upon identification and further characterization these proteins may prove useful in the illumination of *Plasmodium*’s enigmatic cell cycle events.

Although the first transfection did not show integration of the PfPK5-HA into the correct locus, generation of this recombinant gene will serve to confirm data gathered here and reduce false positives in future studies.

Knowledge gained here may aid in rational drug design. PfPK5 substrates or regulators may be specific to *Plasmodium*, an ideal characteristic of a potential drug target. Also, with the structure of PfPK5 determined and the *Plasmodium* genome completed, unmasking PfPK5 interacting partners will facilitate characterization of the interface between the interacting proteins. Targeting the substrate-PfPK5 interface would increase selectivity of putative drugs, a challenging aspect in the design of kinase-targeted drugs; a result of highly conserved kinase domains. In addition to identifying the PfPK5 signaling pathway as a potential drug target, further elucidation of the regulatory mechanisms responsible for *Plasmodium*’s atypical cell cycle phases will promote knowledge of the organism’s ability to thrive in human red blood cells. Ultimately a better understanding of these processes will lead to the generation of new anti-malarials and diminish the threat of this devastating disease.
Works Cited


