CHAPTER THREE

Molecular and Cellular Regulation of PfCDPK4
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3.1 INTRODUCTION

Malaria parasite *P. falciparum* is one of the deadliest parasites, which causes millions of deaths annually and recent emergence of variety of drug resistant strains has made it even more difficult to cope with this parasite. After more than a century of discovery of malaria parasite, we still need to understand the signaling mechanisms and molecular networks operating in the malaria parasite. Calcium, a widely known secondary messenger in eukaryotes, is known to play important role in protozoan parasite, *P. falciparum*. Various vital cellular processes in the parasite like egress of merozoite from erythrocyte (Moreno and Docampo, 2003) and invasion (Vaid et al., 2008), gliding motility of ookinete (Siden-Kiamos et al., 2006), circadian rhythms (Beraldo et al., 2005; Hotta et al., 2000), gametogenesis (Billker et al., 2004) are known to be regulated by calcium. Given the important role calcium plays in malaria life cycle, in depth understanding of signaling pathways regulated by calcium may unravel important events in the life cycle of the parasite.

Calcium interacts with proteins to mediate a wide array of signaling events. One of the common protein motif to which calcium binds is the EF hand motif. EF hand motif consists of a helix-loop-helix fold (*Fig 3.1*). Several parasite proteins have EF hands, which includes a ubiquitous protein Calmodulin (CaM). CaM has four EF hand motifs and each EF hand can bind calcium (*Fig 3.1*). In eukaryotes,
calcium mediates calmodulin dependent signaling by modulating Ca\(^{2+}\)/CaM dependent protein kinases (Swulius and Waxham, 2008).

**Fig 3.1** EF hand motif binds to Ca\(^{2+}\), Calmodulin is made up of four EF hand motifs

Typically, Ca\(^{2+}\)/CaM interacts with these enzymes to convert them from inactive to active state by a series of events. While malaria parasite does not seem to possess classical CaM kinases, it has a family of Calcium Dependent Protein Kinases (CDPKs) (Ward et al., 2004). CDPKs are major targets of calcium in plants and some protozoa, including *P. falciparum*. In plants, CDPKs are known to modulate cell division and differentiation, host symbiont interaction, hormone response, development of tolerance to stress stimuli and of defense responses to pathogens (Harmon et al., 2000; Romeis et al., 2001; Urao et al., 1994) in response to calcium signaling.

**Fig 3.2** The domain architecture of CDPKs. CDPKs possess a Calmodulin like Domain (CLD) which is connected by a Junction domain to the kinase domain.
Typically, in addition to N-terminal kinase domain, CDPKs have four contiguous EF hand motifs at their C-terminal forming a CaM like domain (CLD) (Harper and Harmon, 2005). The two domains are connected via a Junction Domain (JD) (Fig 3.2). The studies done on plant CDPKs suggest the following model for regulation of CDPKs by calcium; in the absence of calcium CDPKs are inactive and the J domain is proposed to interact with the kinase domain (Harper and Harmon, 2005). Upon increase in calcium levels, the CLD interacts with calcium which may result in an interaction with the C-terminal end of J domain (Harmon et al., 1994; Harper and Harmon, 2005). As a result the putative auto-inhibitory region in the N-terminus of the J domain is released from the catalytic domain and resulting in catalytic activation of CDPKs (Harper and Harmon, 2005). The crystal structure of the J-domain with CLD of *Arabidopsis thaliana* CPK-1 (AtCPK1) suggests that the C terminus of J domain forms a α-helix, which interacts with the C-lobe of CLD (Chandran et al., 2006) (Fig 3.3).

![Fig 3.3](image)

**Fig 3.3** Structural model for intramolecular J-CaM-LD of AtCPK1 based on the domain-swapped crystal structure, critical residue for interaction in α-helical J domain, F436 is shown by arrow (Chandran et al., 2006)
Bioinformatic analysis of *P. falciparum* genome suggests that at least six CDPKs may be present in the parasite (Anamika et al., 2005; Aravind et al., 2003; Ward et al., 2004). First five have similar domain architecture like plant CDPKs but in PfCDPK6 one EF hand motif is at the N-terminus of the kinase domain and incomplete Calmodulin like domain, possessing only three EF hand motifs. The role of several CDPKs has been studied in the parasite life cycle. PfCDPK1 has been implicated in phosphorylation of myo-A tail domain-interacting protein (MTIP) *in vitro* (Green et al., 2008; Kato et al., 2008), which is part of glideosome assembly protein (Jones et al., 2006), suggesting that PfCDPK1 may play role in role in invasion or egress related processes in the parasite. The N-terminus of PfCDPK1 has three motifs, a myristoylation motif, Glycine at second position; a cysteine at position 3, serves as a palmitoylation site and a polybasic cluster of amino acids, these all contribute to its membrane attachment (Moskes et al., 2004) and its localization on the parasitophorous vacuole and the tubovesicular system of the parasite (Moskes et al., 2004). PfCDPK3 is expressed in ookinetes (Ishino et al., 2006) and plays a role in the ookinete motility in mosquito midgut (Siden-Kiamos et al., 2006). *P. berghei*, rodent malaria parasite has homologues of all the PfCDPKs except PfCDPK2 (Hall et al., 2005b). In *P. berghei*, PbCDPK6 mutant parasites produced fewer salivary gland sporozoites and displayed enhanced migratory activity with significantly less infectivity for hepatocytes, suggesting that CDPK6 signaling is required for the switch from migration to invasion (Coppi et al., 2007).

*P. berghei* CDPK4 (PbCDPK4) is gametocyte specific protein, more abundant in male gametocytes, with only a small amount present in the female gametocytes.
(Khan et al., 2005). Reverse genetic studies on PbCDPK4 shows that its gene disruption causes severe defects in sexual reproduction and mosquito transmission in *P. berghei* (Billker et al., 2004). Activation by mosquito midgut factor Xanthurenic acid (XA) results in a rapid increase in intracellular Ca$^{2+}$ level in gametocyte, which is transduced by CDPK4 to control cell cycle progression of male gametocytes to S phase and mitosis. PbCDPK4 dependent pathway regulates male gametocyte specific events during differentiation, for instance genome replication and the cytoskeletal rearrangement associated with mitosis and assembly of axonomes that form the motile backbones of the microgametes. PbCDPK4 gene disruption resulted in stalled male gametocyte exflagellation (Billker et al., 2004). These studies highlight the important role of CDPK4 in *Plasmodium* biology and suggested that it may serve as a target for transmission-blocking drugs. The regulatory events involved in the activation of this enzyme have remained unknown. In this study, we have explored the molecular and cellular mechanisms involved in the regulation of PfCDPK4.

### 3.2 MATERIALS AND METHODS

All the reagents stock and working solutions were prepared in milliQ water. The solutions were autoclaved at 121°C (15 psi pressure) for 15 minutes. Most of the fine chemicals were purchased from Sigma (USA) unless stated otherwise. Anti-PfCDPK4 polyclonal antibody was raised in NZW rabbit, using KLH conjugated 15 amino acids synthetic peptide designed from C-terminal region of PfCDPK4 kinase domain. Phosphoinositides were purchased from Calbiochem (USA).
Synthetic peptides used for various studies were custom synthesized by Peptron (South Korea). Reagents and solution preparations have been indexed in List I.

3.2.1 *Plasmodium falciparum* culture

*Plasmodium falciparum* strain 3D7 (MR4, American Type Culture Collection) was used for all the experiments except where gametocyte rich culture was required. For generating gametocytes, 3D7A a variant of 3D7 was used. The parasite was cultured as described below:

3.2.1.1 Preparation of RBCs for culture

Human O⁺ or AB⁺ RBC was obtained from a donor and mixed with heparin (50 units/ml of blood) and centrifuged at 500 g for 10 min with minimum de-acceleration. The supernatant was removed carefully and the pelleted RBCs were washed 3 times with RPMI 1640 to remove serum and buffy coat. Equal amount of RPMI 1640 media was added to packed RBC volume to achieve 50% hematocrit and stored at 4°C till further use.

3.2.1.2 Cryopreservation of *Plasmodium falciparum* cultures

For cryopreservation of *P. falciparum* cultures, mostly ring stage parasites at a high parasitemia were obtained. The parasites were pelleted by centrifugation at 200 g for 5 min with minimum de-acceleration. To the pellet, 1.5 volume of the freezing solution (list I) was added drop-by-drop, while shaking the vial gently; the addition was completed in ~1 min. The medium was then transferred into a sterile cryovial, which was stored in the liquid nitrogen tank.
3.2.1.3 Revival of cryo-preserved *Plasmodium falciparum* cultures

A cryopreserved culture vial was obtained from the liquid nitrogen tank, and thawed quickly at 37°C in a water bath. To the vial, 0.1v of 12% NaCl was added slowly, dropwise, while shaking the tube gently. Subsequently, 10v of 1.6% NaCl was added slowly, dropwise while swirling the tube, followed by centrifugation at 200 g at 20°C for 5 min. The supernatant was discarded and 10v of RPMI 1640 complete media was added, followed by centrifugation at 200 g at 20°C for 5 min. After removal of the supernatant, pelleted parasites were resuspended in complete media at 0.5% hematocrit. Cultures were gassed with 5% CO₂, 3% O₂, and 92% N₂ and maintained at 37°C.

3.2.1.4 Maintenance of *P. falciparum* cultures

*P. falciparum* cultures were maintained as described previously (Trager and Jensen, 1976). Briefly, *P. falciparum* strain 3D7 was cultured at 37°C in RPMI 1640 medium (list I) in O⁺ RBCs supplemented with 10% AB⁺ human serum or 0.5% Albumax II (complete medium). All media were preheated to 37°C and care was taken to minimize the handling time outside the 37°C incubator. Cultures were gassed with 5% CO₂, 3% O₂, and 92% N₂ for 20 seconds and maintained at 37°C.

3.2.1.5 Gametocyte cultures

Fresh stock of parasites was thawed for culture as described above. Thin blood smears were made on the fourth day after setting up the culture. When high parasitemia with “stressed” parasites was observed, culture volume was increased by the addition of medium. At this stage, fresh RBCs were not added to the culture.
medium. The culture volume in 75 cm² culture flasks, was increased to 25 ml from 12 ml. The flasks were kept at 37°C and the medium was prewarmed before use. The flasks were gassed with a mixture of 5% CO₂, 3% O₂ and 92% N₂ for a minimum of 20 seconds at a pressure of around 5 lb/in². The culture medium was changed daily without the addition of RBCs. Blood smears were prepared once or twice a week to check the state of the cultures and the presence of gametocytes. Typically, mature gametocytes were observed after 14-17 days.

3.2.1.6 Giemsa staining of thin blood smear of parasite cultures
Parasite culture was used to make a thin blood film on a glass slide. After air drying, the thin smear was fixed in methanol for about 30 s. A fresh 5 to 10% giemsa solution was prepared in phosphate buffer (list I). The slide was placed in a staining jar and the giemsa solution was poured on the slide for 20 min and subsequently rinsed thoroughly under running tap water. The stained parasites were then observed under a light microscope using 100X objective.

3.2.1.7 Sorbitol-synchronization of P. falciparum
For synchronization, mostly ring stage parasites (10 to 12 h post-invasion) were used. The parasite culture was centrifuged at 200g for 5 min and the supernatant was discarded. To the pellet, 4 ml of 5% sorbitol was added, mixed gently and incubated for 15 min at 37°C. The mix was shaken 2 or 3 times and centrifuged at 200g followed by washing 3 times in complete medium (list I). The culture was then maintained at 5% hematocrit in a 37°C incubator.
3.2.2 Genomic DNA Isolation from Parasite Culture

For DNA isolation from *P. falciparum*, genomic DNA kit from Qiagen (Germany) was used. Isolation was done following manufacturer’s instructions. Briefly, infected erythrocytes (5 ml at 10% parasitemia) were centrifuged at 3,000 g for 2 min. The cells were washed once in cold PBS and resuspended in 1 ml. Following which, 10 μL of 5% saponin (final concentration 0.05%) was added and mixed gently. After lysis, the mix was immediately centrifuged at 6,000 g for 5 min. Further steps were carried out according to the manufacturer’s instructions to isolate genomic DNA. DNA was quantified by measuring absorbance at 260 nm using a UV-spectrophotometer.

3.2.3 Isolation of the parasite RNA

Parasites from synchronized cultures were harvested at different time points of growth to obtain ring, trophozoite and schizont stage parasites. RNA was isolated from these stages by using RNAeasy kit (Qiagen) following manufacturer’s protocol. The concentration of total RNA was determined by measuring the absorbance at 260 nm. Purity of nucleic acid preparations were determined by calculating \( \frac{OD_{260nm}}{OD_{280nm}} \) ratio, a value of near ~1.6-1.8 was taken as a standard of purity. To get stage specific cDNA from RNA, reverse transcription was performed using RT-PCR kit (Invitrogen) that contained random hexamers. Subsequently, the gene of interest was amplified using gene specific primers.
3.2.4 Molecular Cloning of PfCDPK4

To obtain PfCDPK4 gene sequence, BLAST search was done using either TgCDPK1 or the published sequence of other CDPKs in the *P. falciparum* genome sequence. An ORF on chromosome 7 exhibited significant sequence homology with other PfCDPKs. Subsequently, PlasmoDB annotation appeared in the public domain and the gene sequence PF07_0072 matched with the PfCDPK4 sequence identified by us. For PCR amplification, primers were designed on the basis of nucleotide sequence of PF07_0072. Total RNA from asynchronous *P. falciparum* cultures was isolated using RNA easy Kit (Qiagen, Germany) and was used to synthesize cDNA for reverse transcription (RT). Both complimentary and genomic DNA were used as template.

<table>
<thead>
<tr>
<th>Table 3.1: PCR reaction for amplifying PfCDPK4 gene</th>
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<tr>
<td>Template DNA (50 ng)</td>
</tr>
<tr>
<td>Forward Primer (10 µM)</td>
</tr>
<tr>
<td>Reverse Primer (10 µM)</td>
</tr>
<tr>
<td>dNTP's Mix (1.25 mM)</td>
</tr>
<tr>
<td>10 X Polymerase Buffer</td>
</tr>
<tr>
<td><em>Taq</em> polymerase HiFi</td>
</tr>
<tr>
<td>Magnesium Sulphate (MgSO₄) (4 mM)</td>
</tr>
<tr>
<td>Autoclaved water</td>
</tr>
<tr>
<td>TOTAL</td>
</tr>
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</table>

The PCR reaction was carried out using Hi-fi Platinum *Taq* polymerase (Invitrogen) and primers PfCDPK4_F and PfCDPK4_R (see list II) with the following cycling parameters: 94°C for 2 min initial denaturation followed by 30
cycles at 94°C for 30 s, 45°C for 30 s, 68°C for 2 min and final extension at 72°C for 10 min (see table 3.1). PCR products were cloned in pGEM-T easy vector (Promega) and the sequence for the cloned PfCDPK4 gene was obtained by automated DNA sequencing.

3.2.4.1 Ligation
The ligation reaction consisted of 10 ng of vector, appropriate amount of insert (insert:vector ratio :: 3:1), 1x ligation buffer and 1U of T4 DNA ligase (NEB, England). The total volume was made up to 10 µl with autoclaved water. The ligation reaction mixture was incubated at 16°C for 12 hrs.

3.2.4.2 Transformation in E. coli
5 µl of the ligation mix was added to competent cells and mixed gently and the mix was kept on ice for 30 min before giving a heat shock at 42°C for 1 min. The mixture was incubated on ice for 2 min and 900 µl of LB broth was added to each tube. The cells were recovered by centrifugation at 250 rpm at 37°C for 1 h and were plated on LB agar plates containing the appropriate antibiotic(s) and incubated overnight at 37°C.

3.2.4.3 Plasmid DNA Isolation
Plasmid DNA was extracted using commercially available kit (Qiagen, Germany) as per manufacturer’s instructions. For a miniprep, bacterial cell pellet from 5ml freshly grown culture were resuspended in 250 µl buffer P1 containing RNaseA in a microfuge tube, followed by lysis in 250 µl of buffer P2. After the tube was
incubated on ice for 5 min, buffer N3 (350 μl) was added to the mixture and the tube was inverted 4-6 times until mix appeared cloudy. Cell debris was removed by centrifugation at 12000 x g for 10 min and the supernatant was applied to QIAprep spin columns. Columns were centrifuged at 12000 x g for 1 min and the flow through was discarded and columns were washed using 750 μl of 70% ethanol and centrifuged at 12000 x g for 1 min. Additional centrifugation was performed to remove the residual ethanol. The columns were placed in a 1.5 ml microfuge tube and DNA was eluted with autoclaved water or 1 mM Tris-HCl (pH 8.0).

3.2.5 Site directed mutagenesis

All site-directed mutagenesis studies were performed using the QuickChange mutagenesis kit (Stratagene) following the manufacturer's instructions. It is a PCR based method for introducing point mutations, replace amino acids and delete or insert single or multiple amino acids into desired plasmid constructs. Primers containing mutations were designed and PCRs were performed using "wildtype" construct as template. The PCR product was subjected to digestion with DpnI endonuclease, which is specific for methylated DNA. Following DpnI digestion, the parental DNA template gets cleaved and DNA containing desired mutation is selected. The residual mutant nicked DNA was transformed in E. coli DH5α competent cells and the resulting plasmids were isolated and sequenced to confirm incorporation of the desired mutations.
3.2.6 Expression and Purification of Recombinant GST (Glutathione-S-Transferase) fusion PfCDPK4 and its mutants

To facilitate the expression of recombinant GST-CDPK4 or its mutants, the desired regions of enzyme were PCR amplified using pGEMT-PfCDPK4 as template and PCR primers which possessed overhangs for XhoI and SmaI restriction enzymes (see List II). Often, the PCR products were cloned in TA cloning vector pGEMT-easy. Clones in pGEMT-easy vectors were digested with appropriate restriction enzymes to release the inserts. The released inserts were cloned in expression vector pGEX4T-1 to facilitate the expression of recombinant proteins. In some cases, the PCR products were digested directly with restriction enzymes and ligated into expression vectors.

The plasmid DNA for expression was used to transform E. coli BL21-RIL (Stratagene) strain for the expression of GST-PfCDPK4 and its mutants. Protein expression was induced by overnight incubation of cells with 0.1mM IPTG at 18-20°C. Subsequently, cell pellets were suspended in ice cold lysis buffer, containing 50 mM Tris, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 1% TritonX-100, and protease inhibitors (1mM phenylmethylsulfonyl fluoride, 10µg/ml leupeptin, 10µg/ml pepstatin) and sonication was performed for 6 cycles of one minute each. The resulting cell debris was removed by centrifugation at 20,000g for 40 min at 4°C. Fusion proteins from the cell lysates were affinity-purified using glutathione-sepharose resin (Amersham). Briefly, after the protein binding, the resin was washed with lysis buffer, and bound proteins were eluted with 50 mM Tris, pH 8.0 with 10 mM glutathione. Finally, purified proteins were dialyzed against 50 mM
Tris, pH 7.4, 1 mM dithiothreitol, and 10% glycerol. Protein concentration was determined by densitometry analysis of Commassie stained gels. Protein samples were stored at -70°C until further use.

3.2.6.1 Protein Estimation

Protein concentrations were determined using BCA protein estimation kit (Pierce, USA). The assay was performed according to the instructions provided by the manufacturer. Various dilutions of the sample or BSA were made in appropriate buffer and 200 μl of supplied reagent mix (1:50 ratio) was added to each well in a 96 well plate. The plate was incubated at 37°C for 1 h and the absorbance was measured at 540 nm.

3.2.7 SDS PAGE

The protein samples were resolved by SDS PAGE using the Laemmli buffer system (Laemmli, 1970). The protein sample was denatured by boiling at 100°C for 10 min in Laemmli’s buffer (List I). Resolving gel (10%) was prepared in a minigel (Bio-Rad, USA) system alongwith 3% stacking gel and the electrophoresis was carried out at 120 volts for 125 min. The gel was stained with 0.25% Coomassie blue R staining solution for 1h followed by destaining with successive washes of destaining solution. Staining was avoided when gel was used for immunoblotting. Details of reagents used for SDS-PAGE are given in List I.

3.2.8 Assay of Protein Kinase Activity

The catalytic activity of recombinant PfCDPK4 (and its mutants), as well as immunoprecipitated PfCDPK4 from parasite lysate, was 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 6 μg of Myelin Basic Protein. Kinase assays were also performed using "syntide-2" a small peptide substrate (PLARTLSVAGLPGBK) custom synthesized by Pepron, South Korea, and has been used as a substrate for plant CDPKs and CaMKs (Harmon et al., 1994; Hashimoto and Soderling, 1987; Yoo and Harmon, 1996). Reactions were performed in the presence of 2 mM calcium chloride or 2 mM EGTA (0 mM Ca2+) for 40 min at 30°C. When MBP was used as the substrate, reactions were stopped by boiling the assay mix for 5 min in Lammeli’s buffer followed by SDS-PAGE. Phosphate incorporation was adjudged by autoradiography of SDS-PAGE gels. When Syntide-2 was used as substrate, reactions were stopped by spotting the reaction mix on P81 phosphocellulose paper (Millipore). The paper strips were air dried followed by washing with 75 mM ortho-phosphoric acid. Phosphate incorporation was assessed by scintillation counting of the P81 paper. In PfCDPK4 inhibition assays, peptide inhibitors were preincubated with proteins in a kinase assay buffer at 25°C for 30-60 min prior to the addition of substrate and ATP.

3.2.9 Preparation of Parasite Cell Lysates

P. falciparum infected erythrocytes were lysed by the addition of 0.05 % (w/v) saponin to release parasites, followed by a 30 minute incubation on ice. To remove debris and lysed RBCs were washed with cold PBS followed by centrifugation at 8000g. The lysis buffer containing 10 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1x complete protease inhibitor cocktail (Roche Applied Science) was added to the parasite pellet and homogenized by passing the
suspension through a 26 gauge needle. Lysates were cleared by centrifugation at 14,000 g for 30 min at 4°C and supernatant was used for protein estimation using BCA protein estimation kit (Pierce).

3.2.10 Inhibitor Treatment of gametocytes
Parasite cultures were distributed in six well plates (2 ml per well) and pharmacological inhibitors were added at desired concentration. Plates were placed in small gas chambers, gassed and immediately returned to 37°C incubator. The lysates were prepared after ~30 min of the addition of inhibitors.

3.2.11 Generation of anti-PfCDPK4 antisera
A synthetic peptide (KMMTSKDNLNIDIPS) based on the PfCDPK4 sequence was custom synthesized (Peptron Inc.) and conjugated to keyhole limpet hemocyanin via an additional N terminus cysteine residue. It was used to raise polyclonal antisera against PfCDPK4 in rabbit. First immunization was performed using 100 μg of peptide diluted in PBS and mixed 1:1 v/v with Complete Freund’s Adjuvant (CFA). Subsequently, three booster doses of 50 μg each were given on the 14th, 28th, 42nd day post first immunization. Blood was collected from animals on 7th, 21st, 35th, 49th day. Antibody titers were checked by ELISA using recombinant proteins or ovalbumin conjugated peptides as an antigen. In all cases, pre immune sera from the same rabbit were used as control.
3.2.11.1 ELISA

A 96-well microplate was coated overnight at 4°C with ovalbumin conjugated peptide in 100 mM carbonate buffer, pH 9.5 (2 μg/well). The plate was washed 3 times with PBST and blocked with PBS containing 2% BSA (200μl/well) at 37°C for 1 h. Serum samples (diluted in PBS) were added in duplicates (50 (μl/well) at different dilutions (1:100, 1:1000, 1:10,000) and the plate was incubated at 37°C for 1 h. The plate was washed and incubated with HRP-conjugated appropriate antibody (1:10,000 dilution in PBS containing 2% BSA) at 37°C for 1 h. The plate was washed thoroughly with PBST and freshly prepared TMB substrate (100μl/well) was added and the reaction was stopped with 2 N H₂SO₄ (50 (μl/well) and the absorbance at 450 nm was recorded in an ELISA reader.

3.2.12 Western Blot

The proteins separated by SDS-PAGE were transferred from the gel to nitrocellulose membrane using a blotting apparatus (Bio-Rad, USA). In brief, after removal of the stacking gel, the resolving gel was placed over nitrocellulose membrane and sandwiched with Whatman 3 mm filter paper in a cassette. The cassette was submerged in transfer buffer and transfer was carried out at 150 mA for 3h at 4°C. Following the transfer, the membrane was carefully removed from the blotting apparatus and blocked with 3% non-fat dry milk protein for 1h. The membrane was washed thrice with PBST and incubated overnight with the primary antibody at 4°C. Following incubation, the membrane was washed thrice with PBST and incubated with appropriate HRP-labeled secondary antibody at room
temperature for 2h. The nitrocellulose membrane was washed extensively with PBST and developed using chemiluminescence substrate from Pierce (USA).

### 3.2.13 Immunoprecipitation of PfCDPK4 from parasite lysates

Gametocyte rich parasite lysate was prepared using lysis buffer containing phosphatase inhibitors (20μM sodium fluoride, 20μM β-glycerophosphate, and 100μM sodium vanadate). For some experiments, 2mM calcium or 2 mM EGTA was added to the lysis buffer. 100μg of lysate protein was incubated with PfCDPK4 anti-sera (1:100 ratio) for 12 h at 4°C on an end-to-end shaker. Subsequently, 50 μl of protein A+G-Sepharose (Amersham Biosciences) was added to the antibody-protein complex and incubated on an end-to-end shaker for 2 h. The beads were washed with phosphate-buffer saline three times at 4°C and were resuspended in kinase assay buffer that contained phosphatase inhibitors.

### 3.2.14 Immunofluorescence Assay

Thin blood smears of parasite cultures were fixed with chilled methanol for 2 min. After air drying, washing with PBS and permeabilization was done with 0.05 % saponin in 3% BSA/PBS for 15 min, followed by blocking with 3% BSA made in PBS for 1h. Subsequent incubations with primary antibodies were performed for 2h at room temperature or at 4°C overnight. The smears were washed 3x5 times with PBS. The slides were then incubated with appropriate secondary antibodies (labeled either with fluorescein isothiocyanate (FITC) or Texas Red) for 1 hour at room temperature. The slides were washed again with PBS and air dried in the dark. Smears were mounted in glycerol containing mounting media that contained
DAPI (Vector Labs, USA), and stained parasites were visualized using Zeiss Axioimager fluorescence microscope and the images were processed using AxioVision software.

3.2.15 Homology Modeling

The CLD-J domain shares ~51% similarity with the CDPK from Arabidopsis thaliana AtCPK-1. The homology model of CLD-JD was determined using Swiss Model from EMBL. The template model used was CLD-JD of AtCPK-1, which was crystallized as a dimer. The J-domain helices from the two monomers were swapped with each other in this structure (Chandran et al., 2006). Therefore, the initial homology model generated for the complementary CLD-J domain for PfCDPK4 was also a dimer. To understand the interaction of this helix (Gln\textsuperscript{358}-Lys\textsuperscript{371}) with CLD of the monomer, this helix was rotated and translated keeping residues 372-375 as the flexible linker region and superimposed on to the helix from the other monomer, which resulted in the initial model for the CLD-J domain monomer. Initially, these flexible linker residues (372-375) were locally minimized using COOT (Emsley and Cowtan, 2004), and the overall structure was refined with slow cooling using annealing of CNS (Brunger et al., 1998) to remove all the short contacts. Finally, the model quality was checked with the Procheck software (Laskowski et al., 1996). The homology model was generated with the help of Dr. S. Gaurinath, JNU.
3.2.16 Circular Dichroism (CD) spectroscopy

The peptide was dissolved either in MilliQ water or various concentrations of Trifluoroethanol (TFE). CD spectra were obtained on a Jasco J-710 spectropolarimeter with a constant dispersion of 1 nm. Spectra were measured with a time constant of 1s, scan speed of 100 nm/min. Signals were averaged 10 times before each measurement. The peptide concentration was between 185 and 350 μM, and a 0.1-cm path length cell was used.

3.3 RESULTS

3.3.1 Molecular Cloning of PfCDPK4 Gene

PfCDPK4 was amplified using gene specific primers. Both gDNA and cDNA were used as templates, the larger size of the amplicon obtained from gDNA (Fig 3.4) suggested the presence of introns of in PfCDPK4 gene. Both products were cloned in pGEM-T easy vector and sequenced. DNA Sequencing confirmed that PfCDPK4 contains an intron of 347 nucleotides (Fig 3.5), which was similar to the predictions made by PlasmoDb database. PfCDPK4 ORF was found to be 1587 bp and encodes a 328 aa protein. The deduced amino acid sequence was analysed using SMART domain prediction algorhythm. It suggested that PfCDPK4 possesses four EF hand motifs constituting a calmodulin-like domain at the C terminus and an N-terminal serine/threonine kinase domain, characteristic of CDPKs (Fig. 3.6A). The putative catalytic domain of PfCDPK4 possesses all 11 sub-domains that are representative of most eukaryotic protein kinases (Hanks and
Hunter, 1995). PfCDPK4 also has a putative myristoylation signal at its N-terminus (Fig. 3.6B). A similar myristoylation signal in PfCDPK1 was found to be important for its membrane targeting (Moskes et al., 2004). A ~34 amino acid (aa) junction domain links the CLD and the kinase domain.

### 3.3.2 Calcium Stimulates Autophosphorylation and Catalytic Activity of Recombinant PfCDPK4

To study catalytic mechanism of PfCDPK4, it was expressed as a GST fusion protein in E. coli (Fig 3.7). The activity of purified recombinant PfCDPK4 was analyzed using myelin basic protein (MBP) as *in vitro* phosphoacceptor substrate. The recombinant PfCDPK4 was active only in the presence of calcium (Fig 3.8). The recombinant PfCDPK4 also exhibited calcium-dependent autophosphorylation (Fig 3.8A) A small synthetic peptide, syntide-2, which has been used as an *in vitro* substrate for several CDPKs (Harmon et al., 1994; Yoo and Harmon, 1996) and known to be relatively more specific substrate for CDPKs, was effectively phosphorylated by PfCDPK4 in a calcium-dependent manner (Fig 3.8B and C)

### 3.3.3 J-domain is Responsible for PfCDPK4 Regulation

While it is obvious that the kinase domain of PfCDPK4 is responsible for the catalysis and the CLD is important for calcium binding, the role of J-domain needed evaluation. Studies done on plant CDPKs suggest that J-domain is pivotal in the calcium mediated activation of these kinases (Harper and Harmon, 2005). To evaluate the function of PfCDPK4 JD in its regulation, several deletion and
truncation mutants of PfCDPK4 were created (Fig 3.9). The activity of recombinant mutant proteins was determined by performing in vitro kinase assays using syntide-2 as substrate as described above. Firstly, the activity of a deletion mutant ΔJ, which lacks most of the J domain (aa 350 to 379) was assayed (Fig 3.9). Unlike the wild-type PfCDPK4, ΔJ exhibited significant catalytic activity even in the absence of calcium (Fig. 3.10). Since the level of activity of this mutant did not change in the presence of calcium, it is reasonable to propose that the JD negatively regulates PfCDPK4 activity in a calcium dependent manner (Fig. 3.10). Similar effect of JD deletion was also observed on the autophosphorylation of PfCDPK4. While the wild-type enzyme exhibited autophosphorylation only in presence of calcium, ΔJ mutant was autophosphorylated in both the presence and absence of calcium (Fig. 3.11), which fits in well with the activity data.

The JD of plant CDPKs like AtCPK1 (Harper et al., 2002) and GmCDPK (Harmon et al., 1994) controls their activation and shares very similar amino acid sequence. In comparison, PfCDPK4 exhibits an only average sequence homology (~50%) with plant CDPKs (Fig 3.15A). Therefore, regulation by its J-domain was studied in detail. For analysis of the J domain, truncation mutants lacking the CLD and the portions of the J-domain were generated (Fig 3.9) to identify the location of regulatory elements on this domain. A truncation mutant, T369, lacking the CLD, failed to exhibit kinase activity either in the presence or in the absence of calcium (Fig 3.12). A further deletion of the preceding 10aa in truncated mutant T359, also rendered an almost inactive form of PfCDPK4. Strikingly, removal of additional 10 amino acids in mutant T349, from the J-domain caused a dramatic increase in the catalytic activity. In addition, the activity exhibited by this mutant was
independent of calcium (*Fig* 3.12). These data suggested that the stretch between aa 349 and 359 may be responsible for sequestering PfCDPK4 in an inactive state. The interaction of J-domain with the kinase domain was one possibility via which PfCDPK4 may be inhibited as suggested for AtCPK1 (Harper et al., 1994).

### 3.3.4 N-Terminal Region of the J domain has an auto-inhibitory motif

To analyze the role of J domain in PfCDPK4 regulation further, a peptide (Peptide I) corresponding to residues 346-364, which spans almost the entire J-domain, was synthesized and included in kinase assays (*Fig* 3.13A). Peptide I inhibited the activity of PfCDPK4 (*Fig* 3.13Ba) confirming the inhibitory nature of the J-domain (*Fig* 3.10). Significantly, the activity of T349 mutant, which was constitutively active (*Fig* 3.12), was also inhibited by this peptide (*Fig* 3.13Bb). Since this mutant lacks the CLD, it is reasonable to attribute this inhibition to a possible auto-inhibitor motif present in Peptide I. A smaller peptide, Peptide II, corresponding to aa 350-358, was used to further narrow down the inhibitory region (*Fig* 3.13A). This peptide inhibited the activity of a T349 mutant (*Fig* 3.14a) as efficiently as peptide I with IC$_{50}$ ~ 100 µM suggesting that the segment 350-358 may inhibit PfCDPK4 by interacting with the kinase domain. Collectively, these and truncation mutant data (*Fig* 3.12) suggested that these N-terminal residues (aa 350-358) of the J-domain may operate as an auto-inhibitory motif which may operate by interacting with the kinase domain.
3.3.5 C Terminal of the J-domain Controls PfCDPK4 Activation via CLD

We next investigated if the C-terminal region of the J-domain plays a part in PfCDPK4 regulation. For this purpose, peptide III corresponding to residues 357-368 of the J-domain was used (Fig. 3.13A). When added to kinase assays, peptide III inhibited the activity of full-length PfCDPK4 (Fig 3.14C). Strikingly, it failed to influence the activity of T349 mutant (Fig. 3.14B, 3rd bar). Since T349 lacks the CLD, it was possible that this peptide regulates PfCDPK4 via the CLD.

3.3.5.1 Homology Modelling of PfCDPK4 CLD-J domain

While these studies were in progress a crystal structure of the CLD and the J-domain of A. thaliana CDPK1 (AtCPK1) was reported (Chandran et al., 2006). Even though AtCPK1 does not form a dimer in solution, the CLD-J domain of this kinase existed as a dimer in this structure. This structure revealed that the C terminus of the J-domain forms a α-helix, which interacts with the C-lobe of the CLD of the other monomer via a “domain-swap” mechanism (Fig. 3.3). Based on these findings, the C terminus of the AtCPK1 J-domain (aa 433-446) was proposed to interact with its CLD (Chandran et al., 2006). Sequence alignment and homology modeling of the CLD-J-domain of PfCDPK4 suggested that T357-S370 of PfCDPK4 may correspond to the helical region of AtCPK1 J-domain (Fig. 3.15A).

Although the CLDs of the two kinases are highly similar, the J domain shows limited sequence similarity. For instance, only 7 of the 14 aa (~50%) of the putative CLD interacting region are similar (Fig. 3.15A). Therefore, this model was “low resolution” and served only as a guide for further experiments (Fig. 3.15B).
The first support for the predictions made by the model was obtained from experiments done with peptide III, which spans most of the CLD-interacting segment, C terminus of J domain (Fig. 3.13A), as described earlier. The inhibition of PfCDPK4 (Fig. 3.14C), and not T349 (Fig. 3.14B), by this peptide could be attributed to its interaction with the CLD, which is present in PfCDPK4 and not in T349.

3.3.5.2 J domain is α-helical

The homology model of PfCDPK4 JD-CLD (Fig. 3.15B) suggested that the C-terminus of J-domain may be present in α-helical conformation in PfCDPK4. To confirm this, circular dichroism (CD) studies were performed on peptide III, which corresponds to this region of J domain. The CD spectra suggested a random structure for this peptide in water as a large negative ellipticity was observed close to 200 nm (Fig. 3.15C). It is known that the addition of trifluoroethanol (TFE) to an aqueous solution of peptide, which otherwise has a random structure, may stabilize nascent, secondary structural elements (Sonnichsen et al., 1992). The addition of TFE to peptide III aqueous solution resulted in an increase in negative ellipticity at ~204 and 220 nm and a peak at 190 nm (Fig. 3.15C), which was indicative of stabilization of the helical conformer (Woody, 1995). Therefore, it is reasonable to state that the Thr$^{357}$-Met$^{368}$ region has a propensity to form α-helix in a hydrophobic environment, which supports the homology model (Fig 3.15B).
3.3.6 L360 is Critical for J domain CLD interaction

The modeling studies also suggested that Leu\(^{360}\) of PfCDPK4, which corresponds to Phe\(^{436}\) of AtCPK-1, anchors the J-domain to the CLD by interacting with a group of C-lobe hydrophobic residues (Fig. 3.15 A and B). To confirm this, a L360A mutant of PfCDPK4 was generated. In comparison to PfCDPK4, L360A exhibited significantly reduced catalytic activity even in the presence of calcium (Fig 3.16). These results support the proposed model in which Leu\(^{360}\) seems to be critical for exerting the control of CLD over J-domain. Taken together with the peptide III inhibition results (Fig 3.14B and C), these data suggest that the CLD interaction with the C terminus of the J-domain is a key step in PfCDPK4 regulation. Based on these findings, the J-domain can be divided into two segments: an auto-inhibitory region, which resides between aa 350 and 358 and interacts with kinase domain and a CLD-interacting region (aa 357-368).

3.3.7 Autophosphorylation of Thr\(^{234}\) Is Crucial for PfCDPK4 Activation

Some CDPKs are not totally dependent on autophosphorylation for their activation (Zhang et al., 2002). The observations made in (Fig 3.8A) suggested that PfCDPK4 is autophosphorylated in the presence of calcium. To assess the role of autophosphorylation in its regulation, it was important to identify the autophosphorylation site on this enzyme. It is well known that the phosphorylation of the activation loop of most protein kinases results in their activation (Johnson et al., 1996). The role of the activation loop phosphorylation in PfCDPK4 was explored. Sequences of the activation loop, which reside between sub-domains VII and VIII of PfCDPK4, were compared with the corresponding activation loops of
kinases in which the regulatory phosphorylation sites are known. Three possible regulatory autophosphorylation sites in the loop region (Ser\textsuperscript{219}, Thr\textsuperscript{220}, and Thr\textsuperscript{234}) (Fig 3.6B) emerged as likely sites from this exercise. To evaluate this experimentally, two mutants of PfCDPK4 were created: a double mutant S219/T220A and T234A a single point mutant. The S219/T220A double mutant exhibited both autophosphorylation (Fig 3.17A) as well as kinase activity (Fig 3.17B) as observed for the wild-type enzyme ruling out a role of Ser\textsuperscript{219} and Thr\textsuperscript{220} in PfCDPK4 regulation. In comparison, Thr\textsuperscript{234} mutation to A resulted in almost complete loss of PfCDPK4 autophosphorylation (Fig 3.17A), which was accompanied by an inhibition of its catalytic activity (Fig 3.17B). Therefore, it is reasonable to propose that autophosphorylation of Thr\textsuperscript{234} is essential for PfCDPK4 catalytic activation.

3.3.8 A Model for PfCDPK4 Activation by Calcium

Based on the biochemical data, the following mechanism for the activation of PfCDPK4 (Fig 3.18) is proposed: when calcium binds to the CLD of PfCDPK4, it results in its interaction with the C terminus of the J-domain. As a result, constraints are imparted on the auto-inhibitor region of the J-domain resulting in its dissociation from the catalytic domain. These events facilitate the autophosphorylation of the activation loop at Thr\textsuperscript{234}, which ultimately results in PfCDPK4 activation.
3.3.9 Prediction of Regulatory Elements in PfCDPKs

It was worth investigating the presence of regulatory motifs in the JD of other PfCDPKs using the information obtained from PfCDPK4 studies. When sequences of J-domains of PfCDPK1-5 in *P. falciparum* were compared using ClustalW (Fig 3.19), a KLXXΦAXXΦ (Φ= hydrophobic aa, X= any aa) motif was found conserved in almost all PfCDPKs with some minor differences in PfCDPK 1/4 (Fig 3.19). This motif corresponds to the CLD-interacting region of PfCDPK4 (Fig 3.15A). The residues separating KL and the ΦAΦ segments are basic in PfCDPK2, -3, and -5 but are neutral in PfCDPK1 and -4. The other difference between PfCDPK1/4 and other PfCDPKs is in the central -ΦAΦ-segment, whereas the first residue is hydrophobic in PfCDPK2, -3, and -5, it is replaced by an Ala in PfCDPK1 and -4. The core NΦR/KXF pseudosubstrate or autoinhibitor motif is highly conserved among all PfCDPKs. Sequence comparison indicates that two hydrophobic residues, which may provide additional interaction with the catalytic site, precede this motif in PfCDPK2, -3, and -5. A small variation was observed for PfCDPK1 and -4, and this core motif is preceded by only one hydrophobic residue. The subtle differences between PfCDPK1 and -4 and other PfCDPKs may suggest that these enzymes are closest members of this group.

These predictions were tested on PfCDPK1. To this end, peptides I, II, and III, which inhibited PfCDPK4 activity by interacting with the kinase domain and/or CLD, were tested against PfCDPK1 to validate the predictions. These three peptides effectively inhibited PfCDPK1 (Fig 3.20A) supporting the predictions made above. PfCDPK1_T341, a truncation mutant of PfCDPK1 that ends at aa 341, was generated by deleting most of the JD and CLD. Like the T349 mutant of
PfCDPK4, this PfCDPK1 truncation was also active both in the presence and the absence of calcium. Although peptides I and II inhibited this mutant, peptide III did not alter its activity significantly (Fig 3.20B). These studies corroborate well with the results obtained for PfCDPK4 and its T349 mutant (Fig 3.14B and C). Therefore, it is reasonable to suggest that these peptides or the corresponding segments in the J-domain may regulate PfCDPK1 via a mechanism similar to the one proposed for PfCDPK4.

3.3.10 PfCDPK4 is Expressed in Sexual Stages of the Parasite Life Cycle
To determine the stage specific expression of PfCDPK4, firstly, the levels of PfCDPK4 transcripts were assessed by RT-PCR using RNA from various blood stage parasites. PfCDPK4 RNA expression was observed through out the blood stages. For analyzing protein expression, PfCDPK4-specific antisera were raised against a synthetic peptide corresponding to a unique motif in the J-domain. Western blots were performed using protein lysates from different asexual stages as well as the gametocytes. A band corresponding to ~60 kDa, which was consistent with the predicted molecular mass of PfCDPK4, was observed only in the gametocyte lysates (Fig 3.21Ba, right panel). Control blot performed with pre-immune sera did not show any staining (Fig 3.21Ba, left panel) indicating the specificity of the antisera. These data confirmed that PfCDPK4 is expressed mainly in the sexual stages. The presence of PfCDPK4 mRNA in asexual stages (Fig 3.21A) but absence of its protein expression (Fig 3.21Ba) may be suggestive of translational regulation of PfCDPK4.
3.3.11 PfCDPK4 is localized at gametocyte periphery

To study the localization of PfCDPK4 in gametocytes, immunofluorescence studies were performed. IFA studies revealed that antibody staining is mainly on gametocyte periphery (Fig 3.21Bb). It is likely that the presence of a myristoylation signal in this kinase (Fig 3.6B) is responsible for targeting this kinase to the parasite periphery.

3.3.12 Regulation of PfCDPK4 in the Parasite: Role of phospholipase C

It has been demonstrated that the release of calcium from intracellular stores in Plasmodium is controlled by PLC (Garcia, 1999; Gazarini et al., 2003), which is used by the parasite for various important purposes. A strong correlation between increase in the levels of PLC hydrolysis product, inositol 1,4,5-trisphosphate, and gametogenesis has also been reported (Martin et al., 1994), which is suggestive of a role for PLC in this important parasitic process. Given the dependence of recombinant PfCDPK4 on calcium and its role in gamete formation, it was worth exploring whether PLC regulates its activity in the parasite. Gametocytes were treated with PLC inhibitor U73122, which has been used successfully in Plasmodium (Gazarini et al., 2003; Vaid and Sharma, 2006; Vaid et al., 2008), and the activity of immunoprecipitated PfCDPK4 was assayed. In one of the experiments, an cell permeable intracellular calcium chelator, BAPTA-AM, was included (Fig 3.22A). This inhibitor caused a significant decrease in PfCDPK4 activity, which emphasized the importance of intracellular calcium on PfCDPK4 activation. Treatment with the PLC inhibitor, U73122, caused a significant decrease in PfCDPK4 kinase activity. In contrast, U73343, the inactive analogue of
this inhibitor, failed to alter the activity of this kinase (Fig 3.22A). Western blotting confirmed that the level of PfCDPK did not change significantly upon inhibitor treatment (Fig 3.22B). These data suggest that PLC acts as a regulator of PfCDPK4, which is most likely a result of its ability to control levels of free calcium in the parasite.
PfCDPK4 gene was amplified from cDNA or genomic DNA and PCR products were cloned in pGEMT easy vector. The larger size of PCR product obtained with gDNA as template suggested the presence of intron, which was confirmed by DNS sequencing.
Fig.3.5 Sequencing of PCR products with gDNA and cDNA confirmed the presence of 347 bp intron, shown here in underlined italics face. The two exons constitute 1587 bp ORF.
Fig. 3.6 A. The domain architecture of PfCDPK4. Deduced amino acid sequence of PfCDPK4 (panel B) indicated that it possesses a Calmodulin Like Domain (CLD) at the C-terminus which has four EF hand motifs. The kinase domain is present near the N-terminus and a small Junction Domain (JD) separates the two domains. A putative myristoylation signal present at its N-terminal end is highlighted.

B. The deduced amino acid sequence of PfCDPK4 ORF. The catalytic domain of PfCDPK4 has all the 11 kinase subdomains conserved. The putative regulatory phosphorylation sites that reside in the activation loop between subdomains VII and VIII are indicated in italicized bold face. A predicted myristoylation signal present at the N-terminus of PfCDPK4 is underlined and the J domain sequence at the C-terminus is boxed. The four EF hand motifs of the calmodulin like domain are indicated by green boxes.
Fig.3.7 PfCDPK4 was expressed in *E. coli* as GST fusion and purified by affinity chromatography. Lanes: 1. Protein marker, 2. pellet, 3. supernatant, 4. unbound fraction, 5-8. eluted fractions.
Fig.3.8  A. Kinase assay was performed using recombinant GST-PfCDPK4, in the presence of 2mM CaCl$_2$ or 2mM EGTA (absence of Ca$^{2+}$), using 6μg MBP as phosphor-acceptor substrate. Kinase assay mixture was boiled and electrophoresed on SDS-PAGE gel and phosphoimaging was performed. PfCDPK4 shows autoposphorylation only in presence of calcium; it also shows calcium dependent phosphorylation of MBP. (B and C) Similar kinase assays were performed with GST-PfCDPK4 using 150μM syntide-2 as substrate; reaction mix was spotted on phosphocellulose paper and after extensive washes scintillation counting was done. The specific activity is measured in nmoles/mg/min.
Fig.3.9 Schematic diagram illustrating PfCDPK4 mutants created for biochemical studies. Truncation mutants (T) were named based on the number of their C-terminal amino acid. All mutants were expressed as GST-fusion proteins as described for wild-type PfCDPK4. ΔJ is a deletion mutant of PfCDPK4 which lacks the J-domain (aa. 350-379).
Fig. 3.10 The role of J-domain in PfCDPK4 regulation. Equal amount of PfCDPK4 or ΔJ was assayed for catalytic activity using syntide-2 as substrate, in the presence of 2mM CaCl₂ or 2mM EGTA (absence of Ca²⁺). Deletion of J domain results in calcium independent activation of PfCDPK4.
Fig.3.12 Identification of regulatory elements in PfCDPK4 J-domain. Equal amount of truncation mutants (Fig. 3.9) or PfCDPK4 were assayed for catalytic activity in the presence or absence of Ca\(^{2+}\). The kinase activity was determined by measuring phosphate incorporation in syntide-2 as described earlier. A representative of more than three independent experiments is shown. Error bar represents standard error between replicates of the same experiment.
Fig. 3.13 A peptide corresponding to the J-domain inhibits PfCDPK4 activity.

A. The amino acid sequence of the J-domain and of the peptides corresponding to different regions of this domain.

B. Kinase assays were performed using PfCDPK4 (a) or T349 mutant (b). Recombinant enzymes were pre-incubated with different concentrations of Pep I prior to the addition of 200 μM syntide and ATP in a kinase assay mix. Phosphate incorporation in syntide was determined as described above. A representative of three experiments is shown in the figure.
Fig. 3.14. Differential effects of Pep II and Pep III on PfCDPK4 and T349. Pep III (B and C) or pep II (A and B) were incubated with PfCDPK4 (C) or T349 (A and B) in a kinase assay mix. Subsequently, 200 μM syntide-2 was added and the assay was initiated by the addition of ATP. Activity was assessed by measuring syntide phosphorylation as described earlier. Pep II inhibited the activity of T349 (A). While Pep III inhibited PfCDPK4 (C), it did not alter T349 activity (B). Data shown here are presented as mean ± SE of three independent experiments.
The C-terminal region of PfCDPK4 may form an α-helix. (A) Clustal W sequence alignment of J-domains of PfCDPK4 and AtCPK-1 and Soya bean CDPKα (GmCDPKα). The C-terminal region of AtCPK-1 JD, which forms an α-helix (Chandran et al., 2006), and the corresponding region of PfCDPK and GmCDPKα are shadowed in red. Phe⁴³⁶ of AtCPK-1 and the corresponding Leu⁴⁶⁰ of PfCDPK4 are indicated by an asterisk. The arrows indicate start of Calmodulin like domain of each CDPKs. (B) Firstly, a homology model of PfCDPK4 CLD and its J-domain was obtained using the coordinates for the domain-swapped crystal structure of the corresponding domains of AtCPK-1 (Chandran et al., 2006). Based on this structure, a structural model for intramolecular interaction of the J and CLD of PfCDPK4 was constructed (see section 3.2.15), which is shown here. Key residues in the C-lobe of the CLD (magenta) that may form a binding pocket for L360 (yellow) of the J-domain (red), are indicated in ball and stick. (C) CD spectra of PEP III was acquired in water (blue) or increasing concentration of TFE. The concentration of TFE is indicated in the inset.
Fig. 3.16. Leucine 360 plays an important role in PfCDPK4 activation. Kinase assay of equal amount of PfCDPK4 and L360A mutant was performed using 150μM of syntide-2 as substrate in the presence and absence of calcium as described earlier. The L360A mutant was not activated by calcium.
**Fig. 3.17** PfCDPK4 is regulated by autophosphorylation of T234. (A) Equal amounts of PfCDPK4, single point mutant T234A or double point mutant S219/T220A were incubated in the presence or absence of Ca\(^{2+}\) and the kinase assay was performed as described earlier. Reaction mix was electrophoresed on SDS-PAGE gel. Autoradiogram shown here is representative of two independent set of experiments. PfCDPK4 and S219/T220A exhibited autophosphorylation in presence of calcium, but T234A remained unphosphorylated. (B) Similar experiment as in panel (A) except exogenous substrate syntide-2 was added in the reaction to assess the catalytic activity of the mutants. Incorporation of \(^{32}\)P to syntide-2 was measured, in presence or absence of calcium. T234 to A mutation resulted in a significant loss in PfCDPK4 activity.
Fig. 3.18. A model for the regulation of PfCDPK4 by calcium. The J-domain of PfCDPK4 has N-terminal auto-inhibitory motif (green) and CLD interacting region at C-terminal region of J domain (red). In the absence of calcium, autoinhibitory motif interacts with the catalytic domain (blue), keeping the kinase in inactive state (I). The binding of calcium to the CLD promotes interaction between the C-lobe of the CLD and the C-terminus of J-domain (II), which may impart conformational constrains on the autoinhibitor region resulting in its dissociation from the catalytic domain (III). This would make the catalytic site free to autophosphorylate at T234 and interact with its substrates and facilitate their phosphorylation.
Fig. 3.19 Prediction of regulatory elements in PfCDPKs.

Sequences of the J-domain of PfCDPK1-5 were aligned using ClustalW. Based on the data obtained for PfCDPK4 in this work, a putative autoinhibitor motif (black box) and the CLD interacting region (orange box) of CDPKs were predicted. The consensus sequence for these motifs, which emerged from this analysis, is indicated below.
Fig. 3.20 PfCDPK1 may be regulated in a manner similar to PfCDPK4. Wild-type PfCDPK1 (A) or its truncation mutant PfCDPK1_T341 (B) were preincubated with peptides I, II or III, and kinase assays were performed using syntide 2 as substrate as described earlier for PfCDPK4.
Fig. 3.21 PfCDPK4 expression and localization in the parasite.

A. Total RNA isolated from different asexual stages of parasite was used to generate cDNA. RT-PCR was performed to amplify PfCDPK4 followed by agarose gel electrophoresis of the PCR product. EFβ-1 was amplified as control (right panel).

B. (a) Western blot was performed using anti-PfCDPK4 polyclonal antisera. Lysates prepared at different stages of parasite life cycle were probed for PfCDPK4 protein expression: R, ring; T, Trophozoite; S, schizonts; G, gametocyte; M, merozoites. A ~60 KDa band was observed mainly in the gametocyte stage. Antiserum prepared from pre-immune bleed was used as control. (b) Immunofluorescence assays were performed on gametocyte smears using anti-PfCDPK4 antisera and Alexa-594-anti rabbit IgG. Parasite nucleus was stained with Hoechst 33224 (blue).
Fig.3.22  A. Gametocytes were treated with DMSO, 30μM U73122 (PLC inhibitor) or U73343 (inactive analogue), 100 mM BAPTA-AM. Subsequently, PfCDPK4 was immunoprecipitated from protein lysates and PfCDPK4-IP associated kinase activity was assayed using syntide as substrate.

B. Immunoblotting was performed on protein lysates from the experiments described in panel A using anti-PfCDPK4 antisera.
3.4 DISCUSSION

*Plasmodium* genome codes for several signaling molecules like protein kinases, which includes five members that belong to the calcium-dependent protein kinase family (Ward et al., 2004). The CDPKs are typically absent from animals or fungi; interestingly, they are present in protozoan parasites (Harper and Harmon, 2005). However, there are ≈42 CDPK isoforms in *A. thaliana* that are crucial for various aspects of plant physiology (Harper et al., 2004). Calcium regulates a wide variety of important functions in the parasite life cycle, and recent studies have demonstrated that the activity of CDPKs may be critical in carrying out several calcium-regulated processes (Billker et al., 2004; Ishino et al., 2006; Kato et al., 2008; Lin et al., 1996; Zhao et al., 1994). *PbCDPK4* gene disruption stalls cell cycle progression in *P. berghei* male gametocytes and results in reduced sexual reproduction and mosquito transmission (Billker et al., 2004). Another kinase, cGMP-dependent protein kinase from *P. falciparum*, was recently shown to play a role in gametogenesis (McRobert et al., 2008) of *P. falciparum* indicating the importance of signaling events in sexual development of the parasite.

The immunofluorescence studies suggested that PfCDPK4 is mainly present near the gametocyte surface (Fig.3.21Bb). The N-terminal myristoylation signal may be responsible for its cell surface targeting, which is the case with PfCDPK1 (Moskes et al., 2004). It was demonstrated previously that the products of PLC hydrolysis inositol 1,4,5-trisphosphate and diacylglycerol may be involved in exflagellation (Martin et al., 1994; Ogwan'g et al., 1993). It should be noted that a PLC homologue is present in the parasite (*PlasmoDB ID: PF10_0132*). The dependence
of PfCDPK4 activity on PLC in gametocytes correlates well (Fig. 3.22) with a proposed role of PLC and its products in mobilizing parasite calcium (Farias et al., 2005; Gazarini et al., 2003) and that of CDPK4 in gametogenesis (Billker et al., 2004).

It was clear from the activity assays that PfCDPK4 activity was dependent on autophosphorylation. The mechanism of autophosphorylation and dependence on it for catalytic activation can vary among different CDPKs. A phosphoproteomic study involving CDPKs suggested that these kinases can be autophosphorylated on at least five different motifs. Interestingly, several CDPKs do not exhibit autophosphorylation of the activation loop (Hegeman et al., 2006), which indicates the differences in the mechanisms through which these kinases may be regulated. Our results suggest that PfCDPK4 is regulated by autophosphorylation of Thr$^{234}$, which resides in its activation loop (Fig. 3.17). Although autophosphorylation of Ser$^{219}$ and Thr$^{220}$ of PfCDPK4 seems to be non-existent, complementary sites in some CDPKs do get autophosphorylated (Hegeman et al., 2006). It was interesting to note that AtCPK1, which shares similarities with PfCDPK4 in the J-domain regulation, did not exhibit autophosphorylation of the activation loop. Even though PfCDPK1 was shown to be autophosphorylated at a site complementary to Thr$^{234}$ (Hegeman et al., 2006), the role of autophosphorylation in its catalytic activation has not been demonstrated.

Biochemical studies revealed that the J-domain of PfCDPK4 exerts control over its timely stimulation by calcium. It was evident from the calcium-independent
activity of the ΔJ mutant that the J-domain of CDPK4 may regulate PfCDPK4 activation (Fig. 3.10). Studies performed with the J-domain truncation mutants resulted in identification of two functionally distinct regions. The N terminus of the J-domain possesses an NIRQFQS motif (aa 350-358), which acts as a pseudosubstrate or an autoinhibitor. The proposed pseudosubstrate region in AtCPK1 and GmCDPKα is RM/LKQFQS (Harmon et al., 1994; Harper et al., 1994), and it has an extra basic residue at first position, which is missing from PfCDPK4 (Fig 3.15A). Interestingly, this extra basic residue is absent from all PfCDPKs except PfCDPK5 (Fig. 3.19). Peptide II, which corresponds to this motif, inhibited the activity of the constitutively active Thr349 mutant confirming its pseudosubstrate or autoinhibitory nature (Fig. 3.14B). In contrast to Pep II, Pep III, which is complementary to the C terminus of the J-domain, only inhibited the wild-type PfCDPK4 and not the constitutively active T349 mutant (Fig. 3.14B and C). Because T349 lacks the CLD, we conclude that peptide III may regulate PfCDPK4 via its CLD.

The crystal structure of the AtCPK1 J-domain along with the CLD revealed that the C-terminal portion of the JD may interact with the CLD (Chandran et al., 2006). A homology model was generated for the PfCDPK4 CLD-JD, which suggested that Gln358-Lys371 stretch may form a α-helix, which may interact with the CLD. The biochemical results obtained with peptide III, which constitutes a large part of this segment, were consistent with this observation as this peptide inhibited PfCDPK4 and not the constitutively active T349 mutant (Fig. 3.20A and B). Pep III exhibited the propensity to form α-helix (Fig. 3.15C), which provided
further support to the model. It is worth appreciating that, despite only modest homology between the CLD interacting segment of PfCDPK4 and AtCPK1, this motif adopts a helical conformation, which facilitates interaction with CLD via residues like Leu$^{360}$.

These findings led us to propose a model for PfCDPK4 activation, which may also be relevant for other PfCDPKs (Fig. 3.18): the J-domain uses a bipartite motif to interact with the CLD as well as the kinase/catalytic domain. Firstly, calcium binding to CLD results in interaction with the C-terminal region of the J-domain. As a result, the N-terminal autoinhibitory region may be forced to dissociate from the catalytic domain resulting in PfCDPK4 autophosphorylation and catalytic activation.

These studies on PfCDPK4 aided the prediction of regulatory motifs in the J-domain of other PfCDPKs. PfCDPK1 and PfCDPK4 appeared to be the closest among this group. The inhibition of PfCDPK1 by PfCDPK4-JD peptides confirmed that this kinase may also be regulated by the predicted regulatory motifs via a mechanism similar to that of PfCDPK4.

Given the importance of CDPK4 (Billker et al., 2004) and other CDPKs in the development of malaria parasite, this information may prove useful for design of inhibitors against CDPKs.