CHAPTER FOUR

Molecular and Biochemical Characterization of PfPHDK1
4.1 INTRODUCTION

Phosphoinositides (PIPs) are known to play important role in events like cell signaling, membrane trafficking and cytoskeletal dynamics. Phosphoinositides use their phosphorylated inositol group to interact with domains on protein. This makes them well-suited to target specific membrane compartments. Several PIP binding domains have been identified (see section 2.5), which interact with specific phosphoinositides and modulate activity or localization of the proteins which possess them. One of the important PI binding domain is the Pleckstrin Homology (PH) domain (Harlan et al., 1994).

Fig. 4.1 The crystal structure of the PH domain of rat PLC $\delta_1$ bound to IP$_3$ (ball and stick) indicated by arrow. The basic residues in the $\beta_1/\beta_2$ loop of the PH domain interact with the phosphates of phosphoinositide head group (Ferguson et al., 1995).
Pleckstrin homology (PH) domains are \(~\text{120 aa motifs}\) and are found in proteins involved in signaling, cytoskeletal organization, membrane transport, and modification of phospholipids. The PH domain is the 11th most abundant domain class in the human genome (Human-Genome-Project, 2001). These domains have essentially the same tertiary structure, but sequence homology between different PH domains is very low ranging from just \(\sim 7\%\) to \(25\%\) (Fushman et al., 1998). The core PH domain fold consists of a seven-stranded \(\beta\) sandwich capped off by a characteristic C-terminal \(\alpha\)-helix (Fig 4.1). PH domains with specific phosphoinositide recognition properties depend on a sequence motif in the \(\beta1/\beta2\) loop between the first two strands of the \(\beta\) sandwich (Dowler et al., 2000; Isakoff et al., 1998). However, more than \(80\%\) of PH domains do not have this \(\beta1/\beta2\) loop sequence motif (or related sequences), and many have been shown to bind phosphoinositides only weakly and with less specificity (Kavran et al., 1998; Rameh et al., 1997a; Takeuchi et al., 1997).

The PH domains have been broadly classified into four groups (Kavran et al., 1998; Rameh et al., 1997a) Group I binds PI(4,5)P\(_2\), Ins(1,4,5)P\(_3\) and other PIPs; group II interacts with PI (3,4,5)P\(_3\) and Ins(1,3,4,5)P\(_4\) with high affinity and high specificity; group III binds PI(3,4,5)P\(_3\) and PI(3,4)P\(_2\); and group IV shows no binding or has a low affinity for PIPs (Blomberg et al., 1999). PH domains specific for 3-phosphorylated inositol derivatives may represent possible downstream targets of phosphoinositide 3-kinase (PI 3-kinase). For instance, protein kinase B (PKB) interacts with 3\ˈ phosphorylated PIPs via its PH domain, which results in its translocation to the plasma membrane after PI3- kinase activation (Leevers et al., 1999).
To identify targets of the phosphoinositides in the parasite, we screened the *Plasmodium* genome to identify proteins with PIP binding domains. This resulted in the identification of several proteins that possess PIP binding domain which included the PH domain containing proteins. Surprisingly, only one protein kinase (*PlasmoDB ID: PF11_0242*) seems to possess a PH domain, this kinase was named as PH domain containing kinase 1 (PfPHDK1). PfPHDK1 was studied as it represents a putative kinase that may be regulated by PIPs.

### 4.2 MATERIALS and METHODS

The methodology for several experiments described in this section is similar to studies done on PfCDPK4, which are described in chapter 3. Only the additional methods used are described in this section.

#### 4.2.1 Molecular Cloning of PfPHDK1

Using the protein sequence of mammalian PH domain, PlasmoDB database was searched for PH domain containing proteins. This resulted in the identification of several PH domain containing genes, out of these only one gene product (*PF11_0242*) possessed both PH and kinase domain, which was named as PH domain containing kinase 1 (PfPHDK1). PCR primers were designed to amplify (List II) various regions of the gene and PCR products (*Fig 4.4*) were cloned in pMAL-c2X vector. The following cycling parameters were used for PCR amplification: 94°C for 1 min initial denaturation followed by 30 cycles at 94°C for 20 s, 50°C for 45 s, 68°C for 2 min and
final extension at 72°C for 10 min. The PCR primers contained restriction sites for *BamHI* and *XhoI* to facilitate cloning of these fragments in the expression vector pMAL-c2X. The PCR amplifications were done using cDNA as template, which was obtained by performing reverse transcription (RT) as described in chapter 3, section 3.2.3. The location of primer sequence used to amplify various region of PfPHDK1 is indicated (*Fig. 4.4A*) and sequences are given in list II.

### 4.2.2 Maltose Binding Protein (MBP) protein expression

For expression of MBP fusion recombinant proteins, BL21-RIL strain of *E. coli* was used, which was transformed with pMAL-c2X expression plasmids. Typically, the transformed bacterial colonies were grown in LB broth in presence of ampicillin (100 μg/ml) and chloramphenicol (35μg/ml) supplemented with 0.2% glucose. The expression of recombinant protein was induced at mid-logarithmic phase (*A*₆₀₀ value of 0.6) by addition of IPTG at 18°C for 16h. Subsequently, bacterial cells were harvested by centrifugation at 4000g for 15 min and were resuspended in lysis buffer (25 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin). Cell lysates were prepared by sonication of suspension for 8 cycles of 1 min each followed by centrifugation at 26000g for 30 min. Cell free lysates were loaded on pre-equilibrated amylose resin columns followed by washing with 12 bed volumes of the lysis buffer. Subsequently, MBP fusion recombinant proteins were eluted using 50mM Tris, pH 8.0 containing 10 mM maltose. Finally, purified proteins were dialyzed against 50 mM Tris, pH 7.4, 1 mM dithiothreitol and 10% glycerol. Protein fractions were analyzed by SDS-PAGE.
4.2.3 Assay of protein kinase activity

The catalytic activity of recombinant kinase domain of PfPHDK1 and ΔPfPHDK1 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. The assay mixture was incubated for 40 min at 30°C, and reaction was stopped by addition of SDS-PAGE sample buffer. Subsequently, the reaction mixture was electrophoresed and phosphate incorporation in proteins was adjudged by autoradiography of SDS-PAGE gels.

4.2.4 Dot-blot Phosphoinositide Binding Assays

The dot-blot assays to assay PIP binding of proteins was performed as described previously (Jensen et al., 2001; McIntosh et al., 2007). Briefly, various phosphoinositides were spotted on nitro-cellulose membrane and air-dried for 2 h. Subsequently, the membrane was blocked with 3% bovine serum albumin in buffer A (50 mm Tris-HCl, pH 7.4, 150 mm sodium chloride, 0.1% Tween 20) for 3 h. The membrane was incubated with recombinant ΔPfPHDK1 or PH domain (0.5 μg/ml) diluted in blocking buffer, for 12 h at 4 °C. The membrane was washed 5 times with buffer A before 3h of incubation with anti-MBP antibody (NEB, England). Subsequently, the membrane was incubated with horseradish peroxidase-labeled anti-rabbit IgG, and PIP-bound protein was detected by chemiluminescence.
4.2.5 Generation of Transgenic Parasite Lines Expressing Green Fluorescent Protein (GFP) Fusion Proteins

Transfection in *P. falciparum* was done following the protocol described earlier (Wu et al., 1995) with some modifications, as described below:

4.2.5.1 *Cloning of PfPHDK1 fragments in pARL vector:*

To generate parasite expressing GFP chimera with various domains of PfPHDK1, PCR primers were designed to amplify (List II) selective regions of the gene, by introducing a 5' *KpnI* site and a 3' *AvrII* site in the PCR products. The PCR products were digested with *KpnI*/*AvrII* and cloned into *Plasmodium* transfection vector pARL1-a (Marti et al., 2004). The location of primers sequence used to amplify various region of PfPHDK1 is indicated (*Fig. 4.4A*) and sequences are given in list II.

4.2.5.2 *Preparation of DNA for transfection:*

Plasmid DNA was prepared by midiprep kit (Qiagen, Germany) following manufacturer's instruction and ~100µg of plasmid DNA was used for transfection. Ethanol precipitated plasmid DNA was resuspended in 15 µl of sterile 50mM Tris (pH 8.0). Subsequently, 385 µl of sterile cytomix (van den Hoff et al., 1992) was added to plasmid solution (List I). Cytomix and DNA was mixed well and added to parasite infected RBC pellet for transfection.

4.2.5.3 *Transfecting the Parasites:*

A synchronized ring-stage parasite culture with high parasitemia (>8%) was used for transfection and 1-2x10^9 parasitized RBCs were prepared for each transfection.
Infected RBCs (iRBCs) were harvested by centrifugation at 500g for 5 min, resuspended in fresh medium at least 1h before transfection. The electroporation cuvette and the cytomix solution were chilled on ice. iRBCs were pelleted by centrifugation at 1,500 rpm for 5 min in a tabletop centrifuge. The pellet (about 100 to 200 μl) was resuspended in 400 μl of cytomix/DNA mixture and mixed well by pipetting. The parasitised erythrocyte/DNA mixture was then transferred to a GenePulser (BioRad) cuvette (0.2 cm gap) with the help of Pasteur pipette. Electroporation was done at 0.310 kV and 950 μF and the resulting time constant was between 7 and 12 msec. The cuvette was immediately returned to ice for 5 min, and the cells were transferred from the cuvette to a culture flask together with 10 ml of culture medium (the cuvette was rinsed with the culture medium to ensure a complete transfer). The flask was then returned to the incubator and medium was changed after 4 hrs and drug WR99210 was added at a concentration 1nM along with 50 μl of fresh RBCs. Typically, parasite death was seen after 2 days of drug treatment. The drug pressure was kept constant at 1nM till parasites almost disappeared from the culture after a month of transfection. During this period fresh RBCs were added once in a week to maintain 5% hematocrit and medium was changed daily. Parasites reappeared at 1nM drug pressure in a successful transfection and expressed GFP fluorescence. Glycerol stock of parasite lines was made when parasitemia reached 2% at 1nM drug pressure. Drug pressure was further increased stepwise till it reached 8-10 nM.

4.2.6 Fluorescence Microscopy

*P. falciparum* cultures expressing GFP fusion proteins were treated with Hoechst at final concentration 0.5 μg/ml for 15 min at 37 °C prior to imaging. Fluorescence from
Hoechst stained nucleus and GFP fusion proteins was monitored from live parasites, after mounting the samples in culture medium under cover slip on a glass slide, Zeiss AxioVision fluorescence microscope was used to visualize the parasite an AxioVision software was used to analyze the images.

4.1 RESULTS

4.3.1 Identification and cloning of PfPHDK1

BLAST search of PlasmoDB database using mammalian PH domain resulted in identification of several annotated gene products that were predicted to possess the PH domain. One of these “hits”, PF11_0242, possessed both PH and kinase domain, therefore was named PH domain containing kinase 1 (PfPHDK1). In addition, it also possesses two EF hand motifs at the N terminus (Fig. 4.2A). The deduced amino acid sequence of the catalytic domain indicated the presence of all 11 sub-domains (Fig.4.2B) representative of most eukaryotic kinases (Hanks and Hunter, 1995). The sequence of the PH domain of PfPHDK1 was aligned with that of the PH domain of rat PLCδ1 for which the crystal structures are known (Ferguson et al., 1995). This comparison suggested that all 7 β-sheets present in these PH domains may also be conserved in PfPHDK1_PH domain. In addition, the signature C-terminal α-helix, a common feature in PH domains also seems to be conserved in PfPHDK1 (Fig 4.6A).

4.3.2 PfPHDK1 message is present in all parasitic stages

RT-PCR was performed to determine stage specific expression of PfPHDK1. For this purpose, parasites were synchronized and RNA was extracted from various life cycle
stages. Almost similar levels of PfPHKD1 transcripts were observed in ring, trophozoites and schizont stages (Fig. 4.3). Due to unavailability of a good antibody against PfPHDK1, the expression its proteins levels could not be determined.

4.3.3 Expression of PfPHDK1 fragments in E.coli.
PfPHDK1 is predicted to be large protein coding for almost 2265 aa, which makes it almost impossible to express this P. falciparum. For biochemical analysis of this protein, the “business-end” of this molecule was expressed in E.coli. Plasmid DNA constructs were generated by amplifying and cloning PH or the kinase domain in pMAL-c2X vector. In addition, a construct designed to express the PH and kinase domain region was also generated (ΔPHDK1) (Fig. 4.4A) and all fragments were expressed as MBP-fusion proteins in E.Coli. Recombinant proteins were purified by affinity chromatography (Fig. 4.5).

4.3.4 PfPHDK1 interacts with PI(4,5)P2
Eventhough a PH domain was predicted in PfPHDK1, it needed experimental evaluation if this kinase interacts with PIPs. To determine this, a dot-blot assay was performed with ΔPfPHDK1 and a previously identified PI3P interacting protein FCP (McIntosh et al., 2007) was used as a control. The recombinant ΔPfPHDK1 exhibited binding with PI(4,5)P2 (Fig 4.6B,b), and failed to interact with other lipids present on the nitrocellulose membrane. As expected, FCP interacted specifically with PI3P (Fig 4.6B,a). These data demonstrate that PfPHDK1 is a PI(4,5)P2 interacting protein.
4.3.5 **Kinase domain of PfPHDK1 is catalytically active**

To determine if the recombinant PHDK1 was catalytically active, kinase assays were performed using myelin basic protein (MBP) as *in vitro* phosphoacceptor substrate. Both ΔPfPHDK1 and KD phosphorylated MBP *in vitro* demonstrating their catalytic activity (Fig 4.7).

4.3.6 **GFP fusion Proteins with PH domain are Localized near Nucleus**

PfPHDK1 has a PH domain and these domains are well-known to target proteins to various intra-cellular compartments. One of the major interests was to understand the localization and cellular targeting of PfPHDK1 mediated by its PH domain. Various fragments of PfPHDK1 (Fig. 4.4) were cloned in pARL-vector to facilitate their expression in *P. falciparum* with GFP fused to their C-terminal end (Fig. 4.8A). The *P. falciparum* 3D7 lines were transfected with various constructs and put under WR99210 selection. After obtaining transgenic parasites, the expression of various fusion proteins was determined by performing western blot analysis using anti-GFP antibody. Bands corresponding to the expected size of various recombinant proteins were observed at expected size indicating the successful expression of these proteins in *P. falciparum* (Fig. 4.8B).

Next, cellular localization of various PfPHDK1 fragments was determined by live cell microscopy. The PH domain targeted GFP to a subcellular compartment in the vicinity of the nucleus as demonstrated by punctuate staining (Fig. 4.9B). Similar localization was also exhibited by ΔPHDK1-GFP construct (Fig. 4.9A) suggesting that the PH domain may target this kinase to this specific localization. However, transfection with the kinase domain (without the PH domain) resulted in a loss of this
specific targeting as exhibited by cytosolic localization for KD-GFP (*Fig. 4.10*). Collectively, these data demonstrate that the PH domain may target PfPHDK1 to a specific organelle in the parasite. Co-staining with organelle-specific markers may help identify the cellular compartment to which PfPHDK1 is targeted.
Fig 4.2  A. The domain architecture of PfPHDK1. Pf PHDK1 has a C-terminal Ser/Thr kinase domain and a PH domain. In addition, it also has two EF hand motifs at its N-terminus. The location of PCR primers used to clone various regions of PfPHDK1 is indicated.

B. The catalytic domain of PfPHDK1 has all the 11 kinase subdomains found in typical eukaryotic kinases.
Fig 4.3 The expression of PfPHDK1 transcripts in various asexual stages of *P. falciparum* life cycle. Total RNA was isolated from different stages of the parasite and RT-PCR was performed to amplify the PH domain of PfPHDK1 (A) or EFβ-1, which served as a control (B).
Fig. 4.4 A. Generation of PfHDK1 constructs for *P. falciparum* transfection. The regions of PfPHDK1, which were expressed as GFP-fusion protein in the parasite or MBP fusion proteins in *E. coli*, are illustrated.

B. RT-PCR amplification of various regions of PfPHDK1. These PCR products were cloned in pARL or pMAL-c2X vector.
**Fig 4.5** Expression of various recombinant PfPHDK1 domains in *E. coli*. Plasmid DNA of pMAL-c2X vector containing various domains of PHDK1 was used to transform BL21-RIL *E. coli* strain to express these domains as MBP fusion protein. Subsequently, expressed protein was purified by amylase affinity chromatography.
Fig 4.6 The PH domain of PfPHDK1 exhibits binding to PI(4,5)P2.
A. Structure based comparison of amino acid sequences of PH domains of rat PLCδ1 and PfPHDK1. Various secondary structure elements are indicated, which are based on the crystal structure of PLCδ1-PH domain. The identical and similar residues between two PH domains are indicated by * and : respectively.

B. PfPHDK1-PH domain interacts with PI(4,5)P2.
A dot blot assay was performed to determine the affinity of PH domain toward various PIPs. Indicated PIPs were spotted on nitrocellulose paper and incubated with recombinant MBP-PH domain. The bound protein was detected by using anti-MBP antibody. 6XHis-PfFCP (FYVE containing protein) was taken as positive control (Panel a), which is known to interact with PI3P (McIntosh et al., 2007), 6XHis or anti-FCP antibody was used to detect PfFCP.
Fig 4.7 Recombinant MBP-PfPHDK1 exhibits catalytic activity.
Kinase assay was performed using recombinant MBP-ΔPHDK1 and MBP-KD. Equal amount of both the proteins was assayed for catalytic activity, by using 6μg MBP as the phosphor-acceptor substrate. The radiolabelled MBP was detected by phosphorimaging of the SDS-PAGE gel.
Fig 4.8 The expression of various PfPHDK1 domains as GFP fusion protein in *P. falciparum*.

A. Different domains of PfPHDK1 were cloned at the N-terminus of GFP in *P. falciparum* transfection vector pARL vector (Marti et al., 1993).

B. Parasites were transfected with indicated constructs and WR99210 resistant parasites were checked for GFP fluorescence. Protein lysates were prepared from transfected parasite cell lines and subjected to immunoblotting using anti-GFP antisera. Various GFP fusion proteins exhibited mobility at the expected molecular weight.
Fig. 4.9 P. falciparum 3D7 lines were transfected with various PfPHDK1 fragments cloned in pARL-vector. To facilitate their expression in P. falciparum, the transfected parasites were put under WR99210 selection. After obtaining transgenic parasites, cellular localization of various PfPHDK1 fragments was determined by live fluorescent microscopy.

A. ΔPHDK1-GFP fusion protein shows localization pattern similar to PH-GFP (B), which appears to be in specific subcellular compartment.
Fig. 4.10 Kinase domain of PfPHDK1 was cloned in pARL-vector and transfected in *P. falciparum*. KD-GFP fusion protein was expressed and observed by live cell microscopy. Kinase domain (without the PH domain) is localized in the parasite cytoplasm.
4.5 DISCUSSION

Phosphoinositides are major mediators of signaling and trafficking in eukaryotes. The information regarding their role in malaria parasite is almost non-existent. Recently, a FYVE domain containing protein (FCP), which interacts with PI3P, was identified in our laboratory (McIntosh et al., 2007). This protein exhibited interesting localization, which was inside the parasite food vacuole, where it gets targeted via a C-terminal signal sequence (McIntosh et al., 2007). In order to identify other effectors of PIP in the parasite, the PlasmoDB database was searched for proteins which possess PH domains, one of the major PIP binding domains. One of these proteins was PfPHDK1, which also has a protein kinase domain and N-terminal EF hand motifs. The presence of EF hand motifs suggested that it may be regulated by calcium.

The sequence comparison with PLCδ_PH domain suggested that PfPHDK1_PH domain may possess all the secondary structural elements present in PH domains. However, the sequence homology in β5 region was low. Some of the residues of PLCδ_PH that interact with PI(4,5)P2 or I(1,4,5)P3 (Ferguson et al., 1995) were conserved in PfPHDK1_PH domain (Fig.4.6A). These observations corroborated well with PIP binding assay results, which indicated that PfPHDK1_PH also has a preference for PI(4,5)P2 (Fig.4.6B) as is the case with PLCδ_PH. It is important to note that even though the dot blot assay is a good indicator for PIP binding it may not be very sensitive if the interaction is weak. Therefore, other assays like the liposome based method for PIP binding needs to be performed to probe if PfPHDK1_PH domain interacts with other PIPs, albeit weakly.
The targeting of PfPHDK1 in the parasite was studied by transfecting GFP fusion constructs for various domains of PfPHDK1 in the parasite. When the PH domain was present, it targeted the kinase domain to a specific compartment in the parasite, which was adjacent to the nucleus. Upon its removal, the kinase domain was found predominantly found inside the cytoplasm. In addition, the PH domain on its own was found to this specific compartment. To identify this subcellular organelle, co-localization studies using markers for organelles like the apicoplast, mitochondria golgi etc. need to be performed. Nevertheless, these studies establish that the PH domain of PHDK1 is important for its subcellular localization.