Chapter II

Functional and biochemical characterization
Of putative PfOrc2 and PfOrc5 subunits
2.1 Functional Characterization of PfOrc2 and PfOrc5 by yeast complementation analysis

Since the two ORC subunits PfOrc2 and PfOrc5 show weak homology with their eukaryotic counterparts only at the C-terminus, we are interested to determine whether PfOrc2 and PfOrc5 are true functional homologs in vivo. To test this, we performed genetic complementation assay in yeast using swapper strain of *S. cerevisiae* for PfOrc5 (ySPB5.11) in which chromosomal copy of *orc5* gene has been deleted and a copy of the wild type gene has been maintained in a plasmid for cell survival. For PfOrc2 complementation study, we used temperature sensitive strain (JRY 4259) that grows normally at 25° C but it is unable to survive at restricted temperature 37° C.

For complementation assay, we made several constructs in yeast expression vector using either full length PfOrc5 or ScOrc5 or different chimeras of Yeast and *Plasmodium* Orc5. The schematic diagrams of different constructs have been shown in figure 2.1 and the cloning strategy has been described in materials and methods.

2.1.1 Details of Constructs used for PfOrc5 complementation

Initially, all constructs were made in yeast expression vector pRS416 containing a Galactose inducible promoter, terminator sequence, multiple cloning sites (MCS) and *ura* gene as a selection marker. However, the swapper strain of Yeast *orc5*, where *orc5* gene is maintained in an episomal plasmid, also uses *ura* as a selection marker. Chimera constructs were constructed using SOE technique (gene splicing by overlap extension) as discussed in material and methods. Therefore, after cloning into pRS416 vector, the whole cassette containing galactose inducible promoter, insert and terminator sequence was taken out using *SacI*-*KpnI* restriction enzyme and the entire cassette was sub-cloned into
Schematic illustration of yeast constructs for PfOrc5 complementation

Figure 2.1. Schematic diagram of different constructs of PfOrc5, ScOrc5 and Chimera for yeast complementation. Black shaded area in PfOrc5 and ScOrc5 shows conserved homologous region. In Chimera green shaded part represents ScOrc5 and orange shaded part represents PfOrc5.
yeast expression vector pRS314. This vector uses *trp* as a selection marker. Similar strategy was used for all constructs. The strategy for this cloning is depicted in the schematic cartoon below.

These constructs in pRS314 were used for complementation studies. Yeast *orc5* swapper strain was transformed with wild type *ScOrc5*, *PfOrc5* and different chimera constructs as described in material and methods using lithium acetate method and the transformants were grown on plates containing SD -Trp media and incubate at 30°C for 3-4 days. Colonies on -Trp plates were then patched simultaneously on -Trp and -Trp+5FOA (5 Fluoroorotic acid) plates containing galactose and raffinose and incubated at 30°C for 3-4 days to check their viability.

To further confirm the results of complementation assay, we performed serial dilution test on -Trp and -Trp+5FOA plates using different dilutions of all the respective transformants selected previously on the similar plates. For this, serial dilutions (10⁻¹ to 10⁻⁵) of yeast culture were made and further spotted on the plates followed by incubation at 30°C for 3-4 days. Spot test results showed that *ScOrc5* and Chimera Orc5

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Figure 2.2. Yeast complementation assay of PfOrc5. (A) yeast Orc5 swapper strain was transformed with all the constructs, as shown in previous figure, by lithium acetate method as mentioned in material and methods. Transformed colonies were streaked on SD - Trp and −Trp + 5FOA plates and incubated for 3-4 days at 30°C. 1-ScOrc5, 2- PfOrc5, 3-PfOrc5N1, 4-Chimera Orc5, 4-PfOrc5C, 5-ScOrc5N and 5- pRS314. (B) Western blot analysis shows the expression of different constructs used in complementation assay (as indicated on top) using anti-PfOrc5 antibodies. Coomassie stained gel following protein transfer on PVDF membrane is shown as loading control.
survived on –Trp+5FOA plates even at lower dilutions while none other construct including full length PfOrc5 was able to do so in the presence of 5FOA (figure 2.2). 5FOA, used in this experiment is made into toxic compound in the presence of ura gene product and inhibits the growth of episomal plasmid containing wild type orc5 gene under ura selection marker.

2.1.2 Details of Constructs used for PfOrc2 complementation

For the complementation studies of Orc2, several constructs of PfOrc2 and ScOrc2 and chimera of them were made (figure 2.3). All the constructs were made in pRS416 yeast expression vector having ura gene as a selection marker and galactose inducible promoter and terminator. We cloned five DNA fragments in the MCS of pRS416 vector that included ScOrc2, PfOrc2, Chimera Orc2 (having N-terminal part of S. cerevisiae and C-terminal part of P. falciparum Orc2), ScOrc2N and PfOrc2C.

The temperature sensitive Yeast Orc2 strain does not allow expression of functional Orc2 at restrictive temperature, thereby not allowing the yeast cells to survive at 37°C. Only those cells will survive at higher temperature where the episomally maintained plasmid can synthesize the protein that can complement ScOrc2 protein function in vivo.

The whole cassette containing inserts from PRS416 were sub-cloned along with galactose inducible promoter and terminator sequences in pRS314 vector between SacI- KpnI restriction sites having trp selection marker. These constructs were then used for transformation and complementation studies. Yeast orc2 temperature sensitive strain was transformed with these constructs using lithium acetate method as
Figure 2.3 Schematic diagram of different constructs of PfOrc2, ScOrc2 and Chimera for yeast complementation. White shaded area in PfOrc2 and ScOrc2 shows conserved homologous region. Blue shaded part represents ScOrc2 and orange shaded part represents PfOrc2 in Chimera Orc2.
described in material and methods and transformants were grown on SD –Trp media at 25°C for 3-4 days. The colonies on this plate were then streaked on plates containing SD –Trp medium with raffinose and galactose and kept at 25°C and 37°C respectively for 4-5 days.

The complementation results show that all the cells grow at 25°C on –Trp SD medium plate while only ScOrc2 and Chimera Orc2 were able to rescue the cells at restrictive temperature (37°C). All other constructs failed to maintain cell growth at high temperature (figure 2.4).

Cross species complementation of ORC proteins provide strong evidence of conservation of replication protein through species despite differences at the amino acid sequence level.

2.2 Expression and purification of catalytic domain of PfOrc5

Yeast and Human Orc5 show ATP binding activity which is essential for origin firing during DNA replication initiation (Takahashi et al, 2004; Giordano-Coltart et al, 2005). Plasmodium has till now only three confirmed subunits of ORC namely PfOrc1 and two putative subunits, PfOrc2 and PfOrc5. Other three subunits are not identified yet by homology search analysis, although one putative protein shows very weak homology with Orc4 proteins. The presence of fewer ORC subunits in Plasmodium and their lower homology with Yeast and human counterparts raise the issue whether these proteins are the true functional ORC homologs in Plasmodium. One of the hallmarks of Yeast Orc1 and Orc5 proteins is the presence of NTP binding and hydrolysis domains, central to ORC function in origin activation. Interestingly, both PfOrc1 and PfOrc5 contain NTP binding domains. We have earlier reported that recombinant PfOrc1 shows in vitro ATPase activity (Mehra et al, 2005).
Figure 2.4 Yeast complementation assay using *Orc2* constructs. Yeast *Orc2* temperature sensitive strain was transformed with all the constructs, as shown in the figure 2.3, by lithium acetate method as mentioned in material and methods. Transformed colonies were streaked on SD-Trp plates and kept for 3-4 days at 25°C and 37°C respectively for incubation. 1-ScOrc2, 2-PfOrc2, 3-Chimera Orc2, 4-ScOrc2N and 5-PfOrc2C.
To characterize the Nucleotide binding activity of PfOrc5, we tried to express and purify recombinant full length and truncated versions of the protein.

High AT richness (80%) of *P. falciparum* genome attributes to the low complexity regions and the codon biasness in parasite proteins. Therefore, it is extremely difficult to express *Plasmodium* proteins in heterologus systems.

Initially, we tried to express full length PfOrc5 using different bacterial expression vectors without success. We further decided to clone 2.2 kb part of PfOrc5, PfOrc5N1, (amino acid residues 171 to 899, excluding N-terminal low complexity region) that contains conserved Walker A domain and cryptic Walker B domain. We amplified this region by polymerase chain reaction using specific primers (P45 and P46) and cloned in pGEX6p2 *E. coli* expression vector as described in material and methods. GST-tagged fusion protein was purified under native conditions by affinity chromatography using Glutathione agarose beads. The yield of purified protein was quite low. To generate a mutant version of PfOrc5N1, point mutation was introduced specifically in the Walker A ATP binding domain where lysine at position 309 was converted into alanine by site directed mutagenesis. After confirming the sequence of the mutant clone at the WALKER A ATP binding domain (K to A), the mutant protein was also purified under the same experimental conditions (figure2.5).

Since the yield of GST-PfOrc5N1 was not sufficient for Nucleotide binding assay we cloned smaller fragment of PfOrc5 from residues 171 to 421. The protein, termed as PfOrc5N2, contains WALKER A domain. We amplify this 750 bp product by PCR reaction using specific primers P47 and P48. MBP-tagged PfOrc5N2 fusion protein was expressed and purified under native conditions by affinity chromatography using
Figure 2.5 ATPase activity of GST-PfOrc5N1. (A) ATPase assay were performed either using GST- PfOrc5N1 or GST as a control. The positions of radiolabelled ATP and the Released Pi are shown in the figure. (B) The right panel shows the ATPase activity of GST-PfOrc5N1 With the increasing amount of protein. (C) This panel shows the comparison of ATPase Activity of wild and mutant proteins. ATPase assay were performed using 100 ng of GST-PfOrc5Wild or GST-PfOrc5Mutant protein and phosphate release was quantified and plotted graphically with the standard deviation after repeating experiments for three times. The inset shows the purification of the wild and mutant forms of the proteins.
amylose resin as described in material and methods. Mutant form of this protein was generated by site directed mutagenesis as described in material and methods.

### 2.2.1 ATP hydrolysis assay

To find out whether PfOrc5 contains any NTP hydrolysis activity, we performed ATP hydrolysis assay using PfOrc5N1, which contains ATP binding as well as incomplete Walker B ATP hydrolysis domain. GST alone was used as a control under the same experimental conditions. PfOrc5 wild type protein showed ATPase activity as evidenced by the increasing trend of release of Pi (inorganic phosphate) with increasing protein amount whereas GST alone protein did not show any ATPase activity. These results suggest that PfOrc5 protein indeed contains ATP hydrolysis activity that might be important for origin activity in *P. falciparum*.

To further confirm that the ATPase activity associated with PfOrc5 protein was specifically related to PfOrc5 only and not due to any contaminating protein, we compared ATPase activity of PfOrc5N1 wild type protein with the mutant protein. Mutant protein showed drastic reduction up to 60% in its ATPase activity compared to wild type protein under similar experimental conditions (figure 2.5). These observations further confirmed that the ATPase activity is contributed mostly by PfOrc5 protein.

### Nucleotide binding assay

The WALKER A domain of PfORC5 contains G-X-X-G-X-G-K-T/S signature motif, where lysine is expected to interact with the nucleoside triphosphate tail. We investigated the nucleotide binding activity of PfOrc5 by ATP binding assay. Since the yield of PfOrc5N1 was very poor
for nucleotide binding assay we used PfOrc5N2 that contains WALKER A domain and the mutant form of the protein.

For ATP binding assay, ~1 μg of the wild type or the mutant form of MBP-PfOrc5N2 protein was incubated with radio-labeled α-32PdATP in ATP binding buffer and the mixture was further cross linked with UV irradiation as described in material and methods. Samples were resolved in 10% SDS-PAGE and labeled protein was visualized under phosphorimager. Results showed that the wild type MBP-PfOrc5 protein was able to bind ATP under similar experimental conditions whereas mutant protein failed to bind ATP (figure 2.6).

2.3 Characterization of PfOrc2 phosphorylation domain

In eukaryotes, ‘ORC cycle’ is referred to differential regulation of ORC molecules in terms of replication control (Fujita et al, 1998; Liang and Stillman, 1997; Lygerou and Nurse, 1999). Cyclin-dependent kinases (CDKs) are critical for the re-replication control that regulates the activities of components of pre-replication complex once the origin is fired.

Regulation of ORC activity is the premier step in determining the timing of DNA replication. Cell-cycle dependent changes occur in ORC activity as ORC tends to associate or dissociate from chromatin. This “On and off” pattern of ORC molecules is referred as ‘ORC cycle’ (Depamphilis, 2005). Regulating the association of ORC with chromatin is a feasible mechanism for restricting re-initiation event in eukaryotes. In yeast, phosphorylation plays a major role in regulating ORC activity. ScOrc1, ScOrc2 and ScOrc6 proteins are phosphorylated by cyclin dependent protein kinase CDK1 (Cdc28)/Clb1 during G1-S transition and hyperphosphorylated during S-M transition (Nguyen et al, 2001). In mammalian cells, Orc1 gets hyperphosphorylated by cyclinA/CDK1 at
Figure 2.6 (A) Schematic diagram of wild type or different deletion mutants of PfOrc5. Amino acid coordinates are shown on the right. (B) ATP binding assay. One microgram of the MBP- PfOrc5N2 Wild or MBP- PfOrc5N2 mutant proteins were incubated with [α-32P]-dATP and the proteins were further cross linked using UV light as described in material and methods. Upper panel shows the SDS-PAGE Analysis of these proteins. The molecular mass marker are shown on the left. The bottom panel shows the autoradiogram.
G2-M phase which prevents its stable association with chromatin and leads to the subsequent absence of functional ORC-Chromatin sites on metaphase chromatin (Li et al, 2004). In mammalian cells, three different states of Orc1 are found during cell-cycle events. In G1 phase, Orc1 associates with other ORC subunits for pre-RC formation. Then it is released from the chromatin and gets ubiquitinylated during S-phase and it is phosphorylated during M phase (DePamphilis, 2005). The Orc2p is in mammalian system is constitutively phosphorylated while in in S. pombe phosphorylation of Orc2 (Orp2) by Cdc2 kinase occurs in G2/M phase and is important to prevent re-replication (Vas et al, 2001). In Yeast Orc6 is unphosphorylated in G1 phase and gets phosphorylated by CDKs at the onset of S phase only (Li and Herskowitz, 1993). Thus phosphorylation could be a major controlling event that regulates the activity of ORC subunits during cell cycle progression (DePamphilis, 2003, Saha et al, 2006).

Besides ORC subunits, Cdc6, MCMs and Cdt1 are major proteins that are post-translationally regulated by the means of different mechanisms, mainly phosphorylation and ubiquitination (DePamphilis, 2003). Cell cycle stages are difficult to describe in Plasmodium falciparum as nuclear division appears to be asynchronous during asexual blood stages. In order to search for the regulatory mechanism associated with replication and cell cycle control in Plasmodium falciparum, it is vital to look for the key regulatory molecules that may give important insight into the regulation of replication and cell cycle analysis of Plasmodium falciparum.

2.3.1 In silico analysis for putative kinase site in PfOrc2
We are interested to explore whether PfORC subunits can potentially be phosphorylated leading to the modulation of parasite replication. For this purpose, we tried to identify putative cyclin/CDK phosphorylation sites in PfOrc2. The consensus sequence of cyclin/CDK phosphorylation site
Figure 2.7 (A) Schematic representation of PfOrc2 protein illustrating the CDK phosphorylation domain (†). Extreme N-terminus of PfOrc2 possesses the conserved cyclin/CDK phosphorylation motif. (B) Coomassie gel shows purified protein MBP-PfOrc2 fusion protein. Molecular mass marker are shown on the left.
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is (S/T) PX (K/R). Interestingly, in silico analysis of PfOrc2 amino acid sequence suggests the presence of one such putative sequence (SPKK) at the extreme N-terminus of the protein (figure 2.7). Identification of this putative Cyclin/CDK sites will allow us to investigate whether PfOrc2 is truly phosphorylated in-vitro and in-vivo in combination with mammalian and Plasmodium CDK like kinases. Once, we establish the phosphorylation event of PfOrc2, its biological significance for the control of DNA replication could be revealed further.

Recently our lab has identified two such putative cyclin/CDK phosphorylation sites in PfOrc1 and it has been shown that these sites get phosphorylated in-vitro as well as in-vivo (Unpublished data Mehra and Dhar et al). The above studies gave us confidence and platform to further explore the possibilities of PfOrc2 phosphorylation in vitro and in vivo.

2.3.2 Characterization of Kinase domain of PfOrc2 by human Cyclin/CDKs

Based on sequence homology, PfOrc2 contains putative conserved phosphorylation site on its extreme N-terminus. Yeast and human Orc2 are good substrates for S-phase dependent kinases like cyclinA/CDK2 and cyclinE/CDK2. Not much is known regarding cyclin/CDK partners and their possible targets in Plasmodium. To find out whether putative kinase domain in PfOrc2 can be phosphorylated using heterologous cyclin/CDKs, we expressed and co-purified human cyclinA/CDK2 and human cyclinE/CDK2 complexes as described in material and methods. Co-purification of cyclin/CDKs is essential as these cyclins bind to catalytic cleft at one side of CDK2 and induces conformational changes resulting in the active cyclin/CDK complex (figure 2.8). To confirm the activity of bacterially purified hcyclin/CDK2 complex, we performed in vitro kinase reactions using Histone HI as a substrate in the presence of
Figure 2.8 (A) Co-purification of human cyclin A/CDK2 and cyclinE/CDK2 under native conditions. They were co-purified to make active complex as described in material and methods. Upper molecular weight denotes cyclinA and cyclinE (★) while lower molecular mass represents CDK2 (▼). Molecular weight are represented on left panel. (B) CyclinA/CDK2 and cyclinE/CDK2 kinase assay was performed with bacterially purified human cycA/CDK2 and cycE/CDK2 using Histone HI as a substrate as described in material and methods. Upper panel shows the phosphorylation of Histone HI while lower panel shows the Coomassie stained gel to show the presence of Histone.
Figure 2.9 (A) Coomassie stained gel showed purified PfOrc2 wild and kinase mutant proteins as described in material and method with the cartoon of mutated residue (S to A). (B) Kinase assay was performed using human cyclinA/CDK2 as enzyme and MBP-PfOrc2 and its kinase mutant as substrate. (C) Kinase assay was performed using cyclinE/CDK2 as enzyme and MBP-tagged PfOrc2 wild and mutant as substrate as described in material and methods.
γ-32pATP. Following Kinase reactions, products were resolved in 10% SDS-PAGE followed by autoradiography. Both hyclinA/CDK2 as well as hyclinE/CDK2 was able to phopsphorylate Histone HI (figure 2.8). These results suggest that purified hyclin/CDK complexes are active under these experimental conditions. Coomassie gel shows the loading control for the proteins.

In order to find out the phosphorylation status of PfOrc2, N-terminal ~1Kb fragment from PfOrc2 (1-327 residues) was cloned in pMALc2x vector between BamHI- Xhol restriction sites. Protein was expressed in BL21 codon plus cells and purified under native conditions as mentioned in material and methods (figure 2.7 B). A point mutation was introduced to convert conserved Serine into alanine (S9A) by using mutagenic primers as described in material and methods. Both wild type and mutant proteins were purified under similar conditions and subsequently used for phosphorylation studies. To investigate whether putative cyclin/CDK phosphorylation sites in PfOrc2 can be phosphorylated in vitro, kinase reactions were carried out using purified recombinant wild type or mutant form of MBP-PfOrc2 protein as described in material and methods. Wild type MBP-PfOrc2 showed intense signal of phosphorylation in the presence of hyclinA/CDK2 and radiolabelled γ-32ATP, while under similar experimental conditions MBP-PfOrc2 mutant protein showed no such phosphorylation (figure 2.9 B). Kinase experiments were repeated with hyclinE/CDK2 for MBP-PfOrc2 wild or mutant proteins as described in material and methods and similar results were obtained (figure 2.9 C).

These results confirm that the putative cyclin/CDK phosphorylation site in PfOrc2 can be phosphorylated using active cyclin/CDK enzymes. These results also show the specificity of the phosphorylation of PfOrc2
N-terminus since a single point mutation can abolish the phosphorylation event.

### 2.3.3 Biochemical characterization of PfOrc2 kinase domain by hcyclinA/CDK2

To further investigate thoroughly cyclin/CDK dependent kinase reactions, we performed *in vitro* time-dependent and substrate-dependent kinase reactions, using recombinant MBP-PfOrc2 and hcyclinA/CDK2 proteins.

For substrate-dependent phosphorylation of PfOrc2, Kinase reaction was carried out with increasing amount of PfOrc2 in the presence of fixed amount of kinase enzyme. We find that PfOrc2 phosphorylation increases with increasing amount of protein (figure 2.10).

For time dependent phosphorylation of PfOrc2, Kinase reactions were allowed to undergo for different time duration following which the reactions were stopped. The products were resolved in SDS-PAGE and the gel was dried for autoradiography. We find that phosphorylation of PfOrc2 follows a time-dependent kinetics (figure 2.10).

These data confirm that putative kinase domain in PfOrc2 can be phosphorylated at serine residues at N-terminus in the presence of hcyclinA/CDK2. These results further indicate that these sites might be the potential target of *Plasmodium* kinases.

### 2.3.4 *In silico* screening of *Plasmodium* kinases & cyclins for PfOrc2

Cell division cycle in *Plasmodium* is different from fundamental steps of cell cycle in eukaryotes. In *Plasmodium*, cell division is asynchronous and multiple rounds of nuclear divisions take place without the dissolution of nuclear membrane. This unique feature of cell division in
Figure 2.10 Substrate and Time dependent Phosphorylation of PfOrc2 by human cyclins/CDKs (A) Kinase assay was performed using human cyclinA/CDK2 as enzyme with increasing concentration of MBP-Orc2 protein. Coomassie stained gel shows Orc2 protein loading. Phosphorylated population of PfOrc2 (★) was measured by densitometry scanning and values were plotted graphically. Lower band is due to auto-phosphorylation of cyclin A (†). (B) Similarly Kinase assay was performed at different time points using human cyclinA/CDK2 as enzyme with equal amount of MBP-Orc2 and graph was plotted following densitometry scanning of phosphorylated bands. Bottom panel shows the coomassie gel for equal loading of Orc2 protein.
Plasmodium makes it difficult to define cell cycle stages. There is no precise demarcation between G1, S, G2 and M phases of the cell cycle.

In Plasmodium falciparum, different CDKs and cyclins have been identified by BLAST search analysis for known CDKs and cyclins as queries. Though several CDK like kinases have been reported in Plasmodium, except Histone no substrate for CDK like kinases has been assigned yet.

A representation of the expression profiling of CDK-related protein kinases and PfCyclins during the erythrocytic developmental cycle based on microarray profiling data (Bozdech et al, 2003) is shown in figure 2.11. The expression profiling results suggest that Plasmodium CDK like kinases PfPK5, PfCrk-1, PfCrk-5 and Cyclins like Pfcyclin2 and Pfcyclin 4 are mainly expressed during trophozoite (S-phase) and nuclear division cycle. Co-incidence of expression of these proteins during replicating trophozoite stages suggest that the pre-RC members like ORC subunits may serve as targets for these kinases since their expression profile also peaks during this stage.

Our lab has earlier screened different putative CDK like kinase (PfPK5, PfCrk-1 and PfCrk-5) and PfCyclins (PfCyclin1, PfCylin2, PfCyclin3 and PfCyclin4) for PfOrc1 phosphorylation and it has been found that PfPK5 is the main kinase responsible for its phosphorylation (Unpublished data Mehra and Dhar).

2.3.5 Expression and purification of kinases and Cyclins

To study the PfOrc2 phosphorylation in vitro, different Plasmodium CDKs and cyclins were expressed and purified under native conditions as discussed in material and methods. All these constructs were obtained from Prof. Christian Doerig (University of Glasgow).
Figure 2.11 Representation of expression profile of CDK-related protein kinases and cyclins (adapted from Sherman, 2005)
Figure 2.12 Purification of CDK related kinases and cyclins of *Plasmodium falciparum*. Different *Plasmodium* kinases and cyclins were expressed and purified under native conditions as described in material and methods. RINGO (*Xenopus* origin) is an activator of cdc2 related kinases and it can stimulate PfPk5. It was expressed and purified under native conditions as MBP- fusion protein. (*) shows the position of respective recombinant protein.
PfPK5 (~28Kd) and PfPK6 (~35kd) were purified as His6-tagged proteins. PfCRK5 (~70Kd) Kinase was purified as GST-tagged protein. Pfcycl1 (~52Kda), Pfcycl2 (~56Kda), and Pfcycl4 (~56Kda) were expressed and purified as GST tagged fusion proteins whereas Pfcycl3 (~62Kda) was purified as MBP fusion protein.

We also purified an activator of PfPK5, RINGO as a MBP tagged fusion protein. RINGO has been shown previously as an activator for PfPK5 function (MercKx et al, 2003). The purification of all the above proteins is shown in figure 2.11. The major band corresponding to each purified protein is indicated by asterisk (*). The molecular mass of the fusion proteins as shown after SDS-PAGE analysis corresponds with their theoretical molecular mass. These purified proteins were used for in vitro phosphorylation assay for PfOrc2.

2.3.6 In vitro phosphorylation of PfOrc2 by Plasmodium kinases and Cyclin combinations

PfPK6 was first isolated as a novel protein kinase in P. falciparum (Miyata et al, 1999). The catalytic domain of PfPK6 shows 57.3% homology with Cyclin dependent kinase (CDK) and 49.6% homology with mitogen-activated protein kinase (MAPK). PfPK6 is predominantly expressed during trophozoite and schizont stages. It is localized both in the nucleus as well as in the cytoplasm during developmental stages. PfPK6 can be auto-phosphorylated and it can phosphorylate other substrates like Histone HI and ribonucleotide reductase in the absence of a cyclin partner (Bracchi-Ricard et al, 2000).

In an effort to test the ability of PfPK6 as a kinase in in vitro phosphorylation studies, we performed kinase reaction in the presence
and absence of Histone H1 as a substrate as described in materials and methods. The recombinant bacterially expressed PfPK6 can phosphorylate histone as well as itself (figure 2.13). These results confirm that PfPK6 is active in \textit{in vitro} kinase reaction.

Further, to characterize whether PfPK6 can use PfOrc2 as a substrate or not, we performed kinase reaction using recombinant PfPK6 and all four Pfcyclins (Pfccyclins 1–4). The results indicate that PfPK6 is unable to phosphorylate PfOrc2 with either of the cyclin combinations suggesting that kinase specificity of PfPK6 may depend on its right substrate and PfOrc2 may not be the target molecule for PfPK6 (figure 2.13).

In order to screen further \textit{P. falciparum} kinases as potential modulator for PfOrc2 phosphorylation activity, we used another S-phase specific kinase, PfCRK5. PfCRK5 is related to the CDK1/2/3 group of kinases and it shows homology with PfPK6 (unpublished data Nivez et al.). Immunoprecipitation of PfCRK5 from asexual stage parasite extract shows weak Histone H1 phosphorylation activity. Nothing is known about its cyclin partners as well as its potential targets in the \textit{Plasmodium falciparum}.

Initially we tested the activity of PfCRK5 in combination with Pfcyclins using Histone H1 as a substrate. The results indicate that only Pfccyc-1 and Pfccyc-4 can phosphorylate Histone H1. The results suggest that Pfccyc-1 and Pfccyc-4 are the possible cyclin partners to make it an active kinase for Histone H1 substrate.

Further, to find out whether PfOrc2 can serve as a substrate for PfCRK5, we performed kinase reaction in the presence of all four Pfcyclins using MBP-PfOrc2 as a substrate. As it is evident from figure 2.14, PfCRK5/Pfccyclin combination fails to phosphorylate PfOrc2.
Figure 2.13 (A) Autophosphorylation and \textit{in vitro} kinase activity of His-PfPK6 was assayed in a standard kinase reaction using His-PfPK6 alone or His-PfPK6 along with Histone H1 or alone Histone H1 in the presence of \( \gamma^{32} \text{P-ATP} \).  

(B) \textit{In vitro} kinase assay using His-PfPK6 and MBP-PfORC2 as a substrate in combination with Pfcyc1, Pfcyc2, Pfcyc3 and Pfcyc4. Coomassie gel shows equal loading of the MBP-PfORc2 in all the lanes. None of the PfPK6 and cyclin combination was able to phosphorylate PfORc2 protein.
Figure 2.14 Phosphorylation of Histone H1/PfOrc2 substrate by PfCrk5. (A) Kinase assay was performed to check the Histone H1 phosphorylation activity of PfCrk5 in combination with all the four cyclins or Ringo. Coomassie gel showed the equal loading of Substrate in all the lanes. (B) In vitro Kinase assay was performed to check the phosphorylation status of PfOrc2 by PfCrk5 and four cyclins as described in material and methods. Bottom Panel showed Coomassie stained gel as a loading control.
Another CDK related kinase, PfPK5 was identified as a first protein kinase related with CDK1 and CDK5 family of kinases. It shows maximum homology (60%) with CDK1 homologs of higher eukaryotes. Although it shows Histone phosphorylation activity following immunoprecipitation from *Plasmodium* extract, recombinant PfPK5 does not show any Histone phosphorylation activity in the absence of cyclin partner. Monomer of PfPK5 is also not an active form of the enzyme. PfPK5 activity can be stimulated in the presence of PfCyc-l, human cyclin H and RINGO (an activator of CDK2 in vertebrates) (Merckx et al, 2003).

So far, Histone HI is the only substrate known for PfPK5 activity. In order to investigate whether recombinant PfPK5 is active or not in our experimental conditions, we have performed kinase reaction in the presence of all four Pfcyclins as well as RINGO using Histone HI as a substrate. We find that PfPK5 can phosphorylate Histone HI only in the presence of RINGO (figure 2.15). On the contrary, we cannot detect any phosphorylation of Histone HI in the presence of Pfcyclins. Previously it was reported that PfPK5 autophosphorylates in the presence of Pfcyc-1 (LeRoch et al, 2000). However, in our experimental conditions we were unable to detect autophosphorylation of recombinant PfPK5 in the presence of Pfcyc-1. These results suggest that PfPK5 is active only in combination with RINGO.

Further, in order to test its ability to use PfOrc2 as a substrate, we performed the kinase reaction in the presence of MBP-PfOrc2 as a substrate. It is clear from the figure 2.15 that MBP-PfOrc2 is not phosphorylated by PfPK5 in any of the combination.
Figure 2.15 Phosphorylation activity of PfPK5. (A) Standard kinase reaction was performed with PfPK5 in combination with all four cyclins and Ringo using Histone H1 as a substrate. Only PfPK5/Ringo combination is able to phosphorylate Histone H1 as a substrate. Coomassie gel showed equal loading in all lanes. (B) In vitro kinase reaction was performed by adding recombinant proteins (His-PfPK5 in combination with PfCyclins or Ringo) along with PfOrc2 as a substrate. Autorad showed that none of the combination is able to phosphorylate PfOrc2. Bottom Panel (coomassie gel) shows the equal loading of PfOrc2 protein in all the five lanes.
2.3.7 Pull down of kinases from *Plasmodium* extract followed by kinase assay of PfOrc2

*In vitro* kinase assay results indicate that none of the *Plasmodium* Kinases in combination with Pfcyclins are able to phosphorylate PfOrc2 *in vitro*. These results suggest that there is specificity for Pfcyclin/PfCDK combination for the activation of these kinases. Alternatively, it is possible that Pfcyclins used in this study may have unidentified kinase partners that may be required for the formation of active Pfcyclin/PfCDK complex in *P. falciparum*.

In order to find out whether Pfcyclins are capable of capturing cognate PfCDKs from *P. falciparum* extract leading to the formation of active Pfcyclin/PfCDKs complex, we performed pull-down assay using GST or MBP bead containing Pfcyclins as fusion protein and *P. falciparum* extract followed by kinase assay using either PfOrc2 or Histone HI as substrates. The details of pull-down experiments are discussed in materials and methods. The pull-down experiments indicate that all the Pfcyclins can phosphorylate Histone HI as a substrate using *Plasmodium* extract suggesting formation of active cyclin/kinase complex (figure 2.16).

To check whether these Pfcyclins with cognate kinase from *Plasmodium* extract are able to phosphorylate PfOrc2, we also perform similar assay using PfOrc2 as a substrate. We find that PfOrc2 is phosphorylated only in the presence of activated Pfcyclin2, while Pfcyclin1, Pfcyclin3 and Pfcyclin4 fail to phosphorylate PfOrc2 although they are able to phosphorylate Histone HI under similar conditions (figure 2.16). To check the specificity of PfOrc2 phosphorylation by activated Pfcyclin2, we perform similar experiment by taking PfOrc2 wild type or PfOrc2 mutant proteins. We found that only PfOrc2 but not mutant PfOrc2 is phosphorylated by active Pfcyclin2 showing the specificity of the kinase.
reaction (figure 2.16). These experiments indicate that Pfcyclin2 could be the partner of Cognate CDK/cyclin complex that may play role in phosphorylating PfOrc2.

2.3.8 **Immunoprecipitation of PfPK5 from *Plasmodium* extract followed by kinase assay of PfOrc2**

*In vitro* kinase experiment results indicate that none of the Pf kinases in combination with Pfcyclins can phosphorylate PfOrc2. It may be possible that since cognate cyclin/kinase partner are required for their activation, these combinations may not be effective for Orc2 as substrate. Since PfCyclin2 was able to phosphorylate PfOrc2 when activated using *Plasmodium* extract, we were interested to find out whether any of these S-phase specific kinases were able to do so in similar fashion. Our lab has already shown that PfPK5 can phosphorylate another member of ORCs, PfOrc1 from *Plasmodium* extract (Unpublished data Mehra and Dhar).

To explore whether any of the S-phase specific kinases could use PfOrc2 as a substrate, we performed immunoprecipitation of PfPK5 and PfCrk5 from the *Plasmodium* extract using anti-PfPK5 antibody and anti-PfCrk5 antibodies as described in materials and methods. After immunoprecipitation, we performed the kinase assay using wild type MBP-PfOrc2 protein as a substrate. The results show that immunoprecipitated PfPK5 from the *Plasmodium* extract can phosphorylate PfOrc2 but under similar experimental conditions another S-phase specific kinase PfCrk5 fails to phosphorylate PfOrc2. To confirm whether this immunoprecipitated PfPK5 kinase reaction is specific for PfOrc2 domain we have performed the kinase assay with wild type PfOrc2 and mutant PfOrc2. The results show that only wild type PfOrc2 get phosphorylated by PfPK5 but not the mutant PfOrc2 confirming the
**Figure 2.16 Pull down assay from *Plasmodium* extract.** (A) GST-cycl1, GST-cycl2, MBP-cycl3 and GST-cycl4 were purified under native condition and kept bound on respective beads as described in material and methods. Bound beads were incubated with ~150mg of parasite extract from mixed Troph and Schizont stages followed by kinase reaction using Histone H1 as a substrate. All cyclins can phosphorylate Histone H1 as a substrate. (B) Similar reactions were carried out by using PfOrc2 as a substrate under similar experimental conditions. Unlike Histone, Only cyclin2 was able to phosphorylate PfOrc2 under these conditions. (C) To check the specificity of the reaction, kinase reaction with cyclin2 was repeated with PfOrc2 wild type and PfOrc2 mutant protein under similar conditions. Only wild type protein was phosphorylated and not the mutant protein under these experimental conditions.
specificity of phosphorylation of kinase domain of PfOrc2 by PfPK5 enzyme (figure 2.17). These results suggest that PfPK5 may be the potential kinase that acts as kinase enzyme for PfOrc2 as a substrate.

In summary, we have screened three CDK like kinases from *Plasmodium* in combination with four *Plasmodium* cyclins using Histone H1 (as a positive control) and PfOrc2 as a substrate in kinase assays *in vitro*. We observed that PfPK6 and PfCRK5 show differential affinity towards different cyclins (Pfcyc-2 and Pfcyc-4 for PfPK6 and Pfcyc-1 and Pfcyc-4 for PfCRK5) to form active kinase leading to the phosphorylation of control Histone H1. Interestingly, PfPK5 is not active in combination with any of the cyclins. However RINGO, a positive stimulator for mammalian CDK5, activates PfPK5 that can phosphorylate Histone H1. No phosphorylation of PfOrc2 is observed using any of the kinase and Pfcyclin combinations suggesting different substrate specificity of these different kinases.

Finally, pull-down experiments using Pfcyclins or PfPK5 and *Plasmodium* extract confirms the specificity of PfOrc2 phosphorylation by PfCyclin2 and PfPK5. These results clearly suggest that PfOrc2 may serve as a specific substrate for PfPK5 kinase activity during DNA replication and cell-cycle regulation in *Plasmodium*. The results of the kinase assay are summarized in table 2.T.1.
Figure 2.17 Immunoprecipitation from parasite extract using anti-PfPK5 and PfCrk5 antibody followed by kinase assay: (A) Anti-PfPK5 antibody or anti-PfCrk5 antibodies were incubated with *Plasmodium* extract (Troph/Schizont mixed) as described in material and methods. Kinase assay was performed using PfOrc2 as a substrate in kinase buffer using $\gamma^{32}$PATP. Coomassie gel showed the equal loading of PfOrc2 protein in both the lanes. (B) Kinase reaction was performed in similar fashion using anti-PfPK5 antibodies taking PfOrc2 wild and PfOrc2 Kinase mutant as substrate. Bottom panel Coomassie gel showed equal loading in both the cases.
### Table 2.T.1

**In vitro kinase and cyclin screening**

#### A. Activity with HistoneHI

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Cyclin</th>
<th>Substrate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PfCyc-1</td>
<td>PfCyc-2</td>
</tr>
<tr>
<td>1. PfPK5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. PfCRK5</td>
<td>+</td>
<td>-</td>
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</table>

#### B. Activity with PfOrC2

<table>
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<th>Cyclin</th>
<th>Substrate</th>
</tr>
</thead>
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<td>PfCyc-2</td>
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<tr>
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<td>-</td>
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<tr>
<td>2. PfPK6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. PfCRK5</td>
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**In vivo kinase and Cyclin screening**

#### A. PfKinase pull down from *Plasmodium falciparum* extract

<table>
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<tr>
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<th>cyclins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PfCyclin1</td>
</tr>
<tr>
<td>HistoneHI</td>
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<tr>
<td>PfOrC2</td>
<td>-</td>
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#### B. PfCyclin pull down from *Plasmodium falciparum* extract

<table>
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<td>PfPK5</td>
</tr>
<tr>
<td>PfOrC2 wild</td>
<td>++</td>
</tr>
<tr>
<td>PfOrC2 mutant</td>
<td>-</td>
</tr>
</tbody>
</table>

+++ Strong phosphorylation activity - No activity
| ++ | Moderate phosphorylation activity | ND | Not done |
| +  | Weak phosphorylation activity     |    |         |