

# Functional Dissection of the Catalytic Carboxyl-Terminal Domain of Origin Recognition Complex Subunit 1 (PfORC1) of the Human Malaria Parasite *Plasmodium falciparum*<sup>∇†</sup>

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**Origin recognition complex subunit 1 (ORC1) is essential for DNA replication in eukaryotes. The deadly human malaria parasite *Plasmodium falciparum* contains an ORC1/CDC6 homolog with several interesting domains at the catalytic carboxyl-terminal region that include a putative nucleoside triphosphate-binding and hydrolysis domain, a putative PCNA-interacting-protein (PIP) motif, and an extreme C-terminal region that shows poor homology with other ORC1 homologs. Due to the unavailability of a dependable inducible gene expression system, it is difficult to study the structure and function of essential genes in *Plasmodium*. Using a genetic yeast complementation system and biochemical experiments, here we show that the putative PIP domain in ORC1 that facilitates in vitro physical interaction with PCNA is functional in both yeast (*Saccharomyces cerevisiae*) and *Plasmodium* in vivo, confirming its essential biological role in eukaryotes. Furthermore, despite having less sequence homology, the extreme C-terminal region can be swapped between *S. cerevisiae* and *P. falciparum* and it binds to DNA directly, suggesting a conserved role of this region in DNA replication. These results not only provide us a useful system to study the function of the essential genes in *Plasmodium*, they help us to identify the previously undiscovered unique features of replication proteins in general.**

Origin recognition complex subunit 1 (ORC1), the largest subunit among the ORC components is essential for DNA replication initiation in eukaryotes. ORC1 has a regulatory function in DNA replication since it comes on and off chromatin during cell cycle. Human ORC binds to chromatin during G<sub>1</sub> phase of the cell cycle, followed by degradation of ORC1 by a ubiquitin-mediated pathway. ORC1 reappears during M phase, and it binds to DNA at the onset of G<sub>1</sub> phase (21). In mammalian cells, monoubiquitination and phosphorylation may also lead to the subcellular localization of ORC1 to control DNA replication (24). In the case of *Xenopus laevis*, ORC1 is bound to chromatin during early interphase but it is destabilized later with the loading of MCM proteins on chromatin (23). While in *Drosophila melanogaster*, the level of ORC1 is developmentally regulated (2), the murine ORC1 binds to specific locus in the ribosomal RNA in a cell-cycle-dependent manner (29). Interestingly, in the yeast *Saccharomyces cerevisiae*, although ORC is tightly bound to chromatin throughout the cell cycle, another pre-replication complex (pre-RC) protein, CDC6, comes on and off chromatin, ensuring the control of DNA replication during cell cycle (7, 22). The role of *S. cerevisiae* ORC1 (ScORC1) in ORC-DNA binding and modulating ScORC function has been described recently using high-resolution electron microscopy of ScORC (5).

ORC1 proteins consist of two highly conserved domains: the N-terminal regulatory domain that contains the bromo-adjacent homology domain and the C-terminal catalytic domain that contains the AAA<sup>+</sup> ATPase domain. The bromo-adjacent homology domain is involved in the regulation of gene expression through protein-protein interaction (4). It also facilitates the binding of ORC1 to the replication origin (21). The AAA<sup>+</sup> ATPase domain that binds and hydrolyzes ATP is essential for DNA replication in several organisms (11, 27).

*Plasmodium falciparum*, the causative agent of human malaria, contains an ortholog of ORC1/CDC6, although there is no separate CDC6 protein in *Plasmodium*. The homology of *P. falciparum* ORC1 (PfORC1) with other ORC1 counterparts is predominantly confined to the C-terminal region containing the putative nucleoside triphosphate (NTP)-binding and hydrolysis domain (residues 784 to 1014) (see Fig. S1 in the supplemental material). The N-terminal region (residues 1 to 783) and the extreme C-terminal region of PfORC1 (residues 1015 to 1189) exhibit poor homology with other ORC1 counterparts (14, 17) (see Fig. S1 in the supplemental material). The latter domain of PfORC1 (residues 1015 to 1189) may have a unique role in DNA binding since the crystal structure of archaeal (*Aeropyrum pernix* and *Sulfolobus solfataricus*) ORC1/CDC6-like protein along with origin DNA suggests that the extreme C-terminal region of this protein forms a wing-helix domain that binds to DNA (6, 10). Similarly, another member of the pre-RC, Cdc6, also contains a wing-helix domain at the extreme C terminus (15). It remains to be explored further whether the extreme C-terminal region of ORC1 will be responsible for origin DNA binding in eukaryotes.

During the asexual blood-stage *P. falciparum* developmental cycle, PfORC1 is expressed during the ring stage, colocalizes

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with the *P. falciparum* replication foci marker proliferating cell nuclear antigen (PfPCNA1) during the replicating-trophozoite stage, and is degraded completely at the late schizont stage, suggesting its regulatory role in *Plasmodium* DNA replication (12). Interestingly, the presence of a putative PCNA-interacting protein (PIP) motif in PfORC1 (residues 913 to 920) (see Fig. 5A and see Fig. S1A in the supplemental material) further supports the colocalization of PfORC1 and PCNA during DNA replication. The putative PIP domain was identified in different ORC1 homologs, including ScORC1, suggesting its conserved yet unidentified role in DNA replication (12). PCNA interacts with various proteins, like DNA polymerase, Fen1, CDT1, MCM10, etc., with diverse roles ranging from DNA replication to ubiquitination of various proteins leading to their regulation (19).

The presence of a putative NTP-binding domain, a putative PIP motif, and a unique extreme C-terminal region raises the issue of whether these domains have any functional relevance in PfORC1. It is extremely difficult to perform structure-function studies in *Plasmodium* due to the unavailability of a dependable inducible gene expression system. This is due to the time-consuming and poor transfection efficiency in *P. falciparum*. Moreover, an inducible gene expression system often requires expression of the transgene (transactivator) and the gene of interest under different promoters and use of different selectable markers when used episomally, causing considerable hindrance in regulation of gene expression. All of these exercises also may result in leaky expression of the gene of interest instead of tight regulation.

In order to dissect the functional domains of PfORC1, we adopted a yeast genetic complementation approach along with biochemical experiments. Earlier, genetic complementation experiments in yeast were performed for detailed structure-function analysis of *P. falciparum* proteins like dihydrofolate reductase and histone-acetyltransferase GCN5 (9, 28). Using a chimera approach for yeast genetic complementation, we found that the putative NTP-binding domain, the PIP motif, and the extreme C-terminal region of PfORC1 are truly functional in the yeast heterologous system, suggesting their important role in DNA replication.

These findings offer a useful tool to study the structure and function of essential proteins in *P. falciparum* that allows us to identify novel functional domains in ORC1 with a conserved role in DNA replication.

#### MATERIALS AND METHODS

**Parasite culture.** *P. falciparum* strain 3D7 was cultured in human O<sup>+</sup> erythrocytes in RPMI 1640 medium supplemented with 25 mM HEPES, 50 mg/liter hypoxanthine, 0.2% NaHCO<sub>3</sub>, 0.5% Albumax (Invitrogen), 0.2% glucose, and 10 μg/ml gentamicin sulfate. Synchronization of the cultures was achieved by 5% sorbitol treatment.

**ATP-binding assay.** The ATP-binding assay was performed using 1 μg of maltose-binding protein (MBP)-PfORC1C wild-type or mutant protein following the protocol described earlier (12). The proteins were cross-linked with [ $\alpha$ -<sup>32</sup>P]ATP in the presence of UV light followed by precipitation of cross-linked proteins by trichloroacetic acid. The pellet was further washed with acetone containing 0.5% HCl and twice with acetone. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and the radiolabeled ATP-bound protein was visualized using a PhosphorImager.

**Complementation of yeast ORC1 mutant.** Yeast complementation experiments were performed using an *S. cerevisiae* ORC1 “swapper” strain (A1Ay19) in which the chromosomal copy of *ORC1* has been deleted and a wild-type copy is

maintained in a yeast expression vector with a *ura* marker (a kind gift from Steve Bell, MIT). This strain was used for complementation using either wild-type ScORC1 or PfORC1 or different chimera constructs of *S. cerevisiae* and *P. falciparum* ORC1. Details of the methods are described in the experimental procedures part of the yeast complementation section in the supplemental material.

**DNA manipulation.** Full-length or different domains of PfORC1 were amplified by PCR using *P. falciparum* 3D7 genomic DNA as template and specific primers (see Table S1 in the supplemental material). Similarly, ScORC1 was amplified using *S. cerevisiae* genomic DNA and specific primers (see Table S1 in the supplemental material). For a detailed cloning strategy for the wild-type and mutant forms of PfORC1 and ScORC1, see the supplemental material.

**Recombinant protein purification.** For the purification of PfORC1C fusion proteins to perform the ATP-binding assay and electrophoretic mobility shift assay (EMSA), the *Escherichia coli* BL21 Codon Plus strain was transformed with respective recombinant clones. All recombinant proteins were MBP tagged and purified by affinity chromatography using amylose resin. His<sub>6</sub>-tagged proteins were purified using Ni-nitrilotriacetic acid (NTA) agarose beads (Qiagen). Detailed methods for protein purification are described in the supplemental material.

**Gel shift assay.** To investigate DNA binding activity of PfORC1C and other proteins, reactions were carried out in a 20-μl reaction volume containing DNA binding buffer (10 mM Tris-Cl [pH 7.5], 100 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 6% glycerol, 50 μg/ml bovine serum albumin). 30 ng of each protein was incubated in DNA binding buffer containing the appropriate radiolabeled [ $\gamma$ -<sup>32</sup>P]ATP DNA fragment (AT-rich probe, 242 bp, amplified from the PfGyrA sequence using primer set P53 and P54; telomeric probe, 175 bp, amplified from chromosome 3 telomeric DNA sequence of *P. falciparum* using primer set P51 and P52; and GC-rich probe, 242 bp, amplified from the *tel1* gene of plasmid pBR322 using primer set P55 and P56) for 30 min at room temperature. The samples were loaded on 5% native polyacrylamide gel, and the gel was run at 150 V for 5 to 6 h at 4°C and then dried and autoradiographed.

#### RESULTS

**Dissection of functional domains of PfORC1 in the yeast *S. cerevisiae*.** Based on the primary structure analysis, PfORC1 can be broadly divided into three domains. The N-terminal region does not show any homology with ScORC1, and it contains several repeat regions with asparagine and lysine residues (domain I; amino acid residues 1 to 783) (Fig. 1A).

The middle domain of PfORC1 shows maximum homology with ORC1 from different species. This domain (domain II; amino acid residues 784 to 1014) contains the conserved nucleotide binding and hydrolysis motif (residues 815 to 906) and a putative PIP motif (residues 913 to 920) (Fig. 1A).

The extreme C-terminal domain of PfORC1 (domain III; amino acid residues 1015 to 1189) exhibits very poor homology with other ORC1 homologs (Fig. 1A).

It is important to find out whether domain II and domain III have any important role in DNA replication *in vivo*. However, it is difficult to address these questions in *Plasmodium* itself due to the lack of a dependable inducible expression system. Therefore, we adopted a yeast genetic complementation assay to overcome these barriers.

Interestingly, cross-species complementation of ORC1 has not been reported yet. We decided to perform functional complementation in yeast using either full-length or deletion mutants of PfORC1 or chimera constructs of ScORC1 and PfORC1 fusing the N- and C-terminal regions of these respective proteins. We adopted the chimera approach due to the presence of repeat regions at the N terminus of PfORC1 that may affect the expression of full-length PfORC1 in yeast. Recently, a similar chimera approach has worked for the complementation of an ORC5 mutant yeast strain using PfORC5 (12). The N-terminal region of ScORC1 (ScORC1N; residues 1 to

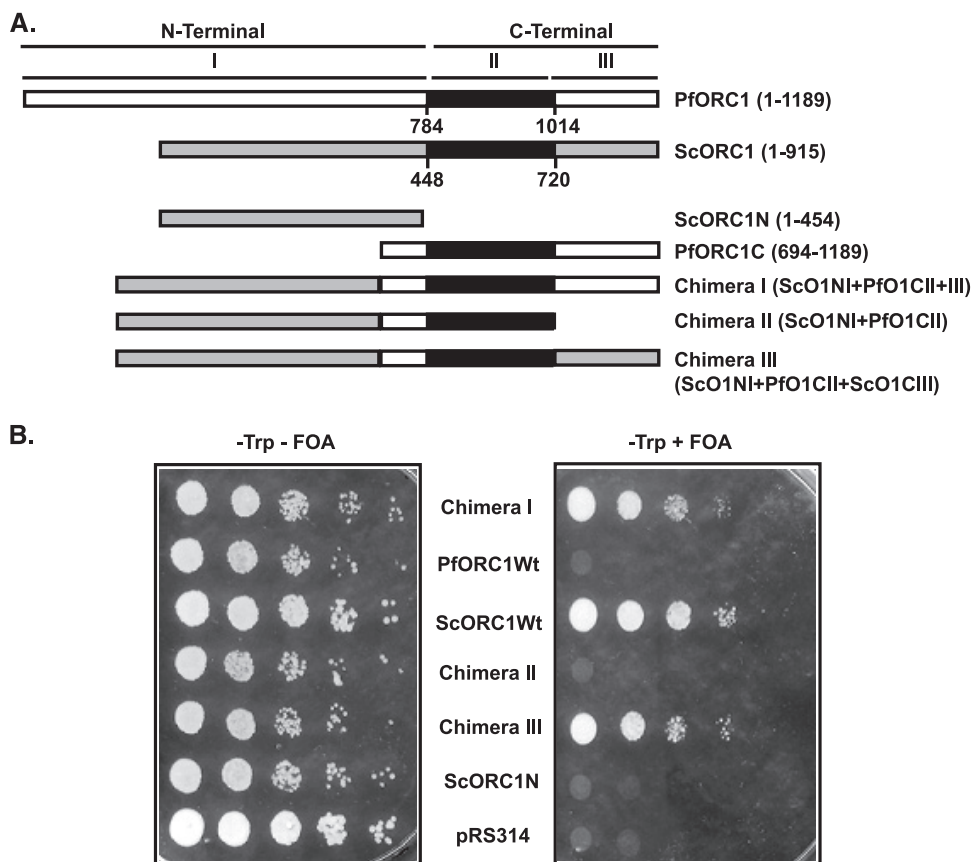


FIG. 1. Complementation of PfORC1 in the *S. cerevisiae* *ORC1* swapper strain. (A) Schematic diagrams of PfORC1, ScORC1, and different chimera constructs of the above two proteins. PfORC1 and ScORC1 can be divided into three parts: the N-terminal regulatory domain (I), the conserved middle domain (II), and the extreme C-terminal domain (III). The black box shows the homology region. In chimera constructs, the gray box shows the ScORC1 region and the open box shows the PfORC1 region. The rationale for chimera construction has been described in the Results section and in the Materials and Methods section in the supplemental material. (B) The *S. cerevisiae* *ORC1* swapper strain was transformed with the constructs shown in panel A, and the growth of these transformed yeast cells was followed by spot test using serial dilutions in either -Trp -FOA- or -Trp +FOA-containing plates. Only ScORC1, chimera I, and chimera III constructs rescued the growth of the mutant yeast strain in the presence of FOA as a selection marker. Control pRS314 vector did not support genetic complementation under the same experimental conditions. Wt, wild type.

454) was fused with the C-terminal region of PfORC1 (PfORC1C; residues 694 to 1189), containing the homology region and the extreme C-terminal region (domains II and III, respectively) to get chimera I (Fig. 1A). While deciding on the boundaries of different domains (I, II, and III) of ScORC1 and PfORC1 for production of chimera constructs, we considered various important features. These include the presence of a suitable restriction enzyme site within the coding region (to avoid creation of an extra restriction enzyme site during fusion) and the secondary structure of both PfORC1 and ScORC1 so that we do not delete helix or  $\beta$ -sheet regions of these proteins. These points are described in detail in the Materials and Methods section of the supplemental material. We also made chimera constructs in which the extreme C-terminal region (domain III) of PfORC1C was deleted (chimera II) or the extreme C-terminal region of PfORC1 (domain III) was replaced with the same region from ScORC1 (chimera III). An *ORC1* mutant haploid strain of *S. cerevisiae* (AIAY19, with the *S. cerevisiae* W303 background; a kind gift from Steve Bell, MIT) (3) with a deletion of chromosomal copy of *ORC1*

and having the wild-type *ORC1* gene in a plasmid containing a *ura3* marker was used for complementation studies. This yeast strain was transformed with plasmid constructs containing various DNA fragments (as shown in Fig. 1A and B) or pRS314 empty vector under a galactose-inducible promoter with a tryptophan marker. Following transformation, the transformants were grown either in the absence or presence of 5-fluoroorotic acid (5-FOA) for the selection of viable yeast cells in minimal media without tryptophan. We find that full-length ScORC1 complements this mutant yeast strain in the presence of 5-FOA, whereas full-length PfORC1 cannot rescue these cells under the same experimental conditions (Fig. 1B). Interestingly, chimera I complements these cells, whereas only the N-terminus region of ScORC1 or the empty vector pRS314 cannot rescue these cells (Fig. 1B). Similarly, PfORC1C cannot complement the yeast *ORC1* mutant strain (data not shown). Surprisingly, we find that chimera II excluding domain III of PfORC1C cannot complement the yeast strain, although chimera III (in which domain III of PfORC1C has been replaced with the same region from ScORC1C) can complement

the mutant yeast strain. These results suggest that the extreme C-terminal region of ORC1 plays a conserved role in DNA replication, although it does not show homology at the amino acid sequence level.

In order to confirm that the chimera I construct is indeed responsible for complementation of the *ORC1* mutant strain, we performed reverse transcription-PCR (RT-PCR) analysis using cDNA derived from either ScORC1 or the chimera I-complemented strains using primers specific for ScORC1 or the chimera I template. The ScORC1-specific primer sets could yield PCR products from ScORC1 template, and the chimera I-specific primers could amplify from the chimera I template only (see Fig. S2A in the supplemental material), suggesting that chimera I was indeed expressed at the mRNA level. The RT-PCRs using control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers show the presence of these transcripts in both samples (see Fig. S2B in the supplemental material).

The inability of PfORC1 to complement the yeast mutant strain may reflect the presence of repeat regions at the N terminus affecting the expression of full-length protein in yeast. Many open reading frames in *P. falciparum* contain several asparagines and lysine and serine-rich repeat regions (26). The heterologous expression of *Plasmodium* proteins is extremely difficult due to the ~80% AT richness of the *Plasmodium* genome and different codon usage in *Plasmodium*.

In order to investigate the expression level of PfORC1 in yeast at both the mRNA level and the protein level, we performed semiquantitative RT-PCR (as explained in Materials and Methods) and Western blot analysis, respectively. Following semiquantitative RT-PCR experiments, we find that the expression of PfORC1 is only ~10% at the transcript level compared to ScORC1 expression, while chimera I is expressed moderately (~50%) (see Fig. S2C and D in the supplemental material). The expression of control GAPDH transcript is found to be similar in all cases (see Fig. S2C, bottom panel, in the supplemental material). The expression of chimera I protein can be detected from yeast lysate obtained from chimera I-transformed yeast cells using anti-PfORC1 polyclonal antibodies, while full-length PfORC1 expression cannot be detected under the same experimental conditions (see Fig. S2E, upper panel, in the supplemental material). The Coomassie-stained gel following protein transfer on membrane shows the equal loading in both lanes (see Fig. S2E, lower panel, in the supplemental material). These results establish that the inability of full-length PfORC1 to complement the yeast ORC1 swapper strain is probably due to very weak or no expression of full-length PfORC1 in yeast.

**The nonconserved extreme C-terminal region of PfORC1 (domain III) is required for DNA binding.** It has been shown earlier that the extreme C-terminal region of ORC1/CDC6 contains a wing-helix region that is important for DNA binding (6). We wanted to know whether the extreme C-terminal region of PfORC1 would show DNA-binding activity, which is central to DNA replication.

For this purpose, we purified recombinant PfORC1C (amino acid residues 689 to 1189) as an MBP fusion protein either with the extreme C-terminal region (domain III) or excluding that region (PfORC1C Tr) (residues 689 to 1022) (Fig. 2A and B). We also purified a series of recombinant proteins as controls for DNA binding experiments. We purified

an unrelated protein, MBP-RINGO (~78 kDa), that stimulates *in vitro* activity of PfPK5 kinase (18). We also expressed and purified the extreme C-terminal region of PfORC1 (PfORC1C1; residues 1022 to 1189) and the N-terminal region of PfORC1 (PfORC1N; residues 1 to 182) as MBP fusion proteins (Fig. 2B). The estimated molecular masses of PfORC1C, PfORC1C Tr, PfORC1C1, and PfORC1N are 83 kDa, 76 kDa, 59 kDa, and 61 kDa, respectively. Alone, MBP (~50 kDa) was purified as a control. These proteins migrate in SDS-PAGE, inconsistently with their deduced molecular mass, except for PfORC1N, which migrates higher than the estimated molecular mass (Fig. 2B). This may be due to an inherent property of this protein that contains leucine heptad repeats. We performed a gel mobility shift assay using these proteins and a region (242 bp) from pBR322 DNA as a radiolabeled probe. We found that a population of labeled DNA was retarded significantly with increasing quantity of MBP-PfORC1C protein compared to the free probe without the addition of the protein (Fig. 2C). Interestingly, PfORC1C Tr protein did not show any such shift. MBP-RINGO (with a similar molecular mass to PfORC1C) and MBP alone also do not show any shift (Fig. 2C) suggesting that the extreme C-terminal region (domain III) of PfORC1 contains DNA-binding activity. However, this domain (PfORC1C1, domain III) alone does not confer DNA binding, suggesting that additional further upstream regions will be required for DNA binding (Fig. 2C). Interestingly, the N-terminal region of PfORC1 (PfORC1N; 1 to 182 residues) does not show any DNA-binding activity under the same experimental conditions (data not shown). Expression and purification of a longer polypeptide from the N terminus of PfORC1 will be required to determine specifically whether the N terminus of PfORC1 has any role in DNA binding. The presence of several asparagines and lysine repeat regions at the N terminus of PfORC1 affects the expression of this region in *E. coli*.

It has been shown recently that endogenous PfORC1 has affinity toward the telomeric region and telomere-associated repetitive elements (16). To find whether recombinant PfORC1C has affinity toward the telomeric region, we have performed a gel shift assay using radiolabeled probe containing the telomeric region, as described earlier (16). We find that the PfORC1C binds strongly to these regions, whereas PfORC1C Tr does not bind under the same experimental conditions (see Fig. S3A in the supplemental material).

In order to investigate whether the endogenous ORC has affinity toward telomere-specific radiolabeled probe, we performed a gel shift assay using *P. falciparum* nuclear extract. We found a specific band shift in the presence of the nuclear extract (see Fig. S3B in the supplemental material). These band shifts show supershift in the presence of anti-PfORC1 antibodies but not in the presence of preimmune sera, suggesting that these band shifts are specific for PfORC1 protein (see Fig. S3B in the supplemental material). We have recently shown that PfORC5, another member of the PfORC family, colocalizes with PfORC1 in the trophozoite stage. To investigate whether these band shifts are due to the presence of PfORC as a complex, we performed a supershift assay using anti-PfORC5 antibodies and preimmune sera. We found similar supershifted bands in the presence of anti-PfORC5 antibodies only (see Fig. S3B in the supplemental material). These

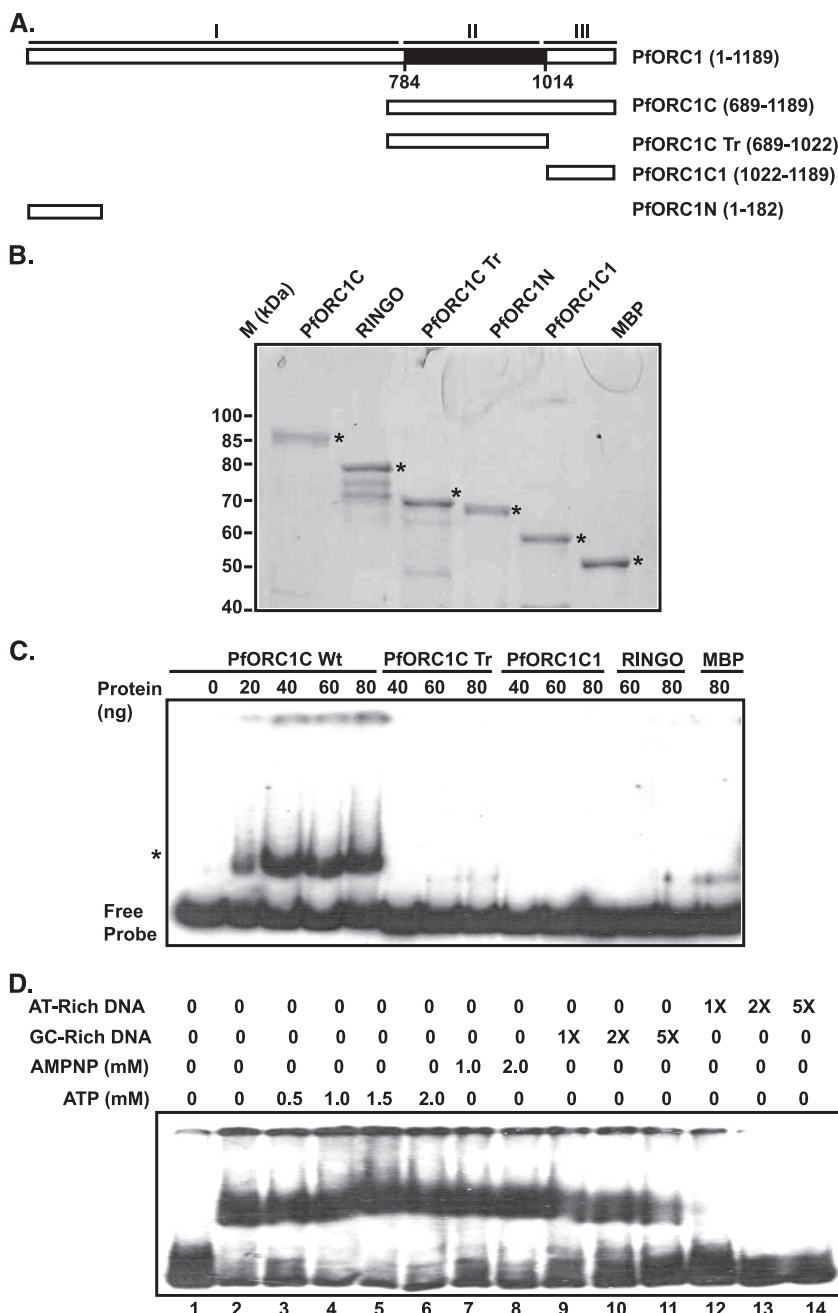


FIG. 2. DNA-binding activity of PforC1 and other deletion constructs by EMSA. (A) Schematic diagrams of PforC1 and different deletion mutants with amino acid coordinates are shown. The black box in PforC1 indicates the maximum homology region with ScORC1 containing the ATPase domain (domain II). (B) The Coomassie gel shows different MBP fusion proteins used in the EMSA. MBP alone, RINGO, and N-terminal PforC1N were used as controls. Asterisks indicate the major bands showing the purified protein. Lane M, molecular mass markers. (C) EMSA was performed using the proteins described above in the presence of radiolabeled AT-rich probe. Only wild-type (Wt) PforC1C shows strong DNA-binding activity (lanes 1 to 8). The asterisk shows the protein-DNA complex compared to the free probe. (D) DNA-binding activity of PforC1C does not depend on ATP hydrolysis. EMSA was performed in the absence or presence of different quantities of ATP or AMPPNP (a nonhydrolyzable analog of ATP) (lanes 1 to 8). The presence or absence of these nucleotides does not show any significant change in DNA-binding activity of PforC1C. PforC1C has affinity toward AT-rich DNA since competition by GC-rich cold DNA does not alter PforC1C DNA-binding activity efficiently, whereas the cold AT-rich DNA shows significant competition even at a 1X concentration (lanes 9 to 14).

findings suggest that the endogenous ORC as a complex has affinity toward the telomeric region.

To investigate whether ATP stimulates the DNA-binding activity of PforC1C, we performed gel mobility shift assays

with various concentrations of ATP or AMPPNP (5'-adenylyl- $\beta$ , $\gamma$ -imidodiphosphate), a nonhydrolyzable analog of ATP. We found that neither ATP nor AMPPNP has a significant effect on the DNA-binding activity of PforC1C (Fig. 2D). Since the

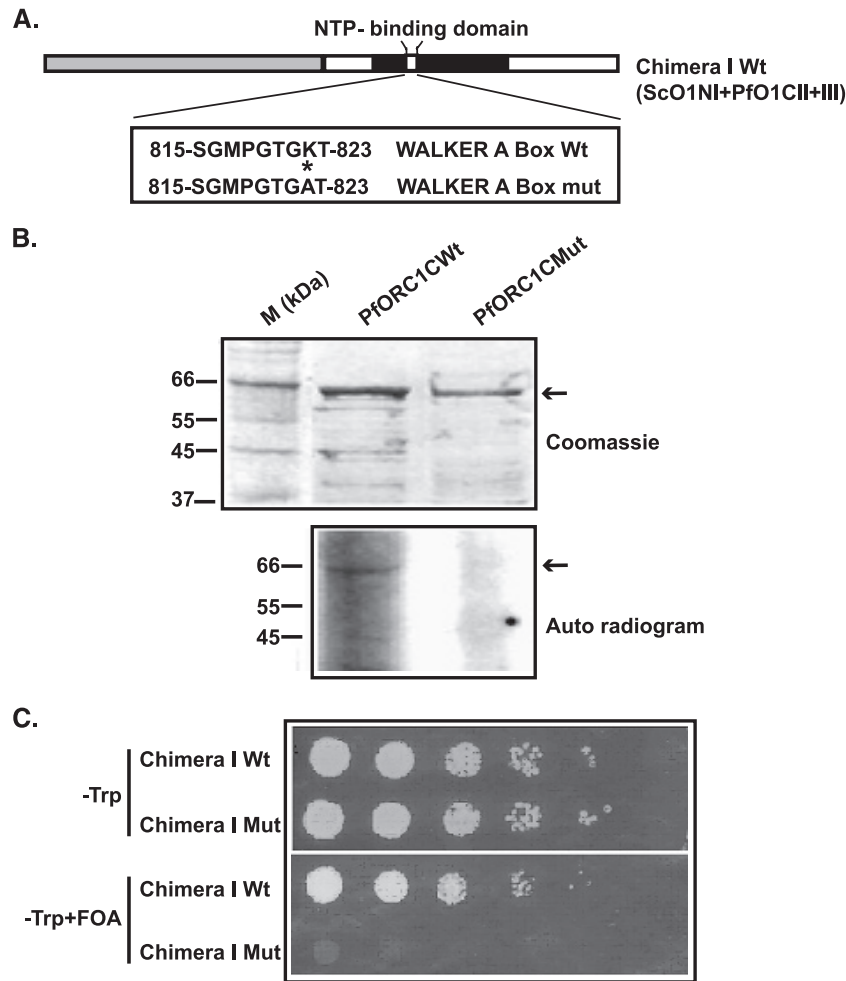


FIG. 3. The putative NTP-binding motif of PfORC1 is active both in vitro and in the heterologous yeast system. (A) Schematic diagram of the chimera I construct (with the N terminus of ScORC1 and the C terminus of PfORC1) showing the wild-type (Wt) or mutant (Mut) form of the NTP-binding domain in the inset box. Asterisks show the mutated residues. (B) ATP-binding assay. One microgram each of MBP-PfORC1CWt or MBP-PfORC1CMut proteins was incubated with  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ , and the mixture was further cross-linked using UV light as described in Materials and methods. The upper panel shows the SDS-PAGE analysis of these UV cross-linked proteins. The molecular mass markers (M) are shown on the left. The bottom panel shows the autoradiogram of the above gel. The arrowhead shows the position of the protein. (C) Genetic complementation assay with chimera I wild-type or mutant constructs in the yeast *ORC1* swapper strain as described above in the absence or presence of FOA. Only the wild type (Wt), not the mutant (Mut) chimera I construct, could complement the mutant yeast strain in the presence of FOA selection.

*Plasmodium* genome is ~80% AT rich, it is logical to assume that PfORC1C might have bias toward the AT-rich DNA. Accordingly, we performed the gel mobility shift assay in the presence of different quantities of cold AT-rich or GC-rich DNA of similar sizes as the radiolabeled probe. The results indicate that the cold GC-rich DNA does not affect the DNA binding affinity of PfORC1C toward the radiolabeled probe significantly, whereas the cold AT-rich competitor DNA can abrogate the DNA binding very efficiently (Fig. 2D), suggesting that PfORC1C indeed has a strong bias for AT-rich DNA.

**The putative ATP-binding motif present in domain II of PfORC1C is essential for ATP binding in vitro and viability in yeast.** The establishment of a functional complementation assay in a yeast *orc1* mutant strain using PfORC1 (domains II and III) allowed us further to dissect the role of the putative ATPase domain present in domain II of PfORC1 (Fig. 3A). It had been shown earlier that this domain is important for cell

viability in yeast. In vitro, ScORC binding to yeast ARS consensus sequences is dependent on the presence of Walker A and B motifs. Although we have reported earlier the ATPase activity of recombinant PfORC1C, the functional significance of this motif has not been characterized in vivo.

To address the issue of functional significance of the putative Walker A motif present in PfORC1C (domain II), we first investigated the ATP-binding activity of PfORC1C in vitro. For this purpose, wild-type PfORC1C and a mutant form of the protein (with a point mutation of K to A in the Walker A domain) were purified as recombinant MBP fusion proteins (Fig. 3A and B). We found that PfORC1C wild-type protein binds to radiolabeled  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ , whereas the mutant protein fails to do so, suggesting that the putative Walker A motif is functionally active (Fig. 3B, lower panel).

The in vitro ATP-binding assay gave us the confidence to test the functional significance of the putative NTP-binding

domain of PfORC1 in vivo. Accordingly, we made a point mutation in the Walker A domain in the chimera I construct and performed the yeast complementation experiments using either the wild-type or mutant form of the chimera I construct. We found that both constructs grow well in minimal media lacking Trp (–Trp), but only the wild-type chimera I and not the mutant form can grow in the presence of 5-FOA (Fig. 3C). These results suggest that the putative Walker A domain present in PfORC1C is indeed important for in vivo function and viability.

**The putative PIP domain present in yeast and *Plasmodium* ORC1 (domain II) plays a conserved role in cell viability.** PCNA is a master regulator of DNA replication and repair processes that involve the direct physical interaction of PCNA with several proteins that include but are not limited to Fen1, DNA polymerase, and MCM10 (19). We discovered earlier the putative PIP motif in PfORC1 and ScORC1 (12). We have also shown that the in vitro interaction between PfORC1 and PfPCNA is dependent on the putative PIP motif (12). Although, it is very encouraging to see the direct in vitro interaction between PfPCNA1 and PfORC1, there is no evidence of an in vivo physical interaction between these proteins in *P. falciparum*.

To address the issue described above, we performed a co-immunoprecipitation experiment using anti-PfORC1 antibodies from the replicating-trophozoite-stage parasite lysate (obtained following DNase I treatment of the parasite nuclei as described in Materials and Methods) followed by Western blot analysis using anti-ORC1 and anti-PCNA antibodies. Both anti-PfORC1 and anti-PfPCNA antibodies could recognize the respective proteins in the parasite lysate following Western blot analysis, whereas the preimmune sera failed to recognize any such band under the same experimental conditions, suggesting the specificity of these antibodies (Fig. 4A and B). Following coimmunoprecipitation experiments, we found that anti-ORC1 antibodies can pull down PfORC1 as well as PfPCNA from replicating-trophozoite-stage parasite lysate, whereas preimmune sera fail to do so under the same experimental conditions (Fig. 4C), suggesting that both PfORC1 and PfPCNA are truly in a complex in *P. falciparum* during active DNA replication. This is consistent with our previously published results showing interaction of another ORC subunit, PfORC5, with PfPCNA by coimmunoprecipitation experiments during the replicative-trophozoite stage (12). A reciprocal coimmunoprecipitation experiment using anti-PfPCNA antibodies for immunoprecipitation and anti-PfORC1 antibodies for immunoblotting was not conclusive (data not shown). It may be due to the poor efficiency of anti-PfPCNA antibodies to pull down the PCNA-ORC1 complex from the trophozoite-stage parasite lysate.

To further confirm the coimmunoprecipitation results, we performed immunocolocalization experiments using anti-ORC1 and anti-PCNA antibodies during the replicating-trophozoite stage and nonreplicating-late-schizont stage. We found that both PfORC1 and PfPCNA show a punctate staining pattern representing replication foci during the trophozoite stage, as described earlier (Fig. 4D) (12). Interestingly, both PfORC1 and PfPCNA foci mostly colocalize with each other during the replicating-trophozoite stage, confirming coimmunoprecipitation data as described above (Fig. 4D, rows 1 to 3). During the late multinucleus schizont stage (in which the in-

dividual nuclei have separated from each other following DNA replication) (Fig. 4D, rows 4 and 5), although bright PfPCNA signals are still visible, the PfORC1 signal is very weak and no distinct colocalization pattern between these proteins can be detected, in contrast to the trophozoite stage. This is also consistent with our previously published results, in which we have shown that PfORC1 is completely degraded during the late schizont stage (12). The colocalization pattern of PfORC1 and PfPCNA during the replicating-trophozoite stage in parasites truly reflects their presence in a complex.

To investigate whether the putative PIP domain in PfORC1C has any functional significance, we used yeast complementation experiments.

In order to find out first whether the putative PIP domain in ScORC1 is truly required for DNA replication and cell viability, we made a PIP domain mutant ScORC1 construct in which all of the conserved residues (in boldface) were changed to alanine (QDIMYNFF to ADIAYNAA) (Fig. 5A). We found that this mutant ScORC1 cannot complement the ScORC1 mutant strain, whereas the wild-type ScORC1 complements the mutant yeast strain efficiently (Fig. 5B). These results strongly suggest that the PIP domain in yeast is essential for its viability.

To further confirm our results, two conserved residues (the extreme Q and F residues) present in the putative PfORC1 PIP domain (QKVLFTLF) were mutated to alanine in the wild-type chimera I construct (Fig. 5A). The yeast ORC1 mutant strain was transformed using the wild-type chimera I or the mutant form along with the wild-type ScORC1 as a positive control and either the N terminus of ScORC1 or the C terminus of PfORC1 as a negative control. We found that the wild-type chimera I construct can complement the yeast ORC1 mutant strain. However, the PIP mutant form fails to complement the yeast strain (Fig. 5B), suggesting the importance of the PIP domain in cell viability and eukaryotic DNA replication in general.

In order to find out whether the mutation in the PIP domain affects the direct interaction between PfORC1 and PfPCNA1, we performed pull-down experiments using MBP-tagged PfORC1C (wild type) or the mutant form of the protein (PfORC1CPIPMut) along with MBP as control protein in the presence of His<sub>6</sub>-tagged PfPCNA1 (Fig. 5C). Pull-down experiments followed by Western blot analysis using anti-PfPCNA1 antibodies show that only wild-type PfORC1C, but not the mutant form nor MBP alone, can bind to PfPCNA1 (Fig. 5C), suggesting a direct interaction between ORC1 and PCNA1 through the PIP domain that may be required for cell viability and DNA replication. The Coomassie-stained gel (Fig., 5C, bottom panel) shows similar loading of wild-type and mutant forms of PfORC1C and the MBP control protein. Pull-down experiments were repeated several times, and similar results were obtained, as shown from another set of experiments in Fig. S3C in the supplemental material.

Furthermore, to validate whether the interaction between ORC1 and PCNA also occurs between the yeast counterparts, we have purified a carboxyl-terminal domain of ScORC1 (residues 401 to 780; ScORC1C) with either the wild-type or mutant PIP motif (ScORC1CPIPMut) as MBP fusion proteins along with ScPCNA as the His<sub>6</sub>-tagged protein (data not shown). Pull-down

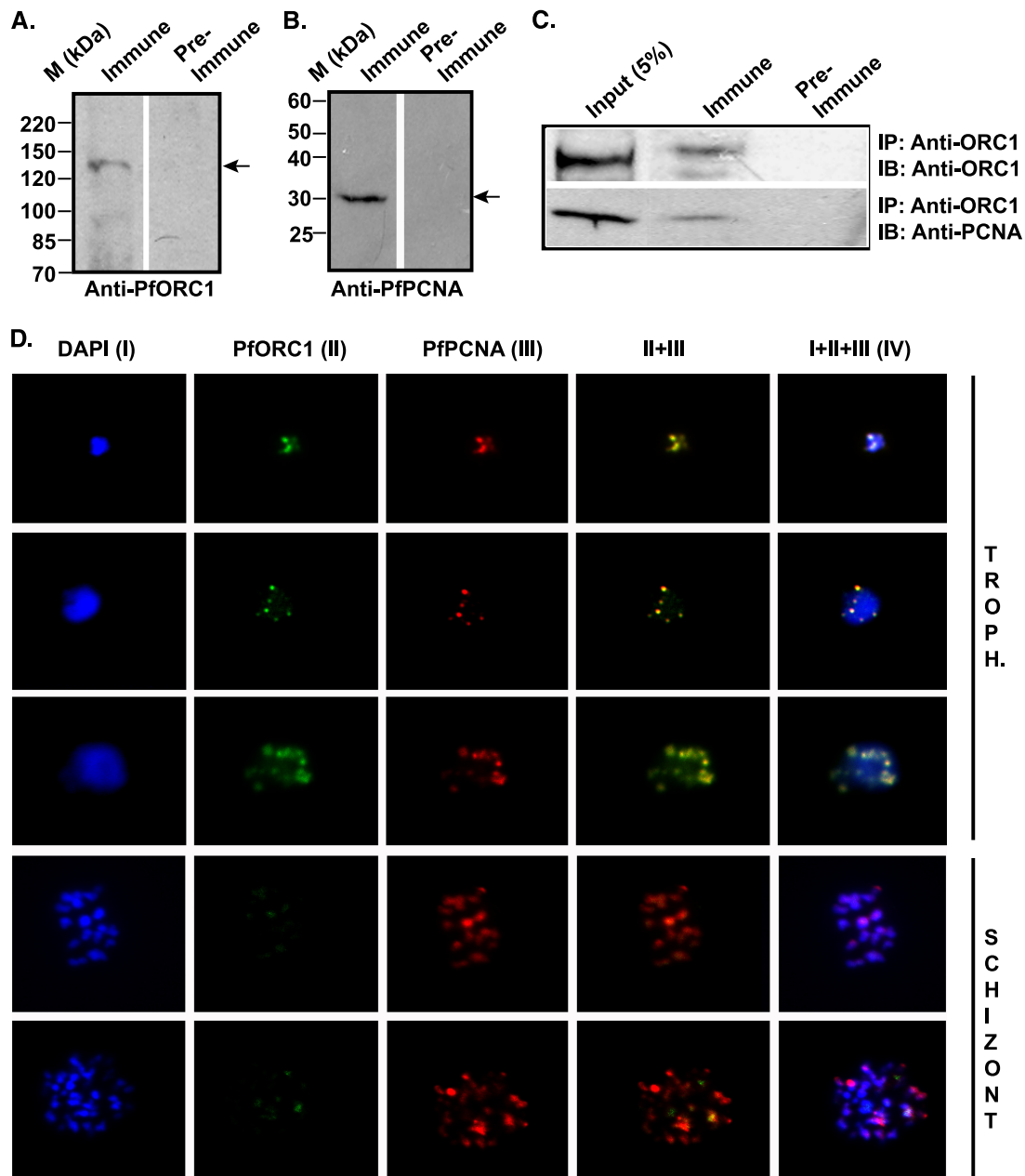


FIG. 4. Interaction between PfORC1 and PfPCNA1. (A) Western blot analysis of *P. falciparum* lysate using anti-PfORC1 antibodies detects a specific band corresponding to PfORC1. Preimmune sera do not recognize any such band. Molecular mass markers (M) are shown on the left. (B) Anti-PfPCNA1 antibodies but not the preimmune sera detect a specific band corresponding to PfPCNA1. (C) Coimmunoprecipitation of PfPCNA1 and PfORC1. DNase I-treated lysate obtained from the replicating-trophozoite-stage parasite was immunoprecipitated (IP) using anti-PfORC1 antibodies followed by immunoblotting (IB) using anti-ORC1 and anti-PfPCNA1 antibodies. Both PfORC1 and PfPCNA1 can be coimmunoprecipitated using specific anti-PfORC1 antisera, but not with preimmune sera. Input lanes are shown on the left. (D) Colocalization of PfPCNA1 and PfORC1 during the replicating-trophozoite stage. A glass slide containing a parasite smear from the replicating-trophozoite stage or schizont stage was treated for immunofluorescence studies as described in Materials and Methods using both anti-PfORC1 and anti-PfPCNA1 antibodies. Distinct colocalization of PfORC1 and PfPCNA1 foci can be detected during the replicating-trophozoite stage (Troph.; rows 1 to 3). Although the expression of PfPCNA can be detected at the multinucleated late schizont stage (rows 4 and 5), the expression of PfORC1 is barely visible as the protein is degraded at the late stage, as described earlier. DAPI (4',6-diamidino-2-phenylindole) (I) shows the nuclei. Column IV represents merged panels I, II, and III.

experiments as described above followed by Western blot analysis using anti-His antibodies clearly indicate that ScPCNA binds to ScORC1C wild-type protein with stronger affinity than ScORC1PIPMut under the same experimental conditions (Fig.

5D), suggesting the importance of the PIP domain for the direct physical interaction between ScORC1 and ScPCNA. The Coomassie-stained gel (Fig. 5D, bottom panel) shows the equal loading of both proteins.



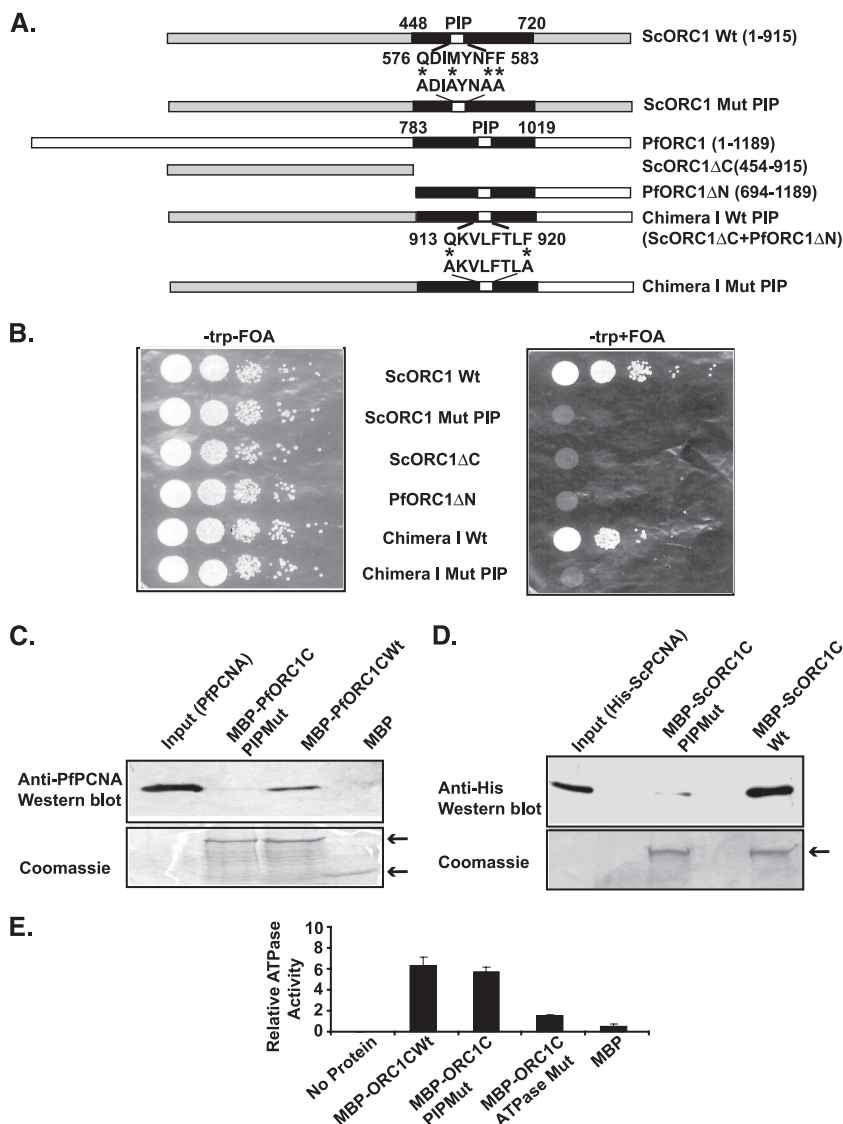


FIG. 5. Role of the PIP domain in cell viability and interaction with PCNA. (A) Schematic diagrams of wild-type (Wt) ScORC1, P<sub>f</sub>ORC1, chimera I, and their different mutant (Mut) forms (as indicated on the right). The sequence and amino acid coordinates for the PIP box are shown. Asterisks show the residues mutated for the study. (B) Yeast complementation assay to show the PIP domain is essential for yeast as well as chimera proteins. The yeast ORC1 swapper strain was transformed with the constructs described above, and the viability of the yeast cells was tested following a spot test after serial dilution in the absence (-FOA) or presence (+FOA) of FOA selection. The results indicate that the wild-type PIP box is important for survival for ScORC1 as well as chimera I construct. (C) Pull-down experiments using beads containing the wild-type or PIP mutant form of MBP-P<sub>f</sub>ORC1C or the MBP control proteins in the presence of His<sub>6</sub>-P<sub>f</sub>PCNA as described in Materials and Methods. Western blot analysis using anti-P<sub>f</sub>PCNA antibodies shows the specific binding of wild-type P<sub>f</sub>ORC1C with P<sub>f</sub>PCNA. The bottom panel shows the Coomassie-stained gel following protein transfer as a loading control. The arrowheads show the purified MBP fusion proteins (top) or MBP alone. (D) Pull-down experiments using yeast proteins. The pull-down experiments were performed using soluble His<sub>6</sub>-ScPCNA protein and the wild-type or PIP mutant form of MBP-ScORC1C protein bound on beads as described above for *Plasmodium* proteins followed by Western blot analysis using anti-His polyclonal antibodies. The results indicate the strong affinity of ScPCNA toward the wild-type ScORC1C compared to the mutant form of the protein. The bottom panel shows the Coomassie-stained gel following protein transfer, and the arrowhead indicates the position of the respective proteins. (E) ATPase activity of different proteins. The ATPase assay was performed as described in Materials and Methods using the wild-type or PIP mutant form of MBP-P<sub>f</sub>ORC1C or MBP-P<sub>f</sub>ORC1C (ATPase mutant) or MBP, and the relative ATPase activity of each protein was plotted accordingly. The results indicate that the activities of the wild-type and PIP mutant forms of P<sub>f</sub>ORC1C do not differ significantly.

The conserved Walker B ATP hydrolysis domain of P<sub>f</sub>ORC1 resides within residues 903 to 906, and the putative PIP motif resides within residues 913 to 920. To test whether the proximity of these domains affects the ATP hydrolysis function of P<sub>f</sub>ORC1 due to the mutation in the PIP domain, we purified

the P<sub>f</sub>ORC1C wild type and P<sub>f</sub>ORC1CPIP mutant and performed ATP hydrolysis assay along with an ATP-binding mutant form of P<sub>f</sub>ORC1C (17) and MBP as control proteins (as described in the Materials and Methods section in the supplemental material). We found that MBP and the P<sub>f</sub>ORC1C

ATP-binding mutant do not show significant ATPase activity, whereas both PfORC1C wild-type and the PfORC1CPIP mutant proteins show similar ATPase activity, suggesting that mutation in PIP domain does not affect the catalytic activity of PfORC1 significantly (Fig. 5E). These results suggest that the inability of chimera I PIP mutant construct to complement the yeast *ORC1* swapper strain is most likely because of the defect in interaction between these proteins and is not due to the effect on the ATPase activity.

## DISCUSSION

The presence of the PIP motif in ORC1 homologs is an important finding (12). Here we show that the mutation in the PIP motif of ScORC1 leads to the loss of viability of yeast cells, suggesting its essential role in DNA replication. Similarly, the non-viability of the PIP mutant form of chimera I-transformed ScORC1 mutant cells confirms that the PIP domain is conserved and functional across the species. The direct interaction between PfPCNA1 and wild-type PfORC1C but not the PIP mutant protein clearly indicates that the PIP motif is truly important for this interaction. These findings set the platform to explore the biological significance of the ORC-PCNA interaction in vivo. We think that ORC foci do not represent the ORC proteins participating in pre-RCs only but rather ORC proteins that bind to PCNA directly via the PIP box at the replication foci. This is consistent with our previously published results showing the reduction of ORC foci in the presence of hydroxyurea, a known replication inhibitor (12). Perhaps the loss of ORC1 (which contains the PIP box) at the late schizont stage explains why the overlap between PfORC5 and PCNA also seems to decline later in the S phase (12).

Alternatively, it remains to be explored further whether the PIP domain in PfORC1 is responsible for the SCF-skp2-cul4-dependent degradation of PfORC1 at the later stages, as has been shown for *cdt1*, another important replication initiation protein conserved in higher eukaryotes (1, 20, 25).

We find that the PIP domain is important for cell survival in yeast and *Plasmodium*. Since there is a basic difference in multiple rounds of rapid DNA replication during the blood stage in *Plasmodium* compared to the single round of DNA replication in the yeast cell cycle, the mechanism and the extent by which PIP domain will modulate PCNA and ORC function in different organisms may differ significantly.

The PfPCNA foci obtained during the late schizont stage may explain its involvement in repair processes like translesion synthesis, as reported earlier in mammalian cells (7, 8, 19). The presence of PCNA at the late stages may also be required for ubiquitination-dependent degradation of CDT1 like licensing factors in *Plasmodium*, important for the control of DNA replication (1). Interestingly, a putative CDT1 homolog has been found in *P. falciparum* recently (S. K. Dhar and A. Pietro, unpublished data).

The conserved function of the extreme C-terminal region of ORC1 with little or no sequence homology is also a novel finding. It has been reported that the archaeal CDC6/ORC1 like protein primarily binds to origin DNA through the wing-helix domain present at the extreme C-terminal region of these proteins (10). It is important to note that unlike higher eukaryotes, archaea contain defined replication origins where

initiation takes place. *S. cerevisiae* ORC binds to ARS consensus sequence in a sequence-specific manner in an ATP-dependent fashion. *Homo sapiens* ORC (HsORC) and *D. melanogaster* ORC (DmORC) also bind to DNA as a complex, and there has been no report so far that an individual ORC subunit may bind to DNA in these systems. Binding of PfORC1 to DNA like archaeal ORC1/CDC6 protein may suggest that *Plasmodium* ORC1 is closer to the archaeal counterpart. In fact, phylogenetic analysis of C-terminal region of PfORC1 with other ORC1 homologs shows that it is closer to archaeal CDC6/ORC1 (data not shown). Surprisingly, *Plasmodium* also does not contain a separate CDC6 like molecule like archaea and it contains fewer ORCs like ORC5 and possibly an ORC2 homolog. The DNA binding capability of PfORC1 thus may become important and significant due to the presence of simpler ORC with fewer components in *Plasmodium*.

The secondary structure analysis of the extreme-C-terminal region of PfORC1C reveals the presence of a helix-turn-helix domain similar to that has been reported in yeast, human, and archaeal CDC6/ORC1 proteins (data not shown) (6). Together with the in vitro DNA-binding activity of the C-terminal region of PfORC1 and the presence of the helix-turn-helix domain at the extreme C-terminal region of PfORC1, in vivo complementation results convincingly establish a global conserved role of this domain between highly divergent species.

It is evident from the in vitro ATP-binding assay and in vivo complementation assay that the putative NTP-binding domain in PfORC1 is functional. PfORC1 shows ATP-independent DNA-binding activity, contrary to *S. cerevisiae* ORC, whose DNA-binding activity is dependent on ATP binding and hydrolysis. PfORC1 may show nonspecific ATP-independent DNA binding in vitro, whereas ATP may play a major role in binding active origins in vivo. The in vivo complementation of the ScORC1 mutant strain with the chimera I construct containing the NTP-binding domain supports this hypothesis.

Does ORC1 play similar role in other protozoan parasites? Recently, an ORC1 homolog has been reported in the kinetoplastid *Leishmania major* that codes for a much smaller ORC1 protein (~434 amino acid residues) than PfORC1 (1,189 amino acid residues) (13). *L. major* contains only one ORC subunit with a conserved ATPase domain. Immunofluorescence analysis of the green fluorescent protein-*L. major* ORC1 (LmORC1)-expressing parasite line shows constitutive nuclear expression of LmORC1 throughout the cell cycle. This is contrary to the regulated expression of PfORC1 during the asexual blood-stage cycle. However, this study does not describe the fate of the endogenous protein due to the non-availability of anti-ORC1 antibodies.

In this study, we have used yeast genetic complementation and biochemical experiments to study essential gene function in *Plasmodium*. We find these techniques very useful to dissect PfORC1 function in vivo and to identify previously undiscovered unique features of ORC1 proteins.

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