

Analogous expression pattern of *Plasmodium falciparum* replication initiation proteins PfMCM4 and PfORC1 during the asexual and sexual stages of intraerythrocytic developmental cycle

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Introduction

Plasmodium falciparum, the deadliest parasite on the earth, affects more than 500 million people worldwide (Ridley, 2002). The increasing trend of antimalarial drug resistance and the lack of an effective vaccine add significantly to the global malaria burden. An in-depth study of molecular basis of parasite life cycle would be a key to development of novel antimalarials. DNA replication initiation, a fundamental step in parasite biology, might be useful in this regard as DNA replication takes place at several points in the parasitic life cycle including both the human and mosquito hosts (White & Kilbey, 1996).

The key step in the DNA replication initiation is the loading of the six-protein origin recognition complex (ORC) at the chromosomal DNA replication initiation sites followed by the concomitant loading of CDC6 (a component of the prereplication complex, conserved in eukaryotes) and hexameric replicative helicase MCM2-7 complex (Kelly & Brown, 2000; Bell & Dutta, 2002). Analysis of *P. falciparum* genomic database (PLASMODB.ORG) reveals the presence of ORC1, ORC2 and all the subunits of MCM proteins, suggesting that some of the important steps in the

Abstract

DNA replication takes place at five different stages during the life cycle of *Plasmodium falciparum* including the human and mosquito hosts. DNA replication initiation, the rate-determining step is poorly understood in *Plasmodium*. Here we show that PfMCM4 and PfORC1, two members of prereplication initiation complex are expressed specifically in the nucleus during the trophozoite and schizont stages of the asexual parasitic life cycle where maximum amount of DNA replication takes place. Further, we show that these proteins are also expressed in gametocytes, where DNA replication also occurs. These results expand our knowledge on these proteins and resolves discrepancies arising from previous studies with respect to the expression pattern of replication initiation proteins during the parasite's life cycle.

DNA replication initiation process might be conserved in this parasite.

At the time of asexual erythrocytic schizogony, multiple rounds of DNA replication take place leading to the formation of 16–32 new parasites from a single parasite-infected red blood cell. During sexual development, the DNA content of *Plasmodium* mature gametocytes reaches between 1C and 2C (Janse *et al.*, 1988), suggesting that selective genome duplication or gene amplification of haploid genome takes place during this process. However, at the onset of gametogenesis in the mosquito midgut, microgametocytes undergo three successive rounds of rapid DNA replication just before exflagellation (Janse *et al.*, 1986). These observations strongly suggest that DNA replication is a rapid but highly co-ordinated process that takes place both at the asexual and sexual stages of the parasitic life cycle including human and mosquito hosts.

Therefore, it is important to characterize the *P. falciparum* replication initiation proteins to investigate their functional role during different developmental stages. There are few reports of characterization of PfORC1 (Li & Cox, 2003; Mehra *et al.*, 2005) and MCM proteins (Li & Cox, 2001; Patterson *et al.*, 2006) in *Plasmodium*. However, some

discrepancies between these reports remain unsolved. For example, PfMCM4 was initially reported to be present at the RNA level only in the sexual stage (Li & Cox, 2001). A recent report shows the presence of PfMCM2, 6 and 7 subunits at the protein level during the asexual stages (Patterson *et al.*, 2006). A recent proteome analysis data of sexual stage-specific parasites confirms the presence of PfMCM4 in the gametocytes (Khan *et al.*, 2005). Like PfMCM4, PfORC1, a member of the preinitiation complex, was initially shown to be present only during the sexual stage (Li & Cox, 2003). In contrast, PfORC1 was shown in another study to be expressed both at the RNA and protein levels in late trophozoites and early schizonts (Mehra *et al.*, 2005). To resolve the issue of stage-specific expression of these proteins, we decided to investigate the expression pattern of PfORC1 and PfMCM4 in asexual parasites and in gametocytes. ORC1 and MCM4 proteins are the key partners of the replication initiation complex (pre-RC). Our studies for the first time show that PfORC1 and PfMCM4 are expressed predominantly in the nucleus during the asexual and sexual stages suggesting their functional role in DNA replication initiation at both the stages.

Materials and methods

Parasite culture

Plasmodium falciparum strain 3D7 was cultured in human erythrocytes in RPMI 1640 medium containing 0.2% NaHCO₃, 10% heat-inactivated pooled human sera type A, gentamycin sulphate (10 µg mL⁻¹) and 0.2% glucose. Parasites were synchronized with 5% sorbitol (Mehra *et al.*, 2005). To lyse the infected erythrocytes, 0.05% saponin was used and parasites were recovered by centrifugation (1000 g) and washed with cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4).

RNA extraction and RT-PCR

RT-PCR analysis was performed essentially following the protocol as described elsewhere (Mehra *et al.*, 2005). The following primers (Table 1) were used for the respective genes: PfMCM4 (PfM4RTFw and PfM4RTRv), PfMCM5 (PfM5RTFw and PfM5RTRv), PfORC1 (PfO1RTFw and PfO1RTRv), PfGAPDH (PfGAPDHFw and PfGAPDHRv). Semi-quantitative analysis of RT-PCR products was performed by quantitating the intensities of the amplified products by densitometry analysis.

DNA manipulations

Plasmodium falciparum MCM4 ORF (PF13_0095) was amplified by PCR using *P. falciparum* strain 3D7 genomic DNA as template and PfMCM4Fw and PfMCM4Rv primer

Table 1. List of primers

Primers	Primer sequences
PfMCM4Fw	5'-CGGAATTCATGGGTACACCAAGAAGAAG-3'
PfMCM4Rv	5'-CGGAATCTTACTTTTTCTTATATAATCC-3'
PfM4RTFw	5'-AGGAGCAGTCGTATTATCAGATAAGG-3'
PfM4RTRv	5'-TTTCTAGCTCTCTGTTGTGTAATATAG-3'
PfM5RTFw	5'-GTTTATGTTTCATGGTGTGTTTG-3'
PfM5RTRv	5'-GCAACAGATGGATCACCTAAC-3'
PfO1RTFw	5'-GTACTCATTTCTATACACCT-3'
PfO1RTRv	5'-AAAGATTATTTTATTTAAC-3'
PfGAPDHFw	5'-ATGCCAAGTAGATGTTGTATG-3'
PfGAPDHRv	5'-TCGTACCATGAAACTAATTTG-3'
PfM4Fw	5'-CGGAATCTTAAAACAAGGAGAAACGCC-3'
PfM4Rv	5'-CGGAATCTCAACAGAGTACAGTGGCTAG-3'

sets (Table 1). For antigen purification, a ~986 bp fragment from the PfMCM4 ORF (nucleotide positions 1182–2168; fragment 'a' in Fig. 1a) was also amplified as above using primers PfM4Fw and PfM4Rv (Table 1), and was cloned in the expression vector pET28a (Novagen) at the *Eco*RI site and subsequently sequenced.

Protein purification and raising polyclonal antibodies

To purify the truncated His₆-PfMCM4 fusion protein (fragment 'a', Fig. 1a), *Escherichia coli* strain BL21 codon plus was transformed with pET28a recombinant plasmid construct. Truncated His₆-PfMCM4 was purified by Ni-NTA (Qiagen) affinity chromatography following the protocol as described elsewhere (Mehra *et al.*, 2005).

To raise polyclonal antibodies against PfMCM4, purified protein was mixed with Freund's adjuvant and mixture was used to immunize two rabbits.

Immunodetection and immunolocalization

For immunodetection, parasite pellets (mostly trophozoites) obtained by saponin lysis were washed with 1 × PBS and then boiled with SDS gel loading buffer and loaded directly on 8% SDS-PAGE gel. Western blot analysis was carried out using anti-PfMCM4 antibodies using standard protocol.

For *in vivo* localization, immunofluorescence analysis was performed following the protocol described elsewhere (Mehra *et al.*, 2005). Anti-PfMCM4, preimmune sera and secondary antibodies (Alexa Flour 488; goat antirabbit IgG) were used at the dilution of 1:3000 and 1:1000, respectively. For IFA in gametocytes, same protocol was followed using the following dilution for the antibodies PfMCM4 (1:400), PfORC1 (1:400), PfNAPS (1:400) and Pfg27 (1:750).

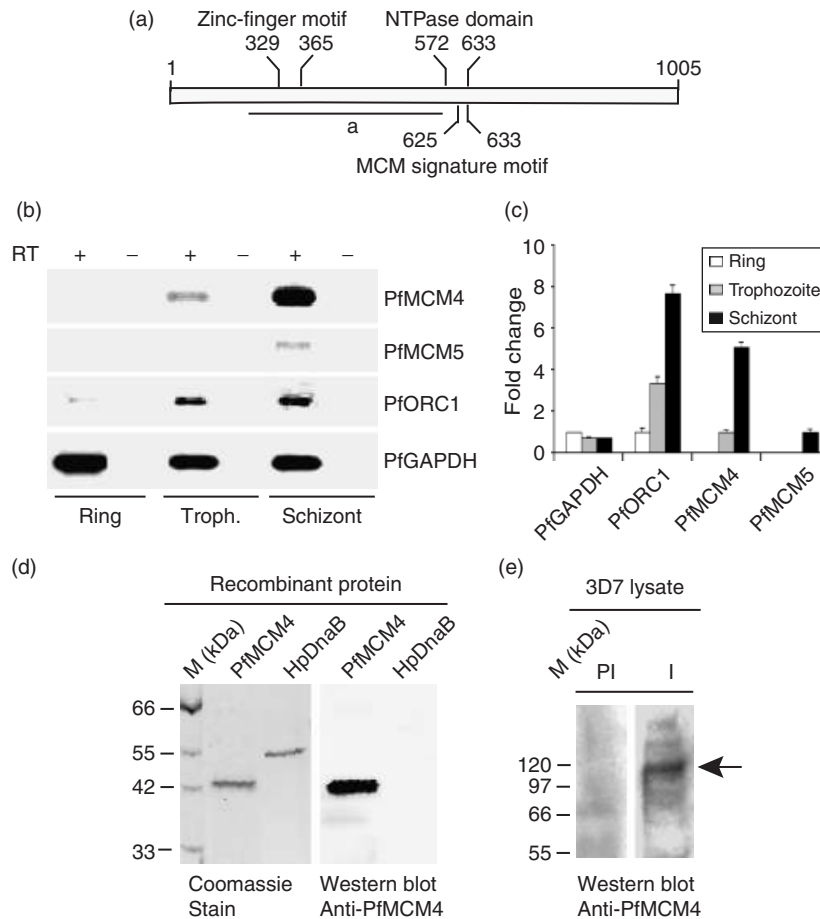


Fig. 1. Stage-specific expression of PfMCM4. (a) Schematic diagram of 1005 amino acid residues long PfMCM4. Different functional domains and their respective positions are marked. The solid line below indicates the region of PfMCM4 that have been expressed and purified for polyclonal antibodies generation. Primers used to amplify this region are shown in Table 1 (PfM4Fw and PfM4Rv). (b) RT-PCR analysis of PfMCM4 at the asexual stages. The reverse transcription (RT) products were made from total RNA isolated from different synchronized erythrocytic stages using Invitrogen kit following the manufacturer's instructions. The RT products were subsequently PCR amplified using primers as shown in the Table 1 and resolved in a 1.5% agarose gel. RT(-) lanes indicates PCR products that were generated using control templates without reverse transcriptase. No products were obtained in the RT(-) lanes. (c) Fold change in the expression of PfMCM4 at the different stages. The relative intensity of each band was calculated using densitometry scanning and the absolute values were obtained by normalizing against background intensity. (d) Specificity of PfMCM4 antibodies. Three hundred nanograms of purified PfMCM4 (truncated) or *Helicobacter pylori* DnaB helicase protein were loaded in SDS-PAGE (as shown in Coomassie-stained gel) followed by Western blot analysis using anti-PfMCM4 antibodies. The antibodies cross-reacted specifically with the recombinant PfMCM4 protein but not with the control HpDnaB protein. (e) 3D7 parasites enriched in late trophozoites/early schizont stages were boiled in the SDS-PAGE loading buffer followed by SDS-PAGE analysis of the lysate and Western blot analysis using either preimmune or immune anti-PfMCM4 sera. A band ~120 kDa was detected only in the immune lane and not in the preimmune lane suggesting that PfMCM4 is expressed at the protein level in the parasites.

Immunoprecipitation

Immunoprecipitation experiments were performed following the protocol as described by Patterson *et al.* (2006) with some modifications. In brief, the antibodies were incubated with protein A Sepharose CL-4B (Amersham) beads for 30 min with constant agitation at 4 °C. Two hundred micrograms of trophozoite stage-specific parasite extract were mixed with this antibody-bead complex and incubated for

1 h at 4 °C in the presence of binding buffer (10 mM TrisCl, pH 7.5, 100 mM NaCl, 0.1% NP-40). The beads were then washed three times with wash buffer containing the same binding buffer except for the addition of 1 M NaCl and 0.5% NP-40. The bound proteins were resolved in SDS-PAGE followed by Western blot analysis either with anti-PfMCM4 antibodies or with commercial antiphosphoserine and antiphosphothreonine antibodies or with anti-PfORC1 antibodies.

Results and discussion

To investigate the stage-specific expression of PfMCM4, we decided to clone, express and raise specific antibodies against this protein. The ORF was amplified by PCR using specific primers (PfMCM4Fw and PfMCM4Rv, Table 1) and 3D7 genomic DNA. Following PCR, a ~3.0 kb fragment was obtained (data not shown). The PfMCM4 PCR product was subsequently cloned into pET28a vector and sequenced. The deduced amino acid sequence confirms previous studies showing that PfMCM4 displays 58–62% similarity and 38–41% identity with the MCM family of proteins across the conserved region (residues 203–995). PfMCM4 contains the conserved NTP binding and hydrolysis domains, MCM signature motif and a putative zinc finger domain (Tye, 1999). The relative positions of these domains are marked in the schematic diagram (Fig. 1a). PfMCM4 shows some unique features not present in other MCM4 members. It contains five unique amino acid inserts ranging from seven to 75 residues that might account for the largest protein containing 1005 amino acid residues among the MCM family of proteins. Further, it is interesting to note that the putative zinc finger domain contains 13 residues between the first two cysteine residues instead of two residues as found in other family members (Li & Cox, 2001). The importance of these residues needs to be explored further.

To find out the expression of PfMCM4 at the transcript level during the asexual erythrocytic stages, semi-quantitative RT-PCR analysis was performed using cDNA isolated from different erythrocytic stages. The PCR reactions were continued for ~25 cycles in order to avoid the saturation of the PCR products. Analysis of the RT-PCR products shows that PfMCM4 is expressed mostly during the schizont stages, although some transcripts could be found in the trophozoite stages (Fig. 1b). The expression pattern of PfMCM4 is similar to that of PfORC1, the expression of which peaks at the schizont stage (Mehra *et al.*, 2005). We also analyzed the expression pattern of PfMCM5, another member of the MCM family of proteins that has not been earlier characterized in *Plasmodium*. We found that the overall expression level of PfMCM5 was low compared with that of PfMCM4 and it could be detected only during the schizont stages where PfMCM4 expression level shows a peak. The low level of PfMCM5 expression can be attributed either to low mRNA abundance or to a less efficient PCR amplification. As an internal control, the specific region of PfGAPDH was also amplified under the same experimental conditions. These results indicate that the expression of PfMCM4 at the mRNA level goes up several folds from ring to schizont, whereas the control PfGAPDH level fluctuates between 1 and 1.7 times under the same experimental conditions (Fig. 1c). Further, to rule out the possibility of any genomic DNA contamination in the RT product, the

mRNA was treated with Dnase I before cDNA synthesis (– RT lanes, Fig. 1b). These results corroborate with the microarray data published by Le Roche *et al.* (2003) and suggest that compared to the PfGAPDH control, PfMCMs and PfORC1 are mostly expressed during the trophozoite and schizont stages where maximum amount of DNA replication takes place. Further, real-time PCR will be required to comment on the exact fold change and abundance of these proteins at the different erythrocytic stages.

To further investigate the expression of PfMCM4 at the protein level, a portion of PfMCM4 ORF (nucleotide positions 1182–2168) was expressed and purified as His₆-fusion protein and injected into two rabbits for immunization as discussed in the materials and methods.

The specificity of the antibodies generated was further analysed by Western blot analysis using recombinant antigen and an unrelated protein *Helicobacter pylori* DnaB helicase. The recombinant protein is nicely recognized by the antisera, whereas it does not cross react with HpDnaB suggesting that anti-PfMCM4 antibodies are specific (Fig. 1d). Similar results were obtained using antibodies generated in both the rabbits. The preimmune sera did not cross react with the purified antigens (data not shown). To investigate the status of PfMCM4 expression at the protein level affinity purified antibodies were used against *P. falciparum* lysate in a Western blot experiment. Immune sera recognized a band around ~120 kDa, whereas the preimmune sera failed to do so (Fig. 1e). The expected molecular mass of PfMCM4 is 117 kDa that is very close to the experimental data. These experiments together suggest that PfMCM4 is expressed both at the transcript and protein level during the asexual blood stages of the parasitic life cycle.

To find out the subcellular localization and stage specificity of PfMCM4 expression during asexual cycle, immunofluorescence assay was performed using thin smears on glass slides prepared from synchronized parasites collected from ring, trophozoite and schizont stages. PfMCM4 immune sera or preimmune sera as primary antibodies and goat antirabbit ALEXA Flour 488 secondary antibodies were used for immunolocalization. The green fluorescence was monitored at each stage using a Nikon fluorescence microscope.

The green fluorescence was detected predominantly in the trophozoite and schizont stage parasites (Fig. 2a). No significant signal was detected in the ring stage parasites, suggesting that PfMCM4 is mostly expressed at the protein level during trophozoite and schizont stages. The expression pattern of PfMCM4 at the protein level nicely matches with the expression pattern of PfMCM4 transcript levels. The expression of PfMCM4 was confined to the nucleus as the DAPI-stained images completely merged with ALEXA green fluorescence. Preimmune sera under the same experimental

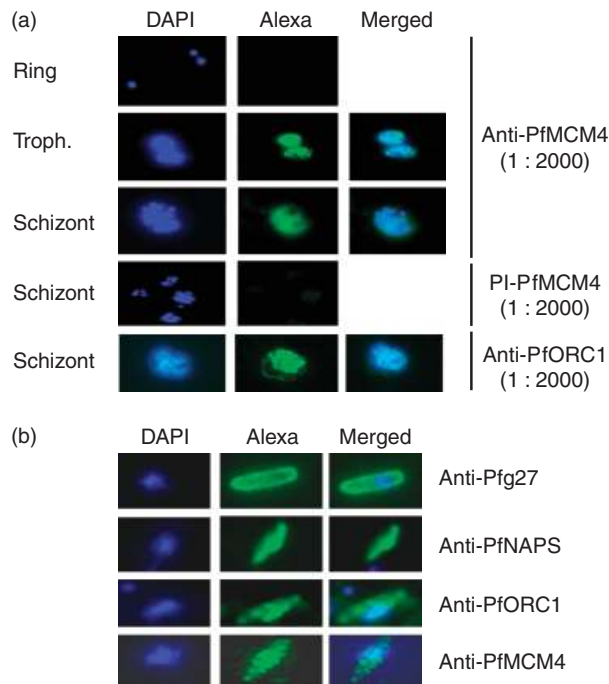


Fig. 2. *In vivo* localization of PfMCM4 and PfORC1 during *Plasmodium falciparum* sexual and asexual stages. (a) The immunofluorescence assay was performed essentially using the protocol described elsewhere (Mehra *et al.*, 2005). In brief, Glass slides containing thin smears of *P. falciparum*-infected erythrocytes from the different erythrocytic stages (as mentioned on the left of the different panels) were incubated with either preimmune or affinity purified PfMCM4 antibodies (1:3000 dilution) followed by green ALEXA Flour 488 conjugated goat antirabbit secondary antibodies. The slides were washed extensively with PBS-T buffer and scanned under Nikon fluorescence microscope for the detection of fluorescence. Strong signals were found in the trophozoite and schizont stage parasites whereas no signal was detected in the ring stage parasites. As a control, preimmune sera failed to detect any signal in the schizont stage parasites. DAPI indicates the nuclear staining in each case. (b) Detection of PfORC1 and PfMCM4 in the gametocytes. *Plasmodium falciparum* clones 3D7A were grown and cultivated for the enrichment of stage III–IV gametocytes essentially following the protocol described elsewhere (Carter *et al.*, 1993). Glass slides containing thin smears of stage III–IV gametocytes were made and the slides were subsequently treated for immunofluorescence as above either using mouse anti-Pfg27 (control for gametocyte stage-specific protein; 1:750 dilution) or rabbit anti-PfNAPS (control for gametocyte stage-specific nuclear protein; 1:400 dilution) or affinity purified rabbit anti-PfORC1 (1:400 dilution) or rabbit anti-PfMCM4 polyclonal antibodies (1:400 dilution) followed by chicken antimouse FITC-conjugated secondary antibodies (in case of Pfg27; 1:1000 dilution) or ALEXA Flour 488 secondary antibodies (against rabbit polyclonal primary antibodies; 1:1000 dilution). DAPI indicates the nuclear staining in each case.

conditions failed to detect any signal in the schizont stage parasites. These results strongly suggest that PfMCM4 is expressed at the transcript level as well as at the protein level during the asexual blood stages of the parasitic life cycle.

Our data are consistent with the recently published data showing the expression of PfMCM2, 6 and 7 mainly during trophozoite and schizont stages (Patterson *et al.*, 2006). PfORC1 was also confined to the nucleus during the schizont stage (Fig. 2a, bottom panel). It has been shown earlier that PfORC1 is not expressed at the protein level during the ring stage (Mehra *et al.*, 2005). These results suggest that the PfORC1 and PfMCM4 proteins follow similar temporal and spatial expression pattern in asexual stages.

We also investigated the expression of these proteins in sexual blood stages (gametocytes). For this purpose, we harvested *P. falciparum* III–IV stage gametocytes and thin smears were made on the glass slides. The typical crescent-shape gametocytes were observed under microscope to ensure the gametocyte stages. Immunofluorescence assays were performed using either with mouse anti-Pfg27 (a gametocyte-specific marker gene) (Lobo *et al.*, 1999) or rabbit anti-PfNAPS (a gametocyte-specific nuclear protein) (Chandra *et al.*, 2005), or anti-PfMCM4 or anti-PfORC1 (Mehra *et al.*, 2005) antisera as primary antibodies and chicken antimouse fluorescein isothiocyanate (FITC)-conjugated secondary antibodies or goat antirabbit ALEXA Flour 488 secondary antibodies (wherever appropriate). Pfg27 showed distinct membrane localization pattern all over the gametocytes as reported earlier (Lobo *et al.*, 1999), whereas PfNAPS showed very strong nuclear signal as shown by Chandra *et al.* (2005). PfORC1 showed strong nuclear staining that perfectly overlapped with the DAPI staining apart from some diffused staining in the cytoplasm. Interestingly, PfMCM4 showed a distinct punctate staining all over the gametocyte although the nuclear signal was more intense than the cytoplasmic signal (Fig. 2b). Pre-immune sera under the same experimental conditions did not show any specific staining (data not shown). These results indicate that both PfORC1 and PfMCM4 are also expressed during the sexual stage of the parasitic life cycle. It is interesting to note that both PfMCM4 and PfORC1 are confined to the nucleus during the asexual stages, whereas these proteins are distributed in the cytoplasm as well as in the nuclei during sexual stage development. Whether these proteins shuttle between nucleus and cytoplasm during gametocytogenesis leading to a gametocyte-specific function remains to be explored further.

The apparent absence or low abundance of these genes both at the transcript level as well as at the protein level during the ring stage and their induction at the later stages clearly suggests that their expression is developmentally regulated.

It has been reported that phosphorylation of MCM proteins is important for subcellular localization and activation (Brown & Kelly, 1998; Lei & Tye, 2001). To investigate the PfMCM4 phosphorylation status, parasite

lysate was immunoprecipitated using anti-PfMCM4 antibodies followed by Western blot analysis using either anti-PfMCM4 or well-established commercially available antiphosphoserine and phosphothreonine antibodies. The results indicate that even though PfMCM4 is nicely immunoprecipitated, antiphosphoserine/antiphosphothreonine antibodies fail to recognize any phosphorylated product of this protein under our experimental conditions (data not shown). Similar results were obtained recently when these phospho-residue specific antibodies were used to detect phosphorylation of PfMCM2, 6 and 7 following immunoprecipitation (Patterson *et al.*, 2006). These results together suggest that either MCM4 phosphorylation does not occur in this system, or that it is too transient to allow detection under our experimental conditions.

The presence of conserved NTP domain, putative Zn finger domain and the MCM signature motif within the ORF and its similar expression profile to other MCMs suggest that PfMCM4 is a true MCM4 homolog that might form a stable hexameric complex together with the other PfMCM subunits. Do PfMCM4 and PfORC1 physically interact with each other? An anti-MCM4 immunoprecipitate did not contain detectable ORC1, as determined by Western blot analysis. In a reciprocal experiment, an anti-ORC1 immunoprecipitate did not contain any MCM4, as determined by Western blot analysis (data not shown). In fact, although these proteins are important partners of the pre-RC, they have not been shown to physically interact with each other in any other system so far.

The presence of PfORC1 and PfMCM4 during asexual and sexual stages suggests its possible role in genome multiplication during erythrocytic schizogony and gametocytogenesis. In addition to a possible role in gametocyte development, it is possible that replication proteins accumulate in male gametocytes to prepare for the three rapid rounds of genome replication that occur during male gametogenesis (exflagellation) that occurs in the mosquito midgut. Our novel findings have implications with respect to the regulatory role of these proteins in the asexual and sexual development of malaria parasites.

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Author contribution

A.G. and P.M. contributed equally to this work.

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