Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular & Biochemical Parasitology 145 (2006) 50–59
MOLECULAR & BIOCHEMICAL PARASITOLOGY

# Pre-replication complex organization in the atypical DNA replication cycle of *Plasmodium falciparum*: Characterization of the mini-chromosome maintenance (MCM) complex formation

Shelley Patterson [a], Claudia Robert [a], Christina Whittle [a], Ratna Chakrabarti [a], Christian Doerig [b], Debopam Chakrabarti [a,*]

[a] *Department of Molecular Biology and Microbiology, University of Central Florida, Orlando, FL 32826, USA*
[b] *INSERM U609, Wellcome Centre for Molecular Parasitology, Anderson College, Glasgow G11 6NU, Scotland, UK*

Received 8 June 2005; received in revised form 9 September 2005; accepted 15 September 2005
Available online 3 October 2005

**Abstract**

The overall organization of cell division in *Plasmodium* is unique compared to that observed in model organisms because DNA replicates more than once per cell cycle at several points of its life cycle. The sequencing of the *Plasmodium* genome has also revealed the apparent absence of many key components (e.g. Cdt1, DDK and Cdc45) of the eukaryotic cell cycle machinery that are responsible for the formation of the pre-replication complex (pre-RC). We have characterized the *Plasmodium falciparum* minichromosome maintenance complex (MCM) that plays a key role in the transition of pre-RC to the RC. Similar to other eukaryotes, the *Plasmodium* genome encodes six MCM subunits. Here, we show that expression levels of at least three of the PfMCM subunits, the homologues of MCM2, MCM6 and MCM7, change during the intraerythrocytic development cycle, peaking in schizont and decreasing in the ring and trophozoite stages. PfMCM2, 6 and 7 subunits interact with each other to form a developmentally regulated complex: these interactions are detectable in rings and schizonts, but not in trophozoites. PfMCM2, 6 and 7 subunits are localized in both cytosolic and nucleosolic fractions during all intraerythrocytic stages of *P. falciparum* development, with increased nuclear localization in schizonts. Only PfMCM6 is associated with the chromatin fraction at all stages of growth. No phosphorylation of PfMCM2, 6 and 7 was detected, but two as yet unidentified threonine-phosphosphorylated proteins were present in the complex, whose pattern of phosphorylation varied during parasite development.


*Keywords:* *Plasmodium falciparum*; MCM; Pre-replication complex; Cell cycle

## 1. Introduction

In spite of spectacular advances in molecular medicine, malaria continues to be a major health problem in much of the developing world, affecting the lives of over 500 million people [1]. A clear understanding of the molecular basis of the malaria parasite cell growth and differentiation is essential to provide a basis for the development of novel chemotherapeutic agents. The malaria parasite has a complex developmental cycle in its mosquito and human hosts and there are several points in the life cycle where DNA replication occur [2,3]. Sporozoites, which are in cell cycle arrest, are injected from the mosquito salivary glands into blood stream of the human host during a blood meal. Upon entering hepatocytes, sporozoites undergo exoerythrocytic schizogony, a process requiring multiple rounds of DNA replication. Merozoites resulting from the exoerythrocytic schizogony invade erythrocytes to initiate the erythrocytic life cycle stages. During the intraerythrocytic growth, the parasite matures through the ring, trophozoite, and schizont stages and undergoes multiple rounds of nuclear division. Chromosomes do not condense, and the nuclear membrane remains intact throughout the nuclear divisions (endomitosis), which results in the formation of a syncytium containing about 16 nuclei. An apparent asynchrony of nuclear division has been observed in individual schizonts [4]. Daughter merozoites are formed through the movement of individual nuclei into merozoite buds [3]. Some of the merozoites undergo cell cycle arrest

*Abbreviations:* MCM, mini chromosome maintenance; ORC, origin recognition complex; CDK, cyclin-dependent kinase

* Corresponding author. Tel.: +1 407 882 2256.
*E-mail address:* dchak@mail.ucf.edu (D. Chakrabarti).

0166-6851/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2005.09.006

to differentiate into male and female gametocytes. The molecular basis for sexual differentiation remains to be elucidated. Ingestion of gametocytes by mosquito initiates further development of gametocytes into gametes, a process that implies, for male gametocytes, rapid cell division to form eight gametes. Zygotes are formed, the only diploid life cycle stage, when male and female gametes are fertilized. In the subsequent ookinete stage meiotic reduction occurs. The ookinete develops into an oocyst and undergoes many rounds of cell division to form sporozoites. Hence, there are five points in the life cycle that involve cell division. In an effort to understand the molecular mechanism of the onset of DNA replication, we have initially focused on the characterization of the pre-replication complex in the intraerythrocytic stage of *Plasmodium falciparum*, which is the causative agent for the most virulent form of the disease.

The basic mechanism of the pre-replication complex formation and the subsequent onset of DNA synthesis are well conserved in eukaryotes [5]. In yeast an origin recognition complex composed of high molecular weight proteins ORC1 through 6 is bound to the origin of replication throughout the cell cycle. It is believed that the ORC acts as a landing pad to nucleate the step-wise assembly of other components, e.g., Cdc6, Cdt1, and MCM (for *m*ini-*c*hromosome *m*aintenance) complex. MCM is a heterohexameric complex composed of MCM2–7 that is thought to function as the replicative helicase [6]. Although the MCM complex is heterohexameric, subcomplexes of various configurations are also detectable [7]. The MCM complex is recruited to the pre-replication complex (pre-RC) after the exit from mitosis and the onset of DNA synthesis. The abundance and subcellular localization of MCM proteins change with the cell cycle [5,6]. In budding yeast MCM proteins are in the nucleus during G1 and S phase whereas in G2 and M they are exported to the cytosol [8]. Although it is expected that the parasite has a tight control mechanism for cell proliferation and differentiation that allows precise duplication of the genome during various points of its complex life cycle, very little is known about the molecular machinery that regulates this process. Only the preliminary characterization of MCM4 and ORC1 subunits has been reported [9,10]. In this paper we report the characterization of PfMCM2, 6, and 7 subunits.

## 2. Materials and methods

### 2.1. Molecular cloning and expression in bacteria

Using the *Saccharomyces cerevisiae* and human protein sequence for the MCM subunits, the PlasmoDB database (plasmodb.org) was screened for matches. The putative ORFs were determined using EditSeq (LaserGene) software. Total RNA was extracted from asynchronous intraerythrocytic *P. falciparum* 3D7 parasites using the RNAgents Total RNA Isolation System (Promega). First-strand cDNA was synthesized using oligo-(dT) primer from 200 ng total RNA using the ProStar RT-PCR Kit (Statagene). Either full-length or fragments of the genes were cloned. The PCR products were purified using the QIAquick Purification Kit (Qiagen). Approximately 0.2 pmol of purified PCR product was treated with T4 DNA Polymerase, cloned into pET30 Ek/LIC vector (Novagen) utilizing the LIC sequence, and transformed into NovaBlue *E. coli* cells (Novagen). Clones with the correct size insert, determined by restriction digestion, were further verified by sequencing using a Beckman Coulter CEQ 2000 XL sequencer. Following sequence verification, the expression plasmid constructs were electroporated into *E. coli* BL21(DE3) cells. The cultures were induced at $0.6A_{600\,nm}$ with 0.5 mM isopropyl-beta-D-galactoside for 6 h at 22 °C.

### 2.2. Purification of recombinant PfMCM proteins and antibody production

Of all the constructs made to express recombinant proteins, only the N-terminus fragment of PfMCM2 (residues 276–466) and the C-terminus fragments of PfMCM6 (residues 658–803) and PfPMC7 (residues 635–814) yielded proteins in the soluble form. The bacterial pellet was resuspended in 1 ml talon extraction/wash buffer (50 mM sodium phosphate, pH 7.0, 300 mM sodium chloride, 10% glycerol) plus mini-complete EDTA-free protease inhibitors (Roche) per 25 ml culture. The cell suspension was lysed using a French Press (three times at 900 psi) followed by brief sonication. The suspension was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. The supernatant was added to 1 ml bed volume of Talon resin and $His_6$-tagged recombinant protein was purified following the manufacturer's batch/gravity-flow column purification protocol (Clontech). Briefly, the talon resin was pre-equilibrated in 1× extraction/wash buffer before adding the clarified lysate supernatant. Binding to the resin was performed at 4 °C for 60 min on a shaker. The unbound sample was removed by two washes with the 1× extraction/wash buffer before being transferred to the column. The two stringent washes, one with the 1× extraction/wash buffer with 5 mM imidazole, the other with 1× extraction/wash buffer with 20 mM imidazole, were passed through the column to remove any non-specific binding proteins. The bound proteins were then eluted with 5 ml elution buffer (50 mM sodium phosphate, pH 7.0, 300 mM sodium chloride, 150 mM imidazole, 10% glycerol). The necessary amount of purified protein was resolved on SDS-PAGE, stained with GelCode® Blue Reagent (Pierce) and excised from the gel to remove contaminating bands. The gel slices were then sent to Cocalico Laboratories (Pennsylvania, USA) for immunization using complete Freund's adjuvant. The animals were subjected to four injections before obtaining the final bleed. Antibodies from the final bleed were affinity-purified on the corresponding recombinant proteins immobilized to nitrocellulose membranes [11].

### 2.3. Parasite extracts

*P. falciparum* 3D7 maintained in a modified erythrocyte culture [12] were synchronized by sorbitol treatment [13]. The infected erythrocytes were treated with 0.1% saponin to release the parasites, which were then washed with phosphate-buffered saline. Cell-free extracts were prepared by resuspending the development stage-specific parasite pellet in M-PER (Pierce) reagent containing protease inhibitor cocktail (Roche), 5 mM