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The malarial CDK Pfmrk and its effector PfMAT1 phosphorylate DNA replication proteins and co-localize in the nucleus☆

Dayadevi Jirage[a], Yueqin Chen[a,1], Diana Caridha[a], Michael T. O'Neil[a], Fredrick Eyase[b], William H. Witola[c], Choukri Ben Mamoun[d], Norman C. Waters[a,e,*]

[a] Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD 20910, USA
[b] United States Army Medical Research Unit-Kenya, Walter Reed Project, Kisumu, MRU 64109, APO, AE 09831-4109, Kenya
[c] Department of Ophthalmology and Visual Sciences, University of Chicago Medical Center, 5835 S. Cottage Grove Ave. N-361, Chicago, IL 60637, USA
[d] Yale School of Medicine, Section of Infectious Diseases, 333 Cedar Street Rm, LMP-1072, PO Box 208056, New Haven, CT 06520-8056, USA
[e] WRAIR Laboratory, Australian Army Malaria Institute, Gallipoli Barracks, Enoggera, Queensland 4051, Australia

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ABSTRACT

Cyclin-dependent kinases (CDKs) have an established role in metazoans and yeast in DNA replication, transcription and cell cycle regulation. Several CDKs and their effectors have been identified in the malaria parasite *Plasmodium falciparum* and their biological functions are beginning to be investigated. Here we report results from the functional characterization of Pfmrk and its effector PfMAT1. We validated the interactions between Pfmrk and PfMAT1 and pinpointed their intracellular location. Co-immunoprecipitation studies demonstrated physical interaction between the two proteins and identified the C-terminal domain of PfMAT1 as the Pfmrk activator domain. Immunofluorescence analyses using GFP and RFP-tagged versions of Pfmrk and PfMAT1, respectively, demonstrated the co-localization of these two proteins to the parasite nucleus. Bacterial two-hybrid screen of a *P. falciparum* cDNA library using Pfmrk as the bait identified two plasmodial DNA replication proteins, PfRFC-5 and PfMCM6, as interactors with Pfmrk. We demonstrate that that these two proteins are substrates of Pfmrk-mediated phosphorylation and that PfMAT1 confers substrate specificity to the Pfmrk kinase complex. Collectively, these data suggest a role for Pfmrk in the nucleus of the parasite presumably in regulation of the DNA replication machinery.

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1. Introduction

Cyclin-dependent kinases are a large group of highly conserved proteins that play a critical role in the regulation of various cellular processes such as cell cycle progression, transcription, DNA replication and repair, apoptosis and differentiation [1]. Active forms of CDKs are heterodimers comprising of a catalytic Ser/Thr kinase subunit and a regulatory subunit, usually the cognate cyclin. Binding of a CDK with its cognate cyclin results in the displacement of a sterically hindering "T-loop" and exposure of residues in the catalytic cleft resulting in a partially active enzyme. The action of CDK activating kinase (CAK) complex then leads to complete activation of the CDK-cyclin complex via phosphorylation of a conserved Thr residue within the "T-Loop" [2]. In metazoans, CDK7 functions as a CAK which phosphorylates and activates cell cycle CDKs. The CAK complex, which is also involved in the process of transcription, consists of CDK7, cyclin H and a third stabilizing partner MAT1 [1]. The MAT1 protein stabilizes and influences the substrate specificity of the CDK7 kinase complex [3]. Sequence analysis and biochemical data have revealed that the MAT1 protein can be divided into three domains with discrete functions: an N-terminal RING finger domain, a central coiled-coil domain and a hydrophobic C-terminal domain [4]. The C-terminal domain interacts with the CDK7–cyclin H complex and stimulates CDK7 kinase activity. The central domain is involved in anchoring the CAK to the TFIIH core, and the N-terminal domain is involved in CTD phosphorylation and transcription activation [4]. The CAK complex has been shown to be localized in the nucleus and its substrates are predominantly nuclear [5,6].

Several of these familiar components of the cell cycle regulatory machinery have been identified and characterized in the malaria parasite *Plasmodium falciparum* [7]. The plasmodial life

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* Corresponding author at: Australian Army Malaria Institute, WRAIR Laboratory, Gallipoli Barracks, Enoggera, Queensland 4051, Australia. Tel.: +1 301 319 9947/ +61 7 3332 4817; fax: +61 7 3332 4800.
E-mail address: norman.waters@us.army.mil (N.C. Waters).
[1] Present address: Key Laboratory of Gene Engineering Key Laboratory for Biocontrol, Zhongshan University, Guangzhou 510275, PR China.

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cycle is characterized by alternation between proliferative and non-proliferative stages. Sporozoites injected into the bloodstream by an infected *Anopheles* mosquito migrate to the liver and invade hepatocytes, producing up to 40,000 merozoites per hepatocyte. These merozoites are then released into the bloodstream after lysis of the infected hepatocyte, and in turn invade erythrocytes, yielding 20–32 daughter merozoites per erythrocyte, which upon release go on to invade more erythrocytes [8]. Although the distinct phases of the malaria cell cycle have not been defined, it is generally accepted that the invading merozoite is in the G1 stage of the cell cycle. At 18 h post-invasion, the ring stage parasite progresses to trophozoite stage and DNA synthesis is thought to be initiated. Several rounds of asynchronous nuclear division ensue the trophozoite stage [9]. The exact molecular mechanisms involved in the regulation of this atypical cell cycle are poorly understood [10], however they are being actively investigated for identification of potential novel drug targets.

Several CDKs, cyclins, and a CDK effector protein have been identified in *P. falciparum* using homology-based PCR and database mining approaches [10]. Pfmrk shares 46% sequence identity with human CDK7 and the recombinant protein phosphorylates RNA Pol II CTD and Histone H1 *in vitro* in the presence of either human cyclin H or Pfcyc-1 [11–13]. PfMAT1, a putative homolog of human MAT1, stimulates the CTD kinase activity of Pfmrk in a cyclin-dependent manner *in vitro* [14]. Although it has been demonstrated that plasmodial CDKs are amenable to inhibitory mechanisms exerted by mammalian CDK inhibitors [15], to date CDK inhibitory proteins have not been identified in *Plasmodium*. Therefore, the regulatory mechanisms for activating and inhibiting CDK activity in *Plasmodium* remain to be unraveled. Furthermore, the substrates of CDKs and how CDKs regulate the malarial cell cycle are not known.

Pfmrk displays highest similarity to mammalian CDK7; however it is unclear if Pfmrk is a functional homologue of CDK7 [13]. In metazoans, CDK7 has a dual function as a CAK and as the kinase element of the general transcription TFIIH complex which phosphorylates the CTD of RNA polymerase II. In *Saccharomyces cerevisiae*, the CDK7 ortholog Kin28 is a CTD kinase and a member of the TFIIH complex but does not possess CAK activity. In *Schizosaccharomyces pombe*, two CAKs have been found—the Mcs6 complex and the Csk1 [16]. Hence, CDK7 orthologs from various metazoans seem to have different biological functions. Therefore, Pfmrk may have a unique role in the cell cycle that may have arisen through *Plasmodium* evolution. In fact, phylogenetic analyses suggested that Pfmrk may not belong to the CDK7 family of CDKs [17,18]. Previous biochemical studies suggested that Pfmrk in association with Pfcyc-1 or Pfcyc-1-PfMAT1 does not possess CAK activity towards the CDK1 ortholog PfPK5 [12,14]. Although Pfmrk may function as a CAK in association with different cyclin subunits or towards different plasmodial CDKs, initial inquiries were raised as to the biological function of Pfmrk in the absence of a *bona fide* CAK activity.

The process of DNA replication is initiated by the action of many enzymes [19]. First, the origin–recognition complex (ORC) is assembled at the replication origin. This is followed by the recruitment of the pre-replication complex (pre-RC) consisting of Cdc6, Cdt1and MCM proteins to the ORC. The MCM2–7 proteins form a hexameric complex that assembles at replication origins during early G1 phase [20]. Coordination of the functional interactions between the MCM2–7 proteins and other components of the pre-RC is mediated by CDKs [19,21]. Subsequently, loading of DNA polymerase occurs and is mediated by the replication factor complex (RFC) [22]. The RFC consists of five members (RFC-1, RFC-2, RFC-3, RFC-4 and RFC-5). The loading and activation of the pre-RC occurs at late G1/early S-phase. Once DNA replication is initiated, the Pre-RC is disassembled thus preventing the re-replication of DNA until the cell cycle resets again—in the subsequent G1 phase. Sequential activation and deactivation of CDKs and other cell cycle regulatory kinases, ensure that the replication of DNA only occurs once per cell cycle [23].

Another malarial CDK, PfPK5, has been proposed to play a role in DNA replication [24,25]. PfPK5 expression and kinase activity levels peak 36 h post-invasion and PfPK5 was shown to co-localize with DNA in the early trophozoite stages, i.e., before or at the onset of DNA synthesis. Two lines of evidence suggest a role for PfPK5 in the S-phase (a) elevated PfPK5-associated kinase activity was detected in parasites treated with the DNA synthesis inhibitor aphidicolin and (b) treatment of parasites with the PfPK5 inhibitors flavopiridol and olomoucine results in decreased DNA replication [26]. Since no obvious NLSs could be detected in the PfPK5 sequence, the possibility exists that it is co-transported into the nucleus along with its cognate cyclin (or other effector) where it regulates DNA replication. Nuclear import/export of CDKs and cyclins has been demonstrated to be a mechanism to regulate cell cycle progression [27].

In this study, we describe the functional characterization of the *P. falciparum* CDK Pfmrk. We have validated its interactions with PfMAT1, demonstrated co-localization to the nucleus and identified components of the DNA synthesis machinery as substrates of this enzyme. Taken together, these results suggest a role for Pfmrk in the DNA replication process.

2. Materials and methods

2.1. Molecular cloning of PfMAT1 deletion mutants

DNA for the construction of PfMAT1 deletion mutants was amplified from a plasmid template of PfMAT1 full-length cDNA in pQE30 [14] with oligonucleotides containing a *BamHI* restriction site. The full-length and truncated PfMAT1 PCR products were digested with *BamHI* (Promega) and cloned into the *BamHI* site of pET15b (Novagen). All the constructs were sequenced to check for orientation and correct reading frame.

2.2. Molecular cloning of MBP-PfMAT1

The MBP-tagged version of PfMAT1 was constructed by amplifying PfMAT1 cDNA with oligonucleotides containing *EcoRI* and *HindIII* restriction sites and cloning the PCR product into pMAL-c2X vector (New England Biolabs).

2.3. Expression and purification of recombinant proteins

Pfmrk (PF10_0141), Pfcyc-1 (PF14_0605), PfMAT1 (PFE0610c), and RNA Pol II CTD were expressed and purified from *Escherichia coli* as GST tagged (Pfcyc-1 and CTD) or 6 × His tagged (Pfmrk and PfMAT1) fusion proteins as previously reported [12,14]. PfRFC-5 was expressed in BL21-CodonPlus-RIL *E. coli* cells, and purified over a nickel chelating column under identical conditions previously described for PfMAT1 [14]. PfRFC-5 purified as a 40 kDa protein as determined by SDS-PAGE. GST-tagged PfMCM6 was purified on a GSTrap HP column (GE Healthcare) using the same protein purification procedures as described for Pfcyc-1. GST-PfMCM6 purified as a110 kDa protein as determined by SDS-PAGE. MBP-PfMAT1 protein was purified on an amylose column (New England Biolabs) according to the manufacturer's instructions (www.neb.com).

2.4. Kinase assays

Kinase assays were performed in filter bottom microtiter plates as previously described [14]. Briefly, 1.5 μg of Pfmrk was assayed in a 50 μl kinase reaction containing kinase buffer (50 mM Tris–HCl pH 7.5, 2.5 mM $MnCl_2$, and 1.0 mM DTT) supplemented with 3.0 μg Pfcyc-1 and various concentrations of substrates. The reaction was started by the addition of an ATP mix containing 12 μM ATP (Sigma)