REVIEW ARTICLE

Regulation of *Plasmodium falciparum* cell cycle involving Cyclins and Cyclin Dependent Kinases

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ABSTRACT

Protein kinases (PKs) are regulators of protein phosphorylation in many infectious diseases, including malaria. However, the cellular functions of majority of PKs in *Plasmodium falciparum* remain unknown. The mechanisms involved in *P. falciparum* cell cycle progress are not fully understood. The activation of cyclin-dependent kinases (CDKs), which constitute a PK family that includes crucial regulators of cell cycle progression in eukaryotes, is strictly being coordinated by the interaction with specific cyclins at well-defined points within the cell cycle. These cyclin/CDK complexes are very well characterised in humans, but little is known in *P. falciparum*. This review expand our understanding of the characteristic of CDKs and cyclins in *P. falciparum*, and paves the way for further investigations on the precise molecular role of these crucial regulatory proteins in mosquito and human. This represents a valuable step towards the elucidation of cell cycle control mechanisms in malaria parasites.

Keywords: Protein Kinase, Cyclins, Cyclin Dependent Kinases (CDKs), *P. falciparum*

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INTRODUCTION

Malaria is a major health and socioeconomic burden for the human population worldwide (1). This disease is caused by parasites called Plasmodium and transmitted by the bite of a female *Anopheles* mosquito. Malaria is primarily located in the tropical and subtropical regions of the world where climates are suitable for transmission, particularly in low socio-economic areas such as Sub-Saharan Africa, which bears over 90% of the malaria burden. Globally, approximately 40% of the world's population (2.4 billion people), mainly in the tropics, is at risk of infection. According to the World Health Organization 2014 report, malaria continues to be a devastating disease killing ~500,000 people annually, of which 90% are due to *P. falciparum*. In 2013, over 198 million cases of human malaria were reported (WHO, 2014) with an estimated 78% of deaths involving children under the age of 5 living in sub-Saharan Africa (2). Although the number of deaths has gradually decreased over the past decade, limited understanding of the parasite's biology is still hindering the urgently needed drug and vaccine development due to increasing number of drug-resistant parasites (3.4).

The life cycle of Plasmodium parasites

The malaria parasite has a complex life cycle involving three distinct proliferative stages that occur inside the mosquito vector and also within the human host. The parasite is transmitted when an infected mosquito vector takes a blood meal and injects the human host with sporozoites present in its saliva. The sporozoites migrate to the liver and invade hepatocytes, where they multiply and differentiate to produce thousands of invasive liver merozoites. This stage is called the exo-erythrocytic schizogony and occurs exclusively in the liver.

Merozoites will be released from the hepatocytes and enter the blood stream. It will then rapidly attached to a red blood cell (RBC). After invading the RBC, parasites undergo a cycle of asexual replication; progressing through the ring stage, maturing into trophozoite forms, and later, developing into multi-nucleated schizonts. This is followed by rupture of the RBC and release of free merozoites, which are able to invade new RBCs and continue the cycle. This is the phase that is responsible for the pathogenesis of malaria, and is readily amenable to in vitro culture and genetic manipulation (5).

Alternatively, some parasites will arrest their cell cycle during the asexual stage and differentiate into sexual forms either into female or male gametocytes; the decision switch to engage into sexual development is taken in the preceding schizont, so that all the progeny of a given schizont will be constituted of either entirely gametocytes, or entirely asexual parasites. In the mosquito midgut, the male gametocytes exit their asexual cell cycle and undergo several rapid rounds of cell division or mitosis in order to form eight haploid

microgametes that will fertilise the female macrogamete to form a diploid zygote. This diploid stage allows for sexual recombination of genetic material and those genes responsible for drug resistance. The zygotes develop into invasive ookinetes that cross the midgut epithelium and mature to form oocysts. The diploid oocyst undergoes meiosis and eventually produced approximately eight thousand haploid sporozoites erupt from the oocyst and migrate to the mosquito salivary glands. Later, the sporozoites migrate to the salivary glands and accumulate until transmitted to the human host. This will be occurred during a blood meal, thus completing the parasite life cycle (5).

The life cycle of all Plasmodium spp. is associated with specific time periods for each developmental stage. Of crucial epidemiological importance is the capacity both of *P. vivax* and *P. ovale* to form hypnozoites which is the dormant stages that is remain in the liver that can be reactivated up to several years after the primary infection (6). The severity of malaria infection changes with age, immunity of the infected individual, the infecting parasite species and also by transmission intensity (7).

Gametocytogenesis

P. falciparum gametocyte formation and maturation are divided into five morphologically distinguishable stages from stage I up until stage V (8). This process takes more than 12 days and is observed by some as a stress response which will allow the parasites to escape from any unfavourable environment. Several factors induce gametocytogenesis, including high density of asexual stages, which is how gametocytes can be produced from a *P. falciparum in vitro* culture (9). Further, it has been suggested that haematological conditions (RBC lysis, anemia and reticulocytes), immune responses and the presence of different parasite clones in the same host (acting as a competitor) can be key factors that favour gametocytogenesis (10).

Gametocytogenesis starts with the invasion of the RBC by a sexually committed merozoite during the asexual stage. Next invasion, the sexually committed ring stage will grow and elongate gradually to occupy the entire capacity of the RBC. When a mosquito ingests these gametocytes during a blood meal, environmental conditions such as the presence of xanthurenic acid (11), a reduction in temperature (of approximately 5°C), and an increase in pH (from 7.2 to 8.0), will initiate gametogenesis (11-13). Both the male microgametocyte and the female macrogametocyte differentiate into gametes and egress from the enveloping human erythrocyte within 10 minutes post-activation (14-15). After 40 hours of post-invasion, stage I gametocytes will be produced. However stage I gametocytes are morphologically undistinguishable from young asexual trophozoites. A small noticeable difference is the presence of multiple small pigmented dots, corresponding to hemozoin vacuoles present throughout the cytoplasm of stage I gametocytes, instead of a single pigment vacuole which is characteristic of the asexual stages (15). Pfs16 could be use a marker for stage I gametocytes and allows discrimination from morphologically similar asexual trophozoites. The expression of Pfs16 can be detected 24 hours post-invasion and continues until mature stage V gametocytes; it is therefore a useful marker that can be used throughout gametocyte development (12).

Gametocyte stages starting from stage II through stage V can be easily distinguished in Giemsa-stained blood smears as their morphology is quite distinct (16). Gametocytes and matured asexual parasites (approximately 20 hours after RBC invasion) are sequestered in a variety of organs, including the kidneys, heart lungs, brain, liver and many other vital organs. Early-stage gametocytes are displayed to be sequestered in the bone marrow and spleen. From stage I to IV gametocytes sequester in host tissues during approximately seven days and mature stage V gametocytes circulate in the peripheral blood till to be taken up by the mosquito during a blood meal (17). *In vitro* early stage V can be observed 8-10 post induction.

Each asexual schizont is able to commit either to asexual replication or sexual development in response to several environment factors such as host immunity, stress from certain anti-malaria drugs, and high density of infected red blood cells (iRBC) (18-20). The genetic factors regulating the induction of sexual differentiation in Plasmodium remain unclear.

Plasmodium Kinome

The life cycle of the malaria parasite suggests a multitude of potential targets (21-23). Several targets in the parasite are now considered for development of novel treatments to cure the disease and block its transmission. For example, the requirement of lipids by the rapidly growing parasite to increase its surface area and size of the internal membranes make lipid metabolic enzymes an attractive target for anti-malarial drugs (24). Likewise, proteases essential for erythrocyte invasion and egress are currently investigated as novel targets (25).

The genome of *P. falciparum* was sequenced in 2002 (26), which facilitated the identification of potential new drug targets, such as invasion-related proteins and cell signaling pathway key components, the protein kinases (PKs) (26-30). Indeed, by having the accessibility to the parasite genome allowed the *P. falciparum* kinome to be determined via bioinformatic analyses (28). The kinome of the malaria parasite has been suggested to be an attractive target for novel antimalarials, as kinase inhibitors that have been successfully used in the treatment of a variety of human diseases including cancers (31). Furthermore, the identification of essential PKs in *P. falciparum* developmental stages of, and for many of them the lack of orthologues in the human kinome, highlighted several PKs as potential drug

targets (32-36). Indeed, recent research reported that a significant portion of the *P. falciparum* kinases do not have orthologues in the human kinome (35.37). The structural divergence of several atypical plasmodial PKs (as compared to human PKs) from the conserved canonical eukaryotic protein kinase domain proposes constitutes a potential source of novel drugs of high specificity and low toxicity (38.46). The sequence of the *P. falciparum* genome was published in 2002, which has opened avenues to study the fundamental of the parasite as well as providing essential information for drug and vaccine development (26). To date, a number of studies focusing on ePKs, including bioinformatic studies on Plasmodium PKs, have been published (28.39.40). The published *P. falciparum* genome sequence permitted the entire complement of *P. falciparum* protein kinases to be fully analyzed (28.40). The parasite possesses ePKs which belong to five of the seven groups mentioned in Table I such as AGC, CK1, CMGC, CamK and TKL. There is no malarial PK clustered in the STE and TK group. The functions of TK are often related to the multicellular mode of life as well as cell-cell signaling pathways. Therefore, it is not surprising to observe the absence of TK in *P. falciparum* as they are also absent in yeast and most unicellular eukaryotes (41). The CMGC group that includes CDK, MAPK, GSK and CDK-like kinases shown to be the largest protein kinase cluster present in apicomplexan parasites. Significantly, the majority of CMGC kinases are involved in the cell cycle progression in mammalian cells. The abundance of these CMGC kinases in the *P. falciparum* kinome could suggest that the variety of the replicative stages present in the parasite life cycle. These kinases might be particularly important due to their involvement in cell cycle progression, cell proliferation and differentiation and therefore may serve as targets for future drug developments (28). PKs, which do not fall into any of the groups and have no orthologue in the human kinome, were classified as orphan kinases. According to a BLASTP and phylogenetic analyses some Plasmodium ePKs show characteristics of more than one group or family, such as PfPK7 (AGC/STE), PfPK6 (CDK/MAPK), Pfcrk-4 (CDK/MAPK) and Pfnek-1 (NIMA/MEK). Characterisation of the complete *P. falciparum* kinome has revealed significant divergence from the kinome of its human host (28.29.34). This is very encouraging as targeting malaria parasite kinases represents an attractive strategy for new drug development with a reduced risk of drug toxicity in humans. (43). Targeting PKs involved in gametocytogenesis is an equally attractive drug target strategy to be used as transmission-blocking drugs. By preventing the parasite's ability to differentiate into gametocytes, this would prevent the transmission of the parasites from humans to mosquitoes, thereby disrupting the malaria life cycle.

Table I: Overview of eukaryotic protein kinases

Group name	Full name/ members
AGC	PKA (cyclic-adenosine-monophosphate-dependent protein kinase), PKG (cyclic-guanosine-monophosphate-dependent protein kinase), PKC (protein kinase C)
CMGC	CDK (cyclin-dependent kinases), GSK3 (glycogen synthase kinase 3), MAPK (mitogen-activated protein kinases), and CLKs (CDK-like kinases),
CamK	Calcium/Calmodulin-dependent kinases
TK	Tyrosine kinases
CK1	Casein Kinase 1
TKL	Tyrosine Kinase-like kinases
STE	STE stands for "sterile", as the first yeast null mutant in these kinases presented a sterile phenotype, This groupincludes enzymes functioning in MAPK pathways.
OPKs	Other Protein Kinases - do not fall into any group but have an ePK-conformant primary strucutre

There are several protein kinase inhibitors have extended the market in the context of cancer chemotherapy. Protein kinases are regulators of protein phosphorylation in many infectious diseases, including malaria (44). However, the cellular functions of the majority of the *P. falciparum* PKs and their regulators remain unknown. This study is concentrated only on the *P. falciparum* cyclin family.

To date, *P. falciparum* CDKs are being proposed as potential drug targets. However, the mechanisms involved in the progression of malaria cell cycle are not well characterised. The activation of CDKs during the cell cycle progression is tightly controlled by the interaction of particular cyclins at a highly specific stages of the cycle, and cyclin/CDK complexes are well characterised in yeast as well as in human. In contrast, very little is known about the role of *P. falciparum*. There are six *P. falciparum* CDKs have been identified such as PfPK6, Pfmrk PfPK5, Pfcrk-1, -3, -4) and CDKs shown to be likely essential for the parasite's erythrocytic cell cycle on the basis of reverse genetic data.

The essential role of CDK/cyclin interactions in cell cycle

According to the classical models, Cyclin D is considered as a cyclin which is responsible for initiation of the cell cycle. It is produced to stimulate CDK4/CDK6 regulating the events in the G0 to G1 phase of the cycle. The complex of CDK4/CDK6-cyclin D initiates the phosphorylation of retinoblastoma proteins family (Rb). This family includes pRb, p107 and p130 that play an important role to control the transcription process by binding and modulating the activity of transcription factors. These factors include the histone deacetylases, E2F family members and also chromatin remodeling which is required for proper completion of G1 as well as S phase (48-49). The precise role of CDK3 is still undiscovered; however, there is evidence that in tumour cells CDK3 binds to cyclin C at G0 stage and could also participate in the pRb phosphorylation during the transition from G0 to G1 phase (50-51). Later in the cell cycle, Cyclin-E will be synthesized and

binds to CDK2 forming a complex of cyclin-E/CDK2, which drives the transition from G1 to G1/S phase. This cyclin E/CDK2 complex is shown to be likely essential for initiating DNA replication by assisting the MCM chromosome maintenance proteins for replication. Once the cells enter S phase cyclin E/CDK2 complex need to be degraded in order to avoid any re-replication of DNA occurred (52). Before the G2/M phase, the newly synthesised cyclin A will binds to CDK2 and eventually to CDK1 to form either cyclin A/CDK2/CDK1 complexes, which regulate the completion of the S phase and initiate the G2/M phase (52). These cyclin A/CDK2/CDK1 complexes share several substrates involved in DNA replication and other proteins involved in cell cycle which required for the duplication of the genetic material. Cyclin A accumulates during the S phase and the binding of cyclin A to CDK1 indicates the transition to G2 phase. Then, during the G phase accumulation of cyclin B/CDK1 allows the inhibition of DNA replication, cell growth and new protein synthesis.

During the G2 phase, cyclin A is degraded by ubiquitin-mediated proteolysis while cyclin B is actively synthesised in order to bind to CDK1; the cyclin B/CDK1 complex is considered likely to be essential for initiating the mitosis phase. CDK1 becomes associated with two main cyclin B isoforms, B1 and B2, and believed to control the G2 to mitosis transition and progression through mitosis. These complexes postulated to have various functions in the cell cycle such as promoting centrosome separation, involved in the chromosomal condensation and other roles involving the progression through mitosis. The degradation of Cyclin B via ubiquitination within anaphase causes the cells to exit mitosis (53).

Furthermore, the rise and fall of CDK/cyclin complex activity may be regulated by interaction with some of the CDK inhibitory subunits (CKIs) (54-55). CKIs families have been identified in mammalian cells, the Cip/Kip family (CDK interacting protein/Kinase inhibitory protein) and the INK4 family (Inhibitor of Kinase 4), which block the activity of CDK/cyclin complexes in the cell cycle progression. The Cip/Kip family includes the gene of p21Cip1, p27Kip1 and p57 that will inhibit or activate by binding to the cyclin or cyclin/CDKs complexes during the G1 phase (56). In contrast, the INK family named p16 INK4a, p19 INK4d p18 and p15, strictly binds to the monomeric CDK, blocks kinase activity thus, inhibits the cell cycle with the absence of G1 phase CDKs, particularly CDK 2, 4 and 6 (57-58).

The unique cell cycle of malaria parasites

In order to complete its complex life cycle, these parasites must tightly regulate its cell division. How the parasite organises the cell cycle within the distinctive developmental stages, and which molecular mechanisms control the cell cycle, are still unresolved questions (34.59). Most attention has been given to the atypical intraerythrocytic schizogony of *P. falciparum*. Merozoites reside in the G1 phase and ring stage parasites, still in G1, prepare for the initiation of S-phase, which begins at the trophozoites stage, about 18 h post-invasion. Schizogony differs dramatically from the classical mitosis process, given that multiple rounds of nuclear divisions occur in the absence of cytokinesis. Further, these nuclear divisions occur asynchronously producing a single multinuclear cell, the schizont, that can contain up to 32 nuclei. Inevitably, this leads to a mixture of daughter nuclei that are in various stages of the division cycle (from mitosis, to G0 or G1 phase) (59). However, the even number of final nuclei (8 to 32) suggests that a synchronous final round of mitosis might occur (60). The schizogony of Plasmodium exhibits other atypical features such as lack of chromosome condensation, maintenance of the nuclear envelope throughout the nuclear division process, and presence of intranuclear spindles (6). These events also take place in other protozoan and fungal organisms, many of which show an intact nuclear envelope throughout the mitosis (61).

The analysis of the function and localisation of cell cycle regulators such as cyclins and CDKs is important in order to investigate the molecular mechanisms of cell cycle control in Plasmodium. To date, various mitotic kinases have been characterised in *P. falciparum*, including CDKs, NIMA and Aurora-related kinases. Some of these proteins are essential for parasite viability, as shown by reverse genetics (33). However, due to non-accessibility to the phenotype, it is impossible to determine the specific point in the cell cycle during which their function is essential (30). Furthermore, if genes could not be knocked out, the accessibility of the locus of interest could be determined by attempting to tag the gene of interest. Thus, the inability to produce a knockout can be proven whether it is due to the essentiality of the gene, rather than inaccessibility of the locus.

Strategies of conditional knock-outs or knock-downs approaches are currently available for *P. falciparum* and can be used to specifically determine the precise cellular role of Plasmodium cell cycle regulators (62).

***P. falciparum* cyclins**

The identification of the *P. falciparum* CDKs encouraged a search for their putative cyclin partners. The availability of *P. falciparum* genomic sequence data in 2002 has opened a new avenue to identify cyclin protein in the parasite. Four Plasmodium cyclins (Pfcyc-1, Pfcyc-2, Pfcyc-3 and Pfcyc-4) have been identified. Pfcyc-1 was the first cyclin to be identified and is closely related to human cyclin H (23% identity). Pfcyc-1, as well as mammalian cyclin H and p25 (the activator of CDK5), activates PfPK5 in vitro. Interestingly, as discussed above, the presence of Pfcyc-1 triggers PfPK5 autophosporylation. In addition, Pfcyc-1 is able

to activate Pfmrk (the Plasmodium CDK with maximal homology to CDK7) (63-64).

Following from the identification of Pfcyc-1, another three additional Plasmodium cyclins (Pfcyc-2, -3 and -4) were characterised (63-64). Pfcyc-1, Pfcyc-3, and Pfcyc4 are small genes, encoding proteins consisting of 327, 229, and 226 amino acids, respectively. In contrast, the Pfcyc-2 gene consists of two exons and potentially encodes a large protein (2281 amino acids) with a molecular weight of 273.5 kDa. The structure of the Pfcyc-2 gene is unique compared to that of other cyclins, and has low homology to mitotic (A and B) cyclins. No kinase activity was found to be associated with immunoprecipitated Pfcyc-2 from parasite extracts, while strong activity was detected in immunoprecipitates of Pfcyc-1, -3 and -4. It remains unclear whether or not Pfcyc-2 is a functional cyclin (63). A recent study has developed a cyclin hidden Markov model (HMM) from a variety of several model species to distinguish any putative cyclins in a extensive series of eukaryotes. It was found that Pfcyc-2 did not show a significant match to HMMs for any of the cyclin specificity and strongly suggests that Pfcyc-2 is not a true cyclin (64). The genes for Pfcyc-1 and Pfcyc-3 consist of one exon whereas the gene for Pfcyc-4 consists of 5 exons.

Compared to the mammalian cell cycle, ring and early trophozoites are equivalent to the G1 phase. However, it is difficult to describe the exact phase for the other parasite stages whether they are equivalent to S, M or the G2 phases. This is due to the asynchronicity of nuclear divisions, leading to a situation where a single schizont contains nuclei at different stages of the division cycle, precluding biochemical studies (65). According to the microarray-based transcriptomic profile from PlasmoDB, Pfcyc-1 mRNA is detectable in ring and early trophozoites stages, which is equivalent to the G1 and S phases of the mammalian cell cycle. It further peaks during late trophozoite stage and schizonts.

Future perspective

Mammalian cells and other organisms such as yeast, Drosophila and Xenopus have been traditionally used to study cell cycle regulation mechanisms. Indeed, the general mechanisms and molecules involved in cell division are assumed to be conserved among eukaryotic organisms. On the basis of this presumption, a similarity-based approach using sequence homology analysis can be used to identify cell cycle control molecules in different organisms.

Most model systems used in cell cycle studies, including humans, yeast, Drosophila and Xenopus, belong to the same phylogenetic lineage, the Opisthokonta. This lineage diverged from the Alveolata lineage (which contains the *Apicomplexa phylum*, of which Plasmodium is a member) early after the emergence of eukaryotes. Given the considerable phylogenetic distance between humans and malaria parasites, predicting the functions of malaria parasite genes and proteins based exclusively on sequence homology. Therefore, the actual role of proteins that are likely to be involved in cell cycle control must be determined experimentally.

Therefore, it appears evident that considerable efforts are required to identify the role of proteins involved in cell cycle regulation during the various stages the Plasmodium life cycle. In future experiments, reverse genetic such as Crispr/Cas9 and a conditional knockdown approach, such as the Rizosyme glms system which has proven to be successful in *P. falciparum*, is an experiment that could be performed to investigate the function of these plasmodial cyclin (72-74). The glmS ribozyme sequence is the knockdown element inserted in the 3′ UTR of the target gene therefore becoming expressed as mRNA. The self-cleaving activity of the ribozyme is dependent on its cofactor, glucosamine. In the presence of the inducer, glucosamine, the ribozyme causes cleavage of the 3′ UTR leading to the degradation of mRNA thus resulting in protein knock-down (72-73).

Subcellular localisation of proteins is an important tool to gather information of the function of a protein. In eukaryotic cells, many protein activities can be assigned to a certain cellular compartment. There are several approaches that could be achieved in order to examine the subcellular localisation of protein of interest such as Western blot, fluorescence microscopy-based techniques and identify protein interaction. By the investigation of the binding partner(s) for the functional domains could help us identify the important interaction in the parasite cell cycle progression. This could assist for further characterization of the cyclin/CDK machinery and the appropriate checkpoint systems in Plasmodium. Together with the developments in the imaging approaches such as super resolution techniques confocal as well as by having improved deconvolution of widefield microscopy, it will aid to improve the method to understand the cell cycles of Plasmodium.

CONCLUSION

To date, very little is known about cyclins in *P. falciparum*, an organism that undergoes a highly atypical cell cycle during its development both in human and in the mosquito hosts. Overall, this project has provided information on cyclin proteins localisation and essentiality. This is a great potential for novel drug targets for Plasmodium biology. By increasing the number of experimental implements may advance our understanding of the human parasite cell cycle thus elucidate the control mechanism for better future treatment.

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