Computational and Structural Biotechnology Journal 19 (2021) 5092–5107

COMPUTATIONAL AND STRUCTURAL BIOTECHNOLOGY JOURNAL

journal homepage: www.elsevier.com/locate/csbj

Review

CDPKs: The critical decoders of calcium signal at various stages of malaria parasite development

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ARTICLE INFO

Article history:
Received 24 April 2021
Received in revised form 31 August 2021
Accepted 31 August 2021
Available online 06 September 2021

Keywords:
CDPK
Calcium signaling
Plasmodium
Malaria
Chemical genetics

ABSTRACT

Calcium ions are used as important signals during various physiological processes. In malaria parasites, *Plasmodium* spp., calcium dependent protein kinases (CDPKs) have acquired the unique ability to sense and transduce calcium signals at various critical steps during the lifecycle, either through phosphorylation of downstream substrates or mediating formation of high molecular weight protein complexes. Calcium signaling cascades establish important crosstalk events with signaling pathways mediated by other secondary messengers such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). CDPKs play critical roles at various important physiological steps during parasite development in vertebrates and mosquitoes. They are also important for transmission of the parasite between the two hosts. Combined with the fact that CDPKs are not present in humans, they continue to be pursued as important targets for development of anti-malarial drugs.

Contents

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https://doi.org/10.1016/j.csbj.2021.08.054

1. Introduction

Calcium (Ca^{2+}) is a ubiquitous secondary messenger used for regulating a variety of physiologically important processes in all unicellular and multicellular organisms [19,29]. The dynamics of intracellular calcium is intricately regulated through constant exchanges between the extracellular environment, cell cytoplasm and calcium stores inside the cell via membrane contact sites [29]. Multicellular organisms respond to various developmental cues and environmental fluctuations through complex signal transduction networks that include protein receptors, enzymes, transcription factors, secondary messengers such as calcium (Ca^{2+}), lipids and hydrogen ions. Among them, Ca^{2+} is involved in regulation of various essential signaling cascades required for multiple physiological events [19,29]. Many Ca^{2+} sensor families such as Calmodulins (CaMs), Calmodulin-like proteins (CMLs), Calcineurin B-like proteins (CBLs) and Ca^{2+}-dependent protein kinases (CDPKs) have been discovered in plants [76,25]. In contrast to multicellular organisms, calcium signal in malaria parasite is mostly perceived and transduced via CDPKs. Interaction of CDPKs with calcium activates the kinase that in turn phosphorylates downstream substrates or mediates interactions with other proteins to form physiologically important high molecular weight protein complexes [25]. CDPKs are expressed in alveolates including apicomplexan parasites, and plants and importantly, are not encoded in human genome.

It has been suggested that malaria parasites and plants shared a common ancestor and diverged millions of years ago into two independent groups [73]. A recent phylogenetic study revealed evolutionary relationships and presence of monophyly in Archaeplastida that suggest early *de novo* gene fusion events between calmodulin gene and a protein kinase gene in the ancestral members giving rise to Calmodulin Fused Kinase (CFK) [24]. Scientific evidence suggests that this event occurred around the same evolutionary timeline in plants and apicomplexan parasites [24]. During the course of evolution, the CFK protein significantly diverged in sequence and structure and co-evolved for optimum functionality. These events are thought to be responsible for the present day CDPKs that continue to persist ever since in single celled eukaryotes, the apicomplexan parasites. The CDPK family is hugely expanded in plants with 34 members in *Arabidopsis thaliana* (*AtCDPKs*) and are reported to play crucial roles in plant development, growth, and stress responses [101]. We have performed primary amino acid sequence alignment of all 34 AtCDPKs with *P. falciparum* CDPKs and arranged the putative orthologues of *P. falciparum* CDPKs in *A. thaliana* according to their similarity index (Table 1). Moreover, phylogenetic analysis of AtCDPKs with PfCDPKs and TgCDPKs show close proximity among Apicomplexan

Table 1
Functional profiling of *Plasmodium* spp. CDPKs and putative orthologues in *Arabidopsis thaliana*. Calcium dependent protein kinases are critical at various stages of *Plasmodium* development. Expression & localization, functional roles of different CDPKs of *P. falciparum* and *P. berghei* are tabulated along with orthologues in *Arabidopsis thaliana*. *AtCDPKs are arranged in the decreasing order of their percentage identity score to the corresponding CDPKs of *Plasmodium* spp. The orthologs are generated according to the alignment of the primary amino acid sequence of *P. falciparum* and *P. berghei* CDPKs using Blast Tool [https://blast.ncbi.nlm.nih.gov/Blast.cgi].

CDPKs	Expression & Localisation	Function in *Plasmodium* spp.	References	Putative Orthologous AtCDPKs*(percentage identity)
P. falciparum				
CDPK1	Asexual (Rings, Trophozoites, Schizonts, Merozoites) Sexual (Gametocytes, Gametes)	Merozoite invasion, Schizont development and egress Male and Female gametogenesis, Mosquito transmission	[84,9,59,5,2,8,55,37,33,79] [6]	AtCDPK29$^{(37\%)}$/23$^{(36\%)}$/21$^{(36\%)}$/9$^{(36\%)}$/26$^{(36\%)}$
CDPK2	Asexual (Trophozoites, Schizonts) Sexual (Gametocytes)	- Male Gametocyte Exflagellation and Mosquito infection	[7] [7]	AtCDPK29$^{(40\%)}$/30$^{(39\%)}$/4$^{(39\%)}$/9$^{(39\%)}$/5$^{(39\%)}$
CDPK3	Sexual (Gametocytes)	–	[69]	AtCDPK33$^{(41\%)}$/29$^{(40\%)}$/9$^{(40\%)}$/4$^{(40\%)}$
CDPK4	- Sexual (Gametocytes)	Merozoite Invasion Male Gametocyte Exflagellation and mosquito transmission	[35] [58,97,112,91,90]	AtCDPK29$^{(40\%)}$/21$^{(39\%)}$/26$^{(37\%)}$/34$^{(37\%)}$
CDPK5	Asexual (Schizonts, Merozoites)	Merozoite egress (Release of Egress-Specific Organelles)	[32][1]	AtCDPK25$^{(39\%)}$/1$^{(38\%)}$/21$^{(37\%)}$/29$^{(37\%)}$/23$^{(37\%)}$
CDPK6	–	–	–	AtCDPK1$^{(32\%)}$/2$^{(32\%)}$/18$^{(31\%)}$/9$^{(31\%)}$
CDPK7	Asexual (Rings, Trophozoites, Schizonts, Merozoites)	Ring to trophozoite transition	[64]	AtCDPK12$^{(42\%)}$/4$^{(40\%)}$/29$^{(40\%)}$/21$^{(38\%)}$
P. berghei				
CDPK1	Asexual (Schizonts) Sexual (Gametocytes, ookinetes, oocysts) Pre-erythrocytic (Sporozoites)	- Zygote development and mosquito transmission, ookinete motility Sporozoite motility and invasion of hepatocytes	[35] [100,35] [45]	AtCDPK29$^{(37\%)}$/26$^{(36\%)}$/6$^{(35\%)}$/21$^{(35\%)}$
CDPK3	Sexual (ookinetes)	Ookinetes motility, microneme secretion, midgut invasion	[102,54,78,108,35]	AtCDPK33$^{(41\%)}$/29$^{(40\%)}$/4$^{(39\%)}$/2$^{(38\%)}$
CDPK4	Asexual (Schizonts) Sexual (Gametocytes) Pre-erythrocytic (sporozoites)	Merozoite Motility and Invasion Cell cycle regulation in male gametogenesis, Sexual reproduction & mosquito transmission, ookinete infectivity, Ookinetes Motility Sporozoite motility and invasion, merosome formation/release from hepatocytes	[35] [12,91,52,36,35] [46,45]	AtCDPK29$^{(40\%)}$/33$^{(39\%)}$26$^{(37\%)}$/21$^{(37\%)}$
CDPK5	Pre-erythrocytic (sporozoites, merosomes)	Sporozoite motility, Sporozoite invasion and Release of hepatic merozoites from merosomes	[45]	AtCDPK4$^{(37\%)}$/2$^{(37\%)}$/17$^{(37\%)}$/29$^{(36\%)}$/34$^{(36\%)}$
CDPK6	Pre-erythrocytic stages (Sporozoites)	Productive invasion of hepatocytes	[28]	AtCDPK25$^{(32\%)}$/16$^{(32\%)}$/30$^{(31\%)}$/28$^{(31\%)}$
CDPK7	–	–	–	AtCDPK2$^{(41\%)}$/4$^{(40\%)}$/29$^{(40\%)}$/33$^{(38\%)}$

CDPKs relative to *A. thaliana* (Fig. 1). Furthermore, our analysis suggests that compared to all other AtCDPKs, AtCDPK16, AtCDPK18 and AtCDPK28 cluster closely with PfCDPKs (Fig. 1, [101].

In plants, CDPKs have been reported to regulate the activity of transcription factors and control various signaling cascades [53,63]. *Nicotiana tabacum* CDPK1 for instance phosphorylates the transcription factor Repression of Shoot Growth (RSG) at Ser-114 thereby facilitating its sequestration in the cytoplasm through interaction with 14–3-3 protein and preventing its translocation to the nucleus [53]. Flowering Locus T (FT) is a critical protein in floral transition (vegetative-to-reproductive phase) that shares homology with phosphatidylethanolamine binding protein (PEBP) family and interacts with phosphorylated (phosphorylation at T282) basic region/leucine-zipper (bZIP) transcription factor called FD [63]. CDPKs have been demonstrated to carry out the phosphorylation of FD and mediate FT-FD complex formation that ultimately leads to floral transition [63]. Similarly, a recent study in malaria parasite suggests a role for *P. falciparum* CDPK1 in transcriptional regulation of gametocyte specific genes [6]. Disruption of CDPK1 led to transcriptional dysregulation of distinct subsets of genes required for transition from asexual to sexual phase of the parasite life cycle.

The CDPK family in malaria parasites is constituted of fewer members as compared to plants. Increases in intracellular calcium level at various developmental stages of malaria parasite are distinctly decoded by effector CDPK proteins that form an intricate part of the calcium signaling cascade. CDPKs are evidently critical for various physiological processes and are therefore indispensable for successful completion of malaria parasite life cycle. In the current review, we present the latest update on the function of CDPKs in malaria parasite life cycle separated into erythrocytic phase, sexual phase and pre-erythrocytic phase. At the end, different strategies previously used to design inhibitors against CDPKs for future anti-malarial drug development are discussed alongside elucidation of functional roles of each CDPK in parasite biology. We have also discussed the use of chemical genetics for functional characterization of CDPKs and approaches for multi-kinase targeting.

2. Domain organization and calcium mediated activation of CDPKs

The *P. falciparum* CDPK family contains 7 members with CDPK1, 2, 3, 4, and 5 conforming to the canonical architecture while CDPK6 and CDPK7 show atypical domain organization along with unusually long amino acid sequences (Fig. 2). The canonical domain organization of CDPK contains four contiguous regions starting with the most variable N-terminal domain followed by an intervening autoinhibitory junction domain (JD) between the N-terminal kinase domain and C-terminal calmodulin-like domain (CaMLD) that contains calcium binding motifs called EF-hands (Fig. 2) [50]. Post-translational modifications of CDPKs are responsible

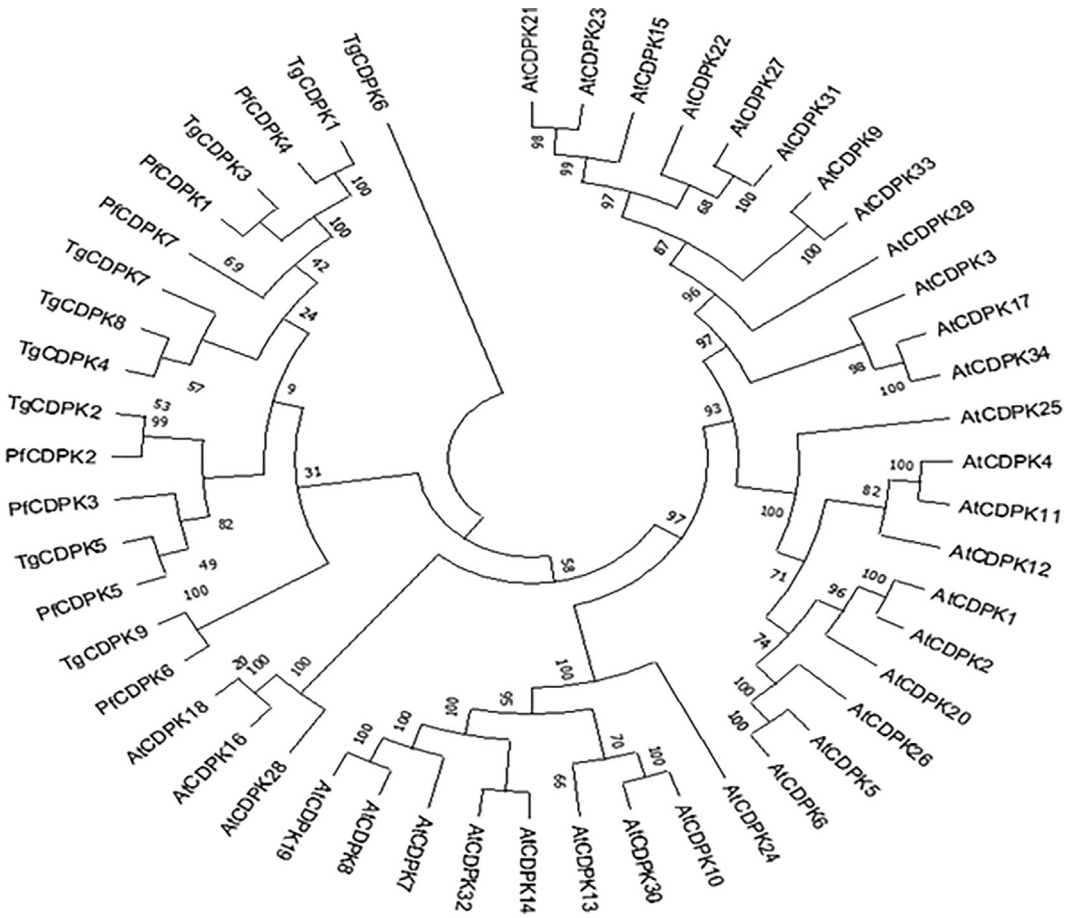


Fig. 1. Phylogenetic relationships of calcium-dependent protein kinases (CDPKs) of *Arabidopsis thaliana*, *Plasmodium falciparum* and *Toxoplasma gondii*. A phylogenetic tree is constructed on the basis of full length primary amino acid sequences of all CDPKs from A. thaliana (34 AtCDPKs), *Plasmodium falciparum* (7, PfCDPKs) and *Toxoplasma gondii* (12 TgCDPKs). Evolutionary history was inferred using Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100replicates) are shown next to the branches. The evolutionary distances are computed using the Poisson correction method and are in the unites of the number of amino acid substitutions per site. This analysis involved 50 amino acid sequences and were analysed by MEGA X software. Compared to other AtCDPKs, AtCDPK16, AtCDPK18 and AtCDPK28 cluster closely with PfCDPKs.

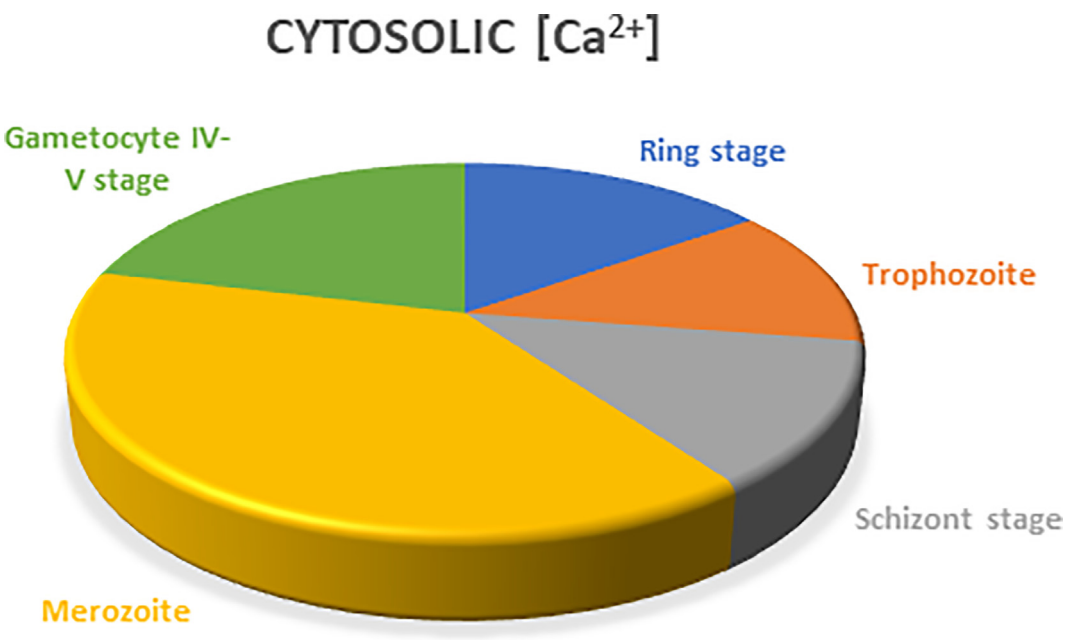
Fig. 2. Domain Organization of *Plasmodium falciparum* calcium dependent protein kinases (PfCDPKs). The canonical architecture of PfCDPKs consists of N-terminal variable region followed by a conserved kinase domain and a calmodulin-like domain containing two pair of EF-hands. The intervening region between the kinase domain and the EF-hands is constituted by a junction domain that allows auto-inhibition of the CDPK activity in absence of calcium ions. CDPK1, 2, 3, 4, and 5 conform to the canonical architecture while CDPK6 and 7 are unusually long proteins with variations in the assembly of EF-hands along the length of the protein. CDPK7 contains a lipid interacting domain called pleckstrin homology (PH) domain.

for modulating the activity, localization and interaction of CDPKs with their cognate substrates [84,79,2]. Myristoylation of a conserved glycine residue (Gly^2) at the N-terminus of PfCDPK1 is critical for its membrane localization in schizonts and merozoites [84]. The glycine residue at the second position is also conserved in *P. falciparum* CDPK2, and CDPK4. The kinase domain is the most conserved domain containing ATP binding motif and an activation loop. A CDPK Activation Domain (CAD) that is formed jointly by JD and CaMLD plays a critical role in overall calcium mediated activation of CDPKs [115]. In plant CDPKs, calcium binding induces conformational changes in CaMLD that allows for release of autoinhibition imparted by CAD on the kinase domain [10]. Interestingly, in apicomplexan CDPKs, calcium mediated activation is associated with release of autoinhibition of the kinase domain by CAD along with allosteric interactions of CAD with kinase and N-terminal domain [116,115].

Distinct functional roles have been assigned to *Plasmodium* CDPKs largely based on their differential expression and intracellular localization in various stages of the parasite development (Table 1). In addition, different CDPKs have different sensitivities to calcium ion concentrations ($[Ca^{2+}]$) and as such, may be optimally active under resting or elevated $[Ca^{2+}]$. Interestingly, partially overlapping functionality of a few members of the CDPK family in rodent-specific malaria parasites suggests co-operativity between different CDPKs to optimize physiological output in the same signaling cascade [45,35]. Under certain conditions, the parasites may adapt themselves for reduced or complete loss in activity of a particular CDPK with concomitant modulations in the gene transcription profile or epistatic changes to counter the loss of the CDPK activity.

3. Calcium dynamics and storage in the malaria parasite

Extracellular calcium plays a critical role during invasion of red blood cells (RBCs) by merozoite. Moreover, calcium is also required for intraerythrocytic development especially during the transition of rings to trophozoites [113](Fig. 3). Calcium concentration show dynamic changes as the parasite develops inside the RBC (Fig. 3) [92] and correlates well with the critical functional roles of different CDPKs especially during transition to the subsequent developmental stages. For example, high $[Ca^{2+}]$ in merozoites is required for activation of CDPK1 and CDPK5 for invasion and egress of the parasite, respectively during blood stages [9,32]. The calcium ion concentration in the RBC is consistently low (~100 nM) [3]. To counter the low calcium availability, parasitized RBCs show markedly increased calcium uptake, perhaps by expressing ion channels of parasite origin on the surface of infected RBCs [30]. Additionally, the parasite uses Ca^{2+} ATPases derived from the plasma membrane of RBCs to pump calcium inside the parasitophorous vacuole [43]. During the start of second infection cycle, the parasitophorous vacuole serves as a calcium store house that is important for the initial establishment of infection in a niche with an unfavorable ionic environment [43]. Depletion of the parasitophorous vacuole calcium level, even for a short duration, is significantly detrimental for intraerythrocytic development of the parasite [43]. Two key parasite organelles responsible for maintaining the intracellular pool of calcium are the endoplasmic reticulum (ER) and acidic compartments [3,15]. A plant-like sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) of the parasite transfers cytoplasmic calcium to the ER [3]. Like in higher eukaryotic cells, the malaria parasite also possesses inositol 1,4,5-trisphosphate (InsP3) sensitive calcium stores, one being the ER and the other being the digestive food vacuole [93,11]. It is widely accepted in the malaria field that protein kinase G (PKG) acts as a unifying signal in controlling calcium surges at different time points of the parasite life cycle. PKG controls the biosynthesis of phosphoinositides including the precursor of InsP3 that mediates calcium release from intracellular stores [16]. Calcium is also stored in alternate locations such as mitochondria [42,98,109], and acidocalcisomes [80]. However, the precise signaling molecules that mediate release of calcium from these organelles remain elusive.



Stage of the parasite	Cytosolic [Ca²⁺]
Ring stage	373nM
Trophozoite	30nM
Schizont stage	310nM
Merozoite	949nM
Gametocyte IV-V stage	522nM

Fig. 3. Calcium concentration across different stages of intraerythrocytic *Plasmodium falciparum* development. Basal level of cytosolic calcium ion concentration according to the proportion at different stages of *P. falciparum* development inside the red blood cells is represented as a pie chart and the absolute values [92] are tabulated underneath.

Calcium is also an important mediator that controls the phenomenon of circadian rhythm. Circadian rhythm is critical for maintaining homeostasis of various physiological processes inside the host. Calcium has been shown to control melatonin induced circadian rhythm in the malaria parasite. Calcium controlled circadian rhythm plays an important role in *in vivo* synchronization of parasite during vertebrate infection [49]. How a surge in intracellular calcium brought about by melatonin is transformed into downstream molecular events associated with parasite growth and development is not completely understood. Whether melatonin induced calcium waves are propagated downstream by CDPKs needs to be investigated.

4. CDPKs and asexual intra-erythrocytic development of malaria parasite

Asexual intra-erythrocytic development of malaria parasite begins with the invasion of a naïve RBC by a free, viable merozoite. Invasion of RBC by a merozoite is a complex, multistep process that necessitates coordinated and sequential release of parasite ligands [104,114] stored in apical organelles called micronemes, rhoptries and dense granules (Fig. 4). Upon successful invasion of host RBC, the merozoite gets surrounded by the host plasma membrane that is extensively modified by the developing parasite as it transforms into ring, trophozoite and schizont stage. The mature schizont bursts releasing 16–32 motile merozoites into the blood stream. These merozoites result in propagation of the infection cycle. Depending on the *Plasmodium* species, the asexual replication cycle could vary from 48 to 72 hr.

CDPKs play critical roles at various stages of the malaria parasite asexual replication cycle inside RBCs (Fig. 4, Table 1). CDPK1 mediates release of micronemes during invasion of RBC by merozoite and as such blocking its activity results in decrease of microneme discharge and subsequent invasion of RBCs [9]. The molecular mechanisms of CDPK1 mediated microneme discharge and invasion are not completely understood. However, existing data suggests it is involved in the phosphorylation of components of motor and inner membrane complexes [48,65]. In addition, cross talk between CDPK1 and Protein Kinase A plays a critical role during the invasion process [65]. A recent study has demonstrated that phosphorylation of RhopH3 is critical for invasion of RBCs, correct localization in rhoptries and discharge during invasion [33]. It has been suggested that phosphorylation of RhopH3 is mediated by CDPK1 [33]. A recent elegant study by More et al., demonstrates formation of a multimeric complex in merozoites involving phosphorylated CDPK1, regulatory subunit of PKA (PKAr) and Pf14-3-3I that plays a critical role in the invasion process and microneme discharge [79]. These studies suggest an essential role for CDPK1 in

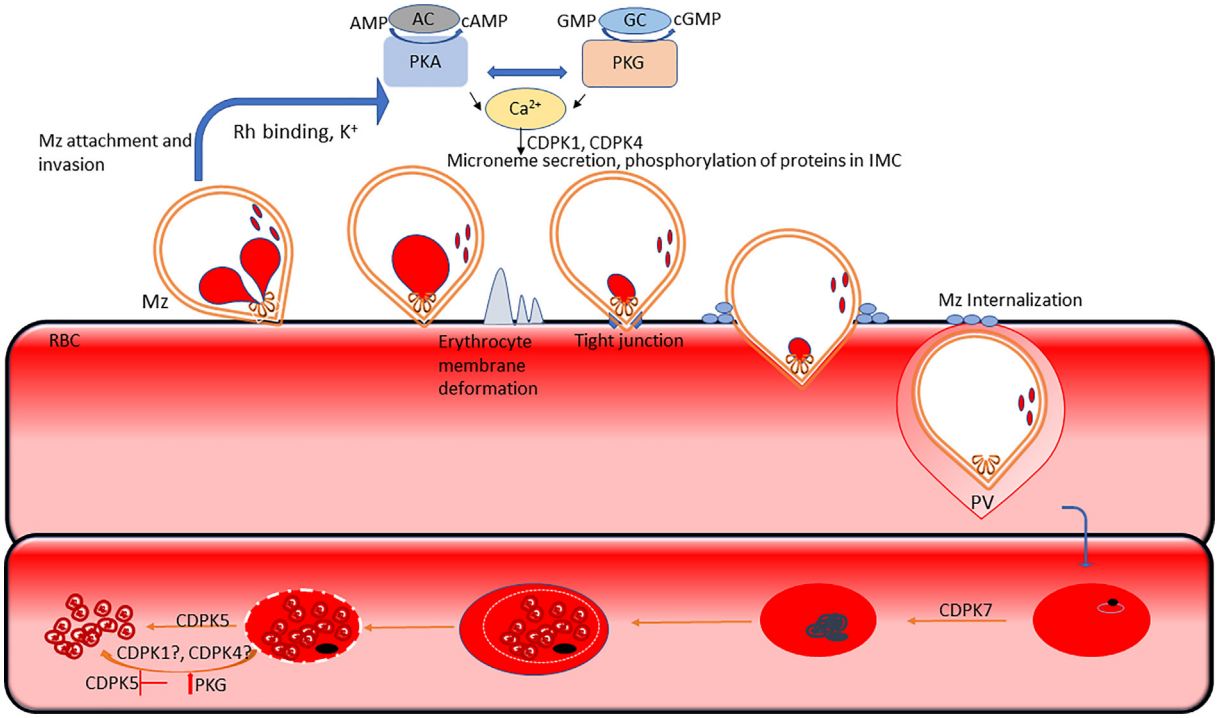


Fig. 4. *Plasmodium falciparum* CDPKs play essential roles during merozoite invasion and parasite development inside red blood cells. Invasion of RBC by merozoite involve different stimuli including specific parasite ligands with cognate host cell receptors (for review see ref. [40,41] and low extracellular K + concentration. These signalling events result in activation of protein kinase A (PKA) and protein kinase G (PKG) through formation of cyclic AMP and cyclic GMP by adenylyl cyclase (AC) and guanylyl cyclase (GC), respectively. The downstream events include cAMP-dependent and cGMP-dependent Ca^{2+} mobilisation from internal stores leading to activation of CDPK1 that results in release of selected apical organelles called micronemes. Release of microneme content is critical for successful invasion of host RBCs. CDPK4 has also been shown to mediate invasion by cooperating with CDPK1 and PKG. CDPK7 plays an important role in transition of parasite from ring stage to trophozoite stage [64] while CDPK5 is critical for release of merozoites from mature schizonts [32]. CDPK5 deficient parasites could be rescued from egress defect by artificial activation of PKG through compensatory mechanisms that may involve other CDPKs such as CDPK1 or CDPK4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

asexual proliferation of the parasite and as such, corroborates well with the inability to delete CDPK1 in the WT parasite background. However, recent studies by Bansal et al., suggest a strategy that employs gradual adaptation of mutant parasites lacking a functional copy of CDPK1 to study its biomolecular properties [7,6]. Reduction in kinase activity of CDPK1 through genetically engineered endogenous mutant CDPK1 with a different gatekeeper residue led to alterations in the expression level of other CDPK family members. Molecular adaptations of mutant CDPK1 expressing *P. falciparum* with reduced kinase activity suggests evolution of compensatory mechanisms by the parasite [8]. Due to pre-adaptation of the parasites for reduced activity of mutant CDPK1, the mutant gene could be successfully knocked out [6] while repeated attempts failed to disrupt the WT *cdpk1* [59,106,6]. Phosphorylation of CDPK1 in *Plasmodium* has been shown to follow PKG dependent signaling pathway. Abrogation of PKG activity through chemical genetics resulted in decrease of CDPK1 phosphorylation implicating PKG as an upstream kinase that directly or indirectly mediates phosphorylation of downstream CDPK1 protein. *In vitro* experiments with recombinant PKG identified S64 and T231 as putative phosphorylation sites in recombinant CDPK1. It has been suggested that the activity of CDPK1 in the parasite is controlled by PKG via phosphorylation of T231 residue in the activation loop of CDPK1. However, phosphorylation of T231 was not observed in the global PKG dependent phosphoproteome *in vivo*. Paradoxically, PKG dependent S64 phosphorylation of CDPK1 that was also identified *in vivo* did not affect the kinase activity of CDPK1. Interestingly, preferential localization of phosphorylated CDPK1 at the apical end of merozoites is indicative that phosphorylation dependent spaciotemporal modulation and formation of higher molecular weight complexes is crucial for parasite invasion of RBCs [2,79]. Overall, molecular details of CDPK1 mediated apical organelle discharge and invasion are not very well understood and needs further investigation.

In addition to the phosphorylation of CDPK1, PKG cooperates with CDPK5 to mediate egress of merozoites from mature schizonts [32,1]. Destabilization-domain mediated down-regulation of CDPK5 was demonstrated to critically affect the egress process [32]. The trapped merozoites within CDPK5 knock-down parasites when released through physical rupture were competent for invasion of fresh RBCs suggesting no role of CDPK5 in invasion or it could also be possible that the residual amount of CDPK5 in the mutant parasites was sufficient for invasion [32]. Interestingly, the block in egress due to CDPK5 down-regulation could be rescued by artificial activation of PfPKG [1]. It has been hypothesized that the basal activity of PKG in CDPK5 down regulated parasites could not allow complete processing of egress related ligands that could only be possible in presence of CDPK5 or artificial upregulation of PKG activity [1]. These results suggest that like CDPK1 [8,6], parasites may also adapt for loss of CDPK5. This hypothesis needs to be further evaluated and if proven would again highlight remarkable adaptability of malaria parasite for loss of important genes. Compensatory adaptations have also been reported in mitogen-activated protein kinase (MAPK) homologues, pfmap-1 and pfmap-2; another *Plasmodium* kinase family [31]. Furthermore, it would be of interest to identify the substrates of CDPK5 in WT parasites and the mutant parasite with decreased level of CDPK5 under normal and PKG upregulated conditions. This may help in understanding the compensatory pathway in CDPK5 down-regulated parasites. These compensatory adaptations are likely to

be missed in experiments that rely on conditional knock-out or instantaneous block in activity of the kinase using pharmacological inhibitors. Taken together, these observations strongly suggest rewiring of calcium effectors that is evident in case of CDPK1 [8,6], CDPK3 [78], CDPK4 [35] and CDPK5 [1].

CDPK4 is predominantly involved in controlling microgametogenesis [36] however, the dependence on CDPK4 for efficient invasion of RBCs became strikingly evident in the background of hypomorphic PKG that is conserved in human and rodent malarias [35]. A genetic screen to identify important interactions between protein kinases during parasite replication within RBC demonstrated critical dependence of the parasite on CDPK4 under conditions where PKG mediated calcium signaling was compromised [35]. Under these conditions, phosphorylation of components belonging to the inner membrane complex was observed suggesting an important role for CDPK4 dependent phosphorylation of certain motor complex proteins [35]. Under normal conditions, PKG mediated increase in intracellular calcium activates CDPK1 and possibly CDPK4 resulting in invasion of RBCs. In the background of hypomorphic alleles of CDPK1 or PKG, the role of CDPK4 becomes critically important as it complements the function of CDPK1 possibly in conjunction with other members of CDPK family [35,8]. It is important to note that viable parasites were obtained with disrupted CDPK4 in both *P. falciparum* and *P. berghei* [59,12,108,44,35] suggesting, perhaps, functional compensation by other kinases during blood stage replication. This emphasizes overlapping roles of distinct CDPKs in physiologically important processes. Furthermore, CDPK2, CDPK3, and CDPK6 are dispensable for asexual proliferation of human malaria parasite, *P. falciparum* [7,106,117]. Apart from the role of CDPKs in invasion and egress of the parasite during blood stages, an atypical member of this family in *P. falciparum*, CDPK7, has been shown to mediate transition of rings to trophozoites [64]. Hence, CDPK family is important in entry, exit and progression of malaria parasite during asexual replication within RBCs. We have tabulated (Table 2) the essentiality status of different CDPKs for asexual blood replication of *P. falciparum* and *P. berghei* based on large scale functional profiling studies [17,117]. Observed differences pertaining to the essentiality of certain CDPKs in blood stage proliferation of *P. falciparum* and *P. berghei* may be attributed to species specific differences and/or *in vitro* and *in vivo* methods, respectively, for culturing the blood stages.

Table 2
Essentiality status of calcium dependent protein kinases of *P. falciparum* [117] and *P. berghei* [17] for asexual blood replication.

Gene Identifier	CDPK	Essentiality	References*
P. falciparum			
PF3D7_0217500	calcium-dependent protein kinase 1	Essential†	[59,106,8,6]
PF3D7_0610600	calcium-dependent protein kinase 2	Dispensable	[7]
PF3D7_0310100	calcium-dependent protein kinase 3	Dispensable	[106]
PF3D7_0717500	calcium-dependent protein kinase 4	Dispensable	[59,35]
PF3D7_1337800	calcium-dependent protein kinase 5	Essential	[32]
PF3D7_1122800	calcium-dependent protein kinase 6	Dispensable	
PF3D7_1123100	calcium-dependent protein kinase 7	Essential	[64]
P. berghei			
PBANKA_0314200	calcium-dependent protein kinase 1	Dispensable	[56,44]
PBANKA_0408200	calcium-dependent protein kinase 3	Dispensable	[102,108,44]
PBANKA_0615200	calcium-dependent protein kinase 4	Significantly slow#	[12,108,44,35]
PBANKA_1351500	calcium-dependent protein kinase 5	Essential	[108,44]
PBANKA_0925500	calcium-dependent protein kinase 6	Significantly slow#	[108,44]
PBANKA_0925200	calcium-dependent protein kinase 7	Essential	[108,44]

* Additional studies that support the essentiality status of CDPKs for blood stage replication.
\# Knock-out parasites were obtained.
† Knock-out was obtained in the mutant parasite background.

5. CDPKs and sexual development of malaria parasites

Malaria parasite undergoes distinct and complex morphological modifications that allow the parasite to survive under different host environment and switch from one host to the next. The role of individual plasmodial CDPKs in the asexual growth of the parasite inside RBCs has been studied appreciably compared to its developmental stages in mosquito and vertebrate hepatocytes. Most of the studies on understanding the function of CDPKs in sexual development of the parasite have been performed on the rodent malaria parasite, *P. berghei*. However, highly conserved amino acid sequences of individual CDPK between different *Plasmodium* species is suggestive of biochemical functional similarities for each enzyme especially in the sexual stage development. It is believed that in all cases, mosquito specific factors play crucial role in formation of gametes inside the mosquito midgut. Two independent studies identified xanthurenic acid as the mosquito factor responsible for exflagellation, the process of microgamete formation [13,38]. Exflagellation has been classically studied under *in vitro* conditions: ambient temperature and alkaline pH (8.0) (for review see [103]. Extracts prepared from different parts of mosquito showed that the requirement for alkaline pH could be substituted but reduced temperature is critical for microgamete formation [14,87,39]. Increase in cytosolic cyclic guanosine monophosphate (cGMP) and intracellular calcium mobilization are essential for successful exflagellation [60,62]. Xanthurenic acid results in activation of guanylyl cyclase in mature gametocytes [82] that in turn leads to increase in intracellular calcium concentration [61,74] thereby leading to the activation of CDPK4 [12] and perhaps other CDPKs as well [7,6](Fig. 5). CDPK4 is critical for regulating key processes during microgametocyte exflagellation such as cell cycle regulation, cytoskeletal rearrangements leading to axoneme and mitotic spindle formation [12,36]. Mimicking the conditions that prevail inside the mosquito midgut increases the activity of PfCDPK4, an ortholog of PbCDPK4 in *P. falciparum* [58]. As such, PfCDPK4 was found to be critical for exflagellation of *P. falciparum* microgametocytes [90] as its ortholog in *P. berghei*. Moreover, CDPK4 is also critical for ookinete infectivity in mosquitoes. Viable ookinetes could only be obtained upon cross-fertilization of macrogametes of CDPK4 KO with microgametes of a parasite strain with P25 and P28 genes deleted [12]. Both these strains are incapable of infecting mosquitoes individually either due to no formation of ookinetes (in case of CDPK4 KO parasites) or formation of unproductive ookinetes (in case of P25/P28 KO parasites). The P25/P28 positive ookinetes obtained from the cross of ♀ CDPK4 KO × ♂Δp25/Δp28 parasites could only establish 1.8% of wt infection in the mosquitoes highlighting possible role of CDPK4 in the ookinete infectivity [12]. In addition to the role of CDPK4 in ookinete infectivity, it is also important for efficient ookinete gliding. The role of CDPK4 in ookinete gliding becomes appreciably significant in the background of hypomorphic PKG. Increase in intracellular calcium downstream of PKG-activation supports phosphorylation of glideosome complex by CDPK1 and CDPK4 that in combination with the activation of CDPK3 results in ookinete gliding and possibly subsequent invasion of midgut cells [35].

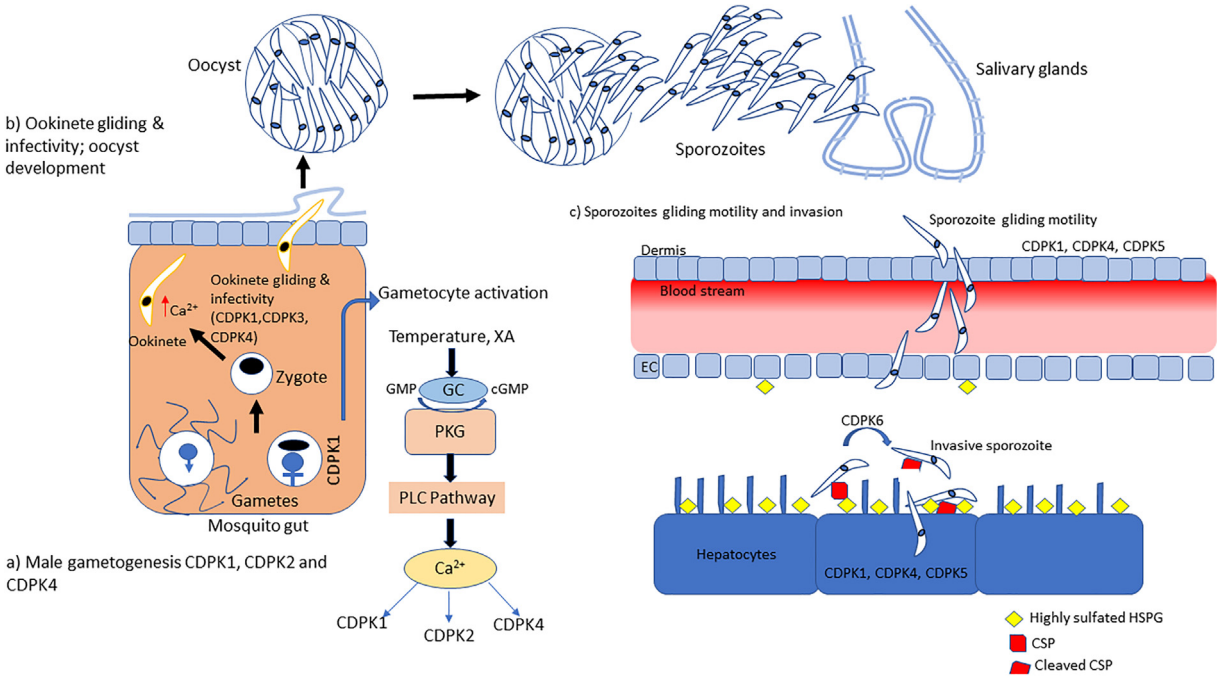


Fig. 5. Sexual and pre-erythrocytic development of *Plasmodium falciparum* and role of CDPKs. a) Mature gametocytes taken up during blood meal by an *Anopheles* mosquito encounter drop in temperature, and xanthurenic acid (XA) that triggers elevation of cGMP levels by guanylyl cyclase (GC) that results in activation of protein kinase G (PKG). Activation of PKG is followed by calcium release from intracellular stores through PLC mediated pathway that involves generation of inositol 1,4,5-trisphosphate. The elevated calcium results in activation of CDPK1, CDPK2, and CDPK4 that play critical roles in gametogenesis. b) Once ookinetes are formed from zygote, CDPK1, CDPK3, and CDPK4 help in gliding motility and invasion of mid-gut epithelium by ookinetes thereby playing a crucial role in ookinete infectivity and oocysts development. CDPK3 is critical for discharge of micronemes in ookinetes. c) Once *Plasmodium* sporozoites are deposited in the dermis, they migrate from the site of inoculation to blood stream to ultimately reach the liver sinusoids. CDPK1, CDPK4, and CDPK5 help the sporozoite in gliding motility. The sporozoites infect hepatocytes by first traversing the endothelial cells (EC) of liver sinusoids and enter the Space of Disse. Sporozoites interact with the hepatocytes due to the presence of highly sulfated heparan sulfate proteoglycans (HSPG) on the surface of hepatocytes. The initial interaction of CSP on the sporozoite surface with highly sulfated HSPG on hepatocytes activates CDPK6 that finally results in proteolytic cleavage of CSP and stronger interaction of CSP with highly sulphated HSPGs. These events result in switching the sporozoites from traversal mode to invasion mode and culminate in productive infection of hepatocyte. CDPK1, CDPK4, and CDPK5 are also important in invasion of hepatocytes by sporozoites however molecular details remain largely unexplored.

Importantly, CDPK1 and CDPK4 individually are redundant for gliding motility of ookinetes [36,90,35].

CDPK1 and CDPK2 are also critical regulators of gametogenesis in malaria parasite. Disruption of CDPK1 and CDPK2 leads to defect in gamete formation. Upon *in vitro* induction of mature gametocytes, the parasites without CDPK1 and CDPK2 are not able to disintegrate the RBC membrane and remain trapped inside the host cells [7,6]. While CDPK2 seems to have a role only in male gametocyte exflagellation [7], CDPK1 seems to be essential for gametogenesis in both male and female parasites [6]. Interestingly, after induction of the CDPK1 KO parasites, no rounding up is evident in the female gametes suggesting that the block is in a step preceding RBC rupture [6]. The exact molecular mechanisms used by CDPK1 and CDPK2 in controlling gametogenesis need to be investigated as it may provide novel targets for drug development. Moreover, it would be interesting to compare the substrates of CDPK1 and CDPK2 in gametogenesis since both enzymes are involved in the same physiological event of male gamete formation along with CDPK4. Therefore, complete understanding of molecular signaling mechanisms associated with the exflagellation process may help in identification and validation of novel drug targets for blocking malaria transmission.

In rodent malaria parasite, CDPK1 has been demonstrated as an important factor that allows translational activation of repressed mRNAs in the zygote stage for proper transformation into ookinetes [100]. A contrasting role for CDPK1 in *P. falciparum* is evident in the asexual stage of the parasite wherein KO of CDPK1 leads to transcriptional dysregulation of gametocyte specific genes [6]. Dysregulated gene expression evident in mutant parasites without CDPK1 in *P. falciparum* and *P. berghei* could be an indirect consequence of transcriptional and translational dysregulation of transcription factor ApiAP2G and AP2-O, respectively. Altogether, these observations strongly implicate role of CDPKs in controlling gene expression patterns across different developmental stages of parasite growth as has been observed in plants [63,86,53].

CDPK3 was shown to be transcriptionally upregulated in the sexual stages of parasite suggesting possible role for this kinase in parasite development inside the invertebrate host [69]. PbCDPK3, a homolog of PfCDPK3 in *P. berghei*, was demonstrated as a critical component for efficient gliding [102] and successful invasion of mosquito midgut epithelium by ookinetes [102,54]. While one study concluded that CDPK3 does not play a role in gliding [54], Siden-Kiamos et al., showed CDPK3 to be a critical factor for gliding motility of ookinetes [102]. They demonstrated that CDPK3 KO ookinetes were unable to be dispersed from the aggregates when co-cultured with insect cells *in vitro* [102]. The block in establishing a productive infection in mosquitoes by CDPK3 KO parasites was shown to be due to inability of the KO parasites to breach the physical barrier of mosquito midgut epithelium [102] perhaps due to inability of microneme discharge in CDPK3 KO parasites [35]. Hence, parasites lacking CDPK3 produce non-functional ookinetes that do not transform into oocysts [108]. Direct injection of CDPK3 KO parasites into the haemocoel allowed the KO parasites to produce infectious salivary gland sporozoites similar to the WT [102]. The CDPK3 KO parasites grew as well as the WT in the asexual stages and did not show any effect up till the conversion of mature female gametocytes to ookinetes [102]. Moreover, the CDPK3 KO sporozoites demonstrated equally efficient motility

and subsequent infectivity in mice [102] suggesting no perceptible role of CDPK3 in these processes.

6. CDPKs and pre-erythrocytic phase of malaria parasite development

Pre-erythrocytic phase of malaria parasite life cycle starts when infectious sporozoites are injected by an infected female *Anopheles* mosquito into the dermis of a naïve individual. The sporozoites are deposited in the skin along with saliva that helps in preventing coagulation of blood during blood-meal. After intradermal deposition, sporozoites traverse through the dermis and endothelial cells by gliding motility that is powered by the conserved actomyosin motor. After traversing through epithelial and endothelial cells, the sporozoites eventually switch from migratory to invasive mode that results in productive infection of the host hepatocytes by formation of exoerythrocytic forms (EEFs) surrounded by an intact parasitophorous vacuole. Parasite develops inside the hepatocyte and undergoes asexual schizogony with formation of hundreds and thousands of hepatic merozoites. As an immune evasion mechanism, hepatic merozoites are released from infected cells as merosomes, packets of hundreds of parasites surrounded by host cell membranes. Merosome packaging protects hepatic merozoites from phagocytic attack by sinusoidal Kupffer cells, and release into the lung microvasculature enhances the chance of successful erythrocyte invasion. Calcium accumulation by the hepatic merozoites ensures low host cytoplasmic calcium concentration that prevents phosphatidyl serine exposure on outer leaflet of the host cells that is a classical signal for cell apoptosis [107]. PKG has been shown to play a role in merosome formation and exit from hepatocytes. Considering that CDPKs are important effectors downstream to PKG signaling at various cross-roads of parasite development, PKG mediated merosome formation, and exit from host cells has been shown to be regulated by different CDPKs [45], discussed below. From the site of inoculation in dermis to hepatocyte invasion, a sporozoite traverses through different cell types until it switches to an invasive mode for productive infection of target hepatocyte. There is enough evidence to suggest that sulfation of heparan sulfate proteoglycans (HSPGs) determines the mode of sporozoite entry of the host cell [96,28]. When host cells are decorated with low sulfated HSPGs, sporozoite show migration phenotype while highly sulfated HSPGs on the surface of host cells allow productive invasion. Interestingly, this correlates well with the sulfation pattern of the host cells that the sporozoites migrate through and invade. Proteolytic processing of major sporozoite surface protein, cirumsporozoite protein (CSP) is also important for switching to invasive phenotype [27]. Interaction of sporozoites with optimally decorated sulfated-HSPGs activates a signaling cascade involving CDPK6 [28]. This CDPK6 mediated signal transduction plays an important role in controlling the switch of sporozoites from migratory to invasive mode and is also interestingly involved in proteolytic processing of CSP [28]. It is worth noting that CDPK6 KO sporozoites show residual invasive phenotype that suggests some level of compensatory mechanism or involvement of an alternative inefficient pathway for invasion [28]. It would be interesting to test the invasion of hepatocytes with conditional knock-out of CDPK6.

Sporozoite motility is also controlled by dynamic changes in intracellular calcium levels [20]. Ca^{2+} flux regulates secretion of adhesins onto the sporozoite surface that mediate attachment to substratum, and turnover of adhesion sites between the sporozoite and the substrate is an important factor that controls the overall efficiency of sporozoite movement and invasion [85]. It is interesting to note that some level of migration also occurs in hepatocytes until the sporozoite establishes a productive infection in a target hepatocyte [81]. The sporozoites need to express optimum levels of invasive ligands on their surface that are conditioned on migration through liver sinusoids and hepatocytes [105]. Taken together, highly sulfated HSPGs present on hepatocytes and endothelial cells of liver sinusoids engage the sporozoites through CSP that initiates a signaling cascade involving CDPK6 along with other kinases that ultimately results in cleavage of CSP and increased avidity of sporozoite interaction with host hepatocytes and culminates in productive invasion of the host cells [28].

An auxin-inducible degron (AID) system has been used to investigate the functional roles of various target genes in the pre-erythrocytic phase of parasite development. AID is a conditional knock down system that allows degradation of the target protein upon addition of the plant hormone, auxin (Indole3-acetic acid (IAA)) [88,94]. The system was initially established in *P. berghei* to study the functional role of a calcium dependent protein phosphatase called calcineurin and CDPK1 [94]. AID mediated depletion of CDPK1 corroborated earlier findings of the critical role of CDPK1 in normal morphology of ookinetes [100,94]. CDPK1 does not seem to have any evident role in sporozoite motility and invasion of hepatocytes [56]. However, AID mediated conditional degradation of CDPK1 had demonstrable and significant effects on the sporozoite motility and invasion of hepatocytes [45]. These observations show that results obtained from two conditional systems may differ due to different efficiencies of target gene knock-down using particular conditional systems. Therefore, data generated from conditional knock down strategies should be interpreted carefully for functional assignment of target genes. Similarly, results obtained from conditional knock down approach and generation of fixed hypomorphic mutants may lead to different functional outcomes. True physiological functions of target CDPKs are most likely to be picked with a robust conditional system, though it may be masked due to compensatory adaptations of the parasite during generation of permanent hypomorphic or null mutants.

AID fused CDPK1, CDPK4 and CDPK5 parasites were used for recovery of viable and infectious sporozoites from salivary glands. Conditional depletion of CDPK1, CDPK4 and CDPK5 resulted in loss of gliding motion without affecting waving and attachment to the substrate [45]. Interestingly, cell traversal by CDPK1 and CDPK5 depleted sporozoite was not affected [45]. However, pharmacological inhibition of WT CDPK4 using bumped kinase inhibitor 1294 (BKI-1294) suggested a role for CDPK4 in sporozoite motility [46]. Furthermore, invasion of HepG2 cells decreased substantially upon depletion of all the three CDPKs individually, suggesting the existence of partially overlapping functions between the 3 kinases during the invasion process (Fig. 4). Blocking the activity of CDPK4 using BKI-1294 [112] in CDPK1 or CDPK5 depleted parasites augmented the decrease in the number of intracellular liver stages 48 h post invasion of hepatocytes [45]. The available evidence suggests functional cooperativity among different CDPKs for regulating sporozoite invasion and subsequent development within hepatocytes. Artificial activation of PKG by augmenting cGMP concentration using a phosphodiesterase inhibitor called zaprinast rescued the defects in CDPK1 and CDPK5 knock down parasites. These findings recapitulate rescue of merozoites by artificial activation of PKG in CDPK5 depleted asexual stage parasites [1] and seems to suggest existence of compensatory adaptations in parasites for hypomorphic alleles [8,35]. Conditional knock-out of PKG (PKG-cKO) resulted in the arrest of parasites inside hepatocytes suggesting a role of PKG in either the formation of hepatic merozoites or their exit from hepatocytes via merosome formation [34,46]. Since CDPK5 acts downstream to PKG in mature schizonts and is a critical regulator of merozoite release from mature schizonts, pleiotropic role of CDPK5 was explored in exit of hepatic merozoites. Depletion of CDPK5 in liver stages reduced the number of merosomes and exit of hepatic merozoites. This corelated well

with a longer pre-patent period observed in the CDPK5 depleted parasite compared to vehicle control. Manually ruptured merosomes resulted in equivalent blood stage infection in both the CDPK5 depleted and control parasites demonstrating that CDPK5 depleted parasites block the exit of hepatic merozoites from merosomes [45].

7. Targeting CDPKs of malaria parasite for drug development and functional characterization

Orthologues of CDPKs are not encoded in human genome. Furthermore, CDPKs are critical for various physiological processes in the malaria parasite lifecycle [57]. Consequently, CDPKs have been considered as potentially good drug targets for the discovery of novel antimalarial agents (see reviews [18,77,83]. High throughput screening has led to the identification of different *Pf*CDPK1 targeting inhibitory scaffolds including 2,3,9-trisubstituted purines [59], indolizines [68] and imidazopyridazines [68,47,4,22,23,66] (Table 3). Among these scaffolds, imidazopyridazines have been extensively studied compared to others. Imidazopyridazines can be classified into two groups based on the type of aromatic linker between the core and the R2 substituent [47]. Compounds containing the pyrimidine linker inhibit growth of *P. falciparum* at the late schizont stage while compounds with a non-pyrimidine linker inhibit trophozoites. Therefore, interruption of *P. falciparum* CDPK enzymes' signal transduction with small molecule inhibitors could be used as a potential strategy for developing effective therapeutic agents for treatment of malaria. Interestingly, poor correlation exists between the *in vitro* inhibition of recombinant CDPK1 protein and inhibition of parasite growth using imidazopyridazines [4]. Chemical genetics approach was used to conclude no perceptible role of CDPK1 in asexual parasite development [47] and inhibitory effect of imidazopyridazines was assigned largely due to inhibition of PKG [47,66] however, existence of compensatory mechanisms could not be ruled out in the transgenic parasites with hypomorphic alleles [8,35].

Although *Plasmodium* CDPKs regulate parasite-specific processes and orthologues are absent in mammalian cells; they still share high amino acid sequences and structural similarities with eukaryotic protein kinases in their ATP-binding sites. Selective targeting of *Plasmodium* CDPKs over mammalian kinases will therefore be important to avoid toxicity caused by off-target effects on host cell signaling or cell cycle regulation [90]. Analysis of protein kinase binding pockets for design of potent and selective kinase inhibitors suggests 5 domains within the ATP binding sites that can be occupied by a compound [70,89]. Of these 5 domains, the more widely reported examples of selective inhibitors are those that target a hydrophobic pocket adjoining an atypically small "gatekeeper residue" [90,67,26,75,111]. Several Apicomplexan CDPKs that are essential for maintenance of fundamental cellular processes and parasite survival have smaller gatekeeper residues creating an enlarged hydrophobic pocket. The enlarged gatekeeper hydrophobic pocket allows the binding of a series of inhibitors called bumped kinase inhibitors (BKIs) that project a bulky C-3 aromatic substituent (Fig. 6). Most of the mammalian kinases have larger gatekeeper residues that precludes the binding of the large C3-aryl substituent of BKIs and thus, these inhibitor BKIs have little to no effect against the host cell kinases.

A set of pyrazolopyrimidine BKI derivatives were shown to potently inhibit PfCDPK4 phosphorylation activities with high selectivity like the TgCDPK1 enzyme on which these compound designs were based (Fig. 6). These series of BKIs were designed using iterative reasoning within the context of X-ray crystallography and computational modeling to guide optimization with functional groups needed to improve potency, selectivity, and pharmacokinetic properties based on BKIs targeting *Toxoplasma gondii* and *Cryptosporidium parvum* CDPK1 (CpCDPK1) [110], 2021, [91,112]. *T. gondii* and *C. parvum* are members of apicomplexan parasites that are known to cause human ailments such as toxoplasmosis [72] and cryptosporidiosis [95], respectively especially in people with weak immune system. Pyrazolopyrimidine based BKIs contain a C-3 bulky aromatic substituent which projects into the hydrophobic pocket adjacent to the gatekeeper position. These derivatives mainly inhibit male gametocytes exflagellation in *P. falciparum* and in *P. berghei*-infected mice. Formation of sporozoites in *Anopheles stephensi* mosquitoes decreased after feeding them on the pyrazolopyrimidine derivatives containing PfNF54 infected human blood. The 5-aminopyrazole-4-carboxamide is another BKI inhibitor series capable of potentially inhibiting PfCDPK4 in the nanomolar range and which concomitantly inhibited exflagellation of male gametocytes [51]. Phenothiazine derivatives were identified as non-ATP-competitive inhibitors of PfCDPK4 among which Trifluoperazine (TFP) was considered to have a high binding affinity and Ki value for PfCDPK4 [21]. TFP binds to the calmodulin-like domain of PfCDPK4 which prevents repositioning of the autoinhibitory J-domain upon binding of Ca^{2+} and thus locking the kinase in its inactive conformation [21]. As CDPK4 has a predominant role in the sexual stage of the parasite, it is mainly considered as a target to inhibit transmission of the parasite between two hosts. The importance of developing an anti-malaria drug based on a transmission blocking strategy cannot be emphasized more since malaria is mostly transmitted to humans through the bites of female *Anopheles* mosquitoes [26].

Rout et al., screened the Malaria Box compounds to find potential inhibitors against CDPK5 [99]. This study led to the identification of MMV687246 that potentially inhibits the kinase activity of recombinant PfCDPK5 [99]. The effect of this compound on the malaria parasite and its specificity for CDPK5 need to be investigated further.

8. Targeting PfCDPKs with the autoinhibitory junction domain

In addition to the screening of small organic compounds as potential drugs for targeting CDPKs, the auto inhibitory junction domain has also been employed for inhibiting cognate CDPKs [5,37,9]. Under basal calcium level, the J domain acts as a pseudosubstrate and inhibits the kinase activity of CDPK by blocking the active site of the enzyme. Binding of Ca^{2+} with EF-hands, triggers a conformational change in CAD that allows new intramolecular interactions and release the autoinhibitory constraints imparted by the J domain on the kinase domain. Using the inhibitory mechanism of the J domain, Flaherty and co-worker designed a strategy to inhibit PfCDPK1 activity using the inhibitory JDD (J domain disruptor) peptide that mimics the C-terminal region of the J domain and thus allosterically inhibits PfCDPK1 by locking it in an inactive conformation [37]. Alignment of the J domains of all *Pf*CDPK proteins shows low conservation at the amino acid level, suggesting that a JDD peptide may be used for selective inhibition of the cognate CDPK. On the basis of these alignments and previous studies, the C-terminal region of the J domain strongly binds and inhibits the activity of CDPK1 [37,9]. A peptide corresponding to the C-terminal region of the J domain was used for inhibiting the kinase activity of CDPK1 [9]. The peptide, when used in increasing concentrations, resulted in a dose dependent decrease in formation of ring stage parasites. This was the first report that directly demonstrated the essential role of CDPK1 in the invasion of RBCs by merozoites. Additionally, controlled, ectopic expression of the complete PfCDPK1 J-domain with a GFP tag was used to understand the functional role of the kinase in the parasite [5].

Table 3
Small organic inhibitors against different *Plasmodium falciparum* CDPKs.

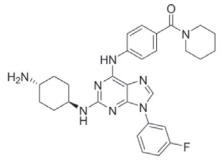
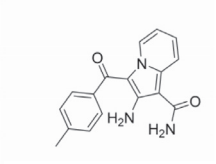
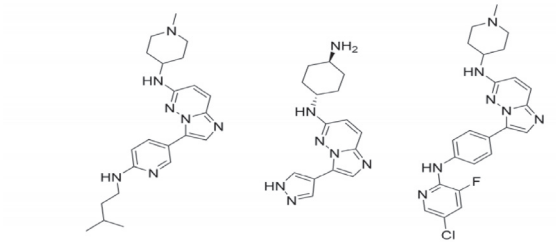
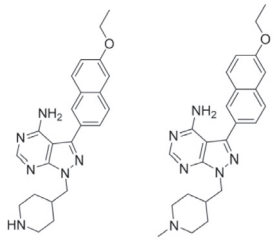
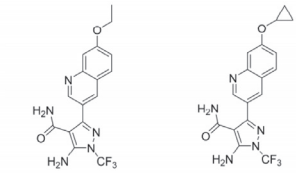
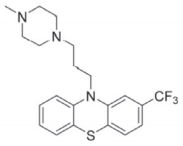
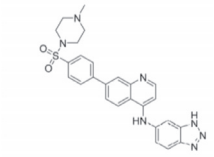
CDPKs	Potential Inhibitor molecules	Reference
*Pf*CDPK1	2,3,9-trisubstituted purines	[59]
		
	Purfalcamine	
	*Pf*CDPK1 IC50 - 17nM	
	Indolizines	[68]
		
	1 (2-amino-3-[(4-methylphenyl) carbonyl]indolizine-1-carboxamide)	
	*Pf*CDPK1 Ki -260 nM	
		[47] [68] [4] [22] [23] [66]
	Imidazopyridazines	
	PfCDPK1 IC50 *13nM* *70nM* *12nM*	
*Pf*CDPK4	Pyrazolopyrimidine derivatives	[91-112]
		
	BKI-1 N-methypiperidine analog of BKI-1	
	*Pf*CDPK4 IC50 4nM 10 nM	
		[51]

Table 3 (*continued*)

CDPKs	Potential Inhibitor molecules	Reference
	 5-Aminopyrazole-4-carboxamide derivatives *Pf*CDPK4 IC50 37 nM 82 nM	[21]
	 trifluoperazine (TFP) PfCDK4 Ki: 150uM	
*Pf*CDPK5	 MMV687246 *Pf*CDPK5 binding affinity -11.6 Kcal/mol	[99]

9. Multi-kinase targeting approach to identify potent anti-plasmodial inhibitors

To develop novel, multi-target anti-plasmodial drug candidates, Lima et al., developed a computational approach which helped in identification of multi-kinase inhibitors against *Pf*CDPK1, *Pf*CDPK4 and PK6 [71]. Shape-based and machine learning models were developed to find the potential target molecule, and the best model was selected for virtual screening of a large number of databases consisting of drug-like molecules. From this study, ten hits were identified, among which LabMol-171, LabMol-172, and LabMol-181 showed potent inhibitory activity against blood stages of multi-drug resistant parasites at nanomolar concentrations. *In vitro* evaluation of these hits against drug-sensitive (3D7) and multidrug-resistant (Dd2) *P. falciparum* parasites showed that these molecules can inhibit the growth of both drug-sensitive (3D7) and multidrug-resistant parasites [71]. BKI-1294 is another example of a multi-targeting inhibitor that has been shown to block malaria transmission with EC_{50} values of 10 nM, 100 nM and 200 nM against PfCDPK4, PfCDPK1 and PfPKG, respectively [46,91,90]. Targeting multiple enzymes with single or multiple agents may help in overcoming compensatory adaptations by malaria parasite and is likely to increase the efficiency of malaria control and elimination efforts.

10. Use of chemical genetics to understand the functional roles of CDPKs

The gatekeeper position in the ATP binding pocket of kinase domains has been exploited for understanding the function of CDPKs using a chemical genetics approach. The gatekeeper position is usually constituted of an amino acid with a bulky side chain. Genetic approaches to substitute the bulky amino acid with smaller gatekeeper residues sensitizes the mutant enzyme for BKIs. Substitution with a smaller gatekeeper residue results in enlargement of the hydrophobic pocket that accommodates the ATP purine group in the ATP-binding site. BKIs contain a bulky C3-aryl substituent that can occupy this enlarged ATP-binding hydrophobic pocket. Amongst the *P. falciparum* CDPKs, CDPK1 and CDPK4 have smaller gatekeeper residues (i.e., serine and threonine, respectively) compared to CDPK2 (methionine), CDPK3 (methionine), and CDPK5 (leucine) that have larger gatekeeper residues. Small gatekeeper residues in CDPK1 and CDPK4 have been exploited to study the functional role of these kinases in the parasite biology. WT CDPK4 containing a serine gatekeeper is inhibited by BKI-1 and BKI-1294, and treatment of mature gametocytes with BKIs leads to defects in male gametocyte exflagellation under both *in vitro* and *in vivo* conditions [91], (Ojo et al., 2014). BKI-1294 was further used to demonstrate role of CDPK4 at multiple stages of

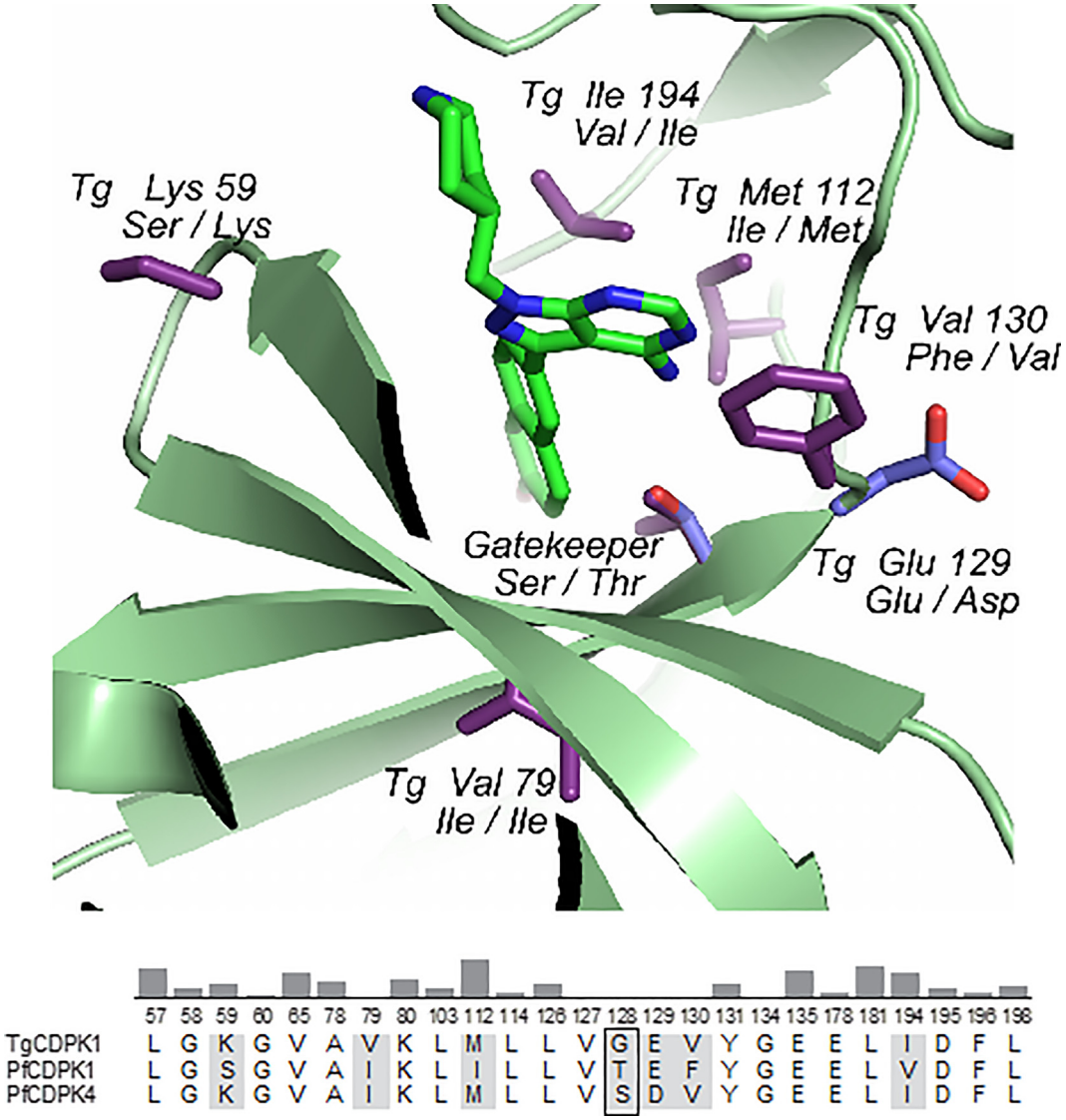


Fig. 6. Structural model to simulate binding motifs of PfCDPK4 enzyme with BKI-1 was based on previous crystal structures of *Tg*CDPK1 co-crystallized with the same inhibitor (PDB3sx9). The 2-ethoxynaphthyl functional group of BKI-1 projected into the hydrophobic pocket adjacent the gatekeeper residue. Some level of conservation within the ATP binding cavity is shown by the alignments of TgCDPK1, PfCDPK1 and PfCDPK4 amino acid residues proposed to interact with BKI-1 are presented below the model image. BKIs designed against TgCDPK1 that also inhibit PfCDPK4 can also have some inhibition of PfCDPK1 based on similarities of residues that contact the compound. The bars at the top represent the relative contribution of atoms in each amino acid moiety to the surface area of within TgCDPK1 binding site. Residues that are not conserved between the 3 enzymes are shaded grey. The gatekeeper residues for the 3 proteins are boxed in. Amino acid residue numbering is based on TgCDPK1 sequence.

genome replication during male gametogenesis [36]. Substitution of smaller gatekeeper in CDPK4 (S147M) imparted resistance against BKI-1294 treatment and results in increased EC_{50} value of exflagellation [90,36]. Furthermore, BKI-1294 was used to show cooperativity between CDPK1 and CDPK4 in controlling gliding motility in ookinetes [35]. Altogether, these studies provided a proof-of-concept that BKIs may be combined along with existing anti-malarial regimen for blocking malaria transmission. Similarly, screening of BKI inhibitors against WT CDPK1 resulted in a BKI that specifically inhibited the WT enzyme while showing an increased IC_{50} value against the mutant CDPK1 that contains a bulky gatekeeper residue [8]. These studies demonstrate the utility of chemical genetics in understanding the physiological function of CDPKs, especially under conditions where genetic manipulation may not be feasible [46,45].

11. Summary and outlook

CDPKs have distinct and partially overlapping functions across some stages of malaria parasite development. This is particularly evident in processes that are crucial for the transmission of the parasite. A conserved set of CDPKs are however employed by zoites for invasion of host cells, and there seems to be less overlap in the requirement of CDPKs, especially during invasion of RBCs by merozoites where CDPK1 plays a critical role. However, the relative contribution of CDPKs may vary at different stages or biological processes. There is a large body of evidence to suggest employment of compensatory adaptations by mutant parasites having hypomorphic alleles of kinases belonging to multigene families. There is a need to identify the functional roles of CDPKs through techniques that do not provide sufficient time for parasites to adapt.

Moreover, a comprehensive set of CDPKs that are employed in a physiological process needs to be deciphered as it would provide a guide on what CDPKs need to be targeted to overcome undesired compensatory events. This emphasizes the need to target two functionally complementary CDPKs rather than one as it may avoid development of resistance by the parasite. Complete understanding of the signaling cascades employing CDPKs would therefore help in designing better strategies for targeting malaria parasites.

CRediT authorship contribution statement

Manish Sharma: Data curation, Formal analysis, Investigation. **Himashree Choudhury:** Data curation. **Rajarshi Roy:** Data curation. **Samantha A. Michaels:** Writing-review & editing. **Kayode K. Ojo:** Data curation, Formal analysis, Writing-review & editing. **Abhisheka Bansal:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Writing-review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We acknowledge the support from Department of Biotechnology (BT/PR28256/MED/29/1313/2018), Science & Engineering Research Board (CRG/2018/002533) and University Grants Commission (F.30-433/2018(BSR)) provided to AB for conducting studies that are directly or indirectly related to the theme of this review. Fellowship provided to MS from Council for Scientific and Industrial Research is also acknowledged. The funders have no role in the design and decision for publishing this review. We also greatly appreciate useful discussion and technical help provided by Ethan A. Merritt. We also acknowledge the feedback received from Louis H. Miller on the initial draft of the review. We regret for not being able to cite studies of all other colleagues working on the subject of the review.

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