Plasmodium berghei Calcium Dependent Protein Kinase 1 Is Not Required for Host Cell Invasion

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Abstract

Plasmodium Calcium Dependent Protein Kinase (CDPK1) is required for the development of sexual stages in the mosquito. In addition, it is proposed to play an essential role in the parasite's invasive stages possibly through the regulation of the actinomyosin motor and micronemal secretion. We demonstrate that Plasmodium berghei CDPK1 is dispensable in the parasite's erythrocystic and pre-erythrocystic stages. We successfully disrupted P. berghei CDPK1 (PbCDPK1) by homologous recombination. The recovery of erythrocytic stage parasites lacking PbCDPK1 (PbCDPK1−) demonstrated that PbCDPK1 is not essential for erythrocytic invasion or intra-erythrocytic development. To study PbCDPK1’s role in sporozoites and liver stage parasites, we generated a conditional mutant (CDPK1 cKO). Phenotypic characterization of CDPK1 cKO sporozoites demonstrated that CDPK1 is redundant or dispensable for the invasion of mammalian hepatocytes, the egress of parasites from infected hepatocytes and through the subsequent erythrocytic cycle. We conclude that P. berghei CDPK1 plays an essential role only in the mosquito sexual stages.

Introduction

Ca2+ signaling plays a crucial role in Apicomplexan parasites. It mediates micronemal protein secretion in Plasmodium [1], Toxoplasma [2–4], Cryptosporidium [5] and Eimeria [6]. In Plasmodium, Ca2+ is a key mediator of egress from the infected erythrocyte [7–10], gametogenesis and ookinete motility in the mosquito [11–13]. It is also implicated in sporozoite invasion of hepatocytes [14].

A major mediator of Ca2+ signaling in Apicomplexans is a family of Calcium Dependent Protein Kinases (CDPK) that is unique to Apicomplexans, plants and some algae. Apicomplexans express five major classes of CDPKs all of which contain Ca2+-binding EF-hand motifs linked to a kinase domain [15]. P. falciparum has seven CDPK homologs belonging to four classes. Plasmodium CDPK1 is conserved in all Plasmodium species and has homologs in Toxoplasma gondii and Cryptosporidium parvum [15]. It is expressed throughout the parasite lifecycle [16,17] suggesting that it has multiple roles in different parasite stages. It is best studied in Plasmodium berghei sexual stages, where CDPK1 controls the transcription of a subset of translationally-repressed mRNAs, and a knock down of P. berghei CDPK1 (PbCDPK1) blocks ookinete development [16]. In asexual stages, CDPK1 is implicated in parasite invasion based on three lines of evidence. First, Plasmodium falciparum CDPK1 (PfCDPK1) is transcriptionally coexpressed with components of the parasite’s actinomyosin motility apparatus and can phosphorylate key components such as the glideosome associated protein 45 (GAP45) and the myosin tail-interacting protein (MTIP) in vitro [17–19]. Second, small molecule and peptide inhibitors of PfCDPK1 block P. falciparum schizontony [17] and micronemal secretion [20], respectively. Third, attempts to obtain P. falciparum and P. berghei parasites with disrupted CDPK1 have failed [Kato et al, 2008; [16]], suggesting that CDPK1 is essential for the parasite’s erythrocytic cycle. CDPK1’s role in sporozoites is yet to be determined. Here we report a comprehensive genetic strategy in P. berghei to examine CDPK1’s function throughout Plasmodium’s lifecycle.

Results and Discussion

CDPK1 is dispensable for the erythrocytic cycle

In order to test CDPK1’s function in the erythrocytic cycle, we attempted to generate a direct knockout of P. berghei CDPK1 (PbCDPK1) (Fig. 1A). Contrary to previous reports [21], we were successful in recovering the knockout parasites (CDPK1−) (Fig. 1B). We confirmed the loss of CDPK1 expression during erythrocytic development in CDPK1− mutant parasites using RT-PCR (Fig. 1C). The recovery of parasites lacking CDPK1 demonstrates that PbCDPK1 is not essential during the erythrocytic cycle. To determine if lack of PbCDPK1 compromises intra-erythrocytic development in the parasite, we monitored the growth rate of CDPK1− erythrocytic stages in mice (Fig. 1D). The parasitemia of PbCDPK1− and wildtype (WT) parasites was similar, suggesting that PbCDPK1 is not essential during the erythrocytic cycle. Therefore, PbCDPK1 function is either redundant or dispensable during erythrocytic invasion, intracellular development and egress. Previously reported failures to obtain CDPK1− mutants may be attributed to technical differences.
A sexual-stage specific knockdown of PbCDPK1 inhibits ookinete development [16]. PbCDPK1 is required for the translational activation of mRNAs in the developing zygote [16]. Consistent with these previous reports, CDPK1- parasites did not form oocysts in the mosquito midgut. The average number of oocysts in the midguts of WT-infected mosquitoes was 37 ± 9 (n = 8) and in CDPK1- infected mosquitoes was 0 (n = 8).

CDPK1 is not Essential in Pre-erythrocytic Stages

PbCDPK1 and one of its putative substrates, MTIP are present in sporozoites [16]. We hypothesized that PbCDPK1 may function in sporozoite invasion of hepatocytes. Since CDPK1- parasites do not complete sexual development in the mosquito, studying the function of PbCDPK1 in pre-erythrocytic stages required a conditional mutagenesis approach.

We generated conditional mutants (CDPK1 cKO) in which oocyst formation and sporozoite development is normal. We used the Flp-FRT system [22] to bypass the requirement for PbCDPK1 in the parasite’s sexual cycle. The PbCDPK1 open reading frame was modified by the addition of flanking FRT sites in parasites expressing FlpL recombinase under the control of the TRAP promoter (FlpL/TRAP) [22] (Fig. 2A, B). In this system, PbCDPK1 is expressed normally during erythrocytic development and the sexual cycle in the mosquito. However, the open reading frame is excised during sporozoite development in the mosquito midgut generating mature sporozoites that lack PbCDPK1 (CDPK1 cKO) (Fig. 2C).

Equal numbers of sporozoites were recovered from salivary glands of CDPK1 cKO-infected and FlpL/TRAP-infected mosquitoes, demonstrating that CDPK1 is not required for parasite invasion of salivary glands. To determine if CDPK1 plays a role in hepatocyte invasion, we used CDPK1 cKO sporozoites to infect the human hepatoma cell line, HepG2. FlpL/TRAP sporozoites were used as controls in this and subsequent experiments. There was no significant difference in the number of liver stages formed by CDPK1 cKO or control sporozoites (Fig. 2D). These results demonstrate that CDPK1 is not essential for the parasite’s invasion of hepatocytes or subsequent intrahepatic development. To study CDPK1’s role in parasite egress from infected HepG2 cells, we determined the number of extracellular merosomes released in CDPK1 cKO and control infected HepG2 cultures. There was no significant difference in the number of merosomes present in both cultures (Fig. 2E). These results indicate that CDPK1 is not essential for parasite development in and egress from hepatocytes.

To determine if CDPK1 cKO sporozoites infect normally in vivo, we infected mice with either CDPK1 cKO or control sporozoites and monitored the appearance of erythrocytic stage parasites. Mice infected with CDPK1 cKO sporozoites had a normal pre-patent period of infection and there was no significant difference in the growth of erythrocytic stage parasites (Fig. 2F). Our results demonstrate that despite being expressed throughout the parasite life-cycle, PbCDPK1’s essential role is in ookinete development. During invasion and egress by asexual stages and sporozoites, PbCDPK1’s function appears to be redundant. Since P. berghei has 6 CDPK homologs and multiple CDPKs are
expressed in the same parasitic stage, it is likely that their functions are overlapping. This model is supported by the limited efficacy in *P. falciparum* cultures of purfalcamine, a potent *in vitro* inhibitor of recombinant PfCDPK1 [17]. Our results suggest that the purfalcamine’s low efficacy in *P. falciparum* cultures may be attributed to PfCDPK1 being non-essential in erythrocytic stage parasites rather than poor pharmacokinetic properties. Failure to disrupt PfCDPK1 may result from a low rate of recombination at the PfCDPK1 locus and may not necessarily reflect an essential function for PfCDPK1. Indeed a specific conditional knock-down PfCDPK1 in erythrocytic stages achieved a very significant...
decrease in protein levels but did not reveal a defect in intraerythrocytic growth [23].

An alternative model to explain our results is that CDPK1 has an essential role in erythrocytic stage invasion of *P. falciparum* but not of *P. berghei*. However, the conservation of the motor components between *P. berghei* and *P. falciparum* suggests that their regulatory mechanism will be similarly conserved. The *Toxoplasma gondii* ortholog of CDPK1 (TgCDPK3) is also dispensable for tachyzoite invasion and motility [24–26]. TgCDPK3 knockout tachyzoites are comparable to wild-type in normal egress. They demonstrate a delay only in ionophore-induced egress. This TgCDPK3-dependent mechanism of tachyzoite egress revealed by ionophore treatment is likely to be limited to a subset of intracellular conditions encountered by *T. gondii* in the host. Support for PICDPK1’s essential role during the erythrocytic cycle was provided by the developmental arrest in early schizogony triggered by the ectopic expression of PICDPK1’s auto-inhibitory junction domain in *P. falciparum* [23]. However, the interpretation of these results is confounded by the potential for non-specific effects. For example, the ectopically expressed junction domain may bind and inhibit related kinases required during intra-erythrocytic development. Future work will focus on distinguishing between the two proposed models using additional genetic tools. Our work reveals redundancy in the *Plasmodium* kinome and will influence future attempts to develop drugs that target multiple CDPK homologs to increase their efficacy.

**Materials and Methods**

**Ethics Statement**

This project was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Animal Care and Use Committee of the New Jersey Medical School (protocol number P132D1113).

**Generation of CDPK1- Parasites**

The CDPK1-targeting plasmid was constructed in pL0001. A 0.6 kb fragment encompassing the 5’ UTR region of PbCDPK1 was amplified using primers 5’GGTACCCTTTTACCTGG-GAAAAGT and 5’CCCGAGGCTTTTACGT-GATTAACCCC. The product was cloned into the pL0001 vector using KpnI an SbfI (underlined). Another 0.6 kb fragment encompassing exon 5 and the 3’UTR region of PbCDPK1 was amplified using primers 5’GGATCCTTCACTTTTTTTCATTTGTTTTT and 5’TACCATTA and inserted into the previously generated plasmid using KpnI (underlined) and EcoRI (underlined). The final insert was released from the targeting construct using NotI and EcoRI. Transfections of the targeting plasmid into PbA TRAP/FplL parasites [22] were carried out using standard methodology. Transfected parasites were selected by pyrimethamine and cloned by limiting dilution. Parasites were passaged into *Anopheles stephensi* mosquitoes.

**Mosquito Cycles**

*Anopheles stephensi* mosquitoes were fed on CDPK1- infected or CDPK1 cKO-infected Swiss-webster mice. CDPK1 cKO-infected mosquitoes were maintained at 20°C until day 14 post bloodmeal and then transferred to 25°C. PbA TRAP/FplL parasites were used as controls. Sporozoites were dissected from their salivary glands at days 18–21 post feeding. Infectivity of salivary glands was similar for cKO and control parasites at approximately 10000–15,000 sporozoites/mosquito. Midguts were dissected from CDPK1- infected mosquitoes at day 9 post blood meal.

**Southern Hybridization and Diagnostic PCR**

Parasite genomic DNA was digested with BamHI and SpeI before transfer to a nylon membrane. The membrane was probed with dioxigenin-labeled exon 5 (DIG High Prime DNA labeling detection kit, Roche Applied Sciences). DNA hybridization was visualized using a chemiluminescent substrate, following the manufacturer’s instructions. The PbCDPK1 genomic locus in CDPK1 cKO erythrocytic stages and sporozoites as queried using primers p1 and p2 5’CATGACTAGCCCATATAAT and 5’GAAAGTTGGGAAATATCCGT.

**Infection of HepG2 Cells and Merosome Assay**

Sporozoites obtained at day 18–21, post-bloodmeal were added to HepG2 cells cultured on collagen-coated coverslips. Cells were fixed at 40 hours or 65 h post-infection (p.i) with 4% paraformaldehyde followed by permeabilization with cold methanol. Infected cells were identified using immunostaining with an anti-Hsp70 LS mAb [27]. Merosomes were obtained from infected cultures by collecting the media at 66–70 h post-infection and counted in a hemocytometer.

**In vivo Infection**

Swiss-webster mice were injected intravenously with CDPK1 cKO or FlpL/TRAP sporozoites (5000 sporozoites/mouse) or erythrocytic stage parasites from CDPK1- or WT (1000 parasites/mouse), Parasitemia was determined daily through microscopic examination of Giemsa stained thin smears.

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Author Contributions
Conceived and designed the experiments: PB. Performed the experiments: SJ KG AM PB. Analyzed the data: PB. Wrote the paper: PB.

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