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Research Brief

Genetic and transcriptional analysis of phosphoinositide-specific phospholipase C in *Plasmodium*

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ABSTRACT

Phosphoinositide-specific phospholipase C (PI-PLC) is a major regulator of calcium-dependent signal transduction, which has been shown to be important in various processes of the malaria parasite *Plasmodium*. PI-PLC is generally implicated in calcium liberation from intracellular stores through the action of its product, inositol-(1,4,5)-trisphosphate, and is itself dependent on calcium for its activation. Here we describe the *plc* genes from *Plasmodium* species. The encoded proteins contain all domains typically found in PI-PLCs of the δ class but are almost twice as long as their orthologues in mammals. Transcriptional analysis by qRT-PCR of *plc* during the erythrocytic cycle of *P. falciparum* revealed steady expression levels that increased at the late schizont stages. Genetic analysis in the *P. berghei* model revealed that the *plc* locus was targetable but that *plc* gene knock-outs could not be obtained, thereby strongly indicating that the gene is essential during blood stage development. Alternatively, we attempted to modify *plc* expression through a promoter exchange approach but found the gene to be refractory to over-expression indicating that *plc* expression levels might additionally be tightly controlled.

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1. Introduction

Changes in the cytosolic calcium levels are an important step in cellular signal transduction. In apicomplexan parasites intracellular calcium signalling has been shown to be required during host cell invasion, egress, motility and parasite differentiation (reviewed in Moreno and Docampo, (2003)). In the malaria parasite *Plasmodium* the presence of a family of calcium dependent protein kinases (CDPKs) (Ward et al., 2004) reflects an expansion of calcium dependent effector molecules and underlines the important role that calcium plays in the parasite. CDPKs are absent from mammalian cells but are also found in plants (Harper and Harmon, 2005).

Another classical effector of intracellular calcium is phosphoinositide-specific phospholipase C (PI-PLC) that hydrolyses the minor membrane lipid phosphatidylinositol-(4,5)-bisphosphate (PIP_2), producing two secondary messengers: inositol-(1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers Ca^{2+} release from intracellular compartments while DAG activates protein kinase C (PKC) (Berridge et al., 2000). In various systems PI-PLC was shown to be activated by calcium alone (Rebecchi and Pentyala, 2000). In metazoans several classes of PI-PLC exist while in unicellular organisms and plants only enzymes of class δ have been described (Fukami et al., 2010; Rhee, 2001; Tasma et al., 2008).

In *Plasmodium* PI-PLC activity was detected upon treatment of infected cells with a calcium ionophore (Elabbadi et al., 1994) and a physiological role for PI-PLC has been proposed during gamete formation (Martin et al., 1994) in both cases shown by an increase of inositol poly-phosphate levels. Using the only commercially available inhibitor of PI-PLC, U73122, several studies suggest a function for PfPI-PLC in erythrocyte invasion by merozoites. PfPI-PLC inhibition prevented increased intracellular calcium levels that are in turn necessary for protein kinase B (PKB) activation by calmodulin (Vaid and Sharma, 2006; Vaid et al., 2008) and secretion of micronemal proteins (Singh et al., 2010). We could recently show through biochemical and cell-biological approaches that PI-PLC activity is implicated in the signal cascade leading to gametocyte activation in the rodent malaria parasite *P. berghei* (Raabe et al., 2011). Here we present a genetic and transcriptional analysis of the *plc* gene and show that *Pfplc* transcription is up-regulated at the very late blood stages, while in *P. berghei* deletion of *plc* or modification of its expression level could not be achieved indicating that PI-PLC activity is essential and possibly tightly regulated during the erythrocytic development.

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2. Materials and methods

2.1. Parasites

P. falciparum parasites (strain 3D7) were propagated in RPMI 1640 (with glutamine and 25 mM HEPES [Invitrogen]) supplemented with 10% of human AB$^+$ serum and A$^+$ erythrocytes at a hematocrit of 2.5% at 37 °C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. For the time course experiment parasites were synchronised by several alternations of gelofusin floatation and 5% D-sorbitol treatment to obtain a culture with a 2 h synchronisation window at 1.6% parasitemia. Gametocytes could not be microscopically detected in these preparations. Three independent cultures were handled in parallel. Samples were harvested every 6 h during 42 h (the duration of the erythrocytic cycle under our culture conditions was about 44 h) and parasites were isolated by treatment with 0.01% saponin in phosphate buffered saline at 4 °C.

P. berghei wild type parasites of the ANKA clone 2.34 were maintained in NMRI mice. Clone 1.7.8 (Billker et al., 2004) expressing the GFP-aequorin fusion protein was used as control strain for Ca^{2+} measurements. Gametocytes purifications, exflagellation assays, intracellular calcium measurements and DNA synthesis during microgametogenesis were performed as described (Billker et al., 2004; Raabe et al., 2009, 2011). This research adhered to the Principles of Laboratory Animal Care and animal studies were approved by the local animal use committee.

2.2. Quantitative real-time PCR

Total RNA was extracted from *P. falciparum* parasite pellets using the NucleoSpin RNAII kit (Macherey Nagel) following manufacturer's instructions and its quality verified by capillary electrophoresis (Agilent). cDNA was obtained from 720 ng of total RNA with the SuperScript III First-Strand Synthesis SuperMix kit for qRT-PCR (Invitrogene). Fragments of 150–200 bp were amplified from total cDNA using a LightCycler® 480 (Roche Diagnostics) with the LightCycler® 480 SYBR Green I Master kit according to the manufacturer's protocol with 3 pmol of each primer in a 10 µl total volume in 384 well plates. Primer sequences were as follows: *plc* (PF10_0132): TTATTGTTGGTCAAAACCATCC and TCGCACAATGG TACAAATCG; seryl-tRNA synthetase (*sts*, PF07_0073) GGGCACAT GGAAAGGATATT and TGCGTTGTTTAAAGCTCCTG; 2-Cys peroxiredoxin (*2cys*, also termed PfTPx1, PF14_0368) TCGACAAGCAAGGTG TTGTT and TCATGGCTACCTTTCCCTTTT. PCR reactions were performed by denaturation for 5 min at 95 °C, 45 cycles of 10 s at 95 °C, 20 s at 60 °C, and 20 s at 68 °C, followed by a melting curve. PCR efficiency (*E*) was determined from the curves obtained from amplifications on serial dilutions of cDNA and were 2.00 (*2cys*), 2.01 (*plc*) and 2.02 (*sts*). The crossing points (Cp) were identified (LightCycler® 480 Software 1.5) and the relative *plc* expression level with respect to the reference gene was calculated using the following formula:

$$[E(plc)^{\mathrm{Cp}(plc)}]/[E(reference)^{\mathrm{Cp}(reference)}].$$

2.3. Transfection constructs

For the generation of a double homologous recombination *plc* knock-out construct, sequences of about 500 bp of the 5′ and 3′ untranslated regions were amplified with primers GGTACCACT ATGTATGCATGAAGCG (KpnI) and GGGCCCCATGAGGAACGCAAAAAA CAC (ApaI) and with GAATTCTTAAAGATTGTGAGATTATGTATG (EcoRI) and GGATCCTGAAGACATTCAAATGCC (BamHI) and cloned as KpnI/ApaI and EcoRI/BamHI fragments in plasmid pBS-DHFR flanking the TgDHFR resistance cassette (Dessens et al., 1999).

Insertion (ends-in) vectors to disrupt *plc* or exchange its 5′ regulatory region were constructed in plasmid pOB-182, which carries the human DHFR resistance cassette and allows expression of GFP-aequorin to detect changes in intracellular calcium levels (Billker et al., 2004). The region for single homologous recombination in the *plc* locus was PCR amplified and cloned as NheI/NotI fragment. In order to generate a unique ApaI site for plasmid linearization this was done in two steps with primer pairs olAR251 AATTGGGCC CAAAATGTGGCTTTTTGGTTTAATG (ApaI)/olAR252 AATTGCGGCCG CATTATTATTCCAATTTTGTGCG (NotI) and olAR271 TTGCTAGCCCTCATGAACCTTAATGAAAATA (NheI)/ olAR258 AATTGGGCCCCCATCTTTGAATATCTAAAAAGG (ApaI) resulting in plasmid pAR06 that was used for generation of a *plc* knock-out by single homologous recombination. Different promoter sequences of about 1 kb were PCR amplified and inserted as XhoI/NheI fragments. *pkg*: CTCGAGTACAATTTTTAACTGTATGCATAAAATTTTATG and GCTAGCTTTTCTTATCTTCCAATCTCGTTAAC, plasmid pAR03(PKG). *ef1α*: olAR259 AATTCTCGAGCCCAGCTTAATTCTTTTCGAGC and olAR260 AATTGCTAGCGGATCCCCCTATGTTTTATAAAATTTTTT, plasmid pAR04(EF1a). Transfection of purified *P. berghei* schizonts was done as described (Dechamps et al., 2010; Janse et al., 2006).

3. Results and discussion

3.1. Analysis of the Plasmodium PI-PLC primary sequence

The *Plasmodium* genome database PlasmoDB v. 7.0 (Aurrecoechea et al., 2009) predicts the presence of a single gene in *P. falciparum* encoding a putative phosphoinositide-specific phospholipase C, PF10_0132. The gene is predicted to consist of 4 exons, resulting in an open reading frame of 4155 base pairs (bp), encoding a protein of 1385 amino acids (aa) and a predicted mass of 164 kDa that we name here PfPI-PLC. This exon/intron structure was experimentally confirmed by sequencing of the corresponding cDNA (accession number HQ676593). The 3′ untranslated region (UTR) extended to 369 bp downstream of the stop codon. Orthologs were identified in other *Plasmodium* species that aligned well with the PfPI-PLC sequence except for the 3′ end of the predicted *P. berghei* sequence (at the time of analysis in PlasmoDB v. 6.3). We therefore sequenced the *Pbplc* cDNA (accession number JF436974) and revealed a four exon structure very similar to the other *Plasmodium* species. The *P. berghei* gene of 4032 bp encodes a predicted protein of 1344 aa (159 kDa), confirming experimentally the predicted gene model (PBANKA_121190) in the current assembly of the *P. berghei* genome.

An alignment of the PI-PLC protein sequences of *P. falciparum*, *P. vivax* and *P. berghei* revealed overall sequence similarities of around 75%. Highly conserved regions alternated with highly divergent sequences (Fig. 1). A search for protein domains identified the presence of all domains that are typical of PI-PLC enzymes of the delta subclass, i.e. the lipid binding pleckstrin-homology (PH) domain (aa position 80-209 in PfPI-PLC), the calcium-binding motif EF-hand (aa 217-304), the catalytic domain consisting of an X-(aa 624-769) and a Y-domain (aa 972-1087) and the calcium/lipid-binding C2-domain (aa 1279-1383). Residues that have previously been shown to be important in the catalytic domain of rat PI-PLCδ1 (Ellis et al., 1998, 1995; Suh et al., 2008) are strictly conserved in the *Plasmodium* PI-PLC sequences (Fig. 1). However, PfPI-PLC is almost twice as long as its mammalian PI-PLCδ counterparts that comprise about 700–800 amino acids. This is due to large insertions (Fig. 1), none of which is predicted to contain a conserved motif or domain. Interestingly however, only the insertion between the Y-and the C2-domain is highly conserved amongst *Plasmodium* species (69–73% sequence identity over 191 residues), and its secondary structure is predicted to be mainly helical. It is tempting to speculate that this *Plasmodium* specific

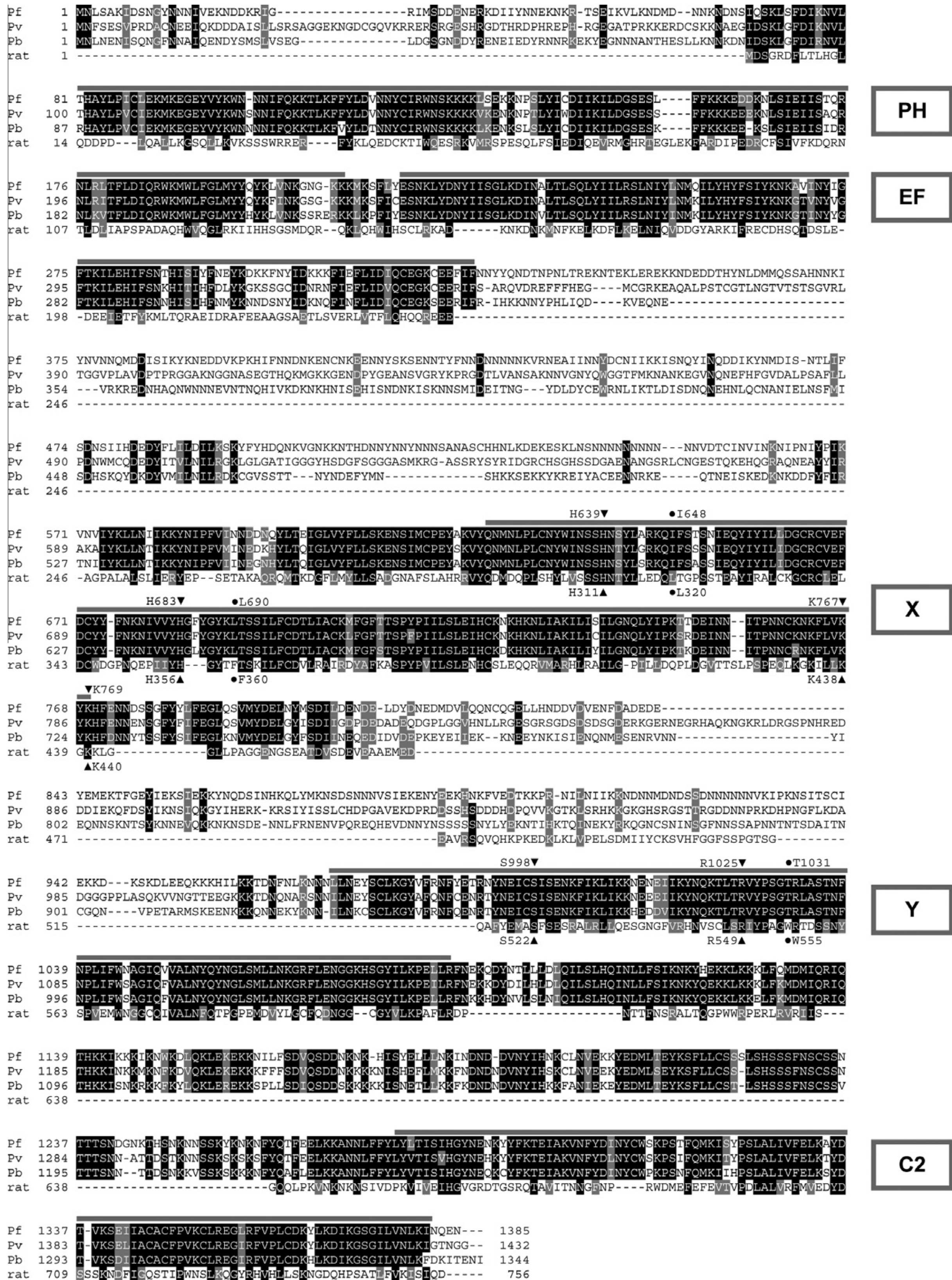

Fig. 1. Sequence alignment of PI-PLC from different *Plasmodium* species with rat PI-PLCδ1. The sequences of *P. falciparum* and *P. berghei* PI-PLC were confirmed by cDNA sequencing while the *P. vivax* sequence (PVX_094895) was taken from PlasmoDB. The indicated domains were identified by InterProScan for the *P. falciparum* sequence. Described important residues in the catalytic domain of rat PI-PLC are labelled with arrowheads and their amino acid positions below the alignment while the corresponding *P. falciparum* positions are given above the alignment. Non-identical residues predicted to be at the hydrophobic ridge at the rim of the active site opening are indicated by circles. The alignment was generated by ClustalW and BoxShade with an identity coefficient of 0.7.

insertion might define an additional domain with as yet unknown function.

3.2. Transcriptional analysis of the plc gene during P. falciparum blood stage development

Two global transcriptome analyses using DNA microarrays indicated that *plc* expression levels are low with little variation during the asexual cycle of *in vitro* cultured *P. falciparum* parasites (Bozdech et al., 2003; Le Roch et al., 2003). We analysed the transcription level of *plc* during the blood stage development by quantitative reverse transcription PCR (qRT-PCR). In a time course experiment of highly synchronised parasites, reactions were performed on identical quantities of total parasite RNA at 6 h intervals post-invasion (p.i.). Two genes were used as internal references: seryl-tRNA synthetase (*sts*) (Mphande et al., 2008; Salanti et al., 2003) and 2-Cys peroxiredoxin/TPx-1 (*2cyc*) (Witola and Ben Mamoun, 2007) (see also www.plasmodb.org for a summary of the expression profiles). When calculating the relative *plc* expression levels, we found similar results with respect to both reference genes. Expression of *plc* remained relatively steady and with respect to the earliest time point at 6 h p.i. a two to threefold down-regulation of *plc* expression at 18 h p.i. and a 3.5 or 10-fold up-regulation in late stage parasites was detected depending on the reference gene (Table 1). The *sts* gene has been described to display an unchanged transcription profile throughout the parasite life cycle (Salanti et al., 2003) and has previously been used for time course experiments (Mphande et al., 2008). Our results confirm that the *sts* gene appears to be the better reference gene for experiments analysing gene expression throughout the erythrocytic development. The here observed *plc* expression profile might indicate that PI-PLC function is important during the very late erythrocytic stages and possibly during the processes of host cell egress and erythrocyte invasion. Indeed, it has recently been shown that a PI-PLC dependent calcium signal is important for secretion of micronemes proteins to the parasite surface in *P. falciparum* merozoites (Singh et al., 2010). Accordingly, treatment of isolated merozoites with the broadly used PI-PLC inhibitor U73122 or with the calcium chelator BAPTA-AM strongly inhibited invasion of red blood cells (Singh et al., 2010) and indicated that PI-PLC might play a crucial role in *Plasmodium* blood stage development.

3.3. Genetic manipulation of the plc gene in P. berghei indicates that the gene is essential

Genetic analysis by genome integration is easier and faster in the rodent parasite *P. berghei* than in *P. falciparum*. We therefore chose this system for a genetic study of the *plc* locus. We first aimed at disrupting the *plc* gene. However, attempts to replace the entire *Pbplc* coding sequence with the resistance marker *Tgdhfr/ts* by double homologous recombination remained unsuccessful in three independent transfections (data not shown). In an alternative knock-out approach we targeted the *Pbplc* locus by single homologous recombination in the beginning of the *plc* open reading frame. The *hdhfr* resistance cassette would in this way be inserted into the *plc* coding sequence after amino acid 368, upstream of the X and Y catalytic domains. However, in three independent experiments this construct did not yield viable knock-out parasites either (data not shown) indicating that either PI-PLC was essential during blood stage development or the locus was refractory to recombination.

We then chose to apply a promoter exchange strategy that would prove that the *plc* locus was targetable and, at the same time, might allow us to modify the *plc* expression level. The targeting vector was designed based on the single cross-over knock-out construct described above. By simply placing an alternative promoter in front of the homology region needed for recombination, integration of the construct places a new promoter in front the *plc* coding sequence (Fig. 2A).

The expression profile of the cGMP-dependent protein kinase gene (*pkg*) resembles the one of *plc* in *P. falciparum* (Bozdech et al., 2003; Le Roch et al., 2003). The *pkg* promoter was therefore used for a "proof of principle" approach. It should be noted however that the detailed expression profiles of these genes are not known for *P. berghei*. Upon transfection pyrimethamine resistant parasites were obtained and were cloned by limiting dilution (clone 3.6). PCR analysis indicated that the construct had integrated correctly into the genome (Fig. 2B), thereby demonstrating that the *plc* locus was generally available for recombination and that the promoter replacement itself was possible. Thus our inability to obtain *plc* knock-out parasites was most likely due the fact that PI-PLC activity is essential during asexual erythrocytic development.

The phenotype of the transgenic clone was analysed with respect to our recent findings that PI-PLC activity is involved in gametocyte activation (Raabe et al., 2011). In comparison to the wild type strain we did not observe significant differences in the intracellular calcium signal (Billker et al., 2004) upon gametocyte activation by increasing concentrations of xanthurenic acid (XA) (Fig. 2C) or in DNA synthesis during microgametogenesis in the presence of XA, the PI-PLC inhibitor U73122, or the ryanodine receptor channel inhibitor dantrolene (Fig. 2D) indicating that PI-PLC-dependent processes in gametocyte activation were not modified to a detectable level. These results were not unexpected and possibly indicate that the *plc* expression profile had not been extensively modified during the analysed stages following the *pkg* promoter exchange.

However, when we aimed at over-expressing the *plc* gene by using the strong promoter of the elongation factor-1alpha gene (*ef1α*) that has become a standard promoter for constitutive protein expression in *P. berghei*, we could not obtain parasites with correct integration in three independent transfections, suggesting

Table 1
Transcriptional analysis of the *plc* gene during *P. falciparum* blood stage development. Given are the relative expression levels of the *plc* gene normalised with respect to either the *sts*- or the *2-cys* gene as reference and the crossing points of the qRT-PCR reactions of the individual genes (see materials and methods for details). The relative *plc* expression level obtained for the 6 h samples was arbitrarily set to one. The other time points are indicated as fold changes with respect to the value at 6 h. All values are the mean and standard deviation obtained from triplicate qRT-PCR reactions each performed on three independent cultures.

Time post-invasion	Relative *plc* expression level		Crossing points		
	Reference gene: *sts*	Reference gene: *2cys*	*plc*	*sts*	*2cys*
6 h	1	1	26.01 ± 0.09	20.60 ± 0.32	18.74 ± 0.08
12 h	0.92 ± 0.41	0.79 ± 0.13	25.67 ± 0.16	20.08 ± 0.54	18.05 ± 0.21
18 h	0.38 ± 0.08	0.46 ± 0.08	26.27 ± 0.33	19.49 ± 0.01	17.86 ± 0.08
24 h	1.32 ± 0.14	1.29 ± 0.23	25.38 ± 0.16	20.39 ± 0.20	18.46 ± 0.16
30 h	1.05 ± 0.25	1.08 ± 0.37	25.06 ± 0.01	19.71 ± 0.37	17.84 ± 0.57
36 h	1.39 ± 0.33	2.76 ± 0.55	25.23 ± 0.30	20.29 ± 0.27	19.41 ± 0.23
42 h	3.47 ± 0.82	9.95 ± 2.42	25.46 ± 0.21	21.82 ± 0.54	21.49 ± 0.53

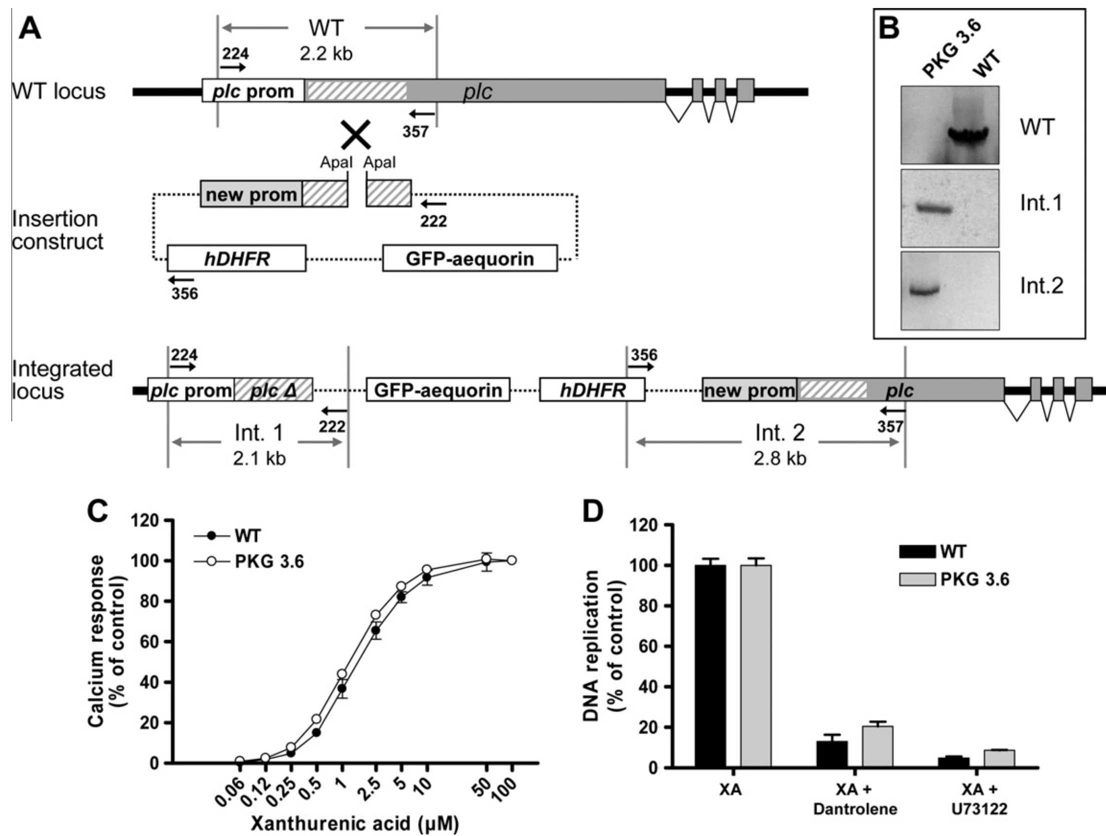

Fig. 2. *plc* promoter replacement. (A) From the top to the bottom are shown the wild type (WT) plc locus of *P. berghei*, the targeting vector for promoter exchange by single homologous recombination, and the locus after integration of the construct. Small numbers and arrows indicate the positions and names of primers used for PCR analysis. The one kilo base region at the beginning of the *plc* gene that has been cloned in the targeting vector is indicated in light gray. The figure is not drawn to scale. (B) PCR analysis of parasites obtained after transfection. Clone 3.6 carries the *pkg* promoter and was obtained by dilution cloning. The primer pairs used are indicated in (A). (C) Calcium response of clone 1.7.8 (wild type strain carrying the aequorin-GFP construct) and *pkg* clone 3.6 in the luminometric Ca^{2+} assay following activation by serial dilutions of XA. Activation with 100 µM XA served as positive control. Error bars indicate STD ($n = 5$), shown is one experiment of three. (D) DNA synthesis in WT and clone 3.6 upon activation by 100 µM XA alone and in presence of 10 µM Dantrolene or 20 µM U73122. Error bars indicate STD ($n = 3$).

that over-expression of *plc* was not supported by the parasite. In addition, several other promoter exchange constructs also did not integrate into the genome (data not shown) indicating that *plc* expression might me tightly regulated by the parasite.

Taken together, the genetic approaches used to analyse PI-PLC function in *P. berghei* strongly indicate that this gene is essential during blood stage development. The *plc* locus was targetable but the gene could not be deleted or disrupted neither by double nor by single homologous recombination. Combined with our finding that transcription of *plc* is relatively up-regulated at the very end of the *P. falciparum* erythrocytic cycle and the published data on a PI-PLC-dependent calcium signal in the activation of PfPKB and the secretion of microneme proteins to the parasite surface in *P. falciparum* merozoites (Singh et al., 2010; Vaid et al., 2008), it is tempting to speculate that the essential role of PI-PLC during the erythrocytic cycle resides in invasion. The promoter exchange strategy was feasible but suggests that the *plc* expression level might be well controlled as indicated by the inability to obtain transgenic parasites, in which the strong ef1α promoter drives the expression of *plc*.

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