Research Brief

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

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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 β 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 β 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 PIPI-PLC in erythrocyte invasion by merozoites. PIPI-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.
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% CO₂, 5% O₂, 90% N₂. For the time course experiment parasites were synchronized 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 macroscopically 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 Ca²⁺ 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 TCGCACAATGGCTACCTTTCCCTTTT. PCR reactions were performed as described (Dechamps et al., 2010; Janse et al., 2006). The crossing points (Cp) were identified by several alternations of gelofusin floatation and 5% D-sorbitol.

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 GTGACCCT ATGTTGATCATGAAAGCC (KpnI) and GGCCGGATCCAGGAAACGAAAAAC AC (ApaI) and with GAATTCTAAAGTGGTGAATGATG (EcoRI) and GGATCCAGGACAATCAG (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 Apal site for plasmid linearization this was done in two steps with primer pairs olAR251 AATTGGGCC AAAATTGCTTTTGTGTTAATG (Apal) and olAR252 AATTGGCGGCC CATATTATCTCAATTGTTGC (NotI) and olAR271 TTGCTAGCTTTTCTTATCTTCCAATCTCGTTAAC, plasmid pAR06(PKG), ef1α: olAR259 AATTTCGACCCCCATTATTTCTG and GCTAGCTTTTCTTATCTCAATTGTTGC, and olAR260 AATTTCGACCCATTATTTCTGAGC and olAR285 AATTTCGACCCCCATTATTTCTG and plasmid pAR04(EF1α). 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 (Aurrecochea 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 PI-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 PI-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 PlcB 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 PI-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-PLCα1 (Ellis et al., 1998, 1995; Suh et al., 2008) are strictly conserved in the Plasmodium PI-PLC sequences (Fig. 1). However, PI-PLC is almost twice as long as its mammalian PI-PLC 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-PLC. 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 (2cys) (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 with respect to both reference genes. Expression of plc has been described to display an unchanged transcription 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 TgΔdhfr/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 xanthenurenic 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
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 be 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 ef1a promoter drives the expression of plc.

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References


