Phospholipases during membrane dynamics in malaria parasites

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A B S T R A C T

Plasmodium parasites, the causative agents of malaria, display a well-regulated lipid metabolism required to ensure their survival in the human host as well as in the mosquito vector. The fine-tuning of lipid metabolic pathways is particularly important for the parasites during the rapid erythrocytic infection cycles, and thus enzymes involved in lipid metabolic processes represent prime targets for malaria chemotherapeutics. While plasmodial enzymes involved in lipid synthesis and acquisition have been studied in the past, to date not much is known about the roles of phospholipases for proliferation and transmission of the malaria parasite. These phospholipid-hydrolyzing esterases are crucial for membrane dynamics during host cell infection and egress by the parasite as well as for replication and cell signaling, and thus they are considered important virulence factors. In this review, we provide a comprehensive bioinformatic analysis of plasmodial phospholipases identified to date. We further summarize previous findings on the lipid metabolism of Plasmodium, highlight the roles of phospholipases during parasite life-cycle progression, and discuss the plasmodial phospholipases as potential targets for malaria therapy.

1. Introduction

Approximately 200 million new malaria cases are recorded per year, resulting in more than 400,000 deaths (World Health Organization, 2016). Antimalarial drug development and vector control strategies have contributed to reduce the malaria burden during the last decade. However, half of the worldwide population remains exposed to malaria, all available antimalarial drugs are meanwhile facing parasite chemoresistance issues and no vaccine is yet commercialized (reviewed in White et al., 2014).

Responsibility for this devastating tropical disease are unicellular parasites of the genus Plasmodium. Currently five Plasmodium species infecting humans are known. Of these, P. falciparum, P. vivax, P. malariae and P. ovale exclusively infect humans, with P. falciparum being responsible for the majority of deaths by malaria. P. knowlesi was originally described as a simian parasite and only recently has emerged as an important cause of human malaria (reviewed in Barber et al., 2017).

Malaria parasites undergo a complex life-cycle starting with their transmission to the human host by blood-feeding Anopheles mosquitoes. Once injected into the human dermis, infective sporozoites immediately target the liver to replicate asymptomatically in hepatocytes, in consequence producing tens of thousands liver stage merozoites. Once released to the blood stream, the merozoites infect red blood cells (RBCs) to begin erythrocytic schizogony. These erythrocytic infection cycles, which last 24–72 h depending on the Plasmodium species, are responsible for the typical symptoms of malaria such as fever, anemia and organ failure (reviewed in Cowman et al., 2016; Haldar and Mohandas, 2009).

During the erythrocytic infection cycle, a proportion of blood stage parasites enter the sexual pathway in response to stress factors, which results in the production of the transmissible intra-erythrocytic gametocyte stages. Following maturation, a process that takes 10 days for

Abbreviations: AMA-1, apical membrane antigen 1; 2-APB, 2-aminoethoxydiphenyl borate; BMP, bis (monoacylglycerol)phosphate; CDP, cytidine diphosphate-choline; cGMP, cyclic guanosine monophosphate; D, aspartate; DAG, diacylglycerol; EBA175, erythrocyte binding antigen 175; ER, endoplasmic reticulum; FASII, fatty acid synthase II; FV, food vacuole; G, glycine; GSSXG, motif characteristic for α/β hydrolases with the conserved amino acid residues of G and of the catalytic active S; H, histidine; HC, host cell; HCM, host cell membrane; IMC, inner membrane complex; IP 3, inositol-(1,4,5)-triphosphate; iRBC, infected RBC; LCAT, lecithin:cholesterol acyltransferase; LPL, lysophospholipid; LPLA, lysophospholipase A; MC, Maurer’s cleft; NL, neutral lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMP, phosphatidylethanolamine N-methytransferase; PI, phosphatidylinositol; PIP 2, phosphatidylinositol 4,5-biphosphate; PKG, cGMP-dependent protein kinase G; PL, phospholipid; PLA, phospholipase A; PLB, phospholipase B; PLC, phospholipase C; PFD, phospholipase D; PIP, patatin-like phospholipase; PPM, parasite plasma membrane; PS, phosphatidylserine; PVAM, parasitophorous vacuolar membrane; RBC, red blood cell; RBCM, RBC membrane; S, serine; ss, stereospecific numbering; sPLA, secretory PLA; TAG, triacylglycerol; TRAP, thrombospondin-related adhesive protein; TVN, tubovesicular network; SM, sphingomyelin; uRBC, uninfected RBC

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gametocytes of *P. falciparum*, the gametocytes are capable of forming gametes immediately after uptake by the blood-feeding *Anopheles* mosquito vector. The gametes fuse to form a motile zygote, termed ookinete, which traverses the gut wall of the mosquito and then transforms into an oocyst, in which the parasite undergoes another round of asexual replication before accumulating as infective sporozoites in the mosquito salivary glands (reviewed in Bennink et al., 2016; Kuehn and Pradel, 2010).

In the human host, *Plasmodium* spends the majority of its time within a host cell (HC), either a hepatocyte or a RBC. This intracellular life-style offers protection against attack by the human immune system and provides the parasite a ready source of nutrients. Growth inside the HC is accompanied by an intense period of membrane biogenesis, including the formation of a vacuolar system that supports expansion and nutrient intake by the parasite as well as the development of daughter cells (reviewed in Vial et al., 2003). This process requires *de novo* synthesis and acquisition but also the degradation of phospholipids (PLs) and neutral lipids (NLs).

While the importance of phospholipases for the lipid turnover during propagation and spread of pathogens has been acknowledged for years (e.g. reviewed in Côtes et al., 2008; Djordjevic, 2010; Flores-Diaz et al., 2016; Kühle and Flieger, 2013; van der Meer-Janssen et al., 2010), surprisingly the phospholipases of malaria parasites as mediators of biomembrane dynamics are hitherto under-investigated. Notwithstanding the limited number of studies that have addressed phospholipase functions to date, they revealed critical roles for these PL-hydrolyzing enzymes during the *Plasmodium* life-cycle. Therefore, this review focuses on the phospholipases of malaria parasites to bring well-deserved attention to this class of hydrolases. The review provides a detailed *in-silico* analysis on putative plasmodial phospholipases, describes their currently known functions and evaluates them as potential drug targets. The review further highlights and summarizes previous findings on the *Plasmodium* lipid metabolism.

2. Membrane dynamics and lipid turnover in plasmodial parasites

During their intracellular development, *Plasmodium* induces substantial changes in the structural and functional properties of the HC. Inside the HC the parasite is encased by a parasitophorous vacuolar membrane (PVM), a membranous compartment that arises by invagination of the RBC membrane during invasion (Fig. 1A). Additionally, tubovesicular networks (TVNs) are formed by the parasite that protrude from the PVM into the erythrocytic cytosol and which may allow for the transport of molecules from the RBC cytosol to the HC surface and vice versa. Such TVNs have previously been described in infected RBCs (iRBCs) and in hepatocytes (e.g. reviewed in De Niz et al., 2016; Sherling and van Ooij, 2016). Further compartments of the exomembrane system in iRBCs are the Maurer’s clefts (MCs) and mobile J-dots. The rapid parasite growth also requires large lipid quantities to maintain cell-internal membrane compartments. These include the parasite plasma membrane (PPM), the food vacuole (FV), the endoplasmic reticulum (ER), the Golgi apparatus, the nucleus, the mitochondrion and apicoplast as well as the apical organelles, i.e. micronemes and rhoptries, and the inner membrane complex (IMC) of the invasive and transmissible life-cycle stages (Figs. 1A and B).

The drastic membrane dynamics during intracellular growth of *Plasmodium* requires a finely regulated lipid metabolism. In general, three types of lipids are distinguished, i.e. PLs, NLs and cholesterol (Table 1). PLs are amphiphilic molecules harboring apolar/hydrophobic long chain fatty acids (FAs) and a polar/hydrophilic part, which is represented by the phosphate and the attached alcohol. By contrast, NLs, such as triacylglycerol (TAG), diacylglycerol (DAG), but also cholesterol, a polycyclic alcohol, are hydrophobic molecules. While the parasite is capable to *de novo* synthesize PLs or NLs, the precursors need to be scavenged from the host or surrounding serum (Fig. 2). FAs, reaction products of phospholipases A (PLA) and lipases, are incorporated from the host serum by blood stage parasites to synthesize PLs or NLs and are essential for parasite survival (Mitamura et al., 2000; Vliekemeyer et al., 2004; reviewed in Vial et al., 2003). *Plasmodium* can also synthesize FAs *de novo* via the fatty acid synthase II (FASII) pathway located in the apicoplast, a relict plastid of algal origin that arose through secondary endosymbiosis (e.g reviewed in van Dooren and Striepen, 2013). FA synthesis via FASII, though, occurs primarily in the mosquito-resident and intrahepatic stages (van Schaijk et al., 2014; Vaughan et al., 2009; Yu et al., 2008).

*Plasmodium* generates PL from polar heads, like choline, ethanolamine or serine (S), which are mainly taken up from the serum (reviewed in Ben Mamoun et al., 2010; Déchamps et al., 2010), whereas phosphatidylinositol (PI) is made by the parasite from inositol that is either taken up from the serum or generated *de novo* from glucose-6-phosphate via inositol-3-phosphate (reviewed in Ramakrishnan et al., 2013). Phosphatidylethanolamine (PE) is synthesized by the parasite via the phosphorylation of ethanolamine obtained from plasma or through decarboxylation of S (Fig. 2). S, in turn, is acquired via direct import or haemoglobin degradation. Phosphatidycholine (PC) is generated from choline by two routes in *Plasmodium*, i.e. the *de novo* cytidine diphosphate (CDP)-choline (Kennedy) pathway and the S decarboxylase/PE methyltransferase (PEMT) pathway (reviewed in Fischer et al., 2012). Another potential PC synthesis pathway is the Lands cycle, in which phospholipase A₂ (PLA₂) removes a FA from PC that has derived from the Kennedy pathway, resulting in the formation of lysoPC. The lysoPC in turn is re-acetylated in a reverse reaction with another FA, resulting in PC with a modified FA residue. Since no lysoPC-acetyltransferase has yet been identified in *Plasmodium*, a potential role of the Lands cycle in plasmodial PC synthesis and PC diversity has to be elucidated.

To date, lipid and membrane compositions of malaria parasites have mainly been studied in the blood stages of *P. falciparum*. This can be explained by the fact that these stages can be cultivated in vitro, harvested at high cell numbers and purified to obtain distinct blood stages. Noteworthy, the majority of studies focused on lipid components of uninfected (u)RBCs versus iRBCs or RBC-freed parasites (here, mostly the trophozoite stages are used for analysis). With the exception of the below discussed apicoplast and microvesicles, the purification of distinct membranous organelles or components of the exomembrane system is not yet possible.

After infection by *P. falciparum*, the total amount of PLs increases approximately 5-fold in the iRBCs (Beaumelle and Vial, 1988; Gulati et al., 2015; Simões et al., 1992; reviewed in Déchamps et al., 2010). In uRBCs, cholesterol and PL are the major lipids, with PL mostly being PC (∼20–40%; percentages differ between studies), PE (∼15–30%), PE plasmalogen (∼15%), sphingomyelin (SM) (∼15%) and phosphatidylserine (PS) (∼10–15%). The membrane composition of RBC-freed malaria parasites is primarily composed of PLs like PC (∼40%) and PE (∼30%). The amount of SM (∼15%) in the parasites is comparable to that of uRBCs, while PS (∼5%) and PE plasmalogen (∼10%) are found at lower concentrations (Botté et al., 2013; Gulati et al., 2015; reviewed in Vial et al., 2003). Cholesterol is almost absent in the membranes of *Plasmodium* parasites, related to its inability to synthesize sterols (reviewed in Déchamps et al., 2010; Vial et al., 2003; Vial and Ancelin, 1992). In iRBCs, relative membrane cholesterol levels decrease inwardly from the RBC membrane (RBCM) via the MC/TVN to the PPM, with cholesterol appearing to travel from RBCM to PVM, but not vice versa (Tokunaga et al., 2014). The reduced susceptibility of iRBCs to cholesterol-binding pore former drugs like streptolydin O that selectively permeabilizes cholesterol-containing membranes underlines the reduced cholesterol content in iRBCs compared to uRBCs (Jackson et al., 2007). TAG and DAG increase by 2- to 5-fold in the parasite during the 48-hours RBC infection (Gulati et al., 2015). These NLs accumulate mostly in the FV, where they appear to be involved in heme detoxification (Gulati et al., 2015; Jackson et al., 2004).

During parasite growth in the iRBC, an increase in lysophospholipids (LPL) can also be detected (Gulati et al., 2015). LPLs, which
in addition to FAs are products of PLA activity, possess single FA chains. They are minor constituents of cell membranes, but can mediate cell signaling, calcium mobilization and protein folding and are further known to promote cell proliferation and migration (reviewed in Grzelczyk and Gendaszewska-Darmach, 2013). Similar to DAGs, TAGs and PLs, the levels of plasmodial bis(monoacylglycero)phosphate (BMP) peak at the end of the erythrocytic replication cycle, when merozoites are released. While BMP has not yet been studied in Plasmodium, the molecule was described to mediate the fusion between the membrane of the vacuolar compartment and host autophagosomes in Leishmania parasites (Schaible et al., 1999), suggesting that BMP might also be involved in vesicle fusion prior to iRBC egress by the merozoites.

During infection, the RBCM also undergoes some remodelling. While the PL composition does not alter significantly, the PLs exhibit an increase in the ratio of unsaturated to saturated FA chains (reviewed in Vial et al., 2003). An exposure of PS on the outer leaflet, known as a final step of apoptosis, was also sometimes observed. These alterations might support iRBC rigidity and cytoadherence of the iRBCs to capillary walls (Brand et al., 2003; Eda and Sherman, 2002).

Cholesterol-rich lipid rafts are located in the iRBCM. It was postulated that host proteins residing in these cholesterol-rich rafts are recruited to the PVM, while non-raft proteins remain in the RBCM (reviewed in Haldar et al., 2001, 2002). However, RBCM-raft proteins like stomatin and band 3 appear to be excluded from parasite-derived membranes, while flotillin-1, -2 and some other RBCM raft proteins are recruited to the PVM (Murphy et al., 2004; reviewed in Murphy et al., 2006).

Both uRBCs and iRBCs release microvesicles into the host serum, which are suggested to arise by blebbing from lipid rafts (Mantel et al., 2013; Nantakomol et al., 2011; reviewed in Mantel and Marti, 2014).
apicoplast (Botté et al., 2013; Tawk et al., 2010). Interestingly, the membrane PLs or the requirement for specific enriched in PIs, particularly PI3-phosphate, as well as in other PLs authors to determine its lipid content. They found that the apicoplast is compared to the RBCM (Gulati et al., 2015).

2013). Isolated microvesicles exhibited enriched PS and PI levels (Mantel et al., 2013; Nantakomol et al., 2011; Regev-Rudzki et al., 2013). The study reported the successful purification of the apicoplast, which has been investigated for its lipid content in more detail, is the plasmodial apicoplast. A recent study reported the successful purification of this plastid, allowing the authors to determine its lipid content. They found that the apicoplast is enriched in PIs, particularly PI3-phosphate, as well as in other PLs having saturated FAs, suggesting limited acyl exchange with other membrane PLs or the requirement for specific physical properties in the apicoplast (Botté et al., 2013; Tawk et al., 2010). Interestingly, the apicoplast also features lipids atypical for plastids, such as SM, ceramides and cholesterol. These lipids were suggested to contribute to changes in multi-membrane properties affecting both permeability and the activity of integral membrane transporter proteins (Botté et al., 2013).

Lipid levels were also investigated for the intraerythrocytic gametocytes. Similar to RBCs infected with asexual blood stages, RBCs containing gametocytes had 6-fold higher lipid levels compared to uRBCs (Tran et al., 2016). This is particularly apparent for ceramides, a subgroup of sphingolipids, which are almost non-existent in the uRBCs, but which increase 6-fold in trophozoite-IRBCs and 9-fold in gametocyte-iRBCs. In this context, a gametocyte-specific ATP-binding cassette transporter was identified, which appears to be involved in the accumulation of NLS, particularly TAGs and DAGs (Tran et al., 2014). The high content of DAGs in gametocytes might be linked to the various signaling pathways that are activated to initiate gametogenesis, once the gametocytes have entered the mosquito midgut. Among others, during gametogenesis the second messengers DAG and inositol-(1,4,5)-triphosphate (IP3) are produced by hydrolysis of phosphatidylinositol-(4,5)-biphosphate (PIP2) through phospholipase C (PLC), leading to a release of calcium from the ER, which in turn activates calcium-dependent protein kinases of the parasite as described below (reviewed in Bennink et al., 2016; Kuehn and Pradel, 2010).

While cholesterol decreases from 50% of total lipid levels in uRBCs to 20% in the trophozoite-iRBCs, the levels increase again during gametocyte maturation with a peak at 30% in mature gametocyte-iRBCs (Tran et al., 2016). Cholesterol increase in mature gametocytes might give rise to the decreasing rigidity of the iRBC membrane in these stages, needed for circulation and passage through the spleen (Tiburcio et al., 2017).
et al., 2012). In opposite, PC levels, which increase from ~ 25 to 35% of total lipid levels following RBC infection, decrease again to 25% in mature gametocyte-iRBCs (Tran et al., 2016). The larger proportion of PC present in trophozoite- compared to gametocyte-iRBCs might reflect the need for these lipids in these stages, for instance to maintain the exo-membrane system.

A previous study investigated the changes in lipid compositions in hepatocytes infected with the rodent malaria parasite P. berghei (Itoe et al., 2014). Upon infection, the NL levels increase, but subside during the late liver phase (approximately 45 hours after infection). Further, an enrichment of PC was observed, as well as a slight increase in SM and ceramides, while the levels of PE, PS and PI decreased. PC was acquired from the HC, among others via lyso-PC, and mouse hepatocytes impaired in de novo PC synthesis via the Kennedy or the PEMT pathway showed reduced infection with *Plasmodium*. The host-derived PC was found in most membranes of the infected hepatocytes, like the host cell membrane (HCM), the PPM and the PVM, where it plays a role in membrane integrity (Itoe et al., 2014). In accordance with these findings, proteins of the *fam-a* variant multigene family of malaria parasites were demonstrated to be transported into the cytoplasm of iRBCs as well as into the parasitophorous vacuole in infected hepatocytes. Most of *Fam-A* family members exhibit a stereogenic acute regulatory-related lipid transfer domain and are capable to transfer PC in vitro, indicating that these proteins might be involved in acquisition of host PC for the synthesis of parasite-derived membranes (Fougère et al., 2016).

3. Phospholipases of plasmodial parasites

Phospholipases are a diverse group of enzymes mediating various cellular functions including membrane synthesis or disruption as well as regulation of signaling or inflammatory responses. Such lipolytic enzymes are classified into groups A, B, C and D corresponding to the different sites of PL hydrolysis (Fig. 3).

PLA and phospholipases B (PLB) target acyl ester bonds, whereas phospholipases C (PLC) and D (PLD) cleave phosphodiester bonds in the molecule. Therefore, typical reaction products of PLAs are free FAs and LPLs. PLAs may be specific for the cleavage of one of the two acyl esters, i.e. for the stereospecific numbering sn-1 or sn-2 positions, and in these cases are designated PLA₁ (EC 3.1.1.32) and PLA₂ (EC 3.1.1.4), respectively. The remaining FA in a LPL may be released by a lysophospholipase A (LPLA) (EC 3.1.1.5). PLB (EC 3.1.1.5) cleaves phospholipids both at sn-1 and sn-2 positions. PLC (EC 3.1.4.10) hydrolyses the glycerol-oriented and PLD (EC 3.1.4.4) the alcohol-oriented phosphodiester bond, thereby releasing DAG and a phosphoalcohol or phosphatic acid and an alcohol, respectively. Additionally, some PLA/ LPLAs possess lipase activity and may liberate FAs from non-PLs such as glycerolipids, and other enzymes may possess PL:sterol-O- acyltransferase activity, which transfers FAs from a PL directly to an acceptor molecule, such as cholesterol (reviewed in Flores-Díaz et al., 2016). It is important to note that also phosphosphingolipids belong to the class of PLs and therefore not only phospholipases targeting glycerophospholipids but also sphingomyelinases (SMases) like SMase C and D, which produce ceramides via SM cleavage, are of biological importance (reviewed in Flores-Díaz et al., 2016).

In order to provide an overview of all established and potential phospholipases of *P. falciparum*, we searched the PlasmoDB database (http://plasmodb.org/plasmo; Aurrecoechea et al., 2009) using the EC numbers 2.3.1.43, 3.1.1.4, 3.1.1.5, 3.1.3.2, 3.1.4.3, 3.1.4.4, 3.1.4.11, 3.1.4.12 and 3.1.4.41, classifying phospholipolytic enzymes. We focused on such proteins which showed domains predicted via the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer et al., 2011, 2015) typically associated with lipolytic enzymes. We identified a total of 22 proteins, i.e. 14 with a predicted α/β hydrolase domain, four with a patatin-like protein domain, one with a PC:sterol-O-acyltransferase domain, one with a PI:PLC domain, one with a sphingomyelin phosphodiesterase domain, and one with a PLA/lipase domain (Fig. 4; Table 2).

The highest number of candidates was found for the α/β hydrolase family. All but two of the 14 putative enzymes were annotated as LPLAs. Ten of these showed a high degree of homology to each other (36–61%), were about 400 amino acids long, contained almost no additional protein sequence outside the predicted α/β hydrolase domain, and possessed no predicted signal peptide. All shared the characteristic GXSXG motif embedding the catalytic active S flanked by glycine (G), as well as the further two members of the catalytic triad, aspartate (D) and histidine (H), typical for this group of enzymes (Arrigoni and Jaeger, 1999). The remaining four of the 14 LPLAs were in between 675 and 921 amino acids long, had substantial additional protein stretches outside of the α/β hydrolase domain and two of them showed a predicted signal peptide. Interestingly, eight out of the 14 putative α/β hydrolases have peak transcript expression in the gametocyte stage (Table 2). Only one of the 10 shorter ones designated as the prodruk activation and resistant esterase PARE (PF3D7_0709700) was more intensely characterized before. The protein was shown to have esterase activity to activate esterified pepstatin, a potent peptidyl inhibitor of malarial aspartyl proteases. *P. falciparum* pepstatin-resistant mutants revealed changes in the *ppare* gene and in the associated esterase activity (Istvan et al., 2017). This suggests that this group of enzymes or at least PARE might release short chain acids from non-PL substrates and rather act as an esterase instead of a lipolytic enzyme. However, the spectrum of enzymatic activity towards a variety of substrates including PLs remains to be comprehensively determined.

Four proteins with a patatin-like protein domain, designated as patatin-like phospholipases (PLPs), were found encoded in the *P. falciparum* genome (PF3D7_0209100, PF3D7_0218600, PF3D7_0924000, and PF3D7_1358000). One of the putative PLPs has a signal peptide and their sizes vary between 679 and 2380 amino acids implying the presence of other protein domains in addition to the PLP domain. PLPs are lipolytic enzymes with an unusual folding topology that differs from classical lipases. PLPs were found both in eukaryotes, like the human cytosolic PLAs, but also in a variety of bacteria, including pathogenic ones (reviewed in Banerji and Flieger, 2004; Kienesberger et al., 2008). For instance the PLPs ExoU and VisD of the lung pathogens
Pseudomonas aeruginosa and Legionella pneumophila, respectively, have been described as crucial secreted virulence factors which are injected by the bacteria into the HC (Phillips et al., 2003; Sato et al., 2003; Shohdy et al., 2005; VanRheenen et al., 2006; reviewed in Banerji and Flieger, 2004; Flores-Díaz et al., 2016). The four plasmodial PLPs exhibit the characteristic lipase motif GXSXG containing the catalytic S. Three of the four PLPs further clearly comprised the second member D of the catalytic S-D dyad, while the fourth enzyme (PF3D7_0924000) possesses several candidates for catalytic Ds (Fig. 4, Table 2). While currently no experimental data are available on the Plasmodium PLPs, a new study in the apicomplexan model parasite Toxoplasma gondii describes an essential role for the PLP TgPL2 (a homolog of Pf3D7_1358000) in maintenance of apicoplast integrity (Lévêque et al., 2017). In the absence of TgPL2, the plastid is rapidly lost and the remaining apicoplasts appear enlarged with abnormal accumulations of membranous structures (Lévêque et al., 2017). TgPL1, in contrast, localizes to cytosolic vesicles and is secreted upon immune stresses. While no phospholipase activity for TgPL1 could be demonstrated, it was shown to be important for parasite survival in activated macrophages (Mordue et al., 2007; Tobin and Knoll, 2012). Another PLP of T. gondii, termed TgPLA2, exhibits a calcium-independent PLA2 activity and is secreted during infection (Cassaing et al., 2000).

We further identified one P. falciparum gene, which encodes a PLA1 harboring a DDHD domain (PF3D7_0814400) characteristic for a metal binding site often seen in phosphoesterases (reviewed in Lev, 2004). The protein also contained a region with a GXSXG motif, which indicated some acylhydrolase/phospholipase activity. Transcript expression of this putative PLA1 peaks in mature gametocytes (Fig. 4; Table 2).

In addition to the potential acyl hydrolases described above, an enzyme with a putative acyltransferase domain is encoded in the genome of P. falciparum (PF3D7_0629300). This protein consisted of 863 amino acids. The acyltransferase domain is located in the C-terminal part of the protein (Fig. 4; Table 2). The protein has a predicted signal peptide and its peak transcript expression was shown to be in the sporozoite stage. The orthologue of this enzyme in P. berghei, PbPL, was shown to support PVM rupture during parasite egress from the hepatocyte as discussed below (Burda et al., 2015). The PLA and membrane lytic activities of PbPL were demonstrated (Bhanot et al., 2005), potential acyltransferase activities have yet to be shown.

Furthermore, two enzymes, which belong to the phosphodiester-cleaving group, are encoded in the genome of P. falciparum. The first, designated PI-PLC, is a protein of 1,385 amino acids (PF3D7_1013500). The protein contains no signal peptide and is expressed in diverse life-cycle stages (Fig. 4; Table 2). Several experimental studies are available and discussed below. The second enzyme (PF3D7_1238600) spans 393 amino acids, comprised a sphingomyelin phosphodiesterase domain,
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(continued on next page)
Table 2 (continued)

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The genes for most of the 22 putative phospholipases were also found in the genomes of other Plasmodium species, such as P. vivax, P. knowlesi and P. berghei, showing their importance for the life-cycle of the Plasmodium genus. The PlasmoGem knockout screen database (http://plasmogem.sanger.ac.uk/phenotyp es; Bushell et al., 2017; Gomes et al., 2015) was searched for the P. berghei homologues of the P. falciparum phospholipases in order to evaluate if these are essential for the erythrocytic replication cycle. Analyses of the blood-stage growth phenotypes revealed that 14 of the putative phospholipases were predicted to be dispensable for erythrocytic replication, while PI-PLC (PBANKA_1013500) and the putative PLA2 (PBANKA_135800) were determined to be essential with significantly reduced relative growth rates (Table 2). Interestingly, the genes of three proteins from the α/β hydrolase family (PF3D7_0731800, PF3D7_1001400, PF3D7_1001600) were not identified in the P. berghei genome (Table 2), indicating that they might be specific to the human pathogenic parasites. The genes PF3D7_1001400 and PF3D7_1001600 are adjacent to each other on chromosome 10 of P. falciparum. Amino acid comparison of PF3D7_1001400 and PF3D7_1001600 shows 55% identity with PF3D7_1001600, lacking the region coding for the N-terminal part including the signal peptide, suggesting that the genes are paralogs that arose via gene duplication.

4. The role of plasmodial phospholipases during life-cycle progression

Phospholipases can contribute to the virulence of many pathogens, such as the bacteria Listeria monocytogenes, L. pneumophila, and P. aeruginosa, by directly affecting pathogen propagation and HC egress by membrane lysis, by depleting/modifying integral PLS or by means of their reaction products, thereby manipulating signaling pathways (reviewed in Flores-Díaz et al., 2016). In Plasmodium, however, the functions of most phospholipases during parasite growth and survival are to date not well known.

Probably the best studied plasmodial phospholipase is PI-PLC (PF3D7_1013500), which was shown to be involved in calcium-dependent signaling pathways leading to merozoite invasion of RBCs, to initiation of gametogenesis and to sporozoite motility. In eukaryotes, PI-PLC hydrolyses the membrane lipid PIP2, thereby releasing the two second messengers IP3 and DAG. While DAG activates protein kinase C (PKC), IP3 triggers the calcium release from intracellular compartments like the ER (reviewed in Berridge et al., 2000). In Plasmodium, however, the functions of most phospholipases during parasite growth and survival are to date not well known.

RBC invasion by merozoites is a complex multi-step process, mediated by specific receptor-ligand interactions. The initial, random contact of the merozoite with the RBC leads to activation of the plasmodial PI-PLC and in consequence to a rise in intracellular calcium. The increased calcium levels then trigger the secretion of microneme-resident proteins like EBA175 or AMA1 and their relocation to the merozoite PPM. At their destination, they support intensified binding of the merozoite to the RBC as well as its reorientation prior to RBC invasion (reviewed in Cowman et al., 2012; Cowman and Grabb, 2006). Inhibition of PI-PLC with the commercial PLC inhibitor U73122 prevents calcium-mediated signaling and thus activation of the plasmodial protein kinase B by calmodulin, in consequence impairing secretion of micronemal proteins (Raabe et al., 2011a, 2011b; Singh et al., 2010; Vaid et al., 2008; Vaid and Sharma, 2006).
The plasmodial PI-PLC is also involved in the signaling pathways initiating gametogenesis, as was shown for *P. berghei* and *P. falciparum* (reviewed in Bennink et al., 2016; Kuehn and Pradel, 2010). Once in the mosquito midgut, the intracellularly encysted gametocytes sense the change in pH of host cells due to the perception of environmental factors present in the gut lumen, triggering their egress from the RBC and their transformation into gametocytes. Initially, a plasmodial guanylyl cyclase becomes activated, leading to the synthesis of cyclic GMP (cGMP) (Carucci et al., 2000; Muhia et al., 2001). The rise of cGMP activates the cGMP-dependent protein kinase G, which regulates the generation of PIP₂ (Alam et al., 2015; Brochet et al., 2014; McRobert et al., 2008). At the same time, PI-PLC is stimulated, which hydrolyses PIP₂ to generate DAG and IP₃ (Martin et al., 1994; Raabe et al., 2011b), resulting in the release of calcium from inner stores (Billker et al., 2004). Downstream of this signaling cascade, the increased calcium levels are sensed by specific calcium-dependent protein kinases, which in turn control DNA replication and protein biosynthesis (Billker et al., 2004; Ojo et al., 2014, 2012; Sebastian et al., 2012).

PI-PLC activity has also been linked to the gliding motility of *Plasmodium* sporozoites (Carey et al., 2014; Kebaier and Vanderberg, 2010). GLiding, a substrate-dependent motility that is specific for apicomplexan parasites and driven by an actin-myosin motor, is required by the malaria parasite to migrate across tissues and through HCs (Kebaier and Vanderberg, 2010; Vanderberg, 1974; Yoeli, 1964; reviewed in Ménard et al., 2013). Mandatory for gliding is the discharge of adhesive proteins, like the thrombospondin-related adhesive protein TRAP, from the micronemes and their relocation to the sporozoite PPM. For the transmembrane protein TRAP it was shown that it engages with the motor complex, while simultaneously binding to HC receptors, resulting in its relocation to the posterior end of the parasite, thereby supporting sporozoite movement along the HC surface (reviewed in Ménard, 2001; Vaughan et al., 2008). During motility, again calcium is the key signaling molecule, coordinating microneme discharge and actin-myosin motor activity. It is postulated that PI-PLC regulates the calcium levels in sporozoites, since both U73122 and the IP₃ receptor blocker 2-APB inhibit sporozoite gliding motility in a dose-dependent manner (Carey et al., 2014).

Besides PI-PLC, one more phospholipase has been functionally characterized in the malaria parasite, i.e. a putative lecithin:cholesterol acyltransferase (LCAT; PFD37_0629300), which was demonstrated to be involved in membrane dynamics during hepatocyte invasion and egress by *P. berghei* parasites. This phospholipase, termed *P. berghei* protein (PbPL), exhibits membrane lytic activity in vitro and following hepatocyte infection, PbPL localizes to the PVM (Bhanot et al., 2005; Burda et al., 2015). Upon genetic knock-out, PbPL-deficient sporozoites lose their infectivity and their ability to cross epithelial cell layers. Also, while the PbPL-deficient parasites undergo replication and develop merozoites, these are unable to egress from the host hepatocyte (Burda et al., 2015). In this context, perforin-like proteins have previously been identified and shown to be important for parasite egress from the HC in a calcium-dependent manner (Deligianni et al., 2013; Garg et al., 2013; Wirth et al., 2014). It is thus suggested that during egress PbPL acts in a conglomerate of different phospholipases or pore-forming proteins. Noteworthy, following PVM rupture, the hepatocyte cytoskeleton degrades, and during this process PIP₂, promoting the linkage between actin and the HCM, disappears from the inner plasma membrane leaflet (Burda et al., 2017). A similar loss was observed for IP₃ and PS, indicating that major modifications of the hepatocyte PLs content occur during egress. While this study is the first one to link phospholipases to HC egress by *Plasmodium*, a role of a LCAT during *T. gondii* replication and egress has also been reported (Pszenny et al., 2016). TgLCAT, which reveals 32–35% identity and 39–43% similarity with LCATs from different *Plasmodium* species, is secreted by the parasite and transforms PC into LPC and therefore exhibits PLA activity. *T. gondii* parasites lacking LCAT are impaired in growth, virulence and egress from the HC (Pszenny et al., 2016).

5. Phospholipases as targets for chemotherapeutics

The need of lipids for growth and proliferation of *Plasmodium* makes the lipid metabolism an attractive target for therapeutic measures (reviewed in Coppens, 2013; Mitamura and Palacpac, 2003; Ramakrishnan et al., 2013). Still, to date little work has been done to validate plasmodial phospholipases as chemotherapeutic targets. As pointed out earlier, a neutral SMase, termed PfNSM, has been identified in *P. falciparum*, which exhibits PLC/SMase activity towards lyso-PC as well as SM. In general, SMases catalyse hydrolysis of SM to ceramide and phosphorylcholine. Scyphostatin, a SMase inhibitor, inhibits the PLC/SMase activity of recombinant PfNSM and blocks parasite growth with an IC₅₀ value of approximately 4 μM (Handa et al., 2002). The physiological and pathological role of the PfNSM in *Plasmodium* remains unknown, but it was speculated that the enzyme is involved in degradation of host-derived SMs to ceramides that in turn modulate cell cycle progression and are used for synthesis of parasite- derived SMs (Handa et al., 2002). SMases were also shown to play an important role in bacterial virulence, e.g. for *Bacillus cereus* (Oda et al., 2014, 2012), *Staphylococcus aureus* (Hayashida et al., 2009; Huseby et al., 2010; Katayama et al., 2013) or *Listeria ivanovii* (Gonzalez-Zorn et al., 1999). The SMase C inhibitor SMY-540 exhibits a strong inhibitory effect against *B. cereus* and significantly reduces lethality of *B. cereus*-infected mice (Oda et al., 2014).

Previous studies also implicated phospholipases as virulence factors in opportunistic fungi such as *Candida albicans*, *C. glabrata*, *Cryptococcus neoformans* and *Aspergillus fumigatus* and thus have been evaluated as potential antifungal targets (reviewed in Djordjevic, 2010; Hossain and Ghandour, 2000; Neely and Ghandour, 2000). For example, in *Candida* studies using PLB-producing as well as PLB-deficient *Candida* strains demonstrated that the PLB-producing strain is capable of penetrating deep into mouse sub-mucosal tissue and gastric mucosal, while the PLB-deficient strain was not invasive (Mükerjee et al., 2001). Treatment with synthetic phospholipase inhibitors exhibiting lipophilic beta-blocking structures in combination with fluconazole, a triazole used for antifungal medication, blocks tissue penetration and prevents death of mice infected with lethal inocula of *C. albicans* (Hänel et al., 1995). Further, the PLB CnPLb1 of *C. neoformans* is important for survival of the fungi in macrophages. CnPLb1 has been evaluated as antifungal drug target in the past, but to date inhibitors such as bis-pyridinium compounds were not effective against the enzyme (reviewed in Djordjevic, 2010).

Besides their roles in virulence of pathogenic microorganisms, phospholipases are assigned to different life-style diseases, such as cardiovascular diseases and atherosclerosis, but also immune disorders and cancer (reviewed in Quach et al., 2014). For example, expression of the human secretory PLAr5 (sPLAr5), a diverse family of phospholipases encompassing 19 members, is increased in breast, lung and prostate cancers compared to control tissues (e.g. Denizot et al., 2005; Sved et al., 2004; Yamashita et al., 1994, 1993; Zhang et al., 2015). The link between PLAr2 and cancer makes these enzymes potential anti-cancer targets (reviewed in Cummings, 2007; Marks et al., 2000; Wang and Dubois, 2006). Although a broad range of PLAr2 inhibitors exist (reviewed in Barroqui et al., 1999; Kokotou et al., 2017; Meyer et al., 2005), current knowledge about the mode of action or toxicity in humans or animal models is limited and their effectiveness as pharmacological agents has yet to be addressed (reviewed in Cummings, 2007; Laye and Gill, 2003). A main drawback in targeting PLAr2 with chemotherapeutics is the wide range of individual PLAr2 isoforms, as well as their physiological roles in healthy cell homeostasis (reviewed in Balsinde et al., 1999; Cummings, 2007).

6. Conclusions

The lipid metabolism of malaria parasites is crucial for intracellular growth as well as for propagation and transmission of the pathogen.
Therefore, enzymes involved in lipid metabolic processes represent prime targets for malaria chemotherapeutics. Despite their important role for PL processing and thus for membrane synthesis and breakdown, to date little is known about the functions of the 22 annotated plasmodial phospholipases. Current data on these PL-hydrolyzing enzymes suggest that some of the enzymes represent important virulence factors involved in HC infection and egress, but also in cell signaling and cell-cell communication. A detailed functional characterization of the plasmodial phospholipases will help to validate these enzymes as potential new targets for antimarial chemotherapy.

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References


