Phosphatidylinositol 3-Phosphate, an Essential Lipid in *Plasmodium*, Localizes to the Food Vacuole Membrane and the Apicoplast†‡

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Phosphoinositides are important regulators of diverse cellular functions, and phosphatidylinositol 3-monophosphate (PI3P) is a key element in vesicular trafficking processes. During its intraerythrocytic development, the malaria parasite *Plasmodium falciparum* establishes a sophisticated but poorly characterized protein and lipid trafficking system. Here we established the detailed phosphoinositide profile of *P. falciparum*-infected erythrocytes and found abundant amounts of PI3P, while phosphatidylinositol 3,5-bisphosphate was not detected. PI3P production was parasite dependent, sensitive to a phosphatidylinositol-3-kinase (PI3-kinase) inhibitor, and predominant in late parasite stages. The *Plasmodium* genome encodes a class III PI3-kinase of unusual size, containing large insertions and several repetitive sequence motifs. The gene could not be deleted in *Plasmodium berghei*, and in vitro growth of *P. falciparum* was sensitive to a PI3-kinase inhibitor, indicating that PI3-kinase is essential in *Plasmodium* blood stages. For intraparasitic PI3P localization, transgenic *P. falciparum* that expressed a PI3P-specific fluorescent probe was generated. Fluorescence was associated mainly with the membrane of the food vacuole and with the apicoplast, a four-membrane bounded plastid-like organelle derived from an ancestral secondary endosymbiosis event. Electron microscopy analysis confirmed these findings and revealed, in addition, the presence of PI3P-positive single-membrane vesicles. We hypothesize that these vesicles might be involved in transport processes, likely of proteins and lipids, toward the essential and peculiar parasite compartment, which is the apicoplast. The fact that PI3P metabolism and function in *Plasmodium* appear to be substantially different from those in its human host could offer new possibilities for antimalarial chemotherapy.

Phosphatidylinositol is a crucial phospholipid in eukaryotic cells. It is a structural membrane lipid, and phosphorylation of the hydroxyl groups of its inositol head group by specific lipid kinases leads to the production of seven different phosphoinositide species, which have been found to be enriched in distinct cellular compartments. They play key roles in a multitude of cellular processes, such as membrane traffic, cell motility, cytoskeletal reorganization, DNA synthesis, the cell cycle, adhesion, and signal transduction (9). Approximately 1% of total lipids in mammalian cells are phosphoinositides, mainly phosphatidylinositol 4-monophosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] (45). Derivatives phosphorylated at the 3 position are considerably less abundant in mammalian cells. Phosphatidylinositol 3-monophosphate (PI3P) is a ubiquitous lipid in eukaryotic cells and is present in small amounts in mammalian cells (classically <15% of PI4P), while PI3P is as abundant as PI4P in the yeast *Saccharomyces cerevisiae* (2). It has been suggested that one of the functions of these lipids is to establish membrane identity (46); PI4P predominates at the Golgi apparatus (33), PI(4,5)P2 at the plasma membrane (62), PI3P on early endosomes (20), and phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2] on late endocytic organelles (48). Certain phosphoinositides have been shown to play important roles in constitutive membrane traffic. PI3P is involved in endocytosis and vesicular trafficking toward the lysosome in yeast and mammalian cells (23, 39). PI3P has also been shown to be implicated in the processes of retrograde trafficking from the endosome to the Golgi apparatus and in autophagy (7, 42). PI(3,5)P2 is found in yeast, mammalian, and plant cells (11) and is essential for protein sorting in multivesicular bodies (38), which are an intermediate compartment for the degradation of cell surface receptors within lysosomes. PI3P is the product of PI3-kinase class III (16), also termed Vps34 (vacuolar protein sorting), and PI(3,5)P2 is synthezed by the phosphorylation of PI3P by PIKfyve (phosphoinositide kinase, FYVE finger containing; Fab1 in yeast) (38). Both enzymes are conserved across eukaryotic evolution from yeast to mammals. Vps34-type enzymes are the only PI3-kinases in unicellular organisms. In contrast, metazoan cells possess three classes of PI3-kinases (I, II, and III), which differ from each other by their activation mode and their substrate specificity (56), leading to the synthesis of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], and PI3P, respectively. The synthesis of PI(3,4,5)P3 and PI(3,4)P2 is controlled by agonist stimulation of cells, resulting in strong fluctuations in their cellular levels.
These phosphoinositides are generally not observed in unicellular organisms. *Plasmodium falciparum* is the causal agent of the most severe form of human malaria. During the symptomatic phase of the disease, the parasite resides within a vacuole in mature erythrocytes, cells that are devoid of protein and lipid biosynthesis and intracellular compartments. In addition to the classically observed organelles of eukaryotic cells, *Plasmodium* contains certain particular compartments. These include the apicoplast, a four-membrane bounded plastid-like organelle derived from an ancestral secondary endosymbiosis event (61), and specialized secretory organelles, rhoptries and micronemes, located at the apical pole and involved in host cell invasion. *Plasmodium* blood stages internalize host cell hemoglobin that is degraded in a specialized compartment, the food vacuole (17). Some characteristics of the food vacuole, such as low pH and the presence of proteolytic enzymes, could qualify it as a lysosome-like organelle (21). However, this peculiar compartment is present only during *Plasmodium* blood stages, is absent from mosquito and liver stages, and is not found in other apicomplexan parasites. Targeting proteins and lipids to these numerous cellular compartments requires sophisticated vesicular trafficking. Certain classical actors in vesicular trafficking in eukaryotic cells, such as Rab GTPases and SNARE-like proteins, are present in *Plasmodium* (3, 44).

Here we addressed the question of whether the second group of classical actors in vesicle transport, i.e., the phosphoinositides PI3P and PI(3,5)P2, are synthesized by the parasite. For this purpose, we established, for the first time, a detailed phosphoinositide profile of *P. falciparum*-infected red blood cells and detected important amounts of PI3P, which we found located at the food vacuole membrane and the apicoplast. In contrast, PI(3,5)P2 could not be detected, indicating possible differences in protein sorting between *Plasmodium* and other eukaryotes. PI3-kinase activity is essential for *Plasmodium*, suggesting that PI3P-dependent functions might be an interesting target for future drug development.

**MATERIALS AND METHODS**

**Parasites.** *P. falciparum* strains 3D7 and 3D7attB (37) were maintained in continuous culture in human leukocyte-free erythrocytes (obtained from the local blood bank). Parasites were cultured at 5% hematocrit in RPMI 1640 supplemented with 2 mM glutamine, 0.1% nonessential amino acids, 10% FCS, and 1.2 ml KCl (2 M). After centrifugation at 3,000 rpm for 5 min, the organic phase was collected and the aqueous phase was washed once with 0.9% NaCl. The cells were resuspended in 500 μl Tris-EDTA (12 M), 1.2 ml chloroform, and 1.2 ml NaCl and were lysed by stepwise addition and intensive vortexing of 2.4 ml of 3D7-infected red blood cells were enriched to 80 to 90% parasitemia using Plasmon (32). For this purpose, 1 volume of packed cells was mixed with 1.4 volumes of 0.2 M sodium phosphate buffer for 90 min and were then washed in PBS-10% fetal calf serum (FCS; Sigma), and infused in 2.3 M sucrose containing 10% polyvinylpyrrolidone before being frozen in liquid nitrogen. Sections were obtained on a Leica Ultracut instrument equipped with an FCS cryoattachment operating at −100°C. Sections were floated successively on PBS-FCS, rabbit anti-PI3-kinase antibodies (Abcam) diluted 1:200 in PBS-FCS, and 10 nm protein A-gold (Utrecht) diluted in PBS to an optical density at 525 nm (OD525) of 0.05, with five 3-min washes of PBS between each step. Sections were then embedded in methacrylate (2%-)uranyl acetate (0.4%) and observed with a Zeiss EM10 electron microscope.

**Labeling, identification, and quantification of phosphoinositides.** Late stages of infected red blood cells were enriched to 80% to 90% parasitemia using Plasmon (32). For this purpose, 1 volume of packed cells was mixed with 1.4 volumes of complete culture medium and 2.4 volumes of Plasmon and was then incubated for 30 min at 37°C, and the late-stage parasites were recovered from the supernatant by centrifugation. Packed cells (volume, 100 μl) were washed once with complete medium and twice with phosphate-free PL buffer (137 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, 30 mM HEPES [pH 7.4]). Cells were labeled with 500 μCi H32PO4 (MP Biomedicals) in 1 ml (final volume) in PL buffer for 1 h at 37°C. Excess radioactivity was removed by three washes with 0.9% NaCl. Parasites were resuspended in 1 ml 0.9% NaCl and were lysed by stepwise addition and intensive vortexing of 2.4 ml methanol (4 volumes), 1.2 ml chloroform, 200 μl HCl (12 M), 1.2 ml chloroform, and 1.2 ml KCl (2 M). After centrifugation at 3,000 rpm for 5 min, the organic phase was collected and pooled with the aqueous phase for 4 ml chloroform. The extracts were dried under a nitrogen stream at room temperature. Dried lipids were resuspended in 40 μl of chloroform-methanol (2:1, vol/vol) and resolved by thin-layer chromatography (TLC) using chloroform-acetic acid-water (80:30:26:24:14:vol/vol) as a solvent. The TLC plate (silica gel 60 with a concentrating zone [Merek]) had been pretreated with 1% potassium oxalate in 2 mM EDTA-methanol (vol/vol) for 15 min and activated at 100°C for 1 h. Spots corresponding to PI monophosphates and PI bisphosphates were visualized using a PhosphorImager scanner and identified by comparison with the migration of authentic standards. The zones encompassing PI monophosphates and PI bisphosphates/PI triphosphates were scraped off separately. The phosphoinositides were then deacylated by the addition of 1 ml of a methanolysis reagent, composed of 40% methanol–methanol–butanol–H2O (26.8:45.7:11.4:16, vol/vol), to the silica powder. After incubation at 53°C for 50 min, the methanolysis reagent was completely evaporated under a nitrogen stream at 37°C. The samples were resuspended in 1.2 ml H2O and were separated by high-performance liquid chromatography (HPLC) using a Whatman Partisphere 5 SAX column (by 125 mm) with guard cartridge anion-exchanger units (reference no. 4641 0005; Whatman) and a gradient of 1 M (NH4)2HPO4 (pH 3.8) and double-distilled water as described previously (41).

**P. berghei PI3-kinase knockout.** To knock out the *P. berghei* PI3-kinase (PhPI3-kinase) gene in *P. berghei* strain ANKA, the recently described PCR-based

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**P. falciparum transfection.** One hundred microliters of 3D7attB-infected red blood cells from a synchronized 5% parasitemia ring-stage culture was washed in Cytomix and added to 300 μl of a plasmid preparation in electroporation cuvettes (Bio-Rad). Parasites were transfected by electroporation (0.31 kV, 950 μF; Bio-Rad Gene Pulser II) and were immediately transferred to culture plates containing 5 ml complete medium and 120 μl fresh red blood cells. Five hours after transfecion, selection was started using 2.5 μM WR99210 and 2.5 μg/ml blasticidin. The medium was changed daily for the first 6 days, and then every 2 days until ring-stage parasites were detected. Cultures were diluted weekly by the addition of 30% fresh red blood cells. Drug pressure was maintained until correct integration at the genomic attb locus was verified.

**Fluorescence microscopy.** For GFP imaging, infected red blood cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (EM Science) overnight at 4°C. After 2 washing steps with PBS, cells were allowed to settle on polylysine-coated 10-well slides (Fisher Scientific) for 1 h at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 5 min. After three washes with PBS, cells were incubated with a primary antibody (rabbit anti-acetyl carrier protein [anti-ACP; kindly provided by G. McFadden] or rabbit anti-chloroquine resistance transporter [anti-CRT; MR4 MRA-308]), both at 1:600 in PBS) for 1 h, washed again three times with PBS, and incubated with a secondary antibody (anti-rabbit Alexa Fluor 488) for 30 min. Cells were washed three times with PBS and were then treated for 5 min with 2 ml Hoechst 33342 (Sigma) diluted with PBS for nuclear staining. Slides were mounted with Vectashield.

**Immunoelectron microscopy.** Highly enriched, late-stage-infected cells were fixed in 2% paraformaldehyde and 0.2 M sodium phosphate buffer for 90 min and were then washed in PBS-10% fetal calf serum (FCS; Sigma), and infused in 2.3 M sucrose containing 10% polyvinylpyrrolidone before being frozen in liquid nitrogen. Sections were obtained on a Leica Ultracut instrument equipped with an FCS cryoattachment operating at −100°C. Sections were floated successively on PBS-FCS, rabbit anti-PI3-kinase antibodies (Abcam) diluted 1:200 in PBS-FCS, and 10 nm protein A-gold (Utrecht) diluted in PBS to an optical density at 525 nm (OD525) of 0.05, with five 3-min washes of PBS between each step. Sections were then embedded in methacrylate (2%-)uranyl acetate (0.4%) and observed with a Zeiss EM10 electron microscope.
method was used (12), by which a gene deletion is achieved by transfection with linear DNA obtained by PCR amplification without prior cloning. In the transfection construct, the resistance gene is flanked by two short regions homologous to the target locus. Two sequential PCRs were performed. Two 500-bp PbPI3K homologous sequences were amplified with primers 161/162 and 168/164 (see Table S1 in the supplemental material). The amplicons obtained were then combined with the linearized pDEFdDHPEA vector (12), which supplied the human dihydrofolate reductase (hDHFR) resistance cassette, and were PCR amplified with primers 161 and 164 to yield a final construct of 3 kb. All PCRs were carried out with the Advantage 2 polymerase mixture (BD Biosciences) and the following amplification program: 95°C for 2 min; 35 cycles of 30 s at 95°C, 45 s at 52°C, and 4 min at 62°C; and a final extension at 62°C for 10 min (51). Ten micrograms of the PCR product was digested with 10 U DpnII for 2 h at 37°C to eliminate any contaminating pDEFdDHPEA plasmid. The DNA was ethanol-precipitated and resuspended for transfection at 2 µg/µL in water.

**Plasmid construction for PbPI3-kinase tagging.** The transfection constructs for C-terminal tagging of the PbPI3-kinase were based on plasmid pDEdH DHPEA (pOB116; kindly provided by Oliver Billker). This plasmid carries the hDHFR gene, conferring resistance to pyrimethamine. For our purposes, the following elements were added: 1.5 kb of the 3' end of the PbPI3K gene with or without the stop codon, either the GFP coding sequence or a triple hemagglutinin (HA) tag fused to the four-cysteine motif (3HA-Cys), and the 3' untranslated region (3' UTR) of the PbPI3K gene. The PbPI3K gene was amplified from genomic DNA with primers 159/160 or 159/274, the 3' UTR with primers 155/156 from plasmid pOB116, the GFP tag with primers 181/182 from plasmid pRON4-GFP (provided by Maryse Lebrun), and the 3HA-Cys tag (34) with primers 161/162 from a 2HA-tag-containing plasmid (see Table S1 in the supplemental material). All constructs were verified by sequencing.

Transfection, plasmid preparations were performed using the Promega Mini-wizard plus kit and were linearized in the center of the PbPI3-kinase sequence by digestion with BclI for 2 h at 37°C. Following ethanol precipitation, the DNA was resuspended in water at 2 µg/µL.

**Transfection of P. berghei.** Transfection was done by following the Amaxa transfection protocol (12). Mature *P. berghei* schizonts were obtained by culturing blood from an infected mouse overnight in complete medium as described previously (55). Schizonts were collected by centrifugation on a Nycodenz density gradient and resuspended for transfection at 2 µg/µL in water.

**RESULTS**

**Phosphoinositide profile of *P. falciparum*-infected red blood cells.** It has been shown previously that *Plasmodium*-infected erythrocytes produce important amounts of phosphatidylinositol mono- and bisphosphates (13); however, their molecular identity had not been determined. Here we analyzed the detailed phosphoinositide profile of *P. falciparum*-infected red blood cells. For this purpose, highly enriched late-blood-stage parasites were metabolically labeled with [32P]phosphate, and the lipids were then extracted and separated by thin-layer chromatography (TLC). Incorporation of [32P]phosphate into the major structural phospholipids phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidyl-inositol was lacking in uninfected erythrocytes but was substantial after *Plasmodium* infection (Fig. 1A). We found that infected erythrocytes (iRBC) produced about 13 times more total phosphoinositides than equal numbers of uninfected erythrocytes (data not shown).

The areas corresponding to phosphatidylinositol mono- and polyphosphates were recovered separately and analyzed by high-performance liquid chromatography (HPLC), allowing identification and quantification of phosphoinositide isomers (41). Uninfected red blood cells produced only PI(4)P and PI(4,5)P2. In contrast, infected red blood cells produced a complex phosphatidylinositol profile; in addition to PI(4)P and PI(4,5)P2, they synthesized large amounts of PI3P and small amounts of PI(3,4)P2 and PI(3,4,5)P3 (Fig. 1B to E). The quantity of PI3P produced was important, representing 29.7% ± 12.9% (mean ± standard error of the mean [SEM]) of total phosphatidylinositol monophosphates (*n* = 8). In contrast, in uninfected red blood cells, PI3P was undetectable even after prolonged labeling (up to 18 h), indicating that its production in infected red blood cells was indeed induced by the parasite. Concerning polyphosphoinositides in infected erythrocytes, PI(4,5)P2 was by far the most abundant, corresponding to 70% ± 11.4% (mean ± SEM) of total phosphoinositides, while PI(3,4)P2, and PI(3,4,5)P3 were present at 0.6% ± 0.3% and 0.4% ± 0.3%, respectively (*n* = 8). Surprisingly, we could not detect PI(3,5)P2, a lipid involved in vesicle transport toward the vacuole/lysosome and present in yeast, plant, and mammalian cells (11).

When phosphoinositides were studied during synchronized parasite maturation of a culture at 10% parasitemia, total phosphoinositide labeling increased about 3-fold between the ring and late stages (Table 1). PI3P levels were relatively low with respect to the other phosphoinositides in ring stages (about 1% of total phosphoinositides) but became more predominant in late stages (about 4%), which may indicate that the function linked to the presence of this lipid is more developed during late parasite stages. The relative abundance of the predominant phosphoinositides PI4P and PI(4,5)P2 also shifted from 19% PI4P and 80% PI(4,5)P2 in rings to 32% PI4P and 64% PI(4,5)P2 in late stages.

Given the surprisingly high levels of PI3P in *P. falciparum*-infected erythrocytes, we were interested in establishing a link between the phosphoinositide profile and PI3-kinase activity, and we analyzed the effect of wortmannin, a well-established inhibitor of PI3-kinases. Highly enriched, late-stage parasite cultures were pretreated with 100 nM wortmannin for 30 min, followed by metabolic labeling in the presence of wortmannin for 1 h. This treatment did not alter the synthesis of labeled structural phospholipids in comparison to mock-treated samples as analyzed by TLC (data not shown). Concerning phosphoinositides, the only significant difference observed was a 58% decrease in PI3P levels, while the levels of the major phosphoinositides, PI4P and PI(4,5)P2, remained unchanged (Fig. 2). This indicated that PI3P production in *P. falciparum*-infected erythrocytes was dependent on a wortmannin-sensitive PI3-kinase and that at the above indicated concentration, wortmannin inhibited PI3-kinase activity specifically, i.e., without affecting other PI-kinases.

The *P. falciparum* genome encodes a PI3-kinase. The *P. falciparum* genome contains one predicted PI3-kinase gene,
PFE0765w, encoding a predicted protein of 2,133 amino acids (aa) containing the characteristic domains of class III PI3-kinases: a calcium/lipid-binding C2 domain (aa 297 to 371), a PI3-kinase family accessory domain (aa 1097 to 1280), and a C-terminal PI3- and PI4-kinase catalytic domain (aa 1867 to 2130) (54) (see Fig. S1 in the supplemental material). However, the predicted P. falciparum protein is substantially longer than any PI3-kinases described so far, in part due to the presence of five different repetitive peptide motifs of six to eight amino acids in the P. falciparum sequence, repeated 5 to 12 times and accounting for about 15% of the total amino acid sequence (see Fig. S1). PI3-kinase orthologues are annotated for other Plasmodium species (PlasmoDB 6.3); however, only

**TABLE 1. Phosphoinositide synthesis during blood-stage development of P. falciparum**

| Phosphoinositide | Radioactivity (cpm)\(b\) at the following stage: |
|------------------|-----------------------------------------------|
|                  | Ring         | Trophozoite | Schizont |
| PI3P             | 11,649       | 146,391     | 184,723  |
| PI4P             | 286,958      | 827,290     | 1,336,574|
| PI(3,4)P(2)      | ND           | 12,823      | 16,750   |
| PI(4,5)P(2)      | 1,076,872    | 2,523,291   | 2,646,058|

\(a\) Highly synchronized cultures at 10% parasitemia were used.

\(b\) Values correspond to counts per minute detected after HPLC analysis in one experiment of two. ND, not detected.
the sequences of the *Plasmodium vivax* (PvPI3K; 1,779 aa) and *Plasmodium chabaudi* (PcPI3K; 1,796 aa) PI3-kinases are complete. A multiple sequence alignment of the three *Plasmodium* PI3-kinases (see Fig. S1) highlighted the fact that sequence conservation was restricted mainly to the three predicted functional domains: amino acid identity was around 86% for the catalytic domain, 64% for the accessory domain, and 34% for the C2 domain (*P. falciparum* versus *P. vivax*/*P. chabaudi*). Repetitive peptide motifs were not detected in PvPI3K and PcPI3K. The catalytic domain of PpPI3-kinase contains the highly conserved motifs that participate in ATP binding and phosphate transfer, in particular the catalytic loop sequence 1987GIGDRHLDN; 1881K, which interacts with the α-phosphate of ATP; and the metal ion binding sites 1995N, 1884D, and 2008D. The sequence following 2008D has been shown to confer substrate specificity (43) and supports the prediction of the sequences of the *Plasmodium* species: amino acid identity was around 86% for the catalytic domain, 64% for the accessory domain, and 34% for the C2 domain (*P. falciparum* versus *P. vivax*/*P. chabaudi*). Repetitive peptide motifs were not detected in PvPI3K and PcPI3K. The catalytic domain of PpPI3-kinase contains the highly conserved motifs that participate in ATP binding and phosphate transfer, in particular the catalytic loop sequence 1987GIGDRHLDN; 1881K, which interacts with the α-phosphate of ATP; and the metal ion binding sites 1995N, 1884D, and 2008D. The sequence following 2008D has been shown to confer substrate specificity (43) and supports the prediction that the *Plasmodium* protein is a class III enzyme; these enzymes are described as having phosphatidylinositol as sole substrate producing PI3P. Furthermore, 1881K, which is essential for kinase activity and is covalently modified in mammalian PI3-kinases by the PI3-kinase inhibitor wortmannin (64), is also present in PpPI3-kinase.

**The PI3-kinase gene is essential in *P. berghei***. To investigate whether PI3-kinase is essential in *Plasmodium* species, we chose to disrupt the *PI3* gene in *P. berghei*. Since no sequence information for the 5′ end of the *PbPI3K* gene was available (corresponding to the first 387 aa of the *P. chabaudi* sequence), we generated a *PbPI3K* knockout construct that, upon double homologous recombination, would lead to deletion of the accessory and catalytic domains, generating a truncated protein without a functional kinase domain (see Fig. S2A in the supplemental material). The transfection construct was generated using the recently described PCR-based method (12). In four independent transfections, pyrimethamine-resistant parasites were obtained, suggesting successful transfection. However, none of the populations showed integration of the recombinant DNA into the *PbPI3K* locus, as analyzed by PCR (data not shown).

In order to verify that targeting of the *PbPI3K* locus was possible, we generated three constructs for single homologous recombination (see Fig. S2B in the supplemental material). Two constructs aimed at tagging PbPI3-kinase at the C-terminal end with either green fluorescent protein (GFP) (plasmid pOB-PbPI3K-GFP) or a triple hemagglutinin (3HA) tag (plasmid pOB-PbPI3K-3HA). A third construct was identical to the 3HA tag plasmid except that the stop codon of the *PbPI3K* open reading frame was maintained (plasmid pOB-PbPI3K-stop-3HA), so that the coding sequence was not changed upon integration, while the same genetic control elements were introduced as with the authentic tagging constructs. Correct genome integration was achieved only with the control construct that maintained the stop codon (see Fig. S2C), while modification of the PI3-kinase sequence by adding a GFP or 3HA tag was unsuccessful in four independent transfections (data not shown). Taken together, these results demonstrated that the *PbPI3K* locus was targetable. However, gene disruption was lethal to blood-stage parasites, as was epitope tagging at the extreme C-terminal end, which likely altered PbPI3-kinase function or stability. These results strongly suggested that PI3-kinase activity is essential in *P. berghei* blood-stage parasites.

To analyze whether PI3-kinase activity is also essential for *P. falciparum* blood-stage development, we studied the effect of PI3-kinase inhibitors on *P. falciparum* growth in vitro. Wortmannin is known to be unstable in complex media and at a neutral pH (66), and accordingly, we could not detect any effect of a single dose as high as 25 μM wortmannin in a standard 2-day *P. falciparum* growth assay (10). However, the alternative PI3-kinase inhibitor LY294002 (59), which is stable under cell culture conditions, interfered with *P. falciparum* growth, with a 50% inhibitory concentration (IC50) of 26 ± 6 μM (mean ± SEM; n = 4), indicating that PI3-kinase might also be essential for *P. falciparum* development.

**Generation of transgenic *P. falciparum* expressing a PI3P-specific fluorescent probe.** We next aimed at monitoring the intraparasitic localization of PI3P in infected red blood cells. For this purpose, we used a fusion of GFP to a tandem repeat of the FYVE (Fab1, YOTB, Vac1, EEAI) domain of the mammalian Hrs protein as a PI3P-specific probe. This construct has been reported to specifically bind PI3P (20) and has been used in various organisms to monitor the intracellular localization of PI3P (22, 58). In order to generate transgenic *P. falciparum* parasites expressing GFP-2×FYVE, a construct was designed in which GFP-2×FYVE was cloned under the control of the moderately strong calmodulin promoter (plasmid pLN-GFP-2×FYVE). The construct was integrated into the genome as a single-copy gene by using the recently described attB/attP site-specific recombination system (37) and strain 3D7attB (Fig. 3A). The same approach was used to generate transgenic parasites that express GFP fused to a mutated version of the FYVE domain (construct GFP-2×FYVEC215S). The cysteine-to-serine mutation at position 215 in both FYVE domains has previously been shown to completely abolish PI3P binding (20). This construct was designed as a control in order to identify and characterize the effect due to specific PI3P binding of GFP-2×FYVE. Stably transfected parasites were obtained less than 3 weeks after transfection, indicating that GFP-2×FYVE expression (wild type or mutated) did not interfere with parasite growth. Cor-
Subcellular localization of PI3P. Transgenic parasites expressing either GFP-2×FYVE or its mutated version GFP-2×FYVEC1255S were analyzed by fluorescence microscopy. For GFP2×FYVE the fluorescent signal was detected as a circular structure surrounding the food vacuole, which was identified by the presence of hemozoin crystals in the corresponding phase images (Fig. 4). Additionally, we observed one or a few dots of bright fluorescence, generally located on the circular structure, and some diffuse cytoplasmic staining. In contrast, fluorescence was diffuse throughout the parasite cytoplasm for GFP-2×FYVE and some diffuse cytoplasmic staining. In contrast, fluorescence bright fluorescence, generally located on the circular structure, images (Fig. 4). Additionally, we observed one or a few dots of

DISCUSSION

The results presented here show that infection of red blood cells by *P. falciparum* leads to profound changes in the phosphoinositide profile. It has long been known that in normal human erythrocytes, the only phospholipids to be labeled upon incubation with radioactive phosphate are phosphoinositides

FIG. 3. Generation of GFP-2×FYVE expressing *P. falciparum* parasites. (A) Schematic representation of the recombination event leading to the integration of GFP-2×FYVE into *P. falciparum* strain 3D7attB. The two plasmids used for cotransfection are represented at the top: plasmid pINT, carrying the integrase expression unit (int) and a neomycin resistance cassette (neo), and plasmid pLN-GFP-2×FYVE, carrying the GFP-2×FYVE fusion gene under the control of the calmodulin promoter and a blasticidin resistance cassette (bsd) flanking the attP site. The attB site is present in the genomic cg6 locus in strain 3D7attB (37). Recombination mediated by the inteegrase between the attB and attP sites yielded strain 3D7attB-GFP-2×FYVE. The primers used in PCR analysis of transgenic parasites are indicated by arrows. Large arrowheads indicate the transcriptional directions of genes and expression cassettes. The diagram is not drawn to scale. (B) PCR analysis of genomic DNA of the transgenic parasite population was performed to confirm the correct integration of GFP-2×FYVE into the *P. falciparum* genome. The relative positions of the primers used are indicated in panel A. Lanes 1 to 3 demonstrate the integration of pLN-GFP-2×FYVE at the attB locus (lane 1, primers 202/251; lane 2, primers 202/211; lane 3, primers 202/242). Lane 4 shows the presence of the blasticidin resistance gene (primers 207/211). Expected fragment sizes were 1,800 bp, 4,260 bp, 4,880 bp and 346 bp for lanes 1 to 4, respectively. M, molecular weight marker, with fragment sizes indicated on the left.
and phosphatidic acid (1). Here we show that concerning phosphoinositides, mature erythrocytes synthesize only PI(4,5)P₂ and its precursor PI4P. Accordingly, the published human erythrocyte proteome revealed the presence of PI4-phosphate-5-kinase type III and diacylglycerol kinase γ, though with very low sequence coverage (26).

Upon infection with *P. falciparum*, the phosphoinositide profile becomes complex, and we could show that *Plasmodium*-

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**FIG. 4.** GFP fluorescence in parasites expressing GFP-2×FYVE or GFP-2×FYVE<sup>C215S</sup>. (A) Cells were fixed and analyzed for GFP fluorescence. Nuclei were stained with Hoechst stain (blue stain in the merged images). (B) Colocalization of GFP-2×FYVE and the food vacuole membrane marker CRT using rabbit anti-CRT. Serial images of a Z-stack acquisition (0.38 μm step) are displayed. Arrowheads indicate protrusions from the food vacuole membrane that are colabeled with CRT and GFP-2×FYVE, while arrows indicate zones of intense GFP-2×FYVE staining in the absence of the food vacuole marker. A corresponding phase-contrast image is shown in the lower left corner.

**FIG. 5.** Colocalization of GFP-2×FYVE with the apicoplast. GFP-2×FYVE expressing parasites at the indicated developmental stages were incubated with rabbit anti-ACP as an apicoplast marker. Nuclei were stained with Hoechst stain (Hoe).
infected red blood cells contain PI3P. The amounts detected in late-stage parasites were important, representing about one-third of total PI monophosphates. During the intraerythrocytic development from rings to schizonts, the proportion of PI3P with respect to total phosphoinositides increased around 4-fold, which may indicate that PI3P function is more developed in late-stage parasites. Synthesis of PI3P was sensitive to wortmannin, a well-known inhibitor of PI3-kinase, thereby establishing a link between PI3P production and PI3-kinase in infected red blood cells. We observed somewhat more than

FIG. 6. Localization of GFP-2×FYVE by cryoimmunoelectron microscopy. (A) Section through a young schizont showing most of the gold label on or near the food vacuole membrane (arrows). Arrowheads point to what should be considered the background. Hz, hemozoin crystals; N, parasite nucleus; RBC, erythrocyte cytosol. (B and C) The membranes of electron-lucent (B) (arrow) and electron-dense (C) (asterisks) vesicles neighboring the food vacuole (Hz) are strongly labeled. Cytostomal vesicles (Cy) are not labeled. (D to E) General (D) and close-up (D′ and E) views of young schizonts showing the strong labeling of the apicoplast, mostly within the lumen. The four membranes of the apicoplast are clearly identified (E, arrow). A, apicoplast; P, parasite.
50% inhibition of PI3P levels in the presence of 100 nM wortmannin. Inhibition at this concentration was considered specific, since production of the two main phosphoinositides, PI4P and PI(4,5)P2, was not influenced.

Our analysis also revealed that the parasite did not produce detectable levels of PI(3,5)P2. This lipid is readily detected in yeast and in mammalian cells, where its abundance has been shown to vary under stress conditions (11). PI(3,5)P2 is synthesized through phosphorylation of PI3P by PIKfyve/Fab1 (18). We analyzed the *Plasmodium* genome database Plasmodb 6.3 for genes coding for the enzymes involved in phosphoinositide metabolism (Fig. 7). PI synthase (63) and PI4P-5-kinase (31) have been biochemically validated. We found candidates for the other enzymes except for PIKfyve/Fab1 PI3-phosphate-5-kinase. Given the lack of a Fab1 orthologue and the observed absence of PI(3,5)P2 synthesis, we conclude that this enzyme activity is indeed missing in *Plasmodium*. In other organisms, PI(3,5)P2 is localized mainly on multivesicular bodies (MVBs) and is implicated in the maintenance of endosome and lysosome/vacuole morphology and in endosome-to-Golgi complex and MVB-to-vacuole trafficking (18, 47). In apicomplexan parasites, MVB-like structures have so far been observed only upon disturbance of endocytic pathways by chemical or mutational manipulations (65), suggesting that endosomal trafficking in *Plasmodium* might be different from the classical scheme described for yeast and mammals.

Surprisingly, we also detected very low quantities of PI(3,4)P2 and PI(3,4,5)P3 in infected erythrocytes. These lipids are generally not found in unicellular organisms, where only class III PI3-kinases are present. A recent publication reports that *P. falciparum* PIPI3-kinase immunoprecipitated from infected red blood cell extracts was capable of phosphorylating PI, PI4P, and PI(4,5)P2 in vitro (54) and could explain the presence of PI(3,4)P2 and PI(3,4,5)P3 that we observed in *P. falciparum*-infected erythrocytes. Our results clearly showed that in intact *P. falciparum*-infected erythrocytes, PI3P is by far the most abundant phosphoinositide phosphorylated at position 3 (87.5% ± 6.1% [mean ± SEM; n = 8]). Interestingly, in our experiments, the synthesis of PI(3,4,5)P3 (and of PI(3,4)P2) was not significantly reduced by wortmannin treatment, indicating that PI3P synthesis might be independent of PI3-kinase activity. This excludes the possibility that PI3P synthesis might be due to a residual silent activity of the human enzyme in red blood cells that becomes activated upon parasite infection, since in this case its synthesis should be sensitive to wortmannin, a potent inhibitor of human class I PI3-kinases. To our knowledge, the only other unicellular organism for which PI(3,4,5)P3 production has been described so far is *Schizosaccharomyces pombe* (36). It has been shown that PI(3,4,5)P3 production in *S. pombe* is mediated not by the sole type III PI3-kinase (52), but by a PI4P-5-kinase, which in a sequential reaction phosphorylates PI3P first to PI(3,4)P2 and then to PI(3,4,5)P3 (36). The situation might be similar in *P. falciparum* (Fig. 7), a possibility additionally supported by the fact that the unique parasite PI4P-5-kinase has been shown to phosphorylate PI3P in vitro (31). It remains to be shown whether PI(3,4,5)P3 plays a physiological role in *Plasmodium* and what this function might be.

The design of fluorescent molecular probes that bind phosphoinositides with high specificity has allowed the imaging of phosphoinositide dynamics in living cells (reviewed in reference 57). A tandem repeat of the FYVE domain of the mammalian Hrs protein fused to GFP (20) has been successfully used in various organisms to visualize PI3P (22, 58). Expressing the same construct in *P. falciparum* allowed us to identify three compartments that are enriched in PI3P: the food vacuole membrane, the apicoplast, and single membrane vesicles. The food vacuole is a compartment with “lysosome-like” functions in *Plasmodium*, and association of PI3P with its membrane is in accordance with the PI3P-mediated function in transport to-
ward the lysosome in other organisms. Indeed, it has very recently been shown that treatment of parasite cultures with the PI3-kinase inhibitors wortmannin and LY294002 inhibited endocytosis within the parasite, resulted in entrapment of hemoglobin vesicles in the parasite cytoplasm, and prevented their fusion with the food vacuole, suggesting that PI3-kinase is involved in endocytosis from the host and the trafficking of hemoglobin in the parasite (54). The authors also reported that PI(3)K in the parasite was in part exported to the red blood cell cytosol. This was demonstrated by using antibodies raised against a recombinant His-tagged PI(3)K fragment showing perfect colocalization with the conserved C-terminal end of var gene products in immunofluorescence analysis. Additionally, PI(3)K activity could be immunoprecipitated from the host cell fraction upon selective membrane permeabilization (54). Our construct was not aimed at exporting GFP-2×FYVE from the parasitic cytosol. We therefore could not test this unexpected observation, and we did not attempt to separate parasite membranes from host cell membranes after metabolic labeling of phosphoinositides because of the prohibiting levels of radioactivity necessary for their identification. Finally, PI(3)K has also been immunolocalized within the food vacuole (54). In addition, the only FYVE domain-containing protein of P. falciparum, which has been shown to bind PI3K in vitro, has also been localized to the lumen of the food vacuole; however, this localization was independent of the FYVE domain, and thus independent of PI3P-binding (35). We did not observe GFP-2×FYVE fluorescence within the food vacuole, but again, our probe was designed to be cytosolic, since this is the usual location of PI3-kinase. So far there has been no proposition as to how PI(3)K exported outside the parasite, an effect that was found to be linked to a default in apicoplast biogenesis, leading to loss of this essential organelle, a phenotype termed “delayed death.” At low expression levels, parasites appeared undisturbed, and GFP-2×FYVE was found at the apicoplast membranes and on electron-dense and electron-lucent vesicles in the vicinity of the apicoplast. These vesicles were shown to carry PI3P and proteins destined for the outermost apicoplast membranes, such as FtsH1 and APT1 (27, 28; Tawk et al., submitted).

In Plasmodium we localized PI3K to the apicoplast; however, we did not observe a delayed death phenotype, which may be due to differences in the expression levels of GFP-2×FYVE and/or the abundance of PI3P in these two apicomplexan parasites. Immunogold particles revealing GFP-2×FYVE were detected mainly in the apicoplast lumen. This finding is intriguing, since the GFP-2×FYVE construct does not contain a signal sequence or apicoplast-targeting sequence, generally required for protein import. Its location in the lumen indicates that the protein is not in a complex with the lipid PI3P anymore. While certain aspects of protein import into the apicoplast are well understood (40, 53), little is known about lipid transport toward the organelle and lipid distribution among the four membranes. One hypothesis might be that GFP-2×FYVE reaches the apicoplast intermembrane space as a consequence of its PI3P-binding capacity and might then be trafficked further independently of lipid interaction. The GFP-2×FYVE-positive vesicles in P. falciparum are similar in size and morphology to the Toxoplasma vesicles shown to carry PI3P and apicoplast membrane proteins, and it is tempting to speculate that PI3P-containing vesicles in Plasmodium might be involved in transport processes, likely of proteins and lipids, toward the essential and peculiar parasite compartment, which is the apicoplast.

PI3-kinase appeared to be essential in Plasmodium. P. falciparum in vitro growth was sensitive to the PI3-kinase inhibitor LY294002. In P. berghei the PbPI3K locus could be targeted successfully but could neither be tagged nor deleted, indicating that PbPI3-kinase was essential at the blood stage. Vps34 orthologues are highly conserved at their C-terminal end, containing only very few amino acids after the catalytic domain (see Fig. S1 in the supplemental material). Mutational analysis in S. cerevisiae revealed that the last amino acids of PI3-kinase are essential for kinase activity (6). On the other hand, C-terminal fusion proteins with GFP or the TAP tag have been described in the literature (24). In general, Vps34 membrane association and activation are mediated by Vps15, a serine/threonine kinase containing HEAT and WD40 repeats and
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10. Desjardins, R. E., C. J. Canfield, J. D. Haynes, and J. D. Chulay. PI(3,5)P2, (ii) the low number of PI3P-interacting proteins, identified. Strikingly, this is a low number compared to the 15 PX domain proteins with PX-domains (PF07_0017 and MAL7P1.108) that interact with the Vps15 orthologue in P. falciparum, which might indicate that their role in chloroquine resistance. Mol. Cell. 68:681–871.

11. Doyle, S. K., F. T. Cooke, M. R. Douglas, L. G. Sayers, P. J. Parker, and R. H. Michell. 1997. Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. Nature 390:187–192.

12. Ecker, A., R. Moon, R. E. Sinden, and O. Billker. 2006. Generation of gene targeting constructs for Plasmodium berghei by a PCR-based method amenable to high throughput applications. Mol. Biochem. Parasitol. 145:262–268.

13. Elabadi, N., M. L. Ancelin, and H. J. Vial. 1994. Characterization of phosphatidylinositol synthase and evidence of a orthologous gene in Plasmodium-infected erythrocytes. Mol. Biochem. Parasitol. 63:179–192.

14. Elliott, D. A., M. T. McIntosh, H. D. Hosgood III, S. Chen, G. Zhang, P. Baeva, and K. A. Joiner. 2008. Four distinct pathways of hemoglobin uptake in the malaria parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. U. S. A. 105:2463–2468.

15. Fido, D. A., T. Nomura, A. K. Talley, R. A. Cooper, S. M. Drekenov, M. T. Ferdig, L. M. Ursos, A. B. Sidhu, B. Naude, K. W. Deitsch, S. X. Su, J. C. Wootten, P. D. Roose, and T. E. Wellemans. 2000. Mutations in the P. falciparum digestive vacuole transmembrane protein PICRT and evidence for their role in chloroquine resistance. Mol. Cell. 68:681–871.

16. Foster, F. M., C. J. Traer, S. M. Abraham, and M. J. Fry. 2003. The phophoinositide (PI) 3-kinase family. J. Cell Sci. 116:3037–3040.

17. Francis, S. E., D. J. Sullivan, Jr., and D. E. Goldberg. 1997. Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. Annu. Rev. Microbiol. 51:97–123.

18. Gary, J. D., A. E. Wurmsner, C. J. Bonangelino, L. S. Weisman, and S. D. Emmr. 1998. Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuole size and membrane homeostasis. J. Cell Biol. 143:65–79.

19. Gauthier, J. M., A. Simonsen, A. D’Arrigo, B. Bremnes, H. Stamp, and R. Aaskael. 1998. FYVE fingers bind PtdIns3P. Nature 394:432–433.

20. Gillibrand, D. L., I. C., S. M. Burda, P. W. Denny, L. G. Tilney, J. M. Gaultier, R. G. Parton, and H. Stenmark. 2000. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. EMBO J. 19:4577–4587.

21. Goldberg, D. E., A. F. Slater, A. Cerami, and G. B. Henderson. 1990. Hemoglobin degradation in the malaria parasite Plasmodium falciparum: an ordered process in a unique organelle. Proc. Natl. Acad. Sci. U. S. A. 87:2931–2935.

22. Hall, B. S., C. Gobernet-Castello, A. Vosk, D. Goulding, S. K. Natesan, and M. C. Field. 2006. TbVps34, the trypanosome orthologue of Vps34, is required for Golgi complex segregation. J. Biol. Chem. 281:27660–27672.

23. Herman, P. K., and S. D. Emmr. 1999. Characterization of Vps34, a gene required for vacuolar protein sorting and vacuole segregation in Saccharomyces cerevisiae. Mol. Cell. Biol. 19:6742–6754.

24. Hul, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson, J. S. Weissman, and E. K. O’Shea. 2003. Global analysis of protein localization in the budding yeast. Nature 426:696–691.

25. Jackson, K. E., N. Klonis, D. J. Ferguson, A. Adisa, C. Dogovski, and L. Tilley. 2004. Food vacuole-associated lipid bodies and heterogeneous lipid environments in the malaria parasite, Plasmodium falciparum. Mol. Microbiol. 54:109–122.

26. Kakkasbivilli, D. G., L. A. Bulla, Jr., and S. R. Goodman. 2004. The human erythrocyte membrane: analysis by ion trap mass spectrometry. Mol. Cell. Proteomics 3:501–509.

27. Karnatak, A., A. Derocher, I. Coppens, C. Nash, J. E. Feagin, and M. Parsons. 2007. Cell cycle-regulated vesicular trafficking of Toxoplasma APT1, a protein localized to multiple apicomplexan membranes. Mol. Microbiol. 63:1653–1668.

28. Karnatak, A., A. E. Derocher, I. C. Coppens, J. E. Feagin, and M. Parsons. 2007. A membrane protease is targeted to the retic plastid of Toxoplasma via an internal signal sequence. Traffic 8:1543–1553.

29. Kohler, S., C. F. Debiche, P. W. Denny, L. G. Tilney, P. Webster, R. J. Wilson, J. D. Palmer, and D. S. Roos. 1997. A plastid of probable green algal origin in Apicomplexan parasites. Science 275:1483–1489.

30. Lambros, C., and J. F. Vanderberg. 1979. Synchronization of Plasmodium falciparum erythrocystic stages in culture. J. Parasitol. 65:418–420.

31. Leber, W., A. Skippen, Q. L. Fivelman, P. B. Bowyer, S. Cocker, and D. A. Baker. 2009. A unique phosphatidylinositol 4-phosphate 5-kinase is activated by ADP-ribosylation factor in Plasmodium falciparum. Nat. Cell. Biol. 10:645–653.

32. Liellier, J., A. Berry, and F. Benoit-Vical. 2005. An alternative method for Plasmodium culture synchronization. Exp. Parasitol. 109:195–197.

33. Levine, T. P., and S. Munro. 2002. Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components. Curr. Biol. 12:695–704.

34. Martin, B. R., B. N. Creamer, S. Adams, and R. Y. Tsien. 2005. Mammalian cell-based optimization of the biarsenical-binding tetracycline moiety for improved fluorescence and affinity. Nat. Biotechnol. 23:1308–1314.

35. McIntosh, M. T., A. A. A. Donaldson, S. Wilson, and P. Sharma. 2000. A plastid of probable green algal origin in Toxoplasma gondii. Mol. Biochem. Parasitol. 109:130–138.

36. Mitra, P., Y. Zhang, L. E. Rameh, M. P. Ivesha, D. McCollum, J. J.
Nunnari, G. M. Hendricks, M. L. Kerr, S. J. Field, L. C. Cantley, and A. H. Ross. 2004. A novel phosphatidylinositol(3,4,5)P3 pathway in fission yeast. J. Cell Biol. 166:205–211.

Nukrach, L. J., R. A. Muhle, P. A. Moura, P. Gosh, G. F. Hatfull, W. R. Jacobs, Jr., and D. A. Fidock. 2006. Efficient site-specific integration in Plasmodium falciparum chromosomes mediated by mycobacteriophage Bxb1 integrase. Nat. Methods 3:615–621.

Odorizzi, G., M. Babst, and S. D. Emr. 1998. Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell 95:847–858.

Odorizzi, G., M. Babst, and S. D. Emr. 2000. Phosphoinositide signaling and the regulation of membrane trafficking in yeast. Trends Biochem. Sci. 25:229–235.

Parsons, M., A. Karnatak, and A. E. Derocher. 2009. Evolving insights into protein trafficking to the multiple compartments of the apicomplexan plastid. J. Eukaryot. Microbiol. 56:214–220.

Payrastre, B. 2004. Phosphoinositides: lipid kinases and phosphatases. Methods Mol. Biol. 273:201–212.

Petiot, A., E. Oger-Denis, E. F. Blommaart, A. J. Meijer, and P. Codogno. 2000. Distinct classes of phosphatidylinositol 3-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J. Biol. Chem. 275:992–998.

Pirola, L., M. J. Zvelebil, G. Bulgarelli-Leva, E. Van Obberghen, M. D. Waterfield, and M. P. Wymann. 2001. Activation loop sequences confer substrate specificity to phosphatidylinositol 3-kinase alpha (PI3Kalpha). Functions of lipid kinase-deficient PI3Kalpha in signaling. J. Biol. Chem. 276:21544–21554.

Quevillon, E., T. Spielmann, K. Brahimi, D. Chattopadhyay, E. Yeramian, and G. Langsley. 2003. The Plasmodium falciparum family of Rab GTPases. Trends Biochem. Sci. 28:535–602.

Vannæsbroeck, B., S. J. Leeners, K. Ahmadi, J. Timms, R. Katso, P. C. Driscoll, R. Woschodski, P. J. Parker, and M. D. Waterfield. 2001. Synthesis and function of 3-phosphorylated inositol lipids. Annu. Rev. Biochem. 70:13–25.

Waller, R. F., and G. I. McFadden. 2007. Protein targeting to the malaria parasite plasma membrane. Traffic 8:166–175.

Vaid, A., R. Ranjan, W. A. Smyth, H. C. Hoppe, and P. Sharma. 2010. PIPEK1, a phosphatidylinositol-3 kinase from Plasmodium falciparum, is exported to the host erythrocyte and is involved in hemoglobin trafficking. Blood 115:2580–2587.

van Dijk, M. R., A. P. Waters, and C. J. Janse. 1995. Stable transfection of malaria parasite blood stages. Science 268:1358–1362.

Vanhæsbroeck, B., S. J. Leeners, K. Ahmadi, J. Timms, R. Katso, P. C. Driscoll, R. Woschodski, P. J. Parker, and M. D. Waterfield. 2001. Synthesis and function of 3-phosphorylated inositol lipids. Annu. Rev. Biochem. 70:13–25.

Watt, S. A., G. Kumar, I. N. Fleming, C. P. Downes, and J. M. Lucocq. 2002. Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C delta1. Biochem. J. 363:657–666.

Wengelnik, K., and H. J. Vial. 2007. Characterisation of the phosphatidylinositol synthase gene of Plasmodium species. Res. Microbiol. 158:51–59.

Wymann, M. P., G. Bulgarelli-Leva, M. J. Zvelebil, L. Pirola, B. Vanhæsbroeck, M. D. Waterfield, and G. Panayotou. 1996. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol. Cell. Biol. 16:1722–1733.

Yam, M., I. Coppens, S. Wormsley, P. Baevova, H. C. Hoppe, and K. A. Joiner. 2004. The Plasmodium falciparum Vps4 homolog mediates multivesicular body formation. J. Cell Sci. 117:3831–3838.

Yuan, H., K. R. Barnes, R. Weisleder, L. Cantley, and L. Josephson. 2007. Covalent reactions of wortmannin under physiological conditions. Chem. Biol. 14:321–328.