# Calcium and a Calcium-Dependent Protein Kinase Regulate Gamete Formation and Mosquito Transmission in a Malaria Parasite

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## Summary

Transmission of malaria parasites to mosquitoes is initiated by the obligatory sexual reproduction of the parasite within the mosquito bloodmeal. Differentiation of specialized transmission stages, the gametocytes, into male and female gametes is induced by a small mosquito molecule, xanthurenic acid (XA). Using a *Plasmodium berghei* strain expressing a bioluminescent calcium sensor, we show that XA triggers a rapid rise in cytosolic calcium specifically in gametocytes that is essential for their differentiation into gametes. A member of a family of plant-like calcium dependent protein kinases, CDPK4, is identified as the molecular switch that translates the XA-induced calcium signal into a cellular response by regulating cell cycle progression in the male gametocyte. CDPK4 is shown to be essential for the sexual reproduction and mosquito transmission of *P. berghei*. This study reveals an unexpected function for a plant-like signaling pathway in cell cycle regulation and life cycle progression of a malaria parasite.

## Introduction

The *Plasmodium* genome (Gardner et al., 2002) encodes a full complement of signaling molecules, allowing the parasite to interact with host cells and to adjust its complex life cycle to the changing environments of vertebrate and mosquito hosts. A notable expansion of proteins containing $Ca^{2+}$ binding EF hand modules in *Plasmodium* relative to yeast (Aravind et al., 2003) reflects the importance of $Ca^{2+}$ as a secondary messenger in apicomplexan parasite-host interactions. In *Toxoplasma gondii*, $Ca^{2+}$ release from intracellular stores governs tachyzoite egress, microneme secretion, motility, and host cell invasion (Carruthers and Sibley, 1999; Lovett and Sibley, 2003; Kieschnick et al., 2001; Moudy et al., 2001) and increasing evidence suggests that simi-

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lar mechanisms operate in invasive stages of *Plasmodium* (Gantt et al., 2000; O.B., unpublished data). In response to host melatonin $Ca^{2+}$ signaling synchronizes *Plasmodium* proliferation in the blood (Hotta et al., 2000) and inhibitor studies show a role for intracellular $Ca^{2+}$ in regulating gamete formation in response to conditions encountered in the mosquito midgut (Kawamoto et al., 1990, 1993).

When considering $Ca^{2+}$ binding effector molecules, it is interesting to note that *Plasmodium*—like plants—lack orthologs of protein kinase C, but has an expanded family of $Ca^{2+}$ dependent protein kinases (CDPKs). CDPKs combine an amino-terminal serine/threonine kinase domain and a carboxy-terminal calmodulin-like domain, composed of four EF hands, in the same molecule. Current evidence suggests that CDPKs may be limited to plants, green algae, ciliates, and apicomplexan parasites. In plants, CDPKs translate $Ca^{2+}$ signals generated by external stimuli into cellular responses, thereby regulating cell division and differentiation, the development of tolerance to stress stimuli and the specific defense responses to pathogens (Harmon et al., 2000; Romeis et al., 2001; Ludwig et al., 2003). The genome of *P. falciparum* encodes a family of six putative CDPKs and one CDPK-related kinase, in which the calmodulin domain is missing (O.B., unpublished data). Three CDPKs of unknown function have been characterized in *P. falciparum* (Zhao et al., 1993; Farber et al., 1997; Li et al., 2000). Their developmental regulation makes these kinases prime candidates for the molecular switches that translate ubiquitous $Ca^{2+}$ signals into appropriate cellular responses at specific stages of the parasite's life cycle.

To explore this hypothesis, we studied $Ca^{2+}$ signaling during gametogenesis in *P. berghei*, a malaria parasite of rodents. Gametogenesis is the process, by which specialized transmission stages circulating in the blood stream, the gametocytes, differentiate rapidly into male and female gametes upon internalization by a susceptible mosquito. Fertilization follows within 15 to 20 min. Only the zygote can then differentiate into a motile, invasive ookinete, which crosses the midgut epithelium and establishes the infection on the haemocoel side of the midgut wall, where it transforms into an oocyst. While circulating in the vertebrate bloodstream, gametocytes are developmentally arrested for many days, yet they respond within seconds to being incorporated into the mosquito blood meal. Both male (micro-) and female (macro-) gametocytes lyse the host erythrocytes in which they reside and round up. Once emerged, females are available for fertilization, whereas microgametocytes enter the cell cycle, replicate their genomes three times within about 8 min and undergo three endomitotic divisions. At the same time, eight axonemes are assembled, each of which becomes the motile backbone of a flagella-like microgamete. In a spectacular process termed exflagellation, microgametes are finally expelled from the residual body of the male gametocyte.

At a permissive temperature of at least 5°C below that of the vertebrate host, gametogenesis is triggered by
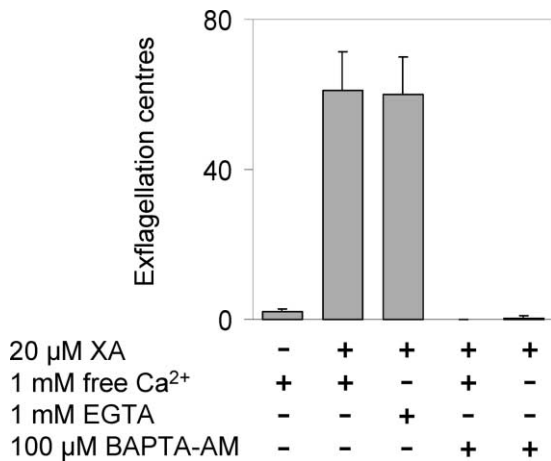
Figure 1. XA-Induced Exflagellation Requires Cellular $Ca^{2+}$

Gametocytes from tail blood of an infected mouse were washed free of serum and preincubated for 10 min with or without 100 μM BAPTA-AM before being stimulated under the conditions shown. After 15 min, exflagellation centers were counted in ten microscopic fields. Mean values and standard deviations of three samples from the same infected mouse are shown.

xanthurenic acid (XA), a small mosquito molecule (Billker et al., 1998). In vitro XA can be replaced by a pH shift from 7.4 to 8.0. The signaling pathways by which these diverse triggers control gametogenesis have not been identified but inhibitor studies show likely roles for protein kinases and different secondary messengers including inositol 1,4,5-trisphosphate ($IP_3$), $Ca^{2+}$, and cyclic nucleotides (Kawamoto et al., 1990; 1993; Martin et al., 1994).

We here show an unexpected role for a plant-like CDPK-dependent signaling pathway in cell cycle regulation and life stage progression in the malaria parasite. We identify and characterize a novel calcium dependent protein kinase, CDPK4, which is transcriptionally upregulated in the male gametocyte. We demonstrate that CDPK4 functions downstream of an XA-induced calcium signal as an essential regulator of cell cycle progression in the male gametocyte and that it is essential for parasite transmission to the mosquito.

**Results**

**Xanthurenic Acid-Induced Exflagellation Requires Cellular $Ca^{2+}$**

In initial experiments, we explored the role of cellular $Ca^{2+}$ in exflagellation using an in vitro assay, in which exflagellation events in *P. berghei*-infected mouse blood were scored microscopically 15 min after addition of 20 μM XA. Medium containing either 1 mM free $Ca^{2+}$ or 1 mM EGTA, a membrane-impermeable $Ca^{2+}$ chelator, supported exflagellation equally well (Figure 1). In contrast, preloading gametocytes with 100 μM of a membrane-permeable $Ca^{2+}$ chelator, BAPTA-AM, blocked exflagellation almost completely. These data suggest that the ability of XA to trigger exflagellation does not require extracellular $Ca^{2+}$, but depends critically on $Ca^{2+}$ stores within the gametocyte or the infected host cell.

**Generation of Transgenic *P. berghei* Strains Expressing a GFP-Aequorin $Ca^{2+}$ Sensor**

The $Ca^{2+}$-dependent bioluminescence of the aequorin protein offers a highly sensitive method to record cytosolic $Ca^{2+}$ peaks from small numbers of cells. Sensitivity can be increased further by exploiting the fluorescence energy transfer that occurs when aequorin is fused to GFP (Baubet et al., 2000). To measure $Ca^{2+}$ in the parasite's cytosol, we generated transgenic reporter strains, in which an expression cassette for a chimeric GFP-aequorin gene was stably integrated into one of two ribosomal loci (Figure 2A) shown previously to have redundant functions in parasite development (van Spaendonk et al., 2001). Two $Ca^{2+}$ reporter strains were generated, one in the gametocyte-producing *P. berghei* strain 2.34 (resulting in clone 1.7.6), and one in the gametocyte nonproducer, strain 2.33 (giving rise to clone 5.2). Genomic integration of the insertion construct was verified by diagnostic PCR analysis (Figure 2B). Clone 1.7.6 produced normal numbers of gametocytes that could be enriched to up to 99% purity (Figure 2C). Live fluorescence microscopy showed expression of GFP-aequorin evenly throughout the cytoplasm of the gametocytes (Figure 2D), which emerged and exflagellated normally. Sex-specific differences in fluorescence intensities, possibly due to differential activity of the *ef1α-a* promoter, enabled us to sort gametocytes into highly enriched male and female populations by FACS (Figure 2F and Franke-Fayard et al., in press). Mixed asexual blood stage parasites of clone 5.2 showed intermediate expression levels of the $Ca^{2+}$ sensor protein (Figure 2E, right panel) when compared to the two gametocyte populations of strain 1.7.6. We also examined asexual blood stages, ookinetes, oocysts, and salivary gland sporozoites of clone 1.7.6 and found GFP-aequorin expressed in all these stages (data not shown).

To test whether the parasite-expressed GFP-aequorin protein was a functional $Ca^{2+}$ sensor, we treated enriched gametocytes and asexual blood stages with pharmacological agents known to raise cytosolic $Ca^{2+}$ levels in *Plasmodium* by different mechanisms (Alleva and Kirk, 2001). A23187, a $Ca^{2+}$ ionophore, and cyclopiazonic acid, an inhibitor of the endoplasmic reticulum $Ca^{2+}$ ATPase, elicited dose-dependent luminescence responses that were comparable in gametocytes (Figure 3A) and asexual parasite stages (Figure 3B) showing that both parasite stages were capable, in principle, of reporting increases in cytosolic $Ca^{2+}$.

**XA Induces a $Ca^{2+}$ Peak Specifically in the Gametocyte**

Stimulation of gametocytes by biologically active concentrations of XA resulted in a 10 s lag phase, followed by a rapid and transient increase in luminescence, which peaked 12-20 s after the beginning of stimulation (Figure 3C). The XA-induced luminescence response in gametocytes was concentration dependent and saturable with respect to XA. In marked contrast, $Ca^{2+}$ levels in mixed asexual parasite stages did not respond to XA over a wide range of concentrations (Figure 3D). Like exflagellation, XA-induced aequorin luminescence in the gametocyte did not require extracellular free $Ca^{2+}$ but was blocked completely by chelation of intracellular $Ca^{2+}$ by BAPTA-AM (Figure 3E). We determined the concentra-
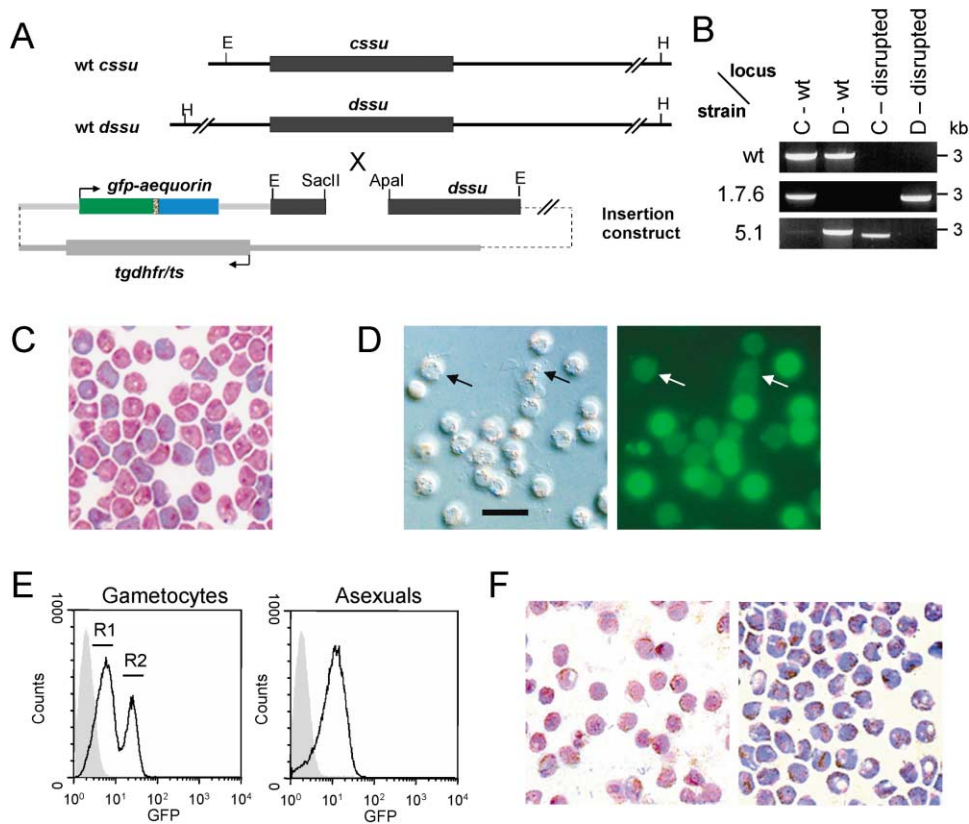
Figure 2. Generation of Transgenic *P. berghei* Strains Expressing a GFP-Aequorin Fusion Protein

(A) Schematic diagram showing the GFP-aequorin insertion construct and the genomic organization of c-type and the d-type small subunit rRNA genes (*cssu* and *dssu*) that are the two potential target loci for genomic integration by a single crossover event.

(B) Diagnostic PCR on genomic DNA with primer pairs specific for the intact and disrupted target loci showing integration of the GFP-aequorin insertion construct, resulting in disruption of the *dssu* target locus in clone 1.7.6 and of the *cssu* locus in clone 5.1.

(C) Gametocytes of clone 1.7.6 can be enriched to >98% purity. A Giemsa-stained smear of enriched gametocytes is shown.

(D) The GFP-aequorin chimeric protein is expressed in enriched gametocytes of clone 1.7.6. A differential interference contrast image (left panel) of live enriched gametocytes 12 min after activation by 20 μM XA is shown next to a GFP fluorescence image (right panel). Arrows point to male gametocytes in the process of exflagellation. Scale bar is equal to 10 μM.

(E) Flow cytometry analysis of GFP-aequorin expression shows two gametocyte populations in clone 1.7.6 (left panel, black line) and homogeneous expression at an intermediate level in enriched trophozoites and schizonts of clone 5.1 (right panel, black line). The gray areas in both panels show background fluorescence of the untransfected recipient strains, 2.34 and 2.33, respectively. R1 and R2, gates used for FACS sorting.

(F) GFP-aequorin is differentially expressed in male and female gametocytes. Gametocytes analyzed in (E) were sorted by flow cytometry into a weakly expressing population (gate R1) of 96% males (left panel), with the remainder being mostly asexual stages and uninfected erythrocytes, and a highly expressing population (gate R2) consisting of >99% females (right panel). Giemsa-stained smears are shown.

tions of XA and of two structurally related compounds, quinaldic acid (QA) and kynurenic acid (KA), that were required to generate a half-maximal $Ca^{2+}$ signal (Figure 3F). $EC_{50}$ values of 3 μM (XA), 110 μM (QA), and 250 μM (KA) were in good agreement with the specific biological activities of these compounds as triggers of exflagellation in *P. berghei* of 9 μM (XA), 80 μM (QA), and 180 μM (KA) (Billker et al., 1998). Extended luminescence recordings over 15 min revealed no further $Ca^{2+}$ peaks during gametocyte differentiation (not shown). These data show that XA activates a signaling pathway specifically in the gametocyte, resulting in a rapid increase in cytosolic free $Ca^{2+}$, which is essential for gametogenesis.

**A Novel $Ca^{2+}$-Dependent Protein Kinase (CDPK4) is a Putative $Ca^{2+}$ Effector in the Gametocyte**
We next asked which $Ca^{2+}$ effector protein regulates gametogenesis. Previous inhibitor studies found exflag-

ellation to be sensitive to the calmodulin inhibitor W-7 (Kawamoto et al., 1990). This drug also inhibits the type of calmodulin domain-containing kinases of the CDPK family that has since been discovered in *Plasmodium* (Farmer and Choi, 1999). A novel member of the CDPK family, PF07_0072, named here CDPK4, emerged from recent proteome studies as an abundant protein kinase that was upregulated in sexual over asexual blood stages in *P. falciparum* (Carlton et al., 2002; Lasonder et al., 2002). The *P. berghei* genome database (ftp://ftp.sanger.ac.uk/pub/pathogens/P_berghei) contains a putative *cdpk4* ortholog, which shares 91% amino acid identity (97% simility) with PfCDPK4 and 73% identity (88% similarity) with *T. gondii* CDPK1 (Kieschnick et al., 2001) (see Supplemental Figure S1 for alignment available at http://www.cell.com/cgi/content/full/117/4/503/DC1). High through-put proteome analyses in the rodent species *P. berghei* and *P. yoelii* suggest CDPK4 is abundantly expressed in both the gametocyte and
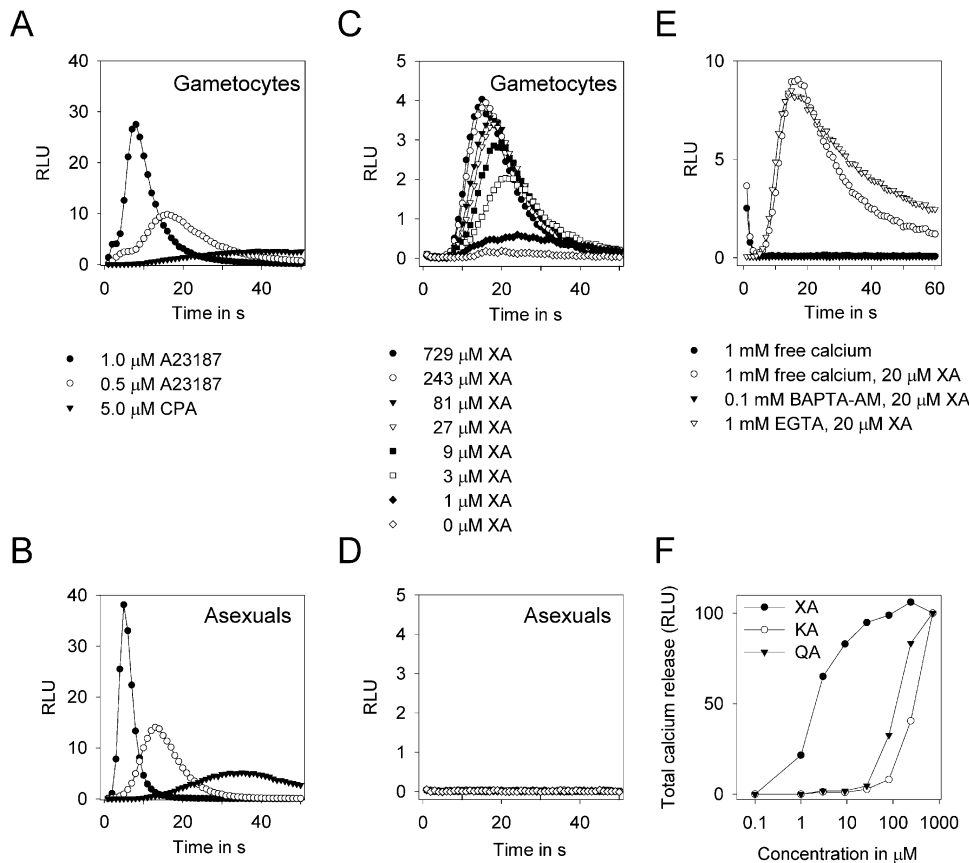
Figure 3. Ca$^{2+}$ Reporter Strains Reveal a Gametocyte-Specific Response to XA

(A) Luminescence responses of purified gametocytes of clone 1.7.6 to the Ca$^{2+}$ ionophore A23187 and the SERCA inhibitor cyclopiazonic acid (CPA) RLU = relative light units.

(B) Luminescence responses of enriched trophozoites of clone 5.1 to the same conditions as in (A). For legend see (A).

(C) XA mobilizes cytosolic Ca$^{2+}$ in purified gametocytes in a dose-dependent manner.

(D) Enriched trophozoites do not respond to the same range of concentrations of XA. For legend to (D), see (C).

(E) The XA-induced increase of cytosolic Ca$^{2+}$ in purified gametocytes is inhibited by BAPTA-AM but does not require free extracellular Ca$^{2+}$. BAPTA-AM treatment involved a preloading period of 10 min in the absence of serum. Control samples were incubated under the same conditions but without BAPTA-AM.

(F) Concentration-dependent light response of purified gametocytes to XA and structurally related compounds, kynurenic acid (KA) and quinaldic acid (QA). A measure for the total Ca$^{2+}$ release was obtained by integrating the light emitted over the first minute of stimulation.

ookinete stages, and that it is also detectable in asexual parasite stages, although at much lower levels (D. Raine, personal communication). Using gene-specific primers, we cloned the *P. berghei cdpk4* coding sequence from gametocyte cDNA and confirmed the predicted two-exon gene model by sequencing. Like other members of the family, the predicted CDPK4 protein consists of a short, variable amino-terminus with putative myristoy-lation site, followed by a conserved serine/threonine kinase domain, a short linker sequence, and a carboxy-terminal calmodulin-like domain composed of 4 typical EF-hands (Figure 4A and Supplemental Figure S1 available on *Cell* website).

**Generation of CDPK4 Knockout Parasites**

To study the function of CDPK4, a vector was designed to replace the entire protein coding sequence of the *cdpk4* gene of *P. berghei* with the DHFR/TS resistance marker from *T. gondii* through homologous recombina-tion in the 5′ and 3′ flanking regions of *cdpk4* (Figure

4B). The gene replacement was verified by diagnostic PCR and Southern blot analysis (Figures 4C and 4D). To investigate expression of the CDPK4 protein, we used a polyclonal rabbit antiserum raised against TgCDPK1, a putative CDPK4 ortholog from *T. gondii* (Kieschnick et al., 2001). This antiserum recognized a single protein band in wt *P. berghei* gametocytes with a mobility of 55 kDa, consistent with the 60 kDa predicted for CDPK4. The CDPK4 protein was expressed predominantly in male gametocytes with some expression occurring also in the female. Equivalent numbers of mixed asexual blood stages of the gametocyte nonproducing strain 2.33 were negative for CDPK4 expression, as were ga-metocytes of the ΔCDPK4 clone (Figure 4E).

**CDPK4 Controls S Phase Entry
in the Male Gametocyte**

Wt and ΔCDPK4 parasites were initially characterized in mice infected by inoculation of infected blood. Asexual replication and gametocyte formation was comparable
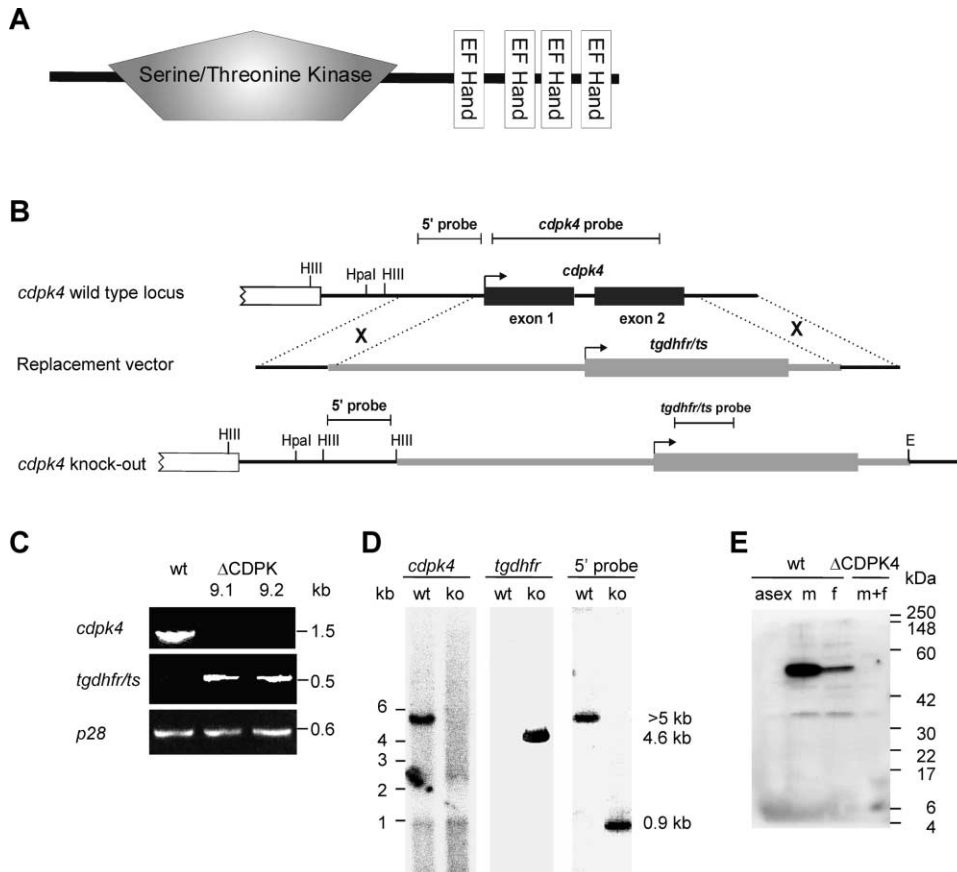
Figure 4. Generation of a *cdpk4* Gene Knockout Parasite, the Analysis of Its Genotype and of CDPK4 Protein Expression

(A) Domain model of CDPK4, showing the predicted amino-terminal kinase domain and the carboxy-terminal calmodulin-like domain composed of four $Ca^{2+}$ binding EF hands.

(B) Schematic drawing illustrating the gene replacement strategy by double homologous recombination, the probes used for DNA hybridization and restriction sites. E = EcoRI, HIII = HindIII.

(C) Diagnostic PCR on genomic DNA showing absence of the *cdpk4* gene and presence of the *tgdhfr/ts* selection marker in two *cdpk4* knockout clones. Control reactions were performed with primers specific for the *p28* gene.

(D) Southern blot analysis of EcoRI/HindIII-digested genomic DNA from wt and *cdpk4* knockout clone 9.1. A *cdpk4* probe recognizes a 5kb fragment only in wt. A *tgdhfr/ts* probe recognizes the expected 4.6 kb HindIII-EcoRI fragment only in the knockout clone. A probe against the 5′ flanking region of *cdpk4* provides evidence for successful gene replacement by recognizing the same fragment as the *cdpk4* probe in wt, while hybridizing to a 0.9 kb HindIII-HindIII fragment indicative of correct integration of the replacement construct in the *cdpk4* knockout clone.

(E) Western blot analysis of CDPK4 protein expression in purified asexual parasite stages (strain 2.33), male (m) and female (f) gametocytes separated by FACS sorting of clone 1.7.6, and mixed (m + f) gametocytes of both sexes of ΔCDPK4 clone 9.1. Lysates from $2 \times 10^6$ parasites were separated in each lane.

in wt and ΔCDPK4 parasites over a nine day infection (Figure 5A). Blood samples were examined daily for the ability of microgametocytes to exflagellate in response to stimulation either by 100 μM XA or by a pH shift to pH 8.0. In blood containing wt gametocytes, exflagellation was abundantly observed under both conditions from day 5 of the infection. In marked contrast, no exflagellation was seen in ΔCDPK4 gametocytes (Figure 5B). Light microscopy indicated that ΔCDPK4 gametocytes were, however, capable of rounding up and lysing their host cell upon activation. A rabbit antiserum that labeled specifically the erythrocyte surface helped us to distinguish between intracellular and emerged gametocytes (Figure 5C). Using this assay, we observed similar levels of emergence in wt and ΔCDPK4 gametocytes upon stimulation by XA (Figure 5D). Transmission

and scanning electron microscopy confirmed that the ability to round up and lyse the host cell remained intact in ΔCDPK4 gametocytes of both sexes (data not shown).

We then examined the mitotic spindles and axonemes that form specifically in the activated microgametocyte. Using a monoclonal antibody against α tubulin, a marked reduction of axoneme-like structures was detected in activated ΔCDPK4 microgametocytes as compared to wt (Figure 5D) and typical mitotic spindles were absent. Microscopic analysis of propidium iodine-stained gametocytes suggested that DNA synthesis in response to activation was blocked in the ΔCDPK4 microgametocyte (Figure 5D). Previous studies using Feulgen staining showed that nonactivated gametocytes contain some DNA in excess of the haploid value, which in the male increases about 6-fold, from 1.5–2N to 8N, upon activa-
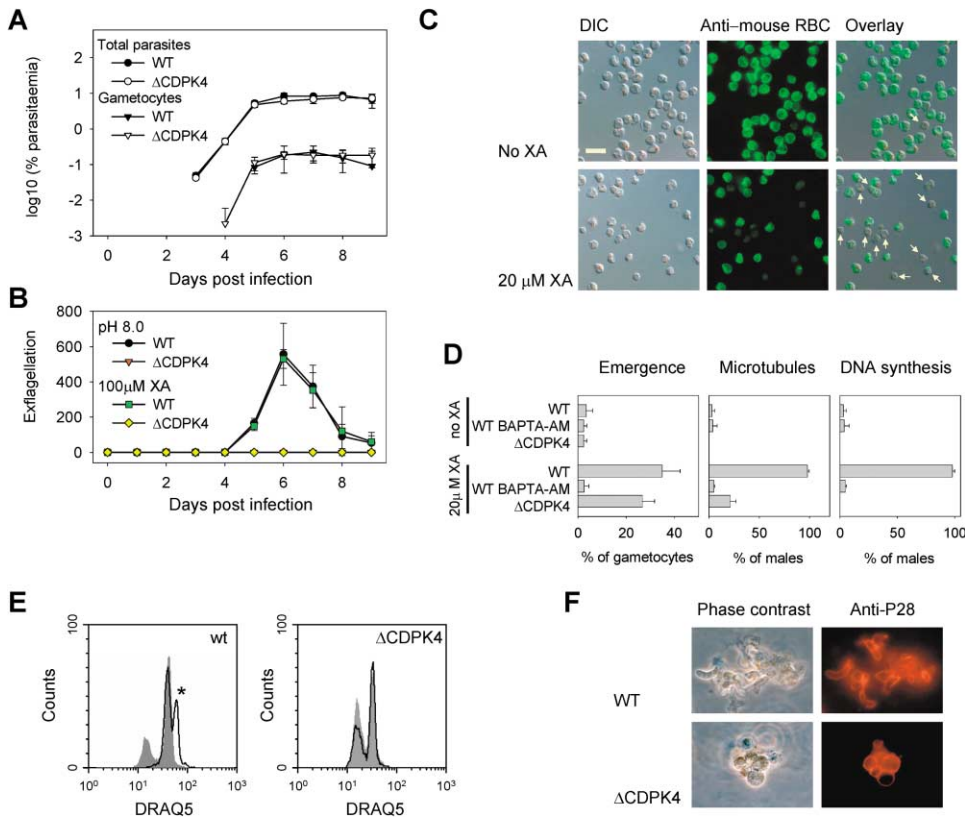
**Figure 5. Phenotypic Analysis of the CDPK4 Knockout Parasite**

(A) Wt and ΔCDPK4 asexual blood stages develop normally in blood-induced infections and produce comparable numbers of gametocytes. Three mice per strain were infected intraperitoneally with 10⁴ parasites and the percentage of infected erythrocytes was monitored daily on Giemsa-stained blood films. Average infection levels and standard deviations are given. Mice were sacrificed after nine days.

(B) ΔCDPK4 gametocytes fail to exflagellate in response to pH 8.0 or 100 μM XA. Blood samples from the same infected mice as in panel (A) were examined daily for the ability of microgametocytes to exflagellate in vitro. Data are given as the mean number of exflagellation centers in 100 microscopic fields. Error bars show standard deviations.

(C) Immunofluorescence labeling of the red blood cell (RBC) surface shows emergence of ΔCDPK4 gametocytes upon stimulation by 20 μM XA. Gametocytes were fixed in 3.7% paraformaldehyde either before or 8 min after stimulation, before microgametocytes began to exflagellate, and stained with an antiserum directed against mouse RBCs, followed by an Alexa488-conjugated secondary antibody. Arrows point to emerged gametocytes. Scale bar indicates 10 μm. DIC, differential interference contrast.

(D) The role of cellular $Ca^{2+}$ and the impact of the CDPK4 knockout were quantified in microscopic assays for gametocyte emergence (as illustrated in C), the formation of axonemes and mitotic spindles and signs of genome replication. To establish the role of cellular $Ca^{2+}$, wt gametocytes were loaded with 100 μM BAPTA-AM for 10 min prior to stimulation. For each experimental condition 100 gametocytes were analyzed. Results are given as means and standard deviations of three experiments with gametocytes from different infected mice.

(E) DNA content of live, purified wt and ΔCDPK4 gametocytes was quantified by flow cytometry using the DNA-specific dye DRAQ5. The gray areas show DNA content of nonstimulated gametocytes, black lines show gametocytes ca. 10 min after stimulation by 20 μM XA. The DNA content of female gametocytes is represented by the highest peak in each panel (reflecting the female-biased sex ratio in *Plasmodium*) and remains unchanged upon stimulation. An asterisk marks the population of replicated males.

(F) Wt and ΔCDPK4 parasites express P28 but only wt macrogametes differentiate into ookinetes. Gametocytes in infected blood were activated and cultured for 24 hr in vitro, when surface expression of P28 was assessed in live cells by direct immunofluorescence staining with the Cy3-labeled monoclonal antibody 13.1.

tion (Janse et al., 1986). To quantify changes in nuclear DNA content, we stained live enriched gametocytes with a membrane permeable DNA dye, DRAQ5, and determined their DNA content by FACS analysis. DRAQ5 staining of nonactivated gametocytes distinguished a weakly and a more strongly stained population of gametocytes (Figure 5E, left panel, gray area), which we identified as male and female, respectively, by exploiting the sex-specific levels of GFP fluorescence of strain 1.7.6 (Figure 2E and data not shown). Wt microgametocytes increased in DNA content by a factor of 5.5 on average within 8 min of activation, whereas the DRAQ5

fluorescence of females remained unchanged (Figure 5E, left panel, black line, the asterisk indicates replicated males). In contrast, no increase in DNA content upon stimulation by XA was observed in ΔCDPK4 gametocytes (Figure 5E, right panel). The relatively strong labeling of macrogametocytes with DRAQ5 was unexpected in view of the previous study by Janse et al. (1986). It was not confirmed using a better characterized DNA dye, Hoechst 33342, and may thus originate from binding of DRAQ5 to RNA that accumulates specifically in the female.

Taken together, these data suggest that CDPK4 is a

specific regulator of cell-cycle progression in the activated gametocyte but that it is not essential for other constituent events of gametogenesis. Host cell lysis, microtubule polymerization, and cell cycle reentry are all strictly $Ca^{2+}$-dependent events that are sensitive to inhibition by BAPTA-AM (Figure 5D). CDPK4 therefore only mediates a subset of the downstream events of the initial $Ca^{2+}$ signal in the gametocyte.

### CDPK4 Is Not Required for Ookinete Formation but Has a Function in Ookinete Infectivity

To further assess the impact of the CDPK4 knockout on female development, we first examined the ability of $\Delta$CDPK4 macrogametes to express the P28 protein, previously known as Pbs21 (Winger et al., 1988). P28 and P25 are two major transmission-blocking vaccine candidates expressed on the surface of the macrogamete, zygote, and ookinete, which are encoded by a tandem array of two paralogous genes. *p28* mRNA accumulates in the macrogametocyte, but translation is strictly suppressed until gametogenesis is induced (Thompson and Sinden, 1994). Wt and $\Delta$CDPK4 parasites both gave rise to cells strongly expressing P28 when cultured for 24 hr following activation by XA (Figure 5F). In the wt strain, most P28-positive parasites had differentiated into ookinetes. In the $\Delta$CDPK4 parasites, ookinetes were absent and P28 was instead expressed on the surface of the emerged macrogametes, which in the absence of male exflagellation remained unfertilized (Figure 5F). CDPK4 is thus not required to lift the translational block on the *p28* mRNA.

Next, we asked whether $\Delta$CDPK4 macrogametocytes could form ookinetes when crossfertilized by a strain capable of exflagellating. As a microgamete donor we chose a parasite strain, in which both the *p28* and *p25* genes had been deleted. This strain produces ookinetes in normal numbers; their infectivity to the mosquito is, however, reduced to 0.5% of wt (Tomas et al., 2001). The lack of P28 in this strain meant that in crosses, ookinetes derived from $\Delta$CDPK4 macrogametes could be identified specifically by their expression of the P28 antigen, allowing ookinete conversion rates to be determined for the female line of the $\Delta$CDPK4 strain. Upon crossfertilization by $\Delta$P28/$\Delta$P25 microgametes, ookinete formation by $\Delta$CDPK4 macrogametes was as efficient as with wt (Supplemental Table S1 available on *Cell* website), showing that $\Delta$CDPK4 macrogametes are fully competent to be fertilized.

We then investigated whether crossfertilization rescued infectivity of the $\Delta$CDPK4 strain to *Anopheles stephensi* mosquitoes. Equal numbers of wt ookinetes and of the P28-positive ookinetes derived from the $\Delta$CDPK4 X $\Delta$P28/$\Delta$P25-cross were offered to mosquitoes in a membrane-feeding apparatus. When oocyst numbers on the midguts of fed mosquitoes were compared 10 days later, infectivity of ookinetes from the cross was found to be reduced to 1.8% of wt (Supplemental Table S1 available on *Cell* website). This significant reduction is probably an underestimate, since some of the oocysts observed in the cross would have been derived from selfing of the $\Delta$P28/$\Delta$P25 strain, which control experiments showed to retain limited infectivity (Supplemental Table S1 available on *Cell* website and Tomas et al., 2001).

### Complementation of the $\Delta$CDPK4 Strain with an Epitope-Tagged *cdpk4* Gene

To complement the $\Delta$CDPK4 strain with an intact copy of the *cdpk4* gene, we constructed an insertion vector that contained the intergenic region upstream of the *cdpk4* gene as targeting sequence for integration, followed by the complete coding sequence of the *cdpk4* gene, to which two copies of a carboxy-terminal c-*myc* epitope tag had been added, followed by the 3′UTR and flanking sequence of the *P. berghei dhfr/ts* gene (Figure 6A). An expression cassette for the human *dhfr* gene conferring resistance to the drug WR99210 was used as a selectable marker in the pyrimethamine-resistant background of the $\Delta$CDPK4 strain. To control for non-specific effects of integration into the target site, we also constructed a vector lacking the CDPK4 coding sequence (empty vector control). Characterization of transgenic clones by Southern blot analysis confirmed stable integration of the complementation vector (Figure 6B). In the case of the CDPK4myc-complemented clone, at least two copies of the complementation plasmid had integrated into the genome (Figure 6B). The protein expression level of CDPK4myc was nevertheless similar to CDPK4 in wild-type gametocytes when equal numbers of cells were compared on Western blots (Figure 6C).

### The *cdpk4myc* Transgene Encodes a Functional $Ca^{2+}$-Regulated Protein Kinase that Restores Mosquito Transmission of the $\Delta$CDPK4 Mutant

For biochemical characterization, we immunoprecipitated the c-*myc* epitope-tagged CDPK4 kinase from lysates of activated gametocytes (Figure 6D). Immune-complexes were then subjected to in vitro phosphorylation assays using artificial substrates. Casein and myelin basic protein were both readily phosphorylated by CDPK4myc in vitro (Figure 6E). As predicted from its primary structure, the kinase activity of CDPK4 was strictly $Ca^{2+}$-dependent. Autophosphorylation of CDPK4myc was observed independently of the presence of substrate and was also $Ca^{2+}$-dependent (Figure 6E and data not shown). No kinase activity was precipitated from gametocyte lysates of the vector-transfected control strain. Complementation of the $\Delta$CDPK4 mutant with CDPK4myc—but not with an empty control vector—restored genome replication in the microgametocyte (not shown). Scanning electron microscopy confirmed that $\Delta$CDPK4 gametocytes emerged successfully from their host cells (Figure 7B) but that the release of microgametes was inhibited unless the mutant was complemented with the *cdpk4myc* gene (Figure 7D). Finally, we examined the overall impact of the CDPK4 knockout on parasite transmission to *A. stephensi*. Oocyst formation was completely suppressed in the $\Delta$CDPK4 strain and in the control-complemented mutant, but wt oocyst numbers were restored by complementation with *cdpk4myc* (Supplemental Table S2 available on *Cell* website). Taken together, these data show that CDPK4 is a $Ca^{2+}$-dependent molecular switch that is essential for the mosquito transmission of malaria parasites by regulating male gametogenesis in response to a XA-induced $Ca^{2+}$ signal.
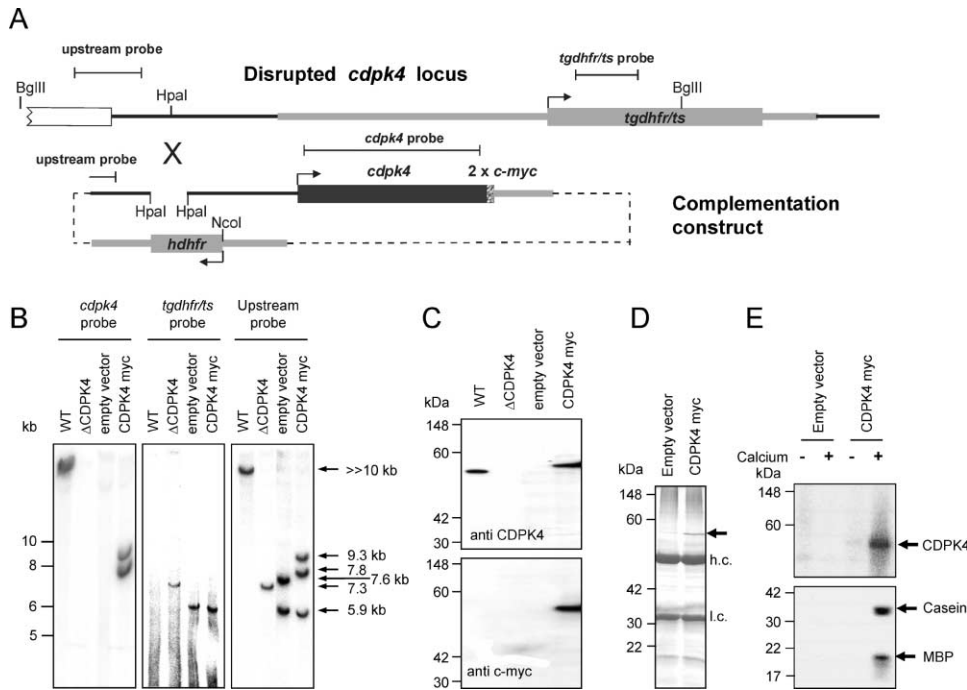
Figure 6. Complementation of the CDPK4 Knockout Clone with Epitope-Tagged CDPK4 and Biochemical Characterization of the Recombinant CDPK4myc Protein

(A) Schematic diagram showing the disrupted *cdpk4* locus and the insertion vector used for complementation.

(B) Southern blot analysis of BglII/NcoI-digested genomic DNA from wt, ΔCDPK4, and complemented clones. A *cdpk4* probe recognizes the expected large DNA fragment in wt and provides evidence for the integration of two copies of *cdpk4myc* in the complemented parasite clone, one on a 9.3 kb fragment spanning from the upstream BglII site to the NcoI site in the first integrated copy, and one on a 7.8 kb fragment indicative of a second inserted copy released as an NcoI-NcoI fragment. All three transgenic clones are recognized by the *tgdhfr/ts* probe, but successful insertion of the complementation construct reduces the size of the fragment from 7.3 to 5.9 kb by introducing an NcoI site. A probe covering part of the gene upstream of *cdpk4* and of the 5′ flanking sequences in the complementation vector additionally reveals a 7.6 kb fragment indicating correct integration of one copy of the empty complementation vector.

(C) Western blot analysis, showing expression of a recombinant CDPK4 protein in the complemented clone that is recognized by mAb 9E10 specific for the *c-myc* epitope. $1 \times 10^6$ purified gametocytes were analyzed per lane.

(D) Coomassie-stained SDS-polyacrylamide gel showing immunoprecipitation of a 57 kDa protein specifically from CDPK4myc-expressing gametocytes. H.c., heavy chain; l.c. light chain of the precipitating mAb 9E10.

(E) Immunocomplex-kinase assay showing $Ca^{2+}$-dependent kinase activity of the CDPK4myc protein. The upper portion of the gel was exposed 10 times longer to reveal autophosphorylation of CDPK4myc.

## Discussion

### XA Triggers Gametogenesis by Mobilizing Cellular $Ca^{2+}$

A role for $Ca^{2+}$ signaling in malarial gametogenesis has previously been suggested on the basis of pharmacological studies showing that exflagellation in *P. berghei* and in *P. falciparum* is sensitive to an inhibitor of $Ca^{2+}$ release from the endoplasmic reticulum (ER) and to a calmodulin antagonist, W-7 (Kawamoto et al., 1990). A GFP-aequorin expressing $Ca^{2+}$ reporter strain has now enabled us to detect changes in gametocyte cytosolic $Ca^{2+}$ and showed that biologically active concentrations of XA induce a rapid, dose-dependent increase in free cytosolic $Ca^{2+}$ after a lag phase of about 10 s. The speed of this response is consistent with the time at which the first ultrastructural changes can be observed in the microgametocyte, i.e., the formation of 8 kinetosomes after 15 s and the assembly of the first mitotic spindle within 1 min of stimulation (Sinden et al., 1976). GFP-aequorin proved a sensitive $Ca^{2+}$ reporter in *Plasmodium* that requires few cells ($10^4$–$10^5$) to measure ago-

nist-induced parasite responses and that can be targeted specifically to the cytoplasm of the intracellular parasite, making measurements independent of host cell lysis during gametogenesis. Further experiments are required to calibrate the bioluminescence response to the $Ca^{2+}$ concentration. Importantly, GFP-aequorin bioluminescence can be limited by the available pool of coelenterazine-aequorin complexes, which become consumed in the process. It is therefore currently unclear whether the XA-induced mobilization of cellular $Ca^{2+}$ is indeed transient, as might be concluded from the kinetic of the luminescence response in Figure 3C, or whether the rapid decline in luminescence after about 20 s is due to sensor depletion.

Our data show that gametogenesis and the XA-induced cytosolic $Ca^{2+}$ signal are independent of extracellular $Ca^{2+}$. This points toward an intracellular $Ca^{2+}$ source, most likely the parasite's ER as a universal $Ca^{2+}$ store. The existence in the gametocyte of a classical pathway involving phosphoinositide-specific phospholipase C, $IP_3$, and $Ca^{2+}$ release from the ER is supported by data from Martin et al. (1994), who observed a rapid
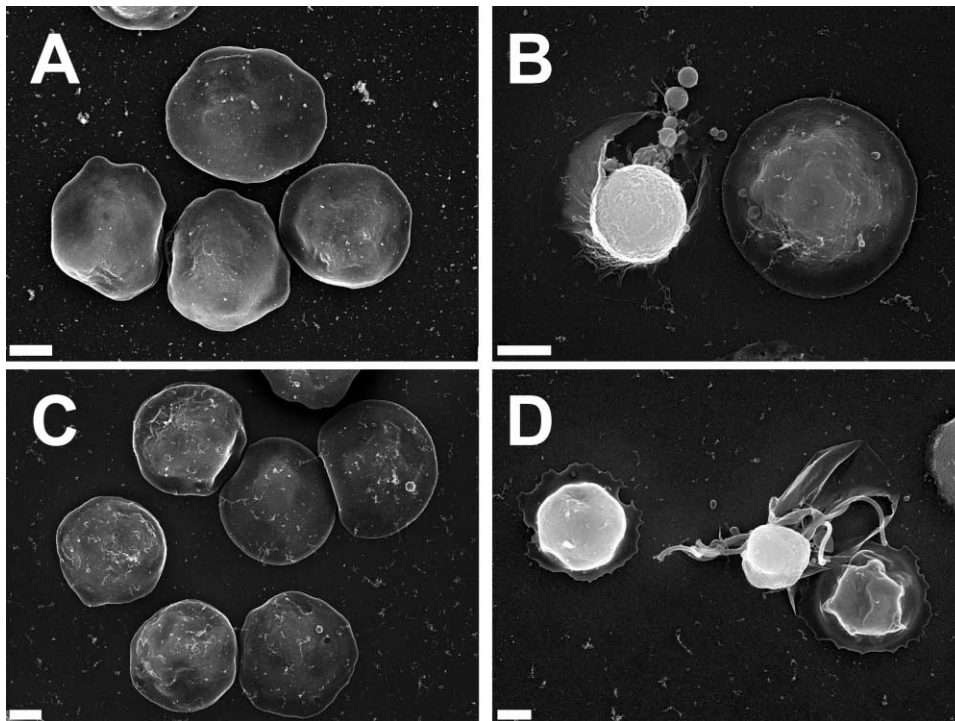
Figure 7. Scanning Electron Micrographs of Emergence and Exflagellation

(A) Purified intraerythrocytic ΔCDPK4 gametocytes, non-activated.

(B) Representative ΔCDPK4 gametocyte fixed 12 min after stimulation by 100 μM XA. The gametocyte shown has rounded up and emerged from its host erythrocyte, from which only a ghost remains.

(C) Purified intraerythrocytic, CDPK4myc-complemented gametocytes, nonactivated.

(D) Representative CDPK4myc-complemented microgametocyte fixed 12 min after stimulation by 100 μM XA. The exflagellating gametocyte remains in contact with the ghost of the erythrocyte from which it has emerged. Scale bar is equal to 2 μm.

increase of phosphoinositide hydrolysis and $IP_3$ generation upon activating *P. falciparum* gametocytes by a pH shift. Whether a unique apicomplexan organelle, the acidocalcisome, is a source of $Ca^{2+}$ during gametocyte activation or whether it is involved in terminating the signal by mopping up released $Ca^{2+}$ (Alleva and Kirk, 2001) needs to be addressed in future studies.

XA is unable to mobilize $Ca^{2+}$ in asexual blood stages suggesting that at least one key element in the upstream signaling pathway is specific to the gametocyte. Using the bioluminescent $Ca^{2+}$ reporter strain, it is now possible to dissect the signaling pathways leading to $Ca^{2+}$ release in greater detail. Future studies will identify a receptor for XA and address the roles of other second messengers. Cyclic nucleotides have long been implicated in regulating gametogenesis (Kawamoto et al., 1990) and a XA-responsive guanylyl cyclase activity has been identified in *P. falciparum* gametocyte membranes (Muhia et al., 2001), which may contribute to the regulation of gametogenesis by acting either upstream or in parallel with $Ca^{2+}$.

We found that BAPTA-AM used at concentrations demonstrably sufficient to block $Ca^{2+}$ mobilization in the gametocyte cytosol inhibits all constituent events of gametogenesis, i.e., cell cycle reentry and cytoskeletal reorganization in the microgametocyte, translational derepression of the *p28* mRNA in the macrogametocyte, and emergence and early changes in cell morphology

in gametocytes of both sexes. An XA-induced increase in free cytosolic $Ca^{2+}$ thus emerged as an essential early regulator for the activation of both micro- and macrogametocytes.

**CDPK4 Is a Master Regulator of Male-Specific Events during Gametogenesis**

Following the initial induction of $Ca^{2+}$ release by XA, different $Ca^{2+}$-dependent signaling pathways are initiated that control different constituent events of gametogenesis. A strictly CDPK4-dependent pathway regulates male-specific events during parasite differentiation, namely genome replication and the cytoskeletal rearrangements associated with mitosis and assembly of axonemes that form the motile backbones of the microgametes. These sex-specific functions of CDPK4 are reflected in its predominantly male expression. Possible substrates for CDPK4 may be found among the cyclins and cyclin-dependent protein kinases, some of which are upregulated in gametocytes (Doerig et al., 1995), or among components of the replication machinery. The quiescent gametocyte contains some DNA in excess of the haploid value (Janse et al., 1986) and its development may thus be arrested in mid S phase rather than in $G_0$. The exact point at which CDPK4 regulates the cell cycle thus remains to be determined. It is tempting to speculate that another member of the CDPK family in *Plasmodium* may function as a female counterpart of

CDPK4 controlling, for instance, the CDPK4-independent translational repression of presynthesized mRNAs in the macrogametocyte. Another constituent event of gametogenesis that is neither sex-specific nor dependent on CDPK4, is the lysis of the host erythrocyte. This is thought to involve vesicles in the gametocyte cytoplasm that discharge their electron-dense content in response to gametocyte activation (Sinden et al., 1996). In eukaryotic cells, the plasma membrane fusion machinery of exocytic vesicles includes synaptotagmins that are regulated directly through elevated $Ca^{2+}$ (Tucker and Chapman, 2002) and a similar direct mechanism may initiate the breakdown of the host cell by the emerging gametocyte. CDPK4 regulates exflagellation induced by either XA or a rise in extracellular pH. This is consistent with previous pharmacological studies on pH-induced gametogenesis (Kawamoto et al., 1990) and suggests that both triggers share a common signaling pathway downstream of calcium release that includes CDPK4.

CDPK4 may have a second function later during sporogonic development. This potential function became evident as a more than 50-fold reduction of infectivity to mosquitoes of ookinetes derived from the crossfertilization of ΔCDPK4 macrogametes by ΔP28/ΔP25 microgametes. The alternative explanation—that reduced infectivity of the crossfertilized ookinetes was due to the absence of *p28* and *p25* alleles in the male gamete—must be rejected, since it is known to be the female gamete that contributes the bulk of presynthesized P28 and P25 mRNA to the zygote, thereby allowing us to recognize crossfertilized ookinetes by their expression of P28. A role of CDPK4 in ookinete infectivity to the mosquito is consistent with the reported function of its likely ortholog in *T. gondii*, TgCDPK1. This kinase is the major CDPK activity in the tachyzoite stage. Pharmacological evidence shows it is essential for tachyzoite motility and host cell invasion (Kieschnick et al., 2001) and that it is involved in parasite egress from the infected host cell (Moudy et al., 2001). Our data suggest that in *Plasmodium* CDPK4 could play a similar role by regulating the invasion of and passage through the mosquito midgut epithelium by the ookinete in addition to regulating microgametogenesis.

## Are CDPKs Regulators of Life Cycle Progression in *Plasmodium*?

CDPKs are a prominent family of signaling kinases in plants, but relatively few have been assigned specific functions as effectors of known signals or upstream regulators of known phosphorylation cascades. In *Plasmodium*, members of the CDPK family are candidates for key regulators of stage-specific cellular responses to cytosolic $Ca^{2+}$, which is emerging as a widespread secondary messenger controlling progression of the parasite through its complex life cycles. Factors likely to contribute to the specificity of parasite responses to different calcium signals include stage-specific expression patterns of CDPK family members (which in the case of CDPK4 we show here to correlate well with function), different substrate specificities, and different sensitivities to $Ca^{2+}$ (Zhao et al., 1994b) and to costimulatory phospholipids (Zhao et al., 1994a). Additional lay-

ers of complexity may be expected from the importance of amino-terminal myristoylation and palmitoylation in targeting CDPKs to different subcellular compartments that is emerging from studies on CDPKs in plants (Dammann et al., 2003). The essential role of CDPKs at different stages of the life cycles of *Toxoplasma* and *Plasmodium*, combined with their absence from the parasites' vertebrate hosts, make members of the CDPK family attractive targets for pharmacological intervention.

## Experimental Procedures

### Parasite Maintenance, Culture, and Purification

The *P. berghei* ANKA strain was used throughout this study. All transgenic parasites were generated in the gametocyte-producing clone 2.34, or the gametocyte nonproducing clone 2.33 of this strain, except for the P25/P28 double gene knockout transgenic parasite (Tomas et al., 2001), which is derived from the ANKA clone 15cy1. Parasites were maintained in Theiler's original outbred mice as described (Sinden et al., 2002) and transmitted by *A. stephensi*, strain SD500, which was reared at 28°C and 75% humidity under a 12 hr light/dark cycle and maintained on a 10% fructose solution. Mosquito transmission was done by feeds on infected mice or by membrane feeding as described (Sinden et al., 2002).

The method for the purification of viable gametocytes was modified from Beetsma et al. (1998). Briefly, mice were pretreated by intra peritoneal injection of 0.2 ml phenylhydrazine (6 mg/ml in PBS) to stimulate reticulocyte formation two days prior to infection with $2 \times 10^7$ parasites. From day four postinfection (p.i.), mice were treated with sulfadiazine at 20mg/L in the drinking water to eliminate asexual parasite stages. On day six p.i. mice were bled into heparin, white blood cells were removed on CF11 cellulose (Whatman) columns, and gametocytes were separated from uninfected erythrocytes on a Nycodenz cushion made up from 48% Nycodenz stock (27.6% w/v Nycodenz in 5.0 mM Tris-HCl [pH 7.20], 3.0 mM KCl, 0.3 mM EDTA) and RPMI1640 medium containing 25 mM HEPES (Sigma), 5% FCS, 4 mM sodium bicarbonate, [pH 7.30]. Gametocytes were harvested from the interphase and washed three times in the appropriate buffer for the subsequent protocol. All manipulations were carried out at 19–22°C.

Exflagellation was quantified microscopically as described (Billker et al., 1997). For ookinete cultures, gametocyte-infected mouse blood was mixed with nine volumes of ookinete culture medium (RPMI1640 containing 25mM HEPES (Sigma), [pH 7.5], 10% FCS, 100 μM xanthurenic acid) and cultured for 20–24 hr at 19°C. P28 expressing parasite stages were quantified by fluorescence microscopy in a haemocytometer, following live staining with Cy3-labeled monoclonal antibody clone 13.1 specific for P28 (Winger et al., 1988). To quantify erythrocyte lysis in live IFA, cells were incubated with a rabbit polyclonal serum against mouse erythrocyte (Rockland, PA) at 1:100, followed by Alexa488-labeled secondary antibody. Parasite α-tubulin was visualized with the TAT1 monoclonal antibody (Woods et al., 1989). Nuclear DNA was stained in fixed, RNaseA-treated parasites using propidium iodide as described (Billker et al., 1997).

### Generation of Transgenic Parasites and Genotype Analysis

Transgenic parasites were generated and genotypes analyzed as described in "Supplemental Experimental Procedures".

### Immunocomplex Kinase Assay

$1 \times 10^8$ gametocytes per parasite strain were purified from two infected mice, activated for 30 s with medium containing 100 μM XA, pelleted by centrifugation for 30 s at 1000*g*, and resuspended in 0.5 ml cold lysis buffer (150 mM NaCl, 25 mM HEPES, 10% glycerol, 1.5 mM $MgCl_2$, 1% NP-40) containing inhibitors of proteases (Complete EDTA-free, Roche) and protein phosphatases (1 mM $Na_3VO_4$, 10 mM NaF). Following lysis for 20 min on ice, insoluble components were removed by centrifugation for 10 min at 20000*g* at 4°C, the supernatant was precleared with 100 μl of proteinA-sepharose slurry (Amersham) for 1 hr and CDPK4myc was precipitated with 3 μg of the anti c-*myc* monoclonal antibody 9E10 coupled to 100 μl proteinA-sepharose slurry at 4°C over night. For the in vitro

phosphorylation assay, immune complexes were washed 3× with lysis buffer and 3× with kinase buffer (50 mM TrisHCl pH 7.4, 1 mM EGTA, 10 mM MgCl$_2$). 5 $\mu$l volumes of the immune complexes were then removed and washed again with kinase buffer supplemented with appropriate concentrations of Ca$^{2+}$, EGTA and artificial substrates (1 mg/ml of myelin basic protein, histone IIIS or dephosphorylated casein; all Sigma, UK), and the kinase reaction was started by addition of ATP (75$\mu$M, 5$\mu$Ci $\gamma^{32}$P-ATP per 20 $\mu$l reaction). After 10 min at 20°C, a period during which no saturation was reached, the kinase reaction was stopped by the addition of SDS sample buffer and heating to 98°C. Labeled substrates were visualized in dried 15% SDS-polyacrylamide gels on a Fuji BAS-1500 phosphorimager.

**Aequorin Reconstitution and Luminometric Ca$^{2+}$ Detection**
Recombinant apoaequorin expressed in transgenic *P. berghei* strains was reconstituted with its luciferin, coelenterazine, to become a functional, Ca$^{2+}$-sensitive luciferase. First, purified gametocytes were washed 3× in coelenterazine loading buffer (CLB), containing PBS, 20 mM HEPES, 20 mM Glucose, 4 mM sodium bicarbonate, 1 mM EGTA, 0.1% w/v bovine serum albumin, [pH 7.25]. Reconstitution was then achieved by shaking approximately 1 × 10$^8$ gametocytes in 0.5 ml CLB, supplemented with 5 $\mu$M coelenterazine-fcp (Biotrend, Germany) for 30 min at 1400 rpm and 20°C. Loaded gametocytes were washed twice in CLB to remove excess coelenterazine and were then suspended in 10 ml CLB. For luminescence measurements, 0.05 ml volumes of the gametocyte suspension, containing 5 × 10$^5$ gametocytes, were automatically injected into the same volume of different test solutions in a 96-well assay plate of an Anthos Lucy 2 luminometer. For each sample 50 luminescence readings were generally taken, each integrated over 1 s, using the "fast kinetics" option of the DAZDAQ-Stingray software. Test solutions for Ca$^{2+}$ luminometry were prepared in CLB by addition of the appropriate amounts of agonists, inhibitors, vehicle, or Ca$^{2+}$ to twice the final concentration. Stock solutions of A23187, cyclopiazonic acid (CPA) and BAPTA-AM (all from Sigma, UK) were made up in dimethyl sulfoxide.

**Flow Cytometry**
GFP fluorescence was analyzed on a Becton Dickinson FACS Vantage SE cell sorter. To analyze DNA content 10$^6$ purified gametocytes were stimulated with medium containing 100 $\mu$M XA or control medium without XA. After 8 min at 19°C, the membrane permeable DNA-specific dye, DRAQ5 (Alexis, UK), was added to a final concentration of 5 $\mu$M and the reaction was transferred to 37°C for 2 min to achieve staining equilibrium. Stained gametocytes were then diluted immediately with ice-cold PBS to prevent exflagellation and DNA content was assessed on a FACSCalibur flow cytometer. All data were analyzed with CellQuest Pro software (BD Biosciences).

**Scanning Electron Microscopy**
Purified gametocytes in RPMI1640, 25 mM HEPES, 4 mM sodium bicarbonate, were allowed to settle for 20 min onto poly-L lysine-coated cover slips. They were then either fixed immediately in 2.5% glutaraldehyde, or stimulated for 5 to 12 min by 100 $\mu$M XA in medium containing 5% FCS before fixation. Cells were postfixed in 0.5% OsO$_4$ and treated with 1% tannic acid, followed by a second OsO$_4$ fixation step. Specimens were coated with a 10 nm layer of gold/palladium and analyzed in a LEO 1550 field emission scanning electron microscope (LEO, Oberkochen, Germany).

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