Malaria, caused by infection with intracellular protozoan parasites of the genus *Plasmodium*, is responsible for 300 million to 600 million clinical cases annually (49), resulting in the deaths of up to 3 million people every year (9, 10). The need for novel intervention strategies is rendered more acute by the increasing evidence shows that CK2 is an attractive target for antimalarial chemotherapeutic intervention.

The parasite life cycle is complex, with a succession of proliferation and differentiation events, in the regulation of which protein phosphorylation is likely to play crucial roles. Reversible phosphorylation of proteins is a major regulatory mechanism in most cellular processes, and approximately 30% of all eukaryotic cellular proteins carry a phosphate group. Derepression of protein phosphorylation underlies various pathologies, including cancers, and protein kinases are considered promising drug targets, comprising as much as 30% of all protein kinases in addition to CK2α (reviewed in reference 6), pointing to a likely role in the integration of numerous signaling pathways. The human genome encodes three CK2α subunits and a single version of the beta subunit. Increasing evidence shows that CK2 is an attractive target for antineoplastic and antiviral drugs (46).

The genome of the *P. falciparum* strain 3D7 (18) has been fully sequenced, allowing the discovery of the entire complement of plasmoidal protein kinases by analysis of the set of predicted peptides (3, 57). A putative CK2α orthologue and two predicted CK2β subunits were identified in these analyses. Here we present the biochemical characterization of the PICK2α and both PICK2β orthologues and demonstrate by using a reverse genetics approach that the catalytic subunit is essential for completion of the erythrocytic asexual cycle of the parasite.

**MATERIALS AND METHODS**

Expression and purification of the three PICK2 subunits. Oligonucleotides were designed to amplify the PICK2α open reading frame (ORF) from *P. falciparum* (clone 3D7A) cDNA. The forward (5’-GGGGGATCCATGTCGGTTAGCTCAATTAATAAA-3’) and reverse (5’-GGGGATCCATTCTAGATCCACT-3’) primers carried BamHI and SalI sites, respectively (underlined). Oligonucleotides were also designed to amplify the PICK2β1 and PICK2β2 ORFs from *P. falciparum* (clone 3D7A) cDNA. The PICK2β1 forward (5’-GGGGGATCCATTCTAGATCCACT-3’) and reverse (5’-GGGGGATCCATTCTAGATCCACT-3’) primers carried BamHI and SalI sites, respectively (underlined). The PICK2β2
sequence has a long N-terminal extension. Oligonucleotides were designed to amplify the PCK2β2 sequence, lacking the N-terminal extension, from P. falciparum (clone 3D7A) cDNA. The forward (5′-GGGGGGCTCATGGAAGC AACAGGTGCTTGGATGTT-3′) and reverse (5′-GGGGGCTCGACTCATTGA CACTTCTCAGAGGAGTTCGG-3′) primers carried BamHI and Sali sites, respectively (underlined). The short version of PCK2β2, lacking the N-terminal extension, was named shPCK2β2. Catalytically inactive (“kinase dead”) PCK2α was obtained by site-directed mutagenesis (K72M) of PCK2α by overlap extension PCR (23). All cloning primers are provided in Table S1 in the supplemental material. All PCR products were verified by sequencing in the vector pGEX-4T3 (GE Healthcare) to generate N-terminal glutathione S-transferase (GST) fusions. The shPCK2β2 sequence was also inserted between the BamHI and NotI sites of the plasmid pQE-30 for expression with an N-terminal His tag. A PET29 vector containing P. falciparum CK2α in frame with a C-terminal His tag sequence was a kind gift from D. Chakrabarti. The pGEX-4T3 constructs were expressed in E. coli BL21 (DE3) cells for 2 h at 20°C with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). GST-tagged proteins were purified on glutathione-agarose beads (Sigma), and His-tagged proteins were purified on Ni2+-agarose beads (Qiagen), following the manufacturer’s recommendations.

Construction of the knockout (KO) plasmid pCAM-BSD-KOPCK2α. A fragment from the PCK2α ORF was amplified by PCR and inserted between the BamHI and NotI sites of the pCAM-BSD plasmid (47), which contains the Aspergillus terreus blasticidin-S-deaminase gene, whose gene product confers resistance to the drug blasticidin. Cloning primers are listed in Table S1 in the supplemental material.

Construction of the complementation plasmid pCHD-PCK2α. A plasmid for in vitro epimonal expression of PCK2α subunits was constructed as follows: the full-length PCK2α coding sequence was first inserted between the BglII and NotI sites of the plasmid pHBG (51) and then transferred into the plasmid pCHD-1/2 (51) by a Gateway LR cloning reaction according to the manufacturer’s instructions (Invitrogen). The plasmid pCHD-1/2 includes a cassette encoding human dihydrofolate reductase, conferring resistance to the antifolate drug WR99210. Parasites that were transfected with both a KO plasmid and a complementation plasmid pCHD-PCK2α under tetracycline control (see below) were selected.

3′-Tagging plasmid. The 3′ end of the PCK2α coding sequence (538 bp, omitting the stop codon) was amplified by PCR using primers incorporating PstI and BamHI restriction sites and inserted between the PstI and BamHI sites of the plasmid pEAS-HT (18), retaining the eIF2α-derived peptide (MSGDEMIFDPTMSKKKKKKKP) (40, 45), and reverse (50-5′ primer: CACTTCTCAGAGGAGTTCGG-3′) primers carried BamHI and Sali sites, respectively (underlined). The short version of PCK2α, lacking the N-terminal extension, was named shPCK2β2. Catalytically inactive (“kinase dead”) PCK2α was obtained by site-directed mutagenesis (K72M) of PCK2α by overlap extension PCR (23). All cloning primers are provided in Table S1 in the supplemental material. All PCR products were verified by sequencing in the vector pGEX-4T3 (GE Healthcare) to generate N-terminal glutathione S-transferase (GST) fusions. The shPCK2β2 sequence was also inserted between the BamHI and NotI sites of the plasmid pQE-30 for expression with an N-terminal His tag. A PET29 vector containing P. falciparum CK2α in frame with a C-terminal His tag sequence was a kind gift from D. Chakrabarti. The pGEX-4T3 constructs were expressed in E. coli BL21 (DE3) cells for 2 h at 20°C with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). GST-tagged proteins were purified on glutathione-agarose beads (Sigma), and His-tagged proteins were purified on Ni2+-agarose beads (Qiagen), following the manufacturer’s recommendations.

Measurement of 50% inhibitory concentrations (IC50s). To test the effect of small molecule inhibitors on PCK2α, kinase activity was measured in the presence of increasing concentrations of these molecules. Stocks of the molecules contained dimethyl sulfoxide or ethanol as a solvent, and negative controls for the reactions were provided by reaction mixtures containing ethanol or dimethyl sulfoxide without the small molecule inhibitor. Kinase reactions were performed by the phosphocellulose method, as detailed above.

Interaction assay. A mixture of 5 μg of each recombinant protein was incubated at 4°C for 30 min in 20 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 0.1% Nonidet P40 (IGEPAL), and 10% glycerol. Glutathione-agarose beads were added to each reaction mixture. The tubes were rotated at 4°C for 1 h; the beads were recovered by centrifugation and washed four times in reaction buffer. Laemmli buffer was added to the beads, which were then heated to 100°C. Samples were separated by SDS-PAGE on 12% acrylamide gels and either stained or transferred to the membrane for Western blot analysis.

Western blot analysis. Western blotting was performed according to conventional protocols. Briefly, samples were separated by SDS-PAGE on a 12% acrylamide gel and blotted onto nitrocellulose. The membranes were blocked against standard methods and incubated with rabbit anti-His antibody (1:1000; Santa Cruz Biotechnology) or subunit-specific antibodies generated in rabbits by BioGenes (Germany) against the PCK2α-derived peptide ADVNII KPKEYYDY. A goat anti-rabbit secondary antibody coupled to horseshad peroxidase was used at a ratio of 1:10,000. Antibody was visualized using the enhanced chemiluminescence system (PerkinElmer).

Parasite culture and transfection. Cultures of the P. falciparum strain 3D7A (54) were maintained at 37°C in RPMI 1640 medium (Gibco) supplemented with 25 mM sodium bicarbonate, 2 mM glutamine, 300 mM hypoxanthine, 10 μg/mL gentamicin, and AlbuMax II (Sigma). Cultures were seeded at 5% hematocrit and maintained at a parasitemia of 1 to 10% with daily changes of medium. The incubator was flushed with a gas mixture containing 5% CO2.

For transfection, asexual blood stage parasites were synchronized by sorbitol treatment (26) to obtain a majority of ring stage parasites. Forty-eight hours later, ring stage parasites were transfected by electroporation with 100 μg of purified plasmid DNA in Cytomix buffer as described previously (16, 17, 47). Blasticidin (2.5 μg/ml) was added to the culture medium to select for transformed parasites. Parasites under double selection had WR99210 (5 μM) added to the culture medium in addition to the blasticidin. Parasites were maintained in this supplemented medium from 2 days posttransfection.

Preparation of parasite protein extract. Parasite cultures were lysed in 0.15% saponin. After centrifugation and washing, the parasite pellets were sonicated in RIPA buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 2 mM MnCl2, and 10 μM ATP) containing 0.075 MBq [32P]ATP (220 TBq/mmol; GE Healthcare), 1 μg of recombinant ATPase, 0.1 mg of substrate. Reactions were carried out at 30°C for 30 min and terminated by the addition of Laemmli buffer samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were dried and exposed for autoradiography.

Kinase assays. Standard kinase reactions (30 μl) occurred in kinase buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 2 mM MnCl2, and 10 μM ATP) containing 0.075 MBq [32P]ATP (370 MBq/ml; GE Healthcare), 1 μg of purified P. falciparum CK2α, and 60 μM of protein. Reactions were carried out at 30°C for 30 min, and then 60 μM of 50% inhibitory concentrations (IC50s).

RESULTS

Bioinformatics. Phylogenetic analysis of P. falciparum protein kinases identified the PlasmoDB (http://plasmodb.org/plasmo) sequence PF11_0096 as that of a CK2α orthologue (3, 57), with 65% amino acid sequence identity to Homo sapiens CK2α. PF11_0096 was therefore named PCK2α. An alignment of PCK2α with CK2α subunits from H. sapiens and Zea mays (see Fig. S1 in the supplemental material) reveals that
PfCK2 possesses all 11 of the subdomains conserved across eukaryotic protein kinases (21, 22) and the majority of the conserved features of CK2 subunits (2). Just downstream from subdomain II is a putative nuclear localization signal, Pro-Val-Lys-Lys-Lys-Lys-Ile, conserved across CK2 homologues. PfCK2 also possesses three invariant residues common to CK2 family members; the ATP binding motif present in most other protein kinases is Gly-X-Gly-X-X-Ser (PfCK2α, Gly50-Ser55). The most highly conserved amino acid motif specific to members of the CK2 family is Asp179-Trp-Gly181 (notation from PfCK2α; most protein kinases display Asp-Phe-Gly at this position). Likewise, Gly203-Pro-Glu205 (notation from PfCK2α) is a common feature of the family, which diverges from the Ala-Pro-Glu motif present in the vast majority of other protein kinases; thus, all three CK2-specific motifs are present in PfCK2α.

Two putative CK2β subunits were identified in *P. falciparum* (57), hereafter referred to as PfCK2β1 and PfCK2β2 (PlasmoDB identifiers PF11_0048 and PF13_0232, respectively). BLASTP searches using the putative PfCK2β1/PfCK2β2 amino acid sequences as queries confirmed their identities as CK2β orthologues. An alignment with the human CK2β sequence (HsCK2β) (Fig. 1) reveals that many of the conserved features of CK2β subunits, including the four cysteine residues responsible for zinc finger formation (12), are present in PfCK2β1 and PfCK2β2 (e.g., Cys117, -122, -145, and -148 for PfCK2β1) (Fig. 1). The human CK2β sequence has a well-documented CK2 phosphorylation site at the N terminus (SSEE). PfCK2β2 possesses several phosphorylatable residues in the N-terminal region that are surrounded by a number of acidic residues, which could therefore be phosphorylated by CK2, and a TESSEE sequence at the C terminus reminiscent of the HsCK2β N-terminal phosphorylation site (MSSEE). The stretch of amino acids found to be necessary for the export of CK2 as an ectokinase (CK2 plus PfCK2β; Fig. 1) reveals that many of the conserved CK2 sequences, leading to the intriguing possibility that PfCK2 may be exported from the parasite. The acidic stretch responsible for downregulation of CK2 activity and association with the plasma membrane (HsCK2β amino acids D55 to D64) (29, 32) is present in PfCK2β1 (D68 to D75) and extended in PfCK2β2 (D207 to E226). This insertion occurs in a region looping out from the main protein structure (12) and is not unique; for example, *Saccharomyces cerevisiae* CK2β has an insertion sequence of 30 amino acids in this location. Along with the insertion region, PfCK2β2 has a highly acidic and repetitive N-terminal exten-

**FIG. 1.** Alignment of PfCK2β1 and PfCK2β2 with *Homo sapiens* CK2β. ClustalW alignments of the following proteins were performed: *Homo sapiens* CK2β (HsCK2β; AAM50092); PfCK2β1 (AAN35637; PlasmoDB PF11_0048; 33% identical to HsCK2β); PfCK2β2 (CAD52554; PlasmoDB PF11_0232; 39% identical to HsCK2β). Note the long N-terminal extension and acidic insertion sequence in PfCK2β2. The sequence of shPfCK2β2 begins (after an artificially introduced initiating methionine) with residue E156, underlined. The cysteine residues thought to hold the zinc finger in place are indicated in bold text and with arrowheads. The potential autophosphorylation site on PfCK2β2 is indicated in bold. The export of CK2 as an ectokinase is mediated by CK2β amino acids 20 to 33 (44) (boxed, along with the equivalent residues in PfCK2β1 and PfCK2β2). The acidic region mentioned in the text is also boxed (D107 to E133 of PfCK2β2).

**FIG. 2.** GST-PfCK2α kinase activity. (A) Autoradiograms (left) and Coomassie blue-stained gels (right) of kinase assays performed with GST-PfCK2α (top) or catalytically inactive GST-K72MPfCK2α (bottom) and the following substrates: lane 1, no substrate; lane 2, α-casein; lane 3, β-casein; lane 4, mixed dephosphorylated caseins; lane 5, myelin basic protein; lane 6, histone H1; lane 7, GST-shPfCK2β2; and lane 8, GST. (B) Autophosphorylation can occur by a transreaction. Left, autoradiogram; right, Coomassie blue-stained gel. The band observed at roughly 66 kDa is the GST-PfCK2α autophosphorylation band, and the band at roughly 40 kDa is the PfCK2α-His autophosphorylation band. Lane 1, GST-PfCK2α; lane 2, GST-K72MPfCK2α; lane 3, PfCK2α-His; and lane 4, GST-K72MPfCK2α plus PfCK2α-His. Radioactive phosphate is incorporated into catalytically inactive PfCK2α (K72MPfCK2α) in the presence of PfCK2α-His, indicating that autophosphorylation can occur by an intermolecular reaction.
and recombinant GST-tagged shPfCK2 with strongest activity toward the caseins (Fig. 2, lanes 2 to 4) is capable of phosphorylating a range of exogenous substrates, /H9251* vitro kinase assay (Fig. 2, top). GST-PfCK2 corresponds to the size of the GST-tagged PfCK2 plates (the 66-kDa band present in each lane in Fig. 2 corresponds on the regression of the enzyme kinetic data for ATP and GTP. The intercept on the y axis gives the negative reciprocal of the $K_m$, and the intercepts on the x axis give the reciprocal of the $V_{max}$. (B) The graph was obtained by linear regression of the enzyme kinetic data for the NEB peptide RRKADDSDDDD. The intercept on the x axis gives the reciprocal of the $K_m$.

FIG. 3. PfCK2α kinetics. The enzyme kinetics of PfCK2α in a Lineweaver-Burke presentation. The experiments were performed in triplicate, the data points represent the means, and the error bars represent three standard deviations. (A) The graph was obtained by linear regression of the enzyme kinetic data for ATP and GTP. The intercepts on the x axis give the negative reciprocal of the $K_m$, and the intercept on the y axis gives the reciprocal of the $V_{max}$. (B) The graph was obtained by linear regression of the enzyme kinetic data for the NEB peptide RRKADDSDDDD. The intercept on the x axis gives the negative reciprocal of the $K_m$.

sion that is not found in other CK2β subunits. The N-terminal region is at the periphery of the 3D structure of the human CK2β peptide (12, 35) and is not a conserved part of the CK2β structure and thus may possibly function as a docking site or region that interacts with binding partners or substrates of the PfCK2 holoenzyme. The human CK2β is phosphorylated at S209 in a cell cycle-dependent manner by p34<sup>cdc2</sup>(19, 30, 33), although the function of this phosphorylation is unknown. Both PfCK2β subunits possess serine residues near the C terminus that could be phosphorylated.

Microarray data available on PlasmoDB reveal that the mRNAs encoding all three subunits are detectable throughout the parasite life cycle (7, 28). Expression of the PfCK2α protein in asexual blood stage parasites was verified by Western blot analysis (see Fig. 7F).

In vitro activity of PfCK2α. Kinase activity of bacterially expressed recombinant GST-PfCK2α was detected by an in vitro kinase assay (Fig. 2, top). GST-PfCK2α autophosphorylates (the 66-kDa band present in each lane in Fig. 2 corresponds to the size of the GST-tagged PfCK2α subunit) and is capable of phosphorylating a range of exogenous substrates, with strongest activity toward the caseins (Fig. 2, lanes 2 to 4) and recombinant GST-tagged shPfCK2β2, a short version of the PfCK2β2 subunit lacking the N-terminal extension (see Materials and Methods) (Fig. 2, lane 7). There was no activity against the GST moiety alone (Fig. 2, lane 8), indicating that the activity in lane 7 was against the beta subunit itself and that the autophosphorylation is against the PfCK2α subunit itself. We did not detect any activity against the PfCK2β1 subunit (data not shown). These observations are consistent with general preferences of CK2 homologues for substrates with highly acidic phosphoacceptor sites (11, 25, 39, 52, 56); several potential such sites are present on PfCK2β. Parallel assays performed with an inactive PfCK2α mutant (Lys72Met; the Lys residue is required for correct orientation of the ATP molecule) were negative for kinase activity, confirming that activity is indeed due to PfCK2α (Fig. 2, bottom). PfCK2α autophosphorylates by a transreaction (Fig. 2B); GST-PfCK2α and PfCK2α-His autophosphorylate (Fig. 2, lanes 1 and 3), while GST-K72MPfCK2α does not (Fig. 2, lane 2) but is phosphorylated in the presence of PfCK2α-His, indicating that at least a proportion of the autophosphorylation of PfCK2α occurs by an intermolecular reaction.

**PfCK2α shares features in common with CK2α from other systems**. A feature often cited as being characteristic of CK2 enzymes is that they have similar affinities for GTP and ATP. PfCK2α has a $K_m$ of 16.7 μM and $V_{max}$ of 6.6 nmol/min for the PfCK2β2 subunit lacking the N-terminal extension (see Materials and Methods) (Fig. 2, lane 7). There was no activity against the GST moiety alone (Fig. 2, lane 8), indicating that the activity in lane 7 was against the beta subunit itself and that the autophosphorylation is against the PfCK2α subunit itself. We did not detect any activity against the PfCK2β1 subunit (data not shown). These observations are consistent with general preferences of CK2 homologues for substrates with highly acidic phosphoacceptor sites (11, 25, 39, 52, 56); several potential such sites are present on PfCK2β. Parallel assays performed with an inactive PfCK2α mutant (Lys72Met; the Lys residue is required for correct orientation of the ATP molecule) were negative for kinase activity, confirming that activity is indeed due to PfCK2α (Fig. 2, bottom). PfCK2α autophosphorylates by a transreaction (Fig. 2B); GST-PfCK2α and PfCK2α-His autophosphorylate (Fig. 2, lanes 1 and 3), while GST-K72MPfCK2α does not (Fig. 2, lane 2) but is phosphorylated in the presence of PfCK2α-His, indicating that at least a proportion of the autophosphorylation of PfCK2α occurs by an intermolecular reaction.
ATP and a $K_m$ of 34.9 $\mu$M and $V_{\text{max}}$ of 2.1 nmol/min for GTP (Fig. 3A). The enzyme displays a number of other features that confirm it as a true member of the CK2 family: (i) PCK2α is able to phosphorylate the CK2 substrate peptide RRRADDSDDDD (NEB), with a $K_m$ of 137.5 $\mu$M (Fig. 3B); (ii) CK2α enzymes are known to have a wide variety of substrates (34), and correspondingly, PCK2α (but not the K72M mutant protein used as a negative control) phosphorylates a number of proteins within heat-inactivated parasite protein extract (data not shown); (iii) the activity of PCK2α is inhibited by the well-established CK2-specific inhibitor TBB (3,4,5,6-tetrabromocatechol), with a similar IC$_{50}$ curve to that of human CK2α (IC$_{50}$ for PfCK2α, 2 $\mu$M; IC$_{50}$ for HsCK2α, 1.5 $\mu$M) (see Fig. 8C); and (iv) it can be recruited by the human CK2β subunit to phosphorylate the eIF2β-derived peptide (40, 45) and the Olig2 protein (27) (Fig. 4). This is in line with the established ability of human CK2, but not CK2α alone, to phosphorylate the substrates used in this experiment (27, 40).

PCK2α and the PCK2β beta subunits interact in vitro. To assess whether the two regulatory PCK2 subunits are able to associate with PCK2α in vitro, all three subunits were expressed in E. coli as His- or GST-tagged proteins and used in pull-down experiments. Mixtures of His- and GST-tagged proteins were prepared, from which proteins were pulled down using glutathione beads. The pulled-down proteins were then subjected to Western blot analysis using an anti-His antibody to detect any bound His-tagged protein that was copurified with the GST-tagged proteins. PCK2α-His was copurified with both GST-tagged beta subunits but not with GST alone (Fig. 5A). The interaction does not significantly alter the $K_m$ for ATP (Fig. 5B) or the phosphorylation of calmodulin or the peptide RRRSDDSDDDDD (data not shown). However, the interaction of the beta subunits with the kinase has functional significance, at least in vitro, since the activity of the kinase toward β-casein is reduced with increasing amounts of GST-PCK2β present in the reaction mixtures (Fig. 5C).

**PCK2α is essential for completion of the erythrocytic asexual cycle.** We next wanted to determine whether PCK2α plays essential functions in parasite survival. To generate a plasmid able to disrupt the PCK2α gene, an internal fragment of the coding sequence, excluding the critical motifs Gly-X-Gly-X-X-X-Gly-Pro-Glu (subdomain VIII, required for structural stability of the C-terminal lobe) (see Fig. S1 in the supplemental material), was amplified and cloned into the transfection vector pCAM-BSD (47), which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPCK2α) into 3D7 parasites, integration was monitored in material), was amplified and cloned into the transfection vector pCAM-BSD (47), which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPCK2α) into 3D7 parasites, integration was monitored in material), was amplified and cloned into the transfection vector pCAM-BSD (47), which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPCK2α) into 3D7 parasites, integration was monitored in material), was amplified and cloned into the transfection vector pCAM-BSD (47), which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPCK2α) into 3D7 parasites, integration was monitored in material), was amplified and cloned into the transfection vector pCAM-BSD (47), which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPCK2α) into 3D7 parasites, integration was monitored in material), was amplified and cloned into the transfection vector pCAM-BSD (47), which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPCK2α) into 3D7 parasites, integration was monitored in material), was amplified and cloned into the transfection vector pCAM-BSD (47), which confers resistance to blasticidin. Integration of this construct (pCAM-BSD-KOPCK2α) into 3D7 parasites, integration was monitored in material).
verify that the PfCK2α locus is indeed recombinogenic, we proceeded to transfec wild-type parasites with a “3’-tagging” construct whose integration was expected not to cause loss of function of the target protein. We readily observed integration of the tagging construct (Fig. 7A to E) and size increase in the PfCK2α protein caused by the HA tag (1.1 kDa) (Fig. 7F). This demonstrates that the locus is accessible to recombination if no loss of function is incurred, as is presumably the case with HA tagging, and therefore strengthens the case that PfCK2α is essential for the parasite’s asexual cycle.

We nevertheless wanted to ascertain that PfCK2α can be disrupted if the enzyme is provided through expression of an extraneous copy of the gene. To this effect, a complementation plasmid was constructed, containing the full-length PfCK2α coding region under the control of the Plhsp86 promoter and preceding a 3’ untranslated region (namely, the Plasmodium berghei dihydrolase reductase terminator sequence). The *P. falciparum* hsp86 gene (PF07_0029) displays a similar mRNA expression profile to the PfCK2α gene (28); therefore, its promoter is presumably appropriate to drive expression of the complementing protein. In parallel with the transfection of the pCAM-BSD-KOPfCK2a plasmid alone, further populations of parasites were cotransfected with both pCAM-BSD-KOPfCK2a and the complementation plasmid. PCR analysis (Fig. 6B, right) showed that disruption of the targeted locus occurred only in the doubly transfected, doubly resistant parasites. Southern blot analysis independently confirmed that integration occurred only in the doubly transfected parasites (Fig. 6C). The 13-kb band that represents the wild-type locus dramatically decreased in the doubly transfected parasites and was undetectable in two clonal lines (E7 and G9) that were derived from this culture by limiting dilution. There are multiple possibilities for the recombination of the KO and complementation plasmids with each other before or after integration, which could account for the additional bands of unexpected size observed (6 kb and 14 kb). The most important observation is that the wild-type band disappears only in the doubly transfected parasites.

Taken together, these data provide strong evidence that PfCK2α is essential to viability of the asexual erythrocytic stage parasites.

**PfCK2α kinase activity is amenable to inhibition.** Using a kinase-directed inhibitor library, we conducted a screen for compounds that inhibit PfCK2α. This screen identified the compounds Rottlerin and ML-7 as inhibitors of PfCK2α. The IC_{50} of these compounds were determined for both PfCK2α and HsCK2α (Fig. 8). While ML-7 inhibits both enzymes with an IC_{50} of 7 μM, Rottlerin exhibits differential effects on the orthologues, inhibiting PfCK2α with an IC_{50} of 7 μM and HsCK2α with an IC_{50} of 20 μM. This result indicates that differential inhibition is possible, despite the high percent identity (65%) between the CK2α amino acid sequences of *P. falciparum* and *Homo sapiens*.

**DISCUSSION**

We have characterized a *P. falciparum* CK2α orthologue and confirmed that the recombinant enzyme exhibits kinase activity in vitro and exhibits features in common with other CK2α enzymes. PfCK2α contains the major motifs conserved across
CK2 catalytic subunits, phosphorylates acidic sequences, interacts with the putative PfCK2α subunits and the HsCK2β subunit, is inhibited by the classic CK2 inhibitor TBB (3,4,5,6-tetrabromobenzotriazole), with a similar IC₅₀ to that of HsCK2α, and is able to utilize GTP or ATP as a cosubstrate. We have also confirmed the identity of two PfCK2α subunits. The N-terminal extension of PfCK2α is unusually long for CK2α proteins, with 160 amino acids before the first conserved residue (Trp161 in PfCK2α). Most CK2α subunits from vertebrates have only eight amino acids prior to this conserved residue (Homo sapiens, Gallus gallus, Mus musculus, Xenopus tropicalis, Bos taurus, and Danio rerio); this N-terminal extension is expanded in yeast (Saccharomyces cerevisiae, 37 residues), trypanosomatids (Trypanosoma brucei, 27 residues; Leishmania major, 21 residues), plants (Arabidopsis thaliana, 100 residues; Oryza sativa, 92 residues), and alveolates (Cryptosporidium parvum, 27 residues; Theileria parva, 34 residues). Within the alveolates, Plasmodium yoelii yoelii (125 residues) and Plasmodium vivax (157 residues) also have long extensions, but the extension of P. falciparum is the longest known. Homo-repeat-containing proteins make up 35.7% of the proteome of P. falciparum, although the majority of these homorepeats are asparagines and lysines (48), unlike the polymers of acidic residues present in PfCK2α. One hypothesis for the function of this extension is the downregulation of the alpha subunit. Polyglutamate is a potent CK2 inhibitor (50), and the N-terminal extension of PfCK2α is rich in polyglutamate and polyspartate. This beta subunit also possesses an insertion of extra acidic residues (including a stretch of 11 consecutive aspartates) (Fig. 1) in the acidic domain known to downregulate CK2 (32). We have not been able to purify PfCK2β with the N-terminal extension, and therefore this hypothesis remains to be tested. However, we showed that the presence of either beta subunit reduces the activity of PfCK2α toward

FIG. 7. PfCK2α can be targeted for recombination. (A) PCR screening for integration in genomic DNA (gDNA) from parasites transfected with pCAM-BSD-HA-PfCK2α revealed the presence of parasites in which integration events had occurred. Lane 1, amplification of the wild-type locus (primers 1 and 2; expected size, 1,176 bp). Lane 2, amplification over the 5’ integration boundary (primers 1 and 4; expected size, 1,973 bp). Lane 3, amplification over the 3’ integration boundary (primers 2 and 3; expected size, 810 bp). Lane 4, amplification of the insert in the pCAM-BSD-KOPfCK2α plasmid (primers 3 and 4; expected size, 610 bp). This culture still contained the wild-type locus, and therefore clonal lines were derived from the culture by limiting dilution. (B) PCR screening for integration in gDNA from one of the parasite clones revealed that the wild-type band had been lost (lane 1, 1,176 bp) and only the integration bands were seen. (D) This clonal line was further analyzed by Southern blotting. (C) A schematic of the chromosomal gene locus, the pCAM-BSD-HA-PfCK2α plasmid, and the recombinant locus, showing the locations of oligonucleotide primers used for the PCR screens (A and B). Oligonucleotide identities are listed in Table S1 in the supplemental material. HindIII sites and the expected sizes of the fragments of gDNA after restriction digestion are shown. (D) Southern blotting. Lanes: 1, untransfected 3D7 parasites; 2, PfCK2αHA clone B3; and 3, PfCK2αHA clone E1. (E) Western blot analysis showing PfCK2α expression in erythrocytic stage parasites. Protein extract from unsynchronized erythrocytic-stage P. falciparum parasites was prepared from wild-type 3D7 parasites (lane 2) and from parasites with a sequence encoding a HA tag incorporated at the 3’ end of the PfCK2α gene locus (lane 3, clone B3; lane 4, clone E1). Protein extract from unparasitized red blood cells (lane 1) and recombinant GST-PfCK2α (lane 5) were included as negative and positive controls. Two identical Western blot analyses were performed with immunopurified rabbit anti-PfCK2α antibodies (right) or with preimmune serum from the same rabbit as a negative control (left). The expected sizes of the proteins are indicated with arrows.
β-casein; such modulation of activity has been seen for other CK2s, often in a substrate-dependent fashion. For example, CK2β stimulates human CK2 activity toward topoisomerase II and p53 and inhibits activity toward calmodulin (6). Thus, the presence of two beta subunit species in *P. falciparum* (whereas there is only one in human cells) is likely to allow exquisite control of the activity of the catalytic subunit. The interactions we detected in vitro between the recombinant catalytic and regulatory subunits suggest that the *P. falciparum* CK2 subunits may form a similar holoenzyme structure to that seen in other organisms, although the stoichiometry of the complex in vivo will require detailed analysis of parasite extracts in non-denaturing conditions.

The existence of a number of discrete subpopulations of mammalian CK2 associated with different cellular compartments has been recognized (36), and it has been proposed that this is mediated by assembly of CK2 subunits as well as interaction with many other proteins. Our observation that the alpha and beta subunits possess putative signals for nuclear localization and protein export, respectively, suggests that in *Plasmodium* spp., like in other eukaryotes, CK2 may localize to a variety of compartments. Work is in progress to address this issue.

CK2α has been shown to be essential for life for a variety of organisms (24, 31, 37). We have demonstrated here that PfCK2α is required for parasite viability. We show that parasites lacking the enzyme are unable to survive or are impaired in their growth rate to such an extent that they are outcompeted by the parasites which retain wild-type genes. Our approach allows us to conclude that PfCK2α plays an important role during erythrocytic schizogony but does not provide any information about the molecular basis for essentiality. We are addressing this issue in a number of ways, including conditional expression based on a destabilization domain (4), localization (see above), and identification of interacting partners. Nevertheless, our data validate PfCK2α as a potential drug target. We have also demonstrated that PfCK2α is amenable to inhibition assays. Active HsCK2α is present in erythrocytes (53); this raises the question of selectivity of antimalarial inhibitors based on PfCK2α inhibition. We demonstrated that a small molecule inhibitor, Rottlerin, has a much lower IC₅₀ for PfCK2α than for HsCK2α. Although we have identified in Rottlerin a compound that can distinguish between the human and plasmodial CK2α enzymes, it is unlikely to represent a suitable starting point for antimalarial drug discovery, since Rottlerin has multiple targets (41) and is too weak and nonspecific an inhibitor even to be used in cellular assays (5). However, we have established that differential inhibition is possible, despite the 65% identity between the primary sequences of PfCK2α and HsCK2α, which suggests that specific inhibition of the plasmodial (versus host) enzyme should be feasible. The level of activity of recombinant PfCK2α is such that the development of a high-throughput assay should be possible, opening the way for screening of chemical libraries as a first step toward antimalarial drug discovery based on PfCK2α inhibition.

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**FIG. 8.** PfCK2α can be distinguished from HsCK2α by small molecule inhibitors. Two small molecules, ML-7 and Rottlerin, were identified in a primary screen as inhibiting the activity of PfCK2α to below 10% of the uninhibited enzyme. The inhibitors Rottlerin and ML-7 were included in increasing concentrations in kinase assays with 25 μM ATP, 36 ng of enzyme, and the peptide RRRDEESDDEE as substrate. Activity was measured using the phosphocellulose assay method, and results were scored as a percentage of the control (no inhibitor). (A) ML-7. (B) Rottlerin. Mean values from two experiments are shown, with the error bars representing the standard deviations. (C) The classical CK2 inhibitor, TBB, has a similar inhibitory profile for PfCK2α and HsCK2α.

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