Functional Analysis of Protein Kinase CK2 of the Human Malaria Parasite Plasmodium falciparum†

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Protein kinase CK2 (casein kinase 2) is a eukaryotic serine/threonine protein kinase with multiple substrates and roles in diverse cellular processes, including differentiation, proliferation, and translation. The mammalian holoenzyme consists of two catalytic alpha or alpha’ subunits and two regulatory beta subunits. We report the identification and characterization of a Plasmodium falciparum CK2α orthologue, PICCK2α, and two PICCK2β orthologues, PICCK2β1 and PICCK2β2. Recombinant PICCK2α possesses protein kinase activity, exhibits similar substrate and cosubstrate preferences to those of CK2α subunits from other organisms, and interacts with both of the PICCK2β subunits in vitro. Gene disruption experiments show that the presence of PICCK2α is crucial to asexual blood stage parasites and thereby validate the enzyme as a possible drug target. PICCK2α is amenable to inhibitor screening, and we report differential susceptibility between the human and P. falciparum CK2α enzymes to a small molecule inhibitor. Taken together, our data identify PICCK2α as a potential target for antimalarial chemotherapeutic intervention.

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 rise of resistance of the parasites (especially Plasmodium falciparum, the causative agent of the most virulent form of the disease) to most available drugs. There is a clear need for further research aimed at identifying novel drug targets (43).

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. Deregulation 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 plasmodial 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 PICCK2α and both PICCK2β 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 PICCK2 subunits. Oligonucleotides were designed to amplify the PICCK2α open reading frame (ORF) from P. falciparum (clone 3D7A) cDNA. The forward 5′-GGGGGATCCATGTCGGTTAGCTCAATTAAAG-3′ and reverse 5′-GGGGGGATCCATGTCGGTTAGCTCAATTAAAG-3′ primers carried BamHI and Sall sites, respectively (underlined). Oligonucleotides were also designed to amplify the PICCK2β1 and PICCK2β2 ORFs from P. falciparum (clone 3D7A) cDNA. The PICCK2β1 forward 5′-GGGGGATCCATGTCGGTTAGCTCAATTAAAG-3′ and reverse 5′-GGGGGGATCCATGTCGGTTAGCTCAATTAAAG-3′ primers carried BglII and Sall sites, respectively (underlined). The PICCK2β2

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‡‡ Downloaded from ec.asm.org at EPLR Scientific information and libraries on April 14, 2010.
sequence has a long N-terminal extension. Oligonucleotides were designed to amplifier the PCK2 β2 sequence, lacking the N-terminal extension, from P. falciparum (clone 3D7A) cDNA. The forward (5'-GGGGGATCCATGGAGC AACAATGCTTTGGATTG-3') and reverse (5'-GGGGGTGCACTCTAGG CACTCTTCAAGGATTCCG-3') primers carried BamHI and Sall sites, respectively (underlined). The short version of PCK2 β2, lacking the N-terminal extension, was named shPCK2 β2. Catalytically inactive (“kinase dead”) PCK2 β2 was obtained by site-directed mutagenesis (K27M) of pGEX-PCK2 β2 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 pGEM-T Easy (Promega) and then subcloned into the plasmid pGEX-4T3 (GE Healthcare) to generate N-terminal glutathione S-transferase (GST) fusions. The shPCK2 β2 sequence was also inserted between the BamHI and NotI sites of the pCAM-βsd 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. Chakraborti. The pGEX-4T3 constructs were expressed in E. coli BL21(DE3) cells for 20 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 a P. falciparum (K0) plasmid pCAM-BS-DOPICK2α. A fragment from the PCK2α ORF was amplified and inserted between the BamHI and NotI sites of the pCAM-βsd plasmid (47), which contains the Aspergillus oryzae glutathione-S-deacetylase 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-PICK2α. A plasmid for in vivo episomal expression of PCK2 β2 subunits was constructed as follows: the full-length PCK2 β2 coding sequence was first inserted between the BglII and NotI sites of the plasmid pHGB (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 were selected under drug pressure (see below).

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 pCAM-βsd-hemagglutinin (HA) plasmid (42).

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 0.057 MBq [33P]ATP (370 MBq/mg, GE Healthcare), 1 μg of recombinant GST-PCK2α substrate. Reactions were carried out at 30°C for 15 min and terminated by the addition of Laemml buffer. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were dried and exposed for autoradiography.

Kinase assays were also analyzed by the phosphocellulose method (20). A final kinase assay volume of 18 μl contained 36 ng of PCK2α, 167 mM of peptide substrate, 50 mM Tris-HCl pH 7.5, 20 mM MgCl2, 25 μM ATP, 40 to 150 μM NaCl, and 0.037 MBq [33P]ATP (220 TBg/mmol, GE Healthcare). Reactions took place for 10 min at room temperature and were terminated by the addition of 60 μl of 4% trichloroacetic acid. Reaction mixtures were centrifuged at 10,000 × g for 15 min, and then 60 μl of supernatant was spotted onto 4- by 4-cm Whatman P81 phosphocellulose paper squares. The squares were washed three times for 15 min in 0.5% orthophosphate, and then the amount of radiolabeled incorporated into the peptide substrate was quantified by scintillation counting. Three peptides substrates were used in this study, the NEP peptide p6012 (RRRADIDSSDD), the custom peptide (RRREDIEESDDE), obtained from NeoMPS, and the eLP2-derivative peptide (MSGDDEMIFDTPSMKDKKKKPK) (40, 45), obtained from GeneCust (Evry, France).

The Ks of PCK2α for ATP and GTP were determined by performing kinase assays with ATP concentrations of 100 μM, 25 μM, 6.25 μM, and 1.5625 μM. Reactions were carried out in triplicate. The [γ-32P]ATP/GTP was added to the unlabeled ATP/GTP and diluted serially to ensure a constant ratio of labeled- to-unlabeled ATP/GTP. The NEP peptide RRADIDSSDD (100 μM) was used as the substrate. The Ks of PCK2α for ATP in the presence or absence of PCK2 β2 were determined by performing kinase assays in triplicate with ATP concentrations of 100 μM, 50 μM, or 25 μM, with the NEP-MS peptide (100 μM) as a substrate. Reaction mixtures contained 36 ng of PCK2α alone or in combination with 36 ng of PCK2 β2/shPCK2 β2.

Measurement of 50% inhibitory concentrations (IC50). 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. Laemml 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 according to the standard protocols 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 ADVNH KPKEYDYY. A goat anti-rabbit secondary antibody coupled to horseradish peroxidase was used at a ratio of 1:10,000. Antigen 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 hematoct on 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 mM) 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 (30 mM Tris, pH 8.0, 150 mM NaCl, 20 mM MgCl2, 1 mM EDTA, 0.5% Triton X-100, 1% Nonidet P40, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine hydrochloride hydrate, and Roche complete cocktail protease inhibitors). The lysates were cleared by centrifugation (15,000 rpm for 15 min at 4°C), and the total amount of proteins in the supernatant was measured using the Bradford assay (8).

DNA extraction and Southern blotting. Parasite cultures were lysed in 0.5% saponin. The parasite pellets were sonicated in cold phosphate-buffered saline and treated with proteinase K (150 μg/ml) and 2% SDS at 55°C for 2 h. The genomic DNA was extracted in phenol/chloroform/isooamyl alcohol (25:24:1) and precipitated in ethanol and 0.3 M sodium acetate. The DNA was digested with HindIII.

Primers used in genotype characterization. For the detection of integration of the plasmid PCK2α into genomic DNA, the following primers were used (see Fig. 6A for numbering and see Table S1 in the supplemental material for the sequences): primer 1, CK2α5primeF; primer 2a, CK2αR; primer 2b, CK2α3primeR; primer 3, pCAM-BSDF; and primer 4, pCAM-BSDR.

For the detection of integration of the plasmid PCK2α-HA, the following primers were used (see Fig. 7D for numbering and see Table S1 in the supplemental material for the sequences): primer 1, CK2αF; primer 2, CK2α3primeR; primer 3, pCAM-BSDF; and primer 4, pCAM-BSDR.

RESULTS

Bioinformatics. Phylogenetic analysis of P. falciparum protein kinases identified the PlasmoDB (http://plasmodb.org/plasmo) sequence PF11_0096 as that of a CK2 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...
FIG. 1. Alignment of PfCK2\(\beta\)1 and PfCK2\(\beta\) with Homo sapiens CK2\(\beta\). ClustalW alignments of the following proteins were performed: Homo sapiens CK2\(\beta\) (HsCK2\(\beta\); AAM50092); PfCK2\(\beta\)1 (AAN35637; PlasmoDB identifier PF11_0048); PfCK2\(\beta\)2 (CAD52554; PlasmoDB identifier PF13_0232; 39% identical to HsCK2\(\beta\)). Note the long N-terminal extension and acidic insertion sequence in PfCK2\(\beta\)2. The sequence of shPfCK2\(\beta\)2 begins (after an artificially introduced initiating methionine) with residue E156, underlined. The acidic stretch responsible for downregulation of CK2 activity and association with the plasma membrane are largely conserved in the PfCK2\(\beta\)2. The stretch of amino acids found to be necessary for the export of CK2 as an ectokinase (CK2, and a TESSEE sequence at the C terminus reminiscent of the HsCK2\(\beta\) has an insertion sequence of 30 amino acids in this location. Along with the insertion region, the acidic region mentioned in the text is also boxed (D107 to E133 of PfCK2\(\beta\)).

PfCK2\(\alpha\) possesses all 11 of the subdomains conserved across eukaryotic protein kinases (21, 22) and the majority of the conserved features of CK2\(\alpha\) subunits (2). Just downstream from subdomain II is a putative nuclear localization signal, Pro-Val-Lys-Lys-Lys-Lys-Ile, conserved across CK2\(\alpha\) homologues. PfCK2\(\alpha\) 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-Gly, whereas in the CK2 family the motif is Gly-X-Gly-X-Ser (PfCK2\(\alpha\), Gly50-Ser55). The most highly conserved amino acid motif specific to members of the CK2 family is Asp179-Trp-Gly181 (notation from PfCK2\(\alpha\); most protein kinases display Asp-Phel as 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 PfCK2\(\alpha\).

Two putative CK2\(\beta\) subunits were identified in *P. falciparum* (57), hereafter referred to as PfCK2\(\beta\)1 and PfCK2\(\beta\)2 (PlasmoDB identifiers PF11_0048 and PF13_0232, respectively). BLASTP searches using the putative PfCK2\(\beta\)1/PfCK2\(\beta\)2 amino acid sequences as queries confirmed their identities as CK2\(\beta\) orthologues. An alignment with the human CK2\(\beta\) sequence (HsCK2\(\beta\)) (Fig. 1) reveals that many of the conserved features of CK2\(\beta\) subunits, including the four cysteine residues responsible for zinc finger formation (12), are present in PfCK2\(\beta\)1 and PfCK2\(\beta\)2 (e.g., Cys117, -122, -145, and -148 for PfCK2\(\beta\)1) (Fig. 1). The human CK2\(\beta\) sequence has a well-documented CK2 phosphorylation site at the N terminus (SSEE). PfCK2\(\beta\)2 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 HsCK2\(\beta\) N-terminal phosphorylation site (MSSEE). The stretch of amino acids found to be necessary for the export of CK2 as an ectokinase (CK2\(\beta\) amino acids E20 to K33) (44) are largely conserved in the PfCK2\(\beta\) 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 (HsCK2\(\beta\) amino acids D55 to D64) (29, 32) is present in PfCK2\(\beta\)1 (D68 to D75) and extended in PfCK2\(\beta\)2 (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* CK2\(\beta\) has an insertion sequence of 30 amino acids in this location. Along with the insertion region, PfCK2\(\beta\)2 has a highly acidic and repetitive N-terminal exten-
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 p34cdc2 (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 ATP, and recombinant GST-tagged shPfCK2α was detected by an in vitro kinase assay with mixtures of human and P. falciparum alpha and beta subunits. (A) Phosphorylation of the cI2β[1-22] peptide (40, 45) by PfCK2α-His or HsCK2α in the presence of ATP and GTP. The intercept on the x axis gives 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 ATP and GTP. The intercept on the y axis gives the negative 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 intercepts on the y 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 RRRADDSDDDDD. The intercept on the x axis gives the negative reciprocal of the $K_m$. 

**FIG. 4.** PfCK2α and HsCK2β interact in vitro. To further test the interactions of the alpha and beta subunits, two substrates that are phosphorylated by the CK2 holoenzyme and not by the CK2α subunit alone were included in kinase assays with mixtures of human and P. falciparum alpha and beta subunits. (A) Phosphorylation of the cI2β[1-177] protein (27) by PfCK2α-His (lanes 1 to 4) or HsCK2α (lanes 5 to 8) alone (lanes 1 and 5) or in the presence of GST-PfCK2β1 (lanes 2 and 6), GST-shPfCK2β2, or HsCK2β was measured by kinase assays, and the amount of radiolabel incorporated into the peptide was counted by scintillation. Results are shown as the means of two experiments, with the error bars representing the standard deviations. (B) Phosphorylation of the GST-Olig2[1-177] protein (27) by PfCK2α-His (lanes 1 to 4) or HsCK2α (lanes 5 to 8) alone (lanes 1 and 5) or in the presence of GST-PfCK2β1 (lanes 2 and 6), GST-shPfCK2β2 (lanes 3 and 7), or HsCK2β (lanes 4 and 8). Top, autoradiogram; bottom, corresponding Coomassie blue-stained gel of the kinase assay.
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 CK2α family: (i) PKC2α is able to phosphorylate the CK2 substrate peptide RRRADDS DDDDDD (NEB), with a similar $K_m$ curve to that of human CK2α (Fig. 3B); (ii) CK2α enzymes are known to have a wide variety of substrates (34), and correspondingly, PKC2α (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 PKC2α is inhibited by the well-established CK2-specific inhibitor TBB (3,4,5,6-tetramobenzotriazole), with a similar $IC_{50}$ curve to that of human CK2α ($IC_{50}$ for PKC2α, 2 $\mu$M; $IC_{50}$ for HS2α, 1.5 $\mu$M) (see Fig. 8C); and (iv) it can be recruited by the human CK2β subunit to phosphorylate the eIF2α-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).

**PKC2α and the PKC2 beta subunits interact in vitro.** To assess whether the two regulatory PKC2 subunits are able to associate with PKC2α 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. PKC2α-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 ATP (Fig. 5B) or the phosphorylation of calmodulin or the peptide RRRADDSDDDDD (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-PKC2β present in the reaction mixtures (Fig. 5C).

**PKC2α is essential for completion of the erythrocytic asexual cycle.** We next wanted to determine whether PKC2α plays essential functions in parasite survival. To generate a plasmid able to disrupt the PKC2α gene, an internal fragment of the coding sequence, excluding the critical motifs Gly-X-Gly-X-X-Ser (subdomain I, involved in anchoring of the ATP molecule) and 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 blastidicin. Integration of this construct (pCAM-BSD-KOPC2α) into 3D7 parasites, integration was monitored in pCAM-BSD (47), which confers resistance to blastidicin. Integration of this construct (pCAM-BSD-KOPC2α) into the genomic locus by single-crossover homologous recombination is expected to result in a pseudodiploid configuration, where both truncated copies will be unable to express a functional enzyme, since both will lack one of the essential motifs (Fig. 6A).

After two independent transfections pCAM-BSD-KOPC2α into 3D7 parasites, integration was monitored in the blastidicin-resistant populations by PCR (Fig. 6B), using
FIG. 6. PICK2α is necessary for erythrocytic-stage parasite viability. 3D7 parasites transfected with pCAM-BSD-KOPICK2a with or without pCHD-PICK2a were analyzed by PCR and Southern blotting. (A) Disruption strategy for PICK2α. The locations of the primers used for PCR screening and the HindIII sites used in Southern blotting are shown. (B) PCR screening of genomic DNA (gDNA) from untransfected 3D7 parasites, two separate pCAM-BSD-KOPICK2α-transfected lines (KOCK2a1 and KOCK2a2), and parasites transfected with both the KO plasmid and the complementation plasmid (KOCK2a + complement). Lane 1, amplification of the wild-type PICK2α locus (primers 1 and 2A; expected size, 1,030 bp). Lane 2, amplification over the 3′ integration boundary (primers 1 and 4; expected size, 677 bp). Lane 3, amplification over the 3′ integration boundary (primers 2B and 3; expected size, 1,060 bp). Lane 4, amplification of the insert in the pCAM-BSD-KOPICK2α plasmid (primers 3 and 4; expected size, 610 bp). Evidence of integration is seen only in gDNA from the doubly transfected parasite culture (KOCK2a + complement). (C) This parasite line was cloned by limiting dilution, and the gDNA was analyzed by Southern blotting. The restriction enzyme HindIII was used to digest the gDNA, and the fragments were analyzed by Southern blotting using BSD and PICK2α as probes. Lanes: 1, untransfected 3D7; 2, KOCK2a1; 3, KOCK2a2; 4, KOCK2a2 plus complement; 5, KOCK2a2 plus complement clone E7; 6, KOCK2a2 plus complement clone G9.

primer combinations that allow discrimination between the episome, the wild-type locus, and the disrupted locus. Only the episome and the wild-type locus were detectable, with no sign of integration even after prolonged culturing (16 weeks); in contrast, we regularly observe disruption of nonessential genes 6 to 7 weeks posttransfection (for an example, see reference 42). This might be due either to the fact that the presence of an intact PfCK2α gene is crucial for parasite asexual multiplication or to the possible nonrecombinogenicity of the locus. To verify that the PfCK2α locus is indeed recombinogenic, we proceeded to transfect 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 PICK2α 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 PICK2α is essential for the parasite’s asexual cycle.

We nevertheless wanted to ascertain that PICK2α 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 Phsp86 promoter and preceding a 3′ untranslated region (namely, the Plasmodium berghei dihydrofolate reductase terminator sequence). The P. falciparum hsp86 gene (PF07_0029) displays a similar mRNA expression profile to the PICK2α gene (28); therefore, its promoter is presumably appropriate to drive expression of the complementing protein. In parallel with the transfection of the pCAM-BSD-KOPICK2α plasmid alone, further populations of parasites were cotransfected with both pCAM-BSD-KOPICK2α 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 PICK2α is essential to viability of the asexual erythrocytic stage parasites. PICK2α kinase activity is amenable to inhibition. Using a kinase-directed inhibitor library, we conducted a screen for compounds that inhibit PICK2α. This screen identified the compounds Rottlerin and ML-7 as inhibitors of PfCK2α. The IC50 of these compounds were determined for both PfCK2α and HsCK2α (Fig. 8). While ML-7 inhibits both enzymes with an IC50 of roughly 3 to 4 μM, Rottlerin exhibits differential effects on the orthologues, inhibiting PICK2α with an IC50 of 7 μM and HsCK2α with an IC50 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 PfCK2 subunits and the HsCK2 subunit, is inhibited by the classic CK2 inhibitor TBB (3,4,5,6-tetrabromobenzotriazole), with a similar IC50 to that of HsCK2, and is able to utilize GTP or ATP as a cosubstrate. We have also confirmed the identity of two PfCK2 subunits.

The N-terminal extension of PfCK2 is unusually long for CK2 proteins, with 160 amino acids before the first conserved residue (Trp161 in PfCK2). Most CK2 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 PfCK2. 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 PfCK2 is rich in polyglutamate and polyaspartate. 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 PfCK2β2 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
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 PfCK2 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 IC_{50} 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 non-specific 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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