PfPK6, a novel cyclin-dependent kinase/mitogen-activated protein kinase-related protein kinase from *Plasmodium falciparum*

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We have isolated a novel protein kinase cDNA, PfPK6, by differential display RT-PCR (DDRT-PCR) of mRNA obtained from different asexual erythrocytic stages of *Plasmodium falciparum*, which shows sequence similarity to both cyclin-dependent kinase (CDK) and mitogen-activated protein kinase (MAPK) family members. The 915 bp open reading frame (ORF) is interrupted by seven introns and encodes a 305-residue polypeptide with a predicted molecular mass of 35848 Da. Several cDNA clones with some of the intron sequences were isolated, indicating alternate or defective splicing of PfPK6 transcripts because the gene seems to be a single copy located on chromosome 13. The similarity of the catalytic domain of PfPK6 to those of CDK2 and MAPK is 57.3 % and 49.6 %, respectively. The signature PSTAIRE (single-letter amino acid codes) CDK motif is changed to SKCILRE in PfPK6. The TXY residues that are phosphorylated in MAPKs for their activation are T¹⁷³PT in

PfPK6. Three size classes of PfPK6 transcripts of 6.5, 2.0 and 1.1 kb are up-regulated during the transition of *P. falciparum* from ring to trophozoite. Western blot analysis suggested the expression of a 35 kDa polypeptide in trophozoites and schizonts. Immunofluorescence studies indicated both nuclear and cytoplasmic localization of PfPK6 in trophozoite, schizont and segmenter stages. *In vitro*, recombinant PfPK6 phosphorylated itself and also exogenous substrates, histone and the small subunit of the malarial ribonucleotide reductase (R2). The kinase activity of PfPK6 is sensitive to CDK inhibitors such as olomoucine and roscovitine. PfPK6 showed a preference for Mn²⁺ over Mg²⁺ ions as a cofactor. The Lys³⁸ → Arg mutant is severely defective in its interaction with ATP and bivalent cations and somewhat defective in catalytic rate for R2 phosphorylation.

Key words: differential display, malaria parasite.

INTRODUCTION

Plasmodium falciparum, the causative agent of human malaria, undergoes distinct morphological changes during the progression through its life cycle in the mosquito and human hosts. The parasite forms known as merozoites are released into the bloodstream after the infection of a hepatocyte, invade erythrocytes, and mature through several distinct forms. The stages of development in erythrocyte are rings (initial stage after invasion), trophozoites (mononucleated metabolically active cells accumulating haemozoin pigment), schizonts (parasites undergoing multiple rounds of DNA replication and nuclear divisions forming a single syncytial cell containing 8–32 nuclei) and segmenters (cells containing individual merozoites that are formed by budding off nuclei and organelles from the parasite mass). A clear understanding of the biochemical changes and molecular switches that regulate the developmental stages of the malaria parasite has not been achieved. Of particular interest would be the elucidation of the molecular mechanisms that produce 8–32 merozoites from a single cell.

Although the correlation between the malaria parasite erythrocytic stages and the classical eukaryotic cell cycle phases is not known, mature trophozoites exhibit peak DNA synthesis and might correspond to S-phase [1]. Homologues of several genes essential for DNA replication and cell cycle progression in other model systems have also been identified in *P. falciparum* [2]. PfPK5, a putative homologue of p34^{cdc2} cyclin-dependent kinase (CDK) required for entry into both S-phase and mitosis in fission

yeast, has been characterized in *P. falciparum* with 60 % identity with human cdc2 [3]. PfPK5 phosphorylates histone H1, and possesses the PSTTIRE motif (single-letter amino acid codes) closely related to the PSTAIRE motif that is known to be implicated in cyclin binding in other CDKs. Furthermore, PfPK5 is activated on phosphorylation of Thr¹⁵⁸, and co-localizes with the DNA stain TOTO1 at the onset of nuclear division [3–5]. Pfcrk1, a homologue of negative cell cycle regulator p58^{GTA}, was isolated in *P. falciparum* by using degenerate oligonucleotides designed against conserved domains of the cdc2 family of genes [6]. Pfcrk3, a kinase showing identity with both CDKs and mitogen-activated protein kinase (MAPK) kinases was also identified similarly (C. Doerig, unpublished work). Pfmrk, a homologue of MO15/CDK7 CDK-activating kinase, has been isolated [7]. Both Pfcrk1 and Pfmrk transcripts accumulate preferentially in gametocytes, the sexually differentiated intra-erythrocytic forms that are responsible for transmission of the parasite to the mosquito [6,7]. The isolation of these kinases implies that, similarly to other organisms, *P. falciparum* might use a large number of cdc2/CDK-related kinases for cell growth and differentiation. However, all of these kinases have been identified on the basis of similarity to database sequences; their actual cellular function or physiological substrates have not been defined yet. The control of cell growth and differentiation is likely to be intimately linked to the regulation of DNA replication. The genes encoding DNA polymerases α and δ and the polymerase δ accessory factor PCNA have been isolated in *P. falciparum* [8,9]. The subunit cDNA species for ribonucleotide

Abbreviations used: DDRT-PCR, differential display reverse-transcriptase-mediated PCR; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated protein kinase; MAPK, mitogen-activated protein kinase; ORF, open reading frame.

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reductase, which plays a key rate-limiting role in DNA synthesis by supplying a continuous balanced supply of dNTPs for the replication and repair of DNA, have also been cloned [1].

In an effort to develop a model for cell cycle progression in the malaria parasite, we have undertaken an initiative to characterize molecules that are involved in regulating the entry into S and M phases. We have used the reverse-transcriptase-mediated PCR-based differential display of mRNA (DDRT-PCR) to identify genes that are up- or down-regulated at various stages of the *P. falciparum* intraerythrocytic cell cycle [10]. One of the cDNA species identified by DDRT-PCR of mRNA samples from parasites undergoing the transition from ring to schizont stages showed identity with CDKs and MAPKs. Here we report the sequence analysis, genomic organization stage-specific expression of this protein kinase, which we have named PfPK6. Overexpression of PfPK6 in *Escherichia coli* allowed us to study the biochemical properties of the recombinant enzyme. In addition, immunolocalization and immunoblot analysis was done to localize PfPK6 protein in various developmental stages.

EXPERIMENTAL

Parasite culture and extract

P. falciparum strain Dd2 was maintained in a modified erythrocyte culture [11,12]. Cultures were synchronized in two steps: (1) purification of schizonts by flotation over 65% (v/v) Percoll (Pharmacia) followed by incubation with fresh erythrocytes (5% haematocrit); (2) the cultures were left to mature into rings and treated with 5% (w/v) D-sorbitol for 15 min at 37 °C [13]. Parasitized erythrocytes were treated with 0.1% saponin to isolate *P. falciparum* cells free from erythrocytes. Cell-free extracts were prepared from *P. falciparum* cell pellet as described previously [14].

Nucleic acid isolation and blot analyses

Genomic DNA and RNA were isolated from *P. falciparum* cells as described [9]. Southern and RNA blots were hybridized with randomly primed ³²P-labelled probes by standard methods [15].

DDRT-PCR

DDRT-PCR was done essentially in accordance with instructions provided with the RNImage kit (GeneHunter Corp.). Total cytoplasmic RNA isolated from synchronized parasites during the transition from ring to schizont stages at 6 h intervals was reverse-transcribed with three single base-anchored 3'-end oligo(dT) primers; 18 random upstream primers (RNImage kit; GeneHunter Corp.) were used in combination with the three downstream primers to amplify cDNA species representing a subset of mRNA species. These primer combinations enabled us to use 54 combinations of primer per sample. Amplified cDNA species were separated on a 6% (w/v) polyacrylamide/urea gel and differences in band patterns were compared; 63 cDNA fragments (150–400 bp) were identified that were regulated differentially during the transition of parasite from trophozoite to schizont stages. These fragments were recovered from gels, re-amplified by PCR, cloned into pGEM-T-Easy cloning vector (Promega) and used for further analysis.

Screening of cDNA library

A *P. falciparum* Dd2 cDNA library in λZAPII (Stratagene) was screened with ³²P-labelled probes as described previously [9,16]. Approx. 2×10^5 plaque-forming units were screened and positive clones were plaque-purified. *E. coli* colonies harbouring recom-

binant pBluescript phagemids were obtained by *in vivo* excision with ExAssist helper phage (Stratagene). Inserts were analysed by restriction analysis and cross-hybridization.

DNA sequence analysis

A capillary electrophoresis-based sequencer (ABI Prism 310 Genetic Analyser; ABI) was used for automated DNA sequencing. A reaction kit from ABI (ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit), ABI Prism 310 capillaries (61 cm × 50 μm internal diam.) and POP-6 polymer for long-read sequencing were used in accordance with the manufacturer's protocol. Raw sequence outputs were processed by DNASTAR sequence analysis software and were subjected to a database search with the BLAST and FASTA algorithms. Multiple sequence alignments were done with the PILEUP and PRETTY programs of the Genetics Computer Group package.

Pulsed-field gel electrophoresis

Chromosomes were separated on a 1% (w/v) fast lane agarose gel (FMC) in 1 × TAE (40 mM Tris/acetate/1 mM EDTA) with a Bio-Rad CHEF DRII system. Gels were run at 100 V for 96 h with a pulse time ramped from 360 to 800 s.

Recombinant PfPK6 expression and purification

Two oligomers (5'-GACGACGACAAGATGAATAGAATTG-3' and 5'-GAGGAGAAGCCCGGTTTAAAAATTATAAG-3') were synthesized (Life Technologies) to amplify the 915 bp coding sequence of PfPK6 into pET30LIC (Novagen). Each primer contained at its 5' end a specific sequence for ligation-independent cloning. They were used in a PCR mixture containing 100 μM dNTPs, each primer at 0.5 μM, 1.5 mM MgCl₂ and 1.25 units of *Taq/Pfu* polymerase (20:1); 30 cycles were performed as follows: five cycles of 95 °C for 10 s/48.8 °C for 30 s/72 °C for 2 min followed by 25 cycles of 95 °C for 10 s/65 °C for 30 s/72 °C for 2 min. The PCR product was then purified on Qiaquick columns (Qiagen) to remove any free nucleotides, eluted in TLowE buffer [10 mM Tris/HCl (pH 8.0)/0.1 mM EDTA], and treated with T4 DNA polymerase in the presence of only dATP to create the 5' overhangs for subsequent cloning. The annealing to the pET-30 LIC vector was performed at 22 °C for 5 min. For screening of recombinant pET30-PfPK6 plasmids, NovaBlue cells (Novagen) were used and once a positive clone was identified and confirmed by sequencing, it was transformed into BL21(DE3) cells for expression.

Overnight cultures of pET30-PfPK6 in BL21(DE3) were diluted 1:50 in fresh medium and incubated at 37 °C until *D*₆₀₀ reached 0.6, when the culture was induced with isopropyl β-D-thiogalactoside (IPTG) (0.5 mM). After induction at 26 °C for 3 h, the cells were harvested and washed once in 50 mM Tris/HCl (pH 7.5); the pellet was stored at –80 °C. To purify recombinant PfPK6, the frozen pellet was resuspended in a buffer containing 20 mM Tris/HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole and 0.1% Nonidet P40. The cell suspension was chilled and sonicated with 10 pulses of 30 s each. The lysate was centrifuged at 30 000 g for 30 min. The resulting supernatant containing soluble proteins was filtered through a 0.45-μm-pore-size low-protein-binding membrane (Gelman Sciences).

Overexpressed PfPK6 was affinity-purified on a nickel column (Novagen), taking advantage of the His₆-tag (hexahistidine tag) attached to the N-terminal of the expressed protein. After elution, the protein was dialysed for 4 h against buffer A [50 mM

Tris/HCl (pH 7.4)/300 mM NaCl] followed by 4 h of dialysis against buffer B [50 mM Tris/HCl (pH 7.4)/15 mM MgCl₂/1 mM dithiothreitol/50 % (v/v) glycerol] and then stored at -80 °C.

Kinase assay

Unless indicated otherwise, the standard kinase assay reaction (20 µl) contained 1 µg of PfPK6 (in buffer B), 15 µM (5 µCi) [γ -³²P]ATP or [γ -³²P]GTP of the same specific radioactivity (3000 Ci/mmol), 15 mM MgCl₂ or MnCl₂, 1 mM dithiothreitol, 5 mM EGTA and 15 µM ATP. Reactions were initiated by adding PfPK6 to the reaction and incubated at 30 °C for 15 min. The kinase reaction was terminated by the addition of Laemmli SDS/PAGE sample buffer; it was then boiled for 5 min and analysed by SDS/PAGE [10 % (w/v) gel] in Tris/glycine buffer, pH 8.3. Dried gels were subjected to autoradiography and analysed with a PhosphorImager (STORM; Molecular Dynamics) where necessary. The catalytic parameters were measured under standard assay conditions and calculated by the Eadie-Hofstee method [17,18].

Site-directed mutagenesis

Mutagenesis was performed as described in the protocol for the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) and confirmed by sequencing.

Western blotting

Partly purified protein extracts [0–40 % -satd (NH₄)₂SO₄ precipitation of the 100 000 g supernatant] of each of the three parasite stages (ring, trophozoite and schizont) were analysed by SDS/PAGE [10 % (w/v) gel], then transferred to Hybond[®] ECL[®] enhanced chemiluminescence membrane (Amersham). The membrane was then probed with PfPK6 rabbit polyclonal antibody (Cocalico Biologicals, Reamstown, PA, U.S.A.) and signals were detected with an ECL[®] Western Blotting Analysis System (Amersham).

Immunofluorescence microscopy

Immunofluorescence microscopy of thin films of erythrocyte infected with *P. falciparum* (Dd2 strain) was done essentially as described [19]. Slides were mounted in PBS/glycerol mounting solution containing nuclear stain (Citifluor with Hoechst 33342) and were viewed on a Zeiss Axioplan 2 fluorescence microscope equipped with a Hamamatsu colour chilled three-charge-coupled-device camera.

Inhibitors and assessment of anti-malarial activities

Chemical inhibitors of CDKs were gifts from Dr Laurent Meijer (CNRS, Roscoff cedex, France). Potential anti-malarial activities of CDK inhibitors were evaluated in synchronous *P. falciparum* Dd2 infected erythrocyte cultures as described previously [1].

RESULTS

Identification of a transcript up-regulated during erythrocytic differentiation of the malaria parasite

Total RNA from synchronized malaria parasites was isolated at 6 h intervals starting from the ring stage of the erythrocytic life cycle and used as a template for DDRT-PCR. cDNA bands whose intensity was up-regulated in the trophozoite were selected for further characterization. One of these bands, a 209 bp

fragment, showed a significant increase in expression in the trophozoite stage (Figure 1A). RNA blot analysis revealed a major transcript of 1.1 kb and two minor transcripts of 2.0 and 6.5 kb (Figure 1B). In addition, there might be several minor transcripts between 2.0 and 1.1 kb size classes, as evident from the broadness of these bands. All size classes of transcripts exhibited expression that peaked in the trophozoites, then decreased progressively with time during maturation of the trophozoites into schizonts. This transcript, which is up-regulated in trophozoites and down-regulated in schizonts, could therefore correspond to a gene regulating S-phase function in *P. falciparum*.

New *P. falciparum* protein kinase related to CDK and MAPK

The sequence analysis of the differentially expressed cDNA fragment showed significant similarity to CDKs and MAPKs. To obtain a full-length cDNA, the partial cDNA was used to screen a λ ZAP phage library generated from *P. falciparum* Dd2 cells. Sequence analysis of a cDNA clone with a 1.1 kb insert revealed an open reading frame (ORF) encoding a protein of 305 amino acid residues, with a predicted molecular mass of 35.8 kDa. The sequence upstream of the methionine start codon is highly AT-rich, typical of *P. falciparum* intergenic regions. In the absence of any apparent splice sequences in this region and having stop codons in all three reading frames, we concluded that this methionine codon is the translational initiation codon. PfPK6 cDNA sequence was aligned with CDK and MAPKs by using Genetics Computer Group programs (Figure 2A). PfPK6 contained a 286-residue catalytic domain similar to that in eukaryotic protein kinases. The identity of PfPK6 ORF with that of human CDK2 and human p38 MAPK was 38 % and 33 % respectively. PfPK6 is also identical with the Pfcrk-3 sequence, which has been recently submitted (by C. Doerig) to the database. The presence of DIKPEN and GTLWYRAPE motifs in subdomains VI and VIII respectively are consistent with the consensus sequence (DLKPEN and GT/SXXY/FXAPE) for serine/threonine kinases [20]. PfPK6 contained 14 of 15 conserved residues found in the catalytic domains of eukaryotic kinases including the GXGXXG (glycine loop) ATP-binding subdomain I motif and the invariant lysine in the catalytic subdomain II [20]. Of these 15 signature residues, only the phenylalanine in the DFG motif in subdomain VII is replaced by a leucine, a conservative change. Several conserved residues whose phosphorylation regulates vertebrate CDK activity, such as Thr¹⁴, Tyr¹⁵, Thr¹⁶¹ and Ser²⁷⁷, are also conserved in PfPK6 (Thr¹⁹, Tyr²⁰, Thr¹⁷³ and Ser²⁸³). Among these, the phosphorylation of Thr¹⁴ and Tyr¹⁵ has been shown to regulate the activity of vertebrate CDKs negatively, whereas phosphorylation of Thr¹⁶¹ is essential for full activation [21]. A number of critical domains known to be important for the binding of CDK-activating CKS proteins are also conserved in PfPK6, e.g. K²⁵ALDKK, R⁸²APE, D²⁷⁷PNYR (Figure 2A).

Interestingly, although PfPK6 contains a motif very similar to the cdc2/CDC28 family consensus sequence EV/IVTLWY in subdomain VIII (TVVTLWY in PfPK6), it lacks the canonical PSTAIRE sequence that has been implicated in binding regulatory cyclin subunits [20,22]. PfPK6 instead contains the sequence SKCILRE at the same location. PfPK6 also exhibits significant sequence identity with MAPKs. However, in comparison with MAPKs, PfPK6 is smaller in size and the TXY motif between kinase subdomains VII and VIII that is involved in the activation by MAPK/ERK kinases (MEKs) through the phosphorylation of both threonine and tyrosine is changed to TPT.

Figure 1 Differential mRNA display and stage-specific expression of PfPK6

(A) Total RNA was extracted from parasites collected at 6 h intervals from a highly synchronized culture during transition from ring to schizonts (a total of five different time points). The 209 bp fragment that yielded PfPK6 is indicated with an arrow. RNA samples were subjected to differential display by using five sets of primer pairs. (B) For Northern blot analysis, 20 µg of RNA was used per lane. Lane 1 (24 h after synchronization) corresponds to the schizont stage, lanes 2 and 3 the transition from schizont to segmenter, lane 4 the ring stage, lane 5 the early trophozoite stage, and lanes 6–8 trophozoite stage parasites. A representative parasite from each time point is pictured in the top panel. The bottom panel shows an ethidium bromide-stained RNA sample, indicating equal loading.

A phylogenetic tree constructed from different CDKs and MAPKs indicated that PfPK6 branches off between the CDK and MAPK family of enzymes, further supporting its relatedness to both groups of kinases (Figure 2B). Molecular modelling of PfPK6 by using the Look[™] program with human CDK2 and murine extracellular signal-regulated protein kinase 2 (ERK2) indicated that PfPK6 seems to be structurally more closely related to cdc2-related kinases than to MAPKs (results not shown).

Genomic organization of PfPK6

While screening the cDNA library for full-length PfPK6, we isolated a few clones containing intron-like sequences. These could have resulted from the presence of gene families with minor variations or pseudogenes. Another possibility could be the existence of defective or alternately spliced transcripts. A Southern blot of *P. falciparum* genomic DNA digested with restriction enzymes with full-length PfPK6 as a probe produced

A

B

Figure 2 Amino acid sequence comparison and phylogenetic analysis

(A) Comparison of the deduced PfPK6 amino acid sequence with human CDK2 (accession number P24941) and P38 MAPK (accession number Q16539) sequences in the National Center for Biotechnology Information database by using the PRETTY program of the Genetics Computer Group package. The protein kinase catalytic domains are indicated by roman numerals as described by Hanks and Hunter [20]. Positions where the amino acids are identical with those in PfPK6 are marked with a dash. The 15 amino acids that are conserved in all eukaryotic kinases are shown in bold and underlined. Asterisks indicate amino acids that are involved in the regulation of CDK activity. The black and grey bars indicate the PSTAIRE motif and the 15-residue insertion in subdomain IV respectively. (B) Phylogenetic tree of CDKs and MAPKs by using the Clustal method (DNASTAR program) with the PAM250 residue weight table. The scale beneath the tree measures the distance between sequences; its units are the number of substitution events. The organisms and the GenBank accession numbers of the sequences are as follows: human, H.s. CDK2, P24941; H.s. CDK1, A29539; H.s. P38 MAPK, Q16539; *Saccharomyces cerevisiae*, S.c. HOG1, S64950; *P. falciparum*, PIPK5, Q07785; PIPK6, AAC61592; P.f. MAPK, JC5153.

only a single band in the autoradiograph (results not shown). This banding pattern is consistent with a single-copy gene for PfPK6. Blots of malaria parasite Dd2 chromosomes separated by pulsed-field gel electrophoresis showed a signal corresponding to chromosome 13. Preliminary Sanger Center sequence data from *P. falciparum* 3D7 confirmed the localization of PfPK6 to chromosome 13 in this strain too. Although this supports the notion of a single-copy PfPK6 gene, it does not exclude the possibility of a multiple copy localized on a single chromosome. Analysis of the genomic sequence from Dd2 and 3D7 strains revealed that unlike most malarial genes containing few or no introns, PfPK6 is arranged in eight exons separated by seven introns.

Enzymic activity of recombinant PfPK6

In an effort to characterize PfPK6 functionally, the ORF of the enzyme was cloned into the T7 polymerase-driven expression vector pET30. On the induction of *E. coli* BL21(DE3) harbouring pET30-PfPK6 with isopropyl β -D-thiogalactoside, a polypeptide with a molecular mass of approx. 40 kDa was overexpressed, which is in agreement with the predicted molecular mass of 35.8 kDa for PfPK6 and 4.5 kDa for the His and S-tag leader sequences. The His₆-tagged fusion protein was purified to near-

homogeneity with a nickel-affinity column (Figure 3A). The recombinant PfPK6 phosphorylated histone H1 as well as itself (Figure 3B). The phosphorylation was completely inhibited by the addition of EDTA (Figure 3B). Interestingly, this phosphorylation of PfPK6 was independent of association with a cyclin. The binding of cyclin to cdc2-related kinases induces the phosphorylation of Thr¹⁶¹, which is a prerequisite for activity [23]. It should be noted that although the recombinant enzyme showed significant activity, the expression of PfPK6 in bacteria would result in a uncomplexed/unphosphorylated enzyme that was potentially less than 1% as active as the native malarial enzyme, in which it is associated with a cyclin. A kinase assay *in vitro* showed that PfPK6 preferred Mn²⁺ to Mg²⁺ as the bivalent cation and ATP to GTP as the phosphate donor (Figure 3C). Because the small subunit (R2) of mammalian ribonucleotide reductase is phosphorylated *in vitro* by p34^{cdc2} [24], we decided to examine whether the malarial recombinant R2 is a substrate for PfPK6 too. As can be seen from Figure 3(D), PfPK6 can indeed phosphorylate malarial R2.

Mutational analysis of recombinant PfPK6

In an effort to establish the identity of PfPK6 cDNA further, we altered the critical Lys residue at position 38 to Arg through site-

Figure 3 Analysis of recombinant PfPK6

(A) Coomassie Blue-stained gel of purified His fusion derivative of PfPK6. Lane MW, molecular mass standards (molecular masses are shown at the left). (B) Recombinant PfPK6 (1 μ g) autophosphorylation was assayed in a standard kinase reaction with [γ - 32 P]ATP without any exogenous substrate (lanes 1 and 2) or with histone H1 (lane 3) and analysed by SDS/PAGE [10% (w/v) gel]. The upper panel was stained with Coomassie Blue, the lower panel shows the corresponding autoradiograph. PfPK6 was autophosphorylated *in vitro* (lane 1), a reaction inhibited by EDTA (lane 2). (C) Effects of various bivalent cations, ATP and GTP on the autophosphorylation of PfPK6. Autophosphorylation of PfPK6 was tested in a standard kinase assay with [γ - 32 P]ATP (lanes 2–5) or [γ - 32 P]GTP (lane 1) and either Mg^{2+} (15 mM, lanes 1 and 2), Mn^{2+} (15 mM, lane 3), Zn^{2+} (1 mM, lane 4) or Ca^{2+} (1 mM, lane 5). The upper part is Coomassie Blue-stained and the lower part is the corresponding autoradiograph. (D) The phosphorylation of R2 subunit by purified recombinant PfPK6 was assayed in a standard kinase assay with [γ - 32 P]ATP. The upper panel is the Coomassie Blue-stained gel, the lower panel is the corresponding autoradiograph. Purified recombinant R2 protein was loaded alone (lane 1) and in the presence of PfPK6 (lane 2). The location of PfPK6 protein is indicated by an arrow in all panels.

Table 1 Catalytic parameters of PfPK6 and its Lys³⁸ $\rightarrow$ Arg mutant

Recombinant wild-type and mutant PfPK6 were purified and assayed under standard assay conditions as described in the Experimental section. The values are means $\pm$ S.E.M. for three reactions.

PfPK6 variant	$K_m^{Mg^{2+}}$ (μ M)	K_m^{ATP} (μ M)	K_m^{R2} (nM)	$100k_{cat}$ (s^{-1})	k_{cat}/K_m^{R2} ($s^{-1} \cdot \mu M^{-1}$)
Wild-type	25 ± 2	15 ± 1.0	510 ± 30	720 ± 38	14.11
Lys ³⁸ $\rightarrow$ Arg	1020 ± 12	1325 ± 16	540 ± 35	125 ± 15	2.31

directed mutagenesis *in vitro* and tested the effect of the mutation on the various catalytic parameters of the recombinant enzyme. On the basis of homology with other protein kinases and structural analysis, this invariant Lys residue in subdomain II has been predicted to be directly involved in the phosphotransfer reaction [20]. Results presented in Table 1 show that the K38R mutant is highly defective in its interaction with ATP as well as with the bivalent cations Mg^{2+} and Mn^{2+} . The mutation also affected the k_{cat} of the enzyme (about one-sixth that of the wild type); however, its affinity (K_m) for the substrate R2 protein remained essentially unchanged. The net result was a decrease to approx. one-seventh in the ratio of k_{cat} to K_m , which resulted in a substantially inactive enzyme.

Effect of chemical inhibitors of CDKs on PfPK6 activity

Kinases are often characterized by their sensitivity to specific diagnostic inhibitors. Several specific inhibitors of CDKs have been described, of which roscovitine [2-(1-D,L-hydroxy-methylpropylamino)-6-benzylamino-9-isopropylpurine] and olomoucine [2-(2-hydroxyethylamino)-6-benzylamino-9-methyl-purine] show a remarkable selectivity for some of the members of the *cdc2*-related kinases [25,26]. In fact, these inhibitors have

been used to classify the CDK family into two groups: CDK1, CDK2 and CDK5 are sensitive, whereas CDK4 and CDK6 are insensitive. Furthermore, CDK1, CDK2 and CDK5 are at least 50-fold more sensitive to roscovitine than are the MAPKs ERK1 and ERK2 [25]. As is evident from Figure 4(A), increasing the concentration of both olomoucine and roscovitine causes the progressive inhibition of autophosphorylation of PfPK6 in a kinase assay *in vitro*. However, the IC_{50} value for roscovitine (30 μ M) is one-sixth that of olomoucine (180 μ M). No inhibition was observed with an inactive isomer, iso-olomoucine [6-benzyl-amino-2-(2-hydroxyethylamino)-7-methylpurine], which served as the negative control. It is of interest that the IC_{50} of PfPK6 for roscovitine is closer to that of ERK1 (30 μ M) and ERK2 (14 μ M) than to that of *cdc2* (0.65 μ M), CDK2 (0.7 μ M) or CDK5 (0.16 μ M).

Roscovitine was also significantly more inhibitory than olomoucine to growth of *P. falciparum* in an erythrocyte culture system. The inhibition of growth of *P. falciparum* in terms of inhibition of DNA synthesis was measured by the incorporation of [3 H]hypoxanthine. As shown in Figure 4(B), the IC_{50} values for inhibition of the malaria parasite growth for roscovitine and olomoucine are 25 and 130 μ M respectively. Iso-olomoucine did not show any significant inhibition.

Stage-specific expression and immune localization of PfPK6 protein

To study the expression of PfPK6 protein *in vivo*, we raised polyclonal antibodies in rabbit with recombinant PfPK6 as the immunogen. Extracts were prepared from synchronized parasites in the ring, trophozoite and schizont stages of erythrocytic growth and were subjected to Western blot analysis with anti-PfPK6 polyclonal antibody. A polypeptide with a molecular mass of approx. 35 kDa was expressed only in the trophozoite and schizont stages (Figure 5A). The molecular mass of PfPK6 protein expressed *in vivo* is in agreement with that deduced from the cDNA sequence.

Figure 4 Effects of CDK inhibitors

(A) Effects of CDK inhibitors on the autophosphorylation of Pfpk6. The inhibitors olomoucine and roscovitine were added to a standard protein kinase assay at various concentrations. Lane 1 is untreated; lanes 2, 5 and 8; 3, 6 and 9; and 4, 7 and 10 are results with inhibitors at 10, 30 and 300 μM respectively. Iso-olomoucine was used as a control. The upper panel is the Coomassie Blue-stained gel and the lower panel the corresponding autoradiogram. (B) Effects of the CDK inhibitors olomoucine (●) and roscovitine (○) on *P. falciparum* growth *in vitro*; iso-olomoucine (△) was used as a control. DNA synthesis (measured by the incorporation of [³H]hypoxanthine; see the Experimental section) was used to monitor malarial parasite growth. The inhibitors and control were used on synchronized (at the ring stage) parasite cultures at 0, 10, 25, 50, 100 and 200 μM, in triplicate. Bars indicate S.D.

Because subcellular localization of CDKs is tightly regulated [27], Pfpk6 localization was analysed by immunofluorescence. Consistent with the Western blotting results was the observation that the expression of Pfpk6 was detected mainly in the trophozoite and schizont stages (Figure 5C); it was also detected in the segmenter stage (Figure 5D) and at a low level in the late ring stage (Figure 5B). Uninfected erythrocytes were not labelled with the antibody. In addition, no fluorescence was observed by using preimmune serum (Figure 5A). Interestingly, Pfpk6 seems to exhibit localization in both the cytoplasm and the nucleus (Figures 5C and 5D).

DISCUSSION

Results reported here provide evidence that Pfpk6 is a novel kinase. The presence of DIKPEN and TLWYRAPE motifs in subdomains VI and VIII suggests that Pfpk6 is a serine/threonine kinase, yet the cofactor requirement of the recombinant Pfpk6 shows that its ability to phosphorylate itself and exogenous substrates is more efficient with Mn²⁺ than with Mg²⁺. This property is usually associated with tyrosine kinases such as epidermal growth factor receptors [28] and tyrosine kinases

phosphorylating insulin [29]. Interestingly, a human Ste20-like serine/threonine protein kinase [30] exhibited a similar co-factor requirement and, like Pfpk6, can use both GTP and ATP as the phosphate donor.

There is a large body of evidence that Pfpk6 activity is a cyclin-independent kinase. (1) The recombinant Pfpk6 shows robust kinase activity in the absence of cyclin. (2) The primary structure of Pfpk6 does not contain any sequence that is related to PSTAIRE. It seems to be unique even among *P. falciparum* CDKs because Pfpk5, the other CDK homologue that has been reported, does contain a PSTAIRE-like sequence (PSTTIRE). Additional *P. falciparum* cdc2-related kinases also contain related motifs: AMTSLRE in Pfcrc-1 (similar to PITSLRE in p58^{GTA}) [6] and NFVLLRE in Pfmrk [similar to NRTALRE for CDK-activating kinases ('CAKs')]. (3) Several other amino acid residues in the N-terminal lobe of CDKs that are involved in cyclin-binding are also absent from Pfpk6; these include [in human CDK1 (Figure 2A)] Glu⁸, Lys⁹, Glu¹², Arg³⁶, Glu³⁸ and Glu⁴². It should be noted that no cyclin has yet been identified in *Plasmodium*.

The mechanism of cyclin-independent kinase activity of Pfpk6 remains to be established. Nevertheless, on the basis of the foregoing, it is safe to say that the major changes that are normally induced by cyclin in vertebrate CDKs must be constitutively present in Pfpk6. Thus the T-loop in Pfpk6 probably does not block the entrance to the catalytic pocket of the enzyme and the ATP might be properly aligned to the substrate. It is noteworthy that Pfpk6 contains a relatively large insertion (Asp⁸⁰–Cys⁹⁴) in the L6 loop that should make this loop approx. 10 residues larger than in the other CDKs (Figure 2A). Because in the CDK structure the L6 loop is juxtaposed against the PSTAIRE helix, it is possible that the extra large L6 loop in Pfpk6 might push the PSTAIRE domain in the same manner as the binding of cyclin would [31,32]. Clearly, a determination of the three-dimensional structure of Pfpk6 will be important in ascertaining the mechanism of its cyclin-independent activity. It will also be important to investigate whether specific *P. falciparum* kinases can additionally phosphorylate Pfpk6. The availability of large amounts of recombinant Pfpk6 will facilitate these studies.

An effective way of analysing the property of an enzyme is to test its sensitivity to specific inhibitors. Inhibitors such as roscovitine and olomoucine show remarkable selectivity and have been used to assign various CDK-related kinases into groups [25]. The IC₅₀ values of Pfpk6 for roscovitine more closely resembled those obtained for the MAPKs ERK1 and ERK2 rather than CDKs; this result also demonstrates another unique property of Pfpk6. It should be noted that the IC₅₀ values of roscovitine and olomoucine for the proliferation of malaria parasite cells is similar to that obtained for kinase assays *in vitro*. IC₅₀ values in culture are usually expected to be higher because the inhibitor has to cross several membrane barriers to reach its site of action. It is possible that the inhibitor sensitivity of autophosphorylation, as measured in our assay, is different from that of the physiological phosphorylation of a substrate. However, this is probably unlikely because these inhibitors localize in the ATP-binding pocket rather than competing with substrate binding [25]. Perhaps a more plausible explanation is that one or more other intracellular targets (possibly another CDK) are more sensitive to these drugs. For example, one possible target could be the malaria CDK homologue, Pfpk5, that showed an IC₅₀ for olomoucine of 15 μM [4] in comparison with 150 μM for Pfpk6.

On the basis of our results, it seems that many factors such as cyclin association and the phosphorylation and dephos-

Figure 5 Western blot analysis and immunolocalization of PfPK6

(A) Western blot analysis of lysates from three stages of the intraerythrocytic growth of *P. falciparum*. The blot was probed with anti-PfPK6 antibody. Parasite extracts were loaded as follows: lane R, ring stage; lane T, trophozoite stage; lane S, schizont stage. (B–E) An asynchronous malarial parasite culture was immunostained with anti-PfPK6 antibody and observed under a Zeiss fluorescence microscope with the FITC channel (top panels) to detect PfPK6 or with the Hoechst channel (middle panels) to reveal the DNA. A superimposition of FITC and Hoechst channels is also shown (bottom panels). (B) Preimmune sera; (C) late ring stage; (D) trophozoite (small nucleus) and schizont (larger multinucleated); (E) segmenter.

phorylation of critical amino acid residues that regulate CDK activity might not be relevant for PfPK6. However, to have a key regulatory role in the cell cycle transition the kinase activity of PfPK6 should oscillate with the cell cycle. Interestingly, an RNA blot analysis demonstrated that the PfPK6 transcript is expressed transiently in trophozoites, the cell stage that might correspond to the onset of classical S phase. Western blot and immunofluorescence studies also showed that PfPK6 polypeptide is absent from the ring stage but could be detected both in trophozoites and schizonts, which correspond to the late G₁, S and M phases of the malaria parasite cell cycle. It therefore seems that the regulation of PfPK6 expression, unlike other CDKs, is at the transcriptional rather than the post-translational level. Subcellular localization by immunofluorescence indicated that PfPK6 is both cytoplasmic and nuclear, unlike nuclear PfPK5 [4]. PfPK5 therefore seems to fit closely to the p34^{cdc2} role as a

regulator of the cell cycle of the malaria parasite. It is tempting to speculate that other CDK-like kinases such as PfPK6 or Pfcrk-1, a developmentally regulated p58^{GTA}-related kinase found in gametocytes [6], might have important separate and distinct roles in cell cycle control. Similarly to differentiating eukaryotic cells [33], the malaria parasite might use different CDK-like enzymes to perform novel stage-specific functions. It is possible that PfPK6 protein is sequestered mostly in the cytoplasm but is imported to the nucleus to participate in any nuclear function at a specific cell stage. Without any discernible nuclear localization signal (NLS) in PfPK6 protein, the protein either has a novel NLS or associates with other proteins carrying a NLS.

Clearly, further studies are needed to characterize the true physiological substrates of PfPK6 and other CDK-related *P. falciparum* kinases to understand their precise roles in regulating the parasite's cell growth and differentiation.

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