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Activation of a *Plasmodium falciparum* cdc2-related Kinase by Heterologous p25 and Cyclin H

FUNCTIONAL CHARACTERIZATION OF A *P. FALCIPARUM* CYCLIN HOMOLOGUE*

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Several *Plasmodium falciparum* genes encoding cdc2-related protein kinases have been identified, but the modalities of their regulation remains largely unexplored. In the present study, we investigated the regulation *in vitro* of PfPK5, a putative homologue of Cdk1 (cdc2) in *P. falciparum*. We show that (i) PfPK5 is efficiently activated by heterologous (human) cyclin H and p25, a cyclin-like molecule that specifically activates human Cdk5; (ii) the activated enzyme can be inhibited by chemical Cdk inhibitors; (iii) Pfmrk, a putative *P. falciparum* homologue of the Cdk-activating kinase, does neither activate nor phosphorylate PfPK5; and (iv) PfPK5 is able to autophosphorylate in the presence of a cyclin. Taken together, these results suggest that the regulation of *Plasmodium* Cdks may differ in important aspects from that of their human counterparts. Furthermore, we cloned an open reading frame encoding a novel *P. falciparum* protein possessing maximal homology to cyclin H from various organisms, and we show that this protein, called Pfcyc-1, is able to activate recombinant PfPK5 *in vitro* with an efficiency similar to that of human cyclin H and p25. This work opens the way to the development of screening procedures aimed at identifying compounds that specifically target the parasite Cdks.

Drug resistance in malaria parasites represents a major public health problem in many parts of the world. The disease affects 400 million people every year and is responsible for 1–2 million deaths a year, mostly among sub-saharan African children (1, 2). Development of new anti-malarials has become an urgent task, which has stimulated research into the basic biology of *Plasmodium falciparum*, the species responsible for the lethal form of human malaria. We are interested in the molecular mechanisms controlling the proliferation and the development of the parasite in the human host, and in this context we investigate enzymes from the parasite involved in cell cycle control and signal transduction.

Progression of the eukaryotic cell cycle is controlled by a family of protein kinases, the cyclin-dependent kinases, whose active forms are composed of a catalytic subunit (Cdk) and a regulatory subunit (cyclin). The temporary association of both subunits results in well defined time windows during which a given Cdk is active and phosphorylates its substrates at the appropriate phase of the cell cycle. Several Cdks and cyclins coexist in eukaryotic cells, with given combinations being responsible for progression of the cell cycle through particular phases. Cdk·cyclin binding is specific in that all cyclins do not bind indiscriminately to all Cdks. Binding of the cyclin causes profound changes in the tertiary structure of the kinase, allowing access of the ATP and protein substrates to the catalytic cleft. A further level of regulation is the phosphorylation status of the catalytic subunit; in most cases cyclin binding alone results only in partial activation or no activation at all. Phosphorylation of a threonine residue in the so-called T-loop (Thr-160 in human Cdk2) by a Cdk-activating kinase (CAK)[1] results in a further modification of the structure of the catalytic subunit, rendering the enzyme fully active (in some cases, prior phosphorylation of Thr-160 is required for cyclin binding). In mammalian cells, the CAK is itself a Cdk, Cdk7, and its activity requires cyclin H (reviewed in Ref. 3–5).

Some Cdks function in cellular processes which are not directly linked with cell cycle control. Thus, Cdk5 has been purified from mammalian brain, where it is involved in the regulation of diverse neuronal functions (6). The Cdk5 kinase subunit is closely related to other Cdks but its activating partner, p25 (derived by proteolytic cleavage from a 35-kDa precursor), is very different from cyclins at the primary structure level, although its predicted three-dimensional structure is similar to that of cyclins (7, 8). p25 is not able to activate other Cdk subunits such as Cdk1 (cdc2), Cdk2, -3, -4, or -6, and thus exhibits a narrow specificity toward Cdk5 (9). Another peculiarity of Cdk5 is that it is not activated by Cdk7 like other mammalian Cdks; instead, it can be activated *in vitro* by casein kinase 1 (10).

Cdk homologues have been identified in all eukaryotes

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AJ245852.

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[1] The abbreviations used are: CAK, Cdk-activating kinase; crk, cdc2-related kinase; ORF, open reading frame; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HRP, horseradish peroxidase; PDBA, phenylboronic acid; contig, group of overlapping clones.

where they have been searched for, including malaria parasites. In line with that of other protozoan parasites (11), the *Plasmodium* genome contains several genes encoding cdc2-related kinase (crk) subunits. The first one to be described was PfPK5, a putative Cdk1 (cdc2) homologue expressed and active during erythrocytic schizogony (12, 13). Another kinase of this group, Pfcrk-1, was subsequently reported as being expressed specifically in gametocytes (14). PfPK6, an enzyme with high homology to both Cdks and the related mitogen-activated protein kinases has recently been identified.[2] A putative homologue of Cdk7, called Pfmrk, has likewise been characterized in *P. falciparum* (15), and the sequences of additional crks have been identified in the context of the *P. falciparum* Genome Project currently under way (for an example, see Ref. 16; for reviews on the cell cycle and protein kinases in parasites, see Refs. 17–19).

The precise function of these enzymes in the life cycle of the parasite still awaits determination. Another point requiring characterization is that of the biochemical mechanisms pertaining to the regulation of the activity of the *Plasmodium* crks. One question in particular comes to mind: do these enzymes require association with a cyclin-like partner and Thr-160 phosphorylation to become active? Here we report that PfPK5 interacts with mammalian p25, and we show that this interaction is accompanied by a strong stimulation of the kinase activity even in the absence of phosphorylation by Pfmrk (15), a *P. falciparum* CAK putative homologue. Furthermore, comparable activation of PfPK5 can be achieved by using mammalian cyclin H instead of p25. Finally, a *P. falciparum* sequence with maximal homology to mammalian cyclin H has been cloned and expressed in *Escherichia coli*. The recombinant protein activates PfPK5 *in vitro*, in terms of both autophosphorylation and substrate phosphorylation. We used the activated enzyme to evaluate the effect of kinase inhibitors, a first step toward the development of a high throughput screening assay for plasmodial crks.

EXPERIMENTAL PROCEDURES

Molecular Cloning—Oligonucleotides were designed to encompass the start and the stop codons of the Pfcyc-1 open reading frame (ORF), which was identified on the contig 19 in the data base of *P. falciparum* chromosome 14 (The Institute for Genome Research web site). They contained *Bam*HI (forward primer: GGGGGGATCCATGAATTATCCAGAAGATAC) and *Sal*I (reverse primer: GGGGGTCGACTTATGATACTTGTAGTTTC) restriction sites near their 5′-end. After amplification with a *Taq* polymerase (Takara), the polymerase chain reaction product was digested with *Bam*HI and *Sal*I prior to insertion into the pGEX-4T3 vector. The cloned ORF was verified by DNA sequencing prior to expression of the recombinant protein.

Expression and Purification of Recombinant Proteins—Expression and purification of GST-p25 (8), GST-Cdk5 (20), and GST-Pfcyc-1 were performed as described previously for GST-Pfmap-2 (21). For His-PfPK5, an overnight culture of the bacterial strain SG13009 harboring the PfPK5 expression construct (12) was diluted 1:50 into 800 ml of 2× YT medium containing 100 μg/ml ampicillin and 25 μl/ml kanamycin. When the A_{600} reached 0.8–1, expression of PfPK5 was induced by the addition of isopropyl-β-thiogalactoside to 0.5 mM, and the culture was grown for an additional 4 h. Cells were collected by centrifugation at 3600 × *g* for 30 min and resuspended in 5 ml of ice-cold sonication buffer (100 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM dithiothreitol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride plus the Complete™ mixture tablet from Roche Molecular Biochemicals) and 100 μg/ml lysozyme. After incubation for 10 min on ice, the cell suspension was sonicated and clarified by centrifugation at 11,300 × *g* for 30 min at 4 °C. The resulting supernatant was applied to 500 μl of packed nickel-nitrilotriacetic acid-agarose beads (Quiagen). After 30 min of incubation at 4 °C, the resin was washed three times with buffer A (sonication buffer plus 10 mM imidazole), three times with buffer B (sonication buffer plus 60 mM imidazole), followed by three additional washes with buffer A. Bound proteins were eluted twice with 250 mM imidazole in elution buffer (50 mM Tris, pH 8, 400 mM NaCl) and concentrated in Centricon tubes (Amicon). Pfmrk and human cyclin H were expressed as polyhistidine fusion proteins in the XL10 GOLD strain of *E. coli* (Stratagene) as described above for PfPK5. For purification of these proteins, the cells were centrifuged and resuspended in a modified sonication buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 5% glycerol, 1% CHAPS, 10 mM β-mercaptoethanol, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride) containing 1 mg/ml lysozyme. The procedure was as described above, except that the bound material was washed using the modified sonication buffer containing 20 mM imidazole. Pfmrk and human cyclin H were eluted using the same sonication buffer containing 250 mM imidazole. Protein concentrations were determined using the Bio-Rad dye reagent according to the manufacturer's recommendations with bovine serum albumin as a standard, and aliquots of purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining.

Kinase Assays—The assays were performed in a standard reaction (30 μl) containing 25 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 2 mM $MnCl_2$ 50 μM ATP/5 μCi of [γ-^{32}P]ATP, and 5 μg of histone H1 (Life Technologies, Inc.). Reactions were initiated by the addition of 0.5 μg each of the recombinant protein kinase and a cyclin partner after both proteins had been allowed to form a complex at 30 °C for 30 min in kinase assay buffer. The kinase reaction proceeded for 30 min at 30 °C, and it was stopped by the addition of Laemmli buffer, boiled for 3 min, and analyzed by electrophoresis on 12% SDS-polyacrylamide gel. The gels were dried and submitted to autoradiography. Phosphorylation of recombinant protein kinases was assayed in the same conditions but without histone H1. Data quantification was obtained by densitometric scanning of autoradiograms or by phosphorimaging.

Inhibition Assays—To test the effect of chemical inhibitors on kinase activity, reactions were performed in the presence of various concentrations of these molecules (stock solutions were at 10 mM in dimethyl sulfoxide), with the negative controls being reaction mixtures containing only the relevant concentration of solvent. Reactions were performed as described above, except that 15 μM ATP/0.05 μCi of [γ-^{32}P]ATP was used per reaction. 25 μl of each reaction were then spotted onto a small piece of Whatman P81 phosphocellulose paper. The paper was washed five times in 1% orthophosphoric acid, and the amount of acid-precipitable radiolabel incorporated in histone H1 was quantified by scintillation counting.

Detection of Protein-Protein Interactions—PfPK5 polyhistidine fusion protein complexed with p25-GST was labeled with horseradish peroxidase by using the Linx horseradish peroxidase (HRP) rapid protein conjugation kit (Invitrogen) as follows: first, PfPK5 (0.5 μg/μl in phosphate buffered saline, 1 mM EDTA, 1 mM PMSF, pH 7.5) reacted with phenylboronic acid (PDBA) *N*-hydroxysuccinimic acid for 1 h at room temperature. A molar ratio of 14:1 (PDBA-*N*-hydroxysuccinimic acid: PfPK5) was used to obtain PDBA-PfPK5, a derivative able to react with salicylhydroxamic acid-activated peroxidase. PfPK5-PDBA was allowed to complex with different amounts of (unmodified) p25 diluted in phosphate-buffered saline (30 min, room temperature). Typically, 60 ng of pfPK5-PDBA reacted with 6–120 ng of p25. Finally, PfPK5-PDBA·p25 complexes reacted with 0.4 μg of salicylhydroxamic acid-activated HRP. Binding of pfPK5 to p25 was demonstrated by a capture enzyme-linked immunosorbent assay, in which the GST tag of p25 was used for the capture of the p25·PfPK5·HRP complexes. A rabbit polyclonal antibody directed against GST was coated onto enzyme-linked immunosorbent assay plates (40 μg/ml in 50 mM carbonate-bicarbonate buffer, pH 8.5) and blocked with 5% bovine serum albumin. PfPK5-HRP·p25 in phosphate-buffered saline + 0.5% bovine serum albumin reacted for 30 min (37 °C) with the coated antibody, the plates were rinsed with phosphate-buffered saline + 0.05% Tween 20, and bound peroxidase was revealed by an enzymatic colorimetric reaction using orthophenyenediamine and H_2O_2 as substrates and quantified at 570 nm in a plate reader.

RESULTS

Activation of PfPK5 by Mammalian Cyclin H and p25—Recombinant PfPK5 has been shown to have a very low level ability to phosphorylate histone H1, a classical Cdk substrate (12). One of us[3] has recently shown that monomeric Pfmrk, a putative *P. falciparum* homologue of the Cdk-activating kinase Cdk7, was able to phosphorylate histone H1, and that this

[2] D. Chakrabarti and C. Doerig, unpublished data.

[3] N. Waters, unpublished data.

basal activity was increased 2-fold in the presence of recombinant human cyclin H (the activating partner of Cdk7) (22). We wanted to determine whether a mixture of Pfmrk and cyclin H would result in PfPK5 phosphorylation and activation *in vitro*. To this effect, we performed kinase assays with histone H1 as a substrate, using diverse combinations of PfPK5, Pfmrk, and human cyclin H. These experiments yielded two unexpected findings: first, the addition of cyclin H alone to the PfPK5 kinase assay was sufficient to activate PfPK5 to a considerable level; histone H1 labeling increased up to 1000-fold (Fig. 1, *lanes 5* and *8*). This activation factor varied with different preparations of recombinant proteins and was difficult to measure accurately because of the extremely low level of basal PfPK5 activity (Fig. 1, *lanes 1* and *8*). We verified that the recombinant cyclin H preparation did not contain any intrinsic kinase activity (*lane 4*). Second, the addition of Pfmrk to a reaction containing PfPK5 and human cyclin H appeared not to cause a significant further increase (other than additive) in histone H1 phosphorylation (*lane 7*), despite the fact that the Pfmrk·human cyclin H was active, as shown by its ability to autophosphorylate and to phosphorylate histone H1 (*lanes 1–3*), as previously reported (see below for a further analysis of Pfmrk effect on PfPK5 activity).

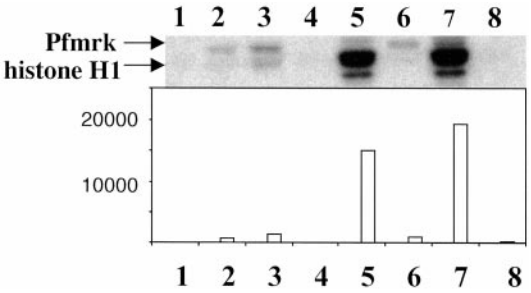

FIG. 1. **Activation of PfPK5 by human cyclin H.** *In vitro* kinase assays were performed by adding 0.5 μg of the following recombinant proteins to a reaction mixture containing the radiolabeled ATP and histone H1 substrates: *lane 1*, no recombinant protein; *lane 2*, Pfmrk; *lane 3*, Pfmrk and human cyclin H; *lane 4*, human cyclin H; *lane 5*, PfPK5 and human cyclin H; *lane 6*, Pfmrk and PfPK5; *lane 7*, PfPK5, Pfmrk and human cyclin H; *lane 8*, PfPK5. *Top panel,* autoradiogram of the gel; *bottom panel,* histogram of the CPM values obtained by phosphorimaging of the histone H1 bands in each lane.

Cyclin H has 25-fold lower affinity *in vitro* for other mammalian Cdks than for Cdk7 (23). Because PfPK5 is clearly more closely related to Cdk1 than to Cdk7, the results described above suggest that the cyclin partner specificity requirements may not be as stringent for the plasmodial enzyme as they are for mammalian Cdks. To test this hypothesis further, we investigated whether or not human p25, whose predicted tertiary structure is similar to that of cyclins despite a divergent primary structure (7), was able to activate PfPK5. Activation by p25 is highly specific toward Cdk5; no activation by this protein was detected on cdc2, Cdk2, Cdk3, Cdk4, or Cdk6 (9). Our results clearly show that p25 is, unexpectedly, a potent activator of PfPK5 (Fig. 2*a*, *lanes 2–4*). By contrast to human Cdk5, which responds to p25 (*lanes 7* and *8*) but not to cyclin H (not shown), PfPK5 appears to be activated to similar levels by both proteins. The autophosphorylation and substrate phosphorylation activities of PfPK6, another *P. falciparum* kinase related to Cdks and mitogen-activated protein kinases, are unaffected by p25 (*lanes 5* and *6*).

Direct Interaction of PfPK5 with p25—Activation of PfPK5 by p25 is dose-dependent and saturatable (Fig. 2, *b* and *c*), which is consistent with a direct and specific interaction between these two proteins. To determine whether such a physical interaction occurs between p25 and PfPK5, we performed an enzyme-linked immunosorbent assay capture assay using 96-well plates coated with an anti-GST antibody. Complexes prepared with various amounts of GST-p25 and a fixed amount of HRP-conjugated PfPK5 were added to the wells, and the amount of peroxidase remaining in the wells after washing was determined. This value increased with the amount of GST-p25 present in the complex sample (Fig. 3). Control wells with no GST antibody, or with different GST fusion proteins in place of

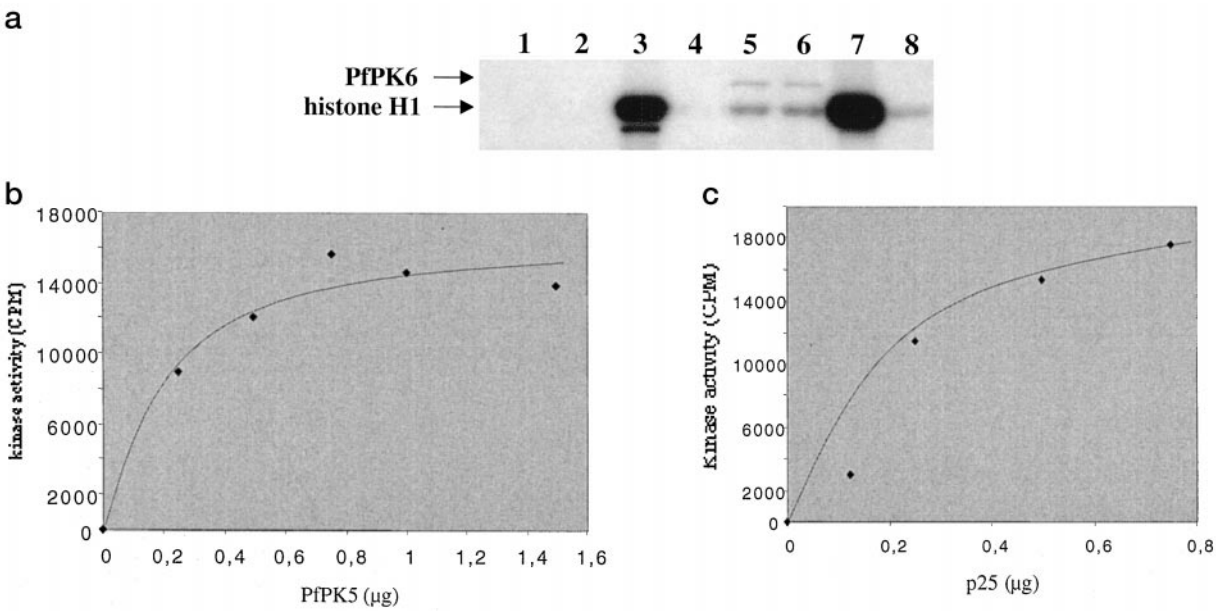

FIG. 2. **PfPK5 activation by p25.** *a*, effect of p25 on the activity of PfPK5, PfPK6, and human Cdk5. *In vitro* kinase assays were performed by adding 0.5 μg of the following recombinant proteins to a reaction mixture containing the radiolabeled ATP and histone H1 substrates: *lane 1*, no recombinant protein; *lane 2*, human p25; *lane 3*, PfPK5 and human p25; *lane 4*, PfPK5; *lane 5*, PfPK6 and human p25; *lane 6*, PfPK6; *lane 7*, human Cdk5 and p25; *lane 8*, human Cdk5. *b,* dose dependence of kinase activity on PfPK5. Kinase assays were performed in the presence of 0.5 μg of human p25 and the indicated amounts of PfPK5, and radiolabel incorporated into histone H1 was measured by scintillation counting after acid precipitation (see "Experimental Procedures"). *c,* dose dependence of kinase activity on human p25. Kinase assays were performed in the presence of 0.5 μg of PfPK5 and the indicated amounts of human p25, and radiolabel incorporated into histone H1 was measured as in *b*. Data in *b* and *c* are from individual experiments, which were repeated with similar results four and three times, respectively.

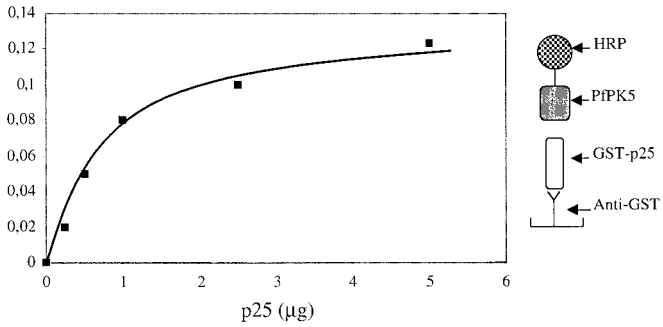

FIG. 3. **Detection of PfPK5·p25 complexes.** An enzyme-linked immunosorbent assay capture assay (see "Experimental Procedures") was performed with excess amounts of immobilized anti-GST antibody, a constant amount of HRP-labeled PfPK5, and various amounts of GST-human p25. Data are from one out of three experiments that gave similar results.

GST-p25, did not give a signal, like the wells where the HRP was added without PDBA-coupled PfPK5 (see "Experimental Procedures"). This demonstrates the formation of a PfPK5·p25 complex under these experimental conditions.

Inhibition Assays using PfPK5·p25 Complexes—Activation of Cdks by cyclins is achieved through a structural rearrangement of the kinase subunit, leading to the reorientation of the ATP molecule in the catalytic cleft, allowing phosphotransfer to occur. The observation that PfPK5 binds to, and is activated by, p25 strongly suggests that this rearrangement occurs in this case as well. One can therefore predict that the activity should be affected by the presence of competitive inhibitors known to target the ATP-binding pocket (24). To test this hypothesis, we performed inhibition assays with a few inhibitors of this class (Fig. 4). Inhibition curves have the expected sigmoid shape and allow the determination of IC_{50} values for the compounds. As could be predicted from the divergence in primary structure between PfPK5 and mammalian Cdks, the IC_{50} of individual compounds is not identical against PfPK5·p25 and Cdk1·cyclin B; for indirubin-3′-monoxime (25) these values are 10 and 0.2 μM, respectively, for purvalanol A (26) they are 8 and 0.004 μM, and for hymenialdisine-01 (27) they are 1 and 0.02 μM.

Cloning and Expression of a P. falciparum Cyclin and Its Ability to Activate PfPK5—No cyclin structural (or functional) homologue has yet been described in malaria parasites, but it can be expected that progress of the *P. falciparum* Genome Project will shed light on this issue. Indeed, while the experiments described above were in progress, we performed a BLAST analysis of the Project data bases, using mammalian cyclins as search sequences. This led us to identify a contig from chromosome 14 carrying an ORF with a BLAST score that warranted further investigation (see Fig. 5*a*). BLAST analysis on the NRprot data base indicated that this ORF was indeed closely related to cyclins, the highest scores being with cyclin H from various organisms. However, the homology was very weak and restricted to a small number of regions of a few amino acids (see Fig. 5*b* for an alignment). The ORF was flanked on both sides by homopolymeric stretches of adenosine and thymidine residues that are characteristic of *P. falciparum* noncoding regions (Fig. 5*a*). Together with the fact that stop codons can be found in all three frames over several hundred base pairs just outside the ORF, this suggests that the latter represents the entire coding region of one polypeptide. The coding sequence was amplified from a *P. falciparum* cDNA library and cloned into the pGEX expression plasmid. After DNA sequence verification, a recombinant protein was produced, purified, and added to *in vitro* kinase assays to determine whether it was able to activate PfPK5 (Fig. 6). Clearly, this protein, which we call Pfcyc-1, is able to stimulate PfPK5 as efficiently as human

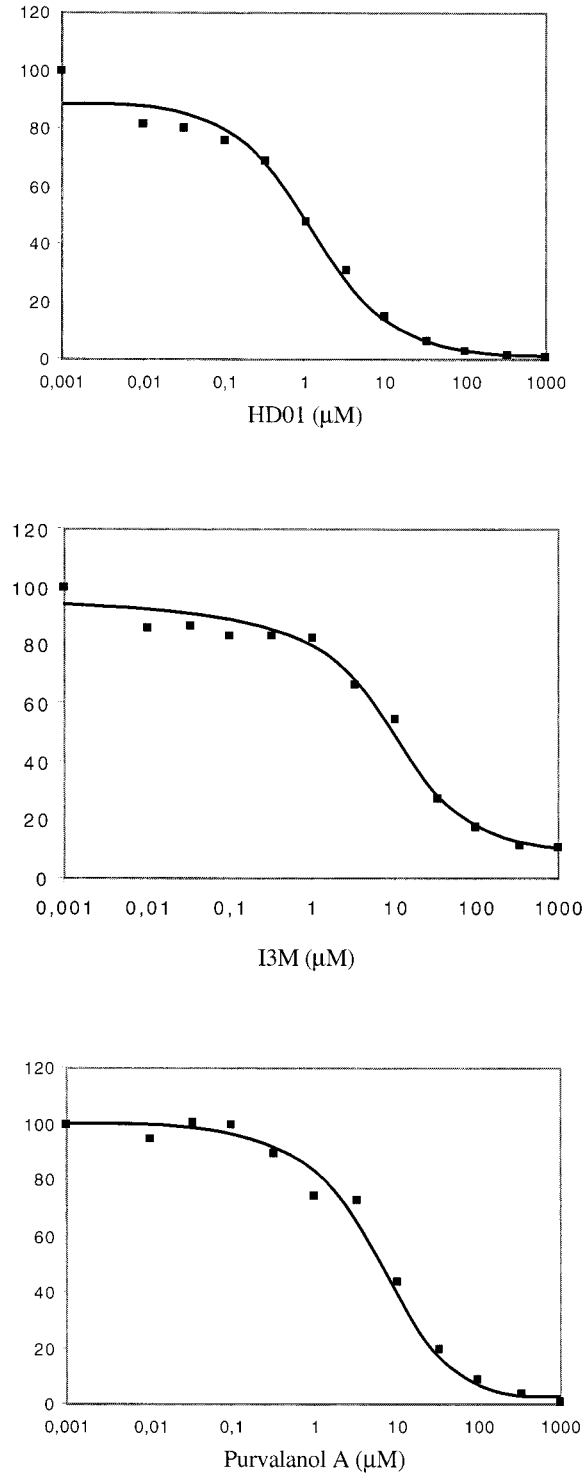

FIG. 4. **Inhibition of PfPK5·p25 activity by Cdk inhibitors.** Indicated amounts of each inhibitor (*top*, hymenialdisine 01 (*HD01*); *middle*, indirubin-3′-monoxime (*I3M*); *bottom*, purvalanol A) were added to *in vitro* kinase assays using histone H1 as a substrate with 0.5 μg each of PfPK5 and human p25. The inhibitors were diluted in Me_2SO in such a way that an equal volume of the solvent was added to all reactions. Values are expressed as the percentage of the activity measured in the absence of inhibitor. The same IC_{50} values have been found in two sets of experiments, each done in duplicate; data in the figure represent the average values from one of these experiments.

cyclin H or p25. We also repeatedly observed low level labeling of an additional band that we attributed to the cyclin subunit (GST-Pfcyc-1, GST-p25, or human cyclin H) when PfPK5 was present (not shown).

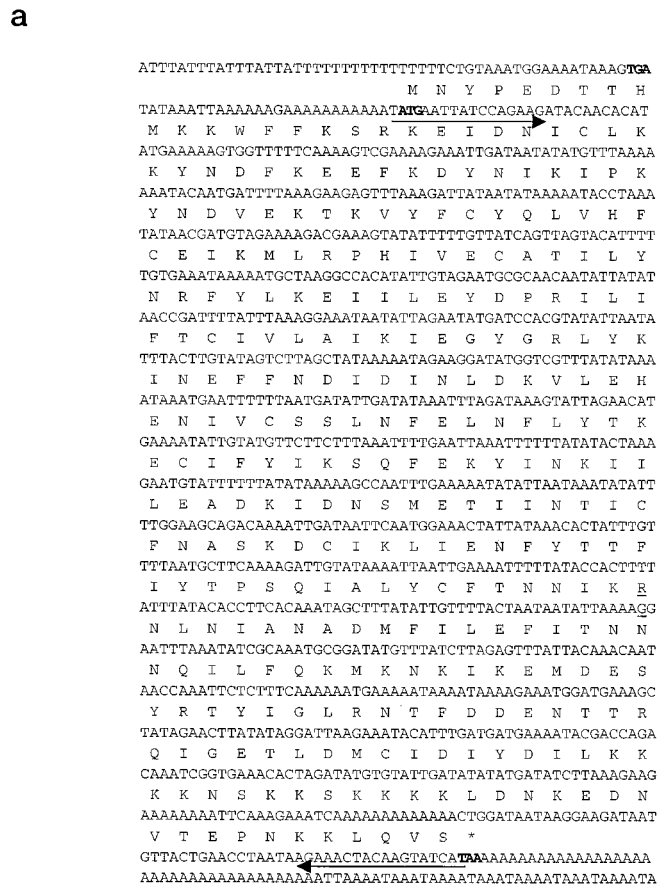

FIG. 5. ***a*. Sequence of the region of contig 19 from *P. falciparum* chromosome 14 encompassing the Pfcyc-1 ORF.** Stop and start codons are indicated in *bold*, and the sequences used for amplification primers are *underlined*. By comparison with the contig sequence, the fragment we amplified from 3D7 DNA had one discrepancy *underlined* in the figure; the codon for amino acid 225 is AAG in the contig and AGG in the amplified fragment, resulting in change from a lysine to arginine. This arginine is conserved in cyclin H from other organisms (see *b*). *b,* alignment of the deduced amino acid sequence of the Pfcyc-1 ORF with the cyclin H sequences from *Homo sapiens* and *Xenopus laevis*. The arginine residue discussed above is *underlined*.

Absence of PfPK5 or PfPK6 Stimulation by Pfmrk·Pfcyc-1 and Autophosphorylation of PfPK5 in the Presence of Pfcyc-1—As Pfcyc-1 is most closely related to cyclin H, which activates the CAKs in other eukaryotes, and as Pfmrk is a putative CAK homologue, we naturally wanted to determine whether the homologous pair Pfmrk·Pfcyc-1 would activate plasmodial crks (we have shown (Fig. 1) that the heterologous pair Pfmrk·human cyclin H did not stimulate PfPK5 activity, but this might conceivably have been because of the heterologous origin of the cyclin). Results of these experiments (not shown, as they mirror the data presented in Fig. 1) indicate that the properties of Pfcyc-1, in terms of its effect on Pfmrk, PfPK5, and PfPK6 activities, are essentially undistinguishable from those of human cyclin H: (i) Pfcyc-1 stimulates histone H1

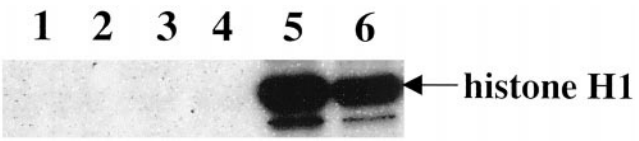

FIG. 6. ***In vitro* activity of Pfcyc-1.** *In vitro* kinase assays were performed by adding 0.5 μg of the following recombinant proteins to a reaction mixture containing the radiolabeled ATP and histone H1 substrates: *lane 1*, no recombinant protein; *lane 2*, Pfcyc-1; *lane 3*, human cyclin H; *lane 4*, PfPK5; *lane 5*, PfPK5 and Pfcyc-1; *lane 6*, PfPK5 and human cyclin H.

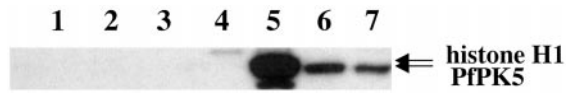

FIG. 7. **Autophosphorylation of PfPK5 in the presence of Pfcyc-1.** *In vitro* kinase assays were performed by adding 0.5 μg of recombinant proteins to a reaction mixture containing radiolabeled ATP; histone H1 was added in *lanes 1–5* but omitted in *lanes 6* and *7*. *Lane 1*, no recombinant protein; *lane 2*, Pfcyc-1; *lane 3*, PfPK5; *lane 4*, Pfmrk; *lane 5*, PfPK5 and Pfcyc-1; *lane 6*, PfPK5 and Pfcyc-1 (no histone); *lane 7*, PfPK5, Pfcyc-1 and Pfmrk (no histone).

phosphorylation by Pfmrk about 2-fold, like human cyclin H does; this effect is small in comparison with that of the human and plasmodial cyclins on PfPK5, where the activation factor is up to three orders of magnitude higher; (ii) the simultaneous presence of Pfcyc-1, Pfmrk, and PfPK5 does not result in a synergistic increase of H1 phosphorylation; and (iii) PfPK6 is not activated by incubation with either Pfmrk·Pfcyc-1 or Pfcyc-1 alone (although we sometimes observed a very small increase of PFPK6 activity (less than 2-fold) in the presence of human cyclin H).

To check whether the lack of synergy between PfPK5 and Pfmrk in the presence of cyclin H was not due simply to a saturation effect, dose-dependent experiments were performed where the effect of variations in the concentration of each kinase on histone H1 phosphorylation was evaluated. These experiments (not shown) confirmed the apparent lack of synergistic activities of PfPK5 and Pfmrk. A more direct way of determining whether Pfmrk can phosphorylate PfPK5 is to measure ^{32}P incorporation in the latter enzyme in the presence of Pfmrk. As PfPK5 migrates very close to phosphorylated histone H1 in polyacrylamide gels, it is difficult to assess the phosphorylation status of the kinase in the presence of the substrate. Therefore, we performed kinase reactions containing diverse combinations of Pfmrk, Pfcyc-1, and PfPK5, without adding histone H1 (Fig. 7). The most striking result of this experiment is that PfPK5·Pfcyc-1 is able to autophosphorylate on the kinase subunit (*lanes 6* and *7*); the GST-Pfcyc-1 recombinant protein, which is much larger (around 60 kDa) than the kinase subunit, was also labeled, to a lower extent than PfPK5. Furthermore, it appears clearly that the addition of Pfmrk does not cause an increase in PfPK5 labeling; on the contrary, we observed a decrease of PfPK5 phosphorylation when Pfrmrk was present (*lane 7*). This is presumably because of competition for Pfcyc-1 between the two kinase subunits.

DISCUSSION

Malaria parasites are not easily amenable to investigations concerning gene function because of the difficulties associated with both classical and reverse genetics approaches in the study of these organisms. The problem is particularly acute with respect to genes that are essential for the erythrocytic asexual cycle, because in available protocols for the generation of null mutants selection of transformed parasites occurs at this stage; this makes it impossible to target a gene required for this process (28). For these reasons, investigations on processes such as cell cycle progression in *Plasmodium* have relied mainly on phenomenological observations and on the cloning and biochemical characterization of the putative homologues of genes involved in cell cycle control in other eukaryotes. This lead on one hand to the description of peculiarities in the cell division process during schizogony, such as crypto-orthomitosis (the maintenance of the nuclear membrane during division) and apparent asynchrony in the division of nuclei within a schizont, and on the other hand to the identification of several genes encoding putative cell cycle regulators (a complete repertoire of such genes will become available as the *P. falciparum* Genome Project nears completion) (reviewed in Refs. 17–18). An obvious task is the integration of these lines of approaches into a coherent picture of *Plasmodium* cell cycle regulation. This forms the context of the present study, which addresses the question of the regulation of *P. falciparum* crks.

Although several plasmodial crks have been identified over the last few years, important questions regarding their function and regulation still require solving. How similar to that of other eukaryotes are the modalities of *Plasmodium* crk regulation? This question has recently been addressed in trypanosomatids, another group of protozoan parasites, and it was shown that mammalian cyclins can interact with a *Trypanosoma cruzi* crk (29). Here, we show not only that such an interaction also occurs between a mammalian cyclin-like protein and PfPK5, a plasmodial putative Cdk1 homologue, but also that this interaction is accompanied by a strong stimulation of PfPK5 kinase activity. PfPK5 is clearly much more closely related to Cdk1 than to Cdk7 (60% identity at the amino acid level with human Cdk1 (12) compared with 35% with Cdk7). Hence the observation that it can be efficiently activated by human cyclin H, which is the activating partner of Cdk7, is unexpected. Also surprising was the observation that human p25 is likewise able to activate PfPK5. That it is unexpected does not arise in this case from considerations about the relatedness between the PfPK5 and the Cdk5 sequences; indeed, PfPK5 turns out to be as closely related, in terms of overall sequence similarity, to human Cdk5 as it is to human Cdk1 (approximately 60% identity in both cases). Instead, the surprise comes from the fact that PfPK5 is activated equally efficiently by two molecules, cyclin H and p25, which in higher eukaryotes activate different catalytic subunits. By contrast, in mammalian cells p25 shows a stringent specificity toward Cdk5 (9) and to our knowledge does not activate other members of the Cdk family. This specificity is at least partially symmetrical, as other cyclins do not activate Cdk5 (in our experiments, Cdk5 activity does not respond to human cyclin H or Pfcyc-1). Incidentally, this suggests that PfPK5 is not the *P. falciparum* orthologue of Cdk1 as previously thought but might instead be a homologue of Cdk5; this is corroborated by our observation that a BLAST search with the PfPK5 amino acid sequence on the *Caenorhabditis elegans* (whose entire complement of Cdks has been characterized) data base gives the putative *C. elegans* Cdk5 homologue as the highest scoring entry (57% identity) before the Cdk1 homologue (53% identity). Nevertheless, taken together our results tend to suggest that PfPK5 may be activated by cyclins *in vitro* in a way that is more promiscuous than mammalian Cdks; could it be that any protein with a cyclin-like tertiary structure is able to activate this kinase? We are planning now to test this hypothesis further, using a larger complement of both heterologous and (as they become available from the *P. falciparum* genome data base) *Plasmodium* cyclins. It is worth mentioning here the ability of Pho85 (a *Saccharomyces cerevisiae* Cdk) to be activated by several different cyclins, which confers to this enzyme a large diversity of functions (30, 31). This aspect of plasmodial Cdk regulation would thus appear to resemble what is found in other lower eukaryotes.

Full activation of most Cdk·cyclin complexes requires phosphorylation of the T-loop threonine (Thr-160 in human Cdk2) by Cdk7·cyclin H, the Cdk-activating kinase. Our experiments show that Pfmrk, a putative homologue of Cdk7, does not activate nor phosphorylate PfPK5, even in the presence of human cyclin H. This is not because of the heterologous (human) origin of the cyclin, because replacing human cyclin H with Pfcyc-1 (a *P. falciparum* protein with maximal homology to cyclin H from various organisms) does not confer activity to Pfmrk. Several possible explanations come to mind: first, Pfmrk may have specific CAK activity on other plasmodial crks; Pfmrk mRNA is more abundant in gametocytes than in asexual parasites, and it is conceivable that it activates some crk different from PfPK5 and present in gametocytes, Pfcrk-1 for example (14). Second, Pfmrk may require another cyclin partner (*i.e.* different from Pfcyc-1) or additional parasite-derived proteins to show CAK activity on PfPK5; in mammalian cells, the majority of Cdk7·cyclin H complexes are found in association with a third protein, Mat1 (5, 32). Third, Pfmrk may not be a CAK at all. In *S. cerevisiae*, the closest relative of Cdk7 (Kin28) is required for RNA polymerase II-dependent transcription and associates with TFIIH, but does not possess CAK activity (3, 5, 33). In this organism CAK activity is provided by Cak1, a kinase unrelated to Cdk7 and that does not require association with activating partners (34).

Although PfPK5 is not a substrate for Pfmrk, it nevertheless becomes phosphorylated in the presence of Pfcyc-1. To our knowledge, such Cdk autophosphorylation has not been documented in other organisms. A very low level autophosphorylation of PfPK5 has been detected in the absence of a cyclin partner (12, 35), but available data are not sufficient to determine whether the same residues are phosphorylated in both cases. It will be interesting to find whether cyclin binding results in autophosphorylation of the T-loop threonine (Thr-158 in PfPK5 corresponding to Thr-160 in human Cdk2). If this were shown to be the case, it would argue in favor of a model of PfPK5 regulation where CAK activity provided by a different kinase would not be required.

Hence the regulation of PfPK5 appears to be notably different from that of its homologues found in higher eukaryotes in that (i) the enzyme seems to be activable by a wider range of cyclin·cyclin-like partners; (ii) when in conjunction with such a partner, it is able to undergo autophosphorylation; and (iii) considerable activation is achieved in the absence of a CAK *in vitro* (the latter property is shared by some Cdk·cyclin pairs of higher eukaryotes, *e.g.* Cdk2·cyclin A (36)). Obviously one cannot exclude at this stage that Pfmrk or yet another enzyme activates PfPK5 by phosphorylation *in vivo*.

It is likely that Pfcyc-1 is only one member of a family of *P. falciparum* cyclin-like proteins. It will soon become possible to address the question of specificity in *Plasmodium* crk·cyclin interactions and to gain information of the temporal expression of these elements during parasite development. It is hoped that this will contribute significantly to the emergence of a better understood picture of cell cycle control in this organism.

Because of the crucial role they are most likely to play in the control of cell division and/or development, plasmodial crks represent attractive potential targets for novel antimalarial agents. Our data (Fig. 4) are consistent with the idea that sequence divergences between parasite crks and human Cdks are likely to result in different affinities for inhibitory molecules. This is supported by the observation that human Cdk1 and its yeast homologue CDC28 have different sensitivities to a given inhibitor (26). Our data indicating that PfPK5·cyclin complexes can be used to measure the IC_{50} of inhibitors pave the way for the development of efficient screening systems aimed at identifying molecules with a specific effect on parasite-encoded kinases, which can then be assayed for *ex vivo* antimalarial activity.

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Activation of a *Plasmodium falciparum* cdc2-related Kinase by Heterologous p25 and Cyclin H: FUNCTIONAL CHARACTERIZATION OF A P. FALCIPARUM CYCLIN HOMOLOGUE

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