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The H89 cAMP-dependent protein kinase inhibitor blocks *Plasmodium falciparum* development in infected erythrocytes

Chiang Syin[1], Daniel Parzy[2], Francois Traincard[3], Irène Boccaccio[4], Manju B. Joshi[1], David T. Lin[1,*], Xiao-Ming Yang[1], Karine Assemat[5], Christian Doerig[6] and Gordon Langsley[7]

[1]*Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA;* [2]*Institut de Médecine Tropical du Service de Santé des Armées, Le Pharo, Marseille, France;* [3]*Unité de Régulation Enzymatique des Activités Cellulaires, FRE CNRS 2364, Département de Biologie Moléculaire, Institut Pasteur, Paris, France;* [4]*INSERM U399, Marseille, France;* [5]*Unité de Biochimie Structurale, Département d'Immunologie, Institut Pasteur, Paris, France;* [6]*INSERM U 511, La Pitié-Salpétrière, Paris, France;* [7]*Laboratoire de Signalization Immunoparasitaire, URA CNRS 1960, Département d'Immunologie, Institut Pasteur, Paris, France*

In *Plasmodium falciparum*, the causative agent of human malaria, the catalytic subunit gene of cAMP-dependent protein kinase (*Pfpka-c*) exists as a single copy. Interestingly, its expression appears developmentally regulated, being at higher levels in the pathogenic asexual stages than in the sexual forms of parasite that are responsible for transmission to the mosquito vector. Within asexual parasites, PfPKA activity can be readily detected in schizonts. Similar to endogenous PKA activity of noninfected red blood cells, the parasite enzyme can be stimulated by cAMP and inhibited by protein kinase inhibitor. Importantly, *ex vivo* treatment of infected erythrocytes with the classical PKA-C inhibitor H89 leads to a block in parasite growth. This suggests that the PKA activities of infected red blood cells are essential for parasite multiplication. Finally, structural considerations suggest that drugs targeting the parasite, rather than the erythrocyte enzyme, might be developed that could help in the fight against malaria.

Keywords: parasite; PKA; inhibition; H89.

The emergence and dissemination of drug-resistant malaria parasites represents one of the most important public health problems in many parts of the world today. New antimalarials are urgently required, whose rational design and development requires the identification of potential therapeutic targets. This in turns rests on a better understanding of the molecular mechanisms controlling the progression of the complex life cycle of malaria parasites, especially *Plasmodium falciparum*, the species responsible for the lethal form of the disease. All *Plasmodium* species are intracellular parasites during infection of their vertebrate hosts. Sporozoites inoculated into a host during a bite by an infected *Anopheles* mosquito soon invade hepatocytes, within which intense asexual division takes place (exoerythrocytic schizogony), yielding up to 40 000 merozoites in the case of *P. falciparum* (http://www.malaria.org). Upon schizont rupture, these merozoites invade red blood cells, where additional rounds of asexual replication occur (erythrocytic schizogony, the phase responsible for the pathogenesis of the disease). Some merozoites, instead of undergoing a further asexual cycle, arrest their cell cycle and differentiate into male or female gametocytes. Unlike asexual forms, these sexual forms are infective to the *Anopheles* vector.

cAMP is involved in the regulation of development of several microorganisms, and cAMP-dependent pathways exist in most eukaryotic cells. For example, in the slime mold *Dictyostelium discoideum*, cAMP acts as a signal for the aggregation and differentiation of cells into a multicellular organism (reviewed in [1]). In most instances, cAMP exerts its action by binding to the regulatory subunit complexed to the catalytic subunit in an inactive holoenzyme of the protein kinase A (PKA or cAMP-dependent protein kinase), thereby releasing the active catalytic subunit (PKA-C), whose substrates can include other protein kinases and transcription factors. PKA activity is also regulated through binding of its natural inhibitor, called PKI. The crystal structure of the catalytic subunit of mouse PKA-C has been resolved and this, combined with mutational analysis, showed that PKI competes with the regulatory subunit for binding to the catalytic subunit [2,3].

Several previous reports suggest a role for cAMP (without directly implicating PKA) in *P. falciparum* asexual stage development and erythrocyte invasion [4,5]. In addition, specific levels of cAMP appear to be important for the induction of gametocytogenesis (the process of gametocyte induction). Treatment of *P. falciparum* cultures with cAMP agonists, or with phosphodiesterase inhibitors such as caffeine and 8-bromo-cAMP, result in an increase of gametocyte induction [6–8]. Moreover, adenylate cyclase activity and cAMP levels have been correlated with the parasite's ability to produce gametocytes [9,10]. A cAMP-dependent histone

Correspondence to G. Langsley, Laboratoire de Signalization Immunoparasitaire, URA CNRS 1960, Département d'Immunologie, Institut Pasteur, 75724 Paris Cedex 15, Paris, France.
Fax: + 33 1 40 61 3185, Tel.: + 33 1 45 68 8922,
E-mail: langsley@pasteur.fr

Abbreviations: PfPKA, *Plasmodium falciparum* protein kinase A; Pfpka-c, cAMP-dependent protein kinase; DAPI, 4′,6-diamidino-2-phenylidole; PKI, protein kinase inhibitor.

**Present address*: Center for Drug Evaluation and Research, FDA, HFD-580, 5600 Fishers Lane, Rockville, MD 20857, USA.

Note: the first three authors contributed equally to this work.

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II-A kinase activity has been detected, and a putative regulatory subunit of 54 kDa observed, in asexual forms of *P. falciparum* [11]. Even though the catalytic subunit of *P. falciparum* (PfPKA-C) has not been identified, the *pka-c* gene from the rodent malaria parasite *Plasmodium yoelii* has been cloned [12].

Given the urgent need for new therapeutic drugs against human malaria, we decided to undertake a study of the cAMP-dependent protein kinase from *P. falciparum*, as both natural and chemical inhibitors of this well characterized enzyme exist. We show that the human malaria parasite has a single *pka-c* gene, whose expression is down-regulated in gametocytes and gametes. The asexual stage specific kinase activity is associated with schizonts and, like the endogenous red blood cell PKA activity, it is sensitive to cAMP and inhibited by PKI and H89. Inhibition studies suggest an essential role for PKA in the intraerythrocytic growth of the parasite. Structural considerations suggest that it might be possible to specifically inhibit the parasite, rather than the erythrocyte enzyme, and such drugs would be viable as new antimalarials.

MATERIALS AND METHODS

Parasite culture and isolation of gametocytes and gametes

P. falciparum clone 3D7 parasites were used throughout the study and gametocytes were isolated from *in vitro* culture as described previously [13]. Mature gametocytes were induced in M199 media (pH 8) to form extracellular gametes/zygotes. For the PKA inhibition studies, two reference *P. falciparum* clones were used, D6 and W2. which are chloroquine sensitive and chloroquine resistant, respectively [14].

Isolation of the *pka-c* gene from *P. falciparum*

Two independent strategies were employed to isolate the *Pfpka-c* gene. A systematic screen of the *P. falciparum* ESTs collection was made for clones displaying homology to PKA-C and an asexual-stage clone (0648c3) was detected using the information at http://parasite.vetmed.ufl.edu/falc.htm. In parallel, degenerate oligonucleotide primers were synthesized and used to amplify *Pfpka-c* gene from *P. falciparum* cDNA using the following conditions: 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min, for 35 cycles in a thermocycler (PerkinElmer–Cetus). Forward [GG(A/T)AC(A/T)GG(A/T)TC(A/T)TT(T/C)GG] and reverse [TT(A/T)GCAAA(A/T)CCAAAATC(A/T)GT] primers were deduced from the conserved PKA motifs GTGSFG and TDFGFAK, respectively [15]. Both the EST (0648c3) and the PCR fragment were sequenced to confirm their identity and then used to screen asexual stage [13] and sexual stage cDNA libraries [16]. Two independent cDNA clones were sequenced and deposited in the databases (GenBank acc. nos AJ224444 and U78291).

Southern and Northern blot analysis

Genomic DNA (4 μg) was digested with *Eco* RI, *Pst* I, *Eco* RV, *Hin*dIII, *Bgl* II and *ClaI*, separated by agarose gel electrophoresis and transferred to nylon (Hybond-N, Amersham). Total RNA (3 μg) was isolated from asexual stages, gametocytes and gametes and loaded onto a 1% agarose gel. RNA was stained by ethidium bromide and transferred to Hybond-N (Amersham). Both Southern and Northern filters were probed with radio-labelled *Pfpka-c* DNA, washed and exposed to film. To ensure comparable loading of total RNA from each developmental stage, the stained gel was visually examined and the Northern blot was re-probed with the unrelated *Pfpast-1* gene [17].

Generation of anti-PfPKA serum and immunofluorescence analysis

The polyclonal anti-(PfPKA-C) serum was generated by immunizing rabbits with peptide corresponding to the C-terminal sequence VPYKPKYKNIFDSSNFE(304–320) conjugated to keyhole limpet hemocyanin through a cysteine at the N-terminus of the peptide (ZYMED Laboratories, Inc., San Francisco, CA, USA). The specificity of the serum was verified by immunopreciptaion of the *in vitro* translation product of the *Pfpka-c* gene and its failure to react with noninfected erythrocytes. Asynchronous blood stage parasites were fixed with acetone prior to incubation with the rabbit antisera used at a 1 : 100 dilution. The parasite nuclei were stained with 4′,6-diamidino-2-phenylidole (DAPI) (Sigma) and the slides read with a Leica DMRB microscope.

In vitro PKA activity assays

Parasites were isolated by centrifugation (15 000 ***g***, 4 °C for 10 min) of infected erythrocytes broken by resuspension in lysis buffer (10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 μM Na_3VO_4, 50 mM NaF, 1 mg·mL^{-1} Pefablock SC^{TM} protease inhibitors (Roche, Mannheim, Germany). Pelleted parasites were washed/centrifuged three times in NaCl/P_i at 4 °C (50 mL) and finally resuspended in 1 mL of lysis buffer (1 mL per 5×10^8 parasites). Parasites were then broken by freeze-thawing (four cycles) and lysates were clarified by centrifugation at 40 000 ***g*** for 90 min. The supernatant was concentrated five-fold in Ultrafree^{TM} (Millipore Corp., Bedford, MA, USA). As a control, noninfected erythrocytes were submitted to the same purification protocol. The protein content of the extracts was determined by the Bio-Rad (Bradford) protein assay. Supernatants were aliquoted and stored at −80 °C for activity assays. PKA activity was assayed by measuring ^{32}P incorporation into the synthetic peptide LRRASLG (Kemptide; Sigma) using a modified protocol [18]. A typical assay was performed in a total volume of 25 μL containing 50 mM Mops pH 7.0, 40 μg·mL^{-1} BSA, 0, 5 mM $MgCl_2$, 300 μM Kemptide, 300 μM [γ-^{32}P]ATP (200–500 c.p.m.·pmol^{-1}), 30 μg·mL^{-1} bestatin and 12.5 μg of proteic extract containing 1.5×10^7 broken parasites originating from 3×10^8 infected erythrocytes or 3×10^8 noninfected erythrocytes. After 20 min at 30 °C, ^{32}P incorporation was measured by spotting 10 μL of the reaction mixture onto P81 phosphocellulose filters (Whatman, Madstone, UK). The filters were washed three times for 5 min in 1% phosphoric acid, once for 5 min in acetone and dried before scintillation counting. Specificity of the reaction was assessed by performing the assay in presence of 40 μM cAMP, or in the presence of 88 μM of the PKA

specific inhibitor peptide PKI_{5-24} [19], or of 80 μM of H89. Under the conditions used, reactions were linear over time and protein amount or parasite content.

Western blot detection of PfPKA-C and human PKA-C

Purified parasites and identically treated healthy erythrocytes were resuspended in denaturating loading buffer at the purification stage preceding the freeze-thawing step (see above). Erythrocyte (1×10^{10}) extract and schizont (8.5×10^7 parasites) extract were submitted to SDS/PAGE analysis as described in [20] using the anti-(PfPKA-C) serum (1 : 200) and an anti-(human PKA-Cα) Ig [PKAα cat (C-20) from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA] diluted to 1 : 1000. The latter Ig was directed against the C-terminal end of the human PKA-Cα subunit that is not conserved in *P. falciparum* (see Fig. 1).

Control Western blots were performed to estimate contamination by human PKA-C of the *Plasmodium* and noninfected erythrocytes extracts submitted to PKA activity testing. A volume of lysate containing 1.5×10^7 parasites (originating from 8×10^8 infected erythrocytes) or 8×10^8 healthy erythrocytes was loaded onto a 10% SDS-polyacrylamide gel. After electrophoretic separation, proteins were transferred onto nitrocellulose and submitted to Western blot analysis as described above using the anti-(human PKA-Cα) Ig.

Inhibition of intraerythrocytic parasite growth with the PKA inhibitor H89

The inhibitor H89 (Sigma) was diluted (100 μM, 50 μM, 25 μM, 10 μM, 2.5 μM, 1 μM and 0.5 μM) in RPMI. The starting parasitemia was between 0.1 and 0.5% in group O^+ erythrocytes and the hematocrit was 1.7% in 11.4% human serum buffered with 25 mM Hepes and 25 mM $NaHCO_3$. A total of 175 μL of this suspension was placed into 96-well plates (Falcon™), together with 25 μL of the different concentrations of H89, giving a final hematocrit of 1.5% in 10% human serum. The plates were shaken in the presence

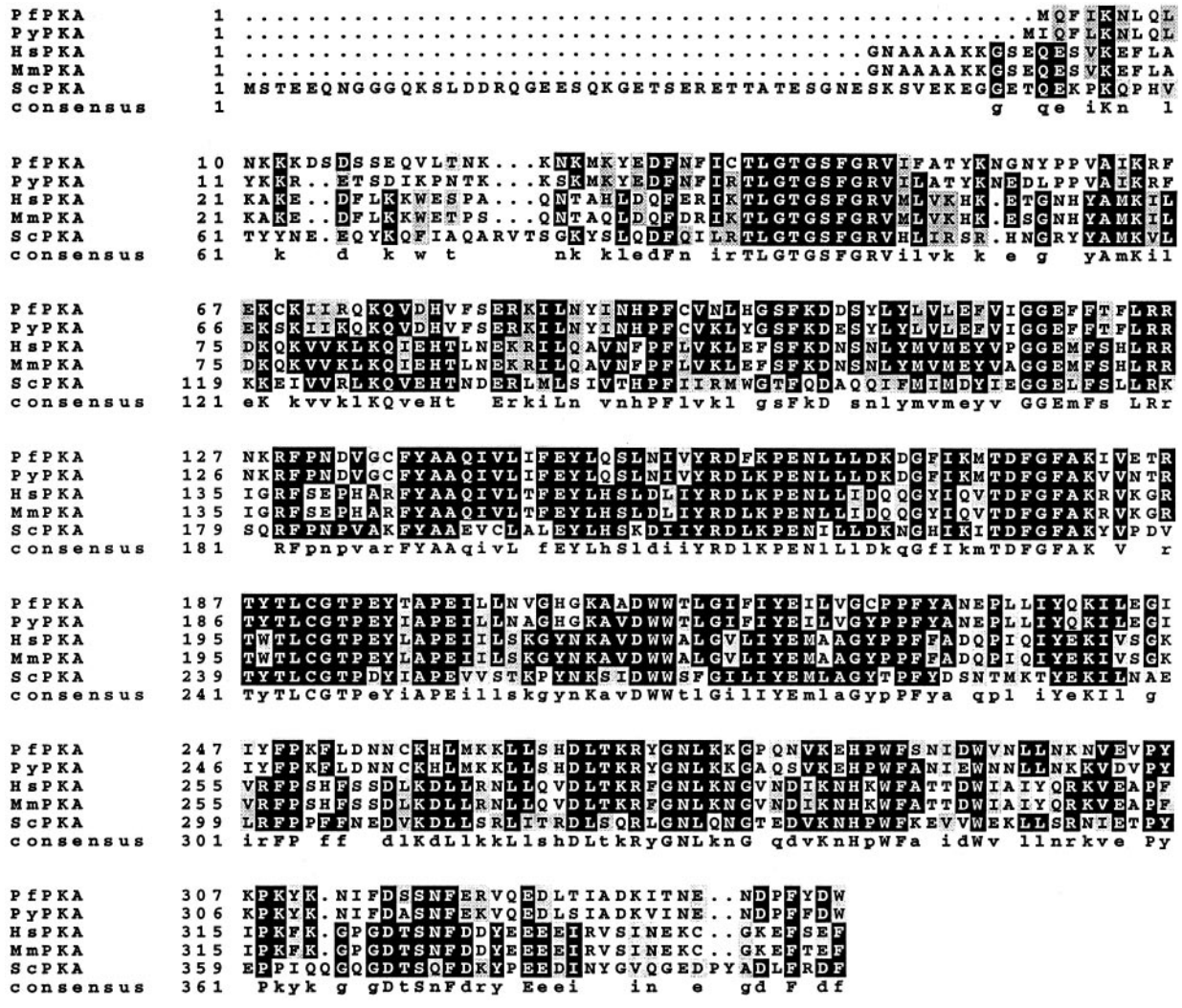

Fig. 1. PfPKA-C shares significant homology with PKA-C subunits from other species. Sequence comparison of PfPKA-C with PKA catalytic subunits from *P. yoelii* (PyPKA), human α subunit (HsPKA), mouse α subunit (MmPKA) and yeast TPK1 (ScPKA). The CLUSTAL W analysis shows (using BOXSHADE) identical residues (black) and similar amino acids (grey) conserved between the different kinases with a consensus indicated when the same residue was found in three or more enzymes. The 15 residues common to most serine/threonine protein kinases are labelled with a dot.

of radiolabeled-hypoxanthine (40 mCi·mL^{-1} or 1 mCi) for 42 h at 37 °C in a humid incubator gassed with 6% CO_2, 10% O_2 and 84% N_2. Forty-two hours after addition of hypoxanthine the plates were frozen at −80 °C and then quickly thawed, causing hemolysis and release of radiolabeled nucleoproteins. These were collected (Filtermate™, Packard Instrument Co.) on standard microplate filters (Unifilter™ GF/B) and when dry, 100 μL of scintillant were added (Microscint™) and the radioactivity counted (c.p.m.·min^{-1}) using a Top Count™ (Packard Instrument Co.) scintillation counter. The results were submitted to linear regression analysis and the dose of H89 giving 50% inhibition was calculated from three independent experiments.

RESULTS

Identification of *Pfpka-c*, a *P. falciparum* homologue of the PKA catalytic subunit gene

Using either specific PCR or EST fragments a number of independent *P. falciparum* cDNA clones encoding a *Pfpka-c* homologue were isolated (see Fig. 1 for the deduced amino-acid sequence). *Pfpka-c* is very closely related (88% identity) to the orthologous gene (*Pypka-c*, acc. no. D45894) from *P. yoelii* [12]. As expected, the 15 signature residues conserved in serine/threonine protein kinases are present in the deduced amino-acid sequence of PfPKA-C, as are most of the residues that are conserved in all PKAs. However, some crucial residues common to PKA catalytic subunits have been substituted (some in a nonconservative manner) in the two plasmodial enzymes, see Fig. 1, and [12].

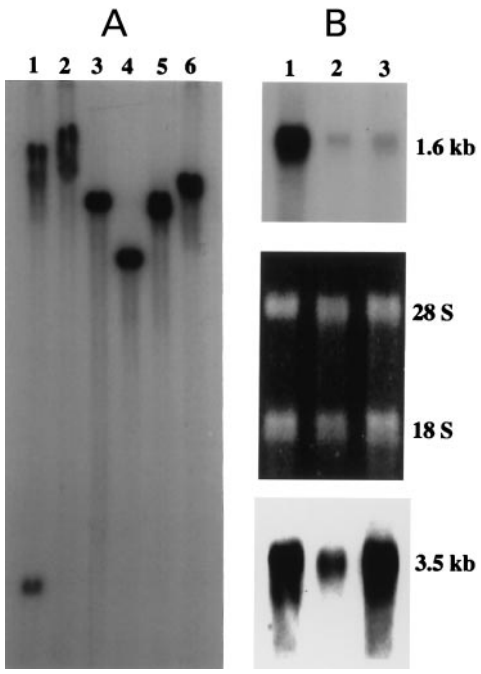

Fig. 2. *Pfpka-c* is a single copy gene expressed strongly in asexual stages and down-regulated in gametocytes and gametes. (A) Southern blot of *P. falciparum* genomic DNA restricted with (1) *Eco* RI; (2) *Pst* I; (3) *Eco* RV; (4) *Hind* III; (5) *Bgl* II (6) *Cla* I. (B) Total RNA (middle panel, ethidium bromide stain) corresponding to asexual stages (1), gametocytes (2) and gametes (3) was hybridized with *Pfpka-c* (top panel) and *PfPast-1* (bottom panel) as a control.

Pfpka-c is a single copy gene whose expression is developmentally regulated

Southern and Northen blotting, combined with PCR analysis on genomic DNA vs. cDNA, indicates that like *Pypka-c* [12], *Pfpka-c* is a multi-intron single copy gene (Fig. 2A, and data not shown). The two bands shown in the *Eco* RI digest (lane 1) are consistent with the presence of an internal site within the coding sequence. The *Pfpka-c* gene is expressed as a 1.6-kb transcript in intraerythrocytic stages and we note that mRNA levels are lower in gametocytes and gametes (compare lanes 1 with lanes 2 and 3 of top panel in Fig. 2B). Clearly, the expression of *Pfpka-c* is down-regulated in gametocytes/gametes (compare top and bottom panels in Fig. 2B).

PfPKA-C activity is highest in intraerythrocytic schizonts

Having demonstrated that *P. falciparum* asexual stage parasites accumulate *Pfpka-c* mRNA, we next examined the different intraerythrocytic stages for the presence of the enzyme. To this end, we raised a specific anti-(PfPKA-C) serum and performed indirect immunofluorescence analysis of *P. falciparum*-infected red blood cells (Fig. 3). This

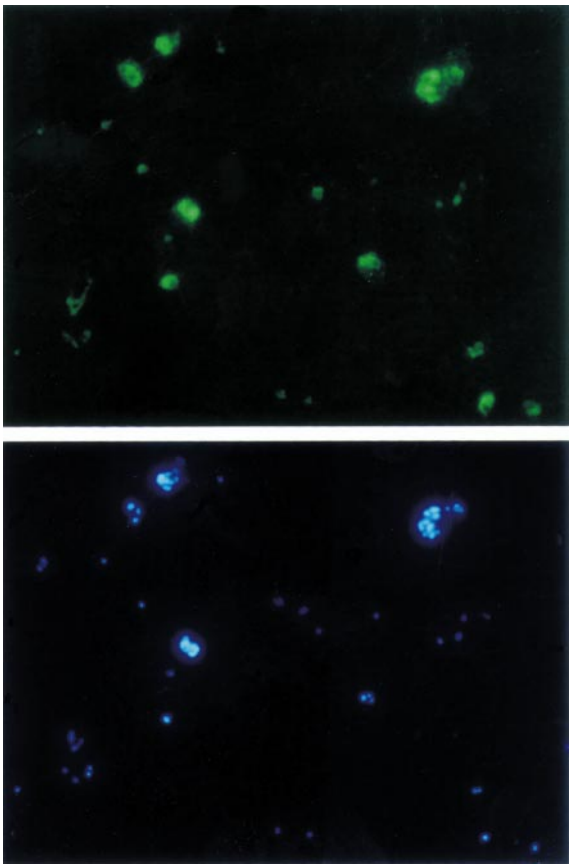

Fig. 3. Antibodies raised to PfPKA-C react preferentially with multinucleated schizonts. Top shows green immunofluorescence staining of intraerythrocytic parasites labelled with anti-(PfPKA-C) Ig and bottom, the nuclei of the same parasites stained blue with DAPI. The strongly DAPI labelled multinucleated schizonts stain strongest with the anti-(PfPKA-C) Ig.

showed that the PfPKA-C protein can be detected in all stages from mononucleated (single small blue dots) rings, to multinucleated (multiple blue dots) schizonts. The anti-(PfPKA-C) Ig appears to have the desired specificity, as it can immunoprecipitate the *in vitro* translated enzyme (data not shown) and fails to react with noninfected (non-DAPI stained) erythrocytes (Fig. 3). Next, we used the anti-(PfPKA-C) Ig together with an anti-(human PKA-Cα) Ig to detect both parasite and human PKA-C in schizont extracts and extracts made from noninfected erythrocytes (Fig. 4). As in the immunofluorescence studies, anti-(PfPKA-C) Ig failed to recognize the human enzyme (lane IIA) and it can be seen that PfPKA-C (lane IIB) migrates more slowly than the human enzyme detected in noninfected red blood cells (lane IA). Interestingly, human PKA-C appears significantly degraded in infected erythrocyte extracts as two bands are observed (lane IB). The faster migrating species probably results from N-terminal cleavage of the enzyme, which is believed to be inactive under physiological conditions [20,21]. Moreover, the predominant degradation product cannot be detected in soluble extracts of freeze-thawed treated parasites (data not shown) and thus, is unlikely to contribute to PKA-C activity measured in schizont extracts. Furthermore, the β-isoform of human PKA-C could not be detected in any red blood cell extract (data not shown) using a specific antibody [Santa-Cruz Biotech. PKAβ cat (C-20)].

We next correlated detection of the PfPKA-C protein by immunofluorescence and Western blot with the presence of a PKA activity in cell extracts. To this end, we synchronized the parasite population and submitted the extracts to a c-AMP-dependent kinase activity in a Kemptide assay (see Materials and methods). The results, shown in Fig. 5, indicate that a cAMP-dependent kinase activity is detectable in multinucleated schizonts (B) at a higher level than in noninfected erythrocyte extracts (A). As expected for a cAMP dependent protein kinase, the activity is inhibited by PKI and slightly increased by cAMP, which suggests the presence of an active regulatory subunit (see Discussion). Moreover, inhibition of the schizont PKA activity *in vitro* by the specific inhibitor H89 (Fig. 5, dotted bars) parallels the effect of H89 observed on infected red blood cells (see below; Table 1). The inset of Fig. 5 shows that the higher activity found in schizonts is not due to increased contamination of the parasite extracts by human PKA-C. Although readily observed by Western blot in total cell extracts (Fig. 4), PfPKA-C was difficult to detect in soluble extracts of schizonts (data not shown). This suggests that most of the parasite enzyme could be lost during preparation of extracts for the Kemptide assays, which might explain why PKA activity of schizonts appears reproducibly to be only twofold that found in red blood cells.

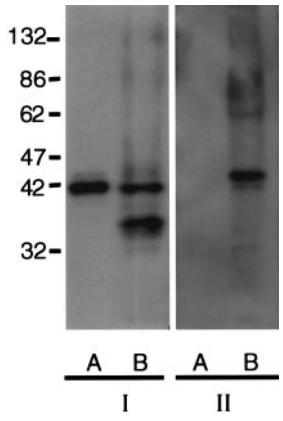

Fig. 4. Western blot detection of PfPKA-C and human PKA-C α in cell extracts. Non-infected erythrocyte extract (A) and schizont total cell extract (B), prepared as described in Materials and methods, were submitted to Western blot analysis using anti-(human PKA-C α) Ig (I) and anti-(PfPKA-C) Ig (II). Molecular masses of marker proteins are expressed in kDa.

Treatment of infected erythrocytes with the PKA inhibitor H89 blocks parasite growth

Having demonstrated that *P. falciparum*-infected erythrocytes have PKA-C activity we then wanted to know if it was necessary for parasite growth within the red blood cell. We used the highly specific PKA inhibitor H89, rather than PKI, as H89 is membrane permeable and can be given to live cells. At reasonably low doses (IC_{50} at less than 3 μM) we observed parasite growth arrest *ex vivo* (Table 1). H89 was equally effective on chloroquine resistant and choloroquine sensitive parasites (2.5 vs. 2.9 μM).

Structural considerations suggest the possibility of developing specific PfPKA inhibitors

Much structural analysis has been performed on mouse PKA-C [3], which displays significant similarity with the parasite enzyme (see Fig. 1). One can see immediately that the catalytic centre (including the ATP-binding site) is strictly conserved, which could suggest that available protein kinase inhibitors based on nucleotide analogues of ATP might not be good candidates for specific PfPKA inhibition. We present in Fig. 6A the 3D structure of mouse the PKA-C α subunit complexed with the inhibitor PKI

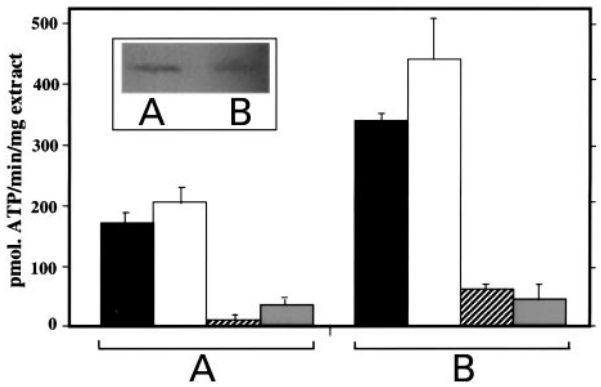

Fig. 5. Developmentally-regulated PKA activity in cellular extracts from schizonts. PKA activity, expressed in pmol of ATP per min and per mg of protein extract, was measured as described in Materials and methods the in absence (black bars) or presence (white bars) of cAMP, in presence of cAMP and PKI (slanted bars), and in presence of cAMP and H89 (dotted bars). Inset: Western blot detection of human PKA-C α subunit in the extracts submitted to PKA activity testing. Cell extracts were from noninfected erythrocytes (A), and from schizonts (B). The standard deviations calculated represent the mean values of five independent experiments.

Table 1. Inhibition (IC_{50}) of intraerythrocytic growth of *P. falciparum* by the PKA inhibitor H89. The inhibition tests were performed on two *P. falciparum* reference clones, where D6 is chloroquine sensitive and W2 is chloroquine resistant. The results are expressed as the minimal concentration at which 50% inhibition of growth was observed with H89 and were calculated following linear regression analysis.

Independent experiment	IC_{50} (μM) Clone CQ-S D6[a]	Clone CQ-R W2[b]
1	1.4	3.2
2	4.6	1.9
3	2.6	2.3
Average concentration	2.9	2.5

[a] Standard deviation 1.6, standard error 0.9, 95% Confidence interval 1.4–4.4 μM. [b] Standard deviation 0.7, standard error 0.4, 95% Confidence interval 2.0–3.0 μM.

(shown in yellow), where we have colour-coded the residues conserved (blue) and variant (red) with PfPKA-C. It is noteworthy that most amino-acid differences between the protozoan and mammalian enzymes are distributed across the molecular surface and importantly, certain variants correspond to amino-acid residues that are in contact with (or in close proximity to) the PKI inhibitor. These include Leu82 in mouse PKA (replaced by Gln in PfPKA) that interacts with a His residue of PKI, and Tyr330 (Val) and Glu328 (Asp), both of which are located close to one of the two conserved Arg residues in PKA substrates. In addition, at the periphery of the PKI-binding cleft, the mouse PKA loop between positions 235 and 245 includes several amino-acid residues that are changed in PfPKA (Figs 1 and 6A). Because we treated infected erythrocytes with H89 we show it (yellow) complexed with the porcine enzyme in Fig. 6B. The conserved amino acids are coloured green, the G-loop as a white ribbon and the C-terminus as a purple ribbon. One can see that the H89 binding site of the parasite enzyme has two variant residues (shown red). In spite of these differences, we have shown that Kemptide, PKI and H89 are PfPKA-C ligands *in vitro*, but clearly differential-binding efficiencies for PKA inhibitors could occur between the mammalian and parasite enzymes *in vivo*. In this context, differential susceptibilities of homologous kinases (yeast CDC28 and starfish cdc2) to the same inhibitor have been reported [22].

DISCUSSION

We have cloned and characterized the catalytic subunit gene for the cAMP-dependent kinase of *P. falciparum.* Following our original deposition of the PfPKA-C sequence in the database, a subsequent report has appeared [23]. Interestingly, *Pfpka-c* gene expression appears to be developmentally regulated, decreasing markedly in sexual stage parasites (Fig. 2). The predominant *Pfpka-c* expression in asexual blood stages described here and by Li and Cox [23] may in fact be restricted to schizonts, as a recent microarray-based study of gene expression also detected *Pfpka-c* transcripts at this stage [24]. The developmental down-regulation of *Pfpka-c* levels in sexual stages may render regulation of the kinase more sensitive to fluctuations in cAMP, thereby explaining the effect of this compound on gametocytogenesis in *P. falciparum* cultures [6–8]. Alternatively, it is possible that the observed effect of cAMP on gametocytogenesis may result from a signalling event involving the PKA activity present in the preceding

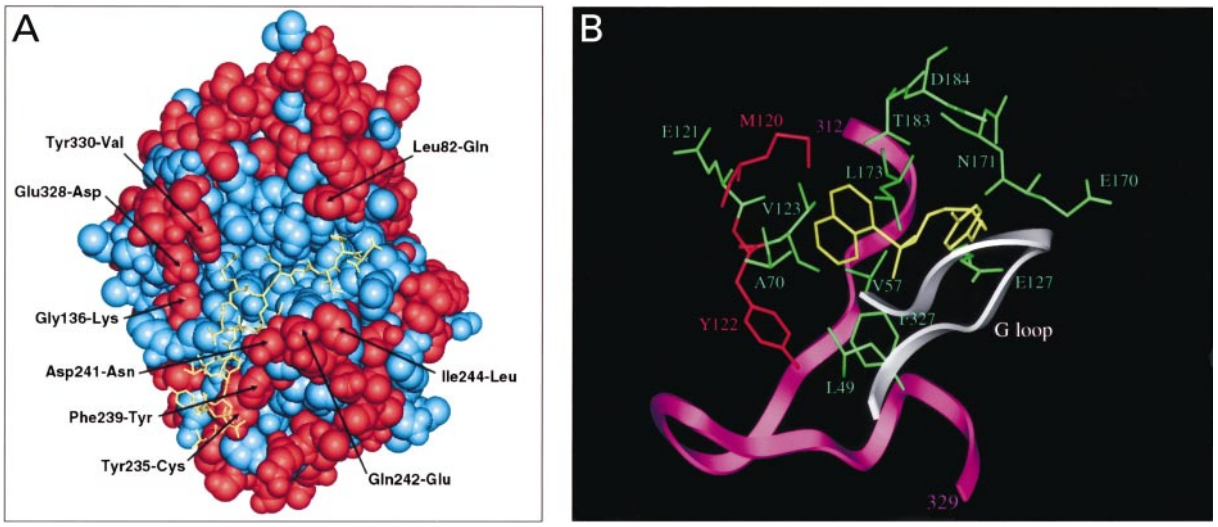

Fig. 6. Divergent residues between PfPKA and mammalian PKA-Cs. (A) Mouse PKA-C α bound to the PKI inhibitor (yellow). Amino-acid residues that differ between the mouse and plasmodial enzymes are coloured red, identical residues are coloured blue. The atomic co-ordinates of the mouse PKA/PKI complex were taken from the RCSB PDB (accession code 1ATP). The figure was made using the program QUANTA (MSI Inc.). (B) Amino acids from porcine PKA-C α implicated in binding to the H89 inhibitor (yellow) according to Engh *et al*. [26]. Amino-acid residues that differ between the porcine and plasmodial enzymes are coloured red, identical residues are coloured green. Part of the G-loop (amino acids 48–57) and of the C-terminal end (amino acids 312-329) are shown as grey and purple ribbons, respectively. The atomic co-ordinates of the bovine PKA/PKI/H89 complex were taken from the RCSB Protein Data Bank (accession code 1YDT). Molecular modelling of the enzyme complex was carried out on a Silicon Graphics O2 wok-station using INSIGHT II 98 software (Biosym/MSI).

schizont. This notion is based on the observation that in *P. falciparum* cultures commitment to sexual differentiation occurs during the asexual cycle producing the merozoites that will develop into gametocytes [25].

Importantly, our ability to ascribe the majority of *Pfpka-c* gene expression and enzymatic activity to asexual schizonts suggests that parasite PKA activity might be necessary for completion of the asexual cycle, or re-invasion of erythrocytes. Although the H89 inhibitor studies (Table 1) support this view, the presence of an endogenous PKA activity in red blood cells hampers clear interpretation, as it is possible that intraerythrocytic growth arrest could be due to a block in erythrocyte, rather than parasite PKA activity. Clearly, differential inhibition of the two kinases will be required to delineate between these two possibilities. In this context, structural comparisons between the parasite and mouse PKA-C enzymes revealed both distinct areas of conserved residues, and divergent regions (Fig. 6). One area of conservation corresponds to the enzymatic reaction centre and the other covers a region that has been proposed to interact with the globular domain of the mouse regulatory subunit PKA-R [2]. A putative regulatory subunit has been biochemically identified in *P. falciparum* extracts [10] and a gene showing significant identity ($\approx 40\%$) to regulatory subunit genes from other species is present in the nonannotated database (http://baggage.stanford.edu/cgi-misc/PlasmodiumSubmission.cgi). This suggests that not only the catalytic mechanism, but also the overall mode of interaction between the catalytic and the regulatory subunits are conserved across the entire family of PKAs and are functional in malaria parasites. The ability of cAMP to stimulate PfPKA activity in the *in vitro* kinase assays (Fig. 5) is consistent with this notion.

For the parasite proliferation studies, the chemical inhibitor H89 was used in preference to the commercially available PKI inhibitor, because unlike PKI, H89 is membrane permeable and can be given to live cells. Moreover, most of the 14 amino acids of the porcine PKA catalytic subunit implicated in the H89 drug binding [26] are conserved, with the exception that M120 and Y122 (porcine numbering) are substituted by L112 and F114 (*P. falciparum* numbering) in the parasite enzyme (Fig. 6B). As expected, given the degree of conservation in the binding site, the PKA activity detected in schizont extracts was inhibited by H89. Although at this concentration (3 μM) H89 also inhibited erythrocyte PKA activity *in vitro,* the presence of two divergent residues in the binding site suggests that variants of H89 might be developed that preferentially inhibit the parasite enzyme. The availability of recombinant active PfPKA-C should facilitate testing of other inhibitors, where a parasite-specific compound could eventually lead to a novel drug for treatment of malaria.

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