REVIEW ARTICLE

cAMP-dependent protein kinase from *Plasmodium falciparum*: an update

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SUMMARY

One of the most important public health problems in the world today is the emergence and dissemination of drug-resistant malaria parasites. *Plasmodium falciparum* is the causative agent of the most lethal form of human malaria. New anti-malarial strategies are urgently required, and their design and development require the identification of potential therapeutic targets. However, the molecular mechanisms controlling the life cycle of the malaria parasite are still poorly understood. The published genome sequence of *P. falciparum* and previous studies have revealed that several homologues of eukaryotic signalling proteins, such as protein kinases, are relatively conserved. Protein kinases are now widely recognized as important drug targets in protozoan parasites. Cyclic AMP-dependent protein kinase (PKA) is implicated in numerous processes in mammalian cells, and the regulatory mechanisms of the cAMP pathway have been characterized. *P. falciparum* cAMP-dependent protein kinase plays an important role in the parasite's life cycle and thus represents an attractive target for the development of anti-malarial drugs. In this review, we focus on the *P. falciparum* cAMP/PKA pathway to provide new insights and an improved understanding of this signalling cascade.

Key words: *Plasmodium falciparum*, malaria, cAMP-dependent protein kinase, PKA, cAMP pathway.

INTRODUCTION

Malaria is one of the most important infectious diseases affecting humans, particularly in developing countries. *Plasmodium falciparum*, an apicomplexan protozoan parasite, is the causative agent of the most lethal form of human malaria. The presence of widespread drug resistance and the lack of a proven vaccine complicate the problem, and limit the available options for effective malaria control. New anti-malarial drugs that interfere with parasite growth are urgently needed, and their rational design and development require the identification of potential therapeutic targets (Renslo and McKerrow, 2006). Detailed knowledge of the molecular mechanisms that control the life cycle of malaria parasites may provide useful information for this purpose (Doerig *et al*. 2008, 2009).

Malaria parasites have a complex life cycle. Infection of humans by *P. falciparum*, the species responsible for the lethal form of human malaria, begins with the bite of an infected *Anopheles* mosquito, which delivers sporozoites to the bloodstream. These cells rapidly gain access to the liver and invade hepatocytes, where they undergo substantial multiplication, generating several thousand merozoites (exo-erythrocytic schizogony). The merozoites invade red blood cells (erythrocytic schizogony), the process responsible for malaria pathogenesis. Some merozoites, however, arrest the cell cycle and differentiate into male or female gametocytes, which are infective to the mosquito. Only after being ingested by the insect, the gametocytes develop into gametes and fuse into a zygote (the only diploid stage). Further development in the mosquito involves a process of sporogony, producing sporozoites that accumulate in the salivary glands and are now ready to infect a new human host. The life cycle of malaria parasites is therefore composed of a succession of developmental stages that vary in their proliferative state (massive cell multiplication during schizogony and sporogony, and cell cycle arrest in sporozoites and gametocytes). These different parasite developmental stages require a high degree of adaptation and strict control of the cellular machinery as well as the coordinated modulation of distinct sets of genes. *Plasmodium* cells regulate these processes by several means, including phosphorylation, transcriptional control, post-transcriptional control and protein degradation. These mechanisms probably involve various interactions between parasite and host

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signalling molecules and may thus represent strategic targets in the fight against malaria (Doerig *et al*. 2009).

The modulation of protein phosphorylation through the antagonistic effects of protein kinases and phosphatases is a major regulatory mechanism of many eukaryotic intracellular processes (Manning *et al*. 2002*a*). The published *P. falciparum* genome sequence and previous studies have revealed that several homologues of eukaryotic signalling proteins are conserved in *P. falciparum* (Gardner *et al*. 2002; Chung *et al*. 2009; Koyama *et al*. 2009), but many of these major mediators have not been characterized for malaria parasites. There is now evidence that protein kinases are essential for the control of the parasite life cycle and that inhibition of such activities can have anti-malarial effects (Doerig *et al*. 2010). Moreover, these enzymes are widely recognized as valuable drug targets for the treatment of several diseases (Johnson, 2007; Grant, 2009). Analyses of the *P. falciparum* kinome have revealed 86 or 99 genes (depending on the study) that encode proteins containing kinase domains; however, their functions, mechanisms of regulation and cellular targets are largely unknown (Ward *et al*. 2004; Anamika *et al*. 2005).

cAMP-dependent protein kinase (protein kinase A, or PKA) is a key signal transduction element in mammalian cells, and the regulatory mechanisms of the cAMP pathway are well known (Taylor *et al*. 2008*b*). However, in *P. falciparum*, components of the cAMP pathway and the precise function of PKA, its downstream target, have not been clearly defined. The cAMP/PKA signalling pathway has attracted interest from a number of research groups, and there are reports of cAMP-dependent protein kinase homologues (PfPKA) in the *P. falciparum* genome (Li and Cox, 2000; Syin *et al*. 2001; Beraldo *et al*. 2005; Doerig *et al*. 2008; Merckx *et al*. 2008*a*; Wurtz *et al*. 2009*b*). The aim of this review is to provide a synthesis of the recent published experimental data on the cAMP/PfPKA signalling pathway, which appears to be essential for parasite growth and survival and, consequently, represents an attractive target for the development of new anti-malarial drugs.

OVERVIEW OF THE cAMP/PKA SIGNALLING PATHWAY

PKA is the best-studied protein kinase, belonging to the AGC group within the eukaryotic protein kinase superfamily (Manning *et al*. 2002*a*, *b*). It was one of the first protein kinases to be discovered, sequenced and cloned, and the resolution of its structure provided the first three-dimensional template for this family (Walsh *et al*. 1968; Shoji *et al*. 1981; Uhler *et al*. 1986; Knighton *et al*. 1991). PKA and cAMP pathways have been implicated in numerous cellular processes, including modulation of other protein kinases, regulation of intracellular calcium concentration and regulation of transcription (Shabb, 2001; Tasken and Aandahl, 2004).

In most organisms, PKA is a heterotetramer composed of 2 regulatory subunits (PKAr), which bind to and inhibit 2 catalytic subunits (PKAc). The cAMP pathway is activated by the binding of a ligand to a membrane-bound G-protein-coupled receptor (GPCR) (7 transmembrane receptor), which interacts with heterotrimeric G-proteins composed of α, β and γ subunits that are bound in the inactive state. The agonist binding triggers a conformational change in the receptor, which catalyses the exchange from GDP to GTP and the dissociation of G_α from $G_{\beta\gamma}$ subunits. Both G_α and $G_{\beta\gamma}$ subunits can modulate the activity of downstream effectors. In particular, Gα subunit proteins are divided into 4 subfamilies ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$) (Hamm, 1998) and a single GPCR can couple to 1 or more families resulting in different cellular responses (Cabrera-Vera *et al*. 2003). When coupling to $G_{\alpha s}$ or $G_{\alpha i}$ proteins, GPCRs either activate or inhibit adenylate cyclase (AC) activity resulting in an increase or decrease in cAMP formation, respectively (Cabrera-Vera *et al*. 2003). The flux through the pathway is controlled by the self-inactivating GTPase activity of the G_α subunit, which hydrolyses GTP to GDP, resulting in re-association of the G_α subunit with the $G_{\beta\gamma}$ subunits and termination of the G protein signalling. The binding of 2 cAMP molecules to each PKAr alters its affinity for the catalytic subunit, resulting in release of the active PKAc. PKAc, a serine/threonine kinase, can phosphorylate many substrates, such as additional protein kinases and transcription factors (Shabb, 2001). The termination of cAMP signalling is conferred by a large superfamily of enzymes known as phosphodiesterase proteins (PDE) that catalyse the degradation of cAMP into 5′AMP (Fimia and Sassone-Corsi, 2001; Lugnier, 2006). The intracellular concentrations of cAMP are therefore regulated by the counterbalancing activities of ACs and PDEs. In addition, the signalling events induced by agonist activation of GPCRs can be counteracted in the cell by intrinsic mechanisms known as the receptor desensitization. Phosphorylation is the most rapid means of GPCR desensitization and is achieved by 2 classes of serine/threonine protein kinases: PKAc directly *via* a feedback regulation and G protein-coupled receptor kinases (GRKs). GRK-mediated receptor phosphorylation promotes the binding of β-arrestins, which not only uncouple receptors from heterotrimeric G proteins but also target many GPCRs for internalization, followed by either recycling or degradation of the receptor (Lefkowitz, 1998; Ferguson, 2001; Hendriks-Balk *et al*. 2008). In mammalian cells, PKA contains 3 catalytic subunit isoforms (Cα, Cβ and Cγ) and 4 regulatory subunit isoforms (RIα, RIβ, RIIα and RIIβ) (Doskeland *et al*. 1993). The tissue-specific expression and assembly patterns of these kinase isoforms are thought to be

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responsible for the diverse cellular responses to cAMP (Taylor *et al*. 1990). PKAc activity can be regulated by binding to protein kinase inhibitor (PKI), its natural endogenous inhibitor (Dalton and Dewey, 2006). PKI inhibits the activity of PKAc by binding to free catalytic subunits of this enzyme and inhibiting the phosphorylation of PKAc substrates (Ashby and Walsh, 1972, 1973). PKI is similar to the PKAr in that both proteins contain amino acid sequences (pseudosubstrate sites) that allow them to bind to the PKAc and inhibit its activity. The functional specificity of PKA is largely dependent on the targeting of the catalytic subunit to specific substrates at precise locations in the cell. This is accomplished by the A-kinase anchoring-protein (AKAP) family, whose members are bound to subcellular structures and recruit PKA *via* interactions with the regulatory subunit (Barradeau *et al*. 2002; McConnachie *et al*. 2006).

P. falciparum cAMP-dependent protein kinase activity has been detected in cytosolic extracts of both the asexual and sexual stages of the parasite, and a putative regulatory subunit has been identified in asexual forms (Kaushal *et al*. 1980; Read and Mikkelsen, 1990; Read and Mikkelsen, 1991*b*), suggesting the existence of the cAMP pathway in the parasite (Fig. 1). The single *P. falciparum* PKA catalytic subunit (*pfpkac*) gene was first isolated and characterized using a PCR-based approach, which identified a DNA fragment that shared high sequence homology with catalytic subunits of the PKA family (Li and Cox, 2000). Subsequent studies of this kinase have illuminated much about its structure and substrate specificity (Syin *et al*. 2001; Sudo *et al*. 2008; Wurtz *et al*. 2009*b*).

The single *P. falciparum* PKA regulatory subunit (PfPKAr) was first identified using BLASTP analyses (Altschul *et al*. 1990) with PKAr subunits from various eukaryotes as queries, and the gene was later cloned and expressed (Ward *et al*. 2004; Merckx *et al*. 2008*b*).

An AC activity, biochemically distinct from that of the host was first measured in *P. falciparum* by Mikkelsen and Read (Read and Mikkelsen, 1991*b*). Two different genes with high homology to ACs (PfACα PF14_0788 and PfACβ MAL8P1.150) have been identified and characterized in *P. falciparum* (Muhia *et al*. 2003; Baker, 2004; Baker and Kelly, 2004; Weber *et al*. 2004). Four putative *P. falciparum* PDEs have now been identified, containing the class I signature motif and sharing approximately 40% amino acid identity (PfPDEα PFL0475w, PfPDEβ MAL13P1.118, PfPDEγ MAL13P1.119 and PfPDEδ PF14_0672). The cyclic nucleotide specificity of the 4 encoded enzymes cannot be predicted on the basis of primary amino acid sequence, but it seems that PfPDEα and PfPDEδ are more specific to cGMP (Yuasa *et al*. 2005; Taylor *et al*. 2008*a*; Wentzinger *et al*. 2008). In the PlasmoDB database, 2 genes coding for a G-protein coupled receptor, putative (PFE1265w) and for a G-protein associated signal transduction protein, putative (PFF0365c) have been found, but no other data are available regarding the function of these two proteins. In addition, Madeira and coworkers have identified 4 putative serpentine receptors in *P. falciparum* (PF11_0321, PFL0765w, PFD1075w, MAL7P1.64), but again, their roles and their implication in the cAMP pathway have not been defined (Madeira *et al*. 2008). They predicted that these receptors could be implicated in sensing extracellular signal and that elucidation of their detailed function may highlight the mechanisms used by the parasite to modulate its life cycle. Results consistent with the presence of heterotrimeric G proteins in *P. falciparum* have also been reported, and it has been suggested that they might be involved in the switch to sexual development (Dyer and Day, 2000). However, until now no gene encoding heterotrimeric G-proteins have been identified in *P. falciparum* genome. On the other hand, it has recently been proposed that the cAMP signalling system of the red blood cell could play a role in malaria infection (Harrison *et al*. 2003). Indeed, host GPCRs and $G_{\alpha s}$ appear to be associated with the parasite vacuole (Lauer *et al*. 2000) and addition of peptides that block the interaction between GPCR and $G_{\alpha s}$ led to decreased parasitaemia (Harrison *et al*. 2003). Finally, until now, only 1 AKAP was annotated in the *P. yoelii* genome (PY04627) (Carlton *et al*. 2002). In *P. falciparum*, an orthologue of this AKAP has been annotated as a conserved protein with unknown function (PFE0640w), but no study has been conducted on this topic.

The knowledge of signal-transduction pathways in *Plasmodium* is fundamental to allow the design of new strategies against malaria. According to these previous data, there is evidence that the cAMP/PKA pathway exists in the malaria parasite. However, a number of important components in this pathway have so far not yet been clearly defined and must be studied more thoroughly: the ligand-receptor complex that initiates the cAMP pathway (GPCRs), the signalling molecules that activate AC (is this mediated by heterotrimeric G proteins in *P. falciparum* or not?), the regulation of this cellular network (do GRK, PKI and feedback PKAc control the mechanisms of regulation in the parasite?), and the downstream targets of PfPKAc.

STRUCTURE OF THE cAMP-DEPENDENT PROTEIN KINASE COMPLEX

cAMP-dependent protein kinase catalytic subunit

The PFI1685w gene in the PlasmoDB database (Bahl *et al*. 2003) has 4 introns and 5 exons, and the protein product, PfPKAc, has a predicted molecular mass of

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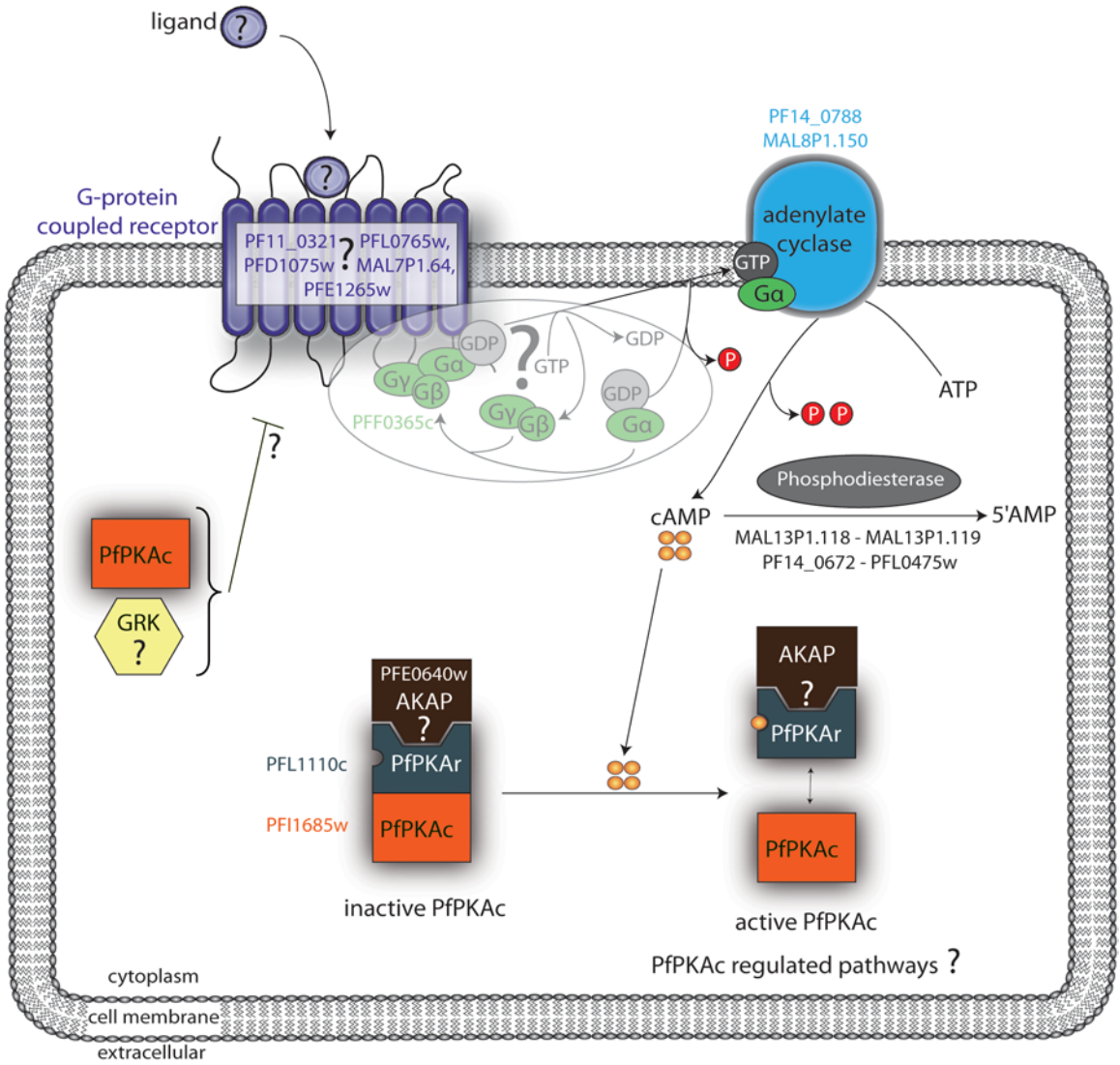

Fig. 1. Schematic model of cAMP/PKA signalling pathway in *Plasmodium falciparum*. This pathway is based on data reported in the literature. All elements that are not known or not proven are associated with a question mark. The pathway begins when an unknown ligand activates a GPCR. The transduction signal is transmitted to G-proteins (not yet identified in *P. falciparum*), which activate adenylate cyclase. The latter allows the production of cAMP from ATP. The cAMP binds to the PfPKAr, which changes its conformation and allows the release of PfPKAc. Once free, PfPKAc phosphorylates unknown substrates using ATP as the phosphate donor. PDEs degrade cAMP and thus limit PfPKAc activation. In *P. falciparum*, the existence of AKAP proteins has not yet been proven, but an orthologue of *P. yoelli* AKAP is annotated in the *P. falciparum* genome as a conserved protein with unknown function (PFE0640w). The signalling events induced by agonist activation of GPCRs can be counteracted in eukaryotes by PKAc directly or by GRKs. However, in *Plasmodium* these mechanisms, as well as GRKs, have not been identified.

40·2 kDa and a calculated pI of 9·11. Comparative analyses using BLASTP revealed that the amino acid sequence of PfPKAc shares about 50% of identity with PKAc from *Homo sapiens* and *Mus musculus* and high homology (88%) with *P. yoelii* PKAc (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment of the amino acid sequences of PKAc from various organisms using T-Coffee method (Notredame *et al*. 2000; Poirot *et al*. 2003) showed strong conservation of the 11 specific kinase subdomains (I-XI) among the different organisms. Several residues required for catalytic activity are also highly conserved (Hanks *et al*. 1988) (Fig. 2A and B and Table 1). A three-dimensional model of PfPKAc was created based on the crystal structure of *Homo sapiens* PKAc alpha subunit (HsPKAc; PDB accession number 2GU8) using the Swiss-Model ternary structure prediction tool (Arnold *et al*. 2006) and Pymol 0.99 software (DeLano, 2008) (Fig. 2B). As expected, and due to a high degree of primary sequence similarity (50% identity) between the two proteins, the PfPKAc model showed structural homology to HsPKAc. The PKAc subunits are bilobal enzymes with 2 major domains (the small N-terminal lobe and the large C-terminal lobe) that are conserved throughout the protein kinase family (Hanks *et al*. 1988; Taylor *et al*. 1990; Hanks and Hunter, 1995; Smith *et al*. 1999; Johnson *et al*. 2001;

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Taylor *et al*. 2008*b*). The smaller N-terminal lobe, which includes subdomains I–IV, is primarily involved in the anchoring and orientation of ATP. This lobe has a predominantly anti-parallel β-sheet structure. The larger C-terminal lobe, which comprises subdomains VI to XI, is mainly composed of α-helices. It serves as a framework for the catalytic machinery and also as a docking scaffold for binding to protein partners that act as substrates or inhibitors. Moreover, the N-terminal lobe can also be involved in docking with some proteins and partners, for example, the A Kinase Interacting Protein (AKIP 1) binds to the N-terminus of PKAc and helps to traffic it into the nucleus (Sastri *et al*. 2005; Taylor *et al*. 2008*b*; Kornev and Taylor, 2010). Table 1 summarizes the important residues, secondary structure and function of each subdomain for both HsPKAc and PfPKAc. We are particularly interested in the sequence and structural differences between HsPKAc and PfPKAc, as the ultimate goal of this study is to design molecules that specifically target the parasite enzyme (Fig. 2B and Table 1). The first divergence concerns subdomain I, which is composed of 2 β-strands and 2 α-helices in PfPKAc but consists of 2 β-strands only in HsPKAc. As shown in Table 1, this subdomain participates in ATP anchoring. Next, in subdomain VI, a β-strand and an α-helix are missing from the PfPKAc model. This domain contains the putative catalytic loop sequence HRDLK*XX*N, which includes an aspartate identified as the candidate catalytic base (Hanks and Hunter, 1995). The last notable divergence concerns subdomain VIII, where the α-helix in HsPKAc is divided into 2 α-helices in PfPKAc. In the same domain, a β-strand was also absent in PfPKAc model as compared to the HsPKAc structure. Moreover, a tryptophan (W^{196}) previously identified as essential for mammalian PKAr binding is not conserved in the PfPKAc subdomain VIII sequence (Y^{187}) (Gibson and Taylor, 1997; Kim *et al*. 2005). Other differences between PfPKAc and HsPKAc were also observed, but these were not located in known functional domains (Table 1).

Despite its high sequence identity and strong structural homology with HsPKAc, PfPKAc presents some interesting differences in essential domains involved in the following functions: (i) ATP anchoring, (ii) mechanisms of substrate phosphorylation and (iii) substrate recognition and/or inhibitor sensitivity. The development of new anti-malarial compounds targeting these domains could allow for the inhibition of the parasite enzyme with low levels of host protein interaction.

cAMP-dependent protein kinase regulatory subunit

The PFL1110c gene in the PlasmoDB database has 2 introns and 3 exons, and the protein product, PfPKAr, has a predicted molecular mass of 50·8 kDa and a calculated pI of 7·49. Comparative analyses using BLASTP revealed that the amino acid sequence of PfPKAr shares ~41% identity with PKAr from *Homo sapiens, Bos taurus* and *Mus musculus* and high homology with *P. knowlesi* strain H PKAr (73%) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A multiple sequence alignment of the amino acid sequences of PKAr from various organisms using the T-coffee program (Notredame *et al*. 2000; Poirot *et al*. 2003) revealed a similar general architectural organization among the various organisms and several residues required for regulatory activity, as well as some interesting differences (Taylor *et al*. 1990) (Fig. 3A and B and Table 2). The nuclear magnetic resonance (NMR) structures of mammalian type I and type II regulatory subunit D/D domains have been solved (Banky *et al*. 1998; Newlon *et al*. 2001), as have the crystal structures of the isolated CBD-A and CBD-B (Diller *et al*. 2001; Su *et al*. 1995). However, no high-resolution structures are available for the full-length regulatory subunit, linker regions or cAMP-free regulatory subunits. Thus, we created a three-dimensional model of PfPKAr based on the crystal structure of a 1–91 deletion mutant of the type I alpha regulatory subunit from *B. taurus* (BtPKAr; PDB Accession number 1RGS) using the Swiss-Model ternary structure prediction tool and Pymol software. The BtPKAr sequence is very close to that of *H. sapiens* PKAr (97% identity), which does not have a described structure.

In most cells, the regulatory subunit is typically a highly asymmetric dimer composed of different domains. The amino-terminal region of the regulatory subunit (1–140) corresponds to the dimerization/docking domain (D/D) responsible for homodimerization. Once dimerized, this region also provides a binding surface for the AKAPs (Banky *et al*. 1998; Newlon *et al*. 2001). The D/D domain of the type I regulatory subunit contains a number of conserved residues that are critical for dimerization and AKAP binding (C^{16}, Y^{19}, V^{20}, N^{24}, I^{25}, L^{36}, C^{37}, L^{48}, Y^{51}, F^{52} and A^{60} in BtPKAr) (Leon *et al*. 1997; Gibson *et al*. 2006) (Fig. 3A and Table 2). This N-terminal sequence, which encodes the regulatory D/D domain found in most regulatory subunits, is not present in the parasite protein, suggesting that it does not undergo regulatory subunit dimer formation as previously reported for *P. falciparum* (Syin *et al*. 2001; Merckx *et al*. 2008*b*) and several other organisms (Mutzel *et al*. 1987; Carlson and Nelson, 1996). While an orthologue of *P. yoelii* AKAP is found in *P. falciparum* genome, no consensus AKAP-binding domain was present in the *P. falciparum* regulatory subunit. This suggests that the parasite uses a mode of binding between AKAP and PfPKAr that is distinct from that of other species (Barradeau *et al*. 2002; McConnachie *et al*. 2006).

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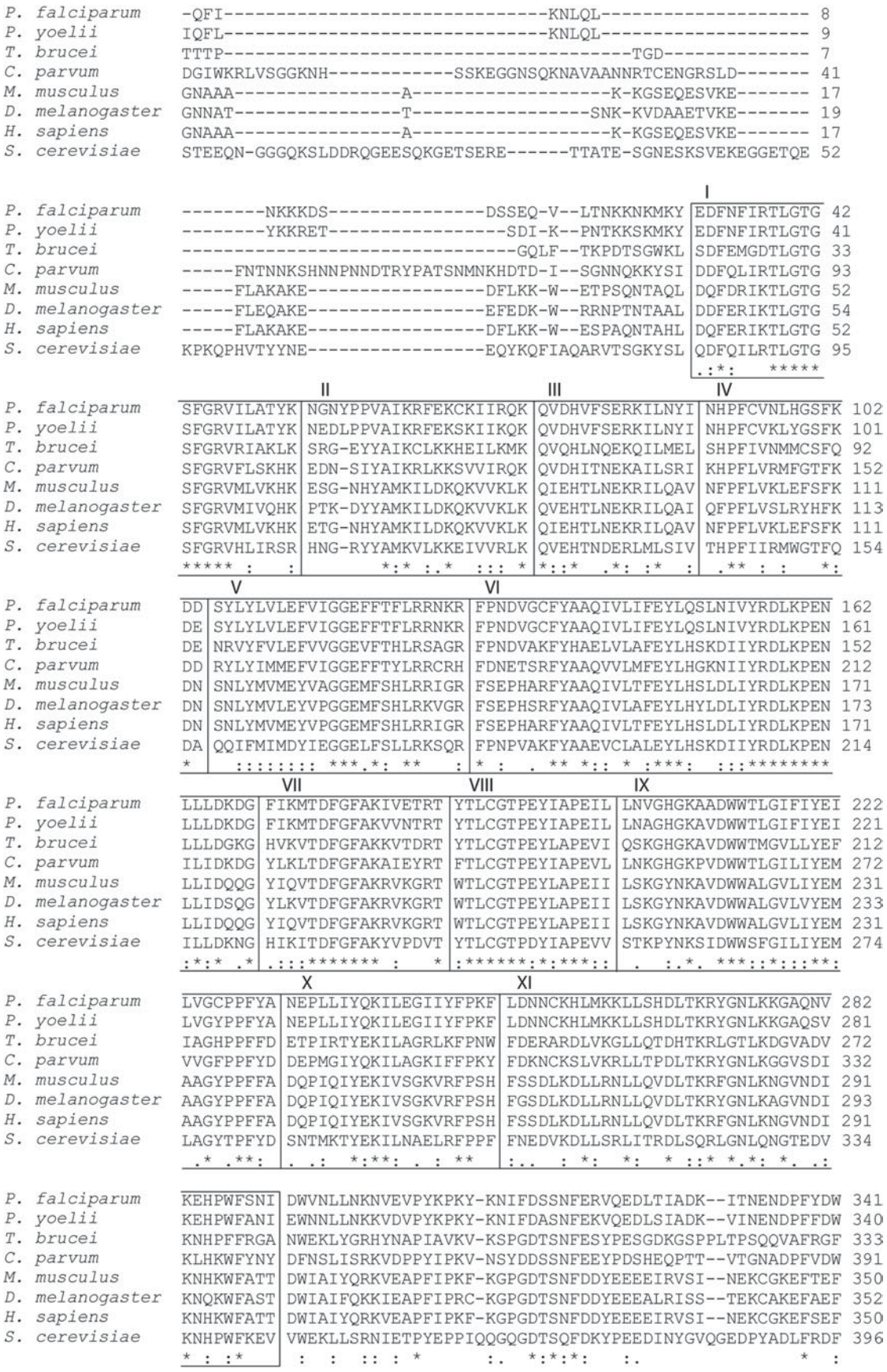

Fig. 2. (Cont.)

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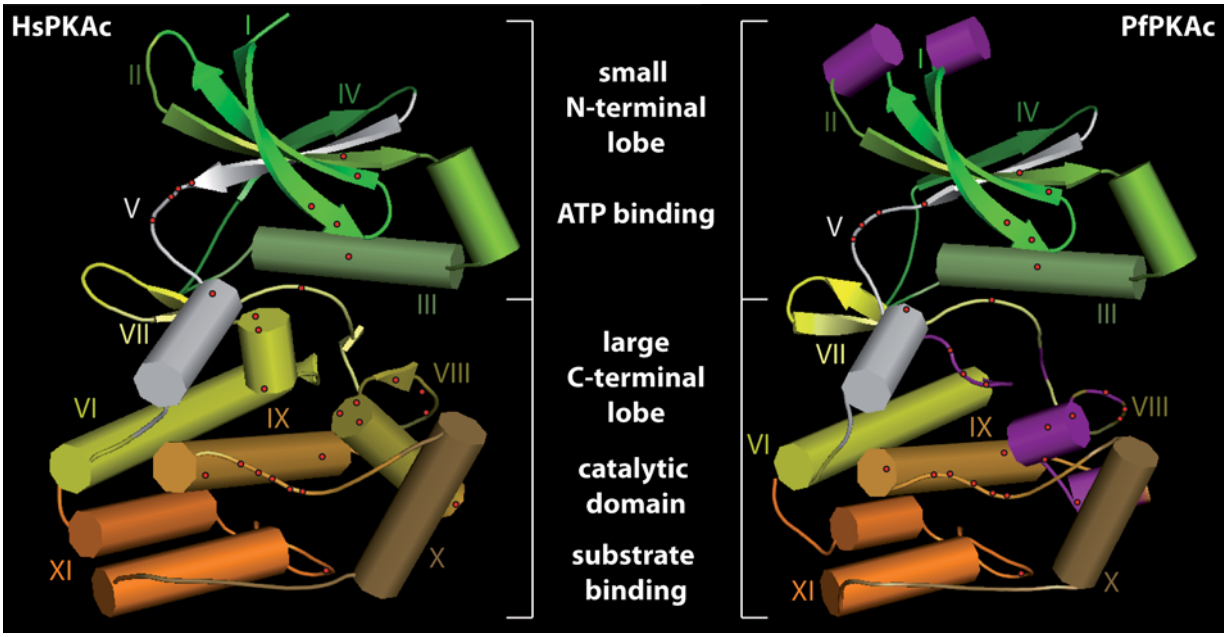

Fig. 2. Amino acid sequence alignments and structural modelling of PfPKAc. (A) Comparison of the PfPKAc amino acid sequence with those of other protein kinase catalytic subunits. *Plasmodium falciparum* (Uniprot Accession number Q7K6A0), *Homo sapiens* (P17612), *Mus musculus* (P05132), *Plasmodium yoelii* (Q7RE33), *Cryptosporidium parvum* (A3FQ39), *Trypanosoma brucei* (Q38DR5), *Saccharomyces cerevisae* (P06244) and *Drosophila melanogaster* (P12370) were aligned using T-coffee 7.38 and CLUSTAL format. The 11 typical subdomains are indicated with boxed regions and Roman numerals. Identical residues (asterisks), conservative substitutions (colons) and semi-conservative substitutions (dots) are also indicated. (B) Structural model of PfPKAc. The PfPKAc structure was modelled on the resolved structure of human PKAc alpha. The different colours represent the 11 subdomains characteristic of protein kinases. The small N-terminal lobe, which is involved in ATP binding, and the large C-terminal lobe, which comprises the catalytic regions and substrate-binding domain, are represented in the figure. α-helices (cylinders), β-sheets (arrows) and turns (lines) are indicated, as are secondary structure differences in PfPKAc (purple) and essential residues in both structures (red dots).

In mammals, the D/D domain is followed by a flexible linker region that contains an inhibitory site. This site, generally located between residues 90 and 100 in the regulatory subunit, binds to and inhibits the active site of the catalytic subunit (Li *et al*. 2000). The amino acid sequence of this region is similar to that of its catalytic subunit substrates and thus can be used to differentiate between type I and type II regulatory subunits. This region includes either a pseudosubstrate site (RR*X*A or RR*X*G in type I regulatory subunits) or an autophosphorylation region with a serine at the phosphorylation site (RR*X*S in type II regulatory subunits) (Taylor *et al*. 1990). The overall architecture of PfPKAr is closer to type I BtPKAr, but it presents a degenerated inhibitory sequence (KR*X*S) containing 1 of 2 important arginines and a serine autophosphorylated site similar to that of type II BtPKAr. Thus, PfPKAr seems to share characteristics with both type I and type II BtPKAr.

The C-terminal end of the mammalian regulatory subunit contains 2 tandem cAMP-binding domains (CBDs), named A and B (Takio *et al*. 1984). Binding of cAMP to the CBD of the regulatory subunit dissociates the catalytic subunit, which becomes catalytically active. These CBDs, which probably resulted from domain duplication, show a strong sequence homology among the different organisms. Comparison of PfPKAr with other mammalian PKAr reveals the existence of these two highly conserved CBDs, which contain essential residues necessary for cAMP binding: a conserved glutamate that binds to the 2′OH of the ribose (E^{200} and E^{324} in BtCBD-A and BtCBD-B, respectively) and a conserved arginine that interacts with the phosphate of cAMP (R^{209} and R^{333} in BtCBD-A and BtCBD-B, respectively) (Taylor *et al*. 1990; Su *et al*. 1995; Berman *et al*. 2005) (Fig. 3A and Table 2). These residues are part of an important motif called the phosphate-binding cassette (PBC) that is present in each of the CBDs (PBC-A and PBC-B). The structure of the truncated PfPKAr revealed strong structural homology with BtPKAr (Fig. 3B). The CBD is a small module, about 120 amino acids in length, which consists of helical domains and an 8-stranded β-barrel where cAMP binds. The essential feature of the β-barrel is the conserved PBC that anchors cAMP and shields it from solvent interactions (Canaves and Taylor, 2002). The PBCs comprise β-strand 6, a short turn of α-helix and β-strand 7. Most of the variability in the CBDs corresponds to the loop between β-strand 4 and β-strand 5 and the C-terminal region of each A

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Table 1. Major structural features of the catalytic domain of PKAc from *Homo sapiens* and *Plasmodium falciparum*

Subdomains	PfPKAc	HsPKAc	Functions
I	$G^{40}G^{42}G^{45}$ 2 β-strands, 2 α-helices	$G^{50}G^{52}G^{55}$ 2 β-strands	ATP fixation
II	K^{63} 1 β-strand, 1 α-helix	K^{72} 1 β-strand, 1 α-helix	essential for optimal kinase activity
III	E^{82} 1 α-helix	E^{91} 1 α-helix	ATP stabilization
IV	ND 1 β-strand	ND 1 β-strand	ND
V	$L^{111}E^{112}F^{113}V^{114}E^{118}$ 1 β-strand, 1 α-helix	$M^{120}E^{121}Y^{122}V^{123}E^{127}$ 1 β-strand, 1 α-helix	linker between the two lobes ATP stabilization / substrate recognition
VI	$D^{157}N^{162}$ E^{161} 1 α-helice, 1 β-strands	$D^{166}N^{171}$ E^{170} 2 α-helices, 2 β-strands	catalytic loop substrate recognition
VII	D^{175} 1 β-strand	D^{184} 2 β-strands	orienting the phosphate of ATP
VIII	E^{199} $Y^{187}L^{189}C^{190}P^{193}I^{196}$ T^{188} 2 α-helices	E^{208} $W^{196}L^{198}C^{199}P^{202}L^{205}$ T^{197} 1 β-strand, 1 α-helix	catalytic loop substrate recognition essential for optimal kinase activity
IX	D^{211} $E^{221}C^{226}P^{227}P^{228}F^{229}Y^{230}$ 1 α-helix	D^{220} $E^{230}Y^{235}P^{236}P^{237}F^{238}F^{239}$ 1 α-helix	stabilization of the catalytic loop substrate recognition
X	ND 1 α-helix	ND 1 α-helix	ND
XI	$R^{271}2$ 2 α-helices	R^{280} 2 α-helices	subdomain stabilization

ND, Not determined.

and B domain. In *P. falciparum*, the 2 CBDs are well conserved; however, there are slight differences between them, especially in the C-terminal area of each CBD, where the α-helices 7 and 8 present in BtPKAr structure are grouped into a single helix in PfPKAr (Fig. 3B).

Analysis of the amino acid sequence and structure of PfPKAr indicates that it shares a number of conserved features with other PKAr proteins. However, there are also several interesting differences, including the degenerate D/D domain and the inhibitory sequence, which is a mixture between type I and type II regulatory subunits.

PHYLOGENETIC ANALYSIS OF PFPKA

Phylogenetic analyses of PKAc and PKAr were performed with sequences from 18 different species, including representatives of Apicomplexa, Euglenozoa and Metazoa phyla. The results are shown in Fig. 4. The methods used to infer the phylogenetic trees are presented in the legend of the figure. The Metazoa clade is fully supported, with 100% bootstrap values in the phylogenetic analyses of both PKAr and PKAc sequences. Within the Metazoa, mammals (*B. taurus, Rattus norvegicus, Mus musculus* and *H. sapiens*) are grouped together (100% bootstrap), and the mammalian relationships are congruent between the two datasets, although with weaker support in the PKAr analysis. Both datasets group the Euglenozoa as a monophyletic clade, with 87% and 100% bootstrap values for PKAc and PKAr, respectively. The Apicomplexa are monophyletic in the PKAr tree and paraphyletic in the PKAc tree. This paraphyly could be explained by the low support for the positions of Apicomplexans *Toxoplasma gondii, Cryptosporidium parvum* and *Babesia bovis* and their long respective branches in both analyses. With both genes, the *Plasmodium* species are grouped in a strongly supported clade (100% bootstrap). The 3 rodent malaria parasites, *P. berghei, P. yoelii* and *P. chabaudi,* form a group with a high bootstrap value that is separated from the human malaria species. Concerning the human parasites, *P. vivax* and *P. knowlesi* are clustered together but *P. falciparum* is either at the root of these species (PKAc) or at the root of the rodent ones (PKAr). The *P. falciparum* branches are the longest among the *Plasmodium* species. The phylogenetic distance between *Plasmodium* species and their vertebrate host (*H. sapiens*) is considerable for both PKAc and PKAr. This information, together with the differences observed in sequences and structures, supports the idea that PfPKA can be specifically inhibited, further establishing this

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protein as an interesting target for anti-malarial compounds.

METHODS TO STUDY THE PKA AND CAMP PATHWAY IN *P. FALCIPARUM*

The biochemical and biological studies of PfPKA has been dominated by the use of either pharmacological inhibitors/activators of members of the cAMP pathway or by molecular strategies.

Biochemical strategies

Two compounds in particular have been widely used to study PKAc function in eukaryotic cells: H89 and KT5720. H89 is an isoquinoline derivative developed from inhibitor H8 (Hidaka *et al*. 1984), while KT5720 belongs to a family of compounds synthesized by the fungus *Nocardiopsis* sp. (Kase *et al*. 1987). Both inhibitors act through similar mechanisms as competitive antagonists of ATP at its PKAc binding site, thus preventing cAMP-dependent phosphorylation of PKAc substrates. However, these two compounds seem to have various non-specific effects, such as inhibiting other protein kinases, sometimes more potently than their intended target (Davies *et al*. 2000; Murray, 2008).

Rp-cAMP, another PKAc inhibitor, acts as a competitive antagonist of cAMP by binding to PKAr (on CBD domain) without dissociating the kinase holoenzyme (Gjertsen *et al*. 1995). In contrast to H89 and KT5720, this compound may not have effects outside the cAMP signalling pathways (Murray, 2008).

PKI peptide, which is an endogenous molecule involved in the regulation of PKA activity, binds to free PKAc and prevents the phosphorylation of PKAc targets. PKI seems to be a more specific PKAc inhibitor than H89 or KT5720, but all three have been widely used to study PfPKA and cAMP signalling in *P. falciparum*. Other inhibitors of the cAMP pathway that specifically target AC, such as MDL-12, SQ22536 and dideoxyadenosine were also used and lead to cAMP depletion, impeding PKAc activation.

Activation of cAMP pathway elements is another way to study the function of PfPKAc and, more generally, the organization of the cAMP pathway in *P. falciparum*. Different molecules targeting PKAc directly or indirectly can be employed. Forskolin, a labdane diterpene produced by the *Coleus forskohlii* plant (Takeda *et al*. 1983), acts by activating the AC enzyme, resulting in an increase of cAMP level and thus allowing PKAc activation. It is interesting to note that AC activity is insensitive to forskolin in asexual blood stages (Read and Mikkelsen, 1991*b*), while ACα in sporozoites seems to be sensitive to forskolin stimulation (Ono *et al*. 2008). Many pathogenic bacteria secrete toxins, such as the cholera and pertussis toxins, that alter the intracellular concentration of cAMP. These toxins disrupt the normal regulation of the cAMP pathway by catalysing the ADP-ribosylation of the heterotrimeric G proteins, which prevents AC inactivation. Thus, AC remains inappropriately active, leading to an increase in cAMP concentration that activates PKAc. Another way to stimulate PKAc activity is to add cAMP analogues such as 8-Br-cAMP or 6-Bz-cAMP directly to the cell. PKAc activity can also be induced by preventing the destruction of cAMP by phosphodiesterases. Some molecules, including IBMX and caffeine, inhibit the action of these enzymes, allowing cAMP to accumulate in the cell thereby leading to the activation of PKAc. It should be noted, however, that these molecules had little or no effect when tested on native PfPDE enzyme activity (McRobert *et al*. 2008), but can be used to clarify and to understand the pathway connections in the parasite.

Molecular strategies

Other molecular techniques have been developed to study the cAMP-signalling pathway, such as RNA interference (RNAi). Briefly, double-stranded RNAs (dsRNA) targeting a specific cellular mRNA can be introduced to the cell to knock down the encoded proteins. To date, RNAi-related genes have not been identified in the *P. falciparum* genome (Ullu *et al*. 2004; Baum *et al*. 2009), it has been suggested that the inhibitory effect of dsRNA might be due to an antisense effect rather than a classical RNAi mechanism (McRobert and McConkey, 2002; Noonpakdee *et al*. 2003; Gunasekera *et al*. 2004; Gissot *et al*. 2005; Rathjen *et al*. 2006). Another possibility is that the proteins involved in RNAi processes (Dicer, RNA-Induced Silencing Complex . . .) could be transported into the parasite from the human host cell (Rathjen *et al*. 2006). Although there has been some controversy surrounding the utility and effectiveness of RNAi in *P. falciparum* (Ullu *et al*. 2004; Baum *et al*. 2009), dsRNAs have been employed to explore the biological function of some *P. falciparum* proteins (Kumar *et al*. 2002; Malhotra *et al*. 2002; McRobert and McConkey, 2002; Pradhan and Tuteja, 2007; Sriwilaijaroen *et al*. 2009).

When the level of *pfpkac* mRNAs is down-regulated using dsRNA, the cAMP pathway is inhibited (Wurtz *et al*. 2009*a*). Moreover, introduction of a non-functioning mutant or over-expression of some proteins using plasmid transfection can be used to specifically perturb signalling pathways (Merckx *et al*. 2008*b*). The major drawbacks to these strategies are the low transfection efficiencies and the complexity of implementing these techniques (Meissner *et al*. 2007). However, given the high specificity of such techniques relative to

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Fig. 3. (Cont.)

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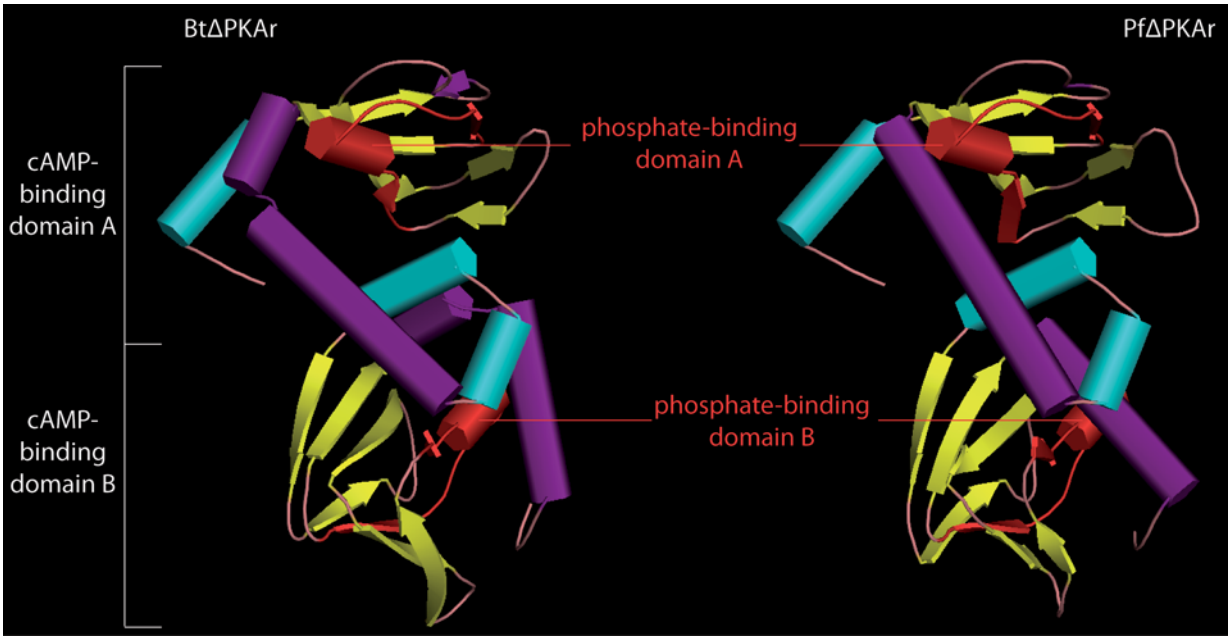

Fig. 3. Amino acid sequence alignments and partial structural modelling of PfPKAr. (A) Comparison of PfPKAr amino acid sequence with other protein kinase regulatory subunits. *Plasmodium falciparum* (Uniprot Accession number Q7KQK0), *H. sapiens* (P10644), *B. taurus* (P00514), *M. musculus* (Q9DBC7), *C. parvum* (A3FPL6), *T. brucei* (Q9GU80), *S. cerevisae* (P07278), *P. knowlesi* (B3LCL9) and *D. melanogaster* (Q9VPA) were aligned using T-coffee 7.38 and CLUSTAL format. The AKAP binding and dimerization domain, inhibitory sequence and cAMP-binding domains A and B are indicated (black boxed regions). Phosphate-binding cassettes A and B (red rectangles), β-sheets (yellow) and α-helixes (turquoise blue) are depicted as in Fig. 2B. Secondary structural element differences between *B. taurus* PKAr and PfPKAr are shown in purple. Identical residues (asterisks), conservative (colons) and semi-conservative substitutions (dots) are also indicated. (B) Structural model of partial PfPKAr. The partial PfPKAr structure was modelled on the resolved structure of *B. taurus* 1-91 deletion mutant type I alpha PKAr. α-helices (blue cylinders), β-sheets (yellow arrows) and turns (lines) are illustrated. Secondary structural differences between PfPKAr and PfPKAc are shown in purple. Phosphate-binding cassettes A and B are shown in red.

pharmacological agents, it is likely that molecular strategies will further improve the understanding of signalling pathways in *Plasmodium*.

BIOLOGICAL FUNCTIONS OF PFPKA

While the downstream targets of PfPKAc have not yet been clearly identified, several reports have suggested that this enzyme seems to play a pleiotropic role during the different stages of the parasite life cycle. First, PfPKAc seems to be involved in asexual parasite development, including erythrocyte invasion and induction of gametocytogenesis (Kaushal *et al*. 1980; McColm *et al*. 1980; Brockelman, 1982; Rangachari *et al*. 1986; Trager and Gill, 1989; Syin *et al*. 2001; Wurtz *et al*. 2009*a*). More recently, several studies have implicated PfPKAc in the following processes: (i) activation of a Ca^{2+} influx (Beraldo *et al*. 2005; Wurtz *et al*. 2009*a*), (ii) regulation of anion transport through the erythrocyte membrane (Merckx *et al*. 2008*b*, 2009), (iii) regulation of apical exocytosis and motility of sporozoites (Ono *et al*. 2008; Kebaier and Vanderberg, 2009) and (iv) mitochondrial protein traffic (Wurtz *et al*. 2009*a*). Thus, PfPKA seems to be a key regulator of *P. falciparum* development and consequently represents an attractive target for the development of anti-malarial compounds.

PfPKA has a key role in the P. falciparum *asexual life cycle*

The catalytic and regulatory subunits of PfPKA are expressed weakly during the ring and trophozoite stages compared to the schizont stage, and *pfpkac* mRNA levels are lower in gametocytes and gametes (Syin *et al*. 2001; Bozdech *et al*. 2003; Ward *et al*. 2004; Wurtz *et al*. 2009*a*). Indeed, PfPKA seems to be essential for parasite growth and survival, as already described in previous studies: (a) H89, which inhibits PfPKAc activity *in vitro*, leads to parasite growth arrest and morphological alteration (Syin *et al*. 2001); (b) the parasite cell cycle is altered after treatment with an activator of PfPKAc (6-Bz-cAMP) (Beraldo *et al*. 2005); (c) transgenic parasites that overexpress PfPKAr have growth defects that can be restored by increasing the levels of intracellular cAMP (Merckx *et al*. 2008*b*) and (d) down-regulation of *pfpkac* mRNA using gene silencing leads to morphological changes in schizont stages and cell cycle arrest (Wurtz *et al*. 2009*a*). Down-regulation of

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Table 2. Major structural features of the regulatory domain of PKAr from *Bos taurus* and *Plasmodium falciparum*

Domains	PfPKAr residues	BtPKAr type I conserved residues	Functions
D/D	None	$C^{16}Y^{19}V^{20}N^{24}I^{25}L^{36}C^{37}L^{48}Y^{51}F^{52}A^{60}$	Dimerization and AKAP binding
Inhibitory sequence	$K^{145}R^{146}X^{147}S^{148}$	$R^{94}R^{95}X^{96}A^{97}$	Binding and inhibition of PKAc
CBD-A	E^{258} R^{267}	E^{200} R^{209}	cAMP binding
CBD-B	E^{376} R^{385}	E^{324} R^{333}	cAMP binding

D/D, Dimerization/docking domain.
CBD, cAMP-binding domain.

pfpkac mRNA using gene silencing is also associated with a compensatory decrease in *pfpkar* mRNA levels, suggesting a transcriptional self-regulation of the PfPKA signalling network. The parasites appear to have tightly controlled mechanisms for self-regulating PfPKA levels to maintain appropriate PKA signalling. This type of self-regulation has also been described in mammalian cells, where, for example, knocking down the regulatory subunit causes a subsequent decrease in levels of the catalytic subunit (Duncan *et al*. 2006). This phenomenon was already proposed for *P. falciparum* because over-expression of the regulatory subunit leads to an increase in *pfpkac* transcript levels (Merckx *et al*. 2008*b*). This PfPKA self-regulation mechanism might exist to counteract the adverse effects caused by changes in the expression levels of either PfPKAc or PfPKAr.

Despite the established importance of PfPKA during parasite growth, its substrates have not been clearly identified. However, several studies presented below have shown that PfPKA is involved in many signal transduction pathways.

Putative role for PfPKA in the induction of gametocytogenesis

Several previous reports have shown that cAMP levels appear to be important for the induction of gametocytogenesis (without the direct involvement of PfPKAc). Kaushal *et al*. (1980) have shown that static cultures of ring stages develop into gametocytes following the addition of 1 mM cAMP. Treatment of *P. falciparum* cultures with cAMP agonists or with phosphodiesterase inhibitors such as caffeine and 8-Br-cAMP results in an increase in gametocyte induction (Brockelman, 1982; Trager and Gill, 1989). Moreover, AC activity and cAMP levels have been correlated with the parasite's ability to produce gametocytes (Read and Mikkelsen, 1991*a*). More recently, Dyer and Day (2000) have shown that the addition of cholera toxin, which prevents AC inactivation, causes an increase in conversion to sexual development. However, to date, no other studies have been conducted to evaluate whether or not PfPKA is involved in the induction of gametocytogenesis.

PfPKA is implicated in sporozoite motility and hepatocyte invasion

Recently, the cAMP pathway was shown to regulate sporozoite motility and hepatic cell invasion by *P. falciparum* sporozoites (Ono *et al*. 2008; Kebaier and Vanderberg, 2009). Sporozoites are deposited in the host's skin by infected mosquitoes and must penetrate cell barriers in the skin and liver sinusoid to reach their target cell, the hepatocyte; once there, they enter in a vacuole and begin the next stage of their life cycle (Fig. 5) (Mota *et al*. 2002; Mota and Rodriguez, 2002; Ejigiri and Sinnis, 2009).

Cell invasion and gliding motility of sporozoites are active processes that are tightly associated with exocytosis of apical organelles in different Apicomplexans (Carruthers *et al*. 1999). Sporozoites possess 2 types of secretory apical organelles: micronemes and rhoptries. Migration through cells activates the exocytosis of sporozoite apical organelles (Mota *et al*. 2002), which results in the release of parasite molecules that are essential for invasion. Two *Plasmodium* sporozoite microneme proteins that have been shown to be released during motility and invasion are circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP) (Khan *et al*. 1992; Spaccapelo *et al*. 1997). Ono *et al*. (2008) have shown that apical exocytosis is induced by increases in cAMP levels in the sporozoites of rodent and human *Plasmodium* species. Increasing the cytosolic cAMP level in *Plasmodium* sporozoites with the addition of the cell-permeable compounds 8-Br-cAMP or forskolin induces apical exocytosis *in vitro*, as measured by an increase in the accumulation of extracellular TRAP at the apical end of sporozoites. Moreover, incubation of sporozoites with AC inhibitors (MDL12330A, SQ22536) prevented sporozoite exocytosis. Thus, it is suggested that the synthesis of cAMP by AC increases sporozoite exocytosis. Indeed, treatment of *P. yoelii* sporozoites with 8Br-cAMP or forskolin was shown to decrease their ability to migrate through monolayers of the hepatoma cell line Hepa 1–6 and increase their ability to invade. The major downstream effector of cAMP is PfPKA, and direct inhibition of its activity by H89 or Rp-cAMP was also able to significantly reduce sporozoite exocytosis, suggesting

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that this process is mediated by PfPKA activation (Ono *et al*. 2008). Pre-treatment of sporozoites with H89 followed by induction of cAMP synthesis by 8-Br-cAMP completely inhibited exocytosis, suggesting that PKA activation occurs after cAMP generation. In the *P. falciparum* genome, 2 genes coding for AC have been identified (ACα and ACβ) (Baker and Kelly, 2004) and their expression demonstrated in sporozoites (Le Roch *et al*. 2003). Deletion of *P. berghei* ACα does not alter parasite growth during blood stages or in the mosquito, but mutant sporozoites are not able to expose the adhesive proteins and their infectivity is reduced by 50% (Ono *et al*. 2008). Re-introduction of ACα in deficient parasites resulted in a complete recovery of exocytosis and infection. These data reveal the importance of cAMP and PfPKA in sporozoite apical-regulated exocytosis, which is involved in hepatocyte infection by sporozoites.

Kebaier and Vanderberg (2009) subsequently focused on motility rather than invasion by using albumin, which triggers motility in *Plasmodium* sporozoites, and evaluated the link between sporozoite exposure to albumin and intracellular signalling pathways, especially cAMP and calcium networks. First, they have shown that parasite intracellular calcium is necessary for sporozoite motility. Use of a calcium chelator (BAPTA-AM) inhibits sporozoite motility, and these effects are reversed by the addition of exogenous calcium (calcium ionophore A23187). Moreover, they have demonstrated that suppression of cAMP synthesis by an AC inhibitor (SQ22536) also leads to a decrease in sporozoite motility. The same results were obtained when the sporozoites were treated with PKA inhibitor H89 or Rottlerin (a non-specific kinase inhibitor). Thus, elevating the concentration of cAMP (by addition of forskolin, IBMX or cAMP analogues) allows for sporozoite motility without the addition of albumin in the medium. This study revealed that calcium and cAMP/PKA pathways act together to promote sporozoite motility.

A highly complex relationship between cAMP/PfPKA and calcium pathways exists in the P. falciparum *asexual life cycle*

Beraldo *et al*. (2005) studied in detail the involvement of cAMP and its target PfPKAc in calcium signalling mechanisms during *P. falciparum* intraerythrocytic development. They demonstrated that melatonin, by activating specific receptors coupled to phospholipase C activation, causes the release of calcium from *P. falciparum* intracellular compartments *in vitro* (Hotta *et al*. 2000). Subsequently, they observed that melatonin increases cAMP levels and PfPKAc activity, suggesting an important role for calcium in the control of cAMP production. When iRBCs were treated with 6-Bz-cAMP or IBMX (PfPKAc activators), there was a change in the parasite cell cycle and an increase in the schizont population, similar to that observed when parasites were treated with melatonin. The effects of melatonin were abolished by adding PfPKAc inhibitors (PKI, H89, Rp-cAMP). These data suggest that cAMP and PKA are key modulators of the *P. falciparum* cell cycle (Fig. 5). Inhibition of phospholipase C by U73122 blocks the melatonin-induced cAMP increase. On the other hand, the addition of exogenous calcium leads to an augmentation of cAMP levels. These data suggest that the increase in cAMP levels is directly caused by an increase in calcium concentration caused by melatonin, rather than by a coupling of the melatonin receptors to AC (Beraldo *et al*. 2005). However, the interplay between cAMP and calcium is not limited to the calcium-dependent activation of cAMP production. In fact, PKA activator 6BZcAMP is able to induce calcium level increases in the parasite, either by mobilizing calcium from intracellular compartments or by stimulating calcium entry from the medium. Beraldo *et al*. (2005) concluded that the modulation of the *P. falciparum* cell cycle by the host hormone melatonin is mediated by 2 second messengers acting together. (1) Melatonin is directly coupled to a classical calcium signalling pathway *via* phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP3). IP3 then diffuses through the cytosol to bind to IP3 receptors on calcium channels in the endoplasmic reticulum, which causes an increase in cytoplasmic calcium concentration. (2) The elevation of calcium concentration initiates an amplification cycle *via* cAMP and PKA, which in turn leads to an increase in cytoplasmic calcium either by calcium release from the endoplasmic reticulum or by stimulating calcium entry from the outside. Thus, there is a highly complex relationship between the calcium and cAMP signalling networks in *Plasmodium*. These data are consistent with another study showing that gene silencing of PfPKAc leads to a down-regulation of members of the calcium/calmodulin pathway, again suggesting an interplay between the cAMP and calcium networks (Wurtz *et al*. 2009*a*).

PfPKA plays a key role in anion transport across the erythrocyte membrane during the P. falciparum *asexual life cycle*

While replicating in red blood cells (RBCs), *P. falciparum* is able to modify the host cell by increasing its permeability to facilitate nutrient uptake and to evacuate potentially dangerous metabolites for the cell (Kirk, 2001). The parasites can alter permeability of the erythrocyte membrane either by over-expressing existing carriers or by creating new permeation pathways (NPP). Egee *et al*. (2002) found

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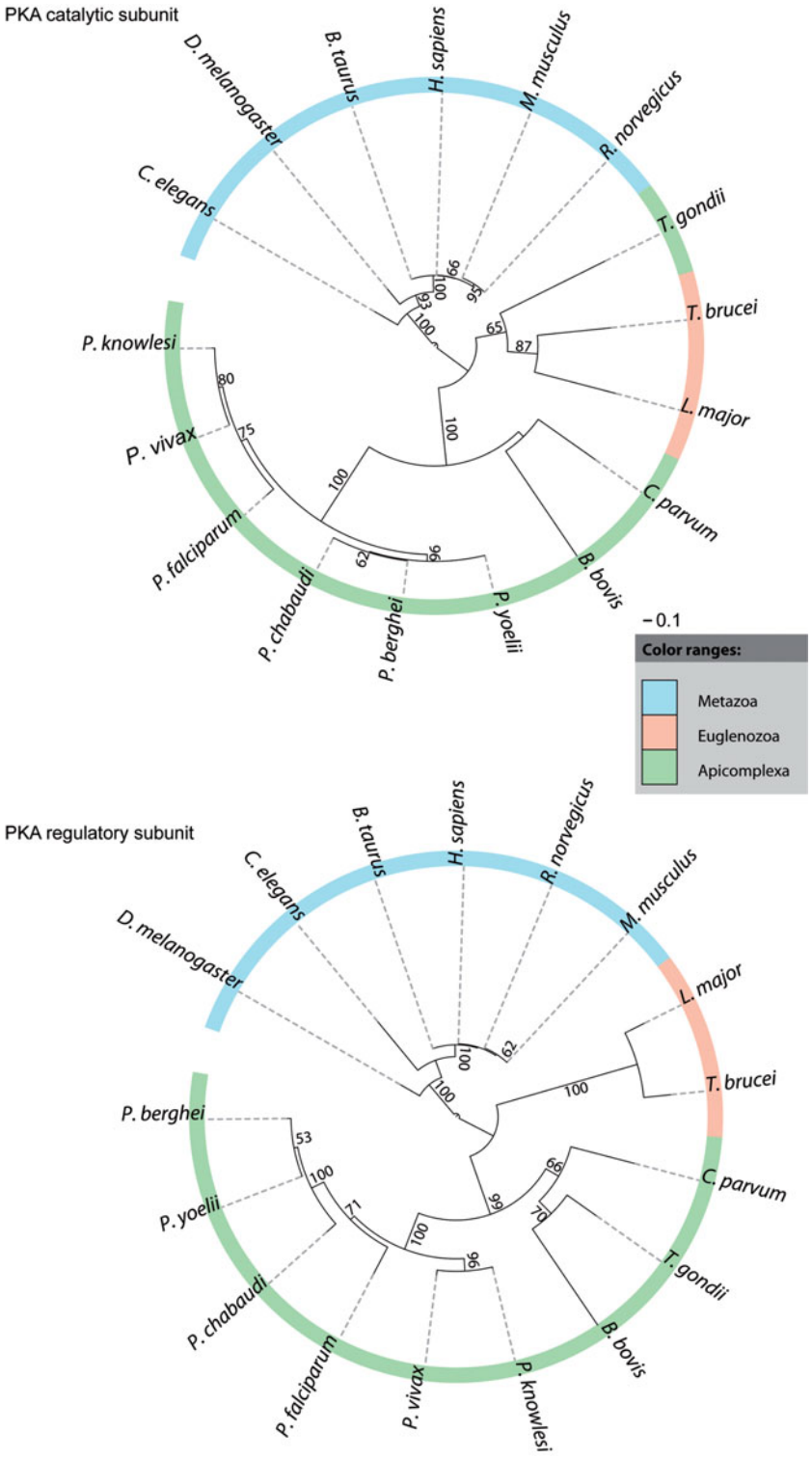

Fig. 4. Phylogenetic tree of cAMP-dependent protein kinase catalytic (A) and regulatory (B) subunits. The PKA protein sequences of 17 species have been obtained from UniProt Knowledgebase (http://www.ebi.ac.uk/uniprot/): *Plasmodium falciparum, P. vivax* (Accession number PKAc A5KE97-PKAr A5K031), *P. yoelii* (PKAr Q7RJ15), *P. knowlesi* (B3L322-B3LCL9), *P. chabaudi* (Q4XMV3-Q4Y575), *P. berghei* (Q4YW20-Q4YNR9), *Babesia bovis* (A7ANK3-A7AU83), *Cryptosporidium parvum, Toxoplasma gondii* (B6KN50-Q9BMY7), *Trypanosoma brucei, Leishmania major* (Q27687-Q4QGD8), *Homo sapiens, Mus musculus, Rattus norvegicus* (A1L1M0-P09456), *Bos taurus* (PKAc P00517), *Drosophila melanogaster* and *Caenorhabditis elegans* (P21137-P30625). Multiple alignments of these sequences were performed using T-coffee (default parameters) (Notredame *et al*. 2000). Selection of the best-fit substitution model was done by a test run with ProTest software on each of the protein alignments (Abascal *et al*. 2005). Phylogenetic relationships among sequences were determined using the maximum likelihood (ML) method implemented in PhyML (Guindon and Gascuel, 2003) using the LG + G model. The additional parameters used in PhyML analyses were as follows: gamma distribution parameter estimated with 4 rates categories, and subtree pruning

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that the anion channels of uninfected erythrocytes could be activated by the addition of bovine PKA and ATP, producing a membrane current similar to that observed in *P. falciparum*-infected red blood cells (iRBCs) and suggesting that the mechanism of regulation used by the parasite may involve phosphorylation steps (Egee *et al*. 2002). Additionally, Merckx *et al*. (2008*b*) recently reported that the addition of exogenous PfPKAr protein to *P. falciparum* iRBCs leads to a down-regulation of whole-cell membrane conductance, probably regulated by cAMP. Thus, exogenous PfPKAr seems to interfere with the parasite-dependent activation of iRBC membrane conductance. Moreover, these authors generated a *P. falciparum* strain over-expressing PfPKAr and observed that the transgenic parasite produces a clearly reduced membrane current, similar to that observed after the addition of exogenous PfPKAr. They also tested whether the over-expression of PfPKAr interferes with NPP activity by using semi-quantitative haemolysis experiments with sorbitol as permeating substrate. The transgenic parasites displayed decreased membrane permeability to sorbitol since the lysis duration was significantly increased compared with control cultures. Thus, the delay observed in haemolysis for the parasites over-expressing PfPKAr suggests that some NPP activity is under the dependence of phosphorylation *via* cAMP dependent protein kinases. In addition, several mechanisms have been proposed regarding the function of PfPKAc during anion conductance regulation (Merckx *et al*. 2009). It seems that PfPKAc regulates anionic conductance, either *via* direct phosphorylation of the channels or indirectly, through phosphorylation of accessory or associated proteins that can be of human or parasite origin (Fig. 5).

Putative role for PfPKA in erythrocyte invasion by merozoites

Several studies have demonstrated roles for the cAMP/PKA and calcium/calmodulin pathways in *P. falciparum* erythrocyte invasion by merozoites (Rangachari *et al*. 1986; Green *et al*. 2008; Wurtz *et al*. 2009*a*). The expression of 6 reticulocyte-binding-like (RBL) homologue genes was found to be down-regulated in response to *pfpkac* mRNA inhibition (Wurtz *et al*. 2009*a*). These genes belong to the invasion/motility pathway and seem to be important for *P. falciparum* invasion of human erythrocytes (Tham *et al*. 2009). To date, only indirect data have demonstrated the potential role of PfPKAc in this specific pathway (McColm *et al*. 1980; Rangachari *et al*. 1986; Syin *et al*. 2001). While PfPKAc seems to be involved *via* the RBL genes in RBC invasion by the parasite, the precise roles of the cAMP/PKA and calcium/calmodulin pathways in this process still need to be elucidated.

Putative role for PfPKA in the regulation of mitochondrial protein traffic

Another unexpected pathway that could involve the cAMP network concerns genes related to mitochondrial functions (Wurtz *et al*. 2009*a*). Many nuclear genes with mitochondrial signal sequences were found to be induced when *pfpkac* expression was inhibited (Wurtz *et al*. 2009*a*). It is well known that parasite mitochondrial activity requires the import of many proteins (van Dooren *et al*. 2006; Torrentino-Madamet *et al*. 2009). PfPKAc may thus regulate part of the mitochondrial protein traffic, as has already been described for mammalian cells (De Rasmo *et al*. 2008).

PROTEIN KINASE CELLULAR SIGNALLING AS A POTENTIAL TARGET FOR THERAPEUTIC INTERVENTION

ATP binding, substrate binding and/or kinase activity are all potential targets for inhibition by drugs designed to block protein kinases. In 2008, 10 protein kinase inhibitors had been approved for clinic use, and there are many more in clinical trials (Johnson, 2009). *P. falciparum* contains members of most of the established protein kinase families (Ward *et al*. 2004). However, the differences between the host and parasite phosphosignalling pathways suggest that specific inhibition of the latter can be accomplished (Leroy and Doerig, 2008). This notion was confirmed by 2 structural studies demonstrating exploitable divergences between host and parasite protein kinases (Holton *et al*. 2003; Merckx *et al*. 2008*a*). In the case of PfPKA, we have shown here that it presents interesting differences in terms of sequence and structure compared to human PKA. These differences involve ATP anchoring, PfPKAc substrate recognition and phosphorylation, sensitivity of PfPKAc to kinase inhibitors and the D/D domain and inhibitory sequence of PfPKAr. Thus, specifically targeting these domains together or separately could be an effective strategy for inhibiting

and regrafting (SPR) tree search method from 5 random starting trees (Hordijk and Gascuel, 2005). Significances of internal branches are indicated as percentages based on 100 bootstrap replications (only bootstrap values >50% are shown). The online tool 'Interactive Tree Of Life' was used for the display and manipulation of the phylogenetic trees (Letunic and Bork, 2007). The phylogenetic analysis included 17 eukaryotes belonging to 3 groups: Metozoa (blue), Euglenozoa (pink) and Apicomplexa (green). The Metazoa group was used as an outgroup in the analyses.

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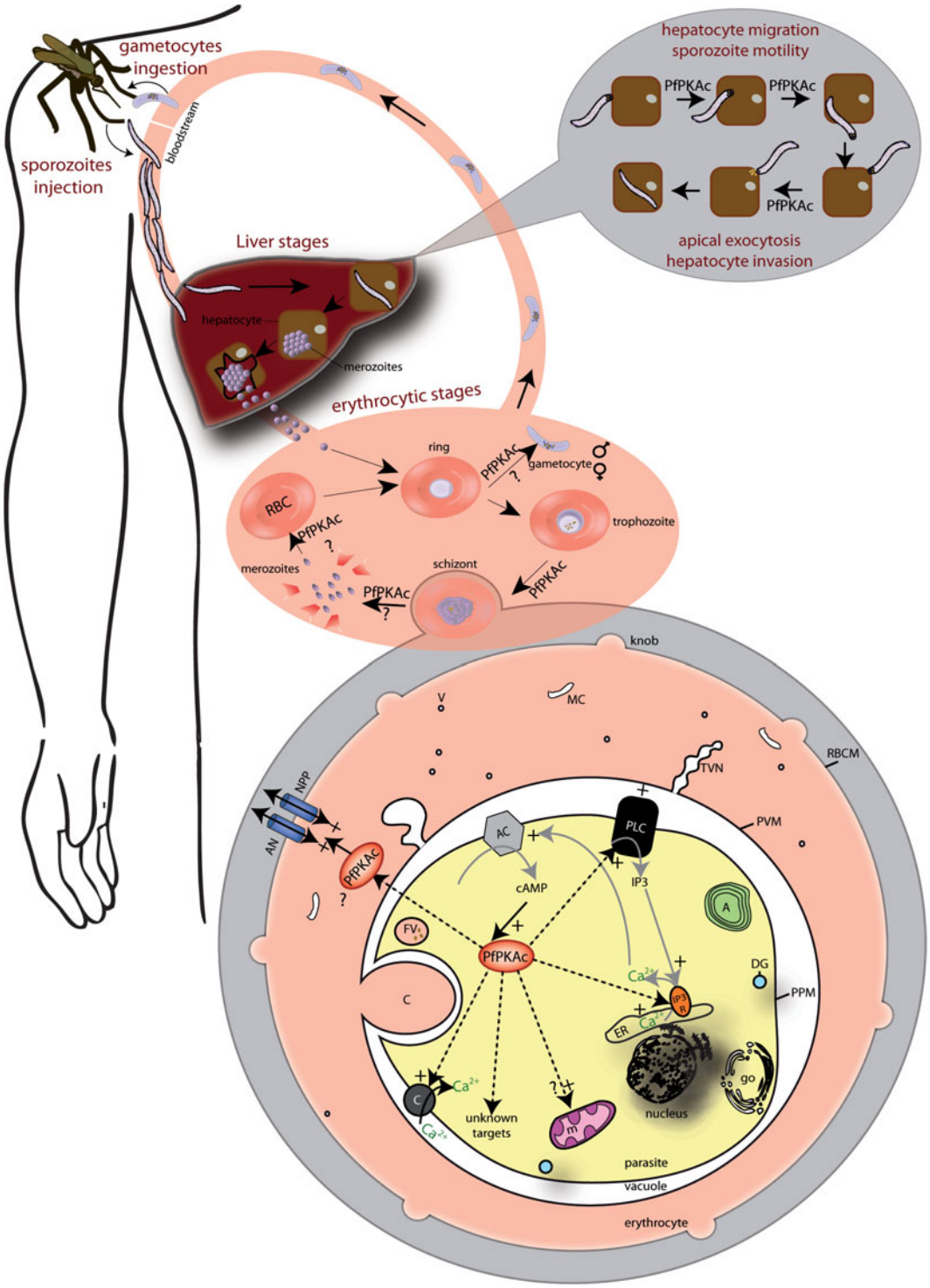

Fig. 5. Schematic model of signalling events including PfPKAc during the *Plasmodium falciparum* life cycle. The *P. falciparum* life cycle requires 2 different hosts, an *Anopheles* mosquito and a human. When an infected mosquito takes a bloodmeal, it injects sporozoites that rapidly move to the liver through the bloodstream. They then progress through the liver cells and ultimately invade a single hepatocyte, where they develop into thousands of merozoites. The cAMP/PfPKAc pathway has been shown to regulate sporozoite motility and the apical exocytosis steps necessary for the invasion of hepatic cells. Next, the hepatocyte bursts and releases the merozoites into the bloodstream, where they invade RBCs. Asexual replication progresses through a series of stages (ring, trophozoite and schizont) that ends with the rupture of the RBC, releasing merozoites that can then reinvade new RBCs. During the asexual life cycle, PfPKAc has been shown to play a role in schizont maturation, merozoite release and in the reinvasion of new RBCs. More specifically, PfPKAc might have different functions in signal transduction events, as follows. (1) A highly complex interplay exists between the cAMP/PfPKAc and Ca^{2+} signalling pathways. First, activation of phospholipase C (PLC) generates inositol 1,4,5-triphosphate (IP3), which binds to its receptor (IP3R) located on the membrane of the endoplasmic reticulum (ER). This leads to calcium release into the cytoplasm that in turns activates AC and induces

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the parasite enzyme without interfering with the equivalent host protein.

Targeting the kinase activity directly

Most of the protein kinase inhibitors currently in clinical trials for cancer therapy are small molecules that compete for the ATP-binding site. A major success in this category of inhibitors is the tyrosine kinase inhibitor imatinib (Gleevec®, Novartis), a potent inhibitor of the constitutively active BCR-ABL fusion protein that is used for the treatment of leukaemia and gastrointestinal stromal tumours (Druker, 2002; Tibes *et al*. 2005). This has been followed by other small molecules such as the epidermal growth factor receptor inhibitors erlotinib (Tarceva™ Genentech) and gefitinib (Iressa™ AstraZeneca), both of which received approval for the treatment of non-small cell lung carcinoma (Modjtahedi and Essapen, 2009). As mentioned above, the ATP-binding site of PfPKAc presents some differences when compared to that of human PKAc, suggesting that the design of molecules specifically targeting the parasite domain may be feasible.

Another strategy to inhibit the activity of a protein kinase is to prevent the translation of its transcripts. This can be achieved using ribozymes, which are modified RNA molecules that can cut other RNA. Ribozymes consist of a central catalytic domain with RNA-degrading activity, flanked by RNA sequences that are complementary to the target mRNA. RPI.4610 (Angiozyme) was the first synthesized ribozyme to be studied in human trials. It is designed to prevent the process of angiogenesis by cleaving the mRNA encoding the VEGF1 receptor (Perabo and Muller, 2007). LErafAON, an antisense oligonucleotide targeting the serine/threonine kinase c-Raf that has been implicated in many cancers, has been tested in phase I clinical trials (Wellbrock *et al*. 2004; Zhang *et al*. 2009). This type of approach could be used to inhibit the expression of PfPKAc by designing ribozymes that specifically target the PfPKAc mRNA without altering human PKAc expression.

Targeting partners of kinases or effectors of the pathway

Rather than directly inhibiting the active enzyme, other strategies can be considered that target either the partners or the upstream and/or downstream effectors of the pathway. One possibility is to target the regulatory subunit of PKA, especially for cancer therapy, as its protein and mRNA levels have been found to be upregulated in a series of transformed cell lines and human neoplasms (Bradbury *et al*. 1994; Miller, 2002). GEM®231 (HYB165, Hybridon) is an 18-mer antisense oligonucleotide targeted against human PKA RIα. Used alone or in combination with other agents, GEM®231 has demonstrated anti-tumour activity in a variety of *in vitro* cancer cells and *in vivo* human tumour xenograft models (Tortora and Ciardiello, 2002; Wang *et al*. 2002). We can imagine designing an antisense oligonucleotide against PfPKAr targeting the C-terminal end of the sequence, as we have demonstrated above a significant difference between PfPKAr and human PKAr in this region.

The AKAPs introduce another level of complexity to PKA signalling and have emerged as key regulators of PKA function. To date, these partners have not yet been well studied but appear as interesting therapeutic targets, particularly in the treatment of severe cardiac pathologies and cancers (Diviani, 2008; Naviglio *et al*. 2009). In the case of *P. falciparum*, the hypothetical AKAP identified by orthology presents only 22% identity with human AKAP18. Thus, we propose specifically targeting either the parasite AKAP or the interaction between AKAP and PfPKAr because, as noted above, the mechanism of interaction between these two partners seems to be different from that of their human homologues.

cAMP synthesis. cAMP binds to the PKA regulatory subunit and leads to the release and activation of the catalytic subunit. The increased calcium concentration initiates an amplification loop *via* cAMP and PKA, which in turns leads to a further increase in cytoplasmic calcium either by stimulating PLC or IP3R directly, or by calcium entry from outside of the cell. (2) cAMP-pathway components are involved in the regulation of anion conductance at the erythrocyte membrane. Despite lacking a *Plasmodium* export element/host targeting motif (PEXEL/HT), PfPKAc can be exported to the host cytosol, where it directly phosphorylates anion channels (AN) and/or eventually participates in NPP (new permeation pathway) formation. Alternatively, PfPKAc could either be exported to the erythrocyte cytosol where it phosphorylates proteins of human or parasite origin, or could phosphorylate an unidentified parasite substrate that is then exported to the host cytosol. Furthermore, if human PKAc is activated by parasite cAMP, it could also contribute to the induction of anion conductance. (3) PfPKAc may regulate some mitochondrial protein traffic. (4) PfPKAc certainly has many other cellular targets that are still unknown. In each erythrocyte cycle, some merozoites arrest their cell cycle and develop into sexual forms (gametocytes). A role for PfPKAc in gametocytogenesis induction has been proposed. The cycle is completed when a mosquito takes its bloodmeal and ingests male (♂) and female (♀) gametocytes. Question marks represent the suggested role of PfPKAc during the various events cited above. Maurer's clefts (MC), vesicles (V), tubovesicular network (TVN), red blood cell membrane (RBCM), parasitophorous vacuolar membrane (PVM), parasite plasma membrane (PPM), dense granule (DG), apicoplast (A), food vacuole (FV), cytostome (C), golgi (go), mitochondria (m), channel (C).

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Interfering with the function of protein kinases can be achieved with substrate-mimicking molecules that compete with the original substrate. By occupying the natural binding site of the substrate, these molecules end the signal transduction events that help maintain the pathogenic state of the cell. The substrate mimic Thymectacin™ (NB1011), which targets thymidylate synthase, an enzyme over-expressed in tumours, has entered clinical trials (Congiatu *et al*. 2005). Therefore, this strategy of inhibiting enzymatic activity has potential clinical applications. However, substrate competitive inhibitors targeting protein kinases have not yet entered clinical trials, and the substrates of PfPKAc have not yet been clearly defined, hampering the development of such molecules.

A different way to restrain protein kinase activity is to block the upstream activation of the pathway using small molecules or antibodies that prevent receptor-mediated signalling. In the case of the PKA signalling pathway, several upstream partners can be targeted to alter kinase function, including G-protein coupled receptors, adenylate cyclase and phosphodiesterase (Wise *et al*. 2002; Zhang *et al*. 2005; Pavan *et al*. 2009). In 2000, 26 of the top 100 pharmaceutical products were compounds that target GPCRs, including salmeterol (asthma), sumatriptan (migraine), ibuprofen (inflammation, pain), rimonabant (obesity), haloperidol (schizophrenia), cabergoline (Parkinson's disease) and many more (Wise *et al*. 2002). Phosphodiesterases play crucial roles in cell signalling and have therefore been the target of clinical drug development for indications ranging from anti-inflammation to memory enhancement. Many drugs targeting such proteins have been developed, including theophylline (asthma), anagrelide (thrombocytosis), milrinone (cardiac failure) and dipyridamole (inhibitor of platelet aggregation) (Wise *et al*. 2002; Zhang *et al*. 2005; Denault *et al*. 2006; Chakrabarti and Freedman, 2008; Emadi and Spivak, 2009). Although many drugs target the cAMP signalling pathway through G-protein coupled receptors or phosphodiesterases, adenylate cyclases have not yet been considered as drug targets. However, the generation of knockout and transgenic animals has revealed that these proteins have crucial roles in numerous biological processes (Pierre *et al*. 2009). Mammalian adenylate cyclases are currently under investigation as potential drug targets, with some compounds already being used in clinics. For example, corlforsin has been approved in Japan for the treatment of heart failure (Ogata *et al*. 2007). These PfPKAc partners could be considered as potential drug targets; however, more work is needed on these genes, which have not yet been well characterized.

Another approach to inhibiting the PKA pathway is the use of cAMP analogues. Between 1960 and 1980, numerous analogues of cAMP were synthesized and screened for their therapeutic potential, especially against diabetes, asthma and cardiovascular diseases. A good illustration is 8-Cl-cAMP which inhibits cancer cell growth through both anti-proliferation and pro-apoptotic mechanisms (Cho-Chung and Nesterova, 2005). This molecule has completed several phase I clinical studies and recently entered phase II clinical trials as an anti-cancer agent (Tortora and Ciardiello, 2002). However, the strategy of using cAMP analogues does not seem feasible in the case of *P. falciparum* because the interactions between cAMP and PfPKAr appear similar to those in humans; therefore, these compounds would not specifically target the parasite. In conclusion, PfPKA and the *P. falciparum* cAMP pathways appear to be relevant biological targets for the therapy and management of malaria.

CONCLUSIONS AND PERSPECTIVES

In spite of its established importance during parasite growth and the different pathways that are associated with it, the substrates of PfPKA have not yet been clearly identified. Merckx *et al*. (2009) identified several PfPKAc substrates in human RBCs and in the *P. falciparum* proteome by using an *in silico* search for phosphorylation sites using PkaPS (Prediction of protein kinase A Phosphorylation Sites, http://mendel.imp.ac.at/sat/pkaPS/pkaPS.html) (Neuberger *et al*. 2007). As the recombinant PfPKAc and PfPKAr proteins are available, different strategies could be employed to identify both the upstream targets and specific inhibitors of this enzyme. First, recombinant PfPKAc could be used to test the protein's capacity to phosphorylate potential parasite/human substrates using the KESTREL (kinase substrate tracking and elucidation) approach (Cohen and Knebel, 2006; Peng *et al*. 2007; Philip and Haystead, 2007). This strategy has been applied successfully to *P. falciparum* to identify the substrates of serine / threonine kinase PfPK9 (Philip and Haystead, 2007). To avoid the use of radioactive molecules, detection of phosphorylated proteins can be achieved using specific fluorescent staining allowing direct, in-gel detection of phosphate groups attached to tyrosine, serine or threonine residues (e.g., ProQ® Diamond, Invitrogen) (Schulenberg *et al*. 2004; Orsatti *et al*. 2009). In addition, the use of protein kinase inhibitors, such as H89, on *P. falciparum* could reveal novel substrates, potential new pathway interconnections and inhibitor specificity by monitoring differences in protein levels and phosphorylation (Davis *et al*. 2006).

Thus recombinant proteins obtained can also be used (i) to produce specific anti-PfPKAc and anti-PfPKAr antibodies to determine the cellular locations of PfPKAc, PfPKAr and their partners (Murtaugh *et al*. 1982; Ray *et al*. 2001), and (ii) to perform high-throughput screening of PfPKAc

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inhibitors and interaction studies with PfPKAr (Merckx *et al*. 2008*b*; Blackwell *et al*. 2009). Clearly, the identification of the upstream signals/molecular events that regulate PfPKAc activity and their downstream targets will be crucial for a more precise understanding of their cellular function and should also provide new targets for drug design.

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