Multiple Splice Variants Encode a Novel Adenyl Cyclase of Possible Plastid Origin Expressed in the Sexual Stage of the Malaria Parasite \textit{Plasmodium falciparum}*

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David K. Muhia‡\$, Claire A. Swales‡, Ursula Eckstein-Ludwig†, Shweta Saran‡, Spencer D. Polley‡, John M. Kelly‡, Pauline Schaap‡, Sanjeev Krishna†, and David A. Baker‡**

From the \#Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, United Kingdom, the \#Department of Cellular and Molecular Medicine, St. George's Hospital Medical School, Cranmer Terrace, London, SW17 ORE, United Kingdom, and the \$Wellcome Trust Biocentre, Dow Street, University of Dundee, Dundee, DD1 5EH, United Kingdom

We report the characterization of an unusual adenyl cyclase gene from \textit{Plasmodium falciparum}, here designated PfAC. The level of mRNA expression is maximum during development of gametocytes (the sexual blood stage of the parasite life cycle). The gene is highly interrupted by 22 introns, and reverse transcriptase-PCR analysis revealed that there are multiple mRNA splice variants. One intron has three alternative 3′-splice sites that confer the potential to encode distinct forms of the enzyme using alternative start codons. Deduced amino acid sequences predict membrane-spanning regions, the number of which can vary between two and six depending on the splice variant. Expression of a synthetic form of two of these variants in \textit{Xenopus} oocytes and in \textit{Dicyostelium} adenyl cyclase-deficient mutants, confirms that PfAC is a functional adenyl cyclase. These results identify a novel mechanism in \textit{P. falciparum} for the generation of multiple isoforms of a key, membrane-bound signaling molecule from a single genomic copy. Comparisons of the catalytic domains of PfAC and a second putative \textit{P. falciparum} adenyl cyclase (PACβ) with those from other species reveal an unexpected similarity with adenyl cyclases from certain prokaryotes including the cyanobacteria (blue green algae). In addition, the presence of an unusual active site substitution in a position that determines substrate specificity, also characteristically of these prokaryotic forms of the enzyme, further suggests a plastid origin for the \textit{Plasmodium} cyclases.

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Adenyl cyclase (AC)\textsuperscript{1} catalyzes synthesis of the signaling molecule cAMP from ATP. The most widely studied form of this enzyme, found in mammals and all higher eukaryotes, has a tandem pair of catalytic domains each preceded by a set of 6 transmembrane domains. The enzyme is activated via heterotrimeric G proteins after binding of an extracellular ligand to a G protein-coupled receptor (1). A soluble form of AC (also containing a pair of catalytic domains) has also been identified in mammals (2). It is G protein-independent and activated by bicarbonate ions (3). In lower eukaryotes and prokaryotes, the overall architecture of ACs varies between species. In all class III enzymes, however (the universal class of purine nucleotide cyclases, which includes guanylyl cyclases (GCs), Ref. 4) there are a number of conserved amino acid residues within the catalytic domain that are vital for enzyme activity (5). Receptor-type ACs have been identified that possess a single transmembrane domain and a single catalytic domain (reminiscent of mammalian GCs) and are activated directly rather than by heterotrimeric G proteins (e.g. in trypanosomes, Refs. 6 and 7). Many of the bacterial ACs have a modular structure with distinct non-catalytic regulatory domains. The filamentous cyanobacterium \textit{Anabaena cylindrica} has an unusual AC (cyaB1) with two membrane-spanning segments. The enzyme also has additional functional regions including a PAS domain (a ubiquitous small molecule receptor) and an allostery cyclic nucleotide-binding domain (8, 9).

In mammals, the membrane-associated ACs (types I-IX) have roles in numerous biological processes including glycogen metabolism (10), olfaction (11), and nerve cell communication (12). Some bacterial pathogen exotoxins comprise ACs (e.g. from \textit{Bacillus anthracis}, the causative agent of anthrax; Ref. 13). In lower eukaryotes, the list of confirmed physiological roles for AC is growing, and CAMP levels are known to control development and differentiation in many species. In the protozoan \textit{Dicyostelium discoideum}, cAMP signaling is extremely complex. One of the ACs (ACA) controls chemotactic aggregation of amoebae under starvation conditions (14), and another (ACG) regulates spor germination (15). In the fission yeast \textit{Schizosaccharomyces pombe}, AC is thought to be important in regulating sexual development (16). In the ciliate \textit{Paramecium}, synthesis of cyclic nucleotides is coupled with ion currents (17); AC activity is associated with the ciliary membranes and is involved in locomotion. Synthesis of cAMP is stimulated by hyperpolarization of ciliary membranes, and this is inhibited by K\textsuperscript{+} channel blockers suggestive of a single unit acting as both a cyclase and an ion channel (18).

In the human malaria parasite \textit{Plasmodium falciparum}, cyclic nucleotide signaling pathways have been implicated in sexual differentiation. Sexual blood stage parasites mediate...
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Cultivation and Purification of F. paracymum Blood Stage Parasites—Gametocyte cultures were set up as described previously (21), although incorporating a synchronization step to enrich for the various developmental stages. Briefly, an early passage of clone D27 (25) was grown to over 5% mixed asexual parasitaemia and used to set up flasks — sites C, 15 min). 

 RNA Isolation and Northern Blotting—The RNA was resuspended in deionized formamide, gel-fragmented, and Northern blotted according to standard procedures (30, 31). Blots (BrightStar™ Plus Nylon membrane, Ambion) were hybridized overnight with radiolabeled probes and visualized using phosphor screens (Kodak) scanned on a Storm Phosphorimag (Amersham Biosciences).

In Vitro mRNA Expression of a Synthetic PfAC—A DNA oligonucleotide containing (5°-Leu30°) of the PfACs sequences was synthesized commercially (Bionexus Inc.) according to the preferred codon usage of Xenopus laevis, cloned into the pCR-Blunt vector (Invitrogen), and sequenced using an ABI 377 automated sequencer. The synthetic gene was then cloned into a BglII site had been incorporated at both ends) into an X. laevis expression vector pSP64T (incorporating a 5°-strong Kozak consensus (CACC)), which contains 6°- and 3°-untranslated X. laevis β-globulin sequences (32). The plasmid construct (with the confirmed orientation) was linearized with Smal, treated with proteinase K, phenol/chloroform extracted, and precipitated with sodium acetate. Capped PFAc cRNA was transcribed using the mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX). After completion of the reaction, the template DNA was removed using DNase I (37°C, 15 min). cRNA was precipitated with LiCl, washed with 70% ethanol, dried, and resuspended in DEPC-treated water.

Expression in X. laevis Oocytes and Measurements of cAMP Levels—X. laevis oocytes were harvested and connected tissue removed with collagenase treatment (2 mg ml⁻¹ for 2 h on a shaker) (33). Stages V to VI oocytes (Dumont, 1972) were selected and microinjected with cRNA (5–55 ng) encoding PfAC or with a comparable amount of DEPC-treated water (~30 nl). Oocytes were incubated in Barth’s solution (33) at 19°C for 3 days. Quantification of in vivo accumulation of cAMP in oocytes was performed using an immunosassay kit (Alexis) according to the manufacturer’s instructions. Briefly, 15 oocytes were sonicated, freeze-thawed in microwaves, and protein precipitated containing 270 μl of 10% trichloroacetic acid. The homogenate was then extracted three times with five volumes of water-saturated ether. Residual ether was removed by heating to 70°C for 20 min, and the samples were stored at –80°C until the assay was performed.

Expression in Dictyostelium ACA-/ACG-null Mutants—Synthetic forms of variants 1 and 3 of PfACs were expressed as yellow fluorescent protein (YFP) fusions to facilitate detection and localization by Western blotting. The open reading frame (ORF) of variants 1 and 3 were amplified from the pCR-Blunt vector using oligonucleotides incorporating a BamHI and XhoI site. The BamHI/XhoI-digested fragment was cloned into the similarly digested vector pB7S, which placed the PfAC variants downstream of the constitutive actin15 promoter and in-frame with the YFP ORF at the C terminus, to generate vectors PFAc1Y and PFAc3Y. An ACA-deficient Dictyostelium cell line (aca-1 aca-) was transformed with PFAc1Y and PFAc3Y by electroporation, and the transformants were selected by growth in HL5 medium in the presence of 100 μg ml⁻¹ of G418 (34).

Western Analysis—Dictyostelium cells were resuspended to 2 x 10⁷ cells ml⁻¹ in 10 mM potassium phosphate buffer, pH 6.2 (KK2), and the lysates size-fractionated on 10% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes, which were incubated overnight at 4°C with a 1:1000 diluted anti-GFP mouse monoclonal antibody (Roche Applied Science). Detection was performed with the ECL kit (Pierce) according to the manufacturer’s instructions, using a 1:2000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Promega) as secondary antibody.

cAMP Accumulation in Intact Cells—Cells were harvested from growth medium, resuspended in KK2 buffer at a concentration of 5 x 10⁷ cells ml⁻¹, and shaken at 200 rpm for 10 min at 22°C. Aliquots (5 μl) of cell suspension were added up to a final volume of 30 μl in microtiter plate wells. Reactions were initiated by addition of 5 mM dithiothreitol (DTT) and terminated by addition of 30 μl of 3.5% perchloric acid. Lysates were neutralized with KHCvo₃, and cAMP levels were determined using the isotope dilution assay (35).

adenyl cyclase assay in Cell Lysates—Cells were resuspended at a concentration of 5 x 10⁷ cells ml⁻¹ in ice-cold lysis buffer (250 mM sucrose in 10 mM Tris-Cl, pH 8.0) and lysed through nucleosome filters (pore size, 3 μm). Aliquots (10 μl) of cell lysate were added to 5 μl of divalent cation solution (MgCl₂, MnCl₂, or water) and incubated at 37°C for 5 min. Reactions were initiated by addition of 5 μl of assay mixture
phylogenies to confirm the results obtained by the above method. Neighbor joining (NEIGHBOR) methods were also used to generate trees associated with SEQBOOT. PROTDIST, FITCH and SEQBOOT are part of the PHYLIP package (37). Maximum parsimony (PROTPARS), and neighbor joining (NEIGHBOR) methods were also used to generate phylogenies to confirm the results obtained by the above method.

RESULTS

Identification of a Gene Encoding a Putative AC and the Presence of mRNA Splice Variants—Conserved motifs within the catalytic domain of ACs from diverse eukaryotic species were used in BLAST searches of P. falciparum preliminary sequence data (Sanger Centre, TIGR, and Stanford University). Initially a single match was found in the chromosome 14 data base (TIGR). However, it was clear that the various motifs were non-contiguous and that the sequence was highly interrupted by introns. RT-PCR was performed on total RNA extracted from mixed blood stage parasites (clone 3D7, see “Experimental Procedures”) to determine the number and position of introns in the sequence. Examination of the upstream and downstream sequence data (TIGR chromosome 14 shotgun, unfinished sequence data) for potential splice sites and extensive RT-PCR analysis revealed a total of 22 exons and 21 introns (Fig. 1) predicting a protein size of 81.2 kDa. Initial RT-PCR analysis indicated that the first in-frame start codon is in exon 5. This suggests that introns 1–4 are situated in the 5′-untranslated region of the gene. However, further RT-PCR analysis of the 5′-region revealed the presence of mRNA molecules corresponding to additional mRNA splice variants. The first in-frame stop codon is in exon 22 indicating the predicted position of the C terminus of the protein.

The majority of the RT-PCR products examined conformed to the above arrangement (variant 1). However, further analysis demonstrated that intron 3 contains alternative 3′-splice sites (Fig. 1B and Fig. 2A). In two instances this results in two small introns (rather than a single large one) and an additional exon (exon 3A, Fig. 1, A and B). Also, there are two versions of the small intron 3 that give rise to different deduced N-terminal protein sequences (variants 2 and 3, Fig. 1, A and B). Intriguingly, in variant 3 all exons are contiguous resulting in a full-length protein of 108 kDa. Variant 2 predicts a protein of intermediate size (98.2 kDa, Fig. 1C). As shown in Fig. 1C, the protein sequences encoded by variants 1, 2, and 3 have differing numbers of predicted transmembrane domains (2, 5, and 6, respectively). The nucleotide sequences towards the 5′-end of variants 1–3 (near to their predicted translation start codons) obtained by sequencing RT-PCR products are shown in Fig. 2A. The precise positions of the introns and the variant intron junctions are shown in relation to the exons. Fig. 2B shows the differences in N-terminal amino acid sequence encoded by the three variants.

Relationship of PfACα to a Second AC from P. falciparum—The majority of the single catalytic domain of PfACα is encoded by exons 16 and 17 toward the C terminus. Surprisingly this sequence is most closely related to an AC from the cyanobacterium Trichodesmium erythraeum (28% identity and 49% similarity over 224 amino acids) as determined by a BLAST search (38). In addition to PfACα, we have also identified a second putative AC (here designated PfACβ) sequence on chromosome 8 in the P. falciparum genome data (plasmodb.org/). PfACβ also has a high degree of relatedness to cyanobacterial ACs. However, it has a double catalytic domain (C1 and C2) at the N terminus, which is topologically more similar to the soluble AC of mammals than to the membrane-bound forms.

Fig. 3 shows an alignment of the catalytic domains of PfACα and PfACβ (C2) with those of the C1 (type V) and C2 (type II)
domains of mammalian AC for which a crystal structure of the heterodimer is available (39, 40). The initial alignment was performed using the ClustalW program (41) but was modified manually to introduce gaps (where possible) between the areas of secondary structure of the mammalian ACs. The catalytic domain of a cyanobacterial cyclase (T. erythraea), and the C2 catalytic domain of the homodimeric, human retinal GC is included in the alignment to emphasize the diagnostic residues that distinguish ACs from GCs. The catalytic domain of a cyanobacterial cyclase, spirochaetes and certain proteobacterial species have only a single catalytic domain containing all the residues required for enzyme activity, whereas PfAC has two catalytic domains (C1 and C2) each containing specific residues required for enzyme activity, whereas PfAC has two catalytic domains (C1 and C2) each containing specific sequences that are conserved in all known examples of soluble ACs (bootstrap value of 100%). By contrast PfAC clusters strongly with putative ACs from other apicomplexan species (bootstrap value 50%). This grouping of the proteins into two distinct lineages of ACs in a phylogenetic analysis (Fig. 4A) is reinforced by the grouping of the proteins into two distinct lineages of ACs in a phylogenetic analysis (Fig. 4A). In this analysis of the catalytic domains, PFACa clusters strongly with putative ACs from other apicomplexan species (bootstrap value of 100%) but also with ACs from prokaryotes including cyanobacteria, spirochaetes and certain proteobacterial species (bootstrap value 50%). By contrast PFACβ groups strongly with all known examples of soluble ACs (bootstrap value of 100%). This grouping of PFACβ reflects a shared double catalytic domain. The group of soluble ACs includes the mammalian bicarbonate sensor (3) together with hypothetical proteins from the mosquito Anopheles gambiae, Chloroflexus aurantiacus (a green non-sulfur bacteria), and the Dictostelium sGCa, which is in fact a GC (42). The phylogenetic analysis also shows that this class of cyclases, despite having a double catalytic domain,

bases. Although both sequences show an unexpectedly high sequence similarity with certain bacterial ACs, it is clear that the two Plasmodium proteins are not closely related to each other. PFACa has only a single catalytic domain containing all the residues required for enzyme activity, whereas PFACβ has two catalytic domains (C1 and C2) each containing specific motifs that would be required for activity. This profound difference between the catalytic domains is reflected in a shared sequence identity of only 19% (using the C2 domain of PFACβ) and 22% (using the C1 domain of PFACβ) at the amino acid level. This is reinforced by the grouping of the proteins into two distinct lineages of ACs in a phylogenetic analysis (Fig. 4A). In this analysis of the catalytic domains, PFACa clusters strongly with putative ACs from other apicomplexan species (bootstrap value of 100%) but also with ACs from prokaryotes including cyanobacteria, spirochaetes and certain proteobacterial species (bootstrap value 50%). By contrast PFACβ groups strongly with all known examples of soluble ACs (bootstrap value of 100%). This grouping of PFACβ reflects a shared double catalytic domain. The group of soluble ACs includes the mammalian bicarbonate sensor (3) together with hypothetical proteins from the mosquito Anopheles gambiae, Chloroflexus aurantiacus (a green non-sulfur bacteria), and the Dictostelium sGCa, which is in fact a GC (42). The phylogenetic analysis also shows that this class of cyclases, despite having a double catalytic domain,
is quite distinct from the membrane-bound G protein-dependent ACs.

A key amino acid residue in the active site, which determines substrate specificity is also indicated in Fig. 4A. Membrane-bound G protein-dependent ACs all have an aspartic acid residue in this position, whereas ACs in the 2 proaryotic clades have a serine/threonine residue. Analysis suggests that this is a defining feature of these 2 classes of cyclases (discussed further below). The alanine residue in the Dictyostelium sequence at this position probably reflects that it is a functional AC. Fig. 4B shows an alignment of ACs from various species showing the two non-contiguous segments of the enzyme active site that are involved in purine binding, and highlighting the residues that define substrate specificity. In all cases the crucial lysine residue (replaced by arginine in Chloroflexus) is present, but the key aspartic acid residue invariant in all membrane-bound G protein-dependent ACs is replaced by a serine or threonine residue in both the soluble ACs (with the paired catalytic domains C1 and C2, like PfACβ) and the ACs with a single catalytic domain (like PfACA). This substitution may define a distinct mechanistic feature shared by these forms of AC.

Expression of a Synthetic PfACA in Xenopus Oocytes and Dictyostelium AC-deficient Mutants Demonstrate That It Is a Functional AC—P. falciparum proteins are notoriously difficult to express in heterologous systems, and problems have been overcome in some cases by changing the extremely A/T-rich codon bias of 43, 44) by in vitro resynthesis of the gene. The entire coding region of the full-length PfACA (variant 3; accession number AY191005) was resynthesized according to the codon bias of X. laevis to facilitate functional expression in oocytes. This system has been used successfully to express functional ACs (33). After incubation of oocytes for 3 days, in vivo accumulation of cAMP was measured. Fig. 5 shows a representative experiment indicating a ~3-fold increase in cAMP levels in oocytes that had been injected with mRNA encoding PfACA compared with the negative controls (p < 0.008 for PfACA compared with either PfHT or DEPC, p = 0.97 PfHT compared with DEPC, Student’s t test). Each experiment (performed three times in triplicate) resulted in a 2.5–3-fold increase in cAMP levels. These results therefore demonstrate that PfACA can catalyze the synthesis of cAMP in the Xenopus oocyte system and indicate that it is a functional AC.

An additional study was initiated in parallel to express variants 1 and 3 in the protozoan D. discoideum (Fig. 6). A mutant cell line in which the endogenous cyclases ACA and ACG had been deleted (aca, acg) was used in these experiments (34). The other Dictyostelium adenylyl cyclase, ACB, shows almost no activity during early development (36). PfACA fragments were expressed as YFP fusions (see “Experimental Procedures”). To confirm that the fusion proteins were of the expected size, lysates of transformed cells were separated by SDS-PAGE, immunoblotted with anti-GFP antibodies (that detect YFP). Bands of expected size, lysates of transformed cells were separated by SDS-PAGE, immunoblotted with anti-GFP antibodies (that detect YFP). Bands of expected size, lysates of transformed cells were separated by SDS-PAGE, immunoblotted with anti-GFP antibodies (that detect YFP). Bands of expected size.
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Fig. 4. Evolutionary relationship of PfACα and PfACβ with other ACs. A, a rectangular cladogram of 27 ACs (single catalytic domain or the C2 domain; corresponding to between α3 and β7/8 of the rat type II enzyme (39, 40), see Fig. 3) with bootstrap values greater than 50% indicated at corresponding nodes. Cyclases with the conventional eukaryotic form of AC group together (bootstrap value of 100%) and have an aspartic acid residue in the crucial position, which determines purine binding specificity. By contrast, ACs (shown in bold) groups strongly with cyclases from other apicomplexa, but also with prokaryotic cyclases, which have a serine/threonine residue at this position (bootstrap value of 50%). The corresponding residue at this position is shown to the right of each species. The soluble ACs form a distinct group (bootstrap value of 100%), which contains the parent enzyme. PfACβ protein (shown in bold). Accession numbers for the proteins used in this analysis are as follows: Mycobacterium, NP_338294; P. falci- parum, NP_704518; Chloroflexus aurantius, ZP_00018205; D. discoidea soluble GC (Sgc), AAK29097; A. gambiense soluble adenyl cyclase (S), EEA10271; Rattus norvegicus soluble AC (S), AP081941; human soluble adenyl cyclase (S), NP_060887; Takifugu rubripes soluble AC (S), AP081941; human soluble adenylyl cyclase (S), NP_060887; T. cruzi soluble AC (S), NP_060887; T. brucei soluble AC, NCBI:120869. An aspartic acid residue acts as a cofactor. This demonstrates that the measured activity cannot be caused by ACβ, which shows highest activity with Mg2+ as a cofactor (34).

PfACα mRNA Expression Is Maximum in the Sexual Stage of the P. falciparum Life Cycle.—Fig. 7 shows the results of a Northern blot containing total RNA preparations from sorbitol-synchronized sexual blood stage parasites and GlnNac-synchronized sexual blood stage parasites. Panel A shows the ethidium bromide-stained agarose gel from which the Northern blot was derived. The blot was hybridized with a probe corresponding to the catalytic domain of PfACα (Fig. 7B). A transcript of ~4,500 nucleotides was detected in the total RNA preparations from sexual blood stage parasites and was maximal during early/mid-stage gametocyte development (stage III; 3 and 5 days old, respectively). The blot was probed with Pf76, a highly expressed sexual stage-specific gene (45), to test for the presence of contaminating sexual stage parasites in the asexual preparations (Fig. 7C). This indicated that the asexual stage preparations also contain low levels of gametocytes, which could account for faint bands in these tracks when probed with PfACα.

The presence of a serine/threonine in this position in the cyanobacterial enzyme (9) is thought to play the same role but in the context of the single amino acid insertion that occurs in this region. The presence of Mn2+ ions, with maximum activity at 1 and 3 mM Mn2+, respectively (Fig. 6C). There was very little activity with Mg2+ as a cofactor (45).

Fig. 5. Levels of cAMP accumulation in Xenopus oocytes injected with PfACα mRNA. Accumulation levels of cAMP in X. laevis oocytes are presented as pmol of cAMP per oocyte. Each experiment was carried out three times in triplicate, and a representative experiment is shown (± S.E.). The negative control consisting of oocytes injected with DEPC-treated distilled water is labeled DEPC; the negative control produced by injecting oocytes with mRNA from a P. falciparum hexose transporter is labeled PHT (33); oocytes injected with mRNA derived from PfACα (variant 3) are labeled PfACα.

Parent cell line accumulated no cAMP (Fig. 6B). Sorbitol reduced rather than stimulated cAMP production, which indicates that neither variant is activated by high osmolality. The CAMP production by variant 3 was somewhat higher than variant 1, but this may reflect different expression levels of the constructs, rather than differences in intrinsic catalytic activity.

AC activity was also measured directly in cell lysates provided with ATP and different concentrations of Mg2+ and Mn2+ ions. Again, no activity could be detected in the acc−/ace− parent. Both variant 1 and variant 3 showed highest activity in the presence of Mn2+ ions, with maximum activity at 1 and 3 mM Mn2+, respectively (Fig. 6C). There was very little activity with Mg2+ as a cofactor. This demonstrates that the measured activity cannot be caused by ACβ, which shows highest activity with Mg2+ as a cofactor (34).
have a regulatory role, since it has been shown to bind DTT in vitro leading to activation of the enzyme (7).

The Occurrence of Splice Variants Encoding PfACα—More than 90% of the examined RT-PCR products conformed to variant 1 in which the first in-frame start codon occurs in exon 5. The presence of introns in the 5′-untranslated region has previously been reported in Plasmodium berghei. Here alternative splicing of a mRNA in distinct life cycle stages gives rise to two distinct transcripts, one of which has introns in the 5′-untranslated region (47). The role of introns in the 5′-untranslated region is not known. All three variants of PfACα are predicted to be integral membrane proteins, consistent with our measurement of native AC activity in gametocyte membrane fractions (data not shown). Variant 1 potentially encodes a short form of the enzyme with just two transmembrane domains; a pattern found in, for example, the germination-specific ACG of D. discoideum (an osmosensor; Ref. 15). However, unlike ACG, PfACα is not activated by high osmolarity.

Variants 2 and 3 are predicted to encode enzymes with five and six transmembrane domains, respectively. It is possible that the three variants of PfACα might, through their differing predicted architectures, respond to different environmental signals each resulting in an appropriate change in intracellular levels of cAMP required for cellular function.

Comparison of the Catalytic Domain of PfACα and PfACβ with Those of Other Cyclases—Crystallography (39, 40), mutagenesis (5, 48), and modeling studies (49) have determined the amino acid residues responsible for catalytic activity and substrate binding in both AC and GC. Furthermore it has also been shown that substrate specificity can be altered by substitution of two residues in the purine-binding pocket (50–52). One of these positions is occupied by a lysine in all known ACs and a glutamic acid in all known GCs. In both PfACα and PfACβ this position contains a lysine. The second position, which determines substrate specificity, is occupied by an invariant aspartic acid residue in all G protein-dependent ACs and most of the remaining eukaryotic ACs. This aspartate is not present in the Plasmodium ACs. Instead, PfACα has a serine and PfACβ a threonine at this position. Prior to this study, this substitution had only been reported in the cyanobacterium Anabaena (9). A more extensive analysis of sequence data bases reveals that this unusual feature is shared with other bacterial species and some lower and indeed higher eukaryotes; for example, a Dictyostelium AC (rAC, Ref. 53), and the mammalian soluble AC (2). Both of these cyclases were shown in Panel A, C, the same blot but stripped and rehybridized with a probe derived from a gametocyte-specific gene (Pf36).

**DISCUSSION**

Identification of Two Genes Encoding Putative *P. falciparum* ACs—Searches for an AC gene in annotations of the recently completed *P. falciparum* genome sequence gave no matches (46). However, our own searches have revealed the presence of two distinct AC genes. The biochemical activity of one of these (PfACα) has now been verified and is the main focus of this study. The large number of introns in PfACα made it particularly difficult to identify, and this has probably led to the lack of functional assignment. For example, the conserved cyclase motifs span four separate exons (exons 15–18). The existence of PfACβ was probably obscured by the presence of an insert of ~120 amino acids, which interrupts the C1 catalytic domain. Otherwise the protein aligns well with known cyclases and contains all the necessary amino acid residues for enzyme activity. The position of this insert (between β3 and α3) corresponds to a shorter insert found in PfACa and also in the two *P. falciparum* GCs (20). A similar insert that occurs in all kinetoplastid ACs (between α3 and β4) has been postulated to

![Fig. 7. Stage-specific expression of PfACα mRNA. A, an ethidium bromide-stained agarose gel containing total RNA extracted from several developmental stages of the parasite life cycle. The left lane contains RNA molecular weight markers, which are labeled. The sample tracks are numbered as follows: asexual ring stage (1), asexual schizont (2), early (3), mid (4), and late (5) stage gametocytes (see “Experimental Procedures”) and mature gametocytes stimulated to undergo exflagellation (6). B, a Northern blot hybridized with a radiolabeled 1-kb fragment derived from cDNA corresponding to the AC catalytic domain (Gln517–Val555) of PfACα. The position of the transcript (~4.5 kb) is indicated with an arrow. The blot was derived from the gel shown in panel A, C, the same blot but stripped and rehybridized with a probe derived from a gametocyte-specific gene (Pf36).](http://www.jbc.org/Downloadedfrom)
tion also correlates with the presence of a single amino acid insert in this highly conserved region. These two associated changes are thought to reflect the structural constraints required for substrate (ATP) binding in this unique, but functional form of the enzyme. This threonine residue in the *Anabaena* cyclase is essential for activity (9). The biochemical significance of this substitution is not known but a reduced affinity for ATP has been observed in the mammalian soluble AC (2). All other amino acid positions in the cyclase catalytic domain (39, 40), which bind the substrate (adenine, ribose, and phosphate moieties), Mg$^{2+}$ ions, or are essential for catalysis (maintenance of the transition state) are conserved in the PfACa and PfACβ sequences.

In G protein-dependent ACs, the catalytic site is formed by the interaction of a C1 and C2 heterodimer resulting in a double pocket, part of which is for ATP binding and the other structurally related part binds forskolin (a non-physiological activator). The motifs required for substrate binding and catalysis are contributed by the C1 and C2 domain, and therefore both are required for activity. In ACs from most lower organisms (e.g. *Dictyostelium* ACG, Ref. 14 and *Trypanosoma cruzi* AC Ref. 6) a homodimer is formed and gives rise to a pair of identical catalytic sites, which accommodate two substrate molecules (49). All the motifs required for substrate binding and catalytic activity are therefore present in a single catalytic domain, but a dimer is required to form two identical active sites. PfACa has the latter conformation and is therefore likely to form a homodimer. PfACβ on the other hand, has two catalytic domains and is related to the mammalian soluble AC rather than the G protein-dependent isomers.

**Developmental Regulation of PfACa**—Northern blot analysis indicates that blood stage expression of PfACa is confined to the sexual forms (gametocytes) and is maximal at stage II–III (54) of gametocyte development. PfACa transcripts were also detected in gametocytes, which had been stimulated to undergo gametogenesis, suggesting that PfAP5 may have a role in subsequent mosquito stages as well as gametocyte development in the human.

The cAMP signaling pathway has been implicated in the initiation of sexual commitment in *P. falciparum* (22, 23). On the basis of temporal expression of mRNA, the present study suggests that PfACa may have a role in sexual development rather than triggering differentiation because we did not detect expression in the asexual blood stages (from which the sexual stages arise). A previous study has reported native AC enzyme activity in *P. falciparum* blood stage parasite preparations (24).

The properties of this enzyme were distinct from that of the host enzyme. For example, their experiments suggested that the enzyme was G protein-independent and showed a marked preference for Mn$^{2+}$ over Mg$^{2+}$. These findings are consistent with both the predicted structure and properties of PfACa expressed in *D. discoideum*.

**Phylogenetic Relationships of P. falciparum ACs**—There is a low level of relatedness between the two *P. falciparum* ACs indicating that they are unlikely to have arisen by a gene duplication event. PfACa is most closely related to single domains from certain cyanobacterial (e.g. *Trichodesmium* and *Anabaena*) and other bacterial species (e.g. spirochaetes). By contrast, the twin catalytic domain structure of PfACβ places it clearly in a novel class of soluble cyclases, which are themselves characterized by a high level of similarity with bacterial ACs. Prior to this study, these soluble cyclases had only been found in mammals and *Dictyostelium*. However, a newly deposited sequence from the green non-sulfur bacteria *Chlororflexus* shows the highest levels of identity with PfACβ. This type of soluble cyclase has not been found in other species including *Drosophila melanogaster* and *Caenorhabditis elegans* despite completion of genome sequencing projects, although intriguingly it has now been detected in the malaria parasite, its mammalian host, and its insect vector, *A. gambiae*.

The relationship of PfACs to the ACs of certain bacteria, including cyanobacteria, was unexpected. It is therefore possible that PfACs may be the product of lateral gene transfer, derived from the non-photosynthetic plastid that is present in *Plasmodium* (termed the apicoplast as it is present all apicoplasts). The apicoplast (a vestigial chloroplast) is thought to be derived from an ancestral symbiotic cyanobacterium (55–57). A number of chromosomally encoded proteins are subsequently targeted to the apicoplast and probably originated from this organelle (46). This hypothesis is supported by examination of unfinished sequence data from other apicomplexans (e.g. *Toxoplasma*: toxodb.org/ToxoDB.shtml and *Eimeria*: www.sanger.ac.uk); their genomes also appear to contain genes encoding this prokaryotic form of AC. It is also conceivable that the soluble lineage (which includes PfACβ) found in mammals arose by lateral gene transfer. The alternative hypothesis is one of selective gene loss from various species including *D. melanogaster*, for which some evidence has been presented (58).

In summary, we have described the first functional AC in *Plasmodium*. Identification of this gene and demonstration of its biochemical activity will be an important step in investigating its biological role in gametocyte development. In addition, the discovery of multiple mRNA splice variants and a second, distantly related isoform provides insight into the complexity of this signaling pathway in the malaria parasite.

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Multiple Splice Variants Encode a Novel Adenylyl Cyclase of Possible Plastid Origin Expressed in the Sexual Stage of the Malaria Parasite *Plasmodium falciparum*

David K. Muhia, Claire A. Swales, Ursula Eckstein-Ludwig, Shweta Saran, Spencer D. Polley, John M. Kelly, Pauline Schaap, Sanjeev Krishna and David A. Baker

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