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## Guanylyl Cyclase Activity Associated with Putative Bifunctional Integral Membrane Proteins in *Plasmodium falciparum*\*

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**We report here that guanylyl cyclase activity is associated with two large integral membrane proteins (PfGC $\alpha$  and PfGC $\beta$ ) in the human malaria parasite *Plasmodium falciparum*. Unusually, the proteins appear to be bifunctional; their amino-terminal regions have strong similarity with P-type ATPases, and the sequence and structure of the carboxyl-terminal regions conform to that of G protein-dependent adenylyl cyclases, with two sets of six transmembrane sequences, each followed by a catalytic domain (C1 and C2). However, amino acids that are enzymatically important and present in the C2 domain of mammalian adenylyl cyclases are located in the C1 domain of the *P. falciparum* proteins and vice versa. In addition, certain key residues in these domains are more characteristic of guanylyl cyclases. Consistent with this, guanylyl cyclase activity was obtained following expression of the catalytic domains of PfGC $\beta$  in *Escherichia coli*. In *P. falciparum*, expression of both genes was detectable in the sexual but not the asexual blood stages of the life cycle, and PfGC $\alpha$  was localized to the parasite/parasitophorous vacuole membrane region of gametocytes. The profound structural differences identified between mammalian and parasite guanylyl cyclases suggest that aspects of this signaling pathway may be mechanistically distinct.**

The life cycle of the human malaria parasite *Plasmodium falciparum* is complex with several stages that differ at both the morphological and biochemical levels. Mosquito transmitted sporozoites migrate to the liver where they undergo asexual multiplication within hepatocytes. Liver forms are then released into the bloodstream where they invade red blood cells. A small proportion of the erythrocytic forms develop into gametocytes, precursors of male and female gametes. When gametocytes are taken up by a mosquito during a bloodmeal, they must first emerge from the red blood cells as gametes before fertilization occurs in the midgut, prior to completion of the insect stages of the parasite life cycle. The emergence of eight motile male gametes from a red blood cell is known as exflagellation. Recent work (1) has identified xanthurenic acid, a metabolite of tryptophan, as a putative *in vivo* gametocyte-activating factor. In both *P. falciparum* and *Plasmodium berghei* there is evidence that cyclic GMP may be involved in the development and emergence of male gametes (2). The parasite signaling pathways involved in these processes remain to be elucidated.

Two key components of signal transduction pathways are adenylyl cyclase (AC)<sup>1</sup> and guanylyl cyclase (GC), enzymes that catalyze the conversion of ATP and GTP to cAMP and cGMP, respectively. Membrane-associated mammalian ACs are activated indirectly following interaction of a ligand with a separate receptor. This in turn binds to an intracellular heterotrimeric GTP-binding protein (G protein). Subunits of the activated G protein then interact with AC stimulating the synthesis of cAMP, which activates cAMP-dependent protein kinase (PKA). PKA phosphorylates a number of proteins; its final destination is often the nucleus where it activates transcription factors, thereby changing the pattern of gene expression. In mammals, nine distinct membrane-localized ACs have so far been characterized (3). They conform to the same basic structure comprising two sets of six hydrophobic transmembrane domains, each of which is followed by a cytoplasmic catalytic region termed C1 and C2, respectively. The amino-terminal portions (C1<sub>a</sub> and C2<sub>a</sub>) are homologous with each other and between species (4). Guanylyl cyclase exists in two main forms. The receptor form (with a single membrane-spanning domain) binds directly to an extracellular ligand leading to activation and production of intracellular cGMP. The soluble, cytosolic form of the enzyme is activated by nitric oxide in

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<sup>1</sup> The abbreviations used are: AC, adenylyl cyclase; GC, guanylyl cyclase; PKA, cAMP-dependent protein kinase; PCR, polymerase chain reaction; bp, base pair(s); ORF, open reading frame; RT, reverse transcription.

the presence of heme (reviewed in Ref. 5). GCs have a variety of roles in higher organisms, for example in photoreceptor signal transduction (reviewed in Ref. 6). GCs have also been identified in several lower organisms including protozoans such as *Dictyostelium* (7), *Paramecium*, and *Tetrahymena* (8, 9). In sea urchins, a membrane-bound GC found on the surface of sperm can act as a receptor for peptides released by the eggs, which influence sperm chemotaxis (reviewed in Ref. 10). cGMP often exerts its function by binding to and activating cGMP-dependent protein kinase, which phosphorylates and regulates the activity of a number of specific proteins.

As part of an investigation of the role of cyclic nucleotides in signal transduction in the malaria parasite, we have isolated two genes from *P. falciparum* that encode integral membrane proteins. These proteins are unusual in that they appear to be bifunctional. The amino-terminal regions have strong similarity to P-type ATPases, and the carboxyl-terminal regions, including domains with GC activity, conform to a structure normally associated with G protein-dependent ACs.

#### EXPERIMENTAL PROCEDURES

**Parasite Culture**—*P. falciparum* clones 3D7A (11), T996 (12), and strain K1 (12) were cultured in flasks with RPMI 1640 medium supplemented with 25 mM Hepes, 0.1 mM hypoxanthine, 10% human serum (A+) as described previously (13). Cultures contained A+ human red blood cells at a 10% hematocrit. Large scale parasite preparations of clone 3D7A were produced using a semi-automated continuous flow apparatus. Gametocytes (stages III–V) were harvested and purified by Percoll gradient centrifugation (13).

**Isolation of Nucleic Acids**—Genomic DNA was isolated from  $10^9$  mixed asexual erythrocytic stage parasites according to standard procedures (14). Briefly, the parasites were first released from red blood cells by treatment with 0.1% saponin in phosphate-buffered saline. The cell pellet was then washed twice with phosphate-buffered saline and incubated in a solution containing  $100 \mu\text{g ml}^{-1}$  proteinase K, 0.5% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 25 mM EDTA at 37 °C for 16 h. DNA was further purified by phenol extraction and ethanol precipitation. Total RNA was isolated from  $5 \times 10^8$  gametocytes (mainly stages III–V) or mixed asexual erythrocytic stage parasites by lysis with 4 M guanidine thiocyanate and CsCl centrifugation (15).

**Amplification of *PfGC* Sequences by PCR**—A 220-bp fragment of the *PfGC* gene was isolated from genomic DNA (strain K1) by PCR using the degenerate primers DC1 (sense) and MS2 (antisense), which were derived from conserved regions of AC/GC genes from diverse species. The reactions (50  $\mu\text{l}$ ) were carried out in 10 mM Tris, pH 8.8, 50 mM KCl, 0.5–5 mM  $\text{MgCl}_2$ , and 1% Triton X-100 with 1  $\mu\text{M}$  of each primer, 200  $\mu\text{M}$  dNTP mix, 1 unit of Biotaq enzyme (Bioline) and 1–50 ng of template DNA. To minimize nonspecific amplification products, the “touchdown” method (16) of thermal cycling was used with the following conditions: first cycle, 94 °C (2.5 min), 41 °C (1 min), and 72 °C (1 min); and second cycle, 94 °C (45 s), 40 °C (1 min), and 72 °C (1 min). The annealing temperature was then decreased by 1 °C each two cycles until it reached 35 °C. 25 cycles were performed under these conditions. A final cycle was then carried out at 94 °C (45 s), 35 °C (1 min), and 72 °C (10 min). Inverse PCR (17) was used to extend the available *PfGC* sequence after library screening (below) failed to isolate the full-length gene. Based on Southern blot data, an *AluI* digest of genomic DNA (1  $\mu\text{g}$ , strain K1) was circularized with T4 DNA ligase, and an overlapping fragment was obtained by inverse PCR using primers IPCR1 and IPCR2.

**Construction of a Genomic DNA Library**—A  $\lambda$ GEM-12 library was constructed with *P. falciparum* (strain K1) genomic DNA using a Promega kit according to the manufacturer’s instructions. Briefly, a *Sau3a* partial digest of genomic DNA was end-filled and ligated with blunt-ended *XhoI* cut  $\lambda$ GEM-12 arms. After ligation, the DNA was packaged *in vitro* using a Packagene Extract (Promega) and plated on *Escherichia coli* strain LE392. The library (containing  $2.7 \times 10^5$  clones) was screened with the original 220-bp PCR product using standard procedures (14), and a genomic clone ( $\lambda$ 1) containing part of the *PfGC* gene was isolated.

**Construction and Screening of Vectorette Libraries**—Vectorette libraries were constructed as described previously (18) using 1  $\mu\text{g}$  of genomic DNA (clone 3D7A) that had been digested with one of several restriction enzymes. This DNA (200 ng) was ligated into blunt-ended Vectorette DNA (Cambridge Research Biochemicals). Based on known

sequence and Southern blot information, PCR was performed using specific primers (AC1–AC9 for *PfGC* $\alpha$ , pvec1–3 for *PfGC* $\beta$ ) and the Vectorette I primer (Cambridge Research Biochemicals). With this procedure we were able to obtain the remainder of the *PfGC* $\alpha$  coding sequence and the cyclase-encoding domain of *PfGC* $\beta$ . In the PCR reactions, the polymerase was added to the tubes in the cycling block when the reaction temperature was at least 80 °C. This “hot start” and the use of annealing temperatures in the range 60–70 °C were important for the success of the reaction.

**Oligonucleotides**—The following oligonucleotides were used: DC1, 5'-GTATATAAAGTAGAAAC(A/T)AT(T/A)GG; MS2, 5'-(T/A)CC(A/G)A-A(T/G)AA(A/G)CA(A/G)TA(T/A)C(T/G)(T/A)GGCAT; IPCR1, 5'-TTATGATCATCAATAAATAC; IPCR2, 5'-TTATTATTTCATATCAGC; AC1, 5'-TGTATCACCATCGATTGTATTAATG; AC2, 5'-CATAATAATTACTTGTATCACCATCG; AC3, 5'-ACGAGTAGGTTCAATACTGGCCAC; AC4, 5'-GGAAATAAACTGCTTGAATACTCC; AC5, 5'-GAATATGCTTCTGTTTAAATTCCTTTTTG; AC6, 5'-TTTTGAATATGCTTCTGTTTAAATTC; AC7, 5'-GGCATTACTATTTCTAAGGCAGC; AC8, 5'-ATGAGCAAGCATCATACATTC; AC9, 5'-TCTTCTTTGCTTTCATATGCATAGG; 5CAT, 5'-CGCGCGAATTCTCTACTTATGTAAGAATCA; 3CAT, 5'-CGCGCGAATTCAAATTCGACTGGCGATCGTT; pvec1, 5'-ATCAATTCGTTTACTATATCTACTATC; pvec2, 5'-TAAACA-GATCCAATCACCACCTG; and pvec3, 5'-ACGTGTGGTTGTAATGT-AGATACC (degenerate positions are marked with slashes).

**Nucleic Acid Analysis**—To isolate chromosome-sized DNA, asexual blood stage parasites were first released from red blood cells by saponin lysis and then washed in TSE buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 50 mM EDTA). The pellet was resuspended in 9 volumes of TSE and then mixed with an equal volume of 1.6% agarose in TSE and pipetted into a gel mold (Bio-Rad). The agarose blocks ( $10^8$ – $10^9$  parasites/block) were incubated in 1% sarcosinate, 0.5 M EDTA, pH 8.0, 2 mg  $\text{ml}^{-1}$  proteinase K for 48 h at 50 °C and then stored at 4 °C. Chromosome separations were performed with 1% agarose gels using a contour-clamped homogeneous field electrophoresis system (CHEF DR11, Bio-Rad). The gels were run in 0.5% TBE buffer at 100 V for 96 h with a pulse time of 360 s ramped to 800 s. Typically, hybridizations were performed at 42 °C in  $6\times$  SSC and 50% formamide with washes in  $1\times$  SSC at 50 °C. For lower stringency experiments, hybridizations were at 50 °C in  $5\times$  SSC with washes in  $2\times$  SSC at room temperature. Northern blots were carried out using standard conditions (15).

**Production of Antibodies against the C2 Catalytic Domain of *PfGC***—Oligonucleotide primers 5CAT and 3CAT were designed to amplify the carboxyl-terminal region of *PfGC* $\alpha$ , Ser<sup>3914</sup>–Leu<sup>4226</sup>, incorporating the C2 catalytic domain. *EcoRI* restriction sites were included in the primers to facilitate insertion of the fragment in-frame with the *Schistosoma japonicum* glutathione S-transferase gene of the pGEX1AT plasmid (Amersham Pharmacia Biotech). Expression of the fusion protein was induced in a 500-ml mid-log phase *E. coli* (strain JM109) culture ( $A_{600} = 0.4$ – $0.5$ ) by the addition of isopropyl-1-thio- $\beta$ -D-galactopyranoside to a final concentration of 1 mM for 2 h. Cells were resuspended in phosphate-buffered saline /1% Triton X-100 and sonicated on ice. The soluble fusion protein was bound to 2 ml of glutathione-agarose (Sigma) on a Poly-Prep column (Bio-Rad) according to the manufacturer’s instructions. Bound protein was eluted by addition of 5 mM reduced glutathione and dissolved in 0.5 ml of normal saline at a final concentration of 2 mg  $\text{ml}^{-1}$ . This was mixed with 0.5 ml of Ribi Adjuvant System (Sigma) resuspended in 1 ml of normal saline. The mixture was homogenized by vortexing, and a New Zealand White rabbit was immunized at six intradermal sites (50  $\mu\text{g}$  each), two intramuscular sites (200  $\mu\text{g}$  each), and two subcutaneous sites (100  $\mu\text{g}$  each). Similar booster injections were given at 3–4-week intervals. Preimmune serum was taken prior to immunization.

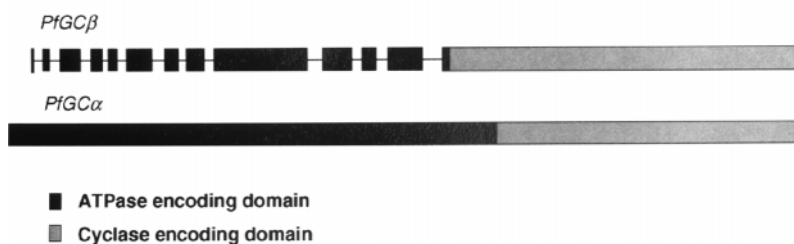
**Immunoelectron Microscopy**—Percoll-purified gametocytes that had been stimulated to undergo gametogenesis were fixed for 30 min with 2% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline, dehydrated in ethanol, embedded in LR White (London Resin Co.), and polymerized at 50 °C for 48 h. Ultrathin sections were collected on piliform-coated nickel grids and incubated with primary antibody and gold-conjugated secondary antibody as described previously (19). The sections were silver enhanced using an Amersham Pharmacia Biotech IntenSE M silver enhancement kit and then stained with a saturated uranyl acetate solution in 30% methanol followed by Reynolds lead citrate solution. The samples were analyzed with a JEOL 1200EX transmission electron microscope at 80 kV and photographed using Agfa Scientia EM film.

**Sequence Analysis**—DNA sequencing was performed using an ABI PRISM 377 DNA sequencer. The reactions were performed with a dye terminator cycle sequencing Ready Reaction Kit (Perkin-Elmer). Pre-



**FIG. 1. Organization of the *PfGC $\alpha$*  and *PfGC $\beta$*  genes.** RT/PCR was used to map introns in both genes (see “Experimental Procedures”). The schematic shows the relative positions of the introns in *PfGC $\beta$*  with respect to the ATPase and cyclase domains. A schematic of the uninterrupted *PfGC $\alpha$*  is also shown. The sizes of each of the 12 introns that interrupt the *PfGC $\beta$*  sequence and also the position with respect to the amino acid sequence are shown. The nucleotide sequences that form the intron splice sites are indicated; capital letters represent exon sequence, and lowercase letters represent intron sequence.

Intron	Size (bp)	Boundaries	Position
1	200	TGgta.....tagAA	Met <sub>9</sub> -Asn <sub>10</sub>
2	137	CCgta.....tagTT	Ser <sub>52</sub> -Leu <sub>53</sub>
3	172	AGgta.....tagGT	Lys <sub>140</sub> -Val <sub>141</sub>
4	111	AAgta.....tagTT	Lys <sub>198</sub> -Ile <sub>199</sub>
5	133	ATgta.....aagCT	Gln <sub>249</sub> -Ser <sub>250</sub>
6	150	AGgta.....tagAA	Ile <sub>363</sub> -Glu <sub>364</sub>
7	104	AGgtt.....tagAA	Arg <sub>443</sub> -Lys <sub>444</sub>
8	123	TCgta.....aagAA	Asn <sub>528</sub> -Gln <sub>529</sub>
9	154	TGgta.....tagAA	Met <sub>886</sub> -Lys <sub>887</sub>
10	120	TGgta.....tagCA	Cys <sub>986</sub> -Lys <sub>987</sub>
11	135	TAgta.....tagTC	Leu <sub>1059</sub> -Ser <sub>1060</sub>
12	241	AGgta.....tagAA	Arg <sub>1193</sub> -Ile <sub>1194</sub>



liminary sequence data for *P. falciparum* chromosome 11 were obtained from the Institute for Genomic Research website. Sequencing of chromosome 11 was part of the International Malaria Genome Sequencing Project and was supported by an award from the NIAID, National Institutes of Health. Sequence data for *P. falciparum* chromosome 13 were obtained from the Sanger Center website. Sequencing of *P. falciparum* chromosome 13 was accomplished as part of the Malaria Genome Project with support by the Wellcome Trust.

**Expression of Recombinant Proteins Corresponding to the *PfGC $\alpha$*  and *PfGC $\beta$*  Catalytic Domains**—The cyclase catalytic domains of both *PfGC $\alpha$*  (C1, Gly<sup>2998</sup>-Val<sup>3329</sup>, and C2, Gln<sup>3956</sup>-Lys<sup>4163</sup>) and *PfGC $\beta$*  (C1, Val<sup>1533</sup>-Tyr<sup>1758</sup>, and C2, Glu<sup>2946</sup>-Ala<sup>3122</sup>) and the catalytic domain of a *Trypanosoma cruzi* AC (ADC-1, Arg<sup>849</sup>-Lys<sup>1169</sup>) were cloned into pTrcHis C (Invitrogen) to give hexahistidine amino-terminal fusion proteins. Expression of the fusion proteins in *E. coli* (strain TP610, AC-deficient) was induced at 30 °C by incubation with 0.1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside for 1 h. Cultures were then lysed by sonication (six 10-s bursts at an amplitude of 20  $\mu$ m using an MSE Soniprep 150) in a solution containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 300 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, 10 mM  $\beta$ -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50  $\mu$ g ml<sup>-1</sup> 1-chloro-3-tosylamido-7-amino-2-heptanone, and 1 mg ml<sup>-1</sup> lysozyme. Lysates were cleared by centrifugation at 15,000  $\times$  g for 20 min at 4 °C. Proteins were affinity purified on Ni<sup>2+</sup>-nitrilotriacetic acid Sepharose according to the manufacturer's instructions (Qiagen).

**Adenylyl/Guanylyl Cyclase Assays**—AC and GC activity was assayed in reaction volumes of 40  $\mu$ l. 30- $\mu$ l aliquots of fusion protein (2–5  $\mu$ g) were added to 10  $\mu$ l of 4 $\times$  reaction buffer (40 mM Tris, pH 8.0, containing either ATP or GTP at 4 mM and 8 mM MgCl<sub>2</sub> or MnCl<sub>2</sub>) and incubated at 30 °C. Reactions were stopped by addition of 10  $\mu$ l of 0.2 M EDTA, pH 8.0, followed by boiling for 2 min. For the *t* = 0 time points, EDTA was added before the protein. cAMP or cGMP concentrations were measured in 96-well plates using a competition assay (20) with purified regulatory subunit of beef heart cAMP-dependent protein kinase as the cAMP-binding protein (PKA-R) or highly specific anti-cGMP antibodies as the cGMP binding protein (21). Briefly, samples were mixed with 20  $\mu$ l of 0.4 mCi of [<sup>3</sup>H]cAMP (48 Ci mmol<sup>-1</sup>; Amersham Pharmacia Biotech) or [<sup>3</sup>H]cGMP (14.8 Ci mmol<sup>-1</sup>) per ml of assay buffer (4 mM EDTA in 150 mM sodium phosphate buffer, pH 7.5) and 20  $\mu$ l of PKA-R or cGMP antibody preparation. After incubation on ice for 90 min, 40  $\mu$ l of activated charcoal (1.25 g of charcoal and 0.5 g of bovine serum albumin in 25 ml of assay buffer) was added to adsorb unbound

[<sup>3</sup>H]cAMP or [<sup>3</sup>H]cGMP. Samples were centrifuged at 3,000  $\times$  g for 15 min at 4 °C. 50  $\mu$ l of supernatant was mixed with 100  $\mu$ l of scintillation fluid, and the radioactivity was measured (22). A standard curve was produced with a linear range from 0–16 pmol cAMP/cGMP.

## RESULTS

**Isolation of *PfGC $\alpha$* , a Gene That Encodes a Protein with Distinct Cyclase and ATPase Domains**—Degenerate oligonucleotide primers were designed based on conserved regions in the catalytic domains of AC/GC from other species (see “Experimental Procedures”). Using these, a 220-bp fragment was amplified by PCR from *P. falciparum* genomic DNA. Data base searches suggested that the product was derived from a purine nucleotide cyclase gene homologue. This fragment was used to screen a genomic DNA library, and a clone was isolated. Sequence analysis of a 5.5-kilobase *Bam*HI-*Eco*RV fragment identified a 1.8-kilobase open reading frame (ORF) corresponding to the 3'-end of a cyclase-like gene, with a putative stop codon and adjacent 3' noncoding sequence. Inverse and Vectorette PCR techniques (see “Experimental Procedures”) were used to extend the sequence toward the 5'-end of the gene. This generated an uninterrupted ORF of approximately 10 kilobases. A putative start codon was then identified following searches of the Institute for Genomic Research *P. falciparum* data base. Verification of the gene structure was obtained by sequencing the corresponding region (Fig. 1), amplified from genomic DNA. In addition the entire sequence of *PfGC $\alpha$*  has been confirmed following release of data from the Institute for Genomic Research data base, which became available during the preparation of this manuscript.

The nucleotide sequence of *PfGC $\alpha$*  comprises a single ORF of 12,678 bp (these sequence data have been submitted to the GenBank™ data base under accession number AJ245435). The A/T content (75.6%) and codon usage conform to that of all *P. falciparum* genes reported to date. The designated termination codon (TAA) of *PfGC $\alpha$*  occurs in a position analogous to that in mammalian-type AC (4, 23–25) and is followed immediately by

an extremely A/T-rich region (95%). Within the ORF, two regions of unusually high A/T content were identified (nucleotides 7350–7530 and nucleotides 9060–9450). To investigate the possibility of introns at these positions and elsewhere in the gene, a series of RT/PCR reactions were carried out. There were no differences in the sizes of the products obtained from RNA (DNase I-treated) and genomic DNA (data not shown). In negative controls where the reaction was performed in the absence of RT, no products were amplified. Using this approach it was shown that PfGC $\alpha$  was uninterrupted by introns.

Analysis of the PfGC $\alpha$  sequence revealed an unexpected finding; the gene had the potential to encode a bifunctional protein in which the amino-terminal domain had high similarity to P-type ATPases (26), and the carboxyl terminus was a structural homologue of G protein-dependent adenylyl cyclases. The nucleotide sequence adjacent to the putative start codon is aaaaATG, which is similar to the consensus sequence upstream of many *P. falciparum* genes (27). The sequence immediately upstream of this is preceded by a highly A/T-rich sequence interrupted by stop codons in all three reading frames.

**Isolation of PfGC $\beta$** —A *P. falciparum*  $\lambda$ GEM-12 library was screened at low stringency with a probe corresponding to the PfGC $\alpha_{C2}$  catalytic domain (see “Experimental Procedures”). A clone containing 1.3 kilobase of sequence with similarity to PfGC $\alpha$  and encompassing an in-frame stop codon was isolated. This gene was designated PfGC $\beta$ . The remainder of the cyclase encoding region (an additional 2000 nucleotides) was obtained from Vectorette libraries (see “Experimental Procedures”). Upstream, an additional open reading frame with a high degree of similarity to P-type ATPases was discovered. The sequence of this region was obtained from the *P. falciparum* genome project (Sanger Center). The cyclase and the ATPase coding regions were found to be separated by 241 bp of A/T-rich sequence. This was shown to be an intron, and both coding regions were found to form a contiguous ORF. Sequence analysis of a series of RT/PCR products obtained using primers specific to both the cyclase and ATPase domains demonstrated the presence of 12 introns in the PfGC $\beta$  sequence. These were all confined to the ATPase encoding domain (Fig. 1). The fully spliced transcript has the potential to encode a bifunctional protein of 3122 amino acids (accession number AJ249165). The 5'-most exon contains an A/T-rich sequence upstream of the designated start codon that has stop codons in all three reading frames. Sequencing of RT/PCR products has confirmed that this A/T-rich sequence forms part of the mature mRNA. The predicted stop codon (position 9367) is located upstream of a region with an extremely A/T-rich composition that has stop codons in all three reading frames. The positions of the designated start and stop codons are consistent with those found in other genes encoding similar P-type ATPases and cyclases, respectively. The sequence of the cyclase-encoding region has been confirmed by chromosome 13 sequence data that were released (Sanger Center) during preparation of this manuscript. Using the Genestream Align global alignment program the sequences of PfGC $\alpha$  and PfGC $\beta$  were found to share 22% identity in their cyclase domains and 19% in their ATPase domains.

**Features of the Cyclase Domains**—The relatedness of both PfGC $\alpha$  and PfGC $\beta$  to GC/AC is concentrated in two regions (PfGC $\alpha_{C1}$  Gly<sup>2999</sup>–Val<sup>3329</sup>; PfC $\alpha_{C2}$ , Gln<sup>3956</sup>–Lys<sup>4163</sup>; PfGC $\beta_{C1}$ , Val<sup>1533</sup>–Tyr<sup>1758</sup>, and PfGC $\beta_{C2}$ , Glu<sup>2946</sup>–Ala<sup>3122</sup>), which have 26–33% sequence identity and 48–52% similarity (28) to the corresponding regions of enzymes from diverse species. These hydrophilic regions correspond to the positions of the catalytic domains. PfGC $\alpha_{C1}$  is characterized by the presence of a long asparagine-rich stretch (amino acids Asn<sup>3082</sup>–Asn<sup>3138</sup>, Fig. 2).

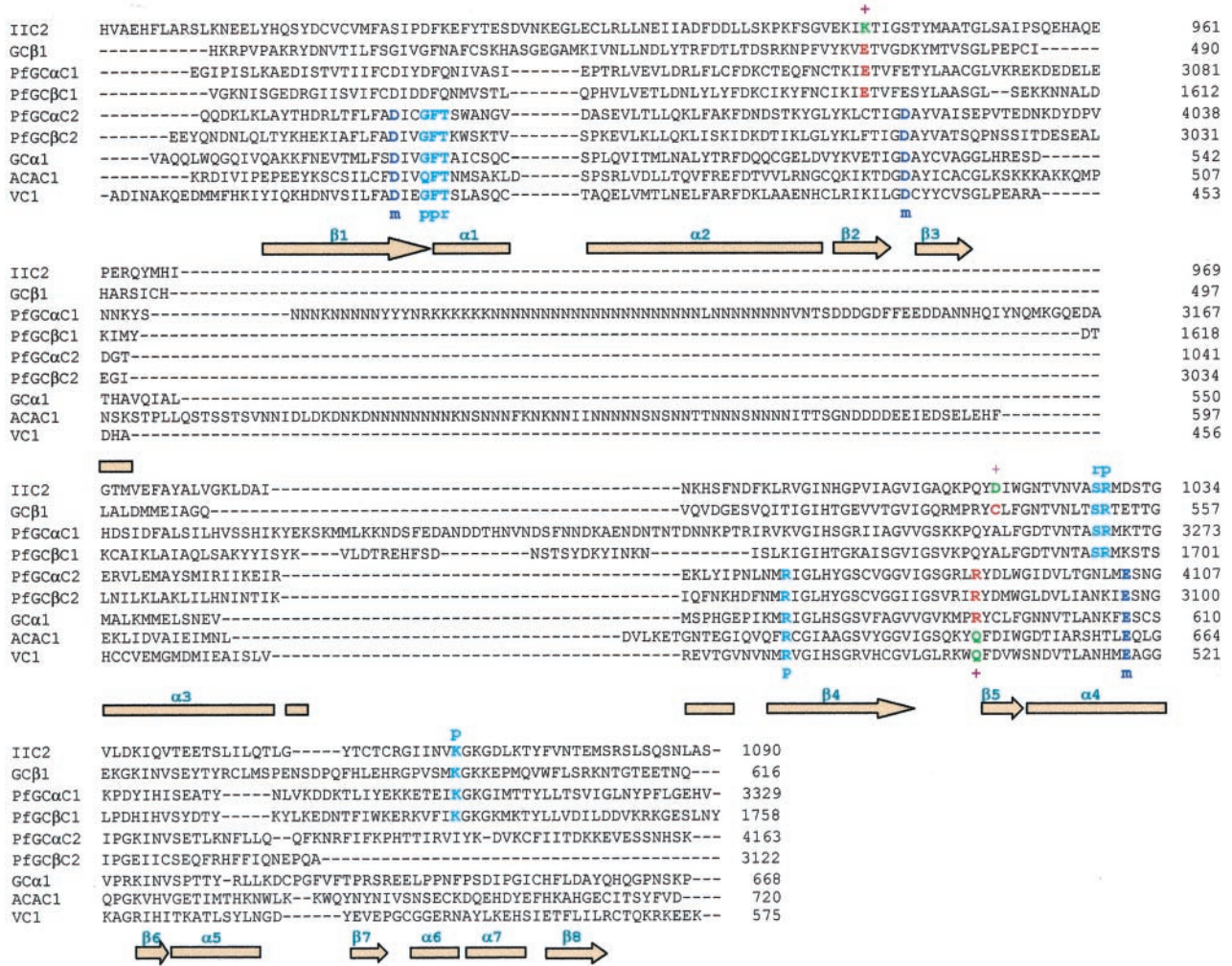
Interestingly, the aggregation-specific adenylyl cyclase (ACA) of the protozoan *Dictyostelium discoideum* has a similar sequence insertion at the same position within the C1 catalytic domain (29). There is a second, shorter insertion in PfGC $\alpha_{C1}$  (Tyr<sup>3186</sup>–Thr<sup>3225</sup>; Fig. 2) that corresponds (in terms of both position and size) to an insert found in all of the trypanosomatid ACs sequenced to date. No such inserts are present in the cyclase catalytic domains of PfGC $\beta$ . Alignment of PfGC $\alpha_{C1}$  with PfGC $\alpha_{C2}$  revealed 23% sequence identity, whereas alignment of PfGC $\beta_{C1}$  and PfGC $\beta_{C2}$  gave 34% identity.

The cyclase catalytic domains of both PfGC $\alpha$  and PfGC $\beta$  are each preceded by highly hydrophobic regions that in membrane-associated mammalian ACs have been predicted to form two sets of six transmembrane domains. The PfGC $\alpha$  and PfGC $\beta$  sequences are also compatible with this type of organization (Fig. 3).

**Features of the ATPase Domains**—The putative ATPase domains of both proteins conform to the P-type (or E1-E2) family of cation transporting ATPases (30). The model for this family of enzymes predicts 10 transmembrane domains arranged as a set of four toward the amino terminus and a set of six near the carboxyl terminus. The amino acid sequences of both PfGC $\alpha$  and PfGC $\beta$  are consistent with this type of structure (Fig. 3). For both PfGC $\alpha$  and PfGC $\beta$  the relatedness to P-type ATPases from diverse species is concentrated in two regions and is summarized below. The amino-terminal domain of PfGC $\alpha$  has 26–32% identity and 48–52% similarity over 250–300 amino acids; for the carboxyl terminus the figures are 28–30% identity and 48–50% similarity. For PfGC $\beta$ , the amino-terminal domain has 23–25% identity and 43–46% similarity; for the carboxyl terminus the figures are 21–23% identity and 46–48% similarity. The membrane-spanning regions of the P-type ATPase domains are more highly conserved than are those of the cyclase domains. This might be expected given the potential functional significance of these transmembrane regions. The consensus phosphorylation site of P-type ATPases (DKTGTLT) is present in PfGC $\alpha$  (Asp<sup>756</sup>–Thr<sup>762</sup>) but not in PfGC $\beta$ . This signature sequence is involved in formation of an aspartyl phosphate intermediate during ATP hydrolysis (31).

**Expression and Subcellular Localization**—Southern analysis of genomic DNA with a probe derived from the region of PfGC $\alpha$  corresponding to the C2 domain produced a hybridization pattern consistent with a single copy gene (data not shown). In accordance with this, when *P. falciparum* (clones 3D7A and T996) chromosomes separated by CHEFE and transferred to nylon membranes were analyzed (the probe corresponded to nucleotides 10,788–11,289), a single band identified as chromosome 11 was detected (Fig. 4). PfGC $\beta$  was localized to chromosome 13. When northern blots of RNA derived from sexual and asexual blood stage parasites were probed with PfGC $\alpha$ , a transcript of approximately 12,000–14,000 nucleotides was detected that was specific to the sexual blood stages (Fig. 4). The sexual stage RNA had been prepared from Percoll-purified gametocytes (mainly stages III–V), and the asexual stage RNA was prepared from an asynchronous culture containing ring, trophozoite, and schizont stages. The same blot was hybridized with a probe derived from the ATPase-encoding domain of PfGC $\alpha$  (nucleic acids 5,901–6,630), and a band of equal size was detected (Fig. 4). A northern blot was also hybridized with a probe corresponding to the cyclase region of PfGC $\beta$ , and a slightly smaller transcript was identified (Fig. 4). The northern blots were hybridized with other probes (data not shown) to demonstrate both the integrity of the RNA preparations and the equivalence of loading as described previously (32, 33). The probes were derived from genes encoding sexual (Pfs16) and asexual blood stage specific proteins (Msp1) and another (cal-





**FIG. 2. Sequence alignment.** Alignment of the catalytic domains of PfGC $\alpha$  and PfGC $\beta$  with those of the mammalian soluble guanylyl cyclases GC $\alpha_1$  and GC $\beta_1$  (49), the C1 domains of both mammalian type V adenylyl cyclase (VC1, (50)) the *Dictyostelium* ACA (29), and the C2 domain of rat type II adenylyl cyclase (IIC2). The known secondary structure of the mammalian type V C1 domain (from a crystallographic study (36)) is indicated by **thick lines** ( $\alpha$  helix) and **arrows** ( $\beta$  sheet) beneath the sequence. The alignment illustrates the reversal of the C1 and C2 domains of the *Plasmodium* proteins in terms of key functional residues compared with the C1 and C2 domains of mammalian adenylyl cyclases. The residues that determine purine binding specificity are marked with a *plus symbol*; *green* residues are indicative of adenine binding, and *red* residues are indicative of guanine binding. Other functionally important residues, determined by mutagenesis and structural studies (discussed in the text) are indicated and have been shown as follows. *Light blue r*, ribose binding; *light blue p*, phosphate binding; *dark blue m*, divalent cation binding. The amino acid residue numbers of each sequence are given on the right. The alignment was produced using the PILEUP program in the GCG package (51).

modulin) expressed in similar amounts in both stages. A rabbit polyclonal antiserum was raised to a recombinant protein corresponding to the carboxyl terminus of PfGC $\alpha$  (amino acids Ser<sup>3914</sup>-Leu<sup>4226</sup>, incorporating the C2 catalytic domain) fused to glutathione *S*-transferase (see “Experimental Procedures”). The serum was used in immunoelectron microscopic examination of sections of red blood cells infected with *P. falciparum*. Antibody binding was visualized by using a colloidal gold-conjugated secondary antibody. Sexual erythrocytic stage parasites (gametocytes) were strongly labeled in the plasma/parasitophorous vacuole membrane region of the parasite (Fig. 5). Only very low levels of gold particles were observed in the gametocyte cytoplasm. Little or no labeling was present on uninfected red blood cells or asexual blood stage parasites (Fig. 5). These sections also contained a small proportion of developing gametes at various stages of emergence from the surrounding red blood cell membrane and parasitophorous vacuole membrane. No gold particles were observed in these forms. Preimmune serum from the rabbit showed little or no labeling at the same concentration as the test serum. An antiserum raised to a control glutathione *S*-transferase fusion protein

(derived from *Schistosoma mansoni*) also gave no significant labeling.

**Guanylyl Cyclase and Adenylyl Cyclase Enzyme Assays**—The regions of PfGC $\alpha$  encoding both the C1 (Gly<sup>2999</sup>-Val<sup>3329</sup>) and C2 (Gln<sup>3956</sup>-Lys<sup>4163</sup>) domains and the regions of PfGC $\beta$  encoding both C1 (Val<sup>1533</sup>-Tyr<sup>1758</sup>) and C2 (Glu<sup>2946</sup>-Ala<sup>3122</sup>) were expressed in *E. coli* strain TP610 (AC-deficient) using an amino-terminal hexahistidine tag system. The selection of these regions was based on the scheme used for expression/crystallization of the rat type II AC C2 domain (25). Coomassie Blue-stained SDS-polyacrylamide gels and Western blots (using a monoclonal antibody that reacts with a vector-derived epitope at the amino terminus of fusion protein) showed distinct bands at the predicted molecular masses (47 kDa for PfGC $\alpha_{C1}$ , 32 kDa for PfGC $\alpha_{C2}$ , 34 kDa for PfGC $\beta_{C1}$ , and 28 kDa for PfGC $\beta_{C2}$ ) of the fusion proteins (data not shown). The fusion proteins, purified by affinity chromatography on Ni<sup>2+</sup>-nitrilotriacetic acid Sepharose columns, were tested for AC or GC activities. Levels of cAMP/cGMP were measured by a competition assay using <sup>3</sup>H-labeled cAMP or cGMP (see “Experimental Procedures”).

The purified fusion proteins were first assayed for AC activ-

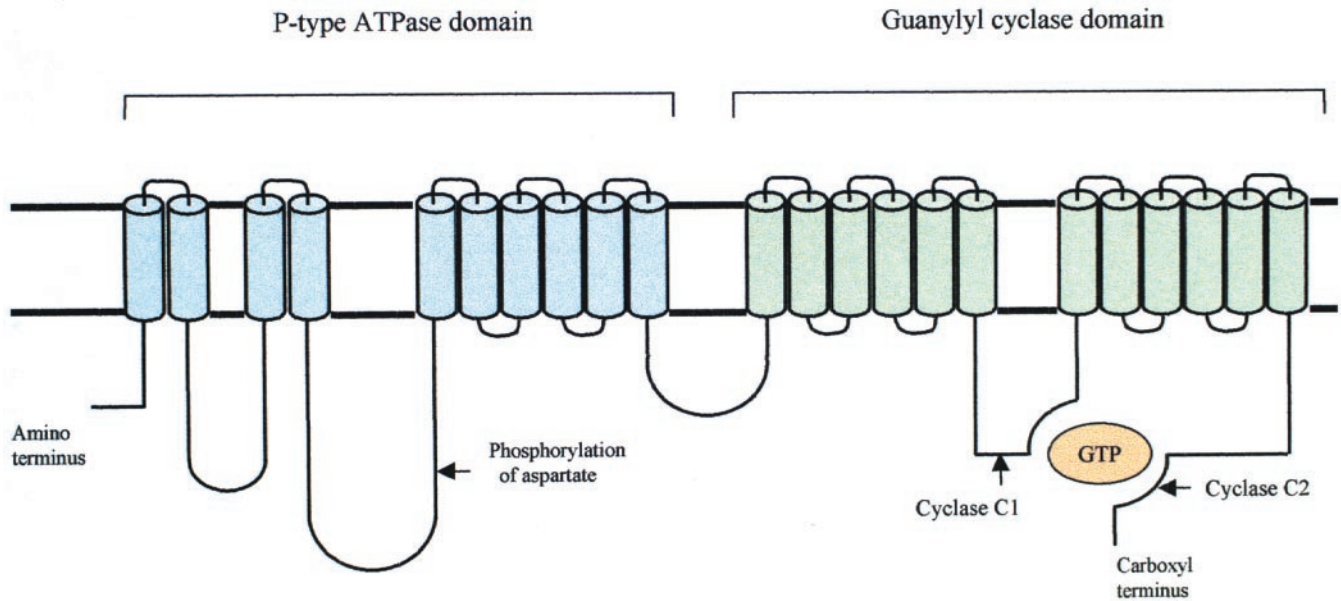


FIG. 3. A model of the proposed structure of PfGC $\alpha$  and PfGC $\beta$ . The deduced amino acid sequences are each consistent with the presence of two sets of six transmembrane regions in the cyclase domain and two further sets of four and six transmembrane regions in the ATPase domain. The cytoplasmic domains of the ATPase are based on a model proposed for a muscle Ca<sup>2+</sup>-ATPase (31, 52).

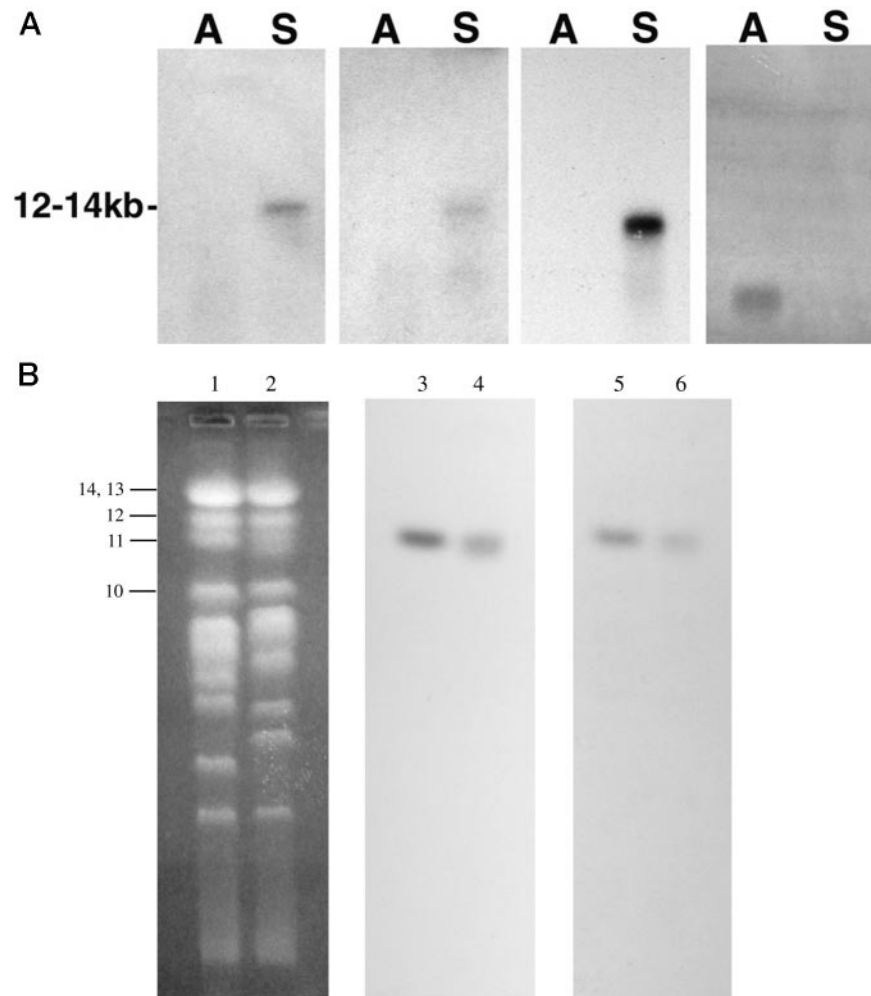


FIG. 4. **Chromosomal location and expression.** A, autoradiograph of a northern blot containing 10  $\mu$ g of total RNA from *P. falciparum* (clone 3D7A) erythrocytic asexual (A) and sexual stage (S) parasites. The four panels from left to right correspond to the respective probes: PfGC $\alpha$  (C2 domain), PfGC $\alpha$  (ATPase domain), PfGC $\beta$  (C2 domain), and *msp1* (a gene expressed specifically in asexual blood stage parasites). The size of the PfGC $\alpha$  gene transcript is indicated. Ethidium staining of agarose gels was used to verify equivalent loading of tracks and integrity of the RNA. B, CHEFE separation of chromosomes from *P. falciparum* clones T996 (lanes 1, 3, and 5) and 3D7A (lanes 2, 4, and 6). Electrophoresis conditions were as described in "Experimental Procedures." Lanes 1 and 2 show an ethidium bromide-stained agarose gel. After transfer to a nylon membrane the DNA was hybridized with PfGC $\alpha$  (lanes 3 and 4) or RESA-2 (lanes 5 and 6). RESA-2 has been localized previously to chromosome 11 (53). Similar results were obtained with the *P. falciparum* K1 strain (data not shown).

ity. No AC activity was obtained using the  $\alpha$ C1,  $\alpha$ C2,  $\beta$ C1, or  $\beta$ C2 proteins derived from these constructs either separately or in combination (Fig. 6). The catalytic domain of a receptor-type AC (ADC-1) from *T. cruzi* was purified and assayed in parallel.

This fusion protein gave a high level of AC activity which was maximized in the presence of Mn<sup>2+</sup> ions (34). We were also able to demonstrate functional complementation of the AC-deficient *E. coli* strain (TP610) with the trypanosome-derived construct,



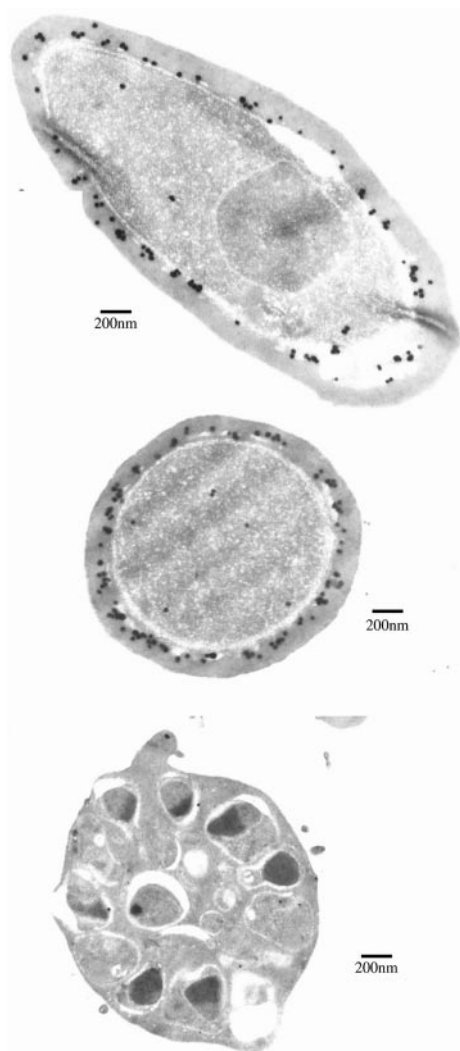


FIG. 5. **Subcellular localization of PfGC $\alpha$ .** Sections of *P. falciparum* (clone 3D7A) erythrocytic stages were incubated with an anti-PfGC $\alpha$  polyclonal antibody (1:3000 dilution, "Experimental Procedures"), and binding was visualized using a gold-conjugated secondary antibody. *Upper panel*, gold particles indicative of antibody binding are associated with the periphery of the gametocyte encapsulated in a red blood cell. Only very low numbers of gold particles can be detected in the cytoplasm. *Middle panel*, a transverse section of a gametocyte showing similar labeling. *Lower panel*, little or no binding of the antibody is detectable with asexual erythrocytic stage parasites. This section shows a schizont with levels of gold particles similar to that obtained in all sections with preimmune serum (data not shown). *Size bars* have been inserted in each panel.

but this was not achievable with the *Plasmodium*-derived constructs (data not shown).

We then tested the fusion proteins for GC activity. Fig. 7A shows that the C1 and C2 domains from the PfGC $\alpha$  gene showed no significant activity over isolates from control bacteria either when tested individually or in combination with each other. However, the fusion proteins derived from the PfGC $\beta$  gene showed moderate levels of activity when tested individually, with  $\beta$ C1 being somewhat more active than  $\beta$ C2. When used in combination, however, considerably elevated levels of activity were obtained (Fig. 7B). Following a brief lag period, cGMP synthesis increased rapidly over the first 10 min before decreasing. To test whether the transient nature of this activity was due to the low stability of the enzyme, we incubated the C1/C2 mixture at 30 °C for 25 min prior to the assay; this resulted in complete loss of activity (Fig. 7C). Addition of forskolin, Ca<sup>2+</sup>, or Ca<sup>2+</sup>/calmodulin to the reaction mixtures did

not affect the level of GC activity (data not shown). In all cases activity was dependent on the presence of Mn<sup>2+</sup> ions. If the divalent cation was replaced with Mg<sup>2+</sup>, no activity could be detected. Proteins from bacteria transformed with control pTrcHis plasmid showed no activity.

#### DISCUSSION

We have isolated two genes from *P. falciparum* that encode proteins with cyclase-like domains similar in structure to the membrane-localized adenylyl cyclases of higher eukaryotes. Unusually, the catalytic regions of at least one of these enzymes has guanylyl cyclase activity. During the preparation of this manuscript, a GC with this type of structure was also described in *Paramecium* (9). An additional remarkable feature found in both the *Plasmodium* and *Paramecium* proteins is that they each have an amino-terminal domain with high similarity to P-type ATPases in terms of sequence and predicted structure. GCs have hitherto fallen into two subclasses: (i) membrane-associated receptor molecules that form homodimers each with a single transmembrane domain and (ii) soluble molecules that form heterodimers. PfGC $\alpha$  and PfGC $\beta$  therefore fall into a third subclass that is membrane-associated (with 12 putative membrane-spanning domains) with a pair of catalytic domains. As in mammalian ACs, their activity is probably mediated by interaction of the C1 and C2 domains.

Northern analysis of mRNA from the erythrocytic stages of *P. falciparum* also indicated that expression of both genes is restricted to gametocytes (Fig. 4). Immunoelectron microscopy localized PfGC $\alpha$  to the plasma/parasitophorous vacuole membrane region of this stage of the life cycle (Fig. 5). In red blood cells, malaria parasites are surrounded by three membranes: the host cell membrane, the parasitophorous vacuole membrane, and the parasite plasma membrane. The latter two membranes are closely opposed, and it is not clear from our electronmicrographs in which membrane PfGC $\alpha$  is located (Fig. 5). However, it can be inferred from the sequence and by comparison with other cyclases that both PfGC $\alpha$  and PfGC $\beta$  are integral membrane proteins. The parasites used for the immunoelectron microscopy study had been stimulated to undergo gametogenesis (see "Experimental Procedures"). The micrographs show parasites at various stages of emergence from the red blood cell and parasitophorous vacuole membranes. No labeling is associated with extracellular gametes, suggesting that PfGC $\alpha$  has a pre-emergence role.

*Structural Comparison of the PfGC $\alpha$  and PfGC $\beta$  Catalytic Domains with Those of Mammalian Adenylyl Cyclases*—The active site of mammalian AC is formed by the interaction of the C1 and C2 domains (35). Recent crystallographic and mutagenesis studies have given insight into the nature of the catalytic site and have identified residues involved in ATP binding (3, 25, 36–38). Based on these studies, models for the analogous binding of GTP within the active site of GC have been derived. From sequence alignment, it can be inferred that the cyclase catalytic domains of both PfGC $\alpha$  and PfGC $\beta$  are different from those of mammalian G protein-dependent ACs in that the C1 and C2 domains appear to be structurally reversed. Several catalytically important residues that are present in the mammalian C2 domain are found only in the C1 domain of the *Plasmodium* molecules (Fig. 2). Likewise, mechanistically important residues present in the C1 domain of the mammalian enzyme are located in the C2 domains of PfGC $\alpha$  and PfGC $\beta$ . For example, in mammalian AC it has been predicted that Arg<sup>398</sup> (type I AC), which approaches the  $\beta$  phosphate of ATP, and Arg<sup>1011</sup>, which interacts with the  $\alpha$  phosphate, are localized in the C1 and C2 domains, respectively (36, 39). The equivalent residues in PfGC $\alpha$  (Arg<sup>4070</sup> and Arg<sup>3268</sup>) and PfGC $\beta$  (Arg<sup>3063</sup> and Arg<sup>1696</sup>) are reversed with respect to their location



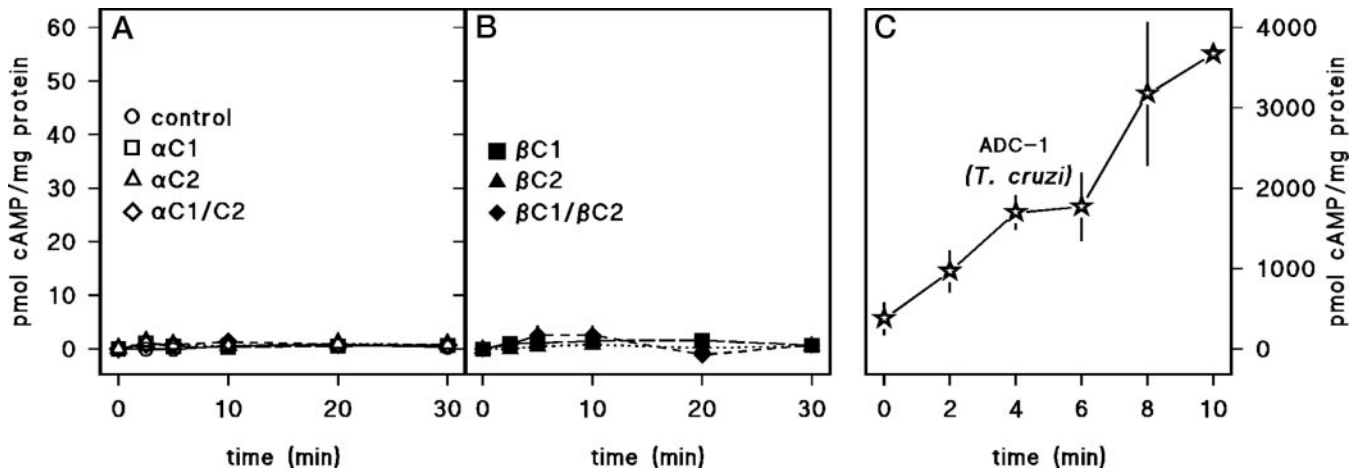


FIG. 6. Adenylyl cyclase enzyme activity assays. The graphs show time courses of cAMP production by the recombinant catalytic domains derived from *T. cruzi* ADC-1 and PfgC $\alpha$  and PfgC $\beta$ . The data represent the means  $\pm$  S.E. of three independent experiments. 2–5  $\mu$ g of affinity purified His $_6$ -tagged fusion proteins were incubated at 30  $^{\circ}$ C for the indicated time periods with 2 mM ATP and 4 mM Mn $^{2+}$  and assayed for cAMP as described under “Experimental Procedures.” Control assays were performed on proteins purified from cultures containing plasmid (pTrcHisC, Invitrogen) with no insert. A, the catalytic domains of PfgC $\alpha$  ( $\alpha$ C1 and  $\alpha$ C2) were assayed both separately and in combination ( $\alpha$ C1/ $\alpha$ C2). B, the catalytic domains of PfgC $\beta$  ( $\beta$ C1 and  $\beta$ C2) were assayed separately and in combination ( $\beta$ C1/ $\beta$ C2). C, the catalytic domain of ADC-1 derived from *T. cruzi* (34) was cloned into pTrcHisC, purified, assayed in parallel, and used as an AC positive control.

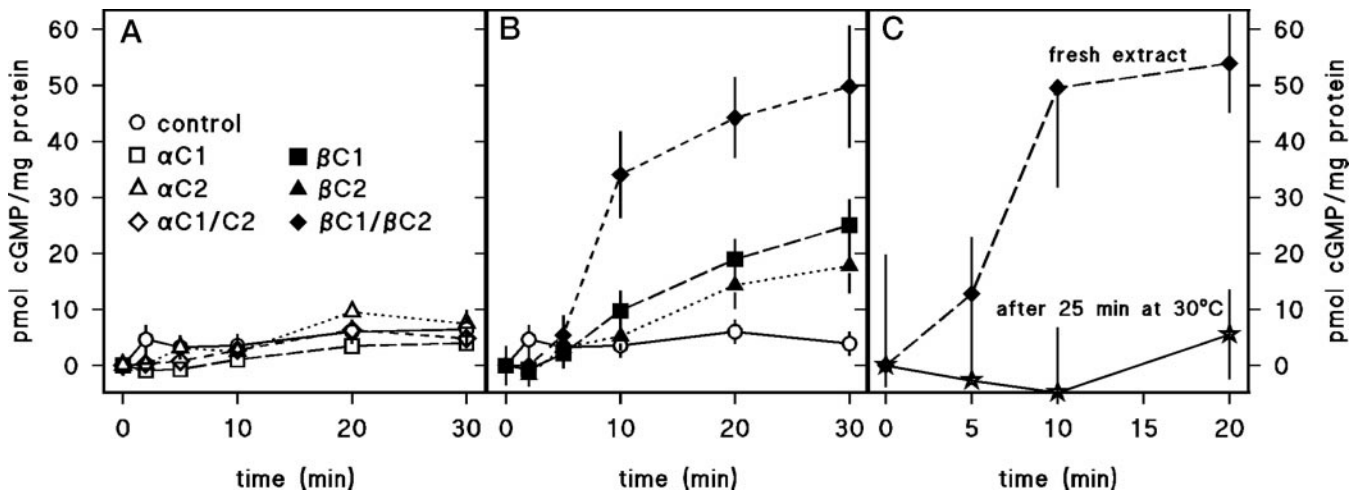


FIG. 7. Guanylyl cyclase enzyme activity assays. The graphs show time courses of cGMP production by the recombinant catalytic domains derived from PfgC $\alpha$  and PfgC $\beta$ . The data represent the means  $\pm$  S.E. of three independent experiments. 2–5  $\mu$ g of affinity purified His $_6$ -tagged fusion proteins were incubated at 30  $^{\circ}$ C for the indicated time periods with 2 mM GTP and 4 mM Mn $^{2+}$  and assayed for cGMP as described under “Experimental Procedures.” Control assays were performed on proteins purified from cultures containing plasmid (pTrcHisC, Invitrogen) with no insert. A, the catalytic domains of PfgC $\alpha$  ( $\alpha$ C1 and  $\alpha$ C2) were assayed both separately and in combination ( $\alpha$ C1/ $\alpha$ C2). B, the catalytic domains of PfgC $\beta$  ( $\beta$ C1 and  $\beta$ C2) were assayed separately and in combination ( $\beta$ C1/ $\beta$ C2). C, a further time course experiment showing the effect of preincubation of  $\beta$ C1/ $\beta$ C2 at 30  $^{\circ}$ C for 25 min.

(Fig. 2). Similarly, three residues in mammalian AC that interact with the ribose moiety of ATP, Thr $^{315}$  (type I C1), Asp $^{354}$  (type I C1), and Asn $^{1007}$  (type I C2), are localized in the opposite catalytic domain in the *Plasmodium* enzymes (Fig. 2). The equivalent positions in PfgC $\alpha$  are Thr $^{3973}$  (C2), Asp $^{4019}$  (C2), and Asn $^{3264}$  (C1), and in PfgC $\beta$  they are Thr $^{2973}$  (C2), Asp $^{3012}$  (C2), and Asn $^{1692}$  (C1). In type I mammalian AC, three negatively charged residues (Asp $^{354}$ , Asp $^{310}$ , and Glu $^{432}$ ) localized in the C1 domain are thought to be involved in Mg $^{2+}$  binding, an essential requirement for enzyme activity (Refs. 3 and 39 and Fig. 2). The corresponding negatively charged residues in PfgC $\alpha$  (Asp $^{4019}$ , Asp $^{3975}$ , and Glu $^{4104}$ ) and PfgC $\beta$  (Asp $^{3012}$ , Asp $^{2968}$ , and Glu $^{3097}$ ) are all in the C2 domain.

**Purine Binding Specificity**—The highly conserved regions of mammalian-type ACs (C1a and C2a) are very similar to the corresponding regions of the catalytic domains of GCs. Mutagenesis and crystallographic studies have shown that a small number of residues are involved in conferring the purine binding specificity of the two enzymes (36, 37, 39, 40). Two separate

studies have demonstrated that the purine specificity of GC can be altered by changing key residues to their counterparts in AC (E925K and C997D of retinal guanylyl cyclase 1, retGC-1 (39); R592Q of soluble GC $\alpha_1$ ; E473K and C541D of soluble GC $\beta_1$  (37)). This results in conversion to an enzyme with an AC activity. These studies indicate a direct interaction between Glu $^{925}$  of GC and the N1 and N2 positions of the guanine ring. In the C1 catalytic domains of both PfgC $\alpha$  and PfgC $\beta$ , the corresponding position is occupied by a glutamic acid residue (Glu $^{3058}$  and Glu $^{1591}$ , respectively) as would be expected in a GC, and not lysine as in AC. However, the *Plasmodium* enzymes are distinct from their mammalian counterparts in that residues in the PfgC $\alpha$  and PfgC $\beta$  positions that correspond to the Cys $^{997}$  (retGC-1), thought to be important for guanine binding specificity, are both alanines (Ala $^{3257}$  and Ala $^{1785}$ , respectively; Fig. 2). In the *Paramecium* GC, the corresponding position is occupied by a serine (9). It has been proposed (36) that the glutamic acid residue (Glu $^{473}$ , GC $\beta$ ) is probably the most important residue for determining GTP binding specific-

ity. This was inferred from studies in which mutated cyclases lacking a glutamic acid at this position were shown to have no GC activity. Modelling studies (36, 37, 39) have also indicated that an arginine residue (Arg<sup>539</sup>, GC $\beta$ , and Arg<sup>592</sup>, GC $\alpha$ ) is important for GTP binding. The arginine residue at this position is conserved in GCs, and the corresponding positions in the C2 domains of both PfGC $\alpha$  and PfGC $\beta$  are also occupied by arginines (Arg<sup>4089</sup> and Arg<sup>3082</sup>, respectively).

**Enzyme Activity of PfGC $\alpha$  and PfGC $\beta$** —Recombinant proteins expressed in *E. coli* corresponding to the C1 and C2 catalytic domains of PfGC $\alpha$  and PfGC $\beta$  were tested for both AC and GC activity. No AC activity was detectable in any of the four malaria-derived recombinant proteins. We were, however, able to demonstrate GC activity in the C1 and C2 domains of PfGC $\beta$  both separately and in combination. The PfGC $\beta$  C1/C2 mixture showed the highest level of cGMP synthesis. No GC activity was detectable in the C1 or C2 domains of PfGC $\alpha$  either separately or in combination. It is not clear why activity was undetectable with PfGC $\alpha$ , but it is possible that in the case of the C1 domain, the asparagine-rich insert that interrupts the domain may interfere with catalysis in the context of this construct. When the respective C1 and C2 domains of the PfGC $\alpha$  and PfGC $\beta$  were mixed with their heterologous partner ( $\alpha$ C1/ $\beta$ C2 and  $\beta$ C1/ $\alpha$ C2), there was evidence of some inhibition of activity of the PfGC $\beta$  domains (data not shown). A similar phenomenon of inhibition between two ACs from *Leishmania donovani* has been reported (41). In this case activity could also be detected in only one of the cyclases.

In the present study, we have demonstrated that although the C1 and C2 domains of PfGC $\beta$  synergize to synthesize cGMP, the individual domains also display activity (Fig. 7). Basal levels of AC activity have been reported in both the C1 and C2 catalytic domains of mammalian AC in the absence of specific activators (35). By analogy, the GC activity that we have demonstrated in this study probably represents only basal activity, because no activator has yet been identified. It is also possible that other parts of the PfGC $\alpha$  and PfGC $\beta$  proteins, not included in the expressed fusion proteins, may have a regulatory role and could be required for optimal activity.

**The Association of Ca<sup>2+</sup> and Guanylyl Cyclase Activity**—Of the P-type ATPases, it is the Ca<sup>2+</sup> pump isoforms that have the highest similarity with the amino-terminal domains of PfGC $\alpha$  and PfGC $\beta$ . This suggests the possibility that Ca<sup>2+</sup> may be involved in the regulation of GC activity in *P. falciparum*. Ca<sup>2+</sup>-dependent GC activity has been demonstrated in a number of systems. For example, in *Paramecium* there is a causal association between Ca<sup>2+</sup> influx and an increase in intracellular cGMP levels (reviewed in Ref. 42). This Ca<sup>2+</sup>-dependent cGMP production is vital to the control of ciliary movement and locomotion. In mammals, retinal GC activity, involved in light activation of photoreceptors, is closely associated with Ca<sup>2+</sup> levels, and it has been shown that specific calcium-binding proteins regulate this activity (Ref. 43; reviewed in Ref. 6). The intimate association of GC activity with a putative Ca<sup>2+</sup> pump in PfGC $\alpha$  and PfGC $\beta$  is intriguing in light of some earlier work on *Plasmodium* (2). This has implicated both Ca<sup>2+</sup> and cGMP in the control of exflagellation. Ca<sup>2+</sup> antagonists (but not Ca<sup>2+</sup> channel inhibitors) strongly inhibit exflagellation, whereas it is enhanced by cGMP and agents that increase levels of cGMP. It will therefore be of considerable interest to investigate whether the linkage of GC activity with a putative Ca<sup>2+</sup> pump ATPase in a single polypeptide is of functional significance.

**Evolutionary Significance**—It can be presumed that the “progenitor PfGC” protein arose from the fusion of two genes, one encoding a P-type ATPase and the second encoding a cyclase. This is not without precedence. There are at least two

other examples in *Plasmodium* of bifunctional proteins that occur as distinct molecules in other organisms. These are the dihydrofolate reductase-thymidylate synthase (44, 45) and dihydropteroate synthetase/pyrophosphokinase (46, 47). The most likely scenario is that the initial fusion event was followed by gene duplication creating two copies that diverged to become PfGC $\alpha$  and PfGC $\beta$ . The fusion event appears to have occurred in a common ancestor of *Plasmodium* and *Paramecium*. This is consistent with classification of the ciliates with apicomplexans and dinoflagellates in the Alveolata (9, 48).

An additional feature of the two *Plasmodium* genes is the presence of 12 introns in the ATPase encoding domain of PfGC $\beta$ , compared with none in the corresponding region of PfGC $\alpha$  (Fig. 1). From a functional viewpoint, it is not immediately obvious why PfGC $\alpha$  and PfGC $\beta$ , two proteins with the same potential activity, should be expressed during the sexual stages of the life cycle. One possibility is that they are expressed differentially during this complex life cycle phase. Another is that they are co-expressed but that they respond to different activators. A greater understanding of this signaling pathway and the manner in which it is regulated is of importance, particularly in the context of novel chemotherapeutic targets. The structural differences that we have identified between *Plasmodium* GC and its mammalian counterparts may be exploitable for the development of drugs capable of blocking transmission of malaria. The recent development of genetic manipulation techniques applicable to malaria parasites will allow this possibility to be explored further.

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## **Guanylyl Cyclase Activity Associated with Putative Bifunctional Integral Membrane Proteins in *Plasmodium falciparum***

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