

The role of two novel regulatory sites in the activation of the cGMP-dependent protein kinase from *Plasmodium falciparum*

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The *Plasmodium falciparum* cGMP-dependent protein kinase (PfPKG) uniquely contains three cGMP binding sites, but also has a 'degenerate' fourth site. The role of each cGMP-binding site in PfPKG activation remains unknown. We have analysed the effect of mutation of each cGMP-binding site (individually and in combination) on PfPKG activation *in vitro*. The most striking result was that mutation of cGMP site 3 resulted in a 10–49-fold increase in the $K_{a(cGMP)}$ value and a 45–55% decrease in maximal activity compared with wild-type. Mutations involving only cGMP-binding sites 1 and 2 had less effect on both the $K_{a(cGMP)}$ values and the maximal activities. These results suggest that, although all three cGMP-binding sites are involved in PfPKG

activation, cGMP-binding site 3 has the greatest influence on activation. A mutation in the fourth, degenerate cGMP-binding site decreased PfPKG maximal activity by 40%, but did not change the $K_{a(cGMP)}$ value for the PfPKG mutant, suggesting that this site does not bind cGMP, but is required for full activation of PfPKG. The distinct activation properties of PfPKG from mammalian isoforms may be exploitable in the design of a parasite-specific inhibitor and development of a novel anti-malarial drug.

Key words: cyclic nucleotide, cGMP-dependent protein kinase, *Plasmodium falciparum*, protein kinase, signal transduction.

INTRODUCTION

cGMP-dependent protein kinase (PKG), a key regulator in the cGMP signalling pathway, mediates a wide range of physiological effects in mammalian cells [1]. To date, two types of mammalian PKG have been reported, type I and type II. Both forms exist as homodimers: each polypeptide comprising a regulatory domain and a catalytic domain. The regulatory domain contains a dimerization domain, an auto-inhibitory domain, auto-phosphorylation sites and two cyclic-nucleotide-binding sites. Recently, PKGs within the apicomplexan group of protozoa have been identified in *Eimeria tenella* (EtPKG), *Toxoplasma gondii* (TgPKG) and the malaria parasite *Plasmodium falciparum* (PfPKG) [2–4]. It has been reported that EtPKG and TgPKG exist as monomers that also occur as two isoforms with alternative translational initiation sites [2,3]. These apicomplexan PKGs uniquely contain three cGMP-binding sites; however, PfPKG contains an additional, degenerate cGMP-binding site (referred to subsequently as the degenerate fourth site).

Despite sharing identical amino-acid sequences within the cGMP-binding sites, mammalian PKG I α and PKG I β exhibit different kinetic properties and cyclic-nucleotide-analogue specificities [5]. The mammalian cGMP-binding sites are distinct from each other in terms of their relative affinity for cGMP, and are therefore designated the cGMP fast- and slow-dissociating sites [6]. It has been shown that substitution of a conserved serine or threonine residue (with an alanine residue) within the cGMP-binding sites leads to distinct effects on PKG activation [7,8]. In our previous work, we identified a *P. falciparum* gene encoding a functional PKG that is predominantly expressed in the ring stage of the parasite life cycle [4]. Apart from the predicted structural differences from the mammalian isoforms, a truncated form

of PfPKG also possessed distinct cyclic-nucleotide-dependent kinase-activation properties. For example, the cGMP analogue, 8-*p*CPT-cGMP, a potent activator of mammalian PKG, inhibits PfPKG activity significantly [4].

In the present study, we have begun to dissect the cyclic-nucleotide-dependent activation mechanism of PfPKG by expression and mutational analysis of a full-length form of the enzyme in *Escherichia coli*. We present an analysis of the contribution of the individual cGMP-binding sites to the activation of PfPKG by mutating conserved residues that are critical to high-affinity cGMP binding. Our results demonstrate that the C-terminal cGMP-binding site (site 3) has the greatest effect on PfPKG activation. We hypothesize that the unique third and fourth cGMP-binding sites resulted from a gene-duplication event, and our results show that the degenerate fourth site is important for maximal activation of the enzyme, but is unlikely to bind to cGMP.

EXPERIMENTAL

Reagents

The PKG substrates Glasstide and histone H1 were obtained from Calbiochem. [γ -³²P]ATP was obtained from Amersham Biosciences. P-81 phosphocellulose paper was obtained from Whatman. The following cGMP analogues were obtained from BIOLOG Life Science Institute (Bremen, Germany): Rp isomer of 8-bromoguanosine 3',5'-cyclic monophosphorothioate (Rp-8-Br cGMPS), 8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate (8-*p*CPT-cGMP); 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP); Sp isomer of 8-(2-aminophenylthio)guanosine-3',5'-cyclic monophosphate (8-APT-cGMP);

Abbreviations used: 8-APT-cGMP, Sp isomer of 8-(2-aminophenylthio)guanosine-3',5'-cyclic monophosphate; 8-Br-cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate; EtPKG, *Eimeria tenella* PKG; 8-*p*CPT-cGMP, 8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PfPKG, *Plasmodium falciparum* PKG; Rp-8-Br-cGMPS, Rp isomer of 8-bromoguanosine 3',5'-cyclic monophosphorothioate; 8-Br-PET-cGMP, β -phenyl-1,*N*²-ethano-8-bromoguanosine-3',5'-cyclic monophosphorothioate; TgPKG, *Toxoplasma gondii* PKG.

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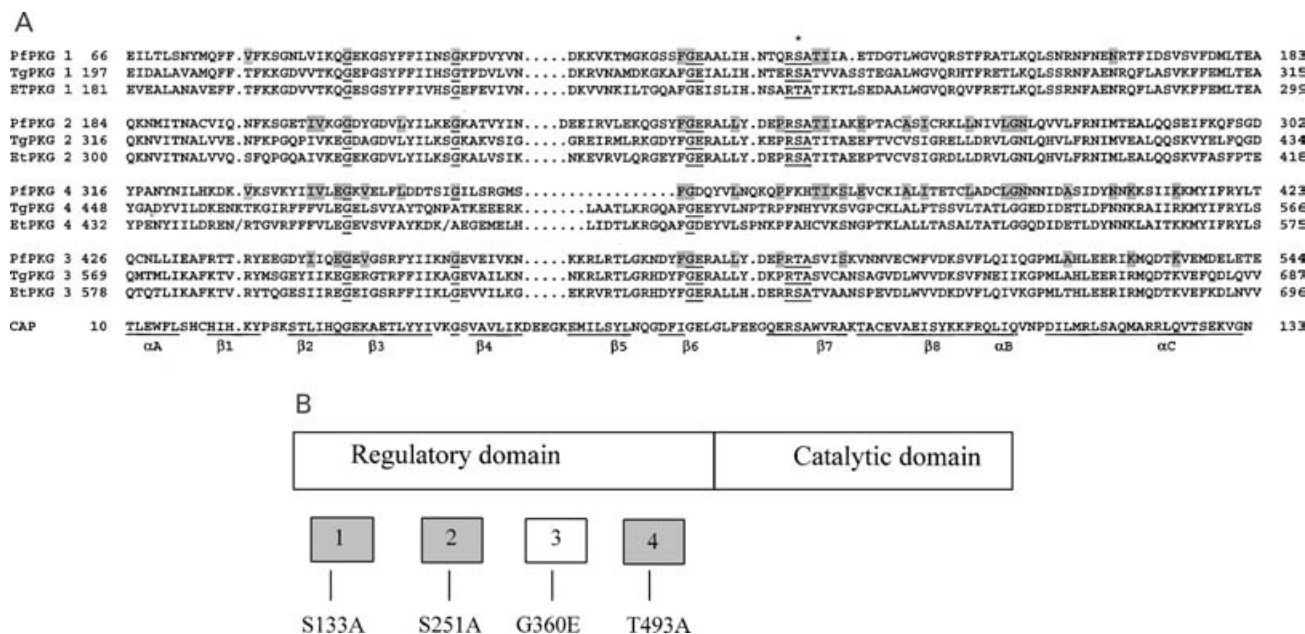


Figure 1 cGMP-binding domains of PfPKG and of other apicomplexan isoforms

(A) Alignment of cGMP-binding domains of PKGs from *P. falciparum*, *T. gondii* and *E. tenella*. The sequence of the cAMP-binding site of CAP (bacterial catabolite gene-activator protein), for which a crystal structure is available [12] is also included in the alignment. The secondary structure of CAP is shown below the sequence. The position of the conserved serine/threonine residues involved in high-affinity binding to cGMP are marked with an asterisk (*). This position was mutated in the three cGMP-binding sites of PfPKG. Other highly conserved residues found in cyclic nucleotide-binding domains are underlined. Residues highlighted in grey show positions that are conserved between the degenerate fourth site and at least one of the three functional cGMP-binding sites. (B) The relative positions of the mutated sites in PfPKG. Three cGMP-binding domains are represented by grey squares. The positions of the serine/threonine residues that were mutated to alanine residues are also shown. The degenerate fourth site is represented as a white square. In this site, a highly conserved glycine residue was mutated to a glutamic acid residue.

β -phenyl-1, N^2 -ethano-8-bromoguanosine-3',5'-cyclic monophosphorothioate (8-Br-PET-cGMP). cGMP, cAMP and 3-isobutyl-methylxanthine, sodium-ATP and all other general chemicals were from Sigma.

Site-directed mutagenesis of PKG

The following primers were designed to mutate conserved residues within the cGMP-binding sites of PfPKG (Figure 1; see below): P1, 5'-gctgcttaattcataataccaagagctgcaactattattcagaa-actgatgg-3'; P2, 5'-gctctactgatgatgaaccaagagctgcaacaatcattgcaaaagaaccaacc-3'; P3, 5'-tatgatgaaccaagagcagctctgttataag-3'; P4, 5'-ggaatgtcttttgaagatcaatattgtaataat-3'. The mutated sites are underlined. Primers P1, P2 and P3 were used to mutate cGMP-binding sites 1, 2 and 3 respectively. P4 was used to mutate the degenerate fourth site (Figure 1; see below). Site-directed mutagenesis was carried out using a QuikchangeTM Multi Site-Directed Mutagenesis kit (Stratagene), according to the manufacturer's instructions. Briefly, the mutation-containing primer was 5'-phosphorylated with T4 DNA kinase for 1 h at 37 °C. PCR was performed in 50 μ l reaction volumes containing 100 ng of recombinant plasmid harbouring the wild-type PfPKG gene, 100 ng of 5'-phosphorylated primer and 2.5 units of QuikchangeTM enzyme blend. The reaction consisted of 30 thermal cycles of 95 °C for 1 min (denaturation), 60 °C for 1 min (annealing) and 65 °C for 13 min (extension). Following PCR, 10 units of *DpnI* restriction enzyme were added to the reaction mixture to digest parental double-stranded DNA. PCR products were precipitated by adding a 1/10 vol. of 3 M sodium acetate and 3 vol. of 100% ethanol to the reaction mixture. After centrifugation, the pellet was dissolved in 20 μ l of sterile deionized distilled water. Purified

PCR product (4 μ l) was then used to transform *E. coli* XL10 Gold competent cells. PfPKG mutants were screened by sequencing using a ABI PRISM[®] BigDye[®] Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI PRISM[®] 377 automated sequencer (Applied Biosystems).

Expression, purification and detection of PKG activity in wild-type and mutant recombinant PfPKG

E. coli BL21 codon plus (Stratagene) was transformed with the pTrcHis expression vector (Invitrogen) containing the wild-type or mutated *PfPKG* gene. Cultures were grown in LB (Luria-Bertani) medium containing 100 μ g \cdot ml⁻¹ ampicillin at 37 °C with shaking at 220 rev./min. When cultures reached the mid-logarithmic phase of growth (D_{600} value of 0.5 where D is attenuation), expression of PfPKG was induced overnight at 18 °C by adding IPTG (isopropyl β -D-thiogalactoside) to a final concentration of 1 mM. The cells were harvested by centrifugation at 400 g for 5 min at 4 °C and were suspended in lysis buffer [50 mM NaH₂PO₄ (pH 8.0), 250 mM NaCl, 7.5 mM imidazole, 2 mM EDTA, 2 mM EGTA and 1% (w/v) Triton X-100], followed by sonication on ice. The lysate was centrifuged at 15000 g for 20 min at 4 °C. The supernatant containing the His-tagged PfPKG was affinity-purified on Ni²⁺-nitrilotriacetic acid agarose under native conditions as recommended by the manufacturer (Qiagen). Eluted proteins were analysed by SDS/PAGE and Western blotting. To detect the phosphotransferase activity of purified PfPKG recombinant proteins, 2 μ l of affinity-purified protein was added to 18 μ l of pre-warmed reaction mixture using a modified version (see below) of the method described previously [4]. After incubation at 30 °C for 30 min, reactions

were terminated by the addition of an equal volume of SDS/PAGE sample buffer. The samples were boiled and then separated on 10% (w/v) polyacrylamide gels, followed by autoradiography or phosphorimaging (Molecular Dynamics). The amount of PfPKG fusion protein was determined by the Bradford test [8a] and densitometry.

Measurement of PfPKG mutant activity relative to that of the wild-type

The activity of PfPKG mutants was determined by measuring the incorporation of a radiolabelled phosphate group from [γ - 32 P]ATP into a peptide substrate (Glasstide). The reaction mixture contained 50 mM Tris/HCl (pH 7.0), 5 mM magnesium acetate, 50 mM 3-isobutyl-1-methylxanthine, 0.2 mg \cdot ml $^{-1}$ BSA, 5 μ M unlabelled sodium-ATP, 8.25 nM [γ - 32 P]ATP, 200 μ M Glasstide and 1 nM–1 mM cGMP. The reaction was initiated by adding 2 μ l of affinity-purified recombinant enzyme. For measurements of PfPKG activation by cyclic nucleotide analogues, cGMP was replaced with a cGMP analogue in the reaction mixture. To examine the effect of a cyclic nucleotide competitive inhibitor on PfPKG mutant activity, 10 μ M–10 mM concentrations of Rp-8-Br-cGMPS were added to the reaction mixture in which the concentration of cGMP was fixed at 10 μ M. The reactions were incubated at 30 °C for 30 min and quenched by spotting onto P81 phosphocellulose paper. After three washes in 1.25% (v/v) phosphoric acid and one acetone rinse, the papers were dried, placed in plastic vials and counted in a scintillation counter (Beckman). All experiments were performed at least twice in triplicate. The specific activity of wild-type and mutant PfPKGs was measured in moles of phosphate group transferred from ATP to the peptide substrate divided by the reaction time and the mass of PfPKG. The effect of Rp-8-Br-cGMPS on PfPKG activity was expressed as an IC $_{50}$ value. Maximal activation (V_{max}) and cGMP concentration causing 50% activation (K_a) of PfPKG were calculated by fitting to a rectangular hyperbola. Co-operativity in cGMP activation was tested using Hill plot analysis.

RESULTS

Site-directed mutagenesis of PfPKG

The cGMP-binding sites of PKGs and cGMP-gated ion channels are structurally related and contain several conserved amino acids, including a serine or threonine residue that is critical for high affinity cGMP binding [9–11]. By comparing the amino-acid sequence of PfPKG with those of PKGs from other organisms, we found that this key residue is conserved in each of the three predicted cGMP binding sites. A serine residue is present in cGMP-binding sites 1 and 2, and a threonine residue in site 3. Figure 1(A) shows an alignment of the predicted cGMP-binding sites of the apicomplexan PKGs identified so far and highlights this conserved residue. The sequence of CAP (bacterial catabolite gene-activator protein), for which a crystal structure is known [12], is also included in the alignment to give an indication of the secondary structure of a cyclic nucleotide binding site. In this study, these three key serine/threonine residues of PfPKG were replaced with an alanine residue by site-directed mutagenesis. Recombinant proteins were generated containing a mutation of one of these residues (single mutants), two of these residues in all three combinations (double mutants) and all three residues (triple mutant). The single mutants were named as follows: McG1 [S133A (Ser 133 \rightarrow Ala) in cGMP-binding site 1]; McG2 (S251A

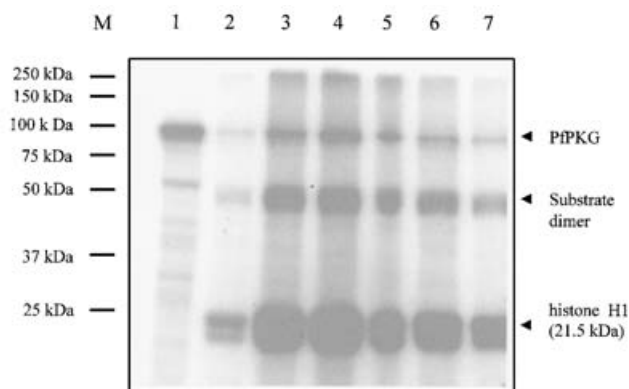


Figure 2 Autoradiogram showing detection of phosphotransferase activity in wild-type and mutant PfPKG recombinant proteins

Expression of recombinant PfPKG mutants was induced with 1 mM IPTG (isopropyl β -D-thiogalactoside) at 18 °C in *E. coli* BL21 codon plus. His-tagged proteins were affinity-purified with Ni $^{2+}$ -nitrilotriacetic acid agarose (Qiagen). Phosphotransferase activity in 2 μ l of affinity-purified recombinant protein was detected by adding the protein kinase substrate histone H1, cGMP and [γ - 32 P]ATP, as described in the Experimental section. The reaction mixtures were analysed by SDS/PAGE and autoradiography. All PfPKG recombinant proteins demonstrated cGMP-dependent activity in terms of autophosphorylation and substrate phosphorylation. Lane M, protein markers; lane 1, wild-type (no Histone H1 substrate); lane 2, wild-type (–cGMP); lane 3, wild-type (+cGMP); lane 4, McG1 (+cGMP); lane 5, McG2 (+cGMP); lane 6, McG3 (+cGMP); lane 7, McG4 (+cGMP).

in site 2) and McG3 (T493A in site 3). In addition, PfPKG contains a degenerate fourth cGMP-binding site which lacks the conserved serine or threonine residues, but contains three conserved glycine residues that occur in all known cGMP-binding sites (Figure 1A). Therefore one of these conserved glycine residues in the degenerate fourth site was replaced by glutamic acid, as previously described [8]. This mutant was named McG4 (G360E; Figure 1B).

Phosphotransferase activity of wild-type and mutant PfPKG recombinant protein

To assess the effect of the specific point mutations on the phosphotransferase activity of PfPKG, affinity-purified recombinant protein derived from the four single mutants (McG1–4) was measured using histone H1 as one of the reaction substrates (the receptor of the phosphate group). The results indicated that all single mutants have phosphotransferase activity that can be stimulated by cGMP (Figure 2). Furthermore, this method demonstrated that the mutant enzymes were still able to direct both autophosphorylation and phosphorylation of the histone H1 substrate. The double and triple mutants showed similar results (see below), suggesting that point mutations in the cGMP-binding sites do not destroy the conformation required for PfPKG activity. The mutant recombinant proteins were then used to quantify PfPKG activation by cGMP analogue activators/inhibitors at different concentrations.

Activation of PfPKG mutants by cyclic nucleotide analogues

Our previous work demonstrated that cAMP, 8-*p*CPT-cGMP and other cGMP analogues showed little or no stimulation of PfPKG activity in an N-terminally truncated form of the enzyme [4]. In the

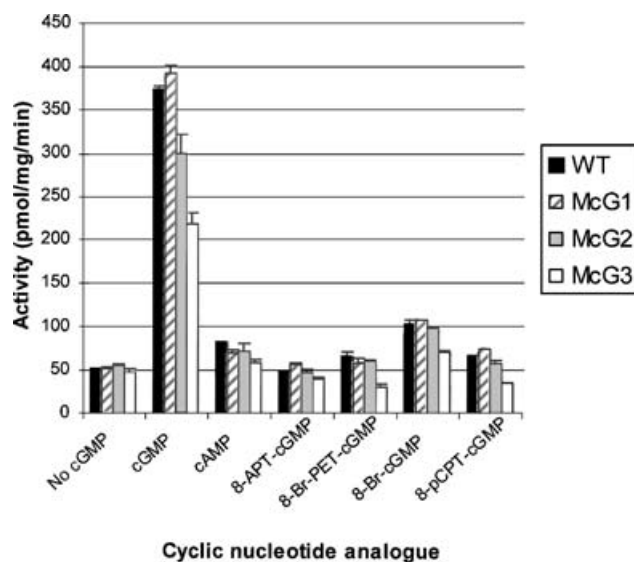


Figure 3 Effects of cyclic nucleotides and cyclic nucleotide analogues on the activation of wild-type and mutant PIPKG

The activities were measured in the presence of 100 μM cyclic nucleotide or analogue as described in the Experimental section. The activity is expressed in moles of the transferred phosphate group/min per mg of recombinant protein. Results are means \pm S.E.M. ($n=3$).

present study, we tested whether or not specific mutations in the cGMP-binding sites of the full-length (wild-type) protein affected the cyclic-nucleotide-dependent activation profile. The activity of PIPKG wild-type and mutants was measured using cGMP, cGMP analogues and cAMP. Figure 3 shows that the single mutants McG1, McG2 and McG3 had the same cyclic nucleotide activation trend as the wild-type PIPKG; namely, cGMP shows the strongest stimulation of activity, 8-Br-cGMP showed slight stimulation, but 8-APT-cGMP, 8-Br-PET-cGMP, 8-pCPT-cGMP and cAMP showed no significant stimulation of PIPKG activity. Double and triple mutants showed the same trend as single mutants (see below). This finding contrasts markedly with results obtained from similar experiments with mammalian PKG isoforms where these analogues all exhibit significant stimulation of activity.

Contribution of the individual cGMP-binding sites to PIPKG activation

The two intramolecular cGMP-binding sites of mammalian PKG have distinct roles in kinase activation. To evaluate the role of the individual cGMP-binding sites in PIPKG activation, enzyme activity was measured in single mutants at different concentrations of cGMP. Compared with wild-type PIPKG, substitution of a conserved serine with an alanine residue in cGMP-binding site 1 (McG1, S133A) only slightly increased the $K_{a(\text{cGMP})}$ value of the mutant McG1, by 1.1-fold. The maximal activity of McG1 was similar to that of the wild-type (Figure 4A; Table 1). Substitution of the conserved serine with an alanine residue in site 2 (McG2, S251A) increased the $K_{a(\text{cGMP})}$ value of McG2 4.1-fold. The maximal activity of McG2 was decreased by 12%. However, substitution of a conserved threonine with an alanine residue in site 3 (McG3, T493A) increased the $K_{a(\text{cGMP})}$ value of the McG3 10.3-fold and the maximal activity of McG3 was decreased by 42%. These data suggest that all three cGMP-binding sites are

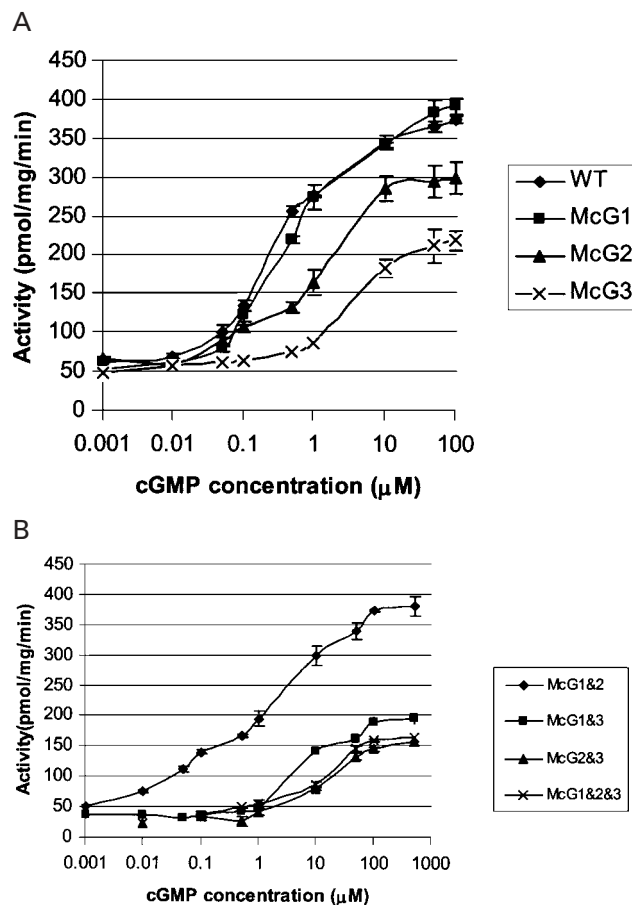


Figure 4 Effects of disruption of the individual cGMP-binding sites on PIPKG activation

Phosphotransferase assays were carried out at different concentrations of cGMP. Reaction mixtures containing the peptide substrate Glasstide, cGMP, unlabelled sodium ATP, [γ - ^{32}P]ATP and recombinant protein were incubated for 30 min at 30 $^{\circ}\text{C}$ (see the Experimental section). (A) Activity measured in wild-type PIPKG and the single mutants McG1, McG2 and McG3. (B) Activity measured in the double mutants McG1/2, McG1/3 and McG2/3, and the triple mutant McG1/2/3. Results are means \pm S.D. for two experiments (each performed in triplicate). The apparent K_a and V_{max} values are shown in Table 1.

Table 1 Summary of the apparent kinetic constants (K_a) for the various cGMP-binding site mutants of PIPKG

The $K_{a(\text{cGMP})}$ and V_{max} values represent the mean of two experiments performed in triplicate \pm S.E.M. The data were analysed using the Kinenort program.

PIPKG mutant	$K_{a(\text{cGMP})}$ (μM)	V_{max} (pmol/mg per min)
Wild-type	0.301 \pm 0.043	303.81 \pm 7.21
McG1	0.327 \pm 0.042	303 \pm 7.02
McG2	1.22 \pm 0.11	245.22 \pm 3.98
McG3	3.086 \pm 0.245	175.25 \pm 2.57
McG1/2	1.09 \pm 0.27	309.04 \pm 12.94
McG1/3	4.66 \pm 1.93	158.42 \pm 8.11
McG2/3	14.71 \pm 2.079	138.06 \pm 4.35
McG1/2/3	13.56 \pm 3.63	145.43 \pm 9.75
McG4	0.217 \pm 0.057	124.98 \pm 5.46

involved in modulating the activation of PIPKG, but disruption of cGMP-binding site 3 has the greatest influence on both the $K_{a(\text{cGMP})}$ value and the maximal activity of PIPKG.

Table 2 Effects of the PKG inhibitor Rp-8-Br-cGMPS on the activation of PfPKG mutants

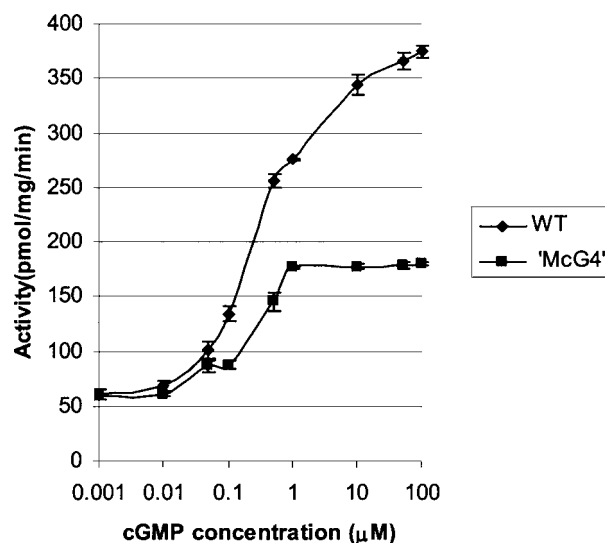
The IC₅₀ values were derived from the mean of two experiments performed in triplicate \pm S.E.M.

PfPKG mutant	IC ₅₀ (mM)
Wild-type	0.325 \pm 0.075
McG1	0.34 \pm 0.097
McG2	0.19 \pm 0.041
McG3	0.093 \pm 0.008
McG1/2	0.123 \pm 0.033
McG1/3	0.105 \pm 0.008
McG2/3	0.098 \pm 0.032
McG1/2/3	0.055 \pm 0.012

To investigate further the relative importance of the three binding sites, multiple cGMP-binding sites of PfPKG were mutated simultaneously. Three double mutants representing each possible combination (of sites 1–3), and one triple mutant were produced. Disruption of sites 1 and 2 simultaneously (McG1/2; S133A/S251A) increased the $K_{a(cGMP)}$ by 3.6-fold, but the maximal activity remained the same (Figure 4B; Table 1). The double mutants McG1/3 (S133A/T493A) and McG2/3 (S251A/T493A), and the triple mutant McG1/2/3 (S133A/S251A/T493A) all displayed dramatically increased $K_{a(cGMP)}$ values: 15.5-fold, 49-fold and 45-fold respectively. The maximal activity in each case was found to have decreased by approx. 45–55% (Figure 4B; Table 1). It is clear that recombinant proteins with a mutation in cGMP-binding site 3 exhibit a significant increase in the $K_{a(cGMP)}$ and a marked decrease in maximal activity. These data demonstrate that cGMP-binding site 3 has the major role in the activation of PfPKG by cGMP, although the other cGMP-binding sites are implicated in a synergistic role in this process. All fusion proteins derived from mutants co-migrated with the full-length (wild-type) PfPKG in non-denaturing polyacrylamide gels, confirming that mutations had not resulted in any detectable gross changes in folding (results not shown). Differences in measured activity can therefore be attributed to the mutated amino-acid residues.

Inhibition of the activity of PfPKG mutants by the competitive cGMP inhibitor Rp-8-Br-cGMPS

To characterize the cyclic nucleotide activation/inhibition profile of PfPKG further, the mutants described above were tested for phosphotransferase activity in the presence of increasing concentrations of the specific PKG inhibitor Rp-8-Br-cGMPS. Mutation of the cGMP-binding sites (especially cGMP-binding sites 2 and 3) of PfPKG altered the inhibition profile with a cGMP competitive inhibitor. Table 2 shows that the IC₅₀ value of Rp-8-Br-cGMPS for the single mutant McG1 is very similar to that for wild-type (0.34 mM). However, the IC₅₀ value of Rp-8-Br-cGMPS for the single mutants McG2 and McG3 decreased to 0.19 and 0.093 mM, respectively. The double and triple mutants also displayed diminished IC₅₀ values with respect to Rp-8-Br-cGMPS. The IC₅₀ of Rp-8-Br-cGMPS for the triple mutant was even less, at 0.055 mM (Table 2). These data suggest that mutation of cGMP-binding sites 2 and 3 can affect the competitive binding between cGMP and Rp-8-Br-cGMPS in the cGMP-binding site. However, mutation of cGMP-binding site 3 had the greatest effect, suggesting further that this site has the most important role in cGMP-binding and activation of PfPKG.

**Figure 5** Effect of disruption of the degenerate fourth cGMP-binding site on activation of PfPKG

The McG4 mutant was produced by replacing a conserved glycine residue in the degenerate fourth cGMP-binding site with a glutamic acid residue (see Figure 1). Phosphotransferase assays containing wild-type and McG4 recombinant proteins were carried out in parallel using different concentrations of cGMP (see the Experimental section). Results are means \pm S.D. of two experiments (each performed in triplicate). The apparent K_a and V_{max} values for the McG4 mutant are shown in Table 1.

The effect of mutation of the degenerate fourth cGMP-binding site on PfPKG activity

An alignment of the cGMP-binding sites of PfPKG with those of TgPKG and EtPKG reveals a high level of conservation between the apicomplexan enzymes. There is also some degree of conservation in the region containing the degenerate fourth site (Figure 1A). Since the degenerate cGMP-binding site does not contain certain critical residues required for high-affinity cGMP-binding (see the legend to Figure 1), theoretically this site should be unable to bind cGMP. To explore the role of this degenerate site in PfPKG catalytic activity, we mutated a glycine residue that is conserved in all known cGMP-binding sites. The conserved glycine residues within cGMP-binding sites are thought to be important in maintenance of their β -barrel structure [13]. Substitution of the conserved glycine with a glutamic acid residue in the degenerate fourth site resulted in an approx. 55% loss of maximal activity of PfPKG, but the $K_{a(cGMP)}$ for PfPKG phosphorylation was not distinct from that of the wild-type (Figure 5; Table 1). These results suggest that this degenerate site does not bind to cGMP, but is required for full cGMP-dependent activation of PfPKG.

Investigation of co-operativity between cGMP-binding sites

A Hill plot of WT PfPKG activation (results not shown) was non-linear, with a gradient > 1 at cGMP concentrations up to 0.5 μ M, and a gradient < 1 at higher concentrations. This indicates that there is positive co-operativity at lower cGMP concentrations and perhaps a repression of activity additionally occurring at the higher concentrations. Closer examination of Figure 4(A) shows that the McG3 and McG2 mutants have lost the high-affinity positively co-operative phase. Furthermore, McG4 (Figure 5) retains the positively co-operative phase, but loses the low-affinity phase.

DISCUSSION

There have been a number of reports that a high-affinity interaction between the hydroxyl side chain of a specific serine or threonine residue and the C2 amino group of cGMP is necessary for PKG activation [7–9]. In the present study, the corresponding conserved serine or threonine residue in the predicted cGMP-binding sites of PfPKG was mutated and expressed in *E. coli*. Our results indicate that mutation of the cGMP-binding sites does not completely destroy the activation of PfPKG by cGMP. However, recombinant proteins with a disrupted cGMP-binding site 3 (single, double or triple mutants) display a dramatic increase in their $K_{a(cGMP)}$ values (10–49-fold). Disruption of sites 1 and 2 has less effect on the $K_{a(cGMP)}$, suggesting that the site proximal to the C-terminal region has the most important role in PfPKG activation. This conclusion is supported by kinase assays performed with PfPKG mutants using the PKG inhibitor Rp-8-Br-cGMPS. Our results are quite distinct from those obtained in similar experiments performed with mammalian PKG isoforms where disruption of the N-terminal binding site (site 1) has the greatest effect on PKG activation [7–9]. Furthermore, mutants containing the disrupted cGMP-binding site 3 decreased the maximal activity of PfPKG by 45–55% compared with wild-type enzyme. The studies with mammalian PKGs did not indicate whether or not the mutation in the cGMP-binding sites had an impact on the maximal activity [7].

Our results are consistent with those obtained with the PKG from the related apicomplexan parasite *Eimeria tenella* [14]. Substitution of a highly conserved arginine with an alanine residue in cGMP-binding sites 1, 2 and 3 in EtPKG and TgPKG increased the $K_{a(cGMP)}$ value 10-fold. Mutation of cGMP-binding site 3 decreased maximal activity by >70%. Mutation of sites 1 and 2 had less effect on the activity of EtPKG and TgPKG; cGMP-binding site 3 was regarded as the key determinant of the activity of EtPKG and TgPKG. However, in the present study, the role of the degenerate fourth cGMP-binding site was also examined by replacing a conserved glycine with a glutamic acid residue. Substitution of the glycine residue (with a glutamic acid residue) in the corresponding position of a mammalian PKG isoform was previously shown to affect activation [8]. As predicted from the amino-acid sequence of this site, our results suggest that this site cannot bind to cGMP. However, the results also show that this conserved glycine residue in the degenerate fourth site is essential for full activation of PfPKG. The role of this degenerate site, however, remains unclear. It is likely that a gene duplication event gave rise to four cGMP sites in an ancestor of PfPKG. This may have then been followed by mutations causing dysfunction of this site and resulting in retention of just three functional cGMP-binding sites. Recent results have shown that a truncated protein containing the degenerate fourth cGMP-binding site has a higher activity than a truncated protein lacking this degenerate fourth cGMP-binding site (W. Deng and D. Baker, unpublished work). Thus the degenerate fourth cGMP-binding site may have a role in stabilizing the conformation of PfPKG and thereby assisting activation of PfPKG by cGMP. It is also possible that this site has evolved an alternative regulatory role in the malaria parasite.

The findings of the present study are broadly in line with those reported for EtPKG [14]. However, the *Eimeria* enzyme (using a *Toxoplasma* expression system) is characterized by a low basal activity and a very high level (>1000-fold) of positively co-operative stimulation by cGMP. In contrast, PfPKG shows a much lower positively co-operative activation by cGMP that appears to be inhibited at higher cGMP concentrations. The explanation for the differences between the *Plasmodium* and *Eimeria* homologues is not known, but it is possible that the N-terminal sequences of

both enzymes could hold the key. The *Eimeria* enzyme has a much longer N-terminal domain than PfPKG (by just over 100 amino acids). In mammalian PKGs, the N-terminus has been shown to have an autoinhibitory role and binding of cGMP releases the enzyme from the influence of this autoinhibition. It is therefore possible that the interactions of the N-terminal domains with the respective catalytic domains of the two apicomplexan enzymes are very different and result in their distinct basal activity and activation properties. It is also possible that an additional cofactor (not present in *E. coli*) is required for full activation of the enzymes.

Expression of full-length mammalian PKGs in *E. coli* has resulted in inactive protein which is believed to be at least partly due to the absence of phosphorylation of a key threonine residue within the catalytic domain that is essential for activity [15]. PKA (cAMP-dependent protein kinase) also requires phosphorylation of the analogous threonine residue for activity, but, like PfPKG, is active when expressed in *E. coli* [16]. In both cases, induction performed at decreased temperature enhanced the amount of soluble protein produced. Alignment of PfPKG with mammalian PKGs and also PKA from mouse reveals a greater similarity with the PKA in this region (including a unique two-amino-acid deletion adjacent to the threonine residue in both PKA and PfPKG). This shared similarity in the primary amino-acid sequence might be the underlying reason for their functionality when expressed in *E. coli* (results not shown).

The human malaria parasite *P. falciparum* accounts for up to 2.7 million deaths worldwide [17]. Drug-resistant malaria parasites have spread to the vast majority of malaria-endemic countries, and disease is therefore very difficult to control [18]. It is widely accepted that protein kinases may be important drug targets in parasitic protozoa; it is likely that *Plasmodium*-specific protein kinase inhibitors can be developed by screening a library of compounds [19]. It has been reported that a derivative of pyridine ('compound 1') is able to inhibit both the growth of *Eimeria* spp. ($IC_{50} > 5 \mu M$) and EtPKG activity (IC_{50} of 1 nM). It is possible, therefore, that EtPKG is a new chicken coccidiosis drug target. This work has demonstrated the feasibility of production of a specific inhibitory compound against apicomplexan PKGs and the amino-acid sequence of PfPKG is very similar to the *Eimeria* homologue. Detailed information on the distinct properties of PfPKG in terms of its cGMP-activation mechanism may prove useful for the design of a new anti-malarial drug.

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