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CYCLIC GMP-DEPENDENT PROTEIN KINASES IN PROTOZOA

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1. ABSTRACT

PKG (cGMP-dependent protein kinase) is a major intracellular receptor for cGMP and mediates a range of downstream physiological functions. Although most studies have concentrated on mammalian isoforms, the advent of whole genome sequencing has lead to identification of orthologues from other diverse species. Many biochemical and regulatory properties and structural features of PKG have been conserved throughout evolution. However, in protozoa there can be variation in the number of cGMP-binding sites and a tendency toward degeneracy of these additional sites. Recent work on the protozoan PKGs is the major focus of this review. Important functional data are emerging for these and other non-mammalian isoforms. Intriguingly, a role for PKG in locomotion is becoming a common functional theme across diverse phyla.

2. INTRODUCTION

The cyclic nucleotides (cAMP and cGMP) are important chemical messengers that are present in all animal species studied to date. They mediate a myriad of cellular functions by binding to and regulating the activity of other molecules. Cyclic nucleotide-dependent protein kinases are an important subgroup of proteins known to be intracellular receptors for cAMP and cGMP. Other proteins such as cyclic nucleotide-gated ion channels, cyclic nucleotide phosphodiesterases, a Rap1 guanine-nucleotide-exchange factor (Epac) (1) and a range of other proteins containing so-called GAF domains (found in cGMP-phosphodiesterases, adenylyl cyclases, and FhlA (formate hydrogen lyase transcriptional activator) are also known to bind cAMP or cGMP. cGMP-dependent protein kinase (PKG) is characterised by the presence of both catalytic and regulatory domains (containing cGMP-binding sites) within a single polypeptide. This feature distinguishes them from their closest relative, cAMP-dependent protein kinase (PKA), in which the catalytic and regulatory subunits are encoded by distinct genes. Much of the work on PKG has been carried out on mammalian isoforms, but orthologues have also been studied in non-mammalian systems. The main focus of this review will be the recent work on protozoan PKGs which has identified isoforms with some key features that are distinct from their mammalian counterparts.

3. A SUBGROUP OF PROTOZOA POSSESS PKGs WITH MORE THAN TWO cGMP-BINDING SITES

Free-living ciliate protozoa such as *Paramecium* and *Tetrahymena* have been grouped taxonomically with the apicomplexan parasites in the Parvkingdom alveolata in a recent re-classification of protozoa (2). The apicomplexa are a large group of protozoan parasites that includes several dangerous human pathogens. An example is the malaria parasite *Plasmodium falciparum* which causes over 1 million deaths in African children each year. *Toxoplasma gondii* infection is serious in immuno-compromised individuals and also in pregnant women, where damage to the foetus can occur. The group also encompasses parasites of veterinary importance such as *Eimeria tenella*, and *Cryptosporidium parvum*.

Some of the enzymatic components of cyclic nucleotide signalling pathways in these organisms have unique structural and biochemical features shared only by other members of the alveolata (3-6). For example, although protozoan PKGs have features characteristic of this protein kinase family, they are all predicted to contain additional (though degenerate in some cases) cGMP-binding sites (Figure 1) (7). All other known PKG sequences reported to date contain two cGMP-binding

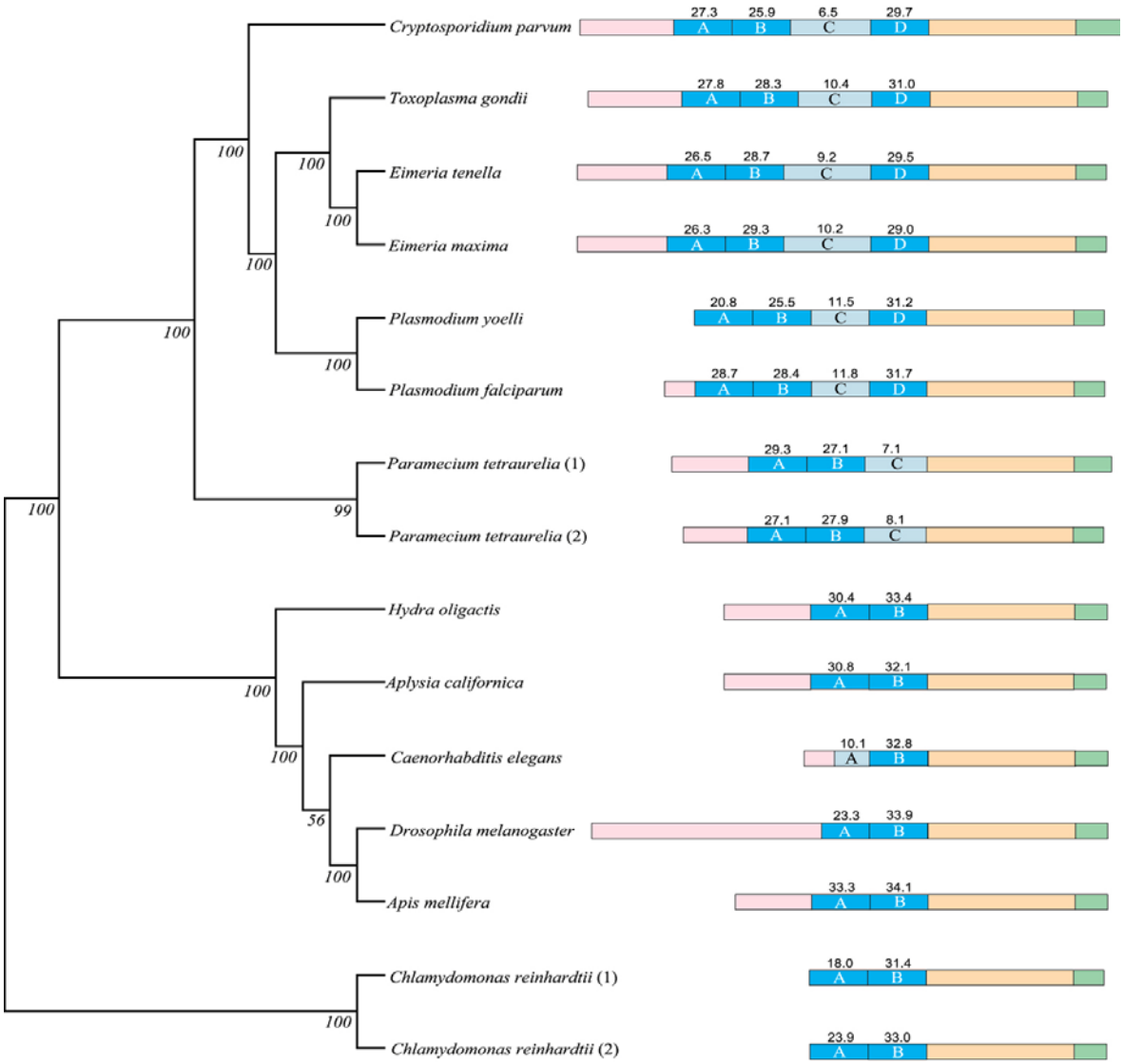

Figure 1. A rectangular cladogram of selected non-mammalian PKG isoforms that are also shown in cartoon form. The kinase catalytic domain is shown in orange, the C-terminal tail in green, the N-terminal segment in pink, the cGMP-binding sites in dark blue and the 'degenerate' sites in light blue. The Prosite profile (CNMP_Binding_3 cAMP/cGMP binding motif) scores are shown above each site. The cGMP-binding sites are labelled A-D. For phylogenetic analysis, sequences were aligned using CLUSTAL W. The alignment was weighted manually to limit the analysis to regions conserved between more than six species. Trees were constructed using the Fitch-Margoliash and Least-Squares Distance Methods program FITCH, after protein distances had been calculated using PROTDIST. Node robustness was assessed by 100 bootstrap replications (data set for bootstrap analysis was generated with SEQBOOT). PROTDIST, FITCH and SEQBOOT are part of the PHYLIP package (47). Maximum parsimony (PROTPARS) and neighbour joining (NEIGHBOR) methods were also used to generate phylogenies to confirm the results obtained by the above method. The tree was displayed using the TREEVIEW package (48). The PKG isoforms in this diagram have the following accession numbers: *Cryptosporidium parvum* AAM20902; *Toxoplasma gondii* AAM27174; *Eimeria tenella* AAM20900; *Eimeria maxima* AAM22643; *Plasmodium yoelii* EAA21741; *Plasmodium falciparum* NP_702235; *Paramecium tetraurelia* (1) CAD79354; *P. tetraurelia* (2) CAD79353; *Hydra oligactis* AAC23588; *Aplysia californica* AAR06171; *Caenorhabditis elegans* (egl-4) NP_741330; *Drosophila melanogaster* (for) P32023; *Apis mellifera* (amfor) AAL93136; *Chlamydomonas reinhardtii* (1; genie.244.5) and *C. reinhardtii* (2; genie.273.7) BAB18104.

sites. Searches of the Prosite database (http://us.expasy.org/tools/scanprosite/) with the protozoan sequences revealed that the apicomplexan PKGs each contain three high probability cyclic nucleotide-binding site profiles (CNMP_Binding_3 cAMP/cGMP binding motif; score >20 implying a close match with the consensus

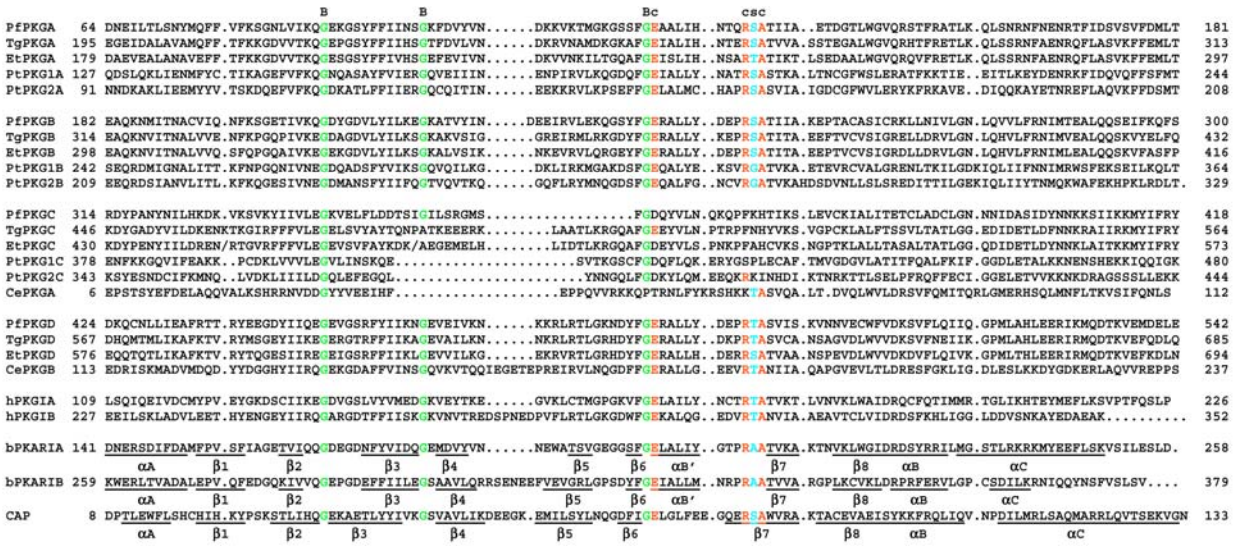

Figure 2. An alignment of the amino acid sequences of cGMP-binding domains in PKG isoforms. The sequences are positioned so that the 'degenerate' sites found in some non-mammalian enzymes are aligned with each other. The sequences of the two cGMP-binding sites of human PKGI-alpha (hPKGIA and hPKGIB) are included for comparison. The secondary structures (indicated below the sequences) of the corresponding regions of the cAMP binding sites of bovine PKA RI and bacterial CAP (for which structural data are available (49) and (50) respectively) are also included. Glycine residues important in maintaining the beta-barrel structure of the binding sites are coloured green and marked with a 'B' above the sequence; residues thought to bind cyclic nucleotide are coloured red and marked with a 'c' above the sequence and those residues involved in determining cyclic nucleotide specificity are coloured light blue and marked with an 's' above the sequence. cGMP-binding sites are named sequentially from the left as A-D. The positions of two short sequence insertions within the *E. tenella* isoform, removed to facilitate alignment, are marked with a '/'. The sequences used have the following accession numbers: *Plasmodium falciparum* NP_702235; *Toxoplasma gondii* AAM27174; *Eimeria tenella* AAM20900; *Paramecium tetraurelia* (PT1) CAD79354; *P. tetraurelia* (PT2) CAD79353; *Caenorhabditis elegans* (egl-4) NP_741330; *Homo sapiens* (cGKI-alpha Q13976; *Bos taurus* (PKAI-alpha regulatory subunit) P00514 and *E. coli* catabolite gene activator protein (CAP) P03020.

sequence). They also have an additional low probability cyclic nucleotide-binding site profile (score 6.5-11.8). The significance of this fourth degenerate site has been addressed by site directed mutagenesis of the *P. falciparum* enzyme (see section 4). BLAST searches also revealed the presence of two unusual putative PKG sequences in *Paramecium* (Figure 1). They appear to represent an intermediate form in that they have two high probability cyclic nucleotide binding sites (score 27.1- 29.3) and a third, apparently degenerate site (score 7.1 and 8.1). Examination of a PKG sequence from the nematode *Caenorhabdites elegans* (egl-4) revealed that one of the two predicted cGMP-binding sites is also somewhat degenerate (Figure 1) as it lacks several residues present in consensus cGMP-binding sites (Prosite cAMP/cGMP binding motif; score 10.1 and 32.8). Additional representatives from diverse non-mammalian phyla are included in Figure 1 and all have 2 high probability cGMP-binding sites.

Figure 1 also incorporates a phylogenetic analysis carried out using representatives of all the major non-mammalian phyla from which PKG sequences are available. Three major clades can be identified within the resulting evolutionary tree. The first contains the protozoan isoforms, the second comprises PKGs from diverse phyla including insects, nematodes, molluscs and cnidarians. The third clade contains two isoforms from the green alga *Chlamydomonas reinhardtii*. The close evolutionary relationship between the apicomplexans and ciliates is reflected by their clustering within a single clade. These are the only groups so far that possess additional cGMP-binding sites in their regulatory domain. All major nodes were strongly supported by bootstrap analysis showing the tree to be robust.

Figure 2 shows an amino acid sequence alignment of the cGMP-binding sites of the protozoan PKGs with those of human PKGI-alpha, the cAMP-binding sites of the bovine regulatory subunit PKAI-alpha and the bacterial catabolite gene activator protein (CAP). The *C. elegans* isoform (egl-4) has also been included in the alignment because the more amino-terminal segment appears to contain a degenerate cGMP-binding site. The secondary structure of the cAMP-binding sites in the regulatory subunit of PKA and CAP, and conserved residues important for structural integrity and cyclic nucleotide-binding are indicated. The degenerate protozoan cGMP binding sites show some conservation of important glycine residues that maintain the beta-barrel structure of the protein. It is possible that the 2 additional sites in apicomplexan isoforms arose by gene duplication followed by degeneration of one. If an alveolate ancestor had 4 sites,

then one of these sites must have been lost in the ciliate isoforms in addition to degeneration of the fourth site

4. FUNCTIONAL ANALYSIS OF INDIVIDUAL cGMP-BINDING SITES OF APICOMPLEXAN PKGs

The relative contributions that the two cGMP-binding sites of mammalian PKG isoforms make to enzyme activation, their dissociation rates (fast and slow), selectivity for cGMP analogues (8) and roles in heterophosphorylation and autophosphorylation (9) have been studied in detail.

The importance of the individual cGMP-binding sites in enzyme activation has also been addressed in some of the protozoan isoforms. It was demonstrated that the *Eimeria tenella* PKG isoform (EtPKG) has 3 functional cGMP-binding sites thought to give rise to unusual activation properties (10). These include an extremely low basal activity, unexpectedly high activation by cGMP (approximately 500-1000 fold, compared to 14-24 fold in a mammalian isoform tested in parallel), significant cooperativity (Hill coefficient, 1.7 compared to 1-1.2) and comparatively weak activation by 8-substituted cGMP analogues. However, analogues modified on the pyrimidine part of the purine showed good levels of activation. The K_{50} (the concentration of cGMP required for half maximal activation) for the enzyme is 2.3 μM. Site directed mutagenesis studies of EtPKG (targeting conserved arginine and glutamate residues), combined with recombinant expression of these mutants in *T. gondii*, showed that the third cGMP-binding site plays an important role in enzyme activation and is highly selective against the cGMP analogue 8-NBD-cGMP (8-[[2-[(7-nitro-4-benzofurazanyl)amino]ethyl]thio]guanosine-3',5'-cyclic monophosphate). Disruption of the third binding site greatly reduced the cooperativity to levels observed in mammalian isoforms (as did replacement of cGMP with 8-NBD-cGMP). This work suggests that the unusual properties of EtPKG are largely attributable to the presence of the additional third cGMP-binding site. It was hypothesised that the role of this additional site may be to provide the parasite with a more rigorous 'off switch' for the inactive enzyme. The functional significance of the third cGMP-binding site was confirmed using PKG assays on tagged recombinant proteins purified from transiently transfected *T. gondii*. Mutant forms of both *E. tenella* and *T. gondii* PKGs (mutated in one or multiple cGMP-binding sites) demonstrated the importance of all three cGMP binding sites for full activation of the enzyme and also the apparently greater influence of the third site (C) (11).

Mutational analysis has also been carried out on the *P. falciparum* PKG. This work utilised purified tagged recombinant proteins expressed in *E. coli* (12). The unusual property of weak activation by 8-substituted cGMP analogues was also noted in the malaria parasite enzyme compared to good activation by pyrimidine-modified analogues (Deng and Baker, unpublished). A key role for the third cGMP-binding site was also demonstrated by mutational analysis. However, the extremely low basal activity and resulting extraordinary stimulation by cGMP obtained with the *E. tenella* and *T. gondii* isoforms were not observed in the *Plasmodium*-derived recombinant proteins. The site-directed mutagenesis experiments in this work targeted a conserved serine/threonine residue involved in cGMP-binding; a strategy that has been used previously (9). Each of the three high probability cGMP-binding sites was disrupted individually and in combination. The results indicated that any mutation (single or multiple) involving the third site had most influence on the $K_{a(cGMP)}$ (cGMP concentration causing 50% activation) and maximal activity, consistent with this site having the primary role in enzyme activation. This study also targeted the degenerate (fourth) site (D) that lacks key cGMP-binding residues, to investigate its potential role in enzyme activation. A conserved glycine residue (corresponding to the position targeted in another study (13) was substituted by glutamate using site-directed mutagenesis (since the conserved serine/threonine used above is not present in the degenerate site). Curiously, a 55% reduction in maximal activity was observed in this mutant enzyme but the $K_{a(cGMP)}$ was not different from the wild type (WT) enzyme. These results suggest that the sequence containing the degenerate site (D) is required for full activation of PfPKG, but does not bind to cGMP. PKG activity measurements in truncated forms of PfPKG incorporating the degenerate site were higher than those lacking this site (Deng and Baker unpublished). This region of the molecule may therefore have a role in stabilizing the conformation of the enzyme and assisting in full activation by cGMP.

These results are broadly consistent with those obtained with other apicomplexan isoforms. However, the *P. falciparum* PKG had a much reduced level of positive cooperativity at low cGMP concentrations that appeared to be repressed at higher concentrations (12). The explanation for the differences in cooperativity between the parasite isoforms is not known. It is possible that significant differences between the N-terminal regions of the enzymes, potentially containing autoinhibitory sequences, may be responsible for differing degrees of repression of activity in the unactivated enzyme.

5. POTENTIAL AUTOINHIBITORY N-TERMINAL SEQUENCES

In mammals, both PKA and PKG are known to be regulated by autoinhibition (reviewed in (14)). This process involves interactions between the catalytic and regulatory subunits/domains. Binding of cyclic nucleotide, thought to cause profound changes in conformation, provides relief from this autoinhibition. It is known that PKAs and PKGs preferentially phosphorylate substrate proteins at sites containing a RRXS/TX consensus sequence. The autoinhibitory sequences that resemble this motif reside in the N-terminal segments of PKG, but autoinhibition is thought to involve additional low affinity contacts. The regulatory subunits of PKA, on the other hand, have more highly conserved, high affinity substrate-like autoinhibitory domains that interact with the catalytic subunit (14). The sequence within the autoinhibitory domain may be autophosphorylated, in which case, the P_0

position is occupied by a serine and is referred to as a 'substrate-like' sequence (14). If the P_0 position is occupied by an alanine or a glycine, it cannot be phosphorylated (See sequence alignment in the text below). In this case, the sequence within the autoinhibitory domain is referred to as a 'pseudosubstrate' sequence. Analysis of mammalian autoinhibitory domains has identified consensus amino acids known to be important in this process. The sequence alignment in the text below shows motifs present in the N-terminal regions of two of the apicomplexan PKG sequences (and one PKA regulatory subunit) that show similarities to the substrate-like and pseudosubstrate sequences of mammalian PKA and PKG isoforms respectively. Despite the low levels of similarity in the N-terminal domains of PKG isoforms between phyla, intriguingly, sequence alignments show that the relative position of this motif in the protozoan PKGs corresponds closely to that found in mammalian isoforms (not shown).

The N-terminal domain of the *E. tenella* PKG is significantly longer than that of the *Plasmodium* isoform (by around 100 residues). It is therefore possible that the interactions between these regions with their respective catalytic domains may differ significantly. This provides a possible explanation for the distinct differences in the basal activities and activation properties of these two enzymes.

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                          P0
BtPKArIIα  89 RFDRRVSVCAET
BtPKArIα   91 GRRRRGAISAEV
PfPKAr    143 IQKKRLSVSAEA
PfPKG      12 ERNKKKAIFSND
EtPKG     127 ERKVQKAIKQQE
BtPKGIβ    72 PRTKRQAISAEP
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In the alignment above, consensus substrate-like or pseudosubstrate motifs found in mammalian cyclic nucleotide-dependent protein kinases may also be functional in protozoan isoforms. Sequences in bold indicate similarity to the consensus substrate sequence (RRXS/TX). The amino acid in position P_0 is either a serine, glycine or alanine. This is followed by a conserved isoleucine or a valine and then often by a serine in those examined functionally. Basic residues are usually found in the P_{-2}, P_{-3} and P_{-5} positions (14). The accession numbers of the sequences in this alignment are: bovine regulatory subunit I-alpha (P00514); bovine PKA regulatory subunit II-alpha (P00515); *Plasmodium falciparum* PKA regulatory subunit (NP_701584); *P. falciparum* PKG (NP_702235); *Eimeria tenella* PKG (AAM20900); *Bos taurus* PKGI-beta (P21136).

6. AUTOPHOSPHORYLATION OF PKG ISOFORMS

In addition to phosphorylation of exogenous substrate proteins, PKGs undergo characteristic autophosphorylation reactions. This property is also maintained by each of the protozoan isoforms examined so far (7; 15). Autophosphorylation of mammalian PKG occurs at multiple sites (at least 6 in PKGI-alpha) in the N-terminal ~100 amino acids of the regulatory domain (16). It has also been reported that phosphorylation of a threonine residue in the catalytic domain of mammalian PKG is essential for activity (17). This may correspond to the phosphorylation event that occurs in the 'activation segment' within the centre of the catalytic domain of other protein kinases, including PKA (18). In some protein kinases, this site is autophosphorylated and in other cases it may be the target of phosphorylation by other kinases. With the *Plasmodium* PKG, most N-terminally truncated recombinant proteins show evidence of autophosphorylation, even $PfPKG_{357-853}$ which lacks the N-terminal 356 amino acids. However, $PfPKG_{416-853}$ which lacks the N-terminal 415 amino acids did not show autophosphorylation (Deng and Baker, unpublished data) suggesting that the most C-terminal autophosphorylation site of PfPKG occurs between residues 357 and 415. This result also implies that PfPKG is not autophosphorylated at the key threonine residue (Thr_{695}) within the catalytic domain.

Interestingly the truncated recombinant protein $PfPKG_{286-853}$ in which the two cGMP-binding sites (A and B) near the N-terminus had been deleted had comparatively high levels of activity and stimulation by cGMP; higher than the WT enzyme in both cases. Furthermore, this recombinant protein had higher levels of activation by cGMP analogues and cAMP than the full length enzyme ($PfPKG_{1-853}$). This isoform is activated approximately 15 fold by 10 μM cGMP compared to 5 fold by 10 μM cAMP, Deng and Baker (unpublished data). The reasons for this are not clear, but it is possible that this form lacks some of the autoinhibitory contacts present in the WT enzyme and can therefore be more fully activated. However, other truncated proteins ($PfPKG_{140-853}$ and $PKG_{357-853}$) had similar levels of activation to the full length PKG. It is possible that folding of $PfPKG_{286-853}$ is optimal in *E. coli* compared with the other truncated forms. Intriguingly, this form could correspond to an alternative isoform that occurs *in vivo,* with different activation properties and a distinct cellular role, perhaps expressed at another stage in the parasite life cycle.

7. BIOCHEMICAL PROPERTIES OF APICOMPLEXAN PKGs

A series of detailed studies by Paul Liberator, Robert Donald and colleagues at the Merck Research Laboratories (USA) have provided important information on the properties of the *Eimeria* and *Toxoplasma* PKGs. Biochemical and genetic analyses have revealed the presence of two distinct forms of the *E. tenella* PKG which are the products of a single gene (15). One of these is soluble, and N-terminal sequencing has shown that it lacks the first 48 amino acids of the full length membrane-associated protein. Evidence suggests that this may arise by a secondary translational initiation event, rather than by protease cleavage or differential splicing (11). Gel filtration has demonstrated the presence of a monomeric form of the enzyme with an apparent molecular weight between 113,000 and 125,000. Western blot analysis has

demonstrated the presence of distinct bands corresponding to the soluble and membrane-bound forms in Triton X-114-depleted and enriched phases respectively (15). Unlike most known PKGs, the EtPKG can function as a monomer. The leucine zipper motif required for homodimerisation found at the N-terminus of many PKGs is absent from the apicomplexan isoforms.

It is known that myristoylation mediates membrane localization of mammalian PKG type II. This facilitates interaction with membrane-associated substrates. An important example is phosphorylation and regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel by PKG (19). Both the *E. tenella* and *T. gondii* PKGs have N-terminal dual consensus acylation signals (M_1**G**A**C**(S/I)SK). Furthermore, it has been demonstrated using site-directed mutagenesis that both enzymes are myristoylated at position G_2 and palmitoylated at position C_4 (11). These modifications have been shown to mediate membrane-association of the full length protein. These acylation signal sequences are not present at the N-terminus of the *P. falciparum* PKG, although there is a series of consensus myristoylation sites in the N-terminal half of the protein (e.g. G_{10}NERNK). Western blots of *P. falciparum* proteins indicated the presence of a faint band migrating ahead of the major species of approximately 100 kDa (7) that may correspond to an additional isoform.

8. APICOMPLEXAN PARASITE PKGs AS SPECIFIC DRUG TARGETS

Identification of the *E. tenella* PKG was the culmination of a meticulous study to identify the precise cellular target of a potent *in vivo* inhibitor (IC_{50} of 1 nM) of this economically destructive parasite of chickens that causes coccideosis (15). The inhibitor, known as compound 1 (a trisubstituted pyrrole, 4-[2-(fluorophenyl)-5-(1-methylpiperidine-4-yl)-1*H*-pyrrol-3-yl]pyridine) was active against a number of apicomplexan parasites. This newly identified drug was tritiated to facilitate identification of its molecular target by column chromatography. A major peak containing a ligand-binding protein of approximately 120 kDa was isolated for further study. Since compound 1 is structurally similar to many ATP-competitive inhibitors of protein kinases, the major tritiated fraction was tested for protein kinase activity which was marginal. However, sequencing of tryptic peptides and synthesis of oligonucleotide primers allowed amplification of cDNAs from both *E. tenella* and *T. gondii* that had significant similarity to a *Drosophila* PKG. Kinase assays were now repeated on purified column fractions in the presence of cyclic nucleotides. This experiment revealed 500-1000 fold stimulation by cGMP. The suggestion that at least one target for compound 1 is a parasite PKG was supported strongly by the observation that kinase activity could inhibited with an IC_{50} of approximately 0.6 nM. A co-purifying PKA activity was around 100-fold less sensitive.

An elegant series of experiments demonstrated conclusively that the parasite PKG is the primary target of compound 1 (20). Firstly, PKG was inferred to be essential. Gene disruption (in this haploid organism) was only possible in the presence of an episomal copy of the gene. Molecular modelling (based on the crystal structure of PKA (21) was used to predict the precise amino acid residues in the catalytic domain which interact with compound 1. This allowed the production of both *E. tenella* and *T. gondii* mutant PKGs with greatly reduced binding affinity by replacing just one of these key residues (T_{761} in *T. gondii* and T_{770} in *E. tenella*) with amino acids with bulky side chains (glutamine and methionine) by site directed mutagenesis. The analogous position had previously been identified as an important determinant in the interaction of specific inhibitors with p38 MAP kinase (22) and substitution could confer insensitivity to this class of compounds. Mutant *T. gondii* disrupted at the PKG locus by transfection were dependent upon the presence of a drug-insensitive copy of the allele for survival in the presence of compound 1. Conversely, mutants rescued by a drug sensitive copy were killed by compound 1. Importantly, the mutant PKGs were otherwise functionally indistinguishable from WT PKG and mutant strains dependent on drug insensitive copies, were as virulent as wild type parasites. Mice infected with WT *T. gondii* were cured by administering compound 1, whereas mice infected with the mutant strain could not be cured by the drug. This provided further evidence that PKG is the primary target for compound 1. Examination of PKGs from many species indicates that the key drug-binding residues are specific to certain protozoan isoforms. This explains the selectivity of compound 1. Analogous substitutions carried out on malaria parasite-derived recombinant enzymes have also dramatically altered drug sensitivity (Deng and Baker, unpublished data).

Protein kinases have been widely studied as targets for novel chemotherapy. STI-571, a tyrosine kinase inhibitor has been used successfully to treat chronic myeloid leukaemia (23; 24). The above study with the *Eimeria* and *Toxoplasma* PKGs represents the first protein kinases to be validated in an animal model of an infectious disease. It will be interesting to discover the functional significance of the interspecies differences in the compound 1 binding site; this is a hydrophobic pocket that overlaps with the ATP-binding site within the catalytic domain of PKGs. Engineering of a drug-insensitive form of PKG by changing a single amino acid immediately raises questions concerning the selection of drug resistance if this drug was used to treat disease. However, at the very least, such compounds represent exquisitely specific tools to investigate the physiological function of parasite PKGs.

9. BIOLOGICAL FUNCTION OF PKGs IN PROTOZOA

Functional studies on protozoan PKGs are at an early stage, however recent experiments have led to interesting findings concerning apicomplexan isoforms. *T. gondii* cell lines containing both mutant *T. gondii* and *E. tenella* PKGs that confer insensitivity to compound 1 have been used to great advantage in addressing the cellular function (25). The broad-specificity protein kinase inhibitor staurosporine has been shown previously to interfere with host cell invasion by *T. gondii* (26). Cell invasion by *T.*

gondii and other apicomplexans initially involves a characteristic gliding motility, followed by cell attachment and penetration. Apical organelles (micronemes) secrete adhesive molecules (e.g. MIC2) that mediate the invasion process (27; 28), that also involves host receptors and the parasite actin-myosin based machinery. Secretion of these microneme adhesins is inhibited by staurosporine. It has also been demonstrated using the protein kinase inhibitor KT5926 (that prevents secretion of MIC2, gliding motility and host cell invasion (29)), that a calcium-dependent protein kinase is involved in these events (30). It has now been shown that compound 1 prevents attachment of *E. tenella* sporozoites and *T. gondii* tachyzoites to host cells, but also inhibits gliding motility and cell invasion. Furthermore, compound 1 inhibits release of the adhesin molecules associated with these processes and evidence suggests that vital calcium signalling events are also blocked (25). Inhibition of gliding motility and cell invasion by compound 1 did not occur in parasites expressing compound 1-insensitive PKG mutants, implying an important role for PKG in these events.

Studies on the cGMP signalling pathway in *Plasmodium* are less advanced, mainly due to the technical limitations of genetic manipulation of *P. falciparum*. However, both cGMP levels and calcium release have been implicated in an important step in male gametogenesis known as exflagellation. Here, eight flagellated male gametes emerge from the encapsulating red blood cell as a pre-requisite to fertilisation. Addition of cGMP or agents that increased cGMP levels promoted exflagellation, whereas inhibitors of calcium release reduced levels of exflagellation (31). It is also known that xanthurenic acid (Xa), a mosquito-derived molecule, can induce exflagellation (32; 33). A calcium-inhibitable guanylyl cyclase activity has been measured in particulate fractions of *P. falciparum* gametocyes that is stimulated upon addition of Xa (34). It is therefore possible that the effects of Xa on exflagellation may be mediated, at least partly, by cGMP. Two unusual, membrane-localised guanylyl cyclases have been identified in *P. falciparum*. They are potentially bifunctional enzymes with N-terminal P-type ATPase-like domains. Though functional guanylyl cyclases, the C-terminal cyclase regions are reminiscent of mammalian adenylyl cyclases, with a pair of catalytic domains each preceded by a set of 6 transmembrane domains (4).

Recently an elegant study has confirmed that Xa induces a rapid rise in cytosolic Ca^{2+} in the mouse malaria parasite *P. berghei*. A specific Ca^{2+}-dependent protein kinase (CDPK4) has been identified as the molecular switch that mediates the cellular effects of the Xa-induced Ca^{2+}-release. Deletion of the gene encoding this protein kinase prevents exflagellation and the phenotype can be rescued by re-introducing a functional copy of the gene (35). An earlier study using ultrastructural examination and cAMP/cGMP-dependent protein kinase inhibitors had concluded that the cGMP pathway may be involved in the later stages of male gametogenesis in *P. berghei*. Protein kinase inhibitors had no effect on the early events in male gametogenesis (such as DNA synthesis and axoneme formation) whereas further development of male gametes was prevented (36). Studies on how the Ca^{2+} and cGMP signalling pathways might interact in the control of exflagellation are underway.

Changes in the environmental ion concentration can reverse the direction of swimming in the ciliate protozoan *Paramecium*. This is brought about by electrophysiological changes in the ciliary membrane that are linked to cellular levels of cyclic nucleotides and reversal of the ciliary beat. The adenylyl cyclase of *Paramecium* has inherent K^+ channel properties (37; 38) whereas cGMP levels are regulated by a Ca^{2+} inward current (39). An additional association between the cGMP signalling pathway and swimming direction in *Paramecium* has been reported. The Atlanta A mutant has a reversed ciliary beat phenotype and addition of purified PKG from wild type cells, restored the direction of swimming suggesting a role for PKG in this process (40).

10. FUNCTION OF PKG ISOFORMS IN OTHER NON-MAMMALIAN SYSTEMS

The physiological function of PKG in some other non-mammalian systems has also been studied in detail. In *Drosophila*, locomotion behaviour of the fruitfly larvae in the presence of food is governed by a PKG isoform known as forager (reviewed in (41)). There are naturally occurring 'rover' and 'sitter' alleles; rover being genetically dominant to the sitter. The names derive from the respective feeding behaviour where rovers travel relatively long distances when feeding. These differences in behaviour are caused by small differences in PKG enzyme activity (12% more in rovers) as a result of greater transcript abundance. Molecular mapping identified the PKG gene, and overexpression of the rover gene in sitters conferred the former behavioural pattern showing that the PKG gene is directly responsible (42). In honey bees, the age-dependent behavioural change from a nurse (who distributes food in the hive) to a foraging way of life is also governed by increased expression of a homologue of the *Drosophila* foraging PKG gene known as *amfor* (43).

The behavioural consequences of mutations in a PKG gene (*egl-4*) of *C. elegans* have been elucidated. Worms show 'roaming' and 'dwelling' states reminiscent of the *Drosophila* larvae; however in this case mutants lacking PKG activity showed increased roaming behaviour as well as growth abnormalities (44). *C. elegans egl-4* mutants also have defects in olfactory behaviour, with a decreased ability to pre-adapt to odour (45). In another study, four different mutant worms with greatly increased body volume all had mutations in *egl-4*. Tissues, such as body wall and intestine, were shown to have increased volume leading to the phenotype, rather than any increase in cell number. These mutants also had an increased life span. Further experiments suggested that the cGMP signalling pathway represses body size and life span (46).

11. SUMMARY

It is perhaps rather surprising that with the relatively small number of non-mammalian species for

which functional data on PKG are available, locomotion is a re-occurring functional theme. These range from responses of *Paramecium* to environmental ionic composition, gliding movement of invasive forms of apicomplexan parasites, to more complex foraging behavioural responses in insects and worms. This may well reflect the few examples studied so far, however it is also possible that in addition to conservation in PKG amino acid sequence and enzyme structure, physiological function may also have been preserved to some extent throughout evolution.

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