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Comparison of moonlighting guanylate cyclases: roles in signal direction?

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Abstract

Over 30 receptor-like kinases contain a guanylate cyclase (GC) catalytic centre embedded within the C-terminal region of their kinase domain in the model plant *Arabidopsis*. A number of the kinase GCs contain both functional kinase and GC activity *in vitro* and the natural ligands of these receptors stimulate increases in cGMP within isolated protoplasts. The GC activity could be described as a minor or moonlighting activity. We have also identified mammalian proteins that contain the novel GC centre embedded within kinase domains. One example is the interleukin 1 receptor-associated kinase 3 (IRAK3). We compare the GC functionality of the mammalian protein IRAK3 with the cytoplasmic domain of the plant prototype molecule, the phytosulfokine receptor 1 (PSKR1). We have developed homology models of these molecules and have undertaken *in vitro* experiments to compare their functionality and structural features. Recombinant IRAK3 produces cGMP at levels comparable to those produced by PSKR1, suggesting that IRAK3 contains GC activity. Our findings raise the possibility that kinase-GCs may switch between downstream kinase-mediated or cGMP-mediated signalling cascades to elicit desired outputs to particular stimuli. The challenge now lies in understanding the interaction between the GC and kinase domains and how these molecules utilize their dual functionality within cells.

Background

cGMP is a key signalling intermediate in eukaryotes and is formed from the enzymatic cyclization and subsequent hydrolysis of GTP catalysed by members of the guanylate cyclase (GC) family. cGMP and GCs are well characterized in vertebrates, invertebrates and lower eukaryotes such as amoeba [1]. In vertebrates and invertebrates, cGMP is a transitory molecule that directly activates its degradation via specific phosphodiesterases and also regulates cyclic nucleotide-gated ion channels and protein kinases [1–3]. The amoeba *Dictyostelium* also uses cGMP as a chemoattractant [1,2]. However defining the roles of cGMP in bacteria, fungi and plants has been controversial [1,4–6] although cyanobacteria contain GCs that are involved in responses to changes in environmental signals [7,8]. Previously it was established that a GC and cGMP system is involved in bacterial encystment in *Rhodospirillum centenum* and by homology in other members of the Alphaproteobacteria [9]. In higher plants, cGMP is now an established second messenger that mediates plant hormone dependent and plant defence responses [6] and novel GCs have been identified that are discussed below. Together these findings indicate that cGMP is a universal signalling molecule in eukaryotic cells with similar roles in at least some prokaryotes.

Guanylate cyclases

GCs in animal cells are either soluble where they form heterodimers that are activated by NO binding at the haem nitric oxide/oxygen-binding (HNOX) site or single transmembrane proteins that have an extracellular ligand-binding site and an active intracellular GC domain (Figure 1). In mammals, there are seven members of membrane-bound GCs (GC-A to GC-G), and these include the natriuretic peptide receptors and photoreceptors [10]. To date, several crystal structures of soluble GCs have been solved providing further insight into their mechanism of action that has recently been reviewed [11,12]. A key attribute is that the canonical eukaryotic GCs are related to type III adenylate cyclases and it is quite remarkable that only a few amino acid changes in the catalytic centre convert a GC into an adenylate cyclase [13–15]. Another key finding is that protein dimerization is essential for GC activity and monomeric subunits are thought to form asymmetric dimers in a head to tail conformation [16,17]. Metal-binding residues and transition state-stabilizing residues are found along the intermolecular interface that provides a catalytic cleft for substrate (GTP) binding [2,16,18,19].

Canonical transmembrane GCs consist of an extracellular ligand-binding domain, a transmembrane domain and an inactive kinase homology domain that is linked to a GC catalytic domain [2,20] (Figure 1). The linker domain that

Key words: guanylate cyclase, interleukin receptor-associated kinase 3 (IRAK3), kinase, phytosulfokine receptor 1 (PSKR1), signal transduction.

Abbreviations: BRI1, brassinosteroid receptor; GC, guanylate cyclase; IRAK, interleukin receptor-associated kinase; MAPK, mitrogen-activated protein kinase; MyD88, myeloid differentiation factor 88; NF-κB, nuclear facror κB; PEPR1, pathogen peptide 1 receptor; PSKR1, phytosulfokine receptor 1; TLR, Toll-like receptor; WAKL10, wall-associated receptor kinase 10.

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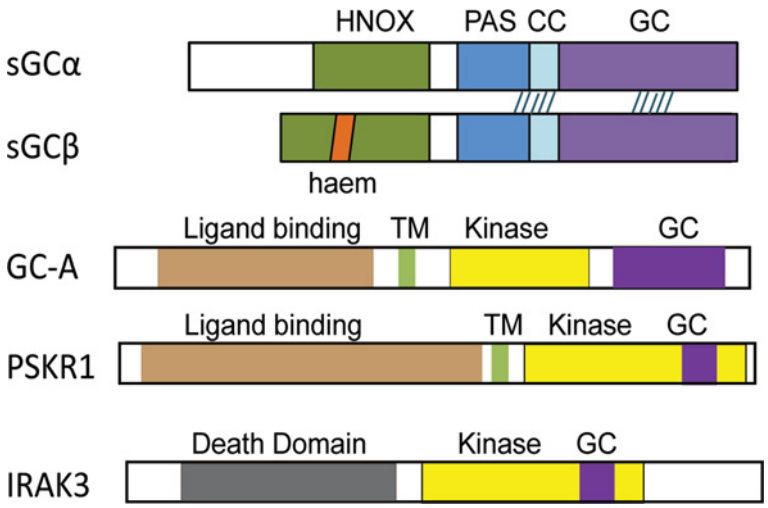
Figure 1 | The domain organization of GCs

The domain organization of animal soluble GCs is compared with canonical transmembrane GCs and two examples of the novel kinase GCs, PSKR1 and IRAK3.



links the kinase homology domain and the GC catalytic domain is a 50–70-amino-acid stretch that has been implicated in receptor dimerization [10,21], which, like soluble GCs, is essential to form the active site cleft for nucleotide binding. The surface groove in the GC domain is thought to rotate following ligand activation to bring the two GC domains into a closed conformation to bind GTP [20]. The kinase homology domain in canonical transmembrane GCs shares sequence similarities with typical protein kinase domains, although the aspartic acid in the HRD domain essential for phosphotransferase activity is often lacking. The kinase homology domains of almost all transmembrane GCs contain an ATP-binding motif and further ATP-binding motifs have been identified in the linker and GC domains that are currently generating a certain degree of discussion, although it is clear that ATP interaction is an important means of regulating the catalytic activity of the GC domain [2,10,17,22,23].

A new class of guanylate cyclases

Although considerable evidence indicated that cGMP had roles in signal transduction in plants, there were no GCs annotated in the model plant *Arabidopsis* genome. Therefore Ludidi and Gehring [24] designed a search motif based on several functionally assigned amino acids in the catalytic centre of known GCs from lower eukaryotes and identified and experimentally confirmed the first molecule with GC activity in higher plants, and homologues have since been found in other plants with roles in light sensing [25]. AtGC1 is a soluble protein with the catalytic GC centre found towards the N-terminus, but does not contain a haem-binding region necessary for NO binding [24]. Another protein AtNOGC1 has since been identified that responds to NO with alterations in its GC activity [26]. The original search motif was relaxed and over 40 receptor-like kinases, including the brassinosteroid receptor (BRI1), phytosulfokine receptor 1 (PSKR1), pathogen peptide 1 receptor (PEPR1) and a wall-associated receptor kinase 10 (WAKL10) [27–31], which all share a similar kinase GC domain structure were identified. The domain architecture of kinase GCs differs from that of characterized animal receptor GCs in that the GC catalytic centre is embedded within the kinase catalytic domain (Figure 1) adjacent to the P activation loop. Consequently, kinase GCs have a very unusual architecture which is distinct from most multidomain proteins that have discrete regions separated by linker sequences or domains [2,32].

If this surprising domain structure is functional in cells, it is likely that natural ligands of the receptor kinase GCs would stimulate cGMP production. Freshly isolated mesophyll protoplasts treated with epibrassinolide (a steroid acting at the BRI1 receptor) or active phytosulfokine peptide (PSK-α) significantly increased cGMP production relative to control protoplasts and inactive non-sulfated PSK [28,32]. cGMP increases occur in a time frame consistent with activating the GC catalytic function of the receptor kinase GCs and so implicate cGMP in early signalling events stimulated by these natural ligands. Overexpression of full length PSKR1 in isolated protoplasts results in raised cGMP levels [28]. A cyclic nucleotide reporter system was used to show that bacteria transformed with the recombinant PSKR1 kinase domain have increased cGMP levels following induction of protein expression [33].

Initial recombinant protein studies were undertaken with a segment of the intracellular domain of BRI1 and revealed cGMP production but not cAMP production [27]. Further to that study, the entire kinase domains of PSKR1, WAKL10 and PEPR1 have been shown to generate cGMP

detectable with both antibody and mass spectrometry studies [28–30]; interestingly the metal ion selectivity of these molecules differed. Overall, the GC activity of the novel kinase GCs was considerably lower than that observed for animal transmembrane GCs which are again lower than soluble GCs. However, a recent study with BRI1 encompassing the kinase domain did not show any cGMP production [34] and these conflicting results remain to be reconciled. Recombinant protein assays can sometimes be difficult to establish when the cofactors are not all known and this may be particularly true when a novel function is in question. The assay used by Bojar et al. [34] used conditions that appeared to favour kinase activity and their construct lacked domains that promote dimerization; together these factors possibly obscured any GC activity especially as it is fairly low. Such considerations are further supported by modelling studies where PSKR1 has been modelled against a kinase or a cyanobacteria GC showing a closed and open GC catalytic centre respectively [31].

Unquestionably, the receptor-like kinases have important roles as kinases in plants where they are involved in regulating growth and development. The intracellular kinase domain of BRI1 has a typical kinase fold structure [34] and homology models of PSKR1 also show typical kinase structure [31] (Figure 2A). The kinase activity of BRI1 has been particularly well characterized and wild-type proteins form homo- and hetero-dimers and cross phosphorylate each other and downstream substrates to activate transcription factors [35]. BRI1 kinase mutants have reduced growth [35], although the weak *bri301* mutant (G989I) that has no detectable kinase activity still retains partial responsiveness to brassinosteroids [36]. Kinase activity of PSKR1 is also partly understood *in vitro* where it has been shown to be inhibited by cGMP [28] and modulated by calmodulin *in vivo* [37]. The others are less well characterized although *in vitro* kinase activity occurs with WAKL10 [29]. The biological data indicates that regulation of the kinase activity is complex with both phosphorylation and other modulatory binding sites generating allosteric changes to switch the kinase activity from off to on. Phosphorylation of the cytoplasmic domain of BRI1 can dramatically alter kinase activity. Phosphorylation in the juxtamembrane domain, C-terminal domain and the active loop are all important in stimulating kinase activity whereas phosphorylation of certain residues (e.g. Ser^{891}) inactivates the kinase [35,38,39]. *In vitro* studies with PSKR1 show that phosphorylation of residues in the juxtamembrane position and active loop are also important for kinase activity while increases in calcium directly inhibit kinase activity (V. Muleya, J.I. Wheeler and H.R. Irving, unpublished work). Calmodulin-binding domains have been identified in both BRI1 [38] and PSKR1 [37] and these also modulate the kinase activity which may play a role in the response in plants. Hence there are several switches that regulate kinase activity and it is feasible that turning the kinase off will possibly stimulate dimer formation that will enhance the GC activity within plant cells and possibly stimulate redeployment of cellular resources. Since plants cannot flee danger, they have evolved an array of defence sensing processes that are intricately linked with their responses to growth signals. Simply put, plants can switch from using valuable and limited nutrient resources for growth to defence if the need arises. Phytosulfokine and its receptor PSKR1 regulate plant growth and development [40,41] and recently a critical role in the plant innate immune response was identified where it attenuates plant growth and redeploys plant resources to fight infection [42,43].

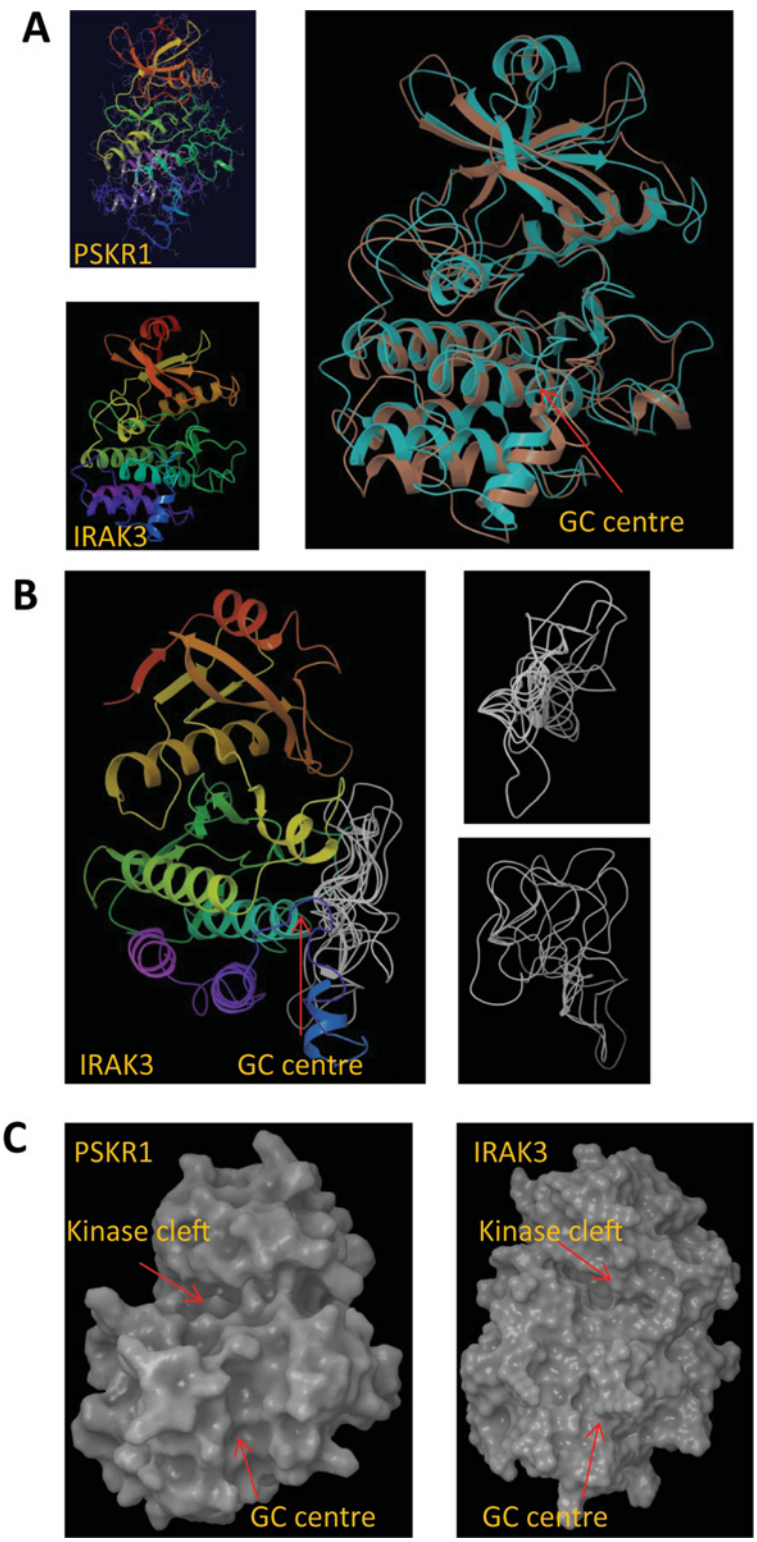
Molecules containing hidden GC catalytic centre signatures are not unique to plants and occur in many other organisms. Our searches revealed annotated kinase molecules with embedded GC catalytic centres in mammals (including humans) indicating that kinase GCs are widespread throughout the tree of life. One of the molecules unearthed using sequence homology-guided bioinformatic data-mining tools was interleukin 1 receptor-associated kinase 3 (IRAK3). Homologues of IRAK3 are found across mammalian species and the catalytic GC centre signature is conserved in these molecules. The function of this GC centre is unknown. IRAK3 is also known as IRAKM as it is predominantly found in macrophages and monocytes where it acts as a negative regulator of the inflammatory cascades stimulated by activating the interleukin 1 (IL-1)/Toll-like receptors (TLRs) [44]. IRAK3 prevents the dissociation of the IRAKs from myeloid differentiation factor 88 (MyD88) and thereby inhibits mitogen-activated protein kinase (MAPK)- and nuclear factor κB (NF-κB)-induced gene transcription leading to decreased inflammation. Recently, it was found that IRAK3 is also able to interact with MyD88–IRAK4 to form an IRAK3 Myddosome to mediate TLR7-induced MAPK/extracellular-signal-regulated kinase (ERK) kinase 3 (MEKK3)-dependent second wave NF-κB activation uncoupled from post-transcriptional regulation [45]. The role of IRAK3 is pivotal to homoeostasis in the innate immune response and preventing development of autoimmune diseases [46]. In some cancers IRAK3 is up-regulated and reduces cell death by inhibiting the immune response [46].

Comparison of IRAK3 with PSKR1

Homology models of the kinase domain of PSKR1 and IRAK3 were developed (see the Supplementary Online Data). The model for PSKR1 was based on its 41.2% identity with the crystal structure of tomato resistance protein Pto (for *Pseudomonas syringae* pv. tomato) kinase (PDB code 3HGK) [47] (Figure 2A). IRAK4 is the only member of the IRAK family that has been crystallized to date. A homology model of IRAK3 was developed based on the IRAK4 (PDB code 2NRU) [48] as this crystal structure was the most complete in terms of the kinase domain and had 28.14% identity with IRAK3 (Figure 2A). Both homology models of IRAK3 and PSKR1 show a typical kinase structure which is in agreement with these molecules being primarily kinase in nature. Although human IRAK3 is a pseudokinase as the conserved aspartic acid in the HRD domain is replaced by Ser^{293} (Supplementary Figure S1) and

Figure 2 | Comparison of PSKR1 and IRAK3 homology models

(**A**) Homology models of PSKR1 and IRAK3 are overlaid following alignment at key amino acid residues detailed in Supplementary Figure S1. The GC centre is indicated as is the extra loop due to the presence of amino acids 336–339 in IRAK3. (**B**) IRAK3 showing ten possible configurations (silver) of the loop containing the C-terminal part of the GC centre of IRAK3 (383–398). In addition, two alternative views of the loop configurations are shown demonstrating the potential for large movements associated with this section of the protein. (**C**) Comparison of the surface topology of homology models of PSKR1 and IRAK 3 showing the kinase cleft and the catalytic GC groove.

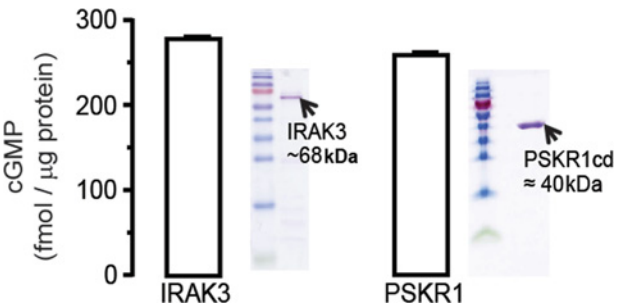


very limited phosphorylation activity of this protein has been reported [49]. Five amino acids that are conserved between the kinase families were used to align the two structures and the histidine residue of the HRD domain of PSKR1 was aligned to the corresponding Cys^{291} of the CGS in IRAK3 (Figure 2A and Supplementary Figure S1). The overall structures have a high degree of similarity (RMSD of 0.72 Å; 1 Å = 0.1 nm) demonstrating the predominant kinase shape. The main differences are that the PSKR1 model does not show an N-terminal α helix, a number of loop configuration differences are seen throughout the model and the C-terminal α helices do not align. The GC centre is in part an internal α-helix, whereas the remainder emerges as a loop (Figure 2A) as shown for the crystal structure of the kinase component of BRI1 [34]. In the homology model of IRAK3 the loop section of the GC centre and the KHLW loop (residues 336–339, see the Supplementary Online Data) can easily adopt alternative structures (Figure 2B). The KHLW (residues 336–339) loop is adjacent to the GC centre section (Figure 2A). There is a possible cation π interaction between Arg^{372} of the GC centre and the adjacent His^{337} whereas Trp^{339} (part of KHLW) sticks out into space and possibly has a role in protein–protein interactions as most nearby amino acids are polar. The loop region of IRAK3 from 383 to 398 includes part of the GC centre and this was refined via iterative molecular dynamics to generate ten possible loop configurations that were overlaid on the IRAK3 model confirming that a number of possible conformations exist that may alter the GC centre configuration (Figure 2B). Surface models of PSKR1 and IRAK3 indicate that the GC centre is associated with a small groove leading into the kinase cleft (Figure 2C). The groove alters with the different loop configurations and potentially with dimerization a catalytic or binding site is developed. Transient dimerization that depends upon interactions at the C-terminus has been observed in PSKR1 (V. Muleya, J.I. Wheeler and H.R. Irving, unpublished work) and it may be that the transient nature of this dimerization is an important regulatory component of the GC response.

Purified recombinant IRAK3 produces equivalent amounts of cGMP as the cytoplasmic domain of PSKR1 *in vitro* (Figure 3). Again these amounts are low and are likely to only have an effect on proteins in very close proximity. More importantly, human embryonic kidney (HEK)-293T cells transfected with IRAK3:GFP fusion constructs have significantly increased levels of cGMP present compared with the GFP transfection controls (L. Freihat, J.I. Wheeler and H.R. Irving, unpublished work) indicating that the GC catalytic centre is active in cells.

Figure 3 | IRAK3 has similar GC activity to PSKR1

The GC activity of recombinant IRAK3 and the cytoplasmic domain of PSKR1 were determined over 5 min in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) (2 mM), 1 mM GTP and 5 mM $MnCl_2$ in 50 mM Tris/HCl (pH 7.8) ($n = 3$). Insets show the typical purified protein following SDS/PAGE separation.



Conclusions and outlook

The novel kinase GCs function predominantly as kinases (or pseudokinases) and this is reflected in their structural properties. However, as discussed above, there is some evidence that they can generate cGMP albeit at low amounts. Recombinant protein studies are possibly hampered by the lack of appropriate cofactors or the inability to turn the kinase activity off. When expressed in cells, there is evidence that the cGMP is generated and this may be due to endogenous factors that down-regulate kinase activity. The GC domain has the catalytic centre present but is likely to require dimerization to generate functional moieties. These domains are truly hidden and not easily recognized as a GC centre. Why are they there? One possibility is that they function as a means to regulate the protein itself as cGMP inhibits PSKR1 [28], suggesting that they are actually a modulatory site rather than a catalytic site. Does the site act as a switch to shift the dynamics of the signal pathway from generating pathway one to pathway two? If this is the case, the low GC catalytic activity may actually represent a micro-autoregulatory site that operates endogenously to switch the protein to interact with alternate proteins. Interestingly, both PSKR1 [42,43] and IRAK3 [45] have recently been identified as molecules at signal transduction branch points.

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