An N-terminally truncated form of cyclic GMP-dependent protein kinase Ia (PKG Ia) is monomeric, autoinhibited, and provides a model for activation

Thomas M. Moon 1,4,6, Jessica L. Sheehe 1,6, Praveena Nukareddy 2, Lydia W. Nausch 1,5, Jessica Wohlfahrt 1, Dwight E. Matthews 2, Donald K. Blumenthal 3, Wolfgang R. Dostmann 1

From the The Department of Pharmacology1, Larner College of Medicine and the Department of Chemistry2, The University of Vermont, 89 Beaumont Ave, Burlington, VT 05405, USA, and the Department of Pharmacology and Toxicology3, The University of Utah, Salt Lake City, UT 84112, USA. Present Address4: The University of Arizona, Department of Chemistry and Biochemistry, Tucson, AZ 85721, USA.

Present Address5: The Nuremberg Hospital Medical School PMU, Department of Physiology, Prof. Ernst-Nathan-Str.1 Nuremburg 90419, Germany.

These authors contributed equally to this work.6

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To whom correspondence should be addressed: Wolfgang R. Dostmann, wolfgang.dostmann@uvm.edu and Thomas M. Moon, thomasmoon@email.arizona.edu

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ABSTRACT

The type I cGMP-dependent protein kinases serve essential physiological functions, including smooth muscle relaxation, cardiac remodeling, and platelet aggregation. These enzymes form homodimers through their N-terminal dimerization domains, a feature implicated in regulating their cooperative activation. Previous investigations into the activation mechanisms of PKG I isoforms have been largely influenced by structures of the cAMP-dependent protein kinase (PKA). Here, we examined PKG Iα activation by cGMP and cAMP by engineering a monomeric form that lacks N-terminal residues 1-53 (Δ53). We found that the construct exists as a monomer as assessed by whole-protein mass spectrometry, size-exclusion chromatography, and small-angle X-ray scattering (SAXS). Reconstruction of the SAXS 3D envelope indicates that Δ53 has a similar shape to the heterodimeric RIIα:C complex of PKA. Moreover, we found that the Δ53 construct is autoinhibited in its cGMP-free state and can bind to and be activated by cGMP in a manner similar to full-length as assessed by surface plasmon resonance spectroscopy (SPR). However we found that the Δ53 variant does not exhibit cooperative activation, and its cyclic nucleotide selectivity is diminished. These findings support a model in which, despite structural similarities, PKG Iα activation is distinct from PKA, and its cooperativity is driven by in trans interactions between protomers.

INTRODUCTION

Cyclic GMP-dependent protein kinases (PKG) are members of the AGC kinase family (PKA/PKG/PKC). They are conserved in eukaryotes, and ubiquitously expressed throughout the human body (1, 2). These kinases regulate critical processes such as vascular tone, cardiac remodeling, and platelet aggregation (1, 3–5). The primary activator of PKG isoforms is the second messenger cyclic-3’,5’-guanosine monophosphate (cGMP) (1). In vascular smooth muscle, activated PKG I phosphorylates intracellular targets — including myosin phosphatase target subunit 1 (MYPT1), regulator of G-protein signaling 2 (RGS2), inositol trisphosphate receptor-associated cGMP-kinase (IRAG), and the large conductance calcium-activated potassium channel (KCa1.1 or BK) — resulting in vasodilation (6–11).
PKG I is expressed as two splice variants that form homodimers \((a/b)\) (2, 12, 13). In both isoforms, each protomer is composed of a dimerization domain formed by a leucine zipper (LZ), followed by an autoinhibitory domain segment, a regulatory domain containing two cGMP-binding sites (A and B), and a catalytic domain (Figure 1a) (14). PKG I\(a\) and I\(b\) have low sequence conservation in their dimerization and autoinhibitory domains, and this difference is thought to both control the activity and response of the kinase to cGMP as well as mediate its targeting to specific substrates (6, 15–20). How the regulatory and catalytic domains interact in the inactive state and communicate to control cooperative activation have remained unsettled questions for the field (21, 22). In the inactive state, it has been hypothesized that the autoinhibitory domain of PKG I occupies the catalytic cleft formed between the N and C-terminal lobes and acts as a pseudo-substrate in a manner similar to the cAMP-dependent protein kinase (PKA) (23).

PKG isoforms share sequence homology with PKA (28% and 41% identity in its regulatory domain and catalytic domain, respectively). Consequently, current models of the relationship between PKG I structure and function have been overwhelmingly influenced by studies of PKA. However, PKA is expressed as separate catalytic and regulatory domains and PKG exists as a single polypeptide chain (24–26). Structural and biochemical studies of PKA have demonstrated an in cis mechanism for PKA activation (27–29). The sequential binding of cAMP to two coupled binding sites (A and B) within the regulatory subunit constitutes the physical basis for cooperativity in both cAMP binding and kinase activation. While it is known that full-length PKG I dimers also exhibit cooperative activation, it is unknown whether cooperativity is driven by the regulatory domain (in a fashion similar to PKA) or whether the dimeric state influences this effect (15, 25, 30).

It has been suggested that the N-terminal domains in the hinge region between the leucine zipper and autoinhibitory domains in PKG I are highly flexible, and structural models of PKG I have depicted the N-terminus protruding from the holoenzyme (regulatory and catalytic domain complex) (14, 23, 31). While it has been widely suggested that dimerization is essential for catalytic function, it has never been experimentally tested in the I\(a\) variant. Informed by previous models, we hypothesized that PKG I\(a\) could be truncated to generate a functional monomer by removing the flexible N-terminus (zipper and hinge). Moreover, we surmised that this construct would retain cGMP-dependent activation due to the inclusion of the autoinhibitory domain. In this study, we provide the biochemical and biophysical characterization of PKG I\(a\) \(\Delta 53\) (subsequently referred to as \(\Delta 53\)) by demonstrating its monomeric architecture, phosphorylation state, cGMP-binding properties, and the kinetic characteristics of its cGMP-dependent activation. Through the use of the \(\Delta 53\) construct, we address the following: 1) whether PKG I\(a\) is inhibited in cis or in trans; 2) how cyclic nucleotide binding and selectivity of the A-site is linked to activation; and 3) a putative mechanism by which cGMP-mediated cooperativity is derived in PKG. Our data indicate that PKG I\(a\) can form an in cis autoinhibited complex. Furthermore, cooperative activation of PKG I\(a\) by cGMP—in contrast to PKA—relies on interactions within the homodimeric regulatory domain. We conclude that cooperativity is driven in part by the N-terminal dimerization domain by localizing PKG monomers within close proximity during cGMP binding to form in trans interprotomer interactions.

RESULTS

Design, Expression, and Purification of \(\Delta 53\) —The design of a monomeric form of PKG I\(a\) was accomplished using a bioinformatic approach by comparing to a previously-solved structure of the homolog, PKA, in a heterodimeric complex with its regulatory domain (Figure 1a-b). The complex of RI\(a\):C (PDBID: 2QCS) was chosen due to the high sequence conservation of its autoinhibitory segment with PKG I\(a\) and the availability of its three-dimensional coordinates (Figure 1c). A multiple sequence alignment of the PKG I\(a\) autoinhibitory fragment with that of known isoforms of PKA was performed. The distance from the first residue (Lys92) to the start of the autoinhibitory sequence in the RI\(a\):C structure was used to determine that the residue equidistant from the autoinhibitory sequence in PKG I\(a\) was Pro56 (Figure 1b). We hypothesized that, in a similar fashion to RI\(a\):C, the autoinhibitory segment preceding the canonical A/GISAE pseudo-
substrate segment would confine the P+1 loop of the catalytic domain and make further contacts with the C-terminal tail, glycine-rich loop, and the F-helix from the large lobe (32, 33). As a consequence, and as to not exclude residues that may be important for autoinhibition, Δ53 was engineered to begin at Ile54.

Following expression and purification of Δ53 (Figure 2a), we examined the kinase by size exclusion chromatography (Figure 2b). Both the apo and cGMP-bound forms exhibited reproducible elution profiles centered at 14.8 mL and 14.7 mL, respectively, indicative of a 70 kDa species according to molecular weight standards (Figure S1). The addition of cGMP appeared to have a negligible effect on the elution volume of the protein, suggesting that dimer formation was not present in the apo or cGMP-saturated samples. Δ53 was further examined by time-of-flight (TOF) mass spectrometry to determine its mass and phosphorylation state (Figure 2c). A mass of 70,511 Da, corresponding to a mono-phosphorylated, monomeric species was observed. We also discovered the presence of a doubly phosphorylated species comprising 20% of the total ion content (70,591 Da). Tandem mass spectrometry analyses of trypsin-digested samples were used to confirm the primary phosphorylation site at T517 (full-length enzyme numbering scheme) located in the activation loop of the catalytic domain (Table 1). This phosphorylation site has been previously identified in full-length PKG Iα, and is required for catalytic activity (34). To confirm this, we also examined preparations of full-length PKG Iα and found a single phosphorylation site corresponding to T517 (Table S1). Based on previous studies, the minor phosphorylation site observed for Δ53 was suspected to reside in the autoinhibitory domain (35, 36). However, the peaks corresponding to the peptide containing the auto-phosphorylated pseudosubstrate sequence, (R)AQGISAEPQTYR(S) (residues 61–72), were of high intensity and suggested no phosphorylations were present.

SAXS Analysis — The results presented thus far indicate that Δ53 exists as a monomer in solution. In an effort to characterize the low-resolution solution structure of the autoinhibited complex, we utilized size exclusion chromatography coupled to small-angle X-ray scattering wherein during analysis single species associated with Δ53 was observed (SEC-SAXS, Table 2). Concurrent Guinier calculation during data collection indicated a constant Rg across the sample peak (Figure 3a). The averaged scattering intensity curve (Iq vs q) for Δ53, which was obtained by averaging frames 410-459 of the SEC-SAXS peak, exhibited a linear Guinier plot (Figure 3b and inset, Table 2). The Guinier indicated the presence of a monodisperse system with no evidence of aggregation. A Kratky plot of the data suggested the presence of a well-folded species (Figure 3c). The P(r) curve calculated from the scattering intensity contained a single peak at ~30 Å that smoothly decayed to a maximum linear dimension (Dmax) of 97 Å (Figure S2). When compared to the P(r) curve of the homologous PKA heterodimer upon which we based our construct, RIIα:C (green), both showed a single peak at 34 Å, but the RIIα:C heterodimer had an extended Dmax of ~130 Å . Comparison of the Δ53 P(r) to that observed for a different PKA heterodimer, RIIβ:C (red), demonstrated that they have nearly identical dimensions (Table S2) (37).

Predicted X-ray scattering from the crystal structures of the PKA RIIα:C, PKA RIIβ:C, and PfPKG from P. falciparum (a monomeric species containing 4 cGMP binding sites and a catalytic domain) was conducted using both CRY SOL and FoXS (Table 2f) (38, 39). The best fit of the experimental SAXS data to the predicted scattering was obtained from RIIα:C using CRY SOL with constant subtraction ($\chi^2 = 0.82$, Figure 3d, Table 2). Using the the RIIβ:C heterodimer in the same analysis resulted in a poorer fit to the experimental data ($\chi^2 = 1.25$). The PfPKG fits were poor using CRY SOL ($\chi^2 = 9.21$), but acceptable with the FoXS server ($\chi^2 = 1.29$, Table 2f, Figure S3). We also found that the Rg values calculated using the predicted scattering curves for both the PKA RIIα:C and RIIβ:C heterodimer crystal structures were in closer agreement with the measured and calculated values for Δ53 (Table 2f). Next, DAMMIF and DAMMIN modeling was implemented. Using SUPCOMB, the resulting averaged and filtered DAMMIF/DAMMIN ab initio three-dimensional envelope accommodated both crystal structure models of the RIIα:C and RIIβ:C heterodimers with little protrusion outside of the envelope (Table 2e, Figure 3e-f). Among the 3D
models examined, these data cumulatively suggest \( \Delta 53 \) adopts a shape most similar to PKA RIz:C.

**Binding and Activation of Full-length PKG I\( \alpha \) and \( \Delta 53 \) with cNMP** — Finally, we studied the activation kinetics of \( \Delta 53 \). No significant differences in basal activity were observed between PKG I\( \alpha \) full-length and \( \Delta 53 \), indicating that the monomeric construct was autoinhibited at levels comparable to previous reports (15, 30). Moreover, the cGMP-dependent activation of \( \Delta 53 \) and full-length PKG I\( \alpha \) were analogous with respect to their maximum velocities of 4.0 and 3.9 \( \mu \text{mol/min*mg} \), respectively (Figure 4Ba). These results indicate that the cGMP-dependent fold-stimulation of \( \Delta 53 \) is indistinguishable from full-length. However, differences between the two constructs were observed with respect to their activation constants and degree of cooperativity. We observed \( K_a \) values and Hill coefficients corresponding to 182 nM (\( n_H=1.6 \)) for PKG I\( \alpha \) full-length and 250 nM (\( n_H=1.0 \)) for \( \Delta 53 \) (\( p<0.0001 \) for both measurements). When we tested the activation profiles of full-length and \( \Delta 53 \) PKG I\( \alpha \) with cAMP, we observed that the full-length enzyme exhibited cooperative activation, while again \( \Delta 53 \) was non-cooperative. Comparisons of the fold-difference in activation by cGMP and cAMP for both enzymes demonstrated that the removal of the N-terminus of PKG I\( \alpha \) reduced the selectivity for cyclic nucleotide from 58-fold (full-length) to 3.5-fold (\( \Delta 53 \)), representing an overall 16-fold decrease in selectivity for cGMP over cAMP.

To probe how binding of cyclic nucleotide correlates with the activities of the enzymes, we measured cGMP and cAMP binding to PKGI\( \alpha \) constructs by surface plasmon resonance (SPR) spectroscopy. Binding of cyclic nucleotides was fit using a two-site binding model since the regulatory domain of PKG I\( \alpha \) contains two cGMP sites per monomer. To determine cooperativity of the binding, we also fit the data using a one-site model with Hill coefficient. PKG I\( \alpha \) full-length exhibited an almost 3-fold weaker affinity for cGMP than \( \Delta 53 \) (\( K_D^{\text{FL}}=7.9 \mu \text{M} \) and \( K_D^{\Delta 53}=2.9 \mu \text{M} \)), when fit with a one-site model (Table 3). However, when fit with a two-site binding model, the ratio dropped to 1.6-fold. Moreover, both constructs displayed negative cooperativity in binding cGMP (\( n_H^{\text{FL}}=0.60 \) and \( n_H^{\Delta 53}=0.74 \)). When cAMP binding was measured for the two constructs, the \( K_D \) values for their high affinity sites increased by over thirty-fold. However, cAMP binding remained negatively cooperative in both enzymes. In binding to either cyclic nucleotide, we found that the low-affinity site demonstrated much weaker binding than the high affinity site.

To distinguish the cyclic nucleotides sites responsible for activation in the full-length PKG I\( \alpha \) kinase, we mutated the glutamic acid residues in the Phe-Gly-Glu (FGE) motif of the phosphate binding cassettes within the A-site (E168G) and the B-site (E292A). Since the glutamate residue is involved in binding to the 2'-hydroxyl from the ribose, mutation of this site in PKA has been shown to disrupt cyclic nucleotide binding (28, 40–43). Mutation of the A-site (\( \text{E}^{168G} \)) completely abolished cGMP-dependent activation of the full-length kinase (Figure S4). These results demonstrate that a functional A-site is necessary for kinase activity. Moreover, when we examined the cGMP-dependent activation of \( \text{E}^{292A} \) PKG I\( \alpha \), we observed cooperative (\( n_H=1.45 \)) stimulation of the kinase with a \( K_a \) of 95 nM. In an effort to further examine the contribution of the N-terminus and the catalytic domain on cyclic nucleotide binding selectivity, we purified two constructs of the regulatory domain of PKG I\( \alpha \). The first construct, \( 1-326 \) PKG I\( \alpha \), contains the N-terminus and cGMP-binding regulatory domain, whereas the second construct, \( 78-326 \) PKG I, contains only the regulatory domain. When we examined binding of cGMP to \( 1-326 \) PKG I\( \alpha \) and \( 78-326 \) PKG I, we found that the one-site model \( K_D \) shifted significantly compared to values observed for full-length PKG I\( \alpha \) and \( \Delta 53 \) (Figure S5). For \( 1-326 \) PKG I\( \alpha \), the two-site binding model showed that the \( K_D \) value for cGMP binding to the A-site increased by 8-fold compared to the full-length enzyme (Table 3). Furthermore, binding of cAMP showed a drastic decrease in selectivity for cGMP over cAMP. The B-site showed a clear selectivity for cGMP over cAMP (4-fold). However, when the N-terminus was excluded in \( 78-326 \) PKG I, we observed no difference in the affinities for cGMP and cAMP in the high affinity A-site. The B-site retained a clear selectivity for cGMP over cAMP (10-fold).
DISCUSSION

PKG activation has been extensively studied by traditional biochemical methods and, more recently, using modern biophysical approaches (18, 24, 25, 44, 45). These investigations sought to determine the architecture of the homodimeric kinase in its basal and activated states, the order and selectivity of cGMP binding, and, by extension, the origin of its cooperativity. The generation of a functional, monomeric form of PKG Iα is a useful tool for investigating the molecular basis of these long-observed biochemical phenotypes.

PKG Iα Δ53 purified from Sf9 cell extracts exists as a mixture of mono- and diphosphorylated forms; no unphosphorylated form was detected. The primary phosphorylation site, T517, was found in high abundance by time-of-flight mass spectrometry (TOF-MS) (Figure 2c, Table 1). Phosphorylation of T517, which is located in the activation loop of the catalytic domain, is essential for catalytic activity (34). In the PKA catalytic domain, the analogous phosphorylation at T197 forms contacts with other activation loop residues, the catalytic loop, and the αC-helix to integrate the active site components (46, 47). It has been hypothesized that phosphorylated T517 in PKG serves a similar function since the lack of phosphorylation or mutation of this residue renders PKG inactive (34). The presence of this phosphorylation site in Δ53 corroborates the cGMP-dependent activation observed by our phosphotransferase assays. In regard to the second, less abundant phosphorylation site, we had hypothesized that this residue would be located in the autoinhibitory domain based on previous published data (35, 36, 48). Analysis of autoinhibitory domain residues suggested S64 and T70 were not phosphorylated (parent peptide Ala61-Arg72; Table 1). Another potential site, T58, has previously been identified as the major site that is autophosphorylated most rapidly in the presence of cGMP in vitro or cAMP in native PKG I preparations isolated from bovine lung (35, 48). Since Sf9 cells endogenously express adenylyl cyclases, we hypothesized that T58 could be the minor phosphorylation site (49, 50). However, our LC-MS/MS data could not confirm the precise location. These results are in agreement with previously published results that observed the phosphorylation at T517, but were also unsuccessful in identifying the second site (23). Furthermore, these results suggest that neither its dimerization through the N-terminal dimerization domain nor exposure to cGMP are required for activation loop phosphorylation.

Despite the presence of these two phosphorylation states, we consistently observe a compact, monomeric, monomeric species in solution by size exclusion chromatography (SEC) (Figure 2b). In addition, the elution profile of Δ53 did not change with the addition of cGMP, suggesting that it maintains its monomeric state even during long-lived exposure. In an effort to reconstruct the three-dimensional shape of inactive Δ53 and confirm its monomeric character, we collected data by SEC-SAXS and compared the reconstructed 3D-envelope to the crystal structures and SAXS data from two PKA heterodimers (PDBID: 2QCS and 4WBB) and PfPKG (PDBID: 5DYK). We found that Δ53 adopts a similar shape to that of the PKA R1α:C heterodimer (2QCS) based on the best fit of the atomistic modeling in CRYSOL to the scattering data (χ^2 0.82) (Figure 3d, Table 2e). In addition, the crystal structure is readily accommodated by the 3D envelope calculated from the Δ53 SAXS data (NSD = 1.06, Figure 4f). These data concurred with previously published results for the apo state of monomeric PKG Iβ Δ1-52 (Table S2) (45). PKG Iβ Δ1-52 contains 18 additional residues upstream of its autoinhibitory domain compared to PKG Iα Δ53. Considering the high overlap between these two truncated constructs in the SAXS analyses, this suggests that the linker in Iβ Δ1-52 adopts a compact conformation relative to the regulatory-catalytic domain complex. Based upon these data, we propose a model wherein each monomer within the dimeric PKG Iα complex can be autoinhibited by its own regulatory domain and autoinhibitory sequence (Figure 5 top).

Finally, we report that Δ53 is activated by cGMP at concentrations similar to the full-length kinase (Figure 4Ba). A previous study by Richie-Jannetta et al. and a more recent study by Kim et al. expressed truncated forms of PKG Iβ (Δ1-53 and Δ1-55) to investigate the influence of the dimeric interface on cGMP-dependent activation (51, 52). Both studies described significant shifts in the activation constants of the truncated Iβ constructs. These results contrast with our observations of PKG Iα. Δ53
does not exhibit an appreciable shift in the activation constant compared to the full-length enzyme. These results support the longstanding conclusion that PKG I\textalpha{} and $\beta$ are enzymatically distinct due to differences in their N-termini (1, 15, 20, 30, 50).

Interestingly, $\Delta 53$ does not exhibit cooperative cGMP-dependent activation (Figure 4Ba). The loss of cooperativity has been previously reported for PKG I$\beta$, wherein full length PKG I$\beta$ ($n_H = 2.1$) becomes noncooperative when the N-terminus is removed (\Delta 1-52 PKG I$\beta$) (30, 52). In addition, we observed that cAMP also cooperatively activates the full-length kinase ($n_H = 1.75$), but not $\Delta 53$ ($n_H = 0.81$). These results suggest that the cooperative activation is mediated by the N-terminus through the facilitation homodimer formation. The N-terminal dimerization domains of PKG I\textalpha{} and I$\beta$ are left-handed, coiled-coil, leucine zipper (LZ) motifs. The LZ is characterized by a heptad repeat of amino acids (abcdefg) where residues $a$ and $d$ are typically hydrophobic and residues $e$ and $g$ are typically hydrophilic. The leucine zipper of PKG I\textalpha{} spans residues 1-47. In addition to its leucine and isoleucine residues, there are also four non-leucine/isoleucine residues at the $d$ positions that provide additional non-hydrophobic interhelical contacts (Phe7, Lys14, Lys28, Cys42) (53). One of these residues, C42, is unique to the I\textalpha{} isoform and forms an interprotomer disulfide bond in the presence of oxidizing agents, such as hydrogen peroxide (54, 55) (Burgoyne et al. 2007. Science. 317(5843):1393-1397; Landgraf et al. 1991. JBC. 266(25):16305-16311). The leucine zipper of PKG I$\beta$ spans residues 4-53, and also contains four non-leucine/isoleucine residues at the $d$ positions (Lys13, Arg20, Lys34, and Tyr48) (18)(Casteel et al. 2010. JBC. 285(43):32684-32688). Although the majority of the non-canonical residues are basic, their differences in position within the helices and the overall discrepancy in length of the helices are reasons why PKG I protomers have not been shown to form heterodimers either in vitro or in vivo.

It is well-characterized that the cGMP A-site in the regulatory domain is the high affinity binding site, and, thus, is the first to bind cyclic nucleotide (24, 26, 44). Our data of PKG I\textalpha{} full-length and $\Delta 53$ agree with these previously published results and indicate a clear selectivity for cGMP over cAMP in the A-site (Table 3). To further test the contributions of the A- and B-sites on PKG I\textalpha{} activity, we measured the activities of $E_{167G}$PKG I\textalpha{} and $E_{292A}$PKG I\textalpha{}. We found that disruption of cyclic nucleotide binding to the B-site did not have an appreciable effect on activity (Figure S4). In contrast, disruption of cyclic nucleotide binding to the A-site completely abolished activity. These data collectively support the importance of the A-site in regulating activation of PKG I\textalpha{}. However, previous studies using isolated regulatory domain constructs have suggested that the A-site is non-selective for cyclic nucleotide (25, 44). These results suggest selectivity for cGMP binding to the A-site must be controlled through regions outside of the regulatory domain.

To investigate the influence of regions outside of the regulatory domain on the cyclic nucleotide-dependent characteristics of PKG I\textalpha{}, we expressed and purified two additional constructs: $1-326$PKG I\textalpha{} and $78-326$PKG I\textalpha{}. PKG I\textalpha{} without its catalytic domain ($1-326$PKG I\textalpha{}) showed a significant decrease in binding affinity for cGMP and loss of selectivity for cGMP over cAMP in the A-site compared to the full-length construct (Table 3). Selectivity was only slightly reduced in the B-site, and the values agree with those measured previously for the isolated B-domain (44). These results differ from the binding and nucleotide selectivity results observed for PKG I\textalpha{} full-length and $\Delta 53$. Thus, these characteristics seem to be integrated into the architecture of the inactive holoenzyme.

The initial comparison of the activation curves for full-length and $\Delta 53$ to the one-site binding model indicates a large disparity between the concentration of cGMP required for full saturation of the A and B-sites and the minimum concentration required for full activation. Early measurements of cyclic nucleotide binding with $^3$H-cGMP observed a significant difference in the $K_D$ values for cGMP binding at 4°C and 30°C (56). Our measurements for cGMP are consistent with these early experiments. When a two-site model is applied to the binding data, we can directly correlate the $K_D$ for the A-site with the $V_{max}$ for activation. This model is reasonable since there are two cGMP binding sites with distinct binding affinities. Furthermore, this effect is observed for both cGMP and cAMP, equally (Figure 4). This implies that full-length PKG I\textalpha{} only requires
that half of the A-sites are occupied to stimulate full activation. These data, which also suggest the B-site is non-essential for kinase activity, are further supported by the fact that the K_D for the B-site (∼90 µM) is higher than estimated intracellular concentrations of cGMP (∼10 nM - 10 µM) (57–59). Moreover, we observe that mutation of the nucleotide binding cassette in the B-site does not appreciably affect activation of the full-length kinase (Figure S4). Thus, we can also conclude that binding studies that are either directed toward the isolated PKG regulatory domains or employ equilibrium-exchange of radiolabeled cyclic nucleotides are limited; and future studies should examine these effects in the context of the intact holoenzymes.

These SPR measurements also indicate that binding of cGMP to both full-length and Δ53 is negatively cooperative (n_H<1), which indicates that binding of cGMP to subsequent sites becomes more difficult with increasing concentrations. These results suggest a lack of avidity between cyclic nucleotide binding domains A and B, and this has been corroborated by recent structural evidence (52). These observations stand in contrast to PKA where the origins of cooperativity have been directly linked to the regulatory domain, wherein binding of cAMP into the B-site enhances binding for the A-site through intra-subunit contacts (27, 28, 32). It has been suggested that the cooperativity observed for PKG activation arises from a similar mechanism (23, 24, 44). However, the loss of cooperative activation in PKG Iα through disruption of the dimer via two mechanisms - 1) by removing residues that allow for N-terminal dimerization (this study) and 2) via mutation of residues forming the knob-nest interface - suggests that this is not the case (25).

Finally, we propose a model based on our findings wherein autoinhibition of PKG Iα is driven in cis. The high affinity A-site provides cyclic nucleotide selectivity through contacts outside of the regulatory domain and cooperative activation of PKG Iα is driven in trans, facilitated by the N-terminus - which ensures monomers are close enough to form necessary interprotomer contacts (Figure 5). These data reinforce our earlier hypothesis that cooperative activation of the kinase is not driven through cooperative binding of cyclic nucleotides to the regulatory domain within the same protomer. Rather, it is driven through an inducible dimeric interface mediated by the switch helix (30, 60). Therefore, we conclude that the mechanism driving cooperativity in PKG Iα lies in the interface between protomers and not between cGMP binding sites within the same protomer.

**EXPERIMENTAL PROCEDURES**

**Sequence Alignments**

Sequences for PKG Iα and PKA regulatory domain isoforms were downloaded from the NCBI and Uniprot repositories (ncbi.nlm.nih.gov; UniProt.org) and uploaded to JalView Desktop (61). The following accession numbers were used: NP_001091982 (PKG Iα Homo sapiens), NP_776861 (PKG Iα Bos taurus), NP_001013855 (PKG Iα Mus musculus), NP_0010999201 (PKG Iα Rattus norvegicus), NP_037313 (PKA RIIα Rattus norvegicus), NP_997637 (PKA RIIα Homo sapiens), NP_001068669 (PKA RIIα Bos taurus), NP_001240819 (PKA RIIα Mus musculus) NP_062137 (PKA RIIα Rattus norvegicus), P12367 (PKA RIIα Mus musculus), NP_001178296 (PKA RIIα Bos taurus), NP_0014148 (PKA RIIα Homo sapiens), P31324 (PKA RIIα Mus musculus), NP_001025191 (PKA RIIα Rattus norvegicus), NP_002727 (PKA RIIα Homo sapiens), P31322 (PKA RIIα Bos taurus). A multiple sequence alignment using CLUSTAL-O with the default parameters was accomplished using the JalView Desktop interface (62).

**Maintenance of Sf9 cells** — Sf9 cells were purchased from Life Technologies. Frozen cell stocks were prepared according to the Growth and Maintenance of Insect Cell Lines Manual (Life Technologies). Typically, one vial containing Sf9 cells (1 x 10^6 cells/vial in freezing medium (80% supplemented Grace’s Insect Medium (Gibco), 10% fetal bovine serum (FBS; Sigma), and 10% DMSO) was suspended in 15 mL Grace’s medium (Life Technologies) supplemented with 5% fetal bovine serum (Sigma) and 10 µg/mL gentamicin (Sigma). Cells were grown in a 75 cm² flask until cells reached 80% confluency. Cells were detached from the flask and suspended in SF900 III medium (Life Technologies) supplemented with 1x lipids (Sigma), 1% poloxamer 81 (Sigma), and 10 µg/mL gentamicin (Sigma). Sf9 cells were maintained in suspension at 27°C, 80 rpm
and passaged every 3.5 days to 1.2 x 10^6 cells/mL.

**Expression of PKG Iα constructs in Sf9 cells** — Full-length PKG Iα (Bos taurus) was amplified and cloned into pFAST Bac-HTA (Invitrogen) as previously described (63). PKG Iα 54-671 (Δ53) was amplified by PCR from bovine PKG Iα 1-671 using forward and reverse primers containing Ncol and XhoI cut sites, respectively: 5′-GCC GAT ATC GCC CCC CGG ACC ACC-3′ (sense); 5′-ATG CCT CGA GAC CTA TTA GAA GTC TAT GTC CCA TCC TGA-3′ (antisense) (Integrated DNA Technologies). The resulting product was cloned into pFastBac-HTA (Invitrogen) using NcoI, XhoI, and DNA ligase (New England Biolabs). Site-directed mutagenesis was performed on the full-length PKG Iα construct in pFAST Bac-HTA to produce E167G and E292A using the following primers: 5′-AAG GTG TTT GGA GGG TTG GCT A-3′ (E167G- sense); 5′-ATA GCC AAC CCT CCA AAC ACT T-3′ (E167G- antisense); 5′-GGA AAA GAT TGG TTT GGA GCG AAA GCC TTG-3′ (E292A- sense); 5′-TTC CCC CTG CAA GGC TTT CGC TCC AAA CC-3′ (E292A- antisense). Transposition of the full-length PKG Iα, mutant, and Δ53 genes into bacmid using DH10 Bac E. coli, confirmation of its insertion by PCR, and transfection into Sf9 cells to produce baculovirus was performed following the Bac-to-Bac Baculovirus Expression System Manual (Life Technologies). Third amplification viruses were used at dilution ratios of approximately 1:500 to express both constructs (virus:Sf9 cells). At 72 hours post-infection, cultures were harvested by centrifugation at 500 x g for 10 min. Pellets were resuspended in lysis buffer (10 mM TES, 300 mM NaCl, 5 mM imidazole, pH 7) at a volume of 20 mL per liter of Sf9 cells, flash frozen in liquid nitrogen, and stored at -80°C.

**Expression of PKG I regulatory domain constructs in E. coli** — The PKG I regulatory domain constructs (78-326PKG I and 1-326PKG I) were amplified by PCR from the full-length PKG Iα gene using forward and reverse primers containing BamHI and EcoRI cut sites, respectively: PKG I-78-f 5′-CGG GAT CCA TGC AGG CAT TCC GGA AGT TCG TGC CCA TCG GAG-3′ (sense), PKGI-1-f 5′-TGG GGA TCC AGC GAG CTG AGA CTA TTA GAA GTC TAT GTC CCA TCC TGA-3′ (antisense). The respective fragments were ligated into BamHI/EcoRI-digested pRSET-A using T4 ligase (NEB). Both constructs were expressed in Rosetta2 E. coli (Novagen). Overnight cultures were used to inoculate 1L of TB supplemented with 50µg/mL ampicillin and 25µg/mL chloramphenicol and subsequently grown at 37°C, 220 rpm to an OD₆₀₀ = 0.8. Expression was induced by the addition of IPTG (1 mM final), and the induced cultures were incubated overnight at 25°C at 220 rpm. E. coli were harvested by centrifugation at 1500 x g for 30 min, and the resulting bacterial pellets were flash frozen in liquid nitrogen and stored at -80°C.

**Purification of PKG Iα constructs** — Protease inhibitors (Roche) were dissolved in 50 mL of lysis buffer and added to 1L of pelleted Sf9 cells containing either PKG Iα full-length, E167G, E292A, or Δ53 (B. taurus). Sf9 cell pellets were thawed on ice for 40 min followed by gentle mixing. Resuspended cell pellets were lysed using a French pressure cell (2 passes at >1,500 bar; SLM-AMINCO) or by passing cells through a 22.5 gauge needle (3 passes).

Bacterial pellets containing 78-326PKG I were resuspended by vortexing in 50 mL of lysis buffer supplemented with protease inhibitors (Roche) and were lysed using a French pressure cell (5 passes at >1,500 bar; SLM-AMINCO).

All cell lysates were clarified by centrifugation at 30,000 x g for 30 min at 4°C and passed through a 0.22 µm PES syringe filter (Millipore). The resulting clarified, filtered lysates were loaded onto a 5 mL prepacked Ni-IMAC column (Bio-Rad) using a P-1 peristaltic pump (GE) and washed with 5 column volumes of lysis buffer and 6 column volumes of mid-wash buffer (lysis buffer supplemented with 30 mM imidazole). Proteins were eluted from the column with elution buffer (lysis buffer supplemented with 250 mM imidazole) using a Profinia FPLC (Bio-Rad). Peak fractions containing PKG Iα were pooled and analyzed by SDS-PAGE. PKG Iα was dialyzed against 4 L of 50 mM MES, 300 mM NaCl, 1 mM TCEP, pH 6.9 using 12-14 kDa MWCO dialysis tubing and 1 L against 50 mM MES, 300 mM NaCl, 1 mM TCEP, 10% glycerol, pH 6.9. Aliquots of PKG Iα were flash frozen in liquid nitrogen and stored at -80°C.

**Analytical size exclusion chromatography (SEC)** — Samples containing 0.5 mg of PKG Iα Δ53 (apo or preincubated with 5 µM cGMP) were loaded
sequentially onto a Superdex 200 10/300 column (GE) connected to an Äkta PURE FPLC (GE) and eluted isocratically in 50 mM MES, 300 mM NaCl, 1 mM TCEP, pH 6.9 (apo) or buffer supplemented with 5 μM cGMP (bound). Peak fractions were examined for the presence of PKG Iα Δ53 by SDS-PAGE. Peaks were analyzed with Unicorn 6.4 (GE) and plotted using DataGraph (Visual Data Tools).

**Phosphotransferase assays** — Activation of PKG Iα constructs by cyclic 3',5'-guanosine monophosphate (cGMP; BioLog) was assessed by measuring phosphorylation of a synthetic peptide substrate (W15, TQAKRRKKSLAMA) with γ-32P-ATP similar to the method described previously (63). Each 100 μL reaction contained 20 μL of 5x MES Mix (250 mM MES, 5 mM MgOAc, 50 mM NaCl, pH 6.9), 10 μL of 100 mM DTT, 10 μL of 10 mg/mL BSA (Cohn’s fraction V; Sigma), 10 μL of 100 μM W15, 10 μL of cGMP (0-5 μM), 20 μL of 5 mM PKG Iα (in 50 mM MES, 1 mM TCEP, pH 6.9), 10 μL of ATP mix (γ-32P-ATP in 1 mM ATP; approximate specific activity = 200 cpm/μL), and 10 μL of H2O. Reactions were performed at 30°C and initiated with the addition of either PKG Iα or ATP mix. Blank reactions were performed by substituting the PKG Iα and cGMP with H2O. Reactions were quenched after 1.5 or 3 min by spotting onto P81 phosphocellulose (Whatman and Reaction Biology Corp.) and washing with 0.8% phosphoric acid. Determination of 32P incorporation into W15 substrate was measured by liquid scintillation. Data were analyzed using Excel (Microsoft) and Prism v7 (GraphPad) and then plotted using DataGraph (Visual Data Tools).

**Measurement of cGMP binding by surface plasmon resonance (SPR) spectroscopy** — SPR measurements were conducted on a SR7500 dual-channel surface plasmon resonance spectrometer connected to an SR7100 autosampler utilizing a standard, dual-channel flow cell (Reichert Technologies). All steps were performed at 25 °C. Gold sensorchips coated with a 1500 nm linear poly-carboxylate hydrogel (HC1500M, Xantec) were installed on the instrument and pre-equilibrated with SPR buffer (50 mM MES, 150 mM NaCl, 1 mM EDTA, 0.05% Tween20, 1 mM TCEP, pH 6.9) at 20 μL/min. Sensorchips were washed sequentially with a solutions of 2 M NaCl and 10 mM NaOH for 5 min each, followed by 2 min with SPR buffer.

The sensorchip was activated for 5 min with a solution of freshly prepared, degassed 100 μM N-hydroxysuccinimide (NHS) and 200 μM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) dissolved in 1 mL of 500 mM MES pH 5.5. PKG constructs were diluted to 1 μM in 20 mM NaOAc pH 5.0 (500 μL) and injected into the left channel for 10 min until 10,000-15,000 μRIU was observed in the difference channel. Excess protein was washed from the surface for 5 min. Any remaining reactive groups on the sensorchips were deactivated by injecting 1 M ethanolamine pH 8.5 into both channels for 5 min and then washing with SPR buffer for 1 min. Solutions of cyclic nucleotides were prepared using the same SPR buffer used during the PKG I coupling steps. During cNMP binding experiments, solutions were injected at 50 μL/min for 1 min (association) and then washed with SPR buffer for 3 min (dissociation). All injected solutions were prepared in clear or amber borosilicate screw-top glass vials with robotic screw-top vial closures (Fisher Scientific). Data were reduced using Scrubber (BioLogic). Data were fit to one-site (with Hill coefficient) and two-site (specific binding) models using GraphPad Prism (GraphPad Software) and plotted using DataGraph (Visual Data Tools).

**Mass Spectrometry** — All LC-MS measurements were performed using a Xevo G2-XS quadrupole time-of-flight (QTOF) mass spectrometer connected to an Acquity ultra performance liquid chromatography system (UPLC) (Waters Corp.) in positive electrospray ionization (ESI) mode. Mass spectrometry of intact protein was achieved using a Jupiter 5-μm 300-Å, 1-mm x 150-mm C4 column (Phenomenex). Conditions: LC flow = 80 μL/min; A = water with 0.1% formic acid; B = acetonitrile; isocratic flow = 0-1 min with 90% A & 10% B, 1-4 min ramp to 10% A and 90% B; Scan range = 500 – 2000 m/z. Approximately 2 pmol of PKG Δ53 was injected per run. The resulting spectra were processed using MassLynx v4.1 and the deconvolution software, MaxEnt (Waters Corp.).

LC-MS/MS was performed to determine specific phosphorylation sites as follows: Two 150-pmol aliquots of dried PKG Δ53 or PKG Iα full-length enzyme were resuspended in 25 μL of 50 mM ammonium bicarbonate buffer (pH 8.5) and 30 μL of 0.02% ProteaseMAX (Promega). A 5-μL
aliquot of sequencing grade trypsin (Promega) was added at a 1:20 ratio (enzyme to substrate). Samples were incubated overnight at 37°C and acidified with formic acid to obtain a final concentration of 0.5%. The samples were evaporated and resuspended in 10 µL of 98% water, 2% acetonitrile, 0.1% formic acid prior LC-MS analyses. UPLC separation of the tryptic peptides was performed on an Acquity HSS T3 1.8-µm, 1.0 x 150 mm column (Waters Corp.). UPLC conditions: LC flow = 50 µL/min; column temperature = 45°C; A = water and 0.1% formic acid; B = acetonitrile and 0.1% formic acid; linear gradient from 98% A and 2% B to 65% A and 35% B over 50 min. Approximately 30 pmol of digested PKG Δ53 was injected. All samples were analyzed by data independent acquisition between 100–2000 m/z using the MSE mode with alternating low (4 V) and high energy (15-40 V) acquisitions. The MSE data were processed using ProteinLynx Global Server v3.0.1 (Waters Corp.) that searched against a consolidated database that included human cGMP-dependent protein kinase 1 (Q13976) and included possible modifications, including phosphorylations.

Size exclusion chromatography-small angle x-ray scattering (SEC-SAXS) — SAXS data pertaining to Δ53 was collected at the Stanford Synchrotron Radiation Lightsource BL4-2 using an MX225-HE detector (Rayonix). A 35 µL sample of 6 mg/mL PKG Iα Δ53 was injected onto a Superdex 200 3.2/30 column (GE) and eluted isocratically using an Et- tan FPLC (GE) at 0.05 mL/min in 50 mM MES, 300 mM NaCl, 1mM TCEP, pH 6.9 supplemented with 5 mM DTT as a radical scavenger. The eluant stream was connected in-line to a 1.5 mm quartz capillary positioned 1.7 m from the detector. Data were collected using a wavelength of 1.127 Å with a 1 second/frame exposure rate at 22°C. A total of 600 frames were collected with a measurement range of 0.0087 - 0.5126 Å⁻¹. Frames 410-459 containing the peak corresponding to Δ53 PKG were averaged and subtracted from the background (45 averaged frames of buffer alone). The data was truncated to exclude the range of 0.0087-0.016 Å⁻¹ due to radiation-induced aggregation and above 0.19 Å⁻¹ for all subsequent analyses. The scattering curve, Guinier and P(r) functions, and Porod volume were calculated using the PRIMUS program suite (64). DAMMIF/DAMMIN/DAMMAVER/DAMFILT was used to generate a total of 20 models, of which 19 were used to calculate the final 3-D envelope. Models were excluded based upon their NSD to the mean using DAMSEL. The excluded model was determined to have a NSD that was two times the standard deviation from the mean of all NSD scores using a cross-correlation matrix created. SUPCOMB was further used to fit the averaged 3-D envelope to existing heterodimeric structures of PKA RIα:C (PDBID: 2QCS), RIβ:C (PDBID: 4WBB), and PfPKG (PDBID: 5DYK) (65–67). Radii of gyration were calculated from the predicted scattering curves of the RIα:C and RIβ:C heterodimers (using PDBID: 2QCS and 4WBB, respectively) using the FoXS and ATSAS servers (https://modbase.compbio.ucsf.edu/foxs/ and https://www.embl-hamburg.de/biosaxs/atsas- online) (38, 39). Data were deposited in the SASBDB using the identifier SASDDS4 (68).

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**Author contributions:** T.M.M. and W.R.D. conceived of and designed the experiments. T.M.M., J.L.S., D.K.B., P.N., L.W.N., J.W., and D.E.M. performed experiments. T.M.M, J.L.S., L.W.N. and J.W. expressed and purified protein. T.M.M and J.L.S. analyzed SPR and enzyme kinetics experiments. D.E.M., J.L.S., and T.M.M. analyzed mass spectrometry data. T.M.M. and D.K.B. analyzed SEC-SAXS data. T.M.M., J.L.S., and W.R.D. wrote the manuscript with contributions from D.E.M. and D.K.B.
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Table 1: Phosphopeptides of Δ53 PKG Iα.

| Precursor MH⁺ (Da) | z | Start | End   | Sequence                             | Modifications | Retention Time (min) | Intensity (x10³) |
|-------------------|---|-------|-------|---------------------------------------|---------------|----------------------|------------------|
| 1320.665          | 2.0| 61    | 72    | (R)AQGISAEPQTYR(S)                    |               | 19.71                | 9,523            |
| 2262.055          | 2.7| 515   | 533   | (K)TWTFGTPYVAPEIILNK(G)               | PO₄-T517      | 44.89                | 1,591            |
| 2182.082          | 2.7| 515   | 533   | (K)TWTFGTPYVAPEIILNK(G)               |               | 42.80                | 123              |
| 2390.150          | 3.0| 514   | 533   | (K)KTWTFGTPYVAPEIILNK(G)              | PO₄-T517      | 41.03                | 260              |
| 2310.173          | 3.0| 514   | 533   | (K)KTWTFGTPYVAPEIILNK(G)              |               | 39.02                | 10               |
Table 2: Small angle X-ray scattering data collection and analysis

(a) Sample details

| Organism          | Bos taurus          |
|-------------------|---------------------|
| Source (Catalogue No. or reference) | Sf9                |
| UniProt sequence ID (residues in construct) | NP_776861 (54-671) |
| Extinction coefficient $\varepsilon (A_{280}, 0.1\% (w/v))$ | 1.069              |
| Molecular mass $M$ from chemical composition (Da) | 70434              |

SEC-SAS

| Loading concentration (mg/ml$^{-1}$) | 6                  |
| Injection volume ($\mu$l)          | 35                 |
| Flow rate (ml/min$^{-1}$)          | 0.05               |
| Average C in combined data frames (mg/ml$^{-1}$) | 0.6               |
| Solvent (solvent blanks taken from SEC flowthrough prior to elution of protein) | 50 mM MES, 300 mM NaCl, 1 mM TCEP, 5 mM DTT, pH 6.9 |

(b) SAS data collection parameters

| Source, instrument and description or reference | SSRL BL4-2 with Rayonix MX225-HE detector |
| Wavelength (Å)                                   | 1.127                                       |
| Beam geometry (size, sample-to-detector distance) | 0.3 x 0.3 mm (1.7 m)                          |
| $q$-measurement range (Å$^{-1}$)                 | 0.0087 - 0.5126                               |
| Absolute scaling method                         | N/A                                          |
| Basis for normalization to constant counts      | Transmission intensity measured by pin diode beamstop |
| Method for monitoring radiation damage, X-ray dose where relevant | fpleplots (available at BL4-2) |
| Exposure time, number of exposures              | 1 sec, 600 images                             |
| Sample configuration including path length and flow rate where relevant | 1.5 mm quartz capillary, 0.05 mL/min |
| Sample temperature (ºC)                         | 22                                            |

(c) Software employed for SAS data reduction, analysis and interpretation

SAS data reduction to sample–solvent scattering, and extrapolation, merging, desmearing etc. as relevant | Sastool |
Calculation of $\varepsilon$ from sequence Protparam (Gasteiger et al, 2005).

Basic analyses: Guinier, $P(r)$, scattering particle volume

Shape/bead modelling

Atomic structure modelling (homology, rigid body, ensemble)

Molecular graphics

### (d) Structural parameters

**Guinier Analysis**

| Parameter                        | Value       |
|----------------------------------|-------------|
| $I(0)$ (cm$^{-1}$)               | 321.37 ± 1.23 |
| $R_g$ (Å)                        | 29.71 ± 0.58 |
| $q$-range (Å$^{-1}$)             | 0.0160 - 0.1904 |
| $qR_g$ max                       | 1.32        |
| Coefficient of correlation, $R^2$| 0.98        |
| $M$ from $I(0)$ (ratio to expected value) | 75814 (1.08) |

**$P(r)$ analysis**

| Parameter                        | Value       |
|----------------------------------|-------------|
| $I(0)$ (cm$^{-1}$)               | 323.9 ± 1.8 |
| $R_g$ (Å)                        | 30.23 ± 0.25 |
| $d_{max}$ (Å)                    | 96.77       |
| $q$-range (Å$^{-1}$)             | 0.0160 - 0.1904 |
| $\chi^2$ (total estimate from GNOM) | 0.979 (0.766) |
| $M$ from $I(0)$ (ratio to expected value) | 72865 (1.03) |
| Porod volume (Å$^{-3}$) (ratio $V_p/M_{calc}$) | 105000 (1.49) |

### (e) Shape modeling results (a complete panel for each method)

**DAMMIF** (default parameters, 20 calculations)

| Parameter                        | Value       |
|----------------------------------|-------------|
| $q$-range for fitting (Å$^{-1}$) | 0.00 - 0.190 |
| Symmetry, anisotropy assumptions | P1, none    |
| NSD (Std Dev), No. of clusters   | 0.904 (0.151), 6 |
| $\chi^2$ value/range             | 0.601-0.602 |
Resolution (Å) (from SASRES) 35 ± 3
M estimate as 0.5 x volume of models (Da) 73473 (1.04)

**DAMMIN** (default parameters)
- $q$-range for fitting ($Å^{-1}$) 0.0160 - 0.190
- Symmetry, anisotropy assumptions P1, none
- $\chi^2$, CORMAP P-values 0.602, 0.999

### (f) Atomistic modelling

| Crystal Structures | PDB: 2QCS | PDB: 4WBB | PDB: 5DYK |
|--------------------|-----------|-----------|-----------|
| PKA RIIα:C heterodimer | PKA RIIβ:C heterodimer | Pf PKG |

**CRYSOL** (with default parameters)
- $q$-range for fitting 0 - 0.1904
- No constant subtraction
  - $\chi^2$ value, P-value 1.29, 0.004 1.83, 0.000 10.35, 0.000
  - Predicted $R_g$ (Å) 29.47 28.75 32.74
  - Vol (Å), Ra (Å), Dro (e Å$^{-3}$) 98423, 1.8, 0.025 94847, 1.8, 0.030 105437, 1.8, 0.00

- Constant subtraction allowed
  - $\chi^2$ value, P-value 0.82, 0.963 1.25, 0.010 9.21, 0.000
  - Predicted $R_g$ (Å) 29.84 29.11 32.74
  - Vol (Å), Ra (Å), Dro (e Å$^{-3}$) 98423, 1.4, 0.033 94847, 1.5, 0.037 105437, 1.8, 0.00

**FoXS** (with default parameters)
- $q$-range for fitting 0.0160 - 0.1904
- $\chi^2$ value 0.89 1.19 1.29
- Predicted $R_g$ (Å) 27.61 26.88 32.35
- $c_1, c_2$ 1.05, 1.19 1.05, 1.79 1.05, -2.00

### (g) Data and model deposition IDs

| SASBDB | SASDDS4 |
Table 3: Binding and Activation of PKG Ia Constructs

| PKG Ia Construct | Binding (SPR) | Activation ($^{32}$PO$_4$-assay) |
|-----------------|--------------|----------------------------------|
|                 | $K_0$ ($\mu$M) | $K_0^{hi}$ ($\mu$M) | $n_H$ | $K_A$ ($\mu$M) | $K_A^{hi}$ ($\mu$M) | $n_H$ | $K_D$ ($\mu$M) | $K_D^{hi}$ ($\mu$M) | $n_H$ |
| Full Length cGMP | 7.9 ± 1.1 | 1.6 ± 0.3 | 6 | 0.60 ± 0.04 | 0.60 ± 0.04 | 12 | 1.58 ± 0.06 | 1.58 ± 0.06 | 6 |
|                 | cAMP        | 387 ± 37 | 51.7 ± 25.4 | 6 | 0.76 ± 0.03 | 0.76 ± 0.03 | 5 | 1.75 ± 0.09 | 1.75 ± 0.09 | 6 |
| ΔS3 cGMP        | 2.9 ± 0.4 | 0.74 ± 0.06 | 5 | 0.76 ± 0.03 | 0.76 ± 0.03 | 4 | 90.2 ± 33.9 | 90.2 ± 33.9 | 6 |
|                 | cAMP        | 297 ± 21 | 36.6 ± 9.2 | 4 | 0.76 ± 0.03 | 0.76 ± 0.03 | 5 | 685 ± 174 | 685 ± 174 | 6 |
| 1-326 cGMP      | 21.3 ± 1.3 | 8.4 ± 2.5 | 6 | 0.81 ± 0.03 | 0.81 ± 0.03 | 4 | 74.4 ± 38.6 | 74.4 ± 38.6 | 6 |
|                 | cAMP        | 64.3 ± 5.3 | 16.9 ± 3.9 | 4 | 0.72 ± 0.03 | 0.72 ± 0.03 | 5 | 509 ± 112 | 509 ± 112 | 6 |
| 78-326 cGMP     | 30.0 ± 1.1 | 3.2 ± 3.1 | 4 | 0.90 ± 0.02 | 0.90 ± 0.02 | 4 | 350 ± 44.4 | 350 ± 44.4 | 6 |
|                 | cAMP        | 340 ± 10 | 4.8 ± 3.1 | 4 | 0.92 ± 0.02 | 0.92 ± 0.02 | 4 | 350 ± 10 | 350 ± 10 | 6 |

The data are represented as the mean ± standard deviation. The data are represented as the mean ± standard deviation.
Figure 1: Design of the Δ53 construct based on sequence homology with PKA R-domains. A) The domain architecture of PKG Iα (charcoal) depicting the truncation of the dimerization domain to generate Δ53 (red) alongside PKA RIα (cyan) and PKA catalytic (grey) domains. The domain architecture is described as D/D: dimerization domain, AI: autoinhibitory domain, cGMP-A/B: cGMP binding sites, SW: switch helix (25), N/C-lobe: catalytic domain. B) A multiple sequence alignment shaded by BLOSUM62 score is shown for PKG Iα relative to PKA regulatory domain isoforms. The autoinhibitory sequences of PKG Iα (red) and PKA:RIα (cyan) are outlined. C) The crystal structure of the RIα:C heterodimer (2QCS) detailing the contacts between the autoinhibitory domain segment beginning at K92 (cyan) and the catalytic domain (grey).
**Figure 2:** Biophysical characterization of Δ53.  
A) Coomassie-stained 12% SDS-PAGE of PKG Iα full-length and Δ53 under denaturing and reducing conditions. B) Size exclusion chromatography traces of Δ53 in its apo (black) and cGMP-saturated (red) forms. C) A raw m/z trace (inset) and the deconvoluted TOF-MS displaying the two predominant masses observed for Δ53 corresponding to singly and doubly phosphorylated species. The mass correlated to the location of an unphosphorylated monomer is also denoted.
Figure 3: SEC-SAXS analysis of PKG Iα Δ53 in the autoinhibited state. A) SEC-SAXS trace of Δ53 depicting scattering intensity (red circles, right axis) and $R_g$ values (black circles, left axis) estimated from Guinier plots of each detector exposure frame. B) The intensity profile ($I_q$ vs $q$) for Δ53 was derived from the averaging of frames 410-459 from panel (a). C) Kratky plot of the scattering profile of Δ53 determined using the averaged scattering data from panel (B). D) The resulting fit and residual plot of the intensity profile ($I_q$ vs $q$) for Δ53 by CRYSOL (allowing constant subtraction) for R1α:C (PDBID: 2QCS, green) and R1β:C (PDBID: 4WBB, red). The 3D envelope of the scattering curve derived from Δ53 (grey balls) fit to the crystal structures of (E) R1α:C (NSD=1.06) and (F) R1β:C (NSD=1.09) heterodimers.
Figure 4: Biochemical characterization of Δ53. A) Domain diagram depicting the constructs used for the activation and binding studies. B) Activation of PKG constructs. Data are represented as the mean ± SD. Ba) Activation of PKG Iα full-length (black) and Δ53 (red) with cGMP. Significant differences in activation by individual cGMP concentrations were confirmed by two-way ANOVA (p<0.005), and denoted by an asterisk. Bb) Normalized activation of PKG Iα (black) and Δ53 (red) with cGMP (solid lines) and cAMP (dotted lines). Shaded areas depict the mean K_d ± SD of the high affinity cyclic nucleotide binding site for cGMP (solid) and cAMP (dashed). C) cNMP binding curves associated with PKG constructs in (A) as measured by SPR spectroscopy. Data are represented as the mean ± SD and fit with a two-site binding model. Ca) Binding curves showing cGMP (solid lines) and cAMP (dotted lines) association with full-length PKG Iα full-length (black), and Δ53 (red). Cb) Binding curves denoting PKG Iα:1-326 (blue) and PKG I:78-326 (green) with cGMP (solid lines) and cAMP (dotted lines).
Figure 5: Putative model of activation of FL and Δ53 PKG Iα. The model depicted hypothesizes that one cyclic nucleotide bound to the high-affinity A-site is sufficient to activate both the full-length and Δ53 PKG Iα constructs. Cooperativity arises the in trans interaction of protomers at the knob/nest site and not from cooperative cyclic nucleotide binding. In the Δ53 construct, cooperativity is not observed and cannot be facilitated due to the lack of dimerization. The organization of the full-length enzyme must also lend itself to generate a cGMP selectivity filter for the A-site based upon our full-length and truncated data.
An N-terminally truncated form of cyclic GMP-dependent protein kinase Iα (PKG Iα) is monomeric, autoinhibited, and provides a model for activation
Thomas M. Moon, Jessica L. Sheehe, Praveena Nukareddy, Lydia W. Nausch, Jessica Wohlfahrt, Dwight E. Matthews, Donald K. Blumenthal and Wolfgang R. Dostmann

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