A Crystal Structure of the Cyclic GMP-dependent Protein Kinase Iβ Dimerization/Docking Domain Reveals Molecular Details of Isoform-specific Anchoring*§

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Darren E. Casteel*, Eric V. Smith-Nguyen‡, Banumathi Sankaran§, Sung H. Roh§, Renate B. Pilz¶, and Choel Kim†

From the †Department of Medicine and Cancer Center and ‡Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093, the ¶Berkeley Center for Structural Biology, Lawrence Berkeley National Laboratory, Berkeley, California 94720, and the §Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

Cyclic GMP-dependent protein kinase (PKG) is a key mediator of the nitric oxide/cGMP signaling pathway and plays a central role in regulating cardiovascular and neuronal functions. The N-terminal ~50 amino acids of the kinase are required for homodimerization and association with isoform-specific PKG-anchoring proteins (GKAPs), which target the kinase to specific substrates. To understand the molecular details of PKG dimerization and gain insight into its association with GKAPs, we solved a crystal structure of the PKG Iβ dimerization/docking domain. Our structure provides molecular details of this unique leucine/isoleucine zipper, revealing specific hydrophobic and ionic interactions that mediate dimerization and demonstrating the topology of the GKAP interaction surface.

As the main effector of the nitric oxide/cGMP signaling cascade, cGMP-dependent protein kinase (PKG) regulates smooth muscle tone, inhibits platelet activation, and modulates neuronal functions (1). In mammalian cells, two different genes encode a soluble type I PKG and a membrane-anchored type II PKG (1). Both enzymes form homodimers through an N-terminal leucine/isoleucine zipper domain. PKG I has two splice variants (α and β) that differ in the first ~100 amino acids, resulting in unique dimerization and autoinhibitory domains. The leucine/isoleucine zipper domain mediates interaction with isotype-specific G-kinase-anchoring proteins (GKAPs), targetting PKG Iα and Iβ to different subcellular compartments and intracellular substrates (2); therefore, we refer to this region as the dimerization and docking (D/D) domain. The domain organization of PKG is shown in Fig. 1. The N-terminal D/D domain is followed by an inhibitory sequence (I5), tandem cyclic nucleotide binding pockets, and the catalytic domain. The D/D domain contains a distinct primary sequence, with a repeating pattern of leucines and isoleucines every seven residues (Fig. 1). This pattern is referred to as a heptad repeat, and the positions of residues are labeled a–g.

Specific binding partners for PKG Iα include the myosin phosphatase targeting subunit (MYPT1) of myosin light chain phosphatase and the regulator of G-protein signaling-2 (RGS-2) (3, 4). Phosphorylation of MYPT1 by PKG Iα activates its phosphatase activity, leading to dephosphorylation of myosin light chain, which desensitizes the contractile apparatus response to calcium, resulting in vasorelaxation (3). RGS-2 functions as a GTPase-activating protein for Gαq subunits of heterotrimeric G-protein complexes, and phosphorylation of RGS-2 by PKG Iα increases its activity toward Gαq, uncoupling downstream signaling from Gαq-linked receptors for vasoconstrictive agents (4). Specific binding partners for PKG Iβ include the inositol triphosphate receptor-associated PKG substrate (IRAG) and the isoform-specific anchoring protein; D/D, dimerization/docking; IS, tandem repeat regions for PKG Iβ dimerization; IRAG, inositol triphosphate receptor-associated PKG substrate; MYPT1, myosin phosphatase targeting subunit; RGS-2, regulator of G-protein signaling-2; TFII-I, transcription factor II-I.

The physiological importance of GKAP interactions with PKG dimers is illustrated by the cardiovascular abnormalities found in transgenic mice expressing a dimerization-deficient form of PKG Iα (9). These mice show a complex cardiovascular phenotype, which includes impaired vasodilatation with systemic hypertension and cardiac hypertrophy despite high basal PKG activity. Because dimerization is necessary for PKG Iα binding to MYPT1, RGS-2, and other interacting proteins, the observed phenotype is thought to be directly linked to the loss of specific protein–protein interactions.

To understand the molecular details of PKG Iβ dimerization and interaction with GKAPs, we solved a crystal structure of a selenomethionine-substituted D/D domain of PKG Iβ. To our knowledge, this represents the first crystal structure for PKG, revealing the unusual features of a non-canonical leucine zipper and the atomic details of the GKAP docking site.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The sequence encoding amino acid residues 4–55 of PKG Iβ was amplified by PCR using the following set of primers: 5’-CATGGATCCT-TGCGGGATTTACAG-TAC-3’ (sense) and 5’-CATGAATTCATGGTCGGATCAC-3’. The on-line version of this article (available at http://www.jbc.org/) contains supplemental Table S1 and Figs. S1–S3.

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‡ The atomic coordinates and structure factors (code 3NMD) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ The on-line version of this article (available at http://www.jbc.org/) contains supplemental Table S1 and Figs. S1–S3.

1 To whom correspondence should be addressed: Dept. of Pharmacology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: ckim@bcm.edu.

2 The abbreviations used are: GKAP, cGMP-dependent protein kinase-anchoring protein; D/D, dimerization/docking; IRAG, inositol triphosphate receptor-associated PKG substrate; MYPT1, myosin phosphatase targeting subunit; RGS-2, regulator of G-protein signaling-2; TFII-I, transcription factor II-I.
The domain organization of PKG Iβ is shown with the D/D domain at the extreme N terminus. The leucine/isoleucine zipper is followed by an inhibitory sequence (antisense). The PCR product was digested with a French Press high pressure cell, and the lysate was cleared by centrifugation at 50,000 × g for 90 min at 4 °C. The supernatant was passed through a 0.45-μm filter. The His-tagged PKG Iβ D/D domain was purified with the automated Profinia™ protein purification system (Bio-Rad) using the “Native IMAC” method as per the manufacturer’s recommendations. The eluate was concentrated by ultracentrifugation and further purified by gel filtration over a Superdex S75 column equilibrated in buffer B.

**Crystallization and Structure Determination**—The purified protein was dialyzed against 20 mM Tris (pH 7.9), 0.3 mM Tris(2-carboxyethyl)-phosphine hydrochloride, and 10% glucose; it was then concentrated to 20 mg/ml by ultracentrifugation. Using Oryx8™ (Douglas Instruments Ltd.), ~500 commercially available conditions were screened for crystallization. Crystals were obtained in 0.1 M sodium citrate (pH 5.6) and 2.5 M hexanol. The crystals were transferred to a cryoprotectant solution (mother liquor containing 15% glycerol) and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at beamline 8.2.1 (Advanced Light Source, Berkeley, CA). Diffraction data were processed and scaled using HKL2000 (10). The final data were integrated and scaled with satisfactory statistics (see Table 1).

The crystal structure of the PKG Iβ D/D domain was determined by single-wavelength anomalous dispersion. Phenix.hys was used to locate the selenium substructure. Subsequent phasing, density modification, and model building were carried out with Phenix.autosol (11). The resulting model was manually completed in Coot (12), and restrained structure refinement implementing TLS refinement (13) resulted in a model with Rwork and Rfree of 20.1 and 24.9%, respectively. The final model was validated using ProCheck (14), and the electrostatic surface was calculated using the Adaptive Poisson-Boltzmann Solver (15). Figures were made using PyMOL (Delano Scientific). We also solved a crystal structure of the native protein; it crystallized in a C2 space group with only one coiled-coil per unit cell. Although the resolution was good (1.9 Å), the R-factors were not satisfactory, and therefore, we did not report the structure (data not shown).

**TABLE 1**

| Data set          | Se-Met Crystal* |
|-------------------|------------------|
| Space group       | C222             |
| Cell parameters (Å) | a = 62.9, b = 77.2, c = 148 |
| Wavelength (Å)    | 0.9795           |
| d_inset (Å)       | 2.27             |
| Total/unique reflections | 132,770/32,149 |
| Average redundancy | 7.1 (7.8)       |
| Anomalous redundancy | 3.7 (4.1)    |
| Completeness (%)  | 99 (100)         |
| R_work (%)        | 23.6 (10.3)      |
| R_free (%)        | 7.4 (26)         |
| Anomalous redundancy | 6.8 (25)        |
| Figure of merit   | 0.30             |
| Refined r’ and f’ values | -9.8/4.2 |
| Resolution range (Å) | 48–2.27         |
| Number of reflection (working/free) | 32085/1620 |
| Number of protein atoms (B-factor, Å²) | 2218 (55) |
| Number of hetero compound atoms (B-factor, Å²) | 38 (63) |
| Number of water atoms (B-factor, Å²) | 85 (55) |
| Root mean square deviation | -0.005 |
| Bond lengths (Å)  | 0.752            |
| Angles (°)        | 20.1/24.9        |
| Ramachandran outliers/favored (%) | 0.984 |

* Numbers in parentheses correspond to the highest resolution shell of data, which was 2.35 to 2.27 Å.

**Dimeric Sites of PKG Iβ**

The domain organization of PKG Iβ is shown with the D/D domain at the extreme N terminus. The leucine/isoleucine zipper is followed by an inhibitory sequence (antisense). The PCR product was digested with BamHI/EcoRI and inserted into a modified pRSSET expression vector to place a hexahistidine tag at the N terminus. Substitution of Met for Lys was accomplished using the QuickChange® II XL site-directed mutagenesis kit (Stratagene) per the manufacturer’s instructions. The construct was sequenced to confirm the presence of the desired mutation and absence of PCR-induced errors.

**Protein Expression and Purification of the Selenomethionine-substituted PKG Iβ D/D Domain**—Minimal medium was prepared according to a protocol developed by the G. and P. Ghosh laboratories. M9 medium salt solution (Bio101) was autoclaved, and 0.3% glucose, 2 mM MgSO₄, and 100 μg/ml ampicillin were added. Each flask with 1 liter of medium was inoculated with 90 μl of overnight starter culture and placed in a 37 °C shaker. When the A₆₆₀ reached 0.6, flasks were removed from the shaker, and an additional 0.3% glucose, 2 mM MgSO₄, and 100 μg/ml ampicillin were added. L-Selenomethionine, L-lysine, L-threonine, and L-phenylalanine were added at 100 mg/liter, and L-leucine, L-isoleucine, and L-valine were added at 50 mg/liter. PKG Iβ D/D synthesis was induced with a final concentration of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The flasks were placed in a shaker at 20 °C and were grown overnight. The flasks were pelleted, and pellets were stored at −20 °C. The pellets were thawed and resuspended in 10 ml/g of bacterial paste in buffer B (5% glycerol, 20 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.3 mM Tris(2-carboxyethyl)-phosphine hydrochloride) with 1 mM phenylmethylsulfonyl fluoride (PMSF). Bacteria were lysed using a French Press high pressure cell, and the lysate was cleared by centrifugation at 50,000 × g for 90 min at 4 °C. The supernatant was passed through a 0.45-μm filter. The His-tagged PKG Iβ D/D domain was purified by ultracentrifugation and further purified by gel filtration over a Superdex S75 column equilibrated in buffer B.
RESULTS AND DISCUSSION

We substituted methionine for Lys41 in the D/D domain of PKG Iβ (amino acids 4–55) so that single-wavelength anomalous dispersion could be used to solve the structure. We previously demonstrated that substitution of Lys41 with glutamic acid did not affect IRAG or TFII-I binding to PKG Iβ (16). Based on these results, we reasoned that the less dramatic K41M mutation would not disrupt dimer formation or affect the IRAG or TFII-I binding surface. We purified the seleno-methionine-substituted D/D domain and obtained crystals that diffracted to 2.2 Å (Table 1). In the crystal structure of the PKG Iβ D/D domain, each unit cell contained five helices (supplemental Fig. S1, labeled A–E), constituting two-and-a-half pairs of coiled-coils. One of the helices, found at the two-fold symmetry axis, was paired with another helix from a crystallographically related molecule (chain E). The equivalent α-carbons of all five helices could be superimposed with a root mean square deviation of less than 1 Å (supplemental Fig. S2A).

The dimer containing chains A and B is shown in Fig. 2A to illustrate the overall structure of the PKG Iβ D/D domain and shows a double-stranded parallel coiled-coil spanning ~70 Å in length and 28 Å in width. The two helices wind around each other to form a left-handed coiled-coil, and the dimer interface covers ~1300 Å² of surface area. Electron densities for the N-terminal His tags were not seen, indicating that they are disordered; because the N termini face the solvent, their presence should not affect the rest of the structure. Residues 4–50 of PKG Iβ are ordered in all five helices within the asymmetrical unit. There are over eight heptad repeats that lie continuously, without any stutters or stammers, with 3.6 amino acids per helical turn, and each helix forms a smooth curvature (supplemental Table S1).

Superhelical and α-helical parameters of the PKG Iβ leucine/isoleucine zipper were calculated using Twister (17) and are similar to the reported values for other coiled-coils (supplemental Table S2). A detailed view of side chain packing in the core of the PKG Iβ leucine/isoleucine zipper is shown in Fig. 2B. Typical leucine zippers, such as the one in the yeast transcription factor GCN4 (18), contain leucines predominantly in position d with position a residues more varied; in contrast, in the PKG Iβ D/D domain, leucines and isoleucines fall predominantly in position a with the majority of position d residues being charged or hydrophilic (Fig. 1). These d position residues are Lys14, Arg21, Lys35, and Tyr49, and the structure reveals how the hydrophobic regions of their side chains participate in interhelical hydrophobic interactions that form the
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interhelical salt bridges. Interhelical salt bridges of coiled-coils within the asymmetric unit of the selenomethionine crystal are shown as viewed looking down the superhelical axes. oA-weighted 2Fα − F maps contoured at 1.5 standard deviations above the mean are shown in blue cage.

FIGURE 3. Details of the interhelical salt bridges. Interhelical salt bridges of coiled-coils within the asymmetric unit of the selenomethionine crystal are shown as viewed looking down the superhelical axes. oA-weighted 2Fα − F maps contoured at 1.5 standard deviations above the mean are shown in blue cage.

FIGURE 4. Details of the GKAP docking site. A, a surface representation of the PKG Iβ D/D domain is shown and colored according to its electrostatic potential (blue = electropositive; red = electronegative). Residues known to mediate GKAP binding are labeled. B, structural alignment of two-and-a-half pairs of coiled-coils in the crystal unit showing side chains involved in GKAP binding.

extensive hydrophobic core. Interestingly, these hydrophilic residues pack in a “knobs-into-holes” fashion (19) with the exception of Tyr49, whose side chain is too bulky. The Tyr49 hydrophobic rings from each helix are stacked on top of each other in a head-to-tail fashion, with the hydrophilic hydroxyl groups facing the solvent (Fig. 2C). At Tyr49, there is a slight distortion of the Ca backbone in one of the helices of each pair, and all residues C-terminal to Tyr49 that are not involved in crystal packing contacts are disordered.

The presence of hydrophilic residues in the core of coiled-coils is known to direct dimer formation and inhibit trimer formation (20). Like leucine and isoleucine residues, their hydrophobic regions pack tightly into the “holes” created by core residues on the neighboring strand. This tight packing is illustrated by the distance between Cβ1 of Leu15 at position a and Cβ1 of Lys14 at position d (3.8 Å), which is comparable with the distance between Cβ1 of Leu47 at position a and Cβ1 of Leu7 at position d (3.6 Å) (Fig. 2B). Arg21 and Lys35 pack in a similarly tight manner. To accommodate their charged groups, the side chains of position d lysine and arginine residues are in a “bent” conformation, pointing toward the outer surface, and their conformation is partly stabilized by interhelical salt bridges.

Interhelical salt bridges are one of the essential features of coiled-coil formation and are required for both stability and specificity (21); they are commonly formed between residues in the g and e’ positions (21, 22). In contrast, our structure shows electrostatic bonds between residues at positions d and e’ (Fig. 3). Specifically, these pairs are Arg21–Asp22 and Lys35–Asp36’. The electrostatic interactions are seen in all helical pairs within the unit cell, independent of crystal contacts, and are preserved in a two-fold symmetry fashion. Spheric constraints imposed by the bulky Arg21 side chain cause the bond length between Arg21–Asp22 (2.7–3.5 Å) to be longer than those between Lys35–Asp36’ (2.3–2.7 Å). The side chain of Asp36’ approaches the guanidinium moiety of Arg21 from the side, making two hydrogen bonds, whereas the side chain of Asp36’ makes a slightly shorter bond with Lys35.

Sequence comparison between PKG Iα and Iβ reveals that residues that form the d–e’ salt bridges in PKG Iβ are relatively conserved in Iα, with basic residues (Lys15 and Lys29) at the d positions followed by acidic residues (Glu16 and Glu29) at the e’ positions (supplemental Fig. S3). Likely, these residues also form interhelical electrostatic pairs.

We have previously demonstrated that PKG Iβ Asp26, Glu29, and Glu31 interacted with positively charged residues within IRAG (16). These residues, plus Glu27, were also involved in the interaction with TFII-I (16). Fig. 4A shows that these residues form a highly electronegative patch on the surface of the PKG Iβ D/D domain. Importantly, a structural alignment of five PKG Iβ helices from the unit cell demonstrates that the rotamer positions of the acidic side chains that make up the GKAP binding surface are conserved (Fig. 4B). The fixed position of the acidic side chains within the folded structure suggests the presence of a preformed, specific, and stable docking surface for GKAP interaction. The interhelical salt bridges flanking the GKAP docking surface likely contribute to its stability.

The crystal structure of the PKG Iβ D/D-domain presented here provides an important step toward understanding the interactions that mediate homodimerization of type I PKGs and the targeting of PKG Iβ to specific subcellular locations and substrates through docking by GKAPs. This work builds on a number of past studies by others that have probed the structure of the PKG I D/D domain using circular dichroism, NMR, and site-directed mutagenesis (23–25). Initial analysis by far-ultraviolet circular dichroism of the N-terminal 39 residues of PKG Iα demonstrated that the structure was stably helical, with the stability interpreted as indicating dimerization (23, 24). NMR studies indicated that the two monomers bound in a parallel
orientation (24). Subsequently, site-directed mutagenesis coupled with gel filtration studies demonstrated the critical role of a position leucine and isoleucine residues in mediating stable dimerization of PKG Iβ (25).

Our crystal structure shows the topology of the GKAP binding site in PKG Iβ, but the molecular details of PKG-GKAP binding will await crystal structures of their complexes. The highly acidic patch seen in PKG Iβ is not present in PKG Iα; two of the acidic residues (Asp26 and Glu31) are substituted by basic residues (supplemental Fig. S3). Although this region in PKG Iα is highly charged, the overall charge distribution and topology are likely quite different from PKG Iβ. As we have previously shown, these amino acid differences explain, at least in part, isotype-specific protein-protein interactions (16). To our knowledge, this work represents the first crystal structure for any domain of PKG; it reveals not only unusual features of a unique leucine/isoleucine zipper but also provides insight into the mechanism of specific GKAP docking.

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