Table S1. Cyclic nucleotide binding affinities of PKG Iβ$_{(219-369)}$ wild-type and mutants.

	K_D ± SEM* (n) direct measurements		EC_{50} ± SEM* (n) competition experiments	
HsPKG Iβ$_{(219-369)}$	**8-Fluo-cGMP**	**8-Fluo-cAMP**	**cGMP**	**cAMP**
Wild type	157 ± 12 nM (5)	12 ± 1 µM (2)	215 ± 13 nM (8)	52 ± 8 µM (5)
Leu296Ala	907 ± 50 nM (2)	> 54 µM (2)	28 ± 1 µM (3)	91 µM (1)
Arg297Ala	9 ± 1 µM (2)	19 ± 1 µM (2)	5 ± 0.5 µM (2)	13 ± 2 µM (2)
Thr317Ala	> 9 µM (2)	> 12 µM (2)	21 µM (1) †	76 µM (1) †
Tyr351Ala	300 ± 40 nM (2)	> 8 µM (3)	6 ± 0.2 µM (2)	130 ± 25 µM (3)

*FP measurements were at least in duplicate. K_D is the dissociation constant, EC_{50} is the half-maximal effective concentration, and SEM is standard error of measurement.

†EC_{50} values measured with SPR competition assay as FP competition was not possible.

Table S2. Measured activation constants of HsPKG Iβ$_{(5-686)}$ wild-type and mutants.

HsPKG Iβ $_{(5-686)}$	K_a ± SEM	
	cGMP	**cAMP**
Wild Type (n=2)	133 ± 32 nM	6 ± 2 μM
Leu296Ala (n=2)	360 ± 65 nM	3.3 ± 1.2 μM
Arg297Ala (n=2)	619 ± 170 nM	5 ± 0.5 μM
Thr317Ala (n=3)	159 ± 29 nM	3.6 ± 0.9 μM
Tyr351Ala (n=2)	430 ± 40 nM	5.5 ± 0.6 μM

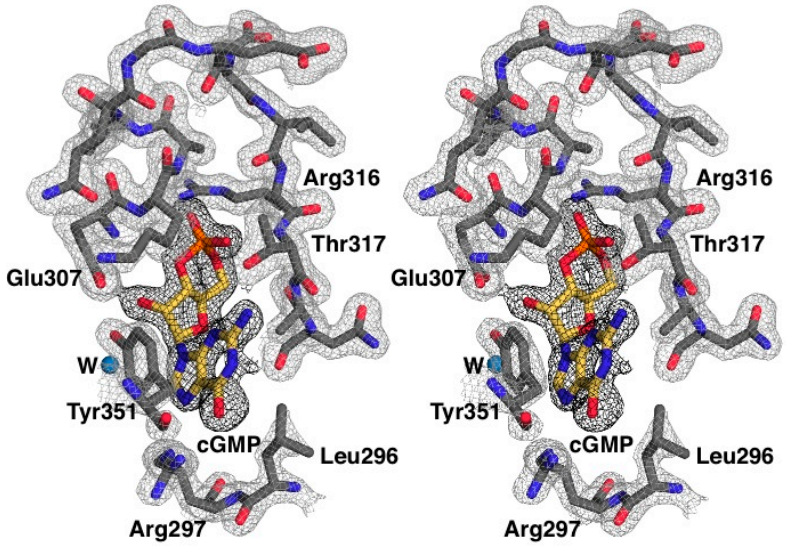

Figure S1. A stereo view of a Fo-Fc omit map showing the electron density for the PBC, Leu296, Arg297, and Tyr351 and cGMP. The map is contoured at σ = 1.0. The cGMP interacting residues are labeled. An ordered water molecule is labeled as W.

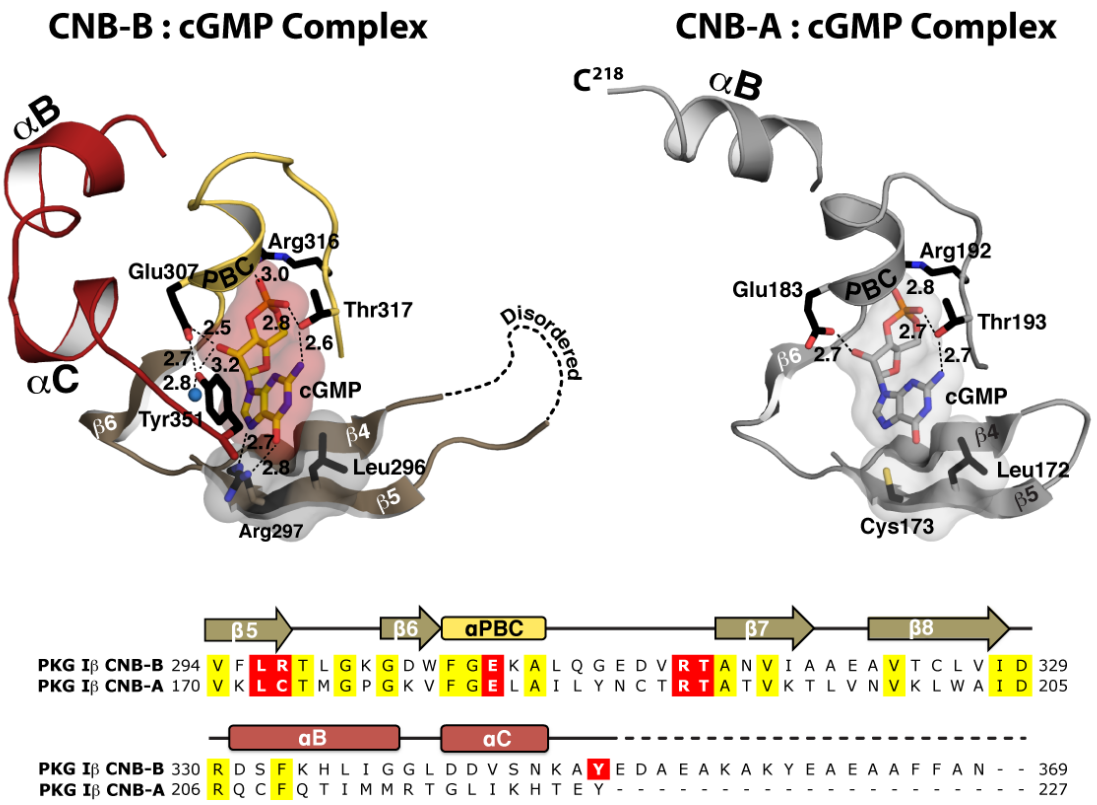

Figure S2. Structural comparison with the PKG Iβ(92-227):cGMP complex. Crystal structures of the PKG Iβ(219-369):cGMP complex and the PKG Iβ(92-227):cGMP complex (PDB code: 3OD0) and the are aligned at the β-barrel and only strand β4 through PBC and the αB and αC helices are shown side by side. The cGMP interacting residues are shown in sticks with their distances to cGMP labeled in Å. In the CNB-B structure, an ordered water molecule near Tyr351 is shown as a blue sphere. Aligned sequences for these regions are shown below with fully conserved residues shaded yellow and cyclic nucleotide binding residues through their side chains are shaded in red. Secondary structural details are indicated above the sequences.

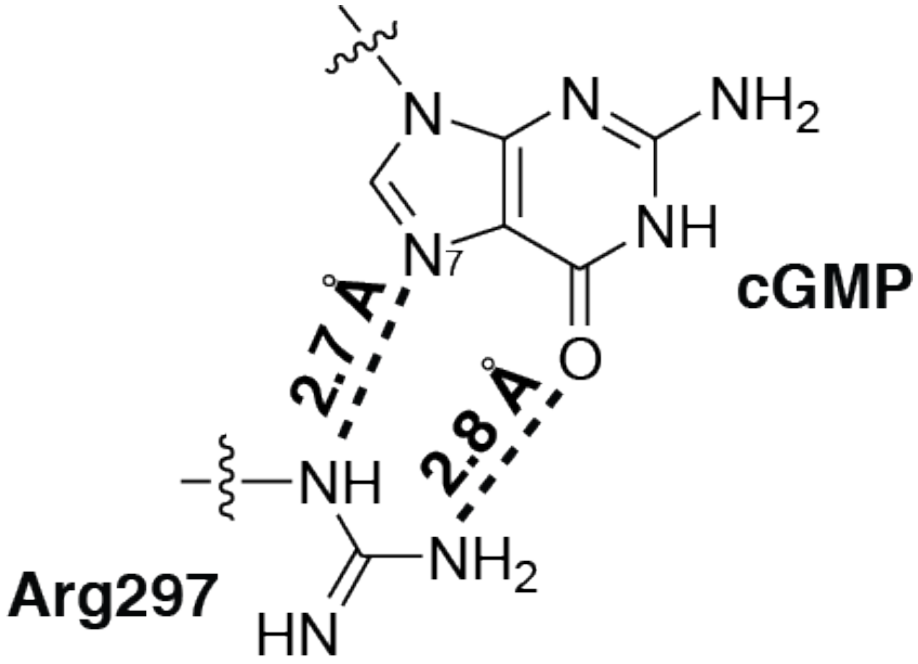

Fig. S3. Arg297 of CNB-B interacts with the guanine moiety of cGMP. In the cGMP-bound structure, Arg297 orients its protonated amine and Nε within hydrogen bonding distance of the carbonyl group and N7 nitrogen on cGMP.

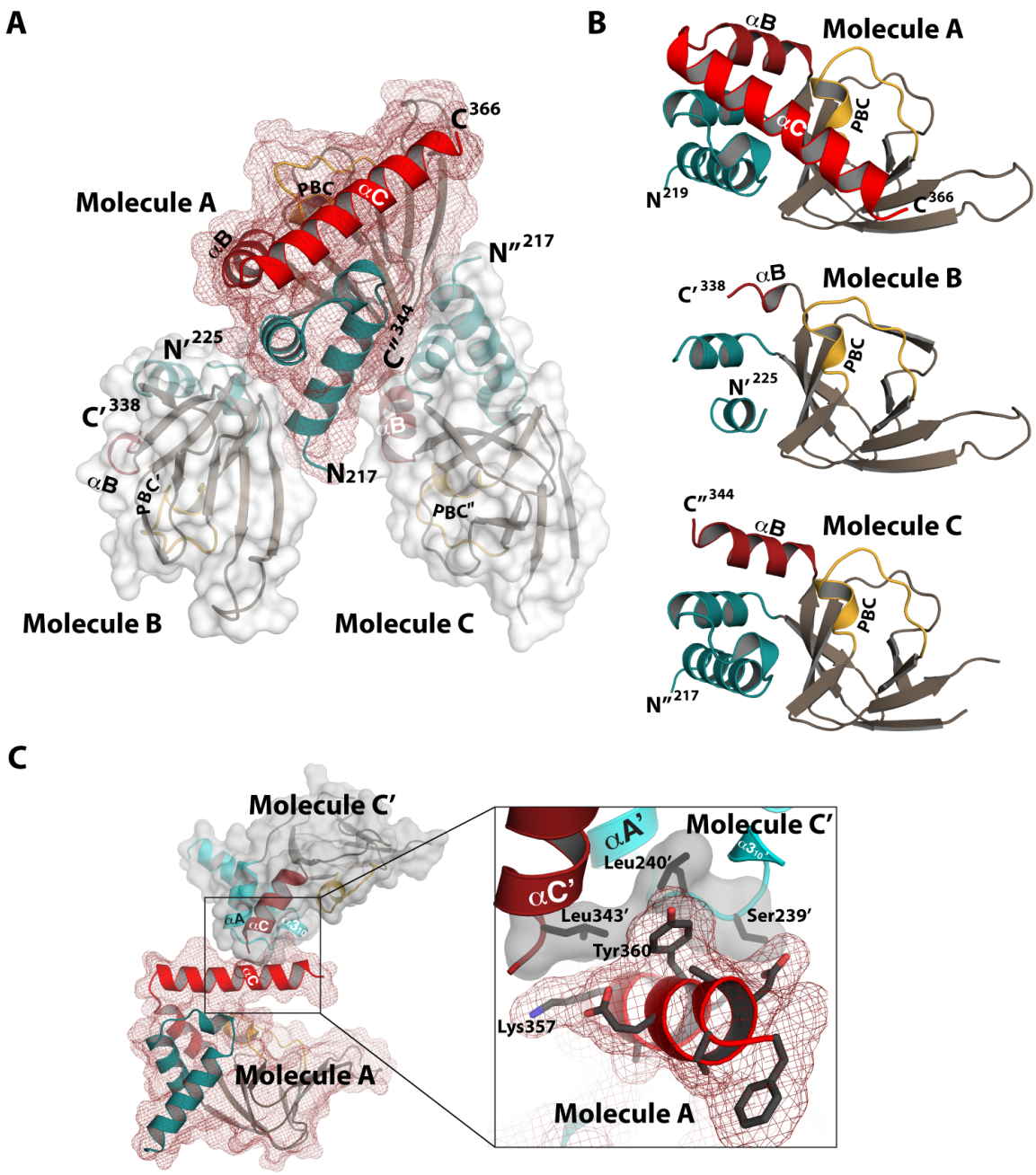

Fig. S4. Apo crystal structure of PKG Iβ (219-369). The crystal packing arrangement within the unit cell of apo crystal of PKG Iβ (219-369) is shown on the left (*A*) and individual molecules are shown on the right (*B*). In panel A, the surface of Molecule A is shown with a red mesh are the others are shown with a gray transparent surface. While the β-barrel was fully ordered in all three molecules, only one molecule (Molecule A,

shown in a red mesh) showed fully ordered helical regions including the N-terminal helices, αB and αC helices. The β-barrel is colored in tan, the N-terminal helices in teal, the phosphate binding cassette (PBC) in yellow and the αB and αC helices in red. The αC helix of Molecule A is ordered due to crystal contacts with a molecule in the adjacent unit cell. (C) The crystal contact between the αC helix of Molecule A and Molecule C' of the adjacent unit cell is shown. Molecule A is shown with its surface in a red mesh and Molecule C is shown with a gray surface. A zoomed in view of the crystal contact is shown on the right, depicting the hydrophobic surface of Molecule C', shown in a transparent gray surface, and its contact with the αC-helix of Molecule A, shown in a red mesh. The hydrophobic arm of Lys357 and the aromatic ring of Tyr360 of molecule A dock onto the hydrophobic surface formed at the loop between the 3_{10} and A-helices and the αC-helix of Molecule C' .

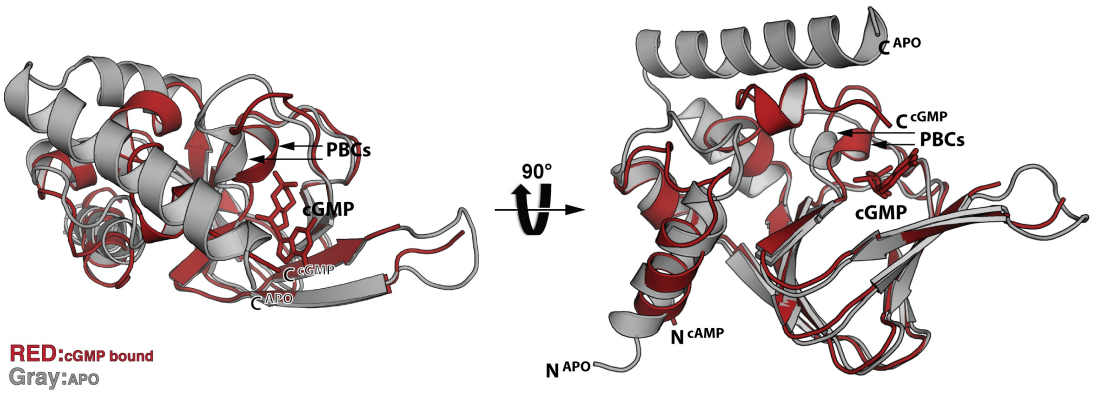

Fig. S5. Structural comparison of apo and cGMP-bound structures. The cGMP-bound structure is shown in red and the apo structure is shown in gray. The β-barrel region shows little change between the two structures, with an overall RMSD of 1.1 Å for 87 Cα atoms.

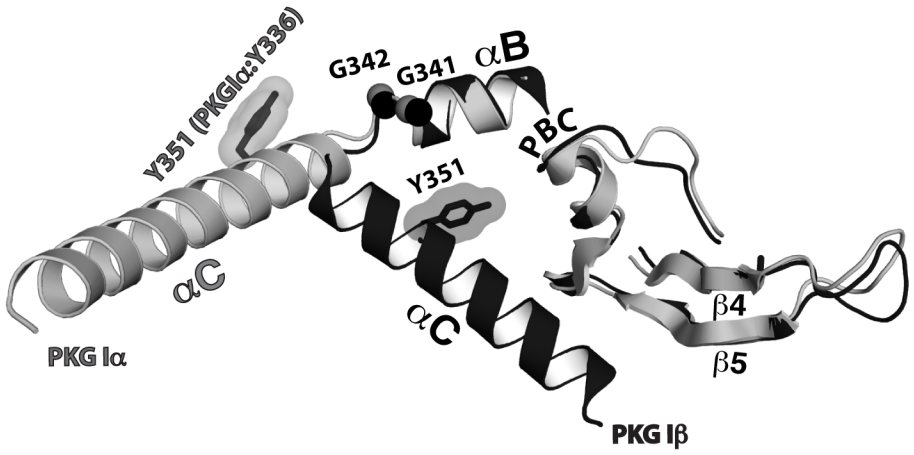

Fig. S6. Structural alignment with PKG Iα. Structures of PKG Iβ 219-369 (in black) and PKG Iα 79-356 (in gray) are aligned at the β barrel. Only strand β4 through PBC and the αB and αC helices are shown. The β barrels of the two structures align well with an rmsd of 1.0 Å for equivalent 82 Cα atoms. Cα atoms of the GlyGly hinge residues (Gly341 and Gly342) are shown in balls, and the capping residue in sticks with transparent surface. In the structure of PKG Iα, residues 331-355 (corresponding to residues 347-371) comprise the ordered αC helix, while in the structure of PKG Iβ, residues 347-366 comprise the ordered αC helix. In the crystal structure of PKG Iα 79-356, the side chain of Tyr336 is not ordered and we model it to show the relative location.

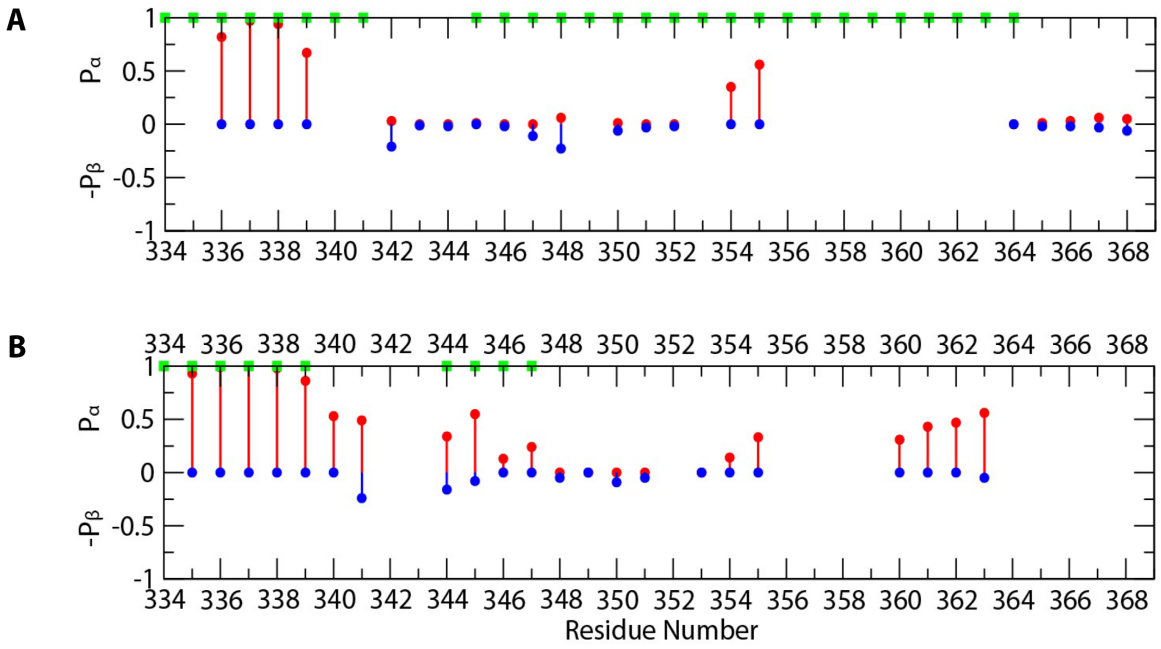

Fig. S7. Secondary structure probabilities computed based on observed chemical shifts for the C-terminal αB and αC elements of (*A*) apo and (*B*) cGMP-bound PKG Iβ 219-369. The α-helix (red points) and β-strand (blue points) probabilities are shown as positive and negative values, respectively, and the α-helix-structured residues observed from the respective X-ray structures are marked as green points with a value of 1. Note that the α-helix-structured residues indicated for the apo-state X-ray structure are based on the one molecule in the unit cell for which the αC helix was resolved.

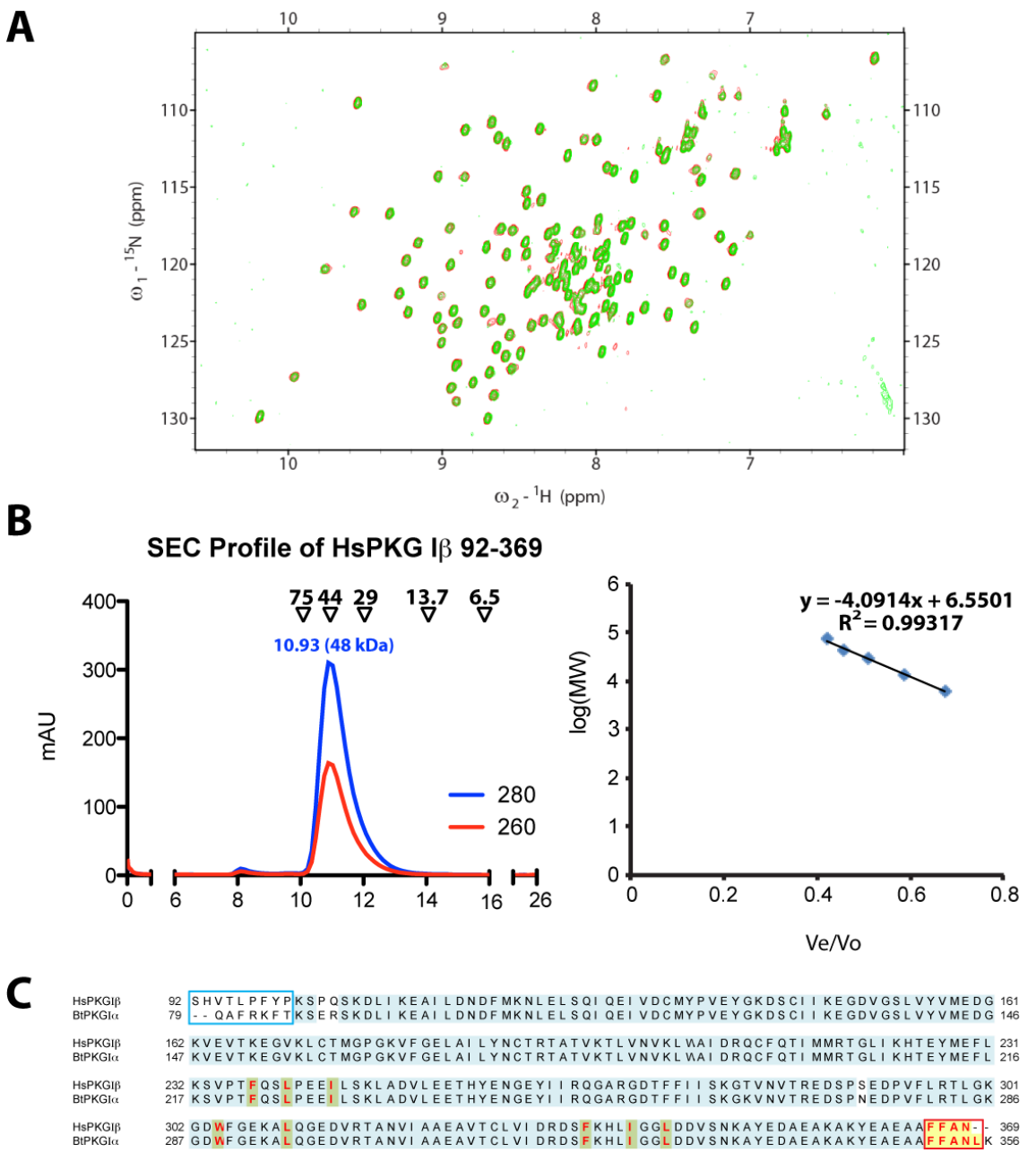

Fig. S8. Oligomeric states of PKG Iβ constructs in solution. (A) ^{1}H,^{15}N-HSQC NMR spectra for apo CNB-B at concentrations of 12μM (green) and 300μM (red). Dilution of the sample results in no significant change in ^{1}H,^{15}N-HSQC spectra. (B) Size exclusion chromatography profile of HsPKG Iβ 92-369. The standard curves for these experiments were obtained from the following proteins: conalbumin, 75 kDa; ovalbumin, 44kDa;

carbonic anhydrase, 29 kDa; ribonuclease A, 13.7 kDa; aprotinin, 6.5 kDa. The formula was derived from the linear regression curve and used to estimate the molecular weight of HsPKG Iβ 92-369. Migration of HsPKG Iβ 92-369 on a size exclusion column is consistent with a 48 kDa monomer, suggesting this construct does not dimerize in solution. (C) Sequence alignment of HsPKG Iβ 92-369 with BtPKG Iα 79–356 (NCBI Reference Sequence: NP_776861.1, PDB: 3SHR). Residues at the N-terminus that differ between the two constructs are boxed in blue. Residues comprising the "nest" assembly described by Osborne et al. are shaded in green and printed in red, while the residues comprising the "knob" assembly are shaded in yellow and printed in red, with a red box indicating alignment of the "knob" assembly in both constructs.