Supplementary Materials for

Molecular Basis of Allosteric Regulation and Pharmaceutical Targeting of Protein Kinase Cβ

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The PDF file includes:

Materials and Methods

Reagents, cell lines, antibodies, plasmids

HEK293F cells (Thermofisher) and MCF7AC1 cells were grown in DMEM with 10% (v/v) Fetal Bovine Serum (Gibco), 50 U μL^{-1} penicillin, 50 $\mu g\ mL^{-1}$ streptomycin, and 0.5 mmol L-1 sodium pyruvate at 37°C in 5% CO_2 atmosphere for adherent growth. Suspension cultures of HEK293F cells were grown in CDM4HEK (Cytiva) media supplemented with 20 mM glutamine, 12 U μL^{-1} penicillin, and 12 $\mu g\ mL^{-1}$ streptomycin at 37°C in 8% CO_2 atmosphere at 135 rpm in a Multitron shaker incubator (HT Infors) for suspension growth. Antibodies used in this study: Anti PKCβ phospho-T500 (Abcam ab5817) Anti-phospho T642 (Abcam ab75657), and anti-phospho S661 (Abcam 192184). DNA encoding PKC constructs was cloned into pMCentr2 (DNASU) and recombined into mammalian protein expression vector pcDNA6.2/N-YFP-Dest using LR Clonase II (Invitrogen). Mutant PKCβI plasmids were generated using the Quickchange kit (Stratagene).

Expression and purification of PKCβI and PKCβII

Recombinant plasmid was mixed with 1 mg/mL polyethyleneimine pH 7.5 (Polysciences, Inc.) in a 1:3 ratio to form a DNA-PEI mixture used to transfect HEK293F cells in suspension culture as described[1] and grown in HyClone HyCell Transfx-H (Cytiva) medium supplemented with 200mM Glutamine (Thermofisher) and 0.075% Pluronic (Gibco 24040-032). Transfected cells were grown for 72 hours at 37ºC, while cell count and protein expression (GFP) level were monitored every 24 hours. PKCβ expressing culture was spun down at 5000xg for 10 minutes, followed by resuspension of pelleted cells in 1X PBS containing 0.1X Roche EDTA-free protease inhibitor cocktail (Sigma). Centrifugation step was repeated to obtain cell pellet that was used directly for protein prep or frozen down at -80ºC for storage.

Thawed HEK293F cells expressing PKCβ were lysed at 4ºC with a lysis buffer solution consisting of 50 mM Tris pH 7.4, 300 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerol phosphate, 1 mM TCEP, 2 mM benzamidine, 2 μg/mL leupeptin, 0.5 mM sodium orthovanadate, 0.5% CHAPS, and 1:100 protease inhibitor cocktail (Sigma P8849) as described[2]. Lysate was sonicated with a Branson sonicator at 50% power for 10 seconds (x2) and spun down in a Lynx 4000 centrifuge (Thermo Fisher) at 15,000 g, 4ºC for 10 minutes. The clarified lysate was bound and recycled 3x over a GFP-enhancer nanobody linked NHS Sepharose (Cytiva 45002965) resin bed[1,3] equilibrated in lysis buffer. Protein bound resin was washed in 3x resin volume with the same lysis buffer. TEV cleavage of the YFP tag was completed overnight by incubating the resin with 1.5x resin volume TEV cleavage buffer containing 50 mM Tris pH7.4, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerol phosphate, 1 mM TCEP, 0.5 mM sodium orthovanadate, and 0.25% CHAPS, supplemented with 0.09 mg/mL TEV protease. Cleaved protein was eluted from the resin using buffer without TEV protease, and the presence of protein in eluted fractions was monitored using a Coomassie-stained SDS-PAGE gel.

Following purification, PKCβ protein was polished on an ÄKTA go FPLC system (Cytiva) to remove TEV protease and further purify protein for biochemical assays and crystallization. TEV eluted protein was diluted (1:3) with a low salt buffer containing 50 mM Tris pH 8.0 and 1 mM TCEP prior to being loaded onto a HiTrap Q HP anion exchange column (Cytiva). A salt gradient was introduced by the gradual addition of buffer supplemented with 1M NaCl at a flow rate of 5 mL min^{-1}. Gradient fraction elution continued to a final of 60% or 600mM NaCl. Eluted fractions containing PKCβ were combined and concentrated to 0.5 mL using a 10K cut off centrifugal filter (Sartorius,Vivaspin), pretreated overnight with 3% PEG 3350 (Jena Biosciences) at 4ºC to avoid protein loss through binding to filter. Concentrated protein was subjected to final purification on a Superdex 200 Increase 10/300 GL column (Cytiva) using 20mM Tris pH 8.0, 100mM NaCl, 2mM $MgCl_2$, and 1mM TCEP at a flow rate of 0.5 mL min^{-1}. All FPLC elution fractions were monitored

for protein presence using Coomassie-stained SDS-PAGE gel. Fractions containing PKC were pooled and concentrated to 10 mg/mL using a 10K centrifugal filter (Sartorius, Vivaspin) for crystallization, or buffer exchanged into storage buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 0.5 mM TCEP, 25% (v/v) glycerol) and stored at -80 ºC prior to use in kinase assays. Typical yield of final, purified PKCβI/II proteins (77kDa) was 3 to 6 mg per litre of culture.

Lambda Phosphatase Reactions

To generate dephosphorylated PKC, 7 µg PKC protein were incubated at 37 °C for 2 hours with 1400U Lambda Phosphatase (New England BioLabs P0753S) and its 1x reaction buffer according to the manufacturer's protocol. As a control, 7 µg PKC protein was incubated under the same conditions in the absence of Phosphatase. After the incubation period, loading dye was added and the reactions were heated to 75 ˚C for 5 min prior to visualization via 8% SDS-PAGE with 0 µM PhosTag or 40µM Phostag (APExBIO).

Crystallization and structure determination of PKCβI and PKCβII

Crystals of PKCβI and PKCβII were grown using the sitting-drop vapor diffusion method by mixing 200 nL of precipitant with 200 nL of protein mixture (PKCβI/II with 1mM AMPPNP). Crystals of PKCβI crystal form 1 and 2 grew in 200–300 mM sodium citrate pH 7–8 and 8–10% (w/v) PEG3350. Crystals of PKCβII grew in 100–200 mM magnesium chloride, 0.1 M MES pH 6.5, and 6–10% (w/v) PEG8000. Crystals grew over a period of 2–6 weeks at 4 °C and were transferred to a cryoprotectant containing crystallization condition supplemented with 25% (v/v) glycerol, then flash frozen in liquid nitrogen. For manganese soaks, the cryoprotectant also contained 1mM $MnCl_2$. X-ray diffraction datasets were collected at the NE-CAT beamlines (24-C and 24-E) at the Advanced Photon Source. X-ray diffraction data were processed and scaled using the HKL2000 suite[4]. Structures were solved via molecular replacement using the C1b, C2, and kinase domains from PDB entry 3PFQ[2] as search models using the PHENIX-PHASER[5]. The C1a domain and other missing regions were built manually using COOT[6], followed by refinement against the high-resolution datasets with PHENIX[7] to produce the final models.

Limited Proteolysis

0.4 mg/ mL PKC protein was digested with 1.4 µg/mL elastase (Promega) in the presence and absence of activating factors (40 µg/mL lipids and 82 µM Ca^{2+}). Additional reaction components or PKC-interacting molecules were added in the concentrations indicated. Reactions were incubated at room temperature for 45 minutes. Protease activity was quenched by adding SDS reducing dye and heating at 70°C for 10 minutes. The full mixture was loaded onto a SurePAGE Bis-Tris 4-12% gel (Genscript) and stained with Coomassie blue to visualize proteins. Gels were photographed using an iBright instrument (Thermofisher). Band intensities for the cleaved and uncleaved protein were quantified using ImageJ, and the % cleaved was determined by dividing the intensity of the cleaved band by the sum of the cleaved + uncleaved bands. IC50 values were calculated by fitting the % cleaved as a function of ENDX concentration to a 4-parameter dose-response model using Graphpad Prism.

Expression and purification of CKAR

pRSET-B-CKAR[8] (Addgene) was transformed into JM109 DE3 *E. coli* (Promega) as described for preparing tandem fluorescent protein reporters[9]. Cultures of pRSET-B-CKAR in JM109 DE3 were grown in 2L of Terrific Broth (Research Products International), supplemented with 300 µL of Antifoam 204 (Sigma-Aldrich) in a LEX-48 Bioreactor (Epiphyte3) at 37°C, until they reached an optical density (OD600) between 3.0 and 4.0. CKAR Protein expression was

induced by the addition of 50 μM of isopropylthio-β-galactoside (IPTG, Goldbio) for 18 hours at 16°C. The bacterial cell pellet was harvested by centrifugation at 6000 g for 20 minutes at 4°C, and frozen at -80°C.

Cell pellets were thawed and lysed in Ni running buffer (20mM Tris pH 7.5, 300mM NaCl, 0.5mM TCEP, 10 mM imidazole) supplemented with 0.1 mg/mL of lysozyme and a 1/2000 dilution of ethanol saturated phenylmethyl sulfonyl fluoride (PMSF, Goldbio) on ice for 30 minutes. Lysate was sonicated at 80% power for three 30-second intervals using a Branson 250 sonifier. Sonicated lysate was centrifuged at 25,000 g for 30 minutes and soluble fraction was passed over Ni-NTA resin (Qiagen) pre-equilibrated with lysis buffer. The resin was washed six times, each with 1 column volume of lysis buffer and CKAR was eluted with Ni running buffer supplemented with 250mM imidazole. Protein was precipitated out of solution by the addition of 2x volume of 4M ammonium sulfate and pellets were collected after centrifugation at 25,000 g for 30 minutes. The protein pellets were redissolved in 2 mL of milli-Q water and polished on a Superdex 200 16/60 column (Cytiva) in 20mM Tris pH 7.5, 300mM NaCl, and 0.5mM TCEP buffer. Protein elution was monitored by absorbance at 280nm, 460nm, and 520nm to identify fractions with full-length CKAR and both YFP and CFP modules. CKAR was further polished by ion exchange chromatography on a Hi-Trap Q HP column (Cytiva) using a 0-50% gradient between low salt (20mM Tris pH 7.5, 3mM DTT) and high salt buffers (20mM Tris pH 8.0, 1M NaCl). CKAR was concentrated and buffer exchanged into PKC Storage Buffer (20mM Tris pH 8.0, 100mM NaCl, 2mM $MgCl_2$, 1mM TCEP, and 25%(v/v) glycerol) using an Amicon 10K concentrator (Millipore). The final CKAR product was quantified using A_{520nm} and an extinction coefficient of 70,000 $M^{-1}cm^{-1}$ for YFP.

CKAR Kinase Assay

Lipid vesicles were prepared fresh by dissolving 10mg/mL bovine brain phosphatidylserine (PS) (Avanti) or/and 1mg/mL 1,2-dioleoyl-glycerol (DAG) (Avanti) in chloroform. Lipids were mixed at the indicated ratio and chloroform was evaporated with a stream of dry air, then lipids were redissolved in water to a final concentration of 40 μg/mL, vortexed for 60s, and sonicated 3 times for 30s at 10% power on a Branson 250 sonifier with a microtip (12840498). Concentrated PKC protein was diluted into storage buffer containing 20mM Tris pH 8.0, 100mM NaCl, 2mM $MgCl_2$, 1mM TCEP, 25%(v/v) glycerol and 1 mg/mL BSA to create a 10X stock based on final assay concentration. Kinase reaction mixtures (100 μL) contained 20mM HEPES pH 7.5, 2mM $MgCl_2$, 1 μM CKAR, 10 nM PKCβ protein and indicated concentrations of ENDX and ATP. Reaction mixtures were pre-heated at 30°C and ATP was mixed in last to initiate the reaction, just prior to placing the reaction plate in a CLARIOstar^{Plus} plate reader (BMG Labtech). Förster Resonance Energy Transfer (FRET) was measured every 5 minutes using a 434-16 excitation filter with 476-16 and 528-16 emission filters[8] at 30°C. Control reactions without PKC were used as a background measurement to correct for fluorophore decay throughout the experiment. The FRET ratio was calculated by dividing the emission measurement at 476nm by 528nm emission. PKC kinase activity was plotted as the change in FRET ratio over time (dFRET/dT).

Z'-LYTE Kinase Assay

Kinase activity was measured using the Z'-LYTE Kinase Assay Kit – Ser/Thr 7 Peptide (ThermoFisher) following the manufacturer's protocol. The reaction mix contained 250 mM HEPES pH 7.5, 50 mM $MgCl_2$, 5 mM EGTA, 0.05% Brij-35, 40 μg/mL PS:DAG lipids, PKC protein at the indicated concentration, drug serial dilution at the respective concentration, 40 μM ATP and 2 μM Z'-LYTE Ser/Thr 7 Peptide Substrate. The peptide substrate and ATP mixture

were added last to initiate the reaction. For reactions without lipids, the 40 μg/mL lipids were substituted with milli-Q H_2O. Reactions were incubated at room temperature for 1 hour. The development solution, created using the manufacturer's instructions, was added and mixed with the kinase reaction prior to placing the plate in the CLARIOstar^{Plus} plate reader (BMG Labtech). The extent of phosphorylation of the peptide substrate is calculated through the ratio of the coumarin emission at 445 nm to the fluorescein emission at 520 nm after a 1-hour incubation with development solution. Kinase activity is plotted as percent activity to the corresponding drug concentration. To determine inhibitor IC50 values, data were fitted to a 4-parameter dose-response model using Graphpad Prism 9. In cases where the IC50 could not be accurately determined due to precipitation of ENDX at very high concentrations, the model was constrained to a bottom value of 0 % and the resulting estimated IC50 value is reported. Extra sum of F squares analysis was used to calculate P values between different drug treatments.

Expression and purification of PKA

DNA encoding PKA catalytic domain (Addgene 14921) was transformed into Rosetta2 cells (EMD) and inoculated in a 2L culture of terrific broth (Research Products International) supplemented with 300 μL Antifoam 204, 100 μg/mL carbenicillin and 34 μg/mL chloramphenicol. Culture was grown using a LEX-48 Bioreactor (Epiphyte) at 37°C and protein expression was induced with 100 μM IPTG overnight at 16°C. *E. coli* culture was pelleted, resuspended in lysis buffer (20 mM Tris pH 7.5, 300 mM NaCl, 10 mM imidazole, and 0.5 mM TCEP) supplemented with 10 mg of lysozyme (GoldBio) and 20 μl of saturated PMSF in ethanol (GoldBio). Cell lysate was incubated on ice for 30 min with occasional mixing and then sonicated by a Branson 250 sonicator at 80% power in five 15-second intervals. Clarified lysate was centrifuged at 25,000*g* for 30 min and passed over a Ni-NTA column equilibrated in lysis buffer. Ni-NTA resin was washed with 5 column volumes lysis buffer and eluted with lysis buffer supplemented with 250 mM imidazole. Protein containing fractions were detected by a color change in a Bradford Assay (99 μl bradford reagent, 1 μl elution fraction), pooled and precipitated with two volumes of 4 M ammonium sulfate followed by centrifugation at 25,000*g* for 30 min at 4°C. Precipitated protein was dissolved in 3-mL Milli-Q water and loaded onto a Superdex S200 size exclusion column equilibrated in 20 mM Tris pH 7.5, 300 mM NaCl, and 0.5 mM TCEP. Fractions were analyzed by SDS-PAGE gel and PKA containing fractions were pooled and diluted 1:5 in Milli-Q water. The pH of the dilution was adjusted to pH 6 by addition of MES powder and loaded onto a Resource 15S cation exchange column (Cytiva) equilibrated in running buffer (20 mM NaH_2PO_4 pH 6.0, 0.1 mM TCEP) and eluted with a linear gradient of 0-100% 1M NaCl in 20 mM NaH_2PO_4 pH 6.0. To increase the pH in PKA–containing fractions 20 mM Tris pH 8.0 was added to pooled protein and protein was then concentrated by Amicon ultrafiltration (Millipore). Concentrated protein was run over a Superdex 200 increase (Cytiva) equilibrated in running buffer (20 mM Tris pH 7.5, 300 mM NaCl, and 0.5 mM TCEP). Purified protein was analyzed by SDS-PAGE gel and buffer exchanged during Amicon centrifugation to PKC storage buffer (20mM Tris pH 8.0, 100mM NaCl, 2mM $MgCl_2$, 1mM TCEP, and 25% (v/v) glycerol).

Small Angle X-ray Scattering

PKCβI was purified and buffer exchanged into SAXS buffer (20mM Tris pH 8.0, 100mM NaCl, 2mM $MgCl_2$, 1mM TCEP, and 1% (v/v) glycerol) using a Superdex Increase 10/300 column (Cytiva). Aliquots of the highest concentration fraction were supplemented with ENDX or TAM to a final concentration of 80 μM, or an equivalent volume of water was added to the inhibitor-free sample. Samples were concentrated briefly with 0.5 mL 10K centrifugal filters (Amicon) that had been pre-blocked and washed (incubated overnight at 4 ˚C in SAXS buffer supplemented with

3% (v/v) PEG400, followed by 3X washing in SAXS buffer with 10s spins in the centrifuge to remove any remaining PEG). Samples were concentrated briefly by centrifugation at 4 ˚C and 8000 g to 17 uM concentration, and concentrator flowthrough was used to prepare diluted samples of 8.5 and 4.2 μM, as well as the buffer subtraction control. SAXS data were collected at the SIBYLS beamline at the Advanced Photon Source using the Mail-in service. Data frames were merged using the SIBYLS Frameslice software as follows: frames 1–8 for S=(0.008742 to 0.091598), frames 1–15 for S= (0.092358 to 175208), and frames 1–33 for S=(0.175968 to 0.446450). Inspection of Guinier plots for the 17 μM samples indicated a small but detectible amount of protein aggregation, whereas none was detectible for the 8.5 and 4.2 μM samples. Data from the 8.5 μM samples was used for analysis in PRIMUS (version 3.03) from the ATSAS suite[10]. Rg was determined using the AutoRg function in Primus. Dmax and real space parameters were determined using GNOM.

Live Cell Imaging

MCF7AC1 cells that stably expressed YFP-PKCβ1 were generated by transfecting plasmid DNA using Lipofectamine 2000 (Thermofisher) according to the manufacturer's instructions, and then following the protocol described in the *Expression and purification of PKCβI and PKCβII* section for generating a stable expression cell line. For microscopy, YFP^+ cells were seeded in 35-mm glass bottom microwell dishes (MatTek Corporation) for at least 24 hrs. Subsequently, cells were incubated with ENDX 1 hour prior to imaging, followed by addition of PMA 15 mins prior to imaging as indicated in figure legends. Where indictaed, phosphatase inhibitors NSC117079 (MedChem Express) or Okadaic Acid (Cayman Chemical) were added 45 mins prior to imaging. Successively, NucRed Live 647 Reagent (Invitrogen) was added for live cell nuclear staining and visualized using a Zeiss-LSM 780 confocal microscope. Confocal images were processed using Carl-Zeiss Blue/Black ZEN 3.0 SR software.

Quantification And Statistical Analysis

Standard statistical analyses were used to distinguish significant from non-significant results as indicated.

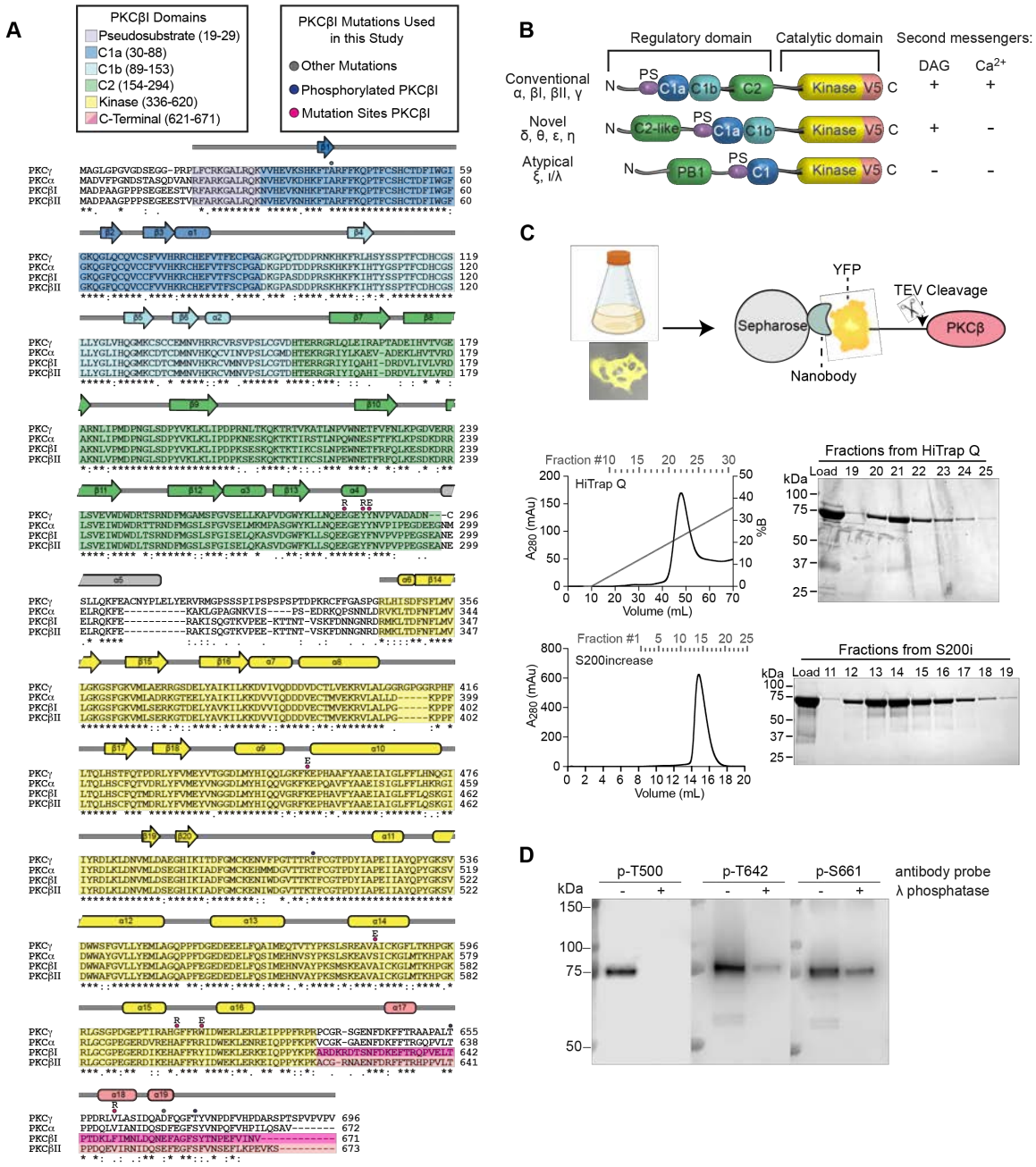


Figure S1.

(A) Sequence alignment of conventional PKCs with domains coloured as indicated. The altered C-terminal tails derived from an alternative splicing event are coloured pink (PKCβI) or salmon (PKCβII).

(B) Domain arrangement and second messenger sensitivity of the three PKC families.

(C) Schematic diagram of YFP-PKCβII protein expression and affinity isolation using the anti-YFP nanobody system. Chromatograms and corresponding Coomassie-stained gels of PKCβII from FPLC purification steps.

(D) Western blots with the indicated phospho-specific PKCβII antibodies detects phosphorylation at all three expected sites (T500 – activation, T642 – turn, and S660 – hydrophobic) in the purified protein.

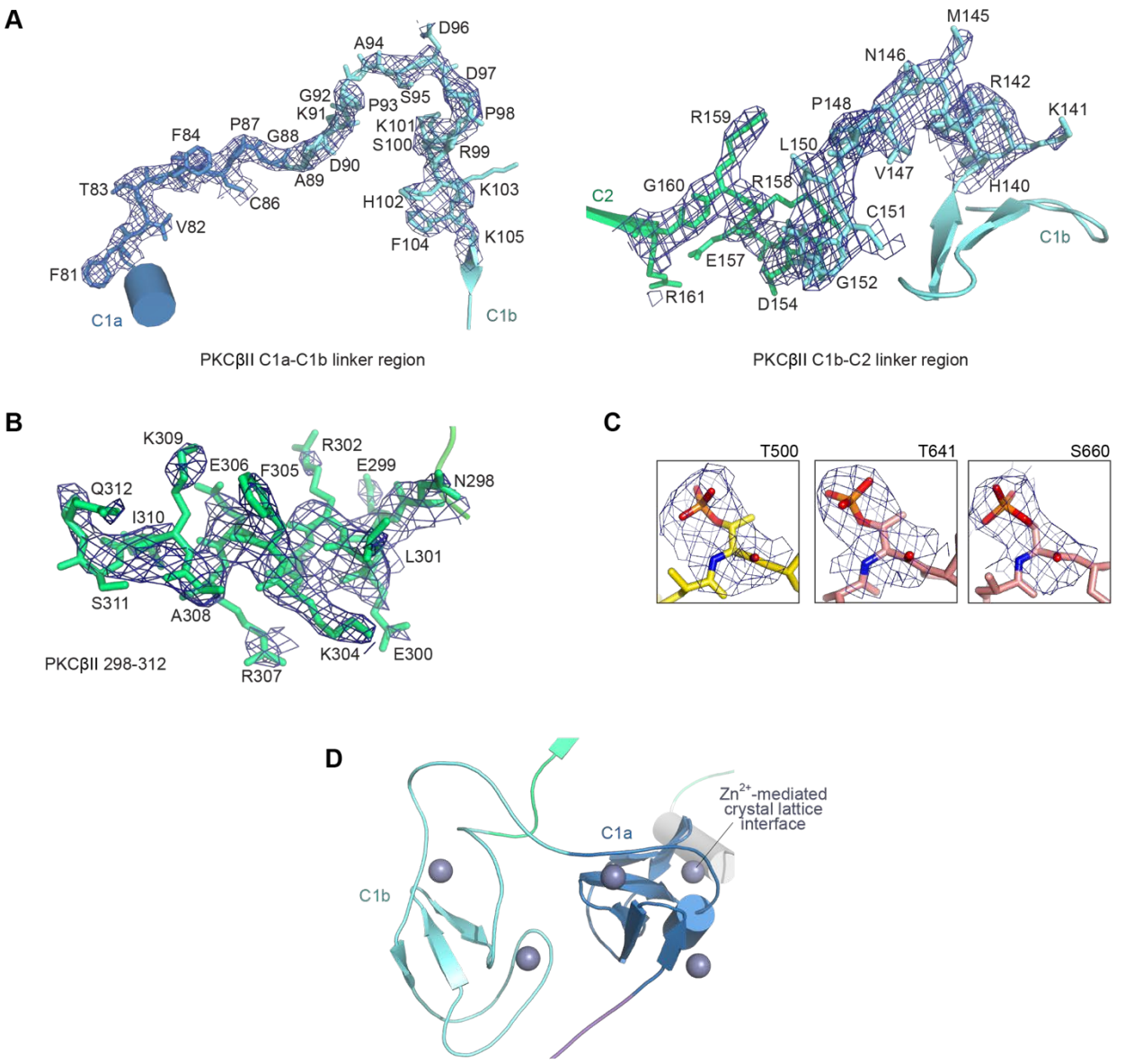


Figure S2.

(A) Experimental $2F_0 - F_c$ map contoured at 1σ indicating continuous electron density for the PKCβII C1a – C1b linker region and C1b – C2 linker region.

(B) Experimental $2F_0 - F_c$ map contoured at 1σ for residues 298 – 312 in the PKCβII C2-kinase domain linker region.

(C) Experimental $2F_0 - F_c$ of the three phosphorylation sites on PKCβII.

(D) Location of Zn^{2+} ions (gray spheres) coordinated within the C1a and C1b domains, as well as a fifth Zn^{2+} ion that is part of the crystal lattice.

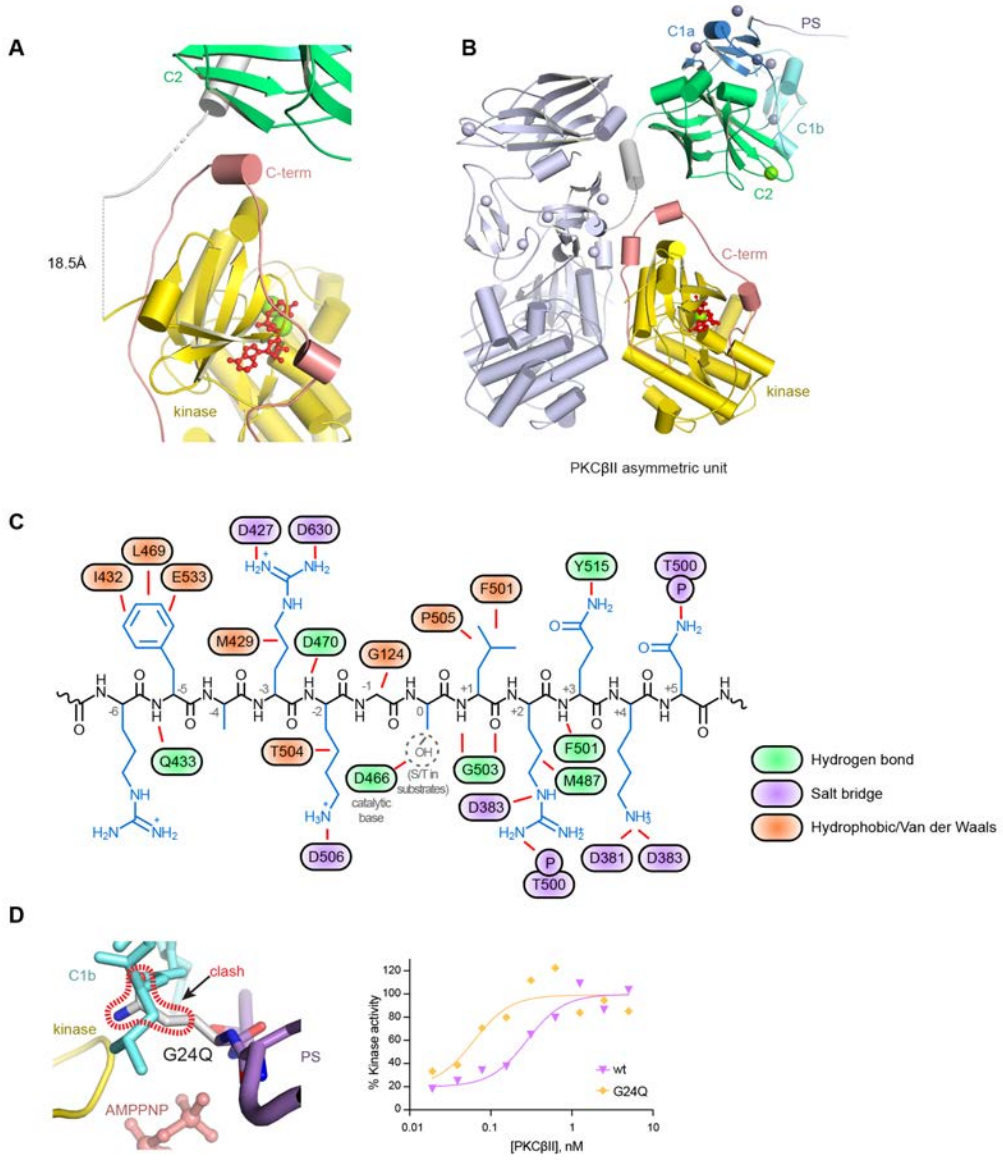


Figure S3.

(A) Measured length of the disordered region between C2 and kinase domain used to define pairing of N-terminal and kinase domains that belong to the same chain.

(B) Domain–swapped arrangement of PKCBII (colored as in Fig. 1A) with an adjacent identical monomer (light purple).

(C) Molecular details of the N-terminal pseudosubstrate side chains (blue) and their specific interactions with the C1b and kinase domains labeled by residues and color-coded (hydrogen bonding, salt bridge, hydrophobic/Van der Waals).

(D) (left) A G24Q mutation (gray) was modelled into the structure of PKCβII, which predicts a significant steric clash (red dashes) with residues from the C1b domain. (Right) Assay of kinase activity of recombinant PKCβII enzymes (WT and G24Q) indicates that G24Q is active at lower concentrations.

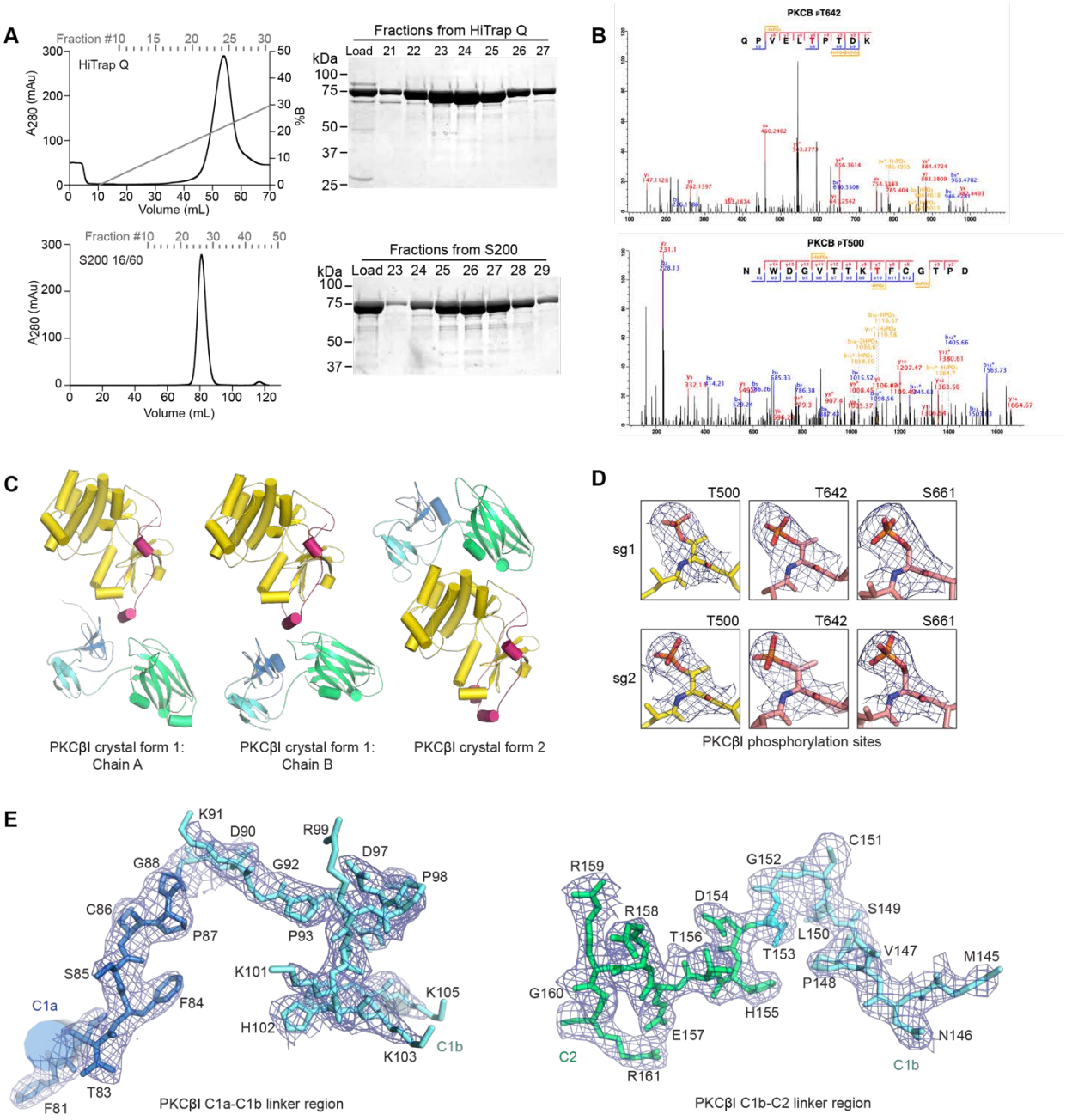


Figure S4.

(A) Ion exchange and size exclusion chromatograms of PKCβI purification and SDS-PAGE analysis of indicated fractions.

(B) Mass spectrometry of purified PKCβI protein detects phosphorylation at T500 and T642.

(C) Individual copies of PKCβI within each crystal form.

(D) Experimental $2F_0 - F_c$ map of phosphorylation sites on PKCβI in each crystal form.

(E) Experimental $2F_0 - F_c$ map contoured at 1σ for the PKCβI C1a – C1b linker region and C1b – C2 linker region.

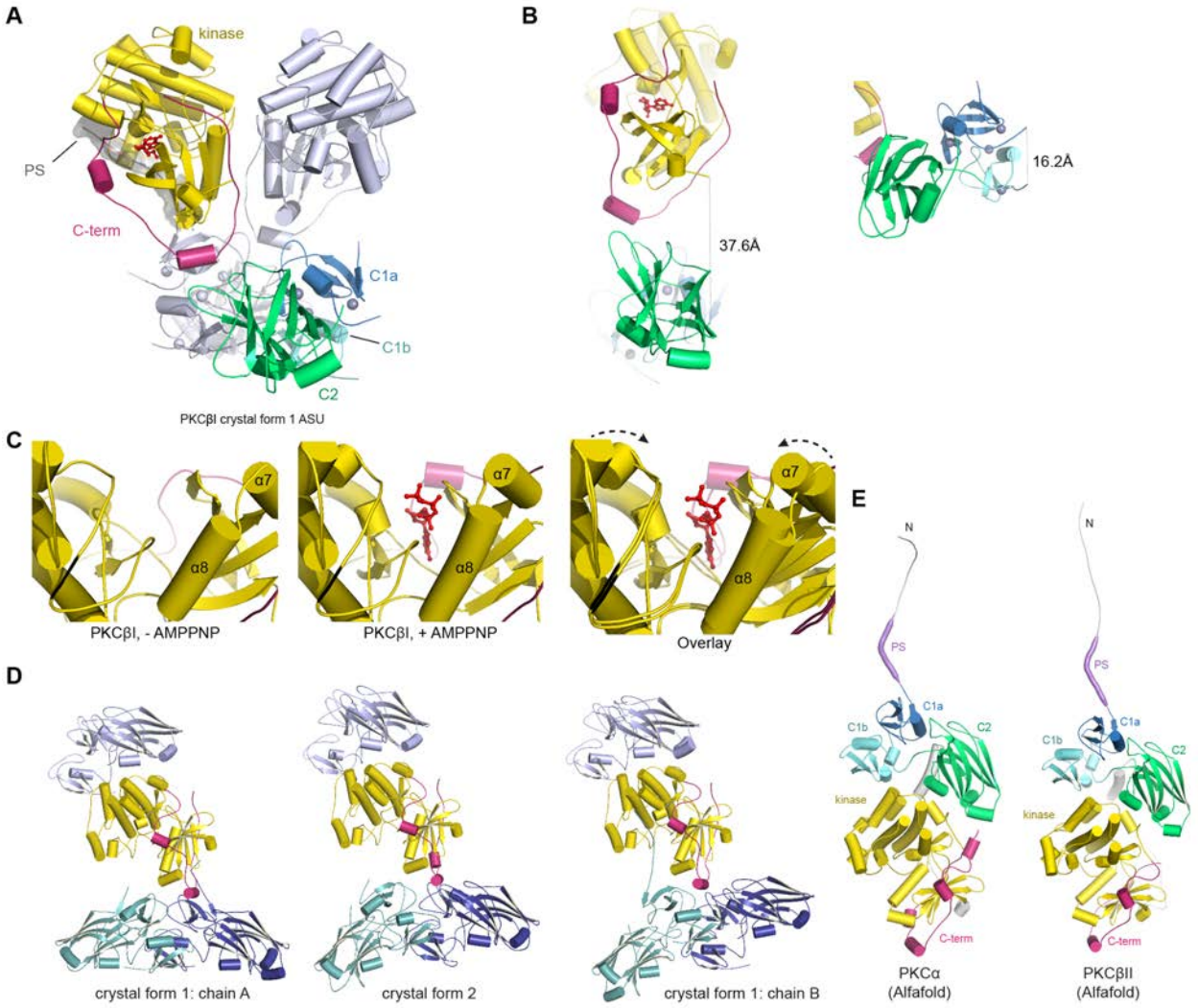


Figure S5.

(A) Pseudosubstrate of the adjacent monomer (light purple) is present in the active site of crystal form 1 (coloured as in Fig. 1A).

(B) Linkers between C1a and C1b domain and C2 and kinase domains are not observed in PKCβI crystal form 1.

(C) PKCβI active site adopts a more closed conformation when occupied by AMPPNP nucleotide and remains open in its absence.

(D) Local symmetry environment of the three copies of PKCβI reveals similar molecular interactions.

(E) AlphaFold-predicted structure of PKCβα and PKCβII show a domain arrangement that is similar to the active form.

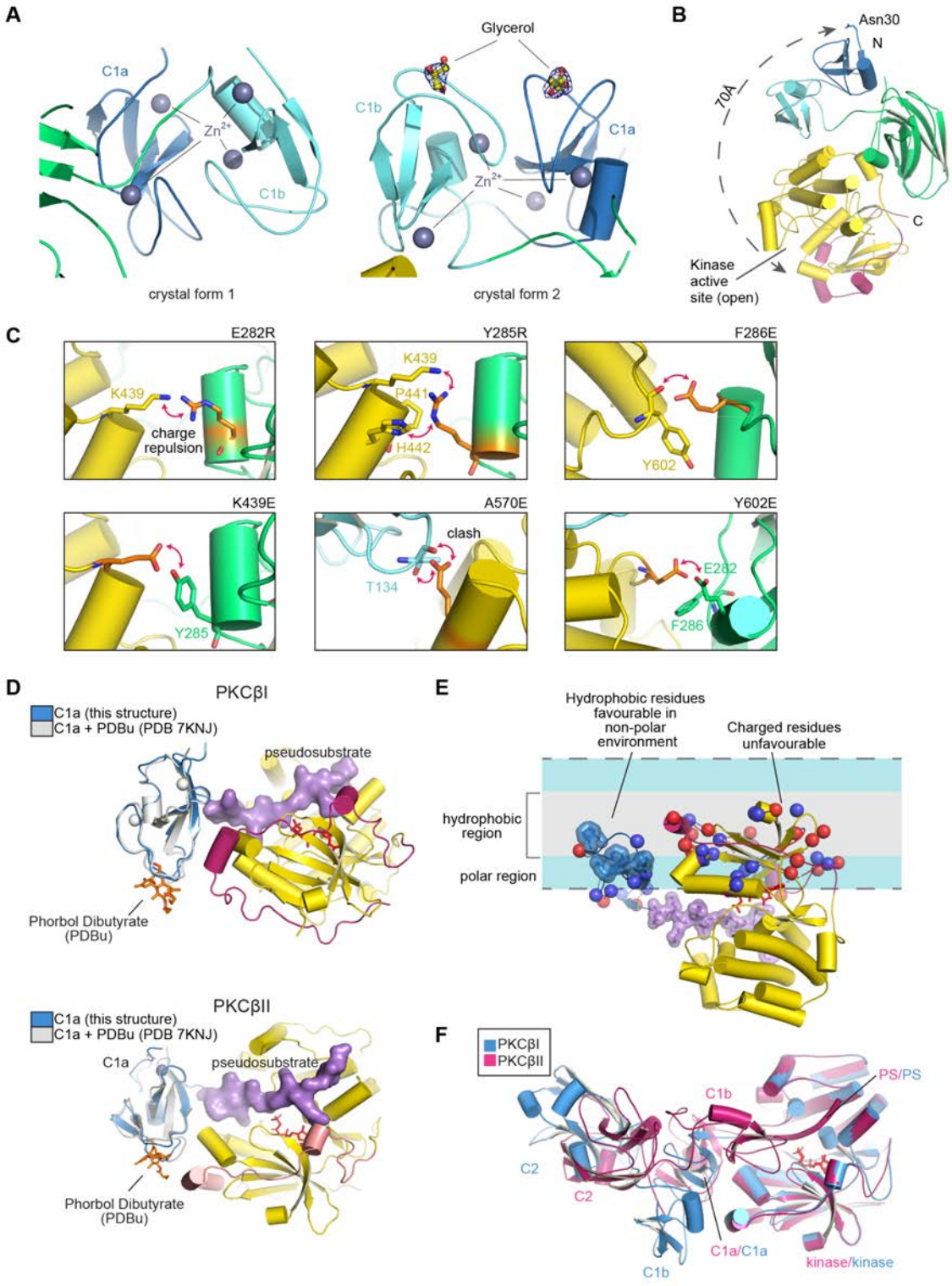
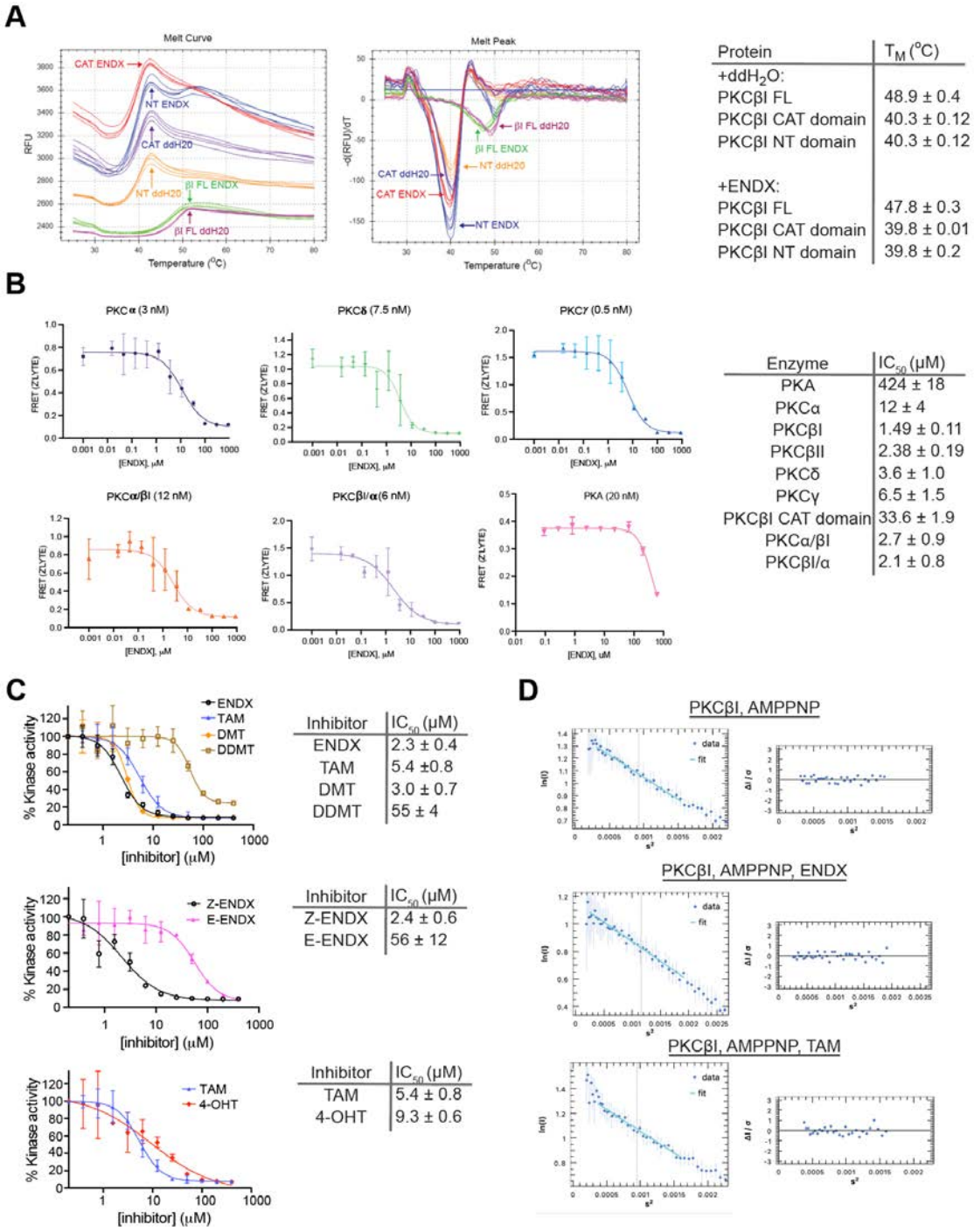


Figure S6.

(A) Ligands coordinate in crystal form 1 and crystal form 2 of PKCβI. DAG binding site of PKCβI is occupied by glycerol molecules in crystal form 2.

(B) 70 Å distance between N-terminal tail and active site prevents re-engagement of the pseudosubstrate.
(C) Modelled mutations that disrupt C2-kinase and C1b-kinase domain interactions in the active conformation of PKCβI.
(D) Aligned crystal structures of the C1a domain with PDBu (PDB 7KNJ) and PKCβI or PKCβII in their auto-inhibited conformation shows PDBu binding does not disrupt the inactive conformation.
(E) Insertion of C1a domain hydrophobic residues into the hydrophobic region of phospholipid bilayer would cause a steric clash between the kinase domain and phospholipid bilayer, which would result in separation of the kinase domain from the pseudosubstrate.



Protein	T_M (°C)
+ddH_2O:	
PKCβI FL	48.9 ± 0.4
PKCβI CAT domain	40.3 ± 0.12
PKCβI NT domain	40.3 ± 0.12
+ENDX:	
PKCβI FL	47.8 ± 0.3
PKCβI CAT domain	39.8 ± 0.01
PKCβI NT domain	39.8 ± 0.2

Enzyme	IC_{50} (μM)
PKA	424 ± 18
PKCα	12 ± 4
PKCβI	1.49 ± 0.11
PKCβII	2.38 ± 0.19
PKCδ	3.6 ± 1.0
PKCγ	6.5 ± 1.5
PKCβI CAT domain	33.6 ± 1.9
PKCα/βI	2.7 ± 0.9
PKCβI/α	2.1 ± 0.8

Inhibitor	IC_{50} (μM)
ENDX	2.3 ± 0.4
TAM	5.4 ±0.8
DMT	3.0 ± 0.7
DDMT	55 ± 4

Inhibitor	IC_{50} (μM)
Z-ENDX	2.4 ± 0.6
E-ENDX	56 ± 12

Inhibitor	IC_{50} (μM)
TAM	5.4 ± 0.8
4-OHT	9.3 ± 0.6

Figure S7.

(A) Differential scanning fluorimetry of 1 μg full-length (FL), catalytic (CAT) domain or the N-terminal (NT) domain of PKCβI or of PKCβII with ddH_2O as negative control or 40 μM ENDX. Presented melt curve and derivative melt plots are shown for each independent measurement. Average melting temperatures (T_M) and standard deviations of each condition are reported.

(B) *In vitro* Z'-LYTE kinase assay of other PKC isoforms and their associated IC_{50} values. Data are mean ± SD. Values for PKCβI and PKCβII are reproduced from Fig. 5C
(C) *In vitro* Z'-LYTE kinase assay of PKCβI in the presence of the indicated inhibitors (DMT = N-desmethyltamoxifen, DDMT = N,N-didesmethyltamoxifen, 4-OHT = 4-hydroxytamoxifen) and their associated IC_{50} values. Data are mean ± SD
(D) (left) Guinier plots and (right) residuals calculated from SAXS data shown in Fig. 5D.

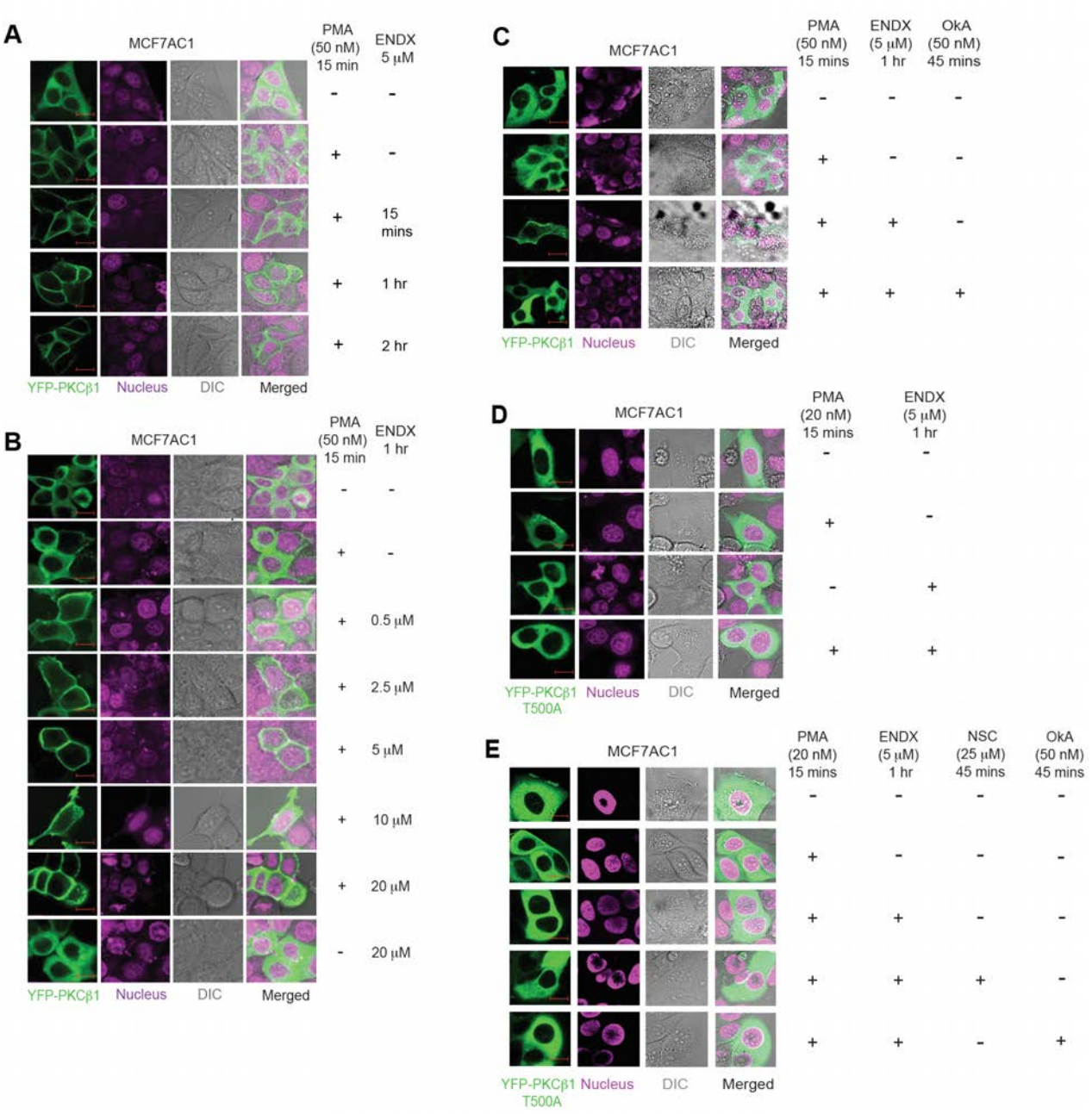


Figure S8.

(A) Confocal live cell microscopy images showing time course of YFP-tagged PKCβI accumulation at the plasma membrane in the presence of indicated compounds. Scale bar = 20 μm.

(B) Confocal live cell microscopy images showing concentration dependence of YFP-tagged PKCβI accumulation at the plasma membrane in the presence of ENDX. Scale bar = 20 μm.

(C) Confocal live cell microscopy images showing the effect of the PP1/PP2A inhibitor okadaic acid on accumulation of YFP-tagged PKCβI in the presence of indicated compounds. Scale bar = 20 μm.

(D) Confocal live cell microscopy images showing YFP-tagged T500A mutant PKCβI does not accumulate at the plasma membrane in the presence of indicated compounds. Scale bar = 20 μm.

(E) Confocal live cell microscopy images showing YFP-tagged T500A mutant PKCβI plasma membrane accumulation is not affected by the indicated phosphatase inhibitors. Scale bar = 20 μm.

Table S1. **X-ray diffraction data collection and structure refinement statistics**

	PKCβII	PKCβI-form 1	PKCβI-form 2	PKCβI-form 1-Mn
PDB Accession #	8SE1	8SE2	8SE3	8SE4
Space group	C 1 2 1	C 1 2 1	C 1 2 1	C 1 2 1
Cell dimensions				
a, *b*, *c* (Å)	130.6, 89.1, 160.7	162.7 157.8, 87.1	82.2, 163.2, 77.3	162.7, 157.9, 85.1
α, β, γ (°)	90, 107.1, 90	90, 113.0, 90	90, 100.4, 90	90, 112.2, 90
Resolution (Å)[a]	50.00-3.35 (3.47-3.35)	50-2.7 (2.80-2.70)	50.00-2.60 (2.69-2.60)	50-2.95 (3.06-2.95)
R_{merge}	0.299 (0.962)	0.141 (2.084)	0.149 (1.485)	0.115 (1.12)
$I / \sigma I$	4.7 (1.5)	15.7 (1.1)	13.2 (1.4)	11.3 (1.3)
$CC_{1/2}$	0.91 (0.46)	0.99 (0.48)	0.99 (0.49)	0.99 (0.48)
Completeness (%)	99.9 (99.3)	99.5 (99.8)	99.2 (99.1)	98.8 (99.0)
Redundancy	3.5 (3.7)	10.6 (10.6)	6.9 (7.1)	3.6 (3.6)
Refinement				
Resolution (Å)	48.75-3.32	49.88-2.70	48.66-2.60	49.70-2.95
No. reflections	25776	55648	30355	41359
R_{work} / R_{free}	0.199/0.256	0.206/0.258	0.197/0.227	0.196/0.252
No. atoms (non-H)	10226	9685	4970	9713
Protein	10077	9657	4871	9657
Ligand/ion	84	27	28	48
Water	65	1	71	8
Average *B* factor (Å^2)	63.31	113.8	78.34	99.5
Ramachandran plot: Favored (%)	91.6	93.4	94.4	94.4
Allowed (%)	8.1	6.4	5.1	5.4
Outliers (%)	0.3	0.2	0.5	0.2
r.m.s. deviations				
Bond lengths (Å)	0.003	0.006	0.002	0.005
Bond angles (°)	0.605	0.863	0.519	0.805

[a]Values in parentheses refer to the highest resolution shell

Table S2. **SAXS data analysis parameters**

Sample	PKCβI, AMPPNP	PKCβI, AMPPNP, ENDX	PKCβI, AMPPNP, TAM
Guinier analysis			
I(0) (cm^{-1})	4.13 ±0.10	3.19 ±0.10	4.08 ±0.13
R_g (Å)	33.4 ±1.2	29.3 ±1.3	32.5 ±1.4
sR_g range	0.47-1.30	0.48-1.28	0.63-1.30
MW [Da], (ratio to calculated)	69745 (0.91)	65076 (0.85)	70400 (0.92)
P(*r*) analysis			
I(0) (cm^{-1})	4.08 ±0.08	3.29 ±0.07	4.09 ±0.08
R_g (Å)	33.1 ±0.7	31.1 ±0.6	32.9 ±0.7
d_{max} (Å)	109	96	109
q range (Å^{-1})	0.0141-0.2436	0.0148-0.2725	0.0171-0.2573

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