Supplementary Information

Supplementary Experimental Methods

Materials

Except where indicated otherwise, all fine chemicals were purchased from Sigma-Aldrich. Synthetic peptides were obtained from the Peptide Synthesis Laboratory at Cancer Research UK (London Research Institute). DNA plasmid pACYC-14-3-3zeta was kindly provided by Alastair Aitken (University of Edinburgh).

Peptide sequences

Peptide sequences are shown below from amino to carboxy terminus with pS denoting phosphorylated serine in single letter amino acid code:

PKCεV3-b-pS346-pS368: Biotin-DRSKpSAPTSPCDQEIKELENNIRKALpSFDNR
PKCεV3-n: DRSKSAPTSPCDQEIKELENNIRKALSFDNR
PKCεV3-pS346: DRSKpSAPTSPCDQEIKELENNIRKALSFDNR
PKCεV3-pS368: DRSKSAPTSPCDQEIKELENNIRKALpSFDNR
PKCεV3-pS346-pS368: DRSKpSAPTSPCDQEIKELENNIRKALpSFDNR
PKCεV3-pS346-pS350-pS368: DRSKpSAPTpSPCDQEIKELENNIRKALpSFDNR
PKCεV3-pS346-G6-pS368: DRSKpSAPTGGGGGGRKALpSFDNR
PKCεV3-pS346-G10-pS368: DRSKpSAPTGGGGGGGGGGRKALpSFDNR
PKCεV3-pS346-G14-pS368: DRSKpSAPTGGGGGGGGGGGGGGRKALpSFDNR
PKCεV3-site1-site1: DRSKpSAPTSPCDQEIKELENNIRSKpSAPNR
PKCεV3-site2-site2: DRKALpSFDTSPCDQEIKELENNIRKALpSFDNR

Stoichiometry of 14-3-3ζ:PKCεV3-b-pS346-pS368

Stoichiometry of binding was determined using the EZ Biotin Quantitation Kit (Pierce). Because HABA absorbs at 500 nm wavelength only when bound to avidin, displacement of HABA by biotinylated peptide binding to avidin allowed calculation of the 14-3-3ζ:PKCεV3-b-pS346-pS368 ratio by measurement of decrease in HABA absorbance at 500 nm. Quantitation was achieved by taking absorbance measurements at 500 nm, subtraction from reference sample, and calculation of the biotin to protein ratio.

Isothermal Titration Calorimetry

Data were fitted using a single site binding model for all peptides except PKCεV3-pS346-pS346 which was fitted with a two site model. The single site model was used for other di-phosphorylated peptides because low binding heats precluded accurate determination of the second enthalpy changes and dissociation constants.

Supplementary Table

Table S1: Data collection and refinement statistics

X-ray source	Rigaku MicroMax-007 HF
Wavelength (/Å)	1.54179
Temperature (/K)	100
Space group	$P2_1$
Cell dimensions a, b, c (/Å)	71.13, 78.16, 108.52
α, β, γ (/º)	90, 90.098, 90
Resolution (/Å)	24.5 - 2.25 (2.45 – 2.25)
Unique reflections	56277 (9922)
Completeness (%)	99.4 (82.4)
Redundancy	4.9 (4.8)
I/σ	19.4 (3.9)
R_{sym} (%)[a]	10.2 (40.6)
Refined twin fraction (Xtriage estimates)	44.2% (H-test 42%, Britton 41%, ML 35%)
Twinning operator	h, -k, -l
R_{work} (%)[b]	17.9
R_{free} (%)[c]	23.5
Mean B-Factors (/Å^2)	
Wilson	39.6
Protein	31.5
Solvent	35.0
Ramachandran Plot (%)	
Favoured	94.6
Allowed	3.8
Outliers	1.6
Rmsd from Target Geometry	
Bond lengths (/Å)	0.006
Bond angles (/º)	0.978
PDB ID	2WH0

[a] $R_{sym} = \Sigma|I_j - \langle I \rangle| / \Sigma I_j$ where I_j is the individual reflection intensity and $\langle I \rangle$ is the average intensity of that reflection.

[b] $R_{work} = \Sigma||F_o| - |F_c|| / \Sigma|F_c|$ where F_o is the observed structure factor amplitude and F_c is the structure factor amplitude calculated from the structure.

[c] R_{free} is calculated identically to R_{work} but with 5.0% of randomly chosen reflections omitted from the refinement.

Data for the outermost shell are in parentheses, one crystal was used for data collection.

Supplementary Figures

Figure S1 Kostelecky et al. 2009

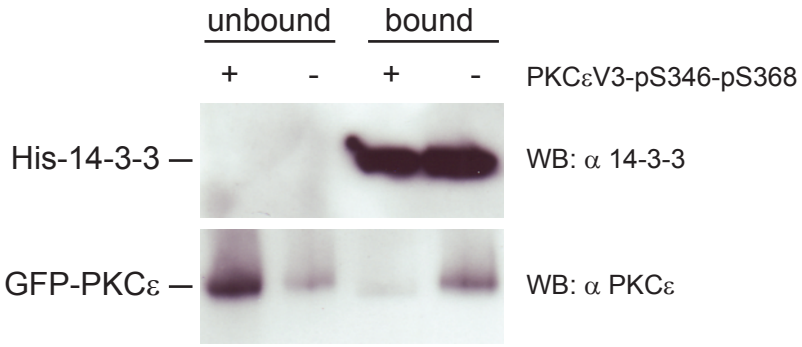

Figure S1: The peptide PKCεV3-pS346-pS368 competes with PKCε for 14-3-3 binding. Hexahistidine tagged 14-3-3ζ (His-14-3-3) was bound to nickel-nitrilotriacetic acid (Ni-NTA) resin and used as an affinity matrix to pulldown of GFP-PKCε from phorbol-12-myristate-13-acetate stimulated HEK-293 cells. Excess peptide PKCεV3-pS346-pS368 was added to half of the Ni-NTA resin after the pulldown, while buffer was added to the other half as a control. The supernatants were collected and are labelled 'unbound' in the Western Blots above. The proteins which remained bound were then eluted from the Ni-NTA resin by addition of 300 mM imidazole (labelled 'bound' in Western Blots).

Figure S2

Kostelecky et al. 2009

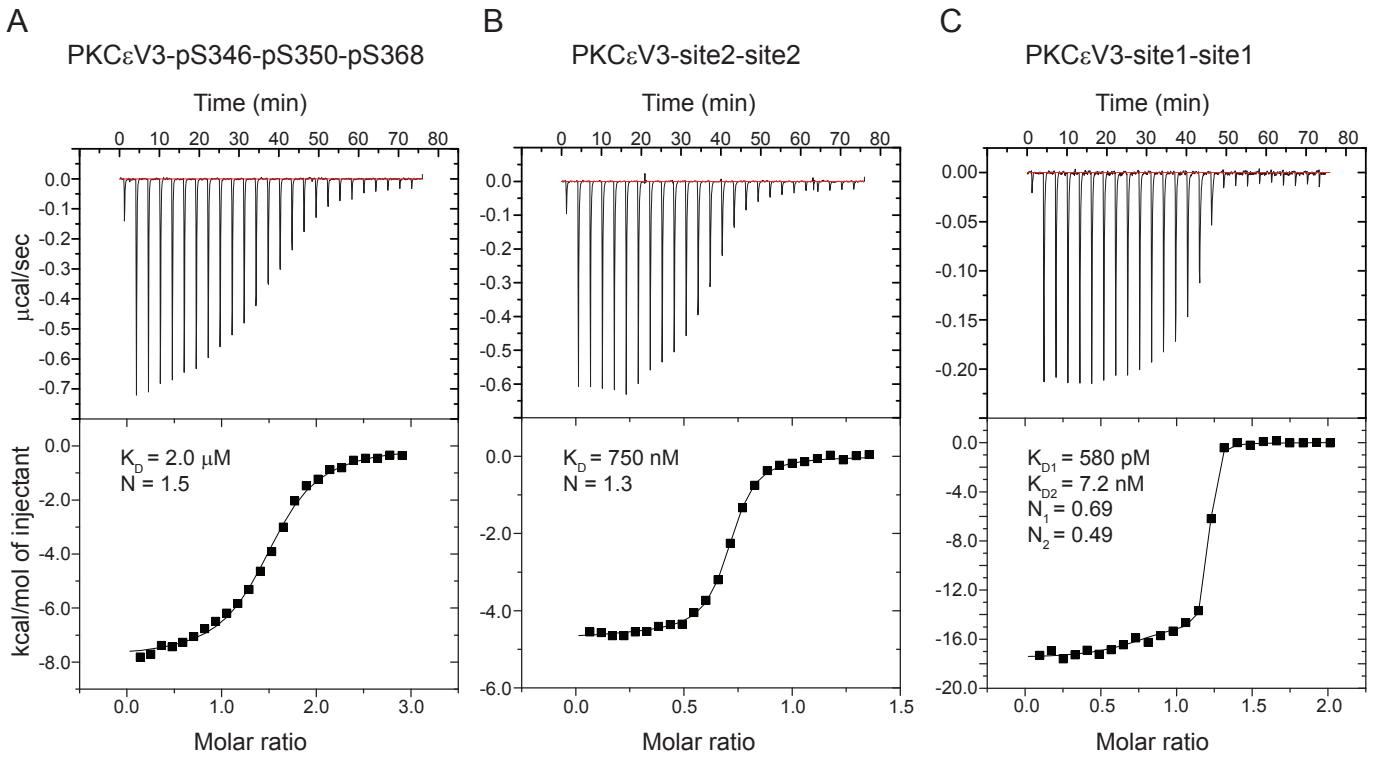

Figure S2: Isothermal titration calorimetry (ITC) binding curves for tri-phosphopeptide and di-phosphopeptides. Top panels show raw ITC data, bottom panels show binding curves fitted with a one site binding model for PKCe-pS346-pS350-pS368 and PKCeV3-site2-site2. The curve for PKCeV3-site1-site1 was fitted with a two binding site model. Data shown are representative of three experiments with independent samples.

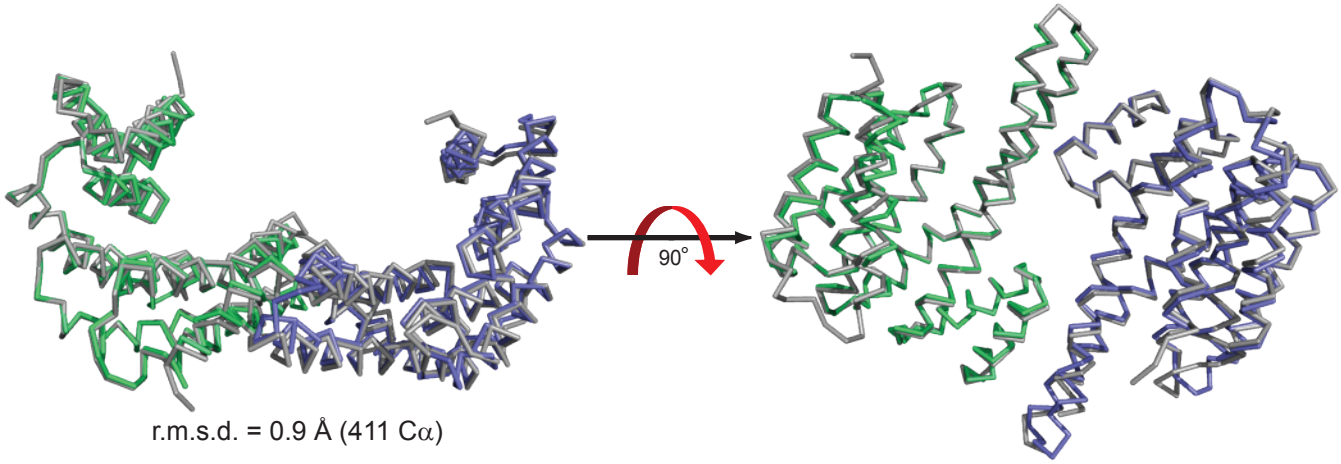

Figure S3: Comparison of 14-3-3ζ:PKCεV3 structure to mode 1-bound 14-3-3ζ. The previously reported structure for 14-3-3ζ bound to a canonical mode 1 peptide (PDB ID 1QJB) is shown in grey. Chains A and B of PKCεV3-bound 14-3-3ζ are shown in green and blue, respectively. The figure was prepared using PyMOL (Delano, 2002).

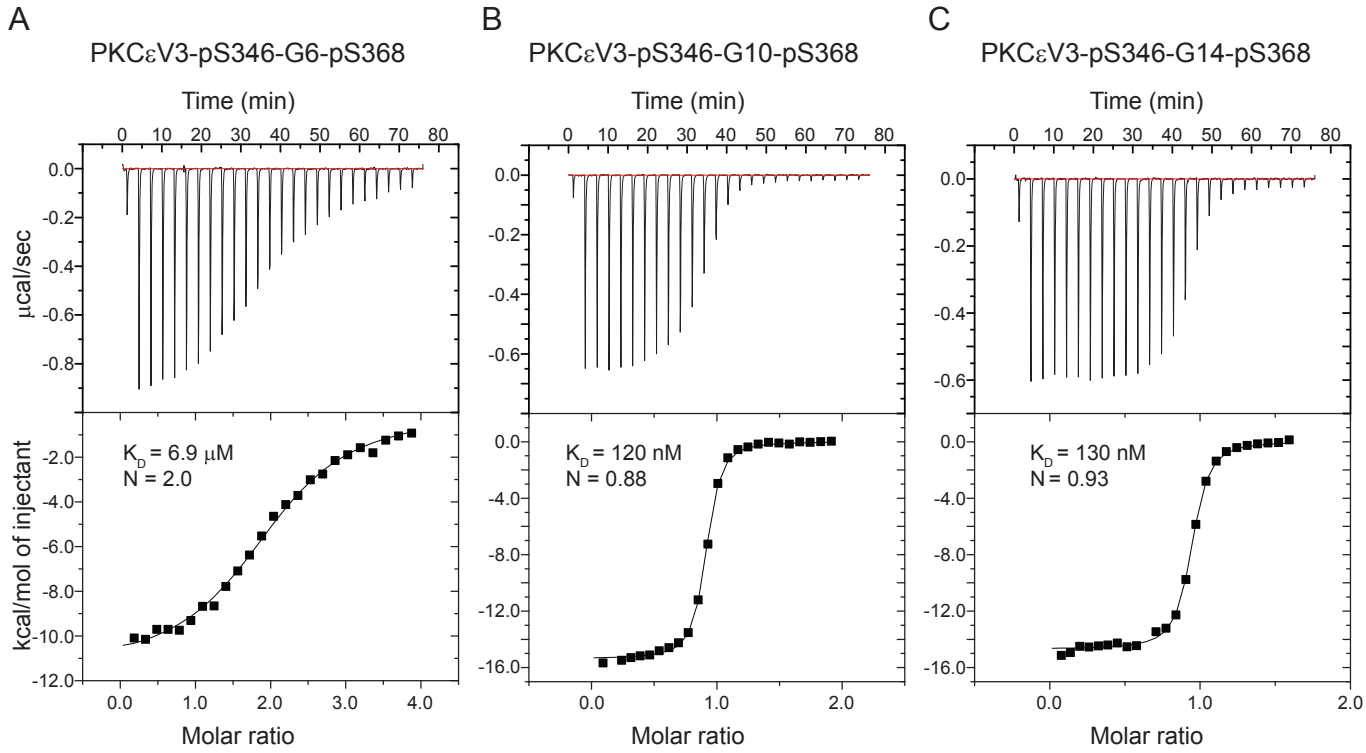

Figure S4: Isothermal titration calorimetry binding curves for peptides with varied linker length. Peptides contained both PKCεV3 14-3-3 binding sites with polyglycine linkers of length 6, 10 and 14 glycine residues, respectively. Top panels show raw data for binding of the peptides to 14-3-3, bottom panels show binding curves fitted with a one site binding model. Data shown are representative of three experiments with independent samples.