Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry. This journal is © The Royal Society of Chemistry 2019

# **Supporting Information**

# Phosphorylation-Dependent Protein Design: Design of a Minimal Protein Kinase-Inducible Domain

 Feng Gao, Blair S. Thornley, Caitlin M. Tressler, Devan Naduthambi, and Neal J. Zondlo\*
Department of Chemistry and Biochemistry University of Delaware
Newark, DE 19716
United States

# Contents

S2. Peptide synthesis, purification, and characterization

S3. Fluorescence spectroscopy

S3. Enzymatic phosphorylation and dephosphorylation of peptides

S4. Cell culture and cell extracts

S6. HPLC analysis of reactions of peptides with enzymes in solution and in cell extracts

S9. NMR spectroscopy

S11. Analysis of EF-Hand-like proteins in the PDB with Glu at residue 9 but lacking Glu at residue 12

#### Peptide synthesis, purification, and characterization

Peptides were synthesized using standard Fmoc solid-phase peptide synthesis (SPPS) with Rink amide resin on a Rainin PS3 peptide synthesizer (Rainin Instruments, Woburn, MA). HBTU was used as a coupling reagent. All peptides were acetylated on the N-terminus and contained C-terminal amides.

To synthesize the phosphorylated peptides, the non-phosphorylated peptide was synthesized with a trityl protecting group on the serine or threonine residue. After SPPS, the trityl group was selectively removed with a solution of 2% TFA (trifluoroacetic acid) and 5% triethylsilane (TES) in CH<sub>2</sub>Cl<sub>2</sub> (3 × 1 min), followed by washing the resin with CH<sub>2</sub>Cl<sub>2</sub> (3×). Phosphitylation was performed under nitrogen by addition to resin of tetrazole (1.35 mmol; 3 mL of 3% tetrazole in acetonitrile) (Transgenomics, Inc., Omaha, NE) and O,O-dibenzyl-N,Ndiisopropylphosphoramidite (500  $\mu$ L, 1.52 mmol) (Alfa Aesar, Ward Hill, MA). The resulting mixture allowed to react with stirring for 5 h. The solution was removed and the resin was washed with DMF (3×), CH<sub>2</sub>Cl<sub>2</sub> (3×), and MeOH (3×). Oxidation was performed with *t*-butyl hydroperoxide (3 mL of a 3 M solution in CH<sub>2</sub>Cl<sub>2</sub>), with the resultant mixture allowed to react with stirring for 1 h. The solution was removed and the resin washed with DMF (3×).

Peptides were subjected to cleavage from the resin and deprotection for 5 h under conditions using Reagent Κ (84%) TFA/4% each of H<sub>2</sub>O/phenol/ standard thioanisole/ethanedithiol). TFA was removed by evaporation and the peptides were precipitated with ether. The precipitate was dissolved in 100 mM phosphate (pH 7.0) buffer and then filtered. All peptides were purified to homogeneity using reverse-phase HPLC on a Vydac C18 semipreparative column ( $10 \times 250$  mm, 5-10  $\mu$ m particle, 300 Å pore) (Grace Vydac, Hesperia, CA). Peptide purity was determined by the presence of a single peak upon analytical HPLC reinjection on a Varian Microsorb MV analytical C18 column (4.6  $\times$  250 mm, 3-5  $\mu$ m particle, 100 Å pore) (Varian, Inc., Palo Alto, CA). The peptides pKID-Min1, pKID-Min2, pKID-Min4, pKID-Min5-PKC, pKID-Min-pThr9, pKID-Min-pThr12, and pKID-Min9-CK1 were purified over 60 minutes using a linear gradient of 0-40% buffer B (80% MeCN, 20% H<sub>2</sub>O, and 0.05% TFA) in buffer A (98% H<sub>2</sub>O, 2% MeCN, and 0.06% TFA). The peptides pKID-Min3 and pKID-Min6 were purified over 60 minutes using a linear gradient of 0-50% buffer B in buffer A. The peptide pKID-Min7-PKA was purified over 60 minutes using a linear gradient of 0-25% buffer B in buffer A.

The identity of each peptide was characterized using ESI-MS (positive ion mode) on an LCQ Advantage (Finnigan) mass spectrometer. Analytical data for the peptides are as follows: non-phosphorylated pKID-Min-pThr9/pKID-Min-pThr12 ( $t_R = 44.3$  min, mass expected (exp.) 1486.7, mass observed (obs.) 1487.6 (M + H)<sup>+</sup>); pKID-Min1 ( $t_R = 40.2$  min, exp. 1030.5, obs. 1031.5 (M + H)<sup>+</sup>); pKID-Min2 ( $t_R = 44.1$  min, exp. 1044.5, obs. 1045.2 (M + H)<sup>+</sup>); pKID-Min3 ( $t_R = 37.0$  min, exp. 1688.9, obs. 845.8 (M + 2H)<sup>2+</sup>); pKID-Min4 ( $t_R = 34.3$  min, exp. 1046.5, obs. 1047.5 (M + H)<sup>+</sup>); pKID-Min5-PKC ( $t_R = 43.5$  min, exp. 1718.9, obs. 860.9 (M + 2H)<sup>2+</sup>); pKID-Min6 ( $t_R = 39.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 808.1 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); pKID-Min7-PKA( $t_R = 52.9$  min, exp. 1599.8, obs. 1176.4 (M + H)<sup>+</sup>); phosphorylated pKID-Min-pThr9 ( $t_R = 49.2$  min, exp. 1565.7, obs. 1567.3 (M + H)<sup>+</sup>); phosphorylated pKID-Min-pThr12 ( $t_R = 51.3$  min, exp. 1565.7, obs. 1589.6 (M + Na)<sup>+</sup>); phosphorylated pKID-Min1 ( $t_R = 36.2$  min, exp. 1109.5, obs. 1110.5 (M + H)<sup>+</sup>); phosphorylated pKID-Min1 ( $t_R = 36.2$  min, exp. 1109.5, obs. 1110.5 (M + H)<sup>+</sup>); phosphorylated pKID-Min1 ( $t_R = 36.2$  min, exp. 1109.5, obs. 1110.5 (M + H)<sup>+</sup>); phosphorylated pKID-Min1 ( $t_R = 36.2$  min, exp. 1109.5, obs. 1110.5 (M + H)<sup>+</sup>); phosphorylated pKID-Min1 ( $t_R = 36.2$  min, exp. 1109.5, obs. 1110.5 (M + H)<sup>+</sup>); phosphorylated pKID-Min1 ( $t_R = 36.2$  min, exp. 1109.5, obs. 1110.5 (M + H)<sup>+</sup>); phosphorylated pKID

pKID-Min2 ( $t_R = 43.9 \text{ min}$ , exp. 1123.5, obs. 1147.5 (M + Na)<sup>+</sup>); phosphorylated pKID-Min3 ( $t_R = 39.4 \text{ min}$ , exp. 1767.9, obs. 885.9 (M + 2H)<sup>2+</sup>); phosphorylated pKID-Min4 ( $t_R = 30.6 \text{ min}$ , exp. 1125.5, obs. 1127.4 (M + H)<sup>+</sup>); phosphorylated pKID-Min5-PKC ( $t_R = 42.8 \text{ min}$ , exp. 1797.9, obs. 900.7 (M + 2H)<sup>2+</sup>); phosphorylated pKID-Min6 ( $t_R = 43.5 \text{ min}$ , exp. 1678.8, obs. 848.3 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); phosphorylated pKID-Min7-PKA ( $t_R = 48.9 \text{ min}$ , exp. 1678.8, obs. 848.3 (M + 2H)<sup>2+</sup> + Na<sup>+</sup>); phosphorylated pKID-Min8 ( $t_R = 32.7 \text{ min}$ , exp. 1185.1, obs. 1186 (M + H)<sup>+</sup>); phosphorylated pKID-Min9-CK1 ( $t_R = 41.3 \text{ min}$ , exp. 1255.5, obs. 1256.2 (M + H)<sup>+</sup>).

#### Fluorescence spectroscopy and dissociation constant determination

The fluorescence spectra for each peptide were collected on a Photon Technology International fluorescence spectrometer model QM-3/2003 with a CW source and a Hamamatsu R928 photomultiplier tube. All experiments were conducted with an excitation wavelength of 280 nm. Data were collected using 10 nm excitation and emission slit widths for all peptides at 10  $\mu$ M. Fluorescence spectra of peptides at 2  $\mu$ M were conducted using 15 nm slit widths on the excitation and emission monochromators. All spectra were acquired at room temperature. Data for pKID-Min1, pKID-Min2, pKID-Min3, pKID-Min4, pKID-Min5-PKC, pKID-Min6, pKID-Min-pThr9, and pKID-Min-pThr12 were collected every 1 nm with an averaging time of 2 s. Data for pKID-Min7-PKA were collected every 1 nm with an averaging time of 1 s. At least three independent binding titrations were conducted for each peptide. All fluorescence experiments were conducted using a 495 nm highpass filter (model 495FG03-25 AM-53074; Andover Corporation, NH) on the emission monochromator.

Peptide solutions were prepared containing buffers with final concentrations of 10 mM HEPES (pH 7.5), 100 mM NaCl, and 2 mM MgCl<sub>2</sub>, with a final peptide concentration of 10  $\mu$ M unless otherwise indicated. Spectra were acquired using 10 mm quartz fluorescence cells (Starna). Terbium titrations were conducted using serial dilutions of a terbium solution, with each terbium solution independently mixed with a solution of peptide. Each emission spectrum represents an independently prepared solution of peptide and metal. The terbium emission band at 544 nm was used to evaluate metal binding.

Data at the emission maximum of 544 nm were plotted against  $Tb^{3+}$  concentration to calculate the final  $K_d$ . Data points indicate the average of at least three independent titrations. Error bars indicate standard error. The data were fit to equation (1) using a non-linear least squares fitting algorithm (KaleidaGraph, version 4.5, Synergy Software), where Q = fluorescence,  $Q_o$  = fluorescence of the apopeptide,  $Q_c$  = fluorescence of the peptide-metal complex,  $M_t$  = total metal concentration,  $K_d$  = dissociation constant, and  $P_t$  = total peptide concentration.

$$Q = Q_0 + (Q_c - Q_0) \frac{\left[(M_t + K_d + P_t) - \sqrt{(M_t + P_t + K_d)^2 - 4(P_t)(M_t)}\right]}{2P_t}$$

#### Phosphorylation by protein kinase C (PKC)

The reaction mixture was prepared to a final volume of 25  $\mu$ L as follows: stock solutions were mixed to yield final concentrations of 500  $\mu$ M ATP, 80  $\mu$ M non-phosphorylated pKID-Min5-PKC, 10  $\mu$ L lipid activator (5  $\mu$ g phosphatidylserine, 0.5  $\mu$ g diacylglycerol, 8 mM MOPS (pH 7.2), 10 mM  $\beta$ -glycerolphosphate, 400  $\mu$ M sodium orthovanadate, 400  $\mu$ M DTT, 400  $\mu$ M

(1)

CaCl<sub>2</sub>), 2 mM MgCl<sub>2</sub>, and 2  $\mu$ L PKC $\alpha$  enzyme solution (25 ng, Promega, catalog #V5261). After incubation at 30 °C for 120 min, the reaction mixture was analyzed by HPLC, fluorescence spectroscopy, and ESI-MS. Fluorescence samples were prepared by adding 75  $\mu$ L buffer (27 mM HEPES (pH 7.5), 270 mM NaCl, 5.3 mM MgCl<sub>2</sub>) and 100  $\mu$ L Tb<sup>3+</sup> solution (160  $\mu$ M) directly to the reaction mixtures. Solutions were heated at 70 °C for 30 minutes before injection on the HPLC, in order to inactivate the enzyme. Fluorescence data represent the average of at least three independent trials.

### Phosphorylation by protein kinase A (PKA)

The reaction mixture was prepared to a final volume of 50  $\mu$ L as follows: stock solutions were mixed to yield final concentrations of 10 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 400  $\mu$ M ATP, 200  $\mu$ M non-phosphorylated pKID-Min7-PKA, 2  $\mu$ L of the diluted PKA enzyme (New England Biolabs catalog # P6000S, 50-fold dilution, 50 units). After incubation at 37 °C for 1 hour, the reaction mixture was analyzed by HPLC, fluorescence spectroscopy, and ESI-MS. Fluorescence samples were prepared by adding 50  $\mu$ L buffer (40 mM HEPES (pH 7.5), 400 mM NaCl, 8 mM MgCl<sub>2</sub>), and 100  $\mu$ L Tb<sup>3+</sup> solution (160  $\mu$ M) directly to the reaction mixtures. Solutions were heated at 70 °C for 30 minutes before injection on the HPLC, in order to inactivate the enzyme. Fluorescence data represent the average of at least three independent trials.

## Dephosphorylation by calf intestine alkaline phosphatase (CIP)

The reaction mixture of phosphorylated pKID-Min7-PKA was prepared to a final volume of 25  $\mu$ L as follows: stock solutions were mixed to yield final concentrations of 20 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM DTT, 80  $\mu$ M phosphorylated pKID-Min7-PKA, 2  $\mu$ L CIP enzyme solution (New England Biolabs, M0290S; the stock enzyme was diluted 10-fold to generate the solution added, 20 units). After incubation at 37 °C for 10 minutes, the reaction mixture was analyzed by HPLC, fluorescence spectroscopy, and ESI-MS. Fluorescence samples were mixed to yield final concentration of 10 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 80  $\mu$ M Tb<sup>3+</sup>. Solutions were heated at 70 °C for 30 minutes before injection on the HPLC, in order to inactivate the enzyme. Fluorescence data represent the average of at least three independent trials.

## Phosphorylation by Casein Kinase 1 (CK1)

The reaction mixture was prepared to a final volume of 25  $\mu$ L as follows: stock solutions were mixed to yield final concentrations of 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.5 mM DTT, 400  $\mu$ M ATP, 80  $\mu$ M non-phosphorylated pKID-Min9-CK1, 2  $\mu$ L CK1 enzyme (New England Biolabs catalog #P6030S, 2,000 units). ATP was supplemented (1  $\mu$ L of a 4,000  $\mu$ M solution) at 90 minutes. After incubation at 30 °C for 3 hours, the reaction mixture was analyzed by HPLC, fluorescence spectroscopy, and ESI-MS. Fluorescence samples were prepared by adding 75  $\mu$ L buffer (27 mM HEPES (pH 7.5), 270 mM NaCl, 5.3 mM MgCl<sub>2</sub>), and 100  $\mu$ L Tb<sup>3+</sup> solution (160  $\mu$ M) directly to the reaction mixtures. Solutions were heated at 70 °C for 10 minutes before injection on the HPLC, in order to inactivate the enzyme. Fluorescence data represent the average of at least three independent trials.

#### **Cell culture and cell extracts**

HeLa cells (ATCC) were cultured in a 37 °C humidified environment containing 5% CO2

in air. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100  $\mu$ g/mL). Serum starvation was achieved by incubation in DMEM containing 0.5% heat in-activated fetal bovine serum for 20 hours prior to harvest. 2 × 10<sup>8</sup> HeLa cells were then trypsinized and centrifuged, followed by resuspension in 2 mL of PBS (8 mM Na<sub>3</sub>PO<sub>4</sub>, 1.5 mM K<sub>3</sub>PO<sub>4</sub>, 2.7 mM KCl and 137 mM NaCl in high purity dH<sub>2</sub>O, pH 7.4). The cells were centrifuged and resuspended in 1 mL of Buffer A (0.4 M HEPES (pH 7.9), 60 mM MgCl<sub>2</sub>, 400 mM KCl, 20 mM DTT, and 8 mM phenylmethylsulfonyl fluoride (PMSF) in H<sub>2</sub>O). The cells were centrifuged and resuspended again in 1 mL Buffer A, followed by incubation on ice for 10 min. The cells were checked for lysis. If lysed, the cells were vortexed for 30 seconds, centrifuged, and resuspended in 1 mL Buffer B (100mM HEPES (pH 7.9), 4.1 M NaCl, 14.7 mM MgCl<sub>2</sub>, 200  $\mu$ M EDTA, 5 mM Dithiothreitol (DTT), 5 mM PMSF in 2.5% glycerol). Cells were incubated on ice for 15 min, and then centrifuged for 5 minutes. The supernatant was divided into aliquots and frozen.

#### Phosphorylation of pKID-Min7-PKA in HeLa cell extracts

The reaction mixtures of pKID-Min7-PKA in HeLa cell extracts were prepared to a final volume of 60  $\mu$ L as follows: stock solutions were mixed to yield final concentrations of 400  $\mu$ M ATP, 200  $\mu$ M  $\beta$ -glycerolphosphate, 200  $\mu$ M sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), 2 mM DTT, 80  $\mu$ M non-phosphorylated pKID-Min7-PKA, and 50  $\mu$ L HeLa cell extracts. 200  $\mu$ M ATP (final concentration of newly added ATP) was supplemented after 30 minutes and after 1 hour of incubation of the reaction mixtures at 37 °C. After 2 hours, the reaction mixture was analyzed by HPLC, fluorescence spectroscopy, and ESI-MS. Solutions for analysis by fluorescence spectroscopy were mixed to yield final concentration of 10 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM MgCl<sub>2</sub>, and 80  $\mu$ M Tb<sup>3+</sup>. Solutions were heated at 70 °C for 30 minutes before injection on the HPLC. Fluorescence data represent the average of at least three independent trials.



HPLC analysis of reactions of peptides with enzymes in solution and in cell extracts

Figure S1. HPLC analysis of phosphorylation of pKID-Min5-PKC by PKC. (top) Coinjection of phosphorylated and non-phosphorylated pKID-Min5-PKC; (bottom) Analytical HPLC chromatogram (UV detection at 215 nm) of the reaction mixture of pKID-Min5-PKC incubated with PKC for 60 minutes, using a linear gradient of 0-50% buffer A (98% H<sub>2</sub>O, 2% MeCN, 0.06% heptafluorobutyric acid (HFB)) in buffer B (80% MeCN, 20% H<sub>2</sub>O, 0.05% HFB) over 60 minutes on a Rainin Microsorb MV C18 column (4.6 × 250 mm, 100 Å pore). Peaks were identified by ESI-MS and by coinjection with the purified non-phosphorylated and phosphorylated pKID-Min5-PKC peptides. Quantification of the volumes of the respective peaks indicates 47% phosphorylation under these conditions.



Figure S2. HPLC analysis of phosphorylation of pKID-Min7-PKA by PKA. (top) Coinjection of phosphorylated and non-phosphorylated pKID-Min7-PKA; (bottom) Analytical HPLC chromatogram (UV detection at 215 nm) of the reaction mixture of pKID-Min7-PKA incubated with PKA for 60 minutes, using a linear gradient of 0-50% buffer A (98% H<sub>2</sub>O, 2% MeCN, 0.06% HFB) in buffer B (80% MeCN, 20% H<sub>2</sub>O, 0.05% HFB) over 60 minutes on a Rainin Microsorb MV C18 column (4.6 × 250 mm, 100 Å pore). Peaks were identified by ESI-MS and by coinjection with the purified non-phosphorylated and phosphorylated pKID-Min7-PKA peptides.



Figure S3. HPLC analysis of dephosphorylation of phosphorylated pKID-Min7-PKA by CIP. (a) Fluorescence of 10  $\mu$ M phosphorylated pKID-Min7-PKA (blue), phosphorylated pKID-optPKA incubated with CIP for 10 minutes (red) with 80  $\mu$ M Tb<sup>3+</sup>; (b) Analytical HPLC chromatogram (UV detection at 215 nm) of the reaction mixture of phosphorylated pKID-Min7-PKA incubated with CIP for 10 minutes, using a linear gradient of 0-50% buffer A (98% H<sub>2</sub>O, 2% MeCN, 0.06% HFB) in buffer B (80% MeCN, 20% H<sub>2</sub>O, 0.05% HFB) over 60 minutes on a Rainin Microsorb MV C18 column (4.6 × 250 mm, 100 Å pore). Peaks were identified by ESI-MS and by coinjection with the purified non-phosphorylated and phosphorylated pKID-Min7-PKA peptides.



Figure S4. HPLC analysis of phosphorylation of pKID-Min9-CK1 by Casein Kinase 1. (top) Co-injection of phosphorylated and non-phosphorylated pKID-Min9-CK1; (bottom) Analytical HPLC chromatogram (UV detection at 215 nm) of the reaction mixture of pKID-Min9-CK1 incubated with CK1 for 3 hours, using a linear gradient of 0-50% buffer A (98% H<sub>2</sub>O, 2% MeCN, 0.06% TFA) in buffer B (80% MeCN, 20% H<sub>2</sub>O, 0.05% TFA) over 60 minutes on a Rainin Microsorb MV C18 column (4.6 × 250 mm, 100 Å pore). Peaks were identified by ESI-MS and by coinjection with purified non-phosphorylated and phosphorylated pKID-Min9-CK1 peptides.



Figure S5. HPLC analysis of phosphorylation of pKID-Min7-PKA in HeLa cell extracts. Analytical HPLC chromatogram (UV detection at 215 nm) of the reaction mixture of pKID-Min7-PKA incubated in HeLa cell extracts for 120 minutes, using a linear gradient of 0-50% buffer A (98% H<sub>2</sub>O, 2% MeCN, 0.06% HFB) in buffer B (80% MeCN, 20% H<sub>2</sub>O, 0.05% HFB) over 60 minutes on a Rainin Microsorb MV C18 column (4.6 × 250 mm, 100 Å pore). Peaks were identified by ESI-MS and by coinjection with the purified non-phosphorylated and phosphorylated pKID-Min7-PKA peptides. Integration of the peak volumes indicates that pKID-Min7-PKA was 61% phosphorylated.

## NMR Spectroscopy



Figure S6. Superposition of the TOCSY spectra of phosphorylated pKID-Min4 in the absence (red) and presence (blue) of 1 equivalent La<sup>3+</sup>. Experiments were conducted at 296 K with 800  $\mu$ M peptide in a solution with 90% H<sub>2</sub>O/10% D<sub>2</sub>O, 5 mM NaCl, and 100  $\mu$ M TSP. The pH of the solutions were adjusted to pH 6.5 prior to the conduct of the NMR experiments. Spectra were acquired using a watergate TOCSY pulse sequence, with 2048 direct and 600 indirect data points, sweep widths of 7184 Hz (12 ppm) in both dimensions, 4 scans per *t*1 increment, and a relaxation delay of 2.0 s.



Figure S7. Superposition of the <sup>1</sup>H-<sup>13</sup>C HSQC spectra of phosphorylated pKID-Min4 in the absence (red) and presence (blue) of 1 equivalent La<sup>3+</sup>. Experiments were conducted at 296 K with 800  $\mu$ M peptide in a solution with 90% H<sub>2</sub>O/10% D<sub>2</sub>O (apo peptide) or 100% D<sub>2</sub>O (metal complex), with 5 mM NaCl and 100  $\mu$ M TSP. The pH of the solutions were adjusted to pH 6.5 prior to the conduct of the NMR experiments. Spectra were acquired using a gradient <sup>1</sup>H-<sup>13</sup>C HSQC pulse sequence, with 2048 direct data points and 400 indirect data points, sweep widths of 5388 Hz (9 ppm) in the direct dimension and 12073 Hz (80 ppm) in the indirect dimension, 64 scans per *t*1 increment, and a relaxation delay of 2.0 s.

# Analysis of EF-Hand-like proteins in the PDB with Glu at residue 9 but lacking Glu at residue 12

In order to identify proteins that might adopt a structure similar to that of a minimal protein kinase-inducible domain that is phosphorylated at residue 9, we examined the PDB for proteins with the following PDB sequence motif:

## DXDXDGXXEXX[ACFGHIKLMNPQRSTVWY]

This minimal motif includes Asp at residues 1, 3, and 5; Gly at residue 6; Glu at residue 9; and any residue other than Asp or Glu at residue 12. The search was conducted with a 90% sequence similarity cutoff. These resultant structures were then manually examined for calcium binding. Proteins lacking electron density at this motif or lacking a bound metal were removed from the analysis. 8 loops were identified (from 5 proteins) with  $\leq 2.0$  Å resolution in which a DXDXDGXXEXX[not D/E] motif bound metal via the 9-residue motif. 7 of these metal-binding loops exhibited bound Ca<sup>2+</sup>. In addition, one exhibited bound Zn<sup>2+</sup>. These motifs had similar conformations to one another (and to classical EF-Hands) in residues 1-7, but had more conformational heterogeneity observed at Glu9.

	prot	ein																boun	d Zn <sup>2+</sup>					
	3vgi1.07 A 2zux 1.32 Å					2zux			2zux			<b>4n2k</b> 1.65 Å				<b>2zuy</b> 1.65 Å			1	<b>4a7k</b> 2.0 Å				
residue 68-76				158-166				-230		369-377			123-131			189-197			336-344		343-351			
1	D	-140	79	D	-95	98	D	-81	85	D	-81	102	D	-95	91	D	-82	87	D	-78	103	D	-88	75
2	κ	-67	-31	V	-80	-18	L	-70	-35	V	-96	-22	Α	-87	-23	L	-73	-31	V	-102	-12	- I	-61	-37
3	D	-92	2	D	-116	-3	D	-97	-9	D	-106	1	D	-91	4	D	-101	-9	D	-118	5	D	-101	2
4	G	60	31	G	80	22	G	80	30	G	82	11	R	66	32	G	81	24	G	80	12	G	72	22
5	D	-97	5	D	-94	1	D	-95	-1	D	-89	4	D	-100	1	D	-82	-6	D	-92	5	D	-95	2
6	G	85	2	G	86	7	G	99	-2	G	87	-14	G	89	1	G	99	1	G	86	-11	G	90	-3
7	N	-122	107	V	-116	132	Q	-124	135	Κ	-101	144	V	-117	157	K	-127	136	ĸ	-103	147	E	-117	135
8	Р	-67	148	V	-70	143	Υ	-72	144	D	-80	138	V	-102	106	A	-74	148	D	-78	137	D	-70	130
9	E	-76	-33	Е	-95	155	Е	-113	174	Е	-105	168	Е	-74	160	E	-120	174	E	-103	162	E	-81	169