Published in final edited form as:

Org Biomol Chem. 2019 April 17; 17(16): 3984–3995. doi:10.1039/c9ob00502a.

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

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Abstract

Protein kinases and phosphatases modulate protein structure and function, which in turn regulate cellular activities. The development of novel proteins and protein motifs that are responsive to protein phosphorylation provides new ways to probe the functions of individual protein kinases and the intracellular effects of their activation and downregulation. Herein we develop a minimal motif that is responsive to protein phosphorylation, termed a minimal protein kinase-inducible domain. The encodable protein motif comprises a 7- or 8-residue sequence (DKDADXW or DKDADXXW), derived from EF-Hand calcium-binding domains, that is necessary but not sufficient for binding terbium, combined with a protein phosphorylation site (Ser or Thr at residue 9) that, upon phosphorylation, completes the metal-binding motif. Thus, the motif binds metal poorly and exhibits weak terbium luminescence when not phosphorylated. Upon phosphorylation, the peptide binds metal with significantly higher affinity and exhibits robust terbium luminescence. Phosphorylation results in up to a 23× increase in terbium luminescence. Minimal phosphorylation-dependent motifs as small as 9 residues (DKDADGWIS) were developed. NMR spectroscopy on this lanthanum(III)-phosphopeptide complex confirmed that binding occurs in a manner similar to that in an EF-Hand, despite the absence of the conserved Glu12 typically present in an EF-Hand. By combining molecular design with known protein kinase recognition sequences, minimal protein kinase-inducible domains were developed that were responsive to phosphorylation by Protein Kinase A (PKA: DKDADRRW(S/pS)I), Protein Kinase C (PKC: DKDADGWI(T/pT)FRRKA), and Casein Kinase 1 (CK1: DKDADDWA(S/pS)I). Phosphorylation by PKA was quantified in HeLa cell extracts, with a 4.4× increase in fluorescence (terbium luminescence) observed at 544 nm. The optimized minimal motif includes alternating aspartate residues at positions 1, 3, and 5, plus binding through the main-chain carbonyl at position 7; a lysine at position 2 to provide electrostatic balance and reduce binding in the absence of phosphorylation; an alanine at residue 4 to promote the α_L conformation observed at that position of the EF Hand; a tryptophan at residue 7 or 8 to sensitize terbium luminescence; and a phosphorylation site with serine or threonine at residue 9. Residues at positions 6; 7 or 8; and 10 or later may be changed to provide kinase specificity. In the CK1-responsive peptide, the acidic residues in the proto-terbium-binding motif are employed as part of the kinase recognition sequence. This work thus presents fundamental rules for the design of compact phosphorylation-responsive terbium-binding motifs, with potential further application to motifs responsive to other protein post-translational modifications.

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Minimal encodable peptides responsive to phosphorylation were developed, based on phosphorylation of Ser/Thr at residue 9 of an EF Hand.

Introduction

Protein kinases are ubiquitous intracellular enzymes in eukaryotes that phosphorylate serine, threonine, and tyrosine residues in proteins, with reversible phosphorylation effected as a result of dephosphorylation by protein phosphatases.^{1, 2} The number of protein kinases and phosphatases (over 650 total in humans), combined with their varied outputs and their complex responses to diverse inputs, has inspired the development of a wide range of approaches to understand the functions of individual protein kinases.³ Primary among these approaches has been the development of peptides and proteins that exhibit an increase in fluorescence as a function of phosphorylation by a specific protein kinase.^{4–14}

We previously described an approach to phosphorylation-dependent protein design, termed protein kinase-inducible domains (pKID).^{15, 16} In this approach, we re-engineered an EF-Hand calcium-binding domain to be responsive to protein phosphorylation, with phosphorylation leading to terbium binding and terbium luminescence.^{10, 17–33} Single EF-Hand motifs bind calcium through alternating Asp or Asn residues at residues 1, 3, and 5; the main-chain carbonyl at residue 7; a polar amino acid with an oxygen-containing side chain at residue 9;³⁴ and a conserved Glu at residue 12, which binds the metal in a bidentate manner.^{35–39} In a typical protein-based EF Hand, two EF-Hand motifs fold against one another to generate a ~50 amino acid structure that can bind 2 calcium atoms with nanomolar affinity. Extensive prior work has shown that single EF-Hands (12–16 amino acids) can bind trivalent lanthanides (generically, Ln^{3+}), including the luminescent lanthanide terbium (Tb^{3+}), with micromolar affinity, while only binding calcium with millimolar affinity. Inclusion of Trp at residue 7 or 8⁴⁰ of an EF Hand renders the peptide luminescent (fluorescent), with excitation of Trp at residue 7 (280–350 nm excitation wavelength) resulting in energy transfer and sensitized emission of terbium (analyzed via fluorescence spectroscopy) at 544 nm.^{41, 42} Sensitized terbium emission is dependent on a close distance between the donor fluorophore (here, tryptophan) and terbium, with a donor fluorophore-metal distance $< 10 \text{ \AA}$ is ideal.^{10, 32, 43–49} The inclusion of tryptophan in an EF Hand motif at or near residue 7, which binds the metal via its main-chain carbonyl, has been shown in numerous examples to yield efficient energy transfer and sensitized terbium emission.

In our initial designs, the conserved Glu12 of an EF Hand was recognized as a potential basis for the design of a phosphorylation-dependent protein switch.^{15, 16} In that work, we employed phosphoserine at residue 12 to mimic the native Glu12, in an inverse of typical approaches to phosphomimetics (using Glu to mimic a phosphoserine). Non-phosphorylated Ser poorly mimics Glu, resulting in poor terbium binding and weak terbium luminescence. In contrast, phosphorylation of Ser12, to generate phosphoserine12, recapitulates the complete EF-Hand ligand sphere, resulting in a large increase in terbium affinity and terbium luminescence. Using this approach, combined with the incorporation of residues for protein kinase recognition, kinase-responsive and phosphorylation-dependent terbium binding and terbium luminescence was achieved, allowing the fluorescence-based detection of protein kinase activity with high signal-to-noise and a large dynamic range in complex solutions, including in cell extracts. This approach was subsequently applied, using appropriate consideration of differences in protein geometry of the resultant metal-binding residues, to the design of protein motifs responsive to tyrosine phosphorylation and to cysteine glutathionylation.⁵⁰

We sought to develop a minimally sized protein motif responsive to phosphorylation. The development of alternative approaches was also expected to increase the range of protein kinases for which sensors could be designed. In a standard EF-hand motif, residue 12 is a Glu, which binds the metal in a bidentate manner. In contrast, residue 9 (typically Ser, Thr, or Asp) binds the metal via a water-mediated contact.^{34, 51} However, in model peptides, Szabo *et al.* found that incorporation of Glu both at residue 9 and at residue 12 of a simple EF hand peptide resulted in enhanced terbium luminescence.⁴⁰ The concept of Tb³⁺ binding via Glu9 and Glu12 was substantially expanded in studies by Imperiali and co-workers.^{52–55} In their design and characterization of high-affinity lanthanide-binding tags, they demonstrated that a Glu9 and Glu12 are capable of simultaneous, direct metal binding (Figure 1) in a context where residue 10 is Gly, which promotes a conformation allowing simultaneous binding of both Glu9 and Glu12. These results suggested the possibility to generate EF-hand-based kinase-inducible protein motifs that employ residue 9 as the site of phosphorylation. That approach is examined herein.

Experimental

Peptide synthesis, purification, and characterization.

Peptides were synthesized by standard solid-phase peptide synthesis. All peptides were acetylated on the N-terminus and contained C-terminal amides. Phosphorylated peptides were synthesized using trityl-protected serine or threonine, with the trityl group selectively removed after peptide synthesis and prior to cleavage from resin and deprotection. Peptides were chemically phosphorylated using standard global phosphorylation methods employing reaction of di-O-benzyl-phosphoramidite with the resultant free Ser or Thr hydroxyls, followed by oxidation of the phosphite to the phosphate with *t*-butyl hydroperoxide. Peptides were purified to homogeneity via reverse-phase HPLC and characterized via mass spectrometry. Details are in the Supporting Information.

Terbium luminescence experiments and determination of dissociation constants of peptide-terbium complexes.

Terbium solutions were prepared by serial dilutions from a freshly prepared stock solution of TbCl_3 . Solutions of peptide in buffer were mixed 1:1 with the terbium solutions, to generate the final solutions used for analysis. Peptide and terbium solution were mixed immediately before fluorescence spectroscopy. Thus, each data point in an individual terbium titration experiment represented an independent solution of peptide and metal. The final solution conditions were 10 mM HEPES pH 7.5, 100 mM NaCl, and 2 mM MgCl_2 in water. Peptide concentrations were 10 μM unless otherwise indicated. Each data point represents the average of at least 3 independent experiments. Error bars indicate standard error. A triad of experiments (on the non-phosphorylated peptide, on the phosphorylated peptide, and on the terbium solution) was conducted in parallel on the same day (typically within the same 4 hour period), in order to minimize systematic errors due to electronic drift, PMT sensitivity, lamp power, or changes in the alignment of the slit widths. Thus, the fluorescence intensities (y-axis in fluorescence plots) are always directly comparable between the non-phosphorylated and phosphorylated forms of a given peptide, but are not necessarily comparable between peptides of different series, due to the conduct of experiments on different peptides at different times. A fluorescence excitation wavelength of 280 nm was used. A 495 nm highpass filter was used before the emission monochromator. The fluorescence data at the maximum of the most intense terbium luminescence emission band at 544 were fit via a non-linear least squares fitting to a 1:1 binding isotherm. Additional details are in the Supporting Information.

NMR spectroscopy.

NMR samples of pKID-Min4 were prepared with a final peptide concentration of 800 μM , in a solution containing 100 μM TSP (3-trimethylsilyl-2,2,3,3- d_4 -propionic acid) and 5 mM NaCl in 90% H_2O /10% D_2O in a total volume of 400 μL . The pH was adjusted to pH 6.5. NMR experiments were conducted in the absence of metal and in the presence of one equivalent of the diamagnetic metal LaCl_3 , in order to examine the changes caused by the formation of the metal-phosphopeptide complex. NMR spectra were recorded on a Bruker AVC 600 MHz NMR spectrometer equipped with a triple resonance cryoprobe. TOCSY and NOESY NMR experiments were conducted for sequential residue assignment and for specific resonance assignments. ^1H - ^{13}C HSQC spectra for the apo peptide were recorded under identical conditions. ^1H - ^{13}C HSQC spectra for the peptide complex with La^{3+} were recorded using solutions in 100% D_2O . Experiments were conducted using La^{3+} due to the paramagnetism of Tb^{3+} (6 unpaired electrons), which results in severe broadening and peak shifts, precluding detailed analysis of the structure of the peptide using the metal employed for optimization.

Enzymatic phosphorylation.

Peptides were enzymatically phosphorylated with PKA, PKC, or CK1 using the suppliers' protocols. After enzymatic phosphorylation, the peptides were diluted into buffer for analysis by fluorescence spectroscopy. After fluorescence experiments, peptides were characterized by HPLC and mass spectrometry to determine the extent of phosphorylation

and to confirm the identity of the phosphorylated peptide. Details are in the Supporting Information.

Results

In order to test the ability to re-engineer an EF-Hand to be responsive to phosphorylation at residue 9, a minimal sequence (pKID-Min-pThr9/pKID-Min-pThr12) was developed that allows the direct comparison of phosphorylation at residue 9 versus at residue 12 (Figure 2, Figure 3).^{56, 57} This sequence used Thr at both phosphorylatable sites. The N-terminal sequence of the EF Hand used the consensus sequence DXNADGWI, including Asn at residue 3, which is observed in EF-Hand peptides with the highest terbium affinities.^{40, 58} EF Hand proteins adopt an α_L conformation (left-handed α -helix) at residue 4 and a (+90°, 0°) conformation at residue 6.^{53, 59} A and G residues are commonly observed at these positions, as these amino acids can readily adopt conformations on the right side of the Ramachandran plot.^{35–39} Residues 7 and 8 adopt an extended conformation, with aromatic and β -branched amino acids most common here. This peptide also employed a Leu at residue 2, to reduce the likelihood of electrostatic repulsion with the metal and to favor the α_R conformation observed at this position. The remaining residues in the peptide (Ala or Lys) were chosen to promote the α -helix which begins at residue 10 in EF-Hand proteins.⁶⁰

These peptides were examined for Tb³⁺ binding and Tb³⁺ luminescence in their non-phosphorylated and phosphorylated states (Figure 4, Table 1). Notably, while both peptides exhibited phosphorylation-dependent Tb³⁺ binding, significantly higher Tb³⁺ affinity was observed for the peptide phosphorylated at Thr9, which resulted in a greater fluorescence difference between the non-phosphorylated and phosphorylated states, as desired. These data indicated that residue 9 could be an effective site for phosphorylation-dependent terbium binding and fluorescence. Indeed, some EF-Hand Ca²⁺-binding motifs include Glu at residue 9 but lack the typical Glu12. For example, Thompson and coworkers recently showed that such a 9-residue Ca²⁺-binding motif exhibited higher Ca²⁺ affinity than the other, typical EF-Hand motifs within protein arginine deaminase (sequence DADRDGVVE, residues 123–131, pdb 4n2k).^{56, 61}

We next examined peptides consisting of only the minimum number of residues (9) necessary for phosphorylation-dependent binding. Peptides were synthesized with both serine (pKID-Min1) and threonine (pKID-Min2) at residue 9, in order to test the role of phosphoacceptor residue on terbium affinity and terbium luminescence. While Ser/Thr protein kinases in general phosphorylate both residues, nature appears to employ Ser and Thr phosphorylation differently in some cases.^{2, 62–64} Most notably, the identity of the phosphorylated residue (Ser versus Thr) is generally conserved evolutionarily at a given site, even when solvent-exposed.⁶² In addition, protein kinases generally have a kinetic preference for one residue over the other, as determined by the identity of the DFG+1 residue.⁶⁴ Finally, Ser and Thr phosphorylation appear to have different structural effects, with Thr phosphorylation inducing a particularly ordered structure.^{65–67} Thus, we sought to identify whether both Ser and Thr could be accommodated within this minimal motif.

Both minimal peptides exhibited phosphorylation-dependent terbium binding and luminescence, with similar terbium affinities for both peptides (Figure 5, Table 1). However, interestingly, the peptide with phosphothreonine exhibited greater maximum terbium luminescence. Terbium luminescence intensity is impacted by interactions of the metal with water molecules, with greater water accessibility leading to reduced fluorescence.^{43, 68} The methyl group of phosphothreonine could potentially impact water dynamics at the metal, as one possible mechanism of the observed increased terbium luminescence.

Having established residue 9 as an effective site for phosphorylation-dependent terbium binding, we examined whether this site was compatible with the incorporation of a complex protein kinase recognition sequence. Protein kinase C (PKC) is a member of a family of related enzymes whose activities are upregulated in cancers.^{69–71} Fluorophore-conjugated peptide-based fluorescent sensors of PKC activity have been generated using only the residues C-terminal to the phosphorylation site.⁷² Thus, we examined a peptide (pKID-Min3) that combined the minimal 9-residue kinase-inducible domain sequence from pKID-Min1 with the PKC C-terminal kinase recognition sequence. The phosphorylated peptide pKID-Min3 exhibited a similar K_d for the terbium complex as the parent phosphorylated peptide pKID-Min1 (Figure 5d, Table 1). More broadly, pKID-Min3 exhibited excellent differentiation in the fluorescence of the phosphorylated peptide compared to the non-phosphorylated peptide, with substantially reduced terbium binding of the non-phosphorylated peptide, suggesting that cationic residues could be particularly effective in selection against binding of the non-phosphorylated peptides. However, the fluorescence of the phosphorylated peptide was relatively modest compared to background or to that of the parent peptide, which would reduce its potential applications, particularly in complex solutions.

The original approach we examined to a minimal protein kinase-inducible domain (pKID-Min-pThr9, pKID-Min1–3) employed Leu at residue 2, in order to minimize electrostatic repulsion with the trivalent cationic metal terbium. In addition, it included Asn at residue 3, based on Asn being observed in the highest affinity EF-Hand Tb^{3+} -binding motifs, where an overall -4 charge in the ligand sphere is optimal.^{40, 51} However, the approach to a minimal protein kinase-inducible domain being developed herein does not include the conserved Glu at residue 12 that binds metal in a bidentate manner. Thus, an Asp at residue 3 might yield improved metal affinity in a minimal motif, compared to a full EF-Hand motif. Moreover, a critical component of a kinase-responsive sensor is the ability to effectively bind metal when phosphorylated, but, importantly, to bind metal poorly when non-phosphorylated. Maximization of Tb^{3+} affinity would be expected to favor metal binding in both the non-phosphorylated and phosphorylated peptides, reducing the effectiveness as a sensor. As such, a cationic Lys at residue 2 could potentially decrease the metal affinity of the non-phosphorylated peptide for the trivalent cationic metal. Lys at residue 2 would improve design effectiveness as long as the impact of this residue in decreasing metal binding was greater on the non-phosphorylated peptide, which lacks a complete metal-binding motif, than on the phosphorylated peptide.

The resultant peptide with Lys at residue 2 and Asp at residue 3 (pKID-Min4) exhibited strong phosphorylation-dependent terbium binding and terbium luminescence (Figure 5ef,

Table 1). pKID-Min4 substantially reduced both the terbium luminescence and the terbium affinity of the non-phosphorylated peptide. Interestingly, pKID-Min4 also exhibited increased affinity of the phosphorylated peptide. We speculate that the cationic Lys at residue 2 can function to electrostatically balance the binding of the -5 ligand sphere (3 Asp plus dianionic phosphoserine) to the trivalent cationic Tb^{3+} .

The structural basis for metal binding in phosphorylated pKID-Min4 was further examined by NMR spectroscopy. NMR experiments were conducted using the diamagnetic lanthanide lanthanum (La^{3+}), which allows NMR analysis without broadening due to paramagnetism. TOCSY and 1H - ^{13}C HSQC spectra were employed to identify the residue-specific effects on metal binding and structure in phosphorylated pKID-Min4. These data (Figure 6, Table 2) indicated that the residues from throughout the EF-Hand motif exhibited changes in chemical shift consistent with the phosphorylated pKID-Min4• La^{3+} complex adopting an EF Hand-like metal-bound structure. Particularly large chemical shift changes were observed in the amide hydrogen of residues 4–9, with the largest change (+1.20 ppm) at the Ile8 amide hydrogen, which is conjugated to the Trp7 carbonyl that binds the metal in a canonical EF Hand. Substantial dispersion was also observed in the metal-bound complex, but not the apo peptide, for the diastereotopic Gly H α ($\delta = 0.43$ ppm in the La^{3+} complex, versus no difference in the apo peptide), and for the diastereomeric Asp5 H β ($\delta = 0.52$ ppm in the La^{3+} complex, versus $\delta = 0.02$ ppm in the apo peptide), consistent with substantial ordering at these amino acids. The largest C α chemical shift changes were observed in Asp3, Ala4, Asp 5, Trp7, and pSer9. In particular, based on ^{13}C chemical shift index (CSI) analysis, the upfield changes in C α chemical shift at Asp3, Asp5, and Asp7, and the downfield changes in chemical shift at Ala4, Gly6, and Ile8 are consistent with expectations for the conformations in the metal-bound form of an EF-Hand.^{73, 74}

The 9-residue peptide pKID-Min4 thus exhibited both phosphorylation-dependent terbium binding and a structure in the metal-bound complex that was consistent with the adoption of a structure similar to that observed in an EF Hand•metal complex. However, this peptide lacked a protein kinase recognition sequence, and as such is not able to function as a sensor of protein kinase activity. In order to develop a minimal kinase-response motif, a variant of this peptide was synthesized to be responsive to phosphorylation by protein kinase C (PKC). This peptide (pKID-Min5-PKC) incorporated a C-terminal FRRKA recognition sequence, which has been previously employed in PKC sensor peptides,^{6, 72} as well as Thr at residue 9.

The peptide pKID-Min5-PKC exhibited strong phosphorylation-dependent terbium luminescence (Figure 7, Table 1).⁷⁵ In addition, the terbium affinity of the phosphorylated peptide was similar to that of phosphorylated pKID-Min4. Notably, these data indicate that the incorporation of the cationic residues in the PKC recognition sequence did not significantly reduce the terbium affinity of the phosphorylated peptide, with the favorable electrostatics of phosphothreonine in the context of the metal-binding loop overcoming the unfavorable electrostatics of Tb^{3+} with the cationic Arg, Arg, and Lys residues C-terminal to phosphothreonine. In addition, this peptide was effectively enzymatically phosphorylated *in vitro* by PKC, with a large fluorescence increase after incubation with PKC. Collectively, these data indicate that a minimal 9-residue protein kinase-inducible domain can be rationally modified to detect protein kinase activity and exhibit strong phosphorylation-

dependent terbium binding and terbium luminescence, as well as be applied to the *in vitro* fluorescence-based detection of enzyme activity.

In order to generalize this approach to the application of minimal protein kinase-inducible domains as sensors of protein kinase activity, we sought to examine the fluorescence-based detection of the activities of other protein kinases. cAMP-Dependent protein kinase (Protein Kinase A [PKA]) exhibits a sequence preference for at least two Arg residues N-terminal to the Ser/Thr phosphorylation site, as well as a preference for multiple hydrophobic residues C-terminal to Ser/Thr.^{76–80} We initially examined a peptide (pKID-Min6) that retains Trp at residue 7, as the ideal position for sensitization of terbium luminescence, and includes Arg at residues 6 and 8 (the –3 and –1 positions relative to the Ser phosphorylation site). This peptide exhibited strong phosphorylation-dependent terbium binding and luminescence (Figure 8, Table 1). These data indicate that the incorporation of multiple cationic residues within the core 9-residue terbium-binding motif was compatible with favorable phosphorylation-dependent terbium binding and terbium luminescence. Indeed, the terbium affinity of phosphorylated pKID-Min6 (overall peptide charge –1) was similar to that of phosphorylated pKID-Min4 (overall peptide charge –4), presumably due to the retention of the same metal-binding residues. However, this peptide exhibited no evidence of phosphorylation by PKA under the conditions examined.

PKA has a strong preference for Arg residues at the –2 and –3 positions relative to the site of phosphorylation (here, Ser9).^{76–79} Therefore, we examined a peptide with Arg at residues 6 and 7. Here, Trp was employed at residue 8 to effect sensitization of terbium luminescence, which has previously been shown to occur only modestly worse at this position than when Trp is at residue 7 of an EF Hand.⁴⁰ The resultant peptide pKID-Min7-PKA exhibited robust phosphorylation-dependent terbium luminescence, albeit with a somewhat lower distinction in terbium binding between the phosphorylated and non-phosphorylated forms of the peptide (Figure 8cd, Table 1). However, in contrast to pKID-Min6, PKA rapidly phosphorylated pKID-Min7-PKA, resulting in the fluorescent detection of protein kinase activity (Figure 8e). This peptide was also rapidly dephosphorylated by phosphatase, resulting in a large reduction in terbium luminescence after incubation with enzyme (Figure 8f). These data indicate that the EF-Hand-based design principles allow the development of a minimal protein kinase-inducible domain with independently identified optimized protein kinase recognition sequences, here the RRXSII sequence of the optimized PKA substrate Kemptide. These data also indicated flexibility in the identity of residue 6 beyond the broadly conserved Gly.

In pKID-Min5-PKC and pKID-Min7-PKA, we demonstrated the development of kinase-responsive peptide motifs that exhibited significant increases in terbium luminescence upon phosphorylation. Both peptides were effectively phosphorylated by the cognate enzymes, resulting in a large increase in fluorescence in the presence of terbium. These peptides incorporated multiple cationic residues either N-terminal or C-terminal to the site of phosphorylation, suggesting generality in the design of minimal protein kinase-inducible motifs responsive to diverse protein kinases that include cationic residues as part of the protein kinase recognition sequence. Notably, numerous other protein kinases have similar

recognition sequences with multiple N-terminal cationic residues, including Akt/PKB, PAK1, CAMK, Pim1, and Aurora Kinase C.^{78, 79, 81}

However, some protein kinases have a preference for sequences with multiple N-terminal anionic residues. The work herein (e.g. pKID-Min6 or pKID-Min7-PKA) or previously (pKID-Abl, Figure 3) demonstrated that the identity of the residue at the non-coordinating positions of the EF Hand (residues 6, 7, or 8) did not substantially impact the phosphorylation-dependent terbium binding of protein kinase-inducible domains. Therefore, we envisioned that we could develop motifs responsive to phosphorylation by kinases that prefer anionic amino acids in the recognition sequence, using a combination of anionic residues at non-metal-binding positions and the native anionic residues at positions 1, 3, and 5 of the EF-Hand (Figure 3).

Casein kinase 1 (CK1) exhibits a preference for multiple anionic residues N-terminal to the site of phosphorylation.⁸² Thus, we examined a variant of pKID-Min4 that included Asp at residue 6 and Ala at residue 8 (pKID-Min8), toward the identification of a motif responsive to CK1 phosphorylation. This motif collectively would include anionic residues at the -3, -4, and -6 positions relative to the site of phosphorylation. This peptide exhibited strong phosphorylation-dependent terbium binding and fluorescence (Figure 9). Indeed, this phosphorylated peptide exhibited the highest terbium affinity of any peptide examined herein, while still binding terbium only modestly in the non-phosphorylated state. These data indicate that the incorporation of an anionic amino acid at residue 6 of an EF Hand does not significantly impact the ability of trivalent terbium to distinguish between the non-phosphorylated (-3 total charge) and phosphorylated (-5 total charge) peptides, presumably because the additional Asp is at a position that does not bind metal. However, pKID-Min8 was not significantly enzymatically phosphorylated by CK1.

CK1 has a preference for a hydrophobic amino acid C-terminal to the site of phosphorylation.⁸² In order to develop a minimal protein kinase-inducible domain that is responsive to phosphorylation by CK1, we examined a peptide (pKID-Min9-CK1) with an additional Ile at residue 10. This peptide exhibited excellent phosphorylation-dependent terbium binding and luminescence (Figure 9cd). In addition, this peptide was phosphorylated by casein kinase 1, with 3.6× greater terbium luminescence after incubation with CK1 (Figure 9e). These data indicate that minimal protein kinase-inducible domains may be developed for responsiveness to protein kinases with diverse kinase recognition sequences, including those with multiple cationic residues or with multiple anionic residues in the protein kinase recognition sequence.

In order to explore the potential applications of the approach described herein, we examined protein kinase activity using a minimal protein kinase-inducible domain in the substantially more complex solution conditions of HeLa cell extracts. The peptide pKID-Min7-PKA was allowed to incubate in unstimulated HeLa cell extracts. Robust terbium luminescence was observed in HeLa extracts incubated with pKID-Min7-PKA (4.4× fluorescence after incubation) (Figure 10). These results in cell extracts could also represent the actions of other kinases in addition to PKA that can act on this recognition sequence. Most broadly, these data indicate that minimal protein kinase-inducible domains can be employed to

examine protein kinase activity with high dynamic range, even in the highly complex solution conditions of cell extracts.

Discussion

We have demonstrated herein the development of a small protein kinase-inducible motif that may be rationally modified to be responsive to serine/threonine kinases with diverse substrate recognition sequences. This approach is based on the combination of the N-terminal sequence (DKDADXW) of an EF-Hand calcium-binding motif, which is necessary but not sufficient for tight metal binding, with a serine or threonine at residue 9, which upon phosphorylation completes the metal-binding motif. Thus, the non-phosphorylated peptide binds terbium poorly and exhibits weak terbium luminescence. In contrast, the phosphorylated peptide exhibits significantly higher terbium affinity and terbium luminescence, with phosphorylation functioning as a switch for metal binding and fluorescence. Using residue 9 as the site of phosphorylation, the positions -3 (residue 6); -2 or -1 (residue 7 or 8, with the other residue Trp to sensitize terbium luminescence); and +1 or greater (residues 10+) can be used as sites to introduce residues to promote protein kinase specificity. This work was applied to develop sequences responsive to the protein kinases PKA, PKC, and CK1.

In the motif responsive to CK1, the Asp at residue 5 functioned both as a metal-binding residue and as a sequence determinant for kinase specificity at the -4 position. The requirement for an Asp at the -4 position to the site of phosphorylation is the primary sequence limitation in minimal protein kinase-inducible domains. The need for Asp at residue 5 renders this specific approach incompatible with a small number of protein kinases that have a strong preference for a non-acidic residue here, such as AMPK, which requires an Arg at the -4 position.⁸³ Moreover, we did not explicitly examine the -5 position (residue 4, here, Ala), which is often a significant determinant for protein kinase specificity. For example, PKD has a strong preference for Leu at the -5 position, while Akt and Pim2 have strong preferences for Arg at the -5 position.^{78, 79} However, surveys of sequences observed in EF-Hand proteins found little sequence conservation at residue 4, including a significant number of proteins with Lys or Arg at residue 4.³⁵⁻³⁹ In addition, data herein demonstrated that minimal kinase-inducible domains may be developed with Arg or Asp at residue 6, which is typically highly conserved as a Gly in an EF-Hand. Furthermore, residue 8 is typically β -branched in EF Hand proteins, but herein Arg and Ala were employed at this position in kinase-responsive and phosphorylation-dependent motifs. We also observed phosphorylation-dependent terbium binding in peptides with Leu at residue 2. Collectively, these results suggest substantial flexibility in the incorporation of residues at the non-coordinating positions, within the minimal sequence motif DXDXDXZZ[pS/pT], where X = any amino acid, and Z = any amino acid at one position with Trp required at the other position to sensitize terbium luminescence. This flexibility is consistent with general observations on calcium-binding motifs, with a DX[DN]XDGX motif recurring in numerous protein contexts.³⁵⁻³⁹

We recently described the development of a short motif (DKDADGWC) responsive to cysteine glutathionylation, with increased Tb³⁺ binding and Tb³⁺ luminescence in the

glutathionylated peptide relative to the unmodified (free cysteine) peptide (Figure 3).⁵⁰ In that design, the additional metal-binding functionality was provided by one or both carboxylates of the glutathione conjugated to cysteine. We have also previously described similar EF-Hand-based phosphorylation-responsive sequences dependent on serine phosphorylation at residue 12, or tyrosine phosphorylation at residue 11 or 15.^{15, 16} Herein, we demonstrate the development of kinase-responsive motifs whose fluorescence is dependent on serine or threonine phosphorylation at residue 9. In all of these cases, the post-translational modification provides an additional anionic ligand (phosphate, carboxylate) to complete the terbium coordination sphere (replacing the native Glu9 or Glu12 of an EF Hand) in a geometrically defined way. This work has provided generalized rules for the rapid development of sequences whose terbium luminescence is dependent on the action of a kinase with a defined recognition sequence. Extensive data have been developed on the recognition sequence preferences of diverse protein kinases. Those data should be readily applicable to the approaches described herein, to allow rapid sensor development for a wide range of protein kinases, including those not currently served by encodable protein kinase sensors. More generally, numerous protein post-translational modifications involve the addition of a negatively charged functional group to the protein. This work suggests the broader possibility of the development of EF-Hand-based fluorescent sensor proteins responsive by design to a significant range of protein post-translational modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank NIH (GM093225) and the University of Delaware for funding of this work.

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Figure 1. Bidentate Glu residues in a Tb^{3+} -binding protein.

(a) Backbone trace of Imperiali's lanthanide-binding tag, LBT1 (PDB: 1tjb),⁵³ with Tb^{3+} (magenta) and the Glu side chains at EF hand positions 9 (blue) and 12 (red) emphasized.

(b) Proposed design of a phosphorylation-dependent terbium-binding motif, utilizing phosphoserine (shown) or phosphothreonine to mimic Glu9 of the EF Hand.

Figure 2. Design of a protein kinase-inducible domain (pKID), a phosphorylation-dependent metalloprotein motif.

(a) Structure of the calcium-binding loop (residues 222–230, sequence DLDGDGKAE) of the polysaccharide lyase YesW from *Bacillus subtilis* (pdb 2zux, 1.32 Å).⁵⁶ Non-metal-binding sidechains were truncated at C β for clarity. Position 9 of the EF Hand (Glu230) is indicated in blue. Ca²⁺ is indicated in magenta. Residues are numbered based on positions within the EF-Hand. This protein contains 3 distinct 9-residue calcium-bound EF-Hand-like structures (DXDGDGXXE) that have a Glu at position 9 that binds the metal in a bidentate manner, and that have a non-metal-binding residue at position 12. A search of the PDB

(structures $\sim 2.0 \text{ \AA}$) using the search motif DXDXDGXXE[not D/E] identified 8 metal-bound loops in non-homologous proteins with this motif; details of the sequences, structures, and torsion angles observed are in the Supporting Information. (b) Phosphoserine (pSer) or phosphothreonine (pThr) mimics the structurally important Glu residue,⁵⁷ whereas the nonphosphorylated residue is a poor Glu mimic. Thus, the peptide with the phosphorylated residue binds Tb^{3+} and (in the presence of Trp at position 7 or 8, which is excited at 280 nm and sensitizes terbium luminescence) exhibits fluorescence at 544 nm. In contrast, the peptide with the non-phosphorylated Ser/Thr binds Tb^{3+} poorly and exhibits weak terbium luminescence.

Figure 3. Peptide sequences.

(a) Sequences of a consensus EF hand; previously designed protein kinase-inducible domains, with an inducible serine (blue) at residue 12 (pKID-PKA) or an inducible tyrosine (blue) at residue 11 (pKID-Abl); a redox-responsive motif (RP1) dependent on cysteine glutathionylation at residue 8; and Imperiali's lanthanide-binding tag (LBT). Residues in red contact metal via the side chain. Tyr or Trp at residue 7 (magenta) contacts the metal via the main chain carbonyl. In an LBT, Gly10 (blue) adopts a (ϕ , ψ = +90°, +0°) restricted conformation to allow simultaneous binding of Glu9 and Glu12. (b) Sequences of peptides synthesized in this study. Residues in blue are sites of phosphorylation. Trp (magenta) serves as the donor chromophore for terbium luminescence. Residues in purple are represent amino acids important for protein kinase recognition. All peptides in this study were acetylated on the N-terminus and contained C-terminal amides.

Figure 4. Fluorescence data of non-phosphorylated and phosphorylated pKID-Min-pThr9 and pKID-Min-pThr12.

(a) Fluorescence spectra of 10 μM non-phosphorylated peptide (red squares), peptide phosphorylated on residue 9 (pKID-Min-pThr9) (blue circles), and peptide phosphorylated on residue 12 (pKID-Min-pThr12) (green diamonds) in the presence of 25 μM Tb^{3+} in 10 mM HEPES (pH 7.5), 100 mM NaCl, and 2 mM MgCl_2 . (b) Tb^{3+} binding isotherms of the peptides (legend as in part (a)). Experiments were conducted with 2 μM peptide and solution conditions as in (a). Data in (a) are shown without background correction, while data in (b) include subtraction of the data with Tb^{3+} alone. The differences in the y-axis also reflect different slit widths used in the spectra in (a) versus the data in (b).

Figure 5. Fluorescence data of non-phosphorylated and phosphorylated peptides pKID-Min1, pKID-Min2, pKID-Min3, and pKID-Min4.

Fluorescence data for the peptides (a,b) pKID-Min1, (c) pKID-Min2, (d) pKID-Min3, and (e,f) pKID-Min4. (a,e) Fluorescence spectra of the non-phosphorylated (red squares) and phosphorylated (blue circles) peptides at 25 μM Tb^{3+} , which exhibits near-maximum differentiation of the non-phosphorylated and phosphorylated peptides for these peptides. (b,c,d,f) Terbium binding isotherms of the peptides. Terbium binding isotherms indicate background-corrected fluorescence, while individual averaged fluorescence spectra are shown without background correction.

Figure 6. NMR Spectroscopy of phosphorylated pKID-Min4 in the absence and presence of La^{3+} .

(a) Fingerprint region of the TOCSY spectra of phosphorylated pKID-Min4 in the absence (red) and presence (blue) of 1 equivalent La^{3+} . (b) $\text{H}\alpha$ -Ca region of the ${}^1\text{H}$ - ${}^{13}\text{C}$ HSQC spectra of phosphorylated pKID-Min4 in the absence (red) and presence (blue) of 1 equivalent La^{3+} . Key peaks are labeled. Resonance assignments are indicated in Table 2. Experiments were conducted with 800 μM peptide in a solution with 5 mM NaCl and 100 μM TSP at 296 K. Experiments in (a) were conducted using a solution in 90% H_2O /10% D_2O , while experiments in (b) were conducted using either a solution in 90% H_2O /10% D_2O (apo peptide) or a solution in 100% D_2O (lanthanum complex).

Figure 7. Fluorescence spectroscopy and enzymatic phosphorylation of pKID-Min5-PKC.

(a) Fluorescence spectroscopy of 10 μ M non-phosphorylated (red squares) and phosphorylated (blue circles) pKID-Min5-PKC with 15 μ M Tb³⁺ in water with 10 mM HEPES, 100 mM NaCl, and 2 mM MgCl₂. (b) Tb³⁺ binding isotherms of non-phosphorylated and phosphorylated pKID-Min5-PKC. Experiments were conducted with 10 μ M peptide and a serial dilution of TbCl₃ under conditions as in (a). (c) Fluorescence spectroscopy to analyze enzymatic phosphorylation of pKID-Min5-PKC, with data prior to addition of kinase (red squares) and after 2 hours incubation at 30 °C with PKC (blue circles), in the presence of 80 μ M Tb³⁺ (2.6 $\times$ terbium luminescence at 544 nm after incubation with PKC). HPLC analysis indicated 47% phosphorylation under these conditions. The relatively modest extent of phosphorylation was likely due to the absence of an Arg N-terminal to the Thr (the -2 and -3 positions have a significant preference for Arg). The increased fluorescence observed in the absence of enzyme in (c) compared to (a) potentially is due to fluorescence of the ATP•Tb³⁺ complex.⁷⁵

Figure 8. Fluorescence spectroscopy and enzymatic phosphorylation and dephosphorylation of pKID-Min6 and pKID-Min7-PKA.

(a-d) Fluorescence data of non-phosphorylated (red squares) and phosphorylated (blue circles) peptides. (a) Fluorescence spectra of pKID-Min6 peptides with 25 μM Tb^{3+} . (b) Tb^{3+} binding isotherm of pKID-Min6 peptides. (c) Fluorescence spectra of pKID-Min7-PKA peptides with 150 μM Tb^{3+} . (d) Tb^{3+} binding isotherm of pKID-Min7-PKA peptides. Terbium binding of phosphorylated pKID-Min7-PKA at 10 μM or 25 μM (shown) peptide concentrations exhibited evidence on non-1:1 complex formation at these concentrations, as indicated by the poor fit of most fluorescence data points to a 1:1 binding equation. In

contrast, at 2 μM phosphorylated pKID-Min7-PKA (inset), the data fit well to a binding equation for a 1:1 complex. (e) Fluorescence spectra of initially non-phosphorylated pKID-Min7-PKA in kinase buffer with 175 μM Tb^{3+} (red squares) prior to addition of protein kinase A (PKA) and (blue circles) after incubation with PKA for 1 hour ($11\times$ increase in fluorescence). HPLC analysis of the solution indicated $> 95\%$ phosphorylation by PKA. (f) Fluorescence spectra of initially phosphorylated pKID-Min7-PKA in phosphatase buffer with 80 μM Tb^{3+} (blue circles) prior to addition of phosphatase and (red squares) after incubation with calf intestine alkaline phosphatase (CIP) for 10 minutes ($> 95\%$ reduction in terbium luminescence). HPLC analysis indicated $> 95\%$ dephosphorylation by CIP. Additional details are in the Supporting Information.

Figure 9. Fluorescence spectroscopy and enzymatic phosphorylation of pKID-Min8 and pKID-Min9-CK1.

(a) Fluorescence spectra of (red squares) non-phosphorylated and (blue circles) phosphorylated pKID-Min 6 in a solution with 32 μM Tb^{3+} . (b) Terbium binding isotherms of (red squares) non-phosphorylated and (blue circles) phosphorylated pKID-Min8. (c) Fluorescence spectra of (red squares) non-phosphorylated and (blue circles) phosphorylated pKID-Min9-CK1 in a solution with 32 μM Tb^{3+} . (d) Terbium binding isotherms of (red squares) non-phosphorylated and (blue circles) phosphorylated pKID-Min9-CK1. (e) Fluorescence spectra of the initially non-phosphorylated peptide pKID-Min9-CK1 with 125

$\mu\text{M Tb}^{3+}$ (red circles) prior to addition of casein kinase 1 (CK1) and (blue circles) after incubation for 3 hours at 30 °C with CK1 (3.6× increase in fluorescence at 544 nm). HPLC data indicated 50% phosphorylation by CK1. Additional details are in the Supporting Information.

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Figure 10. Phosphorylation of pKID-Min7-PKA in HeLa cell lysates.

Non-phosphorylated pKID-Min7-PKA in HeLa lysates (red squares) or in HeLa lysates supplemented with ATP (400 μ M ATP was added to initiate enzyme activity, followed by additional supplementation with 200 μ M ATP (final concentration of added ATP) at 30 minutes and at 1 hour) that was allowed to incubate for 2 hours at 37 $^{\circ}$ C (blue circles). Each solution was then diluted with fluorescence buffer and Tb^{3+} added (final concentrations (in addition to species present in HeLa lysates) 10 μ M peptide, 10 mM HEPES pH 7.5, 100 mM NaCl, 2 mM MgCl_2 , and 80 μ M Tb^{3+}). The data indicate a 4.4 $\times$ increase in terbium luminescence of the peptide in HeLa extracts after incubation for 2 hours. HPLC analysis indicated 61% phosphorylation after incubation for 2 hours in these unstimulated HeLa cell lysates.

Table 1.Tb³⁺ dissociation constants and phosphorylation-dependent changes in fluorescence for peptides

peptide	Tb ³⁺ •peptide complex				fluorescence ^a	at [Tb ³⁺], μM
	phosphorylated	non-phosphorylated				
	<i>K_d</i> , μM	error	Tb ³⁺ •peptide <i>K_d</i> , μM	error		
pKID-Min-pThr9	13	3	229 ^{b,c}	14 ^c	5.7	26
pKID-Min-pThr12	117	9*	229 ^{b,c}	14 ^c	1.6	46
pKID-Min1	16	4	64 ^c	4 ^c	4.7	25
pKID-Min2	10	4	43 ^c	3 ^c	3.0	10
pKID-Min3	33	9	>1000 ^c	—	7.8	50
pKID-Min4	7	2	>1000 ^c	—	15	25
pKID-Min5-PKC	12	7	>1000 ^c	—	23	25
pKID-Min6	18	7	1040 ^c	140 ^c	8.1	50
pKID-Min7-PKA	136 ^d	16	>1000 ^c	—	6.9	150
pKID-Min8	1.2	0.3	265 ^c	6 ^c	9.6	31
pKID-Min9-CK1	4.9	2.6	394	145	17	31

^aThe ratio of the fluorescence (background-corrected) of the phosphorylated peptide over the non-phosphorylated peptide at the indicated Tb³⁺ concentration. The change in fluorescence is inherently dependent on the Tb³⁺ concentration and the relative dissociation constants and maximum terbium luminescence of the peptides, with the maximum change in fluorescence typically observed at a Tb³⁺ concentration somewhat greater than the *K_d* of the phosphorylated peptide (i.e. at a Tb³⁺ concentration where the phosphorylated peptide is mostly bound to Tb³⁺, while the non-phosphorylated peptide still is mostly unbound), except in cases where the phosphorylated and non-phosphorylated peptides had relatively similar dissociation constants.

^bThe non-phosphorylated peptides of pKID-Min-pThr9 and pKID-Min-pThr12 are the same peptide.

^cThis *K_d* was calculated based on the maximum fluorescence of the phosphorylated peptide.

^dThis *K_d* was determined at 2 μM peptide concentration due to evidence of non-1:1 binding behavior at higher peptide concentrations.

Table 2.NMR data (^1H and ^{13}C chemical shifts) of phosphorylated pKID-Min4 in the absence and presence of La^{3+} .

phosphorylated pKID-Min4, no La^{3+}								
residue	H^{N}	$\text{H}\alpha$	$\text{H}\beta$	H_{other}	$^{13}\text{C}\alpha$			
Asp1	8.282	4.54	2.68, 2.56		51.8			
Lys2	8.35	4.20	1.76	2.96, 1.69, 1.61, 1.34	53.7			
Asp3	8.34	4.57	2.73, 2.64		52.0			
Ala4	8.16	4.26	1.40		50.1			
Asp5	8.31	4.58	2.66, 2.64		52.0			
Gly6	8.28	3.92, 3.92			42.7			
Trp7	8.05	4.62	3.27, 3.27	10.29, 7.62, 7.52, 7.25, 7.22, 7.16	55.0			
Ile8	7.71	4.08	1.70	1.34, 1.03, 0.82	57.8			
pSer9	8.50	4.24	4.07, 4.03		54.6			
Ac-		2.03						
-NH ₂	7.55, 7.08							

phosphorylated pKID-Min4 + La^{3+}								
						$\pm \text{La}^{3+}$		
residue	H^{N}	$\text{H}\alpha$	$\text{H}\beta$	H_{other}	$^{13}\text{C}\alpha$	$\delta, \text{H}\alpha$	$\delta, ^{13}\text{C}\alpha$	$\delta, \text{H}^{\text{N}}$
Asp1	8.35	4.57	2.69, 2.54		51.3	+0.03	-0.5	+0.07
Lys2	8.45	4.28	1.84	3.00, 1.78, 1.68, 1.43	53.6	+0.08	-0.1	+0.10
Asp3	8.25	4.66	2.73, 2.68		50.1	+0.09	-1.9	-0.09
Ala4	8.60	4.24	1.46		51.2	-0.02	+1.1	+0.44
Asp5	7.86	4.72	3.09, 2.57		51.0	+0.14	-1.0	-0.45
Gly6	8.12	4.18, 3.75			42.9	0.26, -0.17	+0.2	-0.16
Trp7	8.35	4.97	3.33, 3.13	10.18, 7.73, 7.51, 7.26, 7.23, 7.19	53.3	+0.35	-1.7	+0.30
Ile8	8.91	4.14	1.73	1.45, 1.03, 0.83	58.4	+0.06	+0.6	+1.20
pSer9	8.95	3.99	4.15, 4.05		55.6	-0.25	+1.0	+0.45
Ac-		2.03						
-NH ₂	7.59, 7.19							