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Design of a Protein Kinase-Inducible Domain

Shalini Balakrishnan and Neal J. Zondlo*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Received November 11, 2005; E-mail: zondlo@udel.edu

Reversible phosphorylation of protein hydroxyl groups is a ubiquitous signaling mechanism that renders a limited set of genes capable of cellular specialization and differentiation. In humans, at least 518 genes encode protein kinases and 140 genes encode protein phosphatases, in total accounting for over 2.5% of all human genes.[1] Changes in protein phosphorylation states and kinase activity are associated with many human diseases, most notably cancer.[2]

We sought to use protein design to develop novel protein architectures whose structures are dependent on their phosphorylation state. Previous designs of phosphorylation-dependent protein structure have involved stabilization of multimeric helical bundles via electrostatic, helix dipole, or helix capping interactions.[3] We chose to focus on the design of a monomeric protein motif that undergoes a phosphorylation-dependent structural change.

Ideally, a designed phosphorylation-dependent protein domain would (a) exhibit complete structural switching upon phosphorylation; (b) include a protein kinase recognition sequence; (c) comprise a modular motif that is compatible with different protein kinase recognition sequences; (d) include a fluorescent reporter element for readout; and (e) consist entirely of canonical amino acids, to enable its use as a genetically encoded phosphorylation-dependent protein tag and as a building block in the design of larger phosphorylation-dependent protein architectures.

The key design element is the use of phosphoserine as an inducible mimic of a structurally important Glu residue. Phosphoserine and Glu are approximately isosteric anions, suggesting that replacement of Glu with phosphoserine would generate a motif that would bind metal in a phosphorylation-dependent manner (Figure 1). Indeed, Glu is commonly used as a mimic of phosphoserine.[4] Here, we employ an inverse approach, in which phosphoserine, but not serine, mimics the electrostatic and Lewis base properties of Glu.

This strategy was applied to the design of phosphorylation-dependent EF hand domains. The EF hand contains a simple calcium-binding motif, in which the metal is bound by five side chain groups and one main chain carbonyl (Figure 2). EF hand proteins, such as calmodulin, undergo a significant conformational change to a well-folded helix−loop−helix structure upon calcium binding.[5] Due to the similar electronics and ionic radii of calcium and lanthanides, EF hands effectively coordinate lanthanides,[6] thereby endowing the EF hand with the luminescent, magnetic, and hydrolytic properties of lanthanides.[7]

The EF hand motif accommodates a wide range of residues at most positions. In contrast, residue 12 of the EF hand is nearly invariantly Glu, which binds the metal in a bidentate manner. Replacement of Glu with phosphoserine would generate a motif that would bind metal in a phosphorylation-dependent manner (Figure 1): nonphosphorylated Ser is a poor Glu mimic and should poorly bind metal; in contrast, serine phosphorylation should lead to a tight protein−metal complex. Critically, residues N-terminal and C-terminal to residue 12 are poorly conserved across EF hand proteins, and thus may be tuned to incorporate the recognition sequence of a protein serine/threonine kinase of interest.[8]

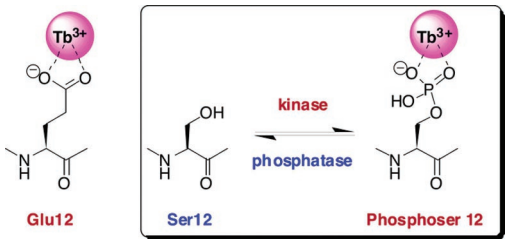

Figure 1. Design of a phosphorylation-dependent motif, indicating the roles of Glu, Ser, and phosphoserine residues.

Figure 2. Left: EF hand Ca^{2+}-binding loop (1cll), with Glu12 emphasized. Right: EF hand consensus sequence and kinase-inducible domain peptide sequences. Side chains of residues in red contact metal. Tyr or Trp (magenta) contacts metal via the main chain carbonyl.

A series of kinase-inducible domain (pKID) peptides was synthesized which comprised an EF hand consensus sequence, a tryptophan at residue 7 to sensitize lanthanide emission,[6c] and the recognition sequence of a kinase important in intracellular signaling (Figure 2). As initial targets, we synthesized peptides containing the minimal recognition sequences for the critical cellular kinases PKA, PKC, and the MAP kinase Erk. These minimal recognition sequences, which include basic or helix-breaking residues that could potentially disrupt lanthanide binding and include residues N-terminal or C-terminal to the target serine, provide a significant test of the generality of the design.

Fluorescence emission spectra revealed that the nonphosphorylated peptides bound Tb^{3+} poorly (Figure 3), displaying very weak terbium luminescence, consistent with the critical role of Glu12 in metal binding. In contrast, all phosphorylated peptides displayed strong fluorescence emission in the presence of Tb^{3+}, indicating the formation of a phosphopeptide−metal complex. Notably, the fluorescence change upon phosphorylation by protein kinase A (Figure 3d) was similar to that observed in nonexpressible kinase sensors and was significantly greater than that of any expressible kinase sensor.[9] All peptides displayed nearly complete dependence on phosphorylation for fluorescence.[10]

Competition experiments were used to confirm site-specific lanthanide binding and to address whether pKID peptides are a general phosphorylation-dependent lanthanide-binding motif. Addition of nonluminescent Ho^{3+} to the phosphorylated pKID-PKC−Tb^{3+} complex resulted in the loss of Tb^{3+} luminescence, consistent with formation of a pKID-PKC−Ho^{3+} complex.[10] In contrast, strong fluorescence and no decrease in affinity of the phosphorylated

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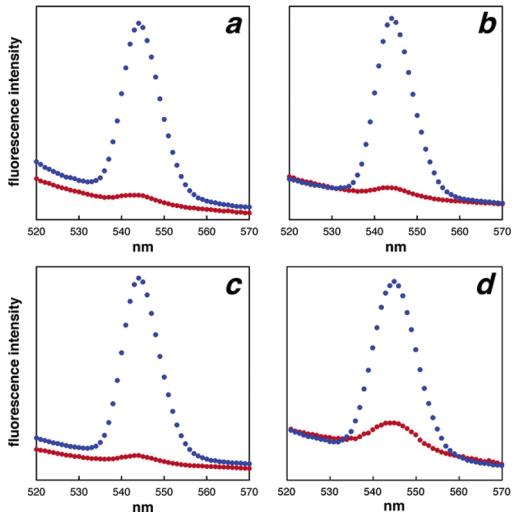

Figure 3. (a−c) Fluorescence spectra of 10 μM (a) pKID-PKA, (b) pKID-PKC, or (c) pKID-Erk when nonphosphorylated (red) or phosphorylated (blue) in the presence of 20 μM Tb^{3+}. Experiments were conducted in buffer containing 5 mM HEPES (pH 7.8) and 100 mM NaCl. (d) Detection of protein kinase A (PKA) activity using pKID-PKA. Fluorescence spectra were collected immediately after addition of PKA (red) and after incubation with PKA (blue). As expected, PKA did not phosphorylate pKID-PKC or pKID-Erk.[10] Fluorescent detection of pKID-PKA phosphorylation by PKA in HeLa extracts was also observed. Full experimental details are in the Supporting Information.

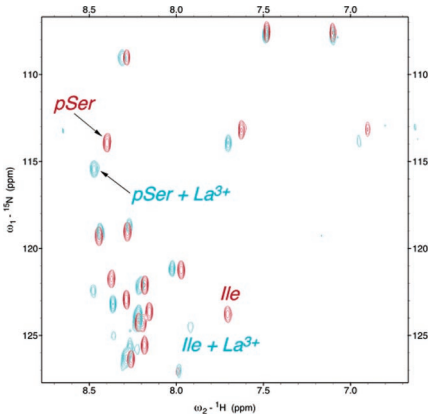

Figure 4. $^1H-^{15}N$ HSQC spectra of phosphorylated pKID-PKC in the absence of metal (red) and in the presence of La^{3+} (cyan). Large changes were observed for the amide resonances of phosphoserine and of Ile8, which is conjugated to the Trp7 carbonyl that contacts metal in an EF hand. Other assignments are in the Supporting Information.

pKID-PKA−Tb^{3+} complex were observed in the presence of 100 μM Ca^{2+} or 2 mM Mg^{2+}, indicating compatibility with cellular Ca^{2+} and Mg^{2+} concentrations.[10]

Metal binding was further characterized by NMR. Addition of paramagnetic Tb^{3+} to phosphorylated pKID-PKC resulted in the disappearance of most signals, consistent with pKID-PKC−Tb^{3+} complex formation and Tb^{3+}-induced paramagnetic relaxation.[10] $^1H-^{15}N$ HSQC spectra (Figure 4) of phosphorylated pKID-PKC in the absence and in the presence of diamagnetic La^{3+} indicated that the metal induced significant changes in chemical shifts, particularly for metal-binding residues, consistent with peptide−metal complex formation.

The work herein is the first example of a designed, small, monomeric, genetically encodable protein sequence whose structure is dependent on phosphorylation. The architecture is potentially applicable to a broad range of serine/threonine kinase recognition motifs, providing a general approach toward the development of designed proteins whose structure and function are under the control of specific protein kinases.

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Supporting Information Available: Experimental procedures, characterization data, NMR and CD spectra, and metal-binding titrations for the peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) See the Supporting Information for details.

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