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Design of an Encodable Tyrosine Kinase-Inducible Domain: Detection of Tyrosine Kinase Activity by Terbium Luminescence

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Tyrosine kinases are critical mediators of intracellular signaling that are misregulated in numerous cancers.[1] Tyrosine kinase inhibitors are therefore emerging as important therapeutics in oncology. In addition, tyrosine kinase signaling is broadly implicated as important in other diseases, including diabetes, heart disease, and viral and bacterial infectivity. The importance of tyrosine phosphorylation in disease has inspired the development of numerous approaches to interrogate tyrosine kinase-mediated signaling.[2]

Considering the central importance of tyrosine kinase activity in cell signaling and disease, we sought to develop new, complementary approaches to investigate tyrosine phosphorylation. An attractive approach would be to employ small protein domains that are responsive to tyrosine kinase activity. For sensor applications, we sought to develop a small, designed, luminescent, genetically encodable protein motif whose structure is dependent on tyrosine phosphorylation.[2,3]

We recently described the design of a protein kinase-inducible domain (pKID), designed peptides that become structured and bind lanthanides when phosphorylated on serine.[3e] The phosphorylation-dependent and kinase-responsive luminescence observed encouraged us to investigate related approaches to design proteins responsive to tyrosine phosphorylation.[4] In our previous design, phosphoserine mimicked a structurally important Glu residue, generating phosphorylation-dependent EF-hand peptides. In the design of a protein dependent on tyrosine phosphorylation, the size differences between phosphotyrosine and Glu would likely preclude an approach involving direct mimicry of Glu by phosphotyrosine. However, the length of the phosphotyrosine side chain suggested an alternative, structure-based approach to phosphorylation-dependent protein design. The conserved Glu_{12} of EF-hand proteins is located within an α-helix.[5] In α-helices and other compact conformations, the side chains of sequential residues may interact to stabilize protein structure (i.e., *i*/*i*+1 interactions). A sufficiently long side chain at the *i* residue could functionally replace an interaction of the *i*+1 residue (Figure 1). This analysis suggests that a phosphotyrosine residue (*i* position, residue 11 of an EF-hand) might replicate the electrostatic and Lewis base properties of a structurally important Glu residue at the *i*+1 position (residue 12).

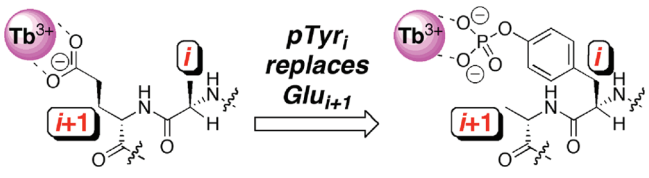

Figure 1. Design of a protein motif dependent on tyrosine phosphorylation. Phosphotyrosine (pTyr) at the *i* position mimics Glu at the *i*+1 position. Nonphosphorylated tyrosine is expected to be a poor Glu mimic.

To test this approach, the model peptide pKID-pTyr11 was synthesized, consisting of a consensus EF-hand sequence with Glu_{12} replaced by Ala; Tyr at residue 11; and Trp_7 to sensitize terbium luminescence (Figure 2).[6] pKID-pTyr11 exhibited phosphorylation-dependent Tb^{3+} binding and luminescence: nonphosphorylated pKID-pTyr11 was nearly nonluminescent in conditions where phosphorylated pKID-pTyr11 exhibited strong terbium luminescence (Figure 3a). Phosphorylated pKID-pTyr11 bound Tb^{3+} with 35-fold greater affinity than did the nonphosphorylated peptide (K_d = 54 ± 9 μM versus K_d = 1900 ± 280 μM). Notably, the Tb^{3+} binding of phosphorylated pKID-pTyr11 was identical in the presence of 100 μM Ca^{2+} and 2 mM Mg^{2+},[7] indicating compatibility of the design with competitive solution conditions. Phosphorylated pKID-pTyr11 bound Tb^{3+} with similar affinity to the EF Hand consensus peptide (EF Hand-E12) with Glu at residue 12 (K_d = 88 ± 8 μM), indicating that phosphotyrosine at the *i* residue effectively mimics Glu at the *i*+1 residue. Notably, phosphorylated pKID-pTyr11 bound Tb^{3+} with far greater affinity than a control peptide with Glu at residue 11 (K_d = 1820 ± 140 μM), indicating that a negatively charged amino acid at residue 11 is not sufficient for the observed metal binding and that the length of the phosphotyrosine side chain is necessary for binding from residue 11. In addition, phosphorylated pKID-pTyr11 also exhibited greater Tb^{3+} affinity than a peptide with phosphotyrosine at residue 12 (K_d = 209 ± 32 μM), consistent with the design.

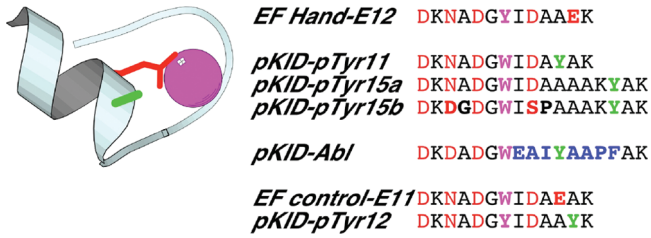

Figure 2. Left: Structure of an EF hand (1cll), with Glu_{12} (red) and the $C_α$−$C_β$ bond of residue 11 (green) emphasized.[5] Right: Sequences of a canonical EF hand, tyrosine kinase-inducible domain peptides, and control peptides. Residues in red contact metal via the side chain; Glu_{12} binds metal in a bidentate manner; residue 9 (Asp or Ser) contacts metal via a water-mediated contact in EF-hand proteins. Trp_7 (magenta) contacts metal via the main chain carbonyl and is a sensitizer for terbium luminescence. Residues in green are sites of phosphorylation. Residues in blue represent an Abl kinase recognition sequence.

The binding of phosphorylated pKID-pTyr11 was further characterized by NMR experiments, which revealed a significant structural change for all residues upon addition of the diamagnetic lanthanide La^{3+}, including chemical shift changes in key metal-binding residues, consistent with the design (Figure 3b−d). Notably, the Ile_8 H^N main chain and the Asn_3 side chain amides, both of which are conjugated to carbonyls that contact metal in EF-hand proteins, as well as the Asp_9 and $pTyr_{11}$ $H_β$ protons, exhibited significant La^{3+}-dependent changes in their chemical shifts. Of particular note is the large increase in dispersion of the diastereotopic Gly_6 and $pTyr_{11}$ $H_β$ chemical shifts, consistent with

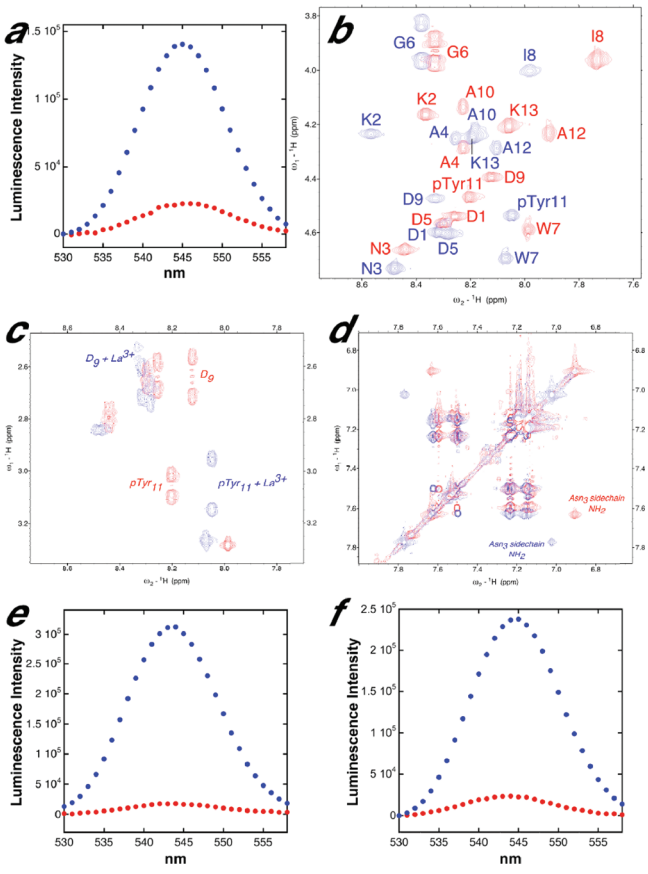

Figure 3. (a, e, f) Luminescence spectra of 10 μM nonphosphorylated (red) and phosphorylated (blue) (a) pKID-pTyr11, (e) pKID-pTyr15a, and (f) pKID-pTyr15b, in the presence of (a, f) 20 μM Tb^{3+} or (e) 80 μM Tb^{3+}. Experiments were conducted in water with 10 mM HEPES (pH 7.8) and 100 mM NaCl with excitation at 280 nm. At the indicated Tb^{3+} concentrations, the phosphorylated peptides exhibited (a) 6.4-fold, (e) 17.6-fold, and (f) 10.1-fold greater luminescence than the nonphosphorylated peptides, respectively. The fold luminescence differentiation between nonphosphorylated and phosphorylated peptides depends on the relative Tb^{3+} affinities of the peptides and on the Tb^{3+} concentration. (b, c, d) TOCSY spectra of phosphorylated pKID-pTyr11 in the absence (red) and presence (blue) of La^{3+} indicating metal-induced peptide folding: (b) H^N-H_α region; (c) H^N-H_β region; (d) aromatic region.

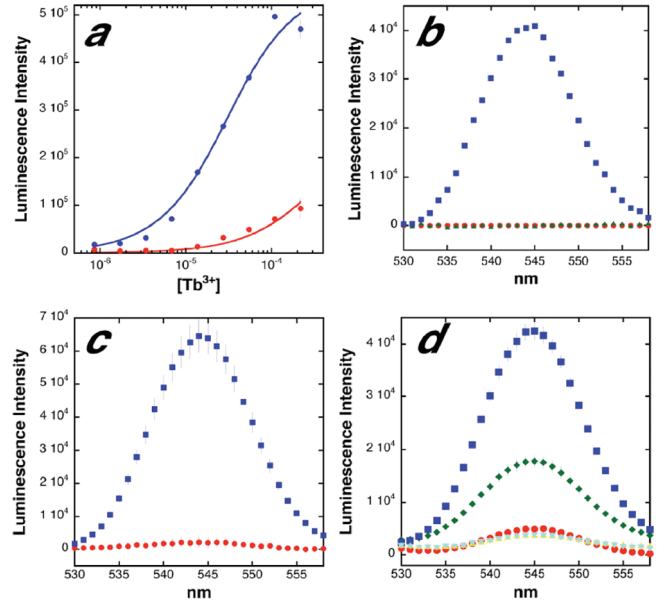

Figure 4. (a) Tb^{3+} binding isotherm of 10 μM nonphosphorylated (red) and phosphorylated (blue) pKID-Abl in 10 mM HEPES (pH 7.5), 100 mM NaCl, and 2 mM $MgCl_2$. (b) Luminescence spectra of 30 μM pKID-Abl with 50 μM Tb^{3+} (final concentrations) after incubation in the absence of Abl kinase (red circles), in the presence of Abl kinase (blue squares; 62% phosphorylated), or in the presence of Abl kinase and Gleevec (green triangles; < 5% phosphorylated); reaction mixtures were incubated for 30 min at 37 °C in buffer containing 10 mM HEPES pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM DTT, and 400 μM ATP, followed by addition of Tb^{3+}. (c) Luminescence spectra of 10 μM phosphorylated pKID-Abl with 20 μM Tb^{3+} prior to (blue squares) and 1 min after (red circles) incubation with the tyrosine phosphatase YOP. (d) Measurement of EGF-induced Abl kinase activity using pKID-Abl in HeLa cell extracts via Tb^{3+} luminescence. Red circles, luminescence of cell extracts, from untreated cells, in the presence of nonphosphorylated pKID-Abl (t_0); green diamonds, luminescence of cell extracts, from untreated cells, after incubation with pKID-Abl (28% phosphorylation); blue squares, luminescence of cell extracts, from cells treated with EGF, after incubation with pKID-Abl (69% phosphorylation); triangles, cell extracts, from untreated (yellow) and EGF-treated (cyan) cells, in the absence of pKID-Abl. The data indicate a 2.4-fold increase in luminescence observed in extracts from the EGF-stimulated cells over those from nonstimulated cells and an 8.6-fold increase in luminescence for pKID-Abl in extracts from EGF-stimulated cells over extracts with nonphosphorylated pKID-Abl. The extent of phosphorylation was determined by HPLC analysis. Error bars indicate standard error.[7]

adoption of an organized structure on addition of La^{3+}. In sum, these data are consistent with side chain organization among EF Hand Ca^{2+}-binding residues upon metal binding.

Tyrosine kinase recognition sites incorporate residues both N-terminal and C-terminal to tyrosine that provide kinase specificity.[8] To develop an approach that might broadly accommodate sequence substitution, to be generally compatible with diverse tyrosine kinase recognition sequences, we synthesized two additional peptides, pKID-pTyr15a and pKID-pTyr15b (Figure 2). In these peptides, Tyr is translated forward one α-helical turn, to residue 15. Due to the geometry of an α-helix, phosphotyrosine at residue 15 should project toward the EF-hand metal-binding site.[9] pKID-pTyr15a and pKID-pTyr15b differ in the identity of residues 3 and 9 in the metal coordination sphere and in the conformational preferences of noncoordinating residues 4 and 10.[10] Modulation of the electrostatics of residues 3 and 9 of the coordination sphere can dramatically impact Tb^{3+} affinity and luminescence in EF Hand peptides.[6]

Both pKID-pTyr15a and pKID-pTyr15b showed phosphorylation-dependent luminescence, indicating that phosphotyrosine$_{15}$ was critical for protein structure (Figure 3e−f).[11] Of these peptides, phosphorylated pKID-pTyr15b exhibited greater Tb^{3+} affinity (K_d = 47 ± 5 μM, compared to K_d = 217 ± 21 μM for pKID-pTyr15a) and a larger difference in Tb^{3+} affinity between the phosphorylated and nonphosphorylated peptides, while phosphorylated pKID-pTyr15a exhibited modestly greater maximum luminescence at Tb^{3+} saturation.[7] These data indicate that the N-terminal sequence in kinase-inducible domain peptides may be used to tune their structural and luminescence characteristics.

To examine the compatibility of tyrosine kinase-inducible domain peptides with a complex kinase recognition sequence, the peptide pKID-Abl was synthesized (Figure 2). pKID-Abl incorporates the critical kinase recognition residues from the optimized Abl substrate peptide Abltide. As observed previously with model peptides, pKID-Abl exhibited strong phosphorylation-dependent Tb^{3+} luminescence (Figure 4a; K_d = 26 ± 6 μM phosphorylated pKID-Abl, versus K_d = 411 ± 38 μM for nonphosphorylated pKID-Abl), suggesting its potential utility as a sensor of Abl kinase activity.[7]

Incubation of nonphosphorylated pKID-Abl with Abl kinase resulted in a significant increase in terbium luminescence (Figure 4b), indicating that pKID-Abl may be used as a sensor of Abl kinase activity.[12] Luminescent detection of kinase inhibition by the Abl

inhibitor Gleevec was also readily observed using pKID-Abl, suggesting that kinase-inducible domain peptides are also effective for screening protein kinase inhibitors.

Tyrosine phosphatases have critical roles in the temporal dynamics of cell signaling via their role in dephosphorylating kinases and kinase substrates.[13] Changes in tyrosine phosphatase activities are associated with numerous human diseases, including diabetes, obesity, inflammatory diseases, and some cancers. Accordingly, inhibitors of tyrosine phosphatases have received increasing attention as possible therapeutics. To examine the potential of a tyrosine kinase-inducible domain peptide to detect tyrosine phosphatase activity, phosphorylated pKID-Abl was incubated with the tyrosine phosphatase YOP. pKID-Abl was an effective sensor of tyrosine phosphatase activity, with a large and rapid reduction in terbium luminescence observed due to dephosphorylation of $pTyr_{11}$ by YOP (Figure 4c).

Finally, to examine the use of pKID-Abl to analyze changes in cellular kinase activity, pKID-Abl was incubated with untreated HeLa cell extracts and with HeLa cell extracts stimulated with epidermal growth factor (EGF), which induces Abl kinase activity (Figure 4d). The large dynamic range of tyrosine kinase-inducible domains resulted in strong differentiation of treated and untreated cells and the ready identification of induced cellular tyrosine kinase activity. More generally, these results suggest compatibility of kinase-inducible domain peptides with highly complex solution conditions. In sum, these results indicate that tyrosine kinase-inducible domains may be broadly applied toward the luminescent detection of tyrosine kinase and tyrosine phosphatase activities.

We have described the design of a small, genetically encodable protein sequence whose metal binding and luminescence depend on its tyrosine phosphorylation state. The designed tyrosine kinase-inducible domain comprises a motif that should be compatible with diverse tyrosine kinase recognition sequences and a broad range of applications in tyrosine kinase signaling.

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Supporting Information Available: Experimental procedures, characterization data, NMR spectra, CD spectra, and Tb^{3+}-binding titrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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