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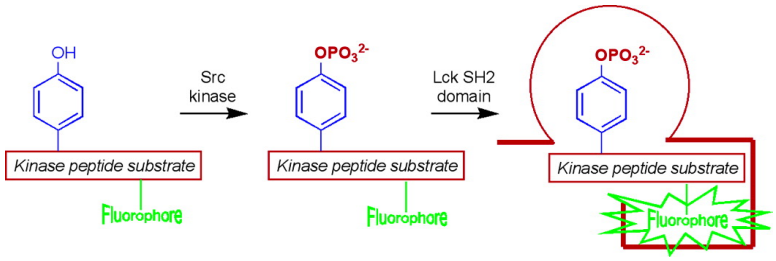

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Phosphorylation-Driven Protein−Protein Interactions: A Protein Kinase Sensing System

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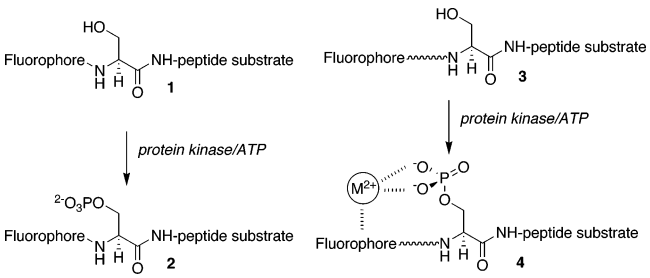
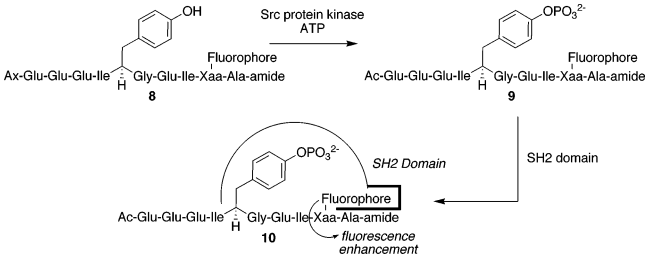
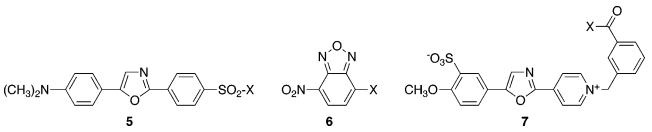
Protein kinases comprise a large family of signaling enzymes that enable the cell to respond to both extracellular and intracellular environmental events. Although the general role played by these enzymes is well recognized, the contributions made by individual protein kinases to specific cellular actions have proven difficult to decipher. In particular, a not uncommon problem is the inability to directly correlate kinase action with some given cellular event of interest. Recently, however, several fluorescent reporters of protein kinase activity have been described, thereby enabling observation of the activity of these enzymes within the context of cellular behavior. Two general strategies have emerged for the design of kinase fluorescent indicators. Several investigators have described genetically encoded proteins comprised of a protein kinase phosphorylation sequence fused to a FRET pair of two spectrally distinct analogues of green fluorescent protein (GFP).[1] Protein kinase catalyzed phosphorylation of the GFP_1-(protein kinase phosphorylation sequence)-GFP_2 substrate induces FRET changes up to 30%. A second group of kinase probes are comprised of fluorescently labeled peptides that, upon phosphorylation, display fluorescence changes that are as much as severalfold in magnitude.[2,3] The latter include peptides containing an environmentally sensitive fluorophore directly appended to the phosphorylatable residue (e.g., **1** → **2**)[2] as well as divalent metal-ion-dependent constructs (e.g., **3** → **4**)[3]. However, the strategies depicted by both **1** and **3** lack generality to the protein kinase family and their substrates as a whole since the peptide-appended fluorophore occupies a fixed spatial relationship with respect to the residue that suffers phosphorylation. We describe herein a new strategy to sense protein kinase activity that eliminates the need for spatial constraints within the active site directed peptide substrate. Furthermore, we have found that several different fluorophores can be employed with this strategy.



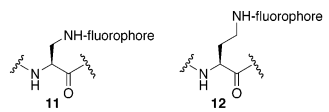
A number of environmentally sensitive fluorophores, such as **5**−**7**, have been described. For example, the dapoxyl derivative **5** displays both a shift in its emission wavelength as well as an enhancement in fluorescence quantum yield as a function of decreasing solvent polarity.[4] We reasoned that a fluorescently labeled protein kinase peptide substrate could recapitulate these attributes in an aqueous milieu if, following phosphorylation, the peptide became embedded within a hydrophobic environment (Scheme 1). Several protein-binding domains are known that recognize phosphorylated serine- and tyrosine-containing sequences, including 14−3−3[5a] and SH2[5b] domains, respectively, and sensors for the latter[6] have been described. In this study, we examined the ability of the Lck SH2 domain to bind to the Src kinase phosphotyrosine peptide product **9** and thereby selectively enhance fluorescent intensity relative to its unphosphorylated counterpart **8**.

Scheme 1. Selective Sensing of the Protein Kinase Catalyzed Phosphorylated Product





The three-dimensional structures of several Lck/phosphopeptide complexes have been described.[7] Although molecular modeling highlighted a number of potential binding pockets that could offer a relatively lipophilic environment, it was far from clear where the fluorophore should be appended on the peptide in order to ensure SH2-induced fluorescence enhancement while maintaining efficient Src kinase catalyzed phosphorylation. Consequently, we prepared a library of peptides in which the three fluorophores **5**−**7** were attached to (L)-2,3-diaminopropionic acid (Dap) **11** and (L)-2,4-diaminobutyric acid (Dab) **12**. These six distinct fluorophore−Dap/Dab residues were positioned at four different sites along the peptide backbone, as depicted below (note, the residues on the N-terminal side of position P are required for Tyr phosphorylation by Src kinase, but do not interact with the SH2 domain).



Ac-Glu-Glu-Glu-Ile-Tyr-Gly-Glu-Ile-Glu-Ala-$NHCH_2CH_2SH$
P +1 +2 +3 +4

The library was prepared via parallel synthesis using a previously described disulfide-linked Tentagel resin (see Supporting Information). Following solid phase synthesis of the primary sequence, the

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Table 1. Fold Change in Fluorescence Intensity in the Src Kinase Catalyzed Phosphorylation of Peptide Substrates as a Function of Fluorophore Attachment Site[a]

	Fluorophore Attachment Site			
fluorophore	+1	+2	+3	+4
Dap-5	0.6	2.4	3.3	2.3
Dap-5	0.6	2.4	3.3	2.3
Dap-6	1.6 (1.6)[a]	NC[b]	1.3	1.3
Dap-5	0.6	2.4	3.3	2.3
Dap-7	1.4	1.8	1.6	1.4
Dab-5	2.4	1.6	3.6	4.1 (7.2)[a]
Dab-6	1.3	1.4	1.9	1.7
Dab-7	1.5	1.7	2.1	1.6

[a] All peptides contain the C-terminal $-NH(CH_2)_2SH$ moiety, except for the $-NH_2$ derivatives indicated by parentheses. [b] No fluorescence change.

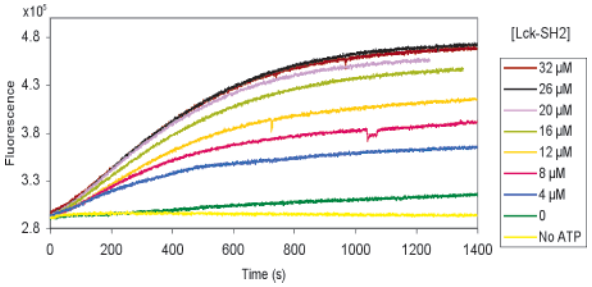

Figure 1. Fluorescence change as a function of [Lck SH2].

side chain amine of the Dap or Dab residue was selectively deprotected and subsequently modified with the appropriate activated forms of **5**, **6**, and **7**. The remaining protecting groups on the peptide were then removed with trifluoroacetic acid (TFA), the peptide resin extensively washed to eliminate the last traces of TFA, and the peptide cleaved from the resin with assay buffer (which contained dithiothreitol). The fluorescent response of the individual library members to Src catalysis in the presence Lck SH2 was subsequently examined in detail.

As is evident from Table 1, the dapoxyl fluorophore positioned off the +3 and +4 sites on the peptide substrate (Dap-5 and Dab-5) produced the largest changes in fluorescent behavior. We decided to examine two peptides (**13** and **14**) in greater detail to assess whether the mechanism proposed in Scheme 1 is correct. Both peptides were resynthesized on the Rink resin and purified by HPLC. In addition, the phosphotyrosine version of **13** was enzymatically prepared. The K_D of the phosphorylated peptide **13**/Lck SH2 domain complex is 2.1 ± 0.2 μM. If the SH2 domain is responsible for the fluorescence change induced by Src kinase catalyzed phosphorylation, then the Lck SH2 domain concentration should influence the observed fluorescence response. This experiment was performed by fixing the peptide concentration at 16 μM and varying the Lck SH2 domain concentration from 0 to 32 μM (Figure 1). The reactions were initiated by the addition of ATP. When only buffer was added to "initiate" the reaction (i.e., no ATP), the fluorescence of the mixture remained unperturbed. Furthermore, in the absence of Lck SH2 domain, ATP addition to initiate the reaction furnished an exceedingly modest change in fluorescence intensity (<5%). By contrast, increasing concentrations of SH2 domain produced increasing enhancements in fluorescence intensity. Above an Lck SH2 concentration of 16 μM, the change in fluorescence intensity began to level off, which is in keeping with the notion that the interaction between phosphopeptide and Lck SH2 domain was approaching saturation. In addition, no fluorescence change was observed when the reaction was performed in the presence of the known Lck SH2 domain ligand Ac-pTyr-Glu-Glu-Ile-Glu (50 μM) (Supporting Information). This suggests that the fluorophore-appended phosphorylated peptide is binding to the known ligand binding site of the Lck SH2 domain. Furthermore, addition of PTP1B, a phosphotyrosine phosphatase, to the reaction at the same time as ATP blocked the fluorescence change. Finally, addition of PTP1B after completion of the Src kinase catalyzed reaction reduced the fluorescence intensity to the starting value (Supporting Information). These experiments demonstrate that the phosphorylation status of the peptide is essential for the observed change in fluorescence as is the presence of the Lck SH2 domain. Interestingly, when an analogous series of experiments were performed with the amide-capped peptide **14**, the observed fluorescence change (7.2-fold) was significantly larger than that exhibited by its library counterpart (4.1-fold). This appears to be a consequence of the $-NH(CH_2)_2SH$ tail that is present on the library members. Both peptides **13** and **14** exhibit V_{max} (1.4 ± 0.1 and 1.5 ± 0.1 μmol/min·mg, respectively) and K_m (32 ± 0.5 and 4.8 ± 0.8 μM, respectively) values comparable to the best Src kinase peptide substrates.[8]

The strategy outlined herein provides flexibility with respect to the site of fluorophore attachment to the peptide framework, thereby enabling the creation of sensing systems for protein kinases with demanding sequence specificities. In addition, given the availability of many different environmentally sensitive fluorophores with a range of photophysical properties,[9] it should be possible to generate orthogonal kinase sensing systems and thereby allow simultaneous monitoring of two or more protein kinases. These studies, as well as the construction of unimolecular cell-permeable constructs, are in progress.

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Supporting Information Available: Experimental details of protein kinase sensor synthesis and characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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