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J. Am. Chem. Soc., **2003**, 125 (47), 14248-14249• DOI: 10.1021/ja0380502 • Publication Date (Web): 04 November 2003

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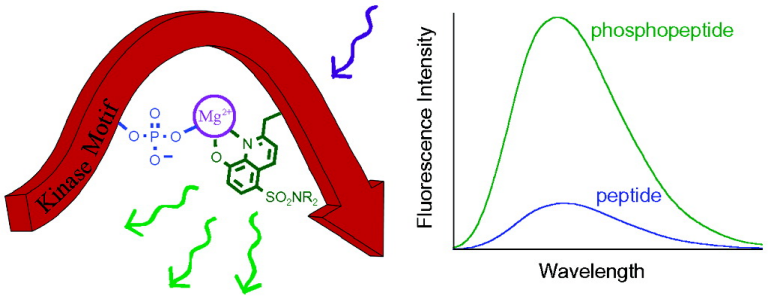

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Published on Web 11/04/2003

Versatile Fluorescence Probes of Protein Kinase Activity

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Protein kinases play a pivotal role in all aspects of cellular regulation.[1] This enzyme class constitutes nearly 1 in 50 of all human genes,[2] and its members orchestrate complex and interwoven signal transduction pathways. Because of the centrality of protein phosphorylation as a regulatory process, kinase inhibitors are in demand as therapeutic agents for many diseases,[3] especially cancer.[4] Sensitive and widely applicable methods for monitoring protein kinase activity will provide valuable tools to identify inhibitors and to unravel complex signaling cascades.

Traditional assays for kinase activity monitor product formation discontinuously and rely either on radioactive labeling or on the use of expensive and specialized biological reagents such as phosphopeptide-specific antibodies. Continuous fluorescence assays are ideal, and specific kinase substrates that exhibit large fluorescence increases upon phosphorylation will be extremely useful for high-throughput screening of inhibitors or substrates, detection of activity in cell extracts or enzyme purifications, and, ultimately, spatial or temporal localization of kinase activity within a cell. Several assays for monitoring kinase activity continuously have been reported. Approaches include use of environment-sensitive fluorophores adjacent to the phosphorylated residue,[5,6] placement of fluorescence resonance energy transfer (FRET) partners flanking a sequence that undergoes a conformational change upon phosphorylation,[7] and signaling via metal chelation between the newly introduced phosphate and an external[8] or internal[9] chelator. Unfortunately, a majority of these sensors demonstrate very modest fluorescence changes, which limits their applicability. A notable exception is the 1.5−2.5-fold increase in fluorescence reported in the probes developed by Lawrence and co-workers.[6,9] However, these probes, with fluorophores adjacent to the phosphorylated residue or very large fluorophores, may interfere with recognition by, and reactivity with, certain kinases, and therefore there is considerable scope for the continued development of novel chemical approaches for assaying protein kinase activity.

Herein, we report the design and use of a versatile kinase activity reporter scaffold. We demonstrate that this scaffold functions in enzyme assays wherein the phosphorylated serine is located on the N- or C-terminus of the kinase recognition motif. In addition, we report that the concept may also be applied to peptide sequences that are phosphorylated on either threonine or tyrosine.

The key elements of the reporter as well as its ability to function both N- and C-terminal to a kinase recognition motif are depicted in Figure 1. The fluorescence signal is generated by the Sox amino acid[10] contained in the reporter motif, which undergoes chelation-enhanced fluorescence upon binding Mg^{2+}. The Sox residue is separated from the residue to be phosphorylated by a β-turn sequence that preorganizes a binding site[11] for Mg^{2+}. The β-turn sequence consists of two amino acids, most generally a proline and glycine, although the residue other than proline can be an additional specificity determinant for the kinase. In addition, the reporter contains a kinase recognition motif that can be varied to target a desired kinase. This design requires that only three additional amino acids be appended to the recognition sequence. The key feature of the design is that Mg^{2+} binding prior to phosphorylation is weak (100 mM < K_D < 300 mM), whereas after phosphorylation affinity is augmented (4 mM < K_D < 20 mM) and a considerable proportion of phosphopeptide exists in the bound, fluorescent state in the presence of 10 mM Mg^{2+}.

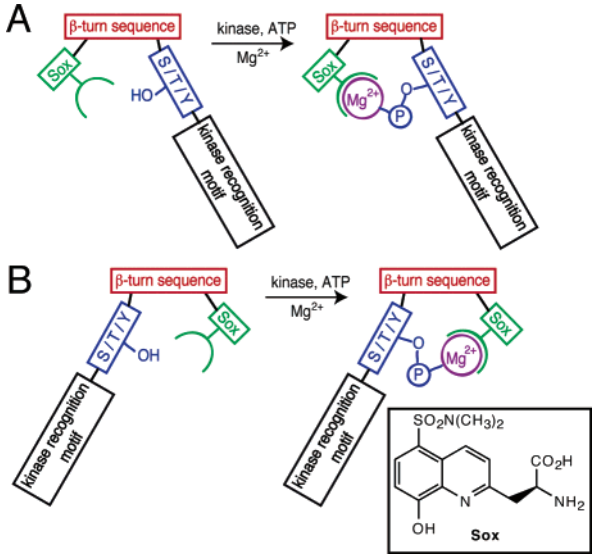

Figure 1. Schematic representation of fluorescent kinase probes. (A) Design for C-terminal kinase recognition motif. (B) Design for N-terminal kinase recognition motif. Inset: Structure of Sox amino acid.

Peptide substrates, containing the reporter motif for Protein Kinase C (PKC) and cAMP-dependent protein kinase (PKA), as well as corresponding synthetic product peptides were prepared by Fmoc-based solid-phase peptide synthesis. Peptide identities and purities were confirmed via electrospray mass spectrometry and high-performance liquid chromatography (HPLC) analysis. The increase in fluorescence intensity upon complete phosphorylation was obtained by comparison of the synthetic phosphorylated and unphosphorylated peptides (Table 1). A 3−5-fold increase in fluorescence intensity is obtained upon phosphorylation with these probes, which is the greatest change thus far for this type of probe. The exact magnitude of the fluorescence increase varies with the substrate, and it is due to the difference in binding affinities for Mg^{2+} present in the reaction mixture between the substrate and product peptides. In general, a larger difference in binding affinities results in a larger percent increase in fluorescence intensity.

The new probes have been implemented for assaying typical protein kinase substrates. The kinase reactions are monitored with excitation at 360 nm and emission at 485 nm. The fluorophore, Sox, is small, causing minimal perturbation of the substrate affinities, and it is resistant to photobleaching. With typical Mg^{2+} concentrations (10 mM), neither substrate nor product peptides are

10.1021/ja0380502 CCC: $25.00 © 2003 American Chemical Society

Table 1. Kinetic and Fluorescence Properties of Protein Kinase Substrates Containing Kinase Sensing Motif

Target Kinase	Phosphorylated Residue	Substrate Sequence[a]	K_M (μM)[b]	V_{max} (μmol/min/mg)[b]	Fluorescence Increase[c]
PKC_α	Ser	Ac-Sox-Pro-Gly-Ser*-Phe-Arg-Arg-Arg-NH_2	8.6 ± 2.9[d]	5.9 ± 1.9[d]	470%[e]
PKA	Ser	Ac-Leu-Arg-Arg-Ala-Ser*-Leu-Pro-Sox-NH_2	1.8 ± 0.5[f]	3.7 ± 1.6[f]	300%[e]
PKC_α	Thr	Ac-Sox-Pro-Gly-Thr*-Phe-Arg-Arg-Arg-NH_2			280%[g]
Abl	Tyr	Ac-Sox-Pro-Gly-Ile-Tyr*-Ala-Ala-Pro-Phe-Ala-Lys-Lys-Lys-NH_2			400%[g]

[a] Residue that is phosphorylated is marked (*). [b] K_M and V_{max} values were obtained from initial slopes, corrected appropriately for substrate and product fluorescence. An average of four values from separate Hanes plots is reported. [c] Corresponding kinase assay conditions with 10 mM Mg^{2+}, 360 nm excitation, 485 nm emission. [d] Assay conditions: 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 mM ATP, 1 mM DTT, 0.5 μg/mL phosphatidylserine, 0.1 μg/mL diacylglycerol, 0.7 nM PKC_α, 30 °C. [e] From slope (units/μM) of product and substrate concentration versus fluorescence intensity. [f] Assay conditions: 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 40 units PKA, 30 °C. [g] From single peptide solutions (10 μM).

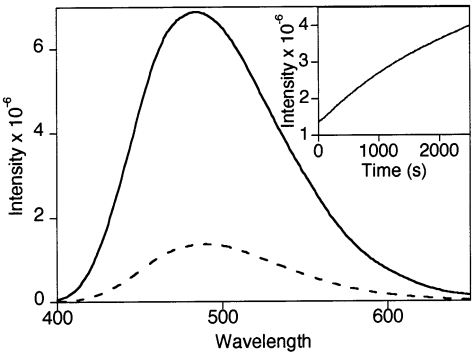

Figure 2. Spectra of peptide substrate (— —) and phosphopeptide product (—) (10 μM each) in PKC assay buffer containing 10 mM $MgCl_2$. Inset: Fluorescence intensity over the reaction time-course of Ac-Sox-Pro-Gly-Ser-Phe-Arg-Arg-Arg-NH_2 (10 μM) with PKC_α.

saturated with Mg^{2+} so that the difference in affinities produces a difference in fluorescence intensity (Figure 2).[12] Upon reaction with the appropriate kinase, an increase in fluorescence intensity over time is observed as expected (Figure 2 inset), and the increase upon complete phosphorylation matches the values reported in Table 1. At intermediate time points, the extent of product formation predicted from the fluorescence signal was confirmed by HPLC and electrospray mass spectrometry. Importantly, initial rate experiments verify that these peptides are good substrates for their respective enzymes (Table 1), demonstrating that the reporter motif does not interfere with reactivity of the substrate. K_M and V_{max} values were determined from fluorescence measurements.[13] In addition, biologically relevant metal ions do not interfere with the sensing of kinase activity at physiological concentrations.

Peptides containing phosphothreonine and phosphotyrosine also exhibit a several-fold increase in fluorescence intensity when compared to their unphosphorylated analogues (Table 1), thus establishing that this general approach will also be amenable for the development of assays for other protein kinase enzymes.

In conclusion, we have developed a widely applicable, versatile, continuous fluorescence assay for protein kinase activity. It is immediately applicable to high-throughput screening assays. Improvements in these probes are underway to increase the Mg^{2+} affinity of the phosphopeptide, signal intensity, and scope of their use.

Acknowledgment. This research was supported by the NIH (GM64346 Cell Migration Consortium) and the NSF (CHE-9996335). Award of an NSF graduate research fellowship for M.D.S. is gratefully acknowledged.

Supporting Information Available: Experimental procedures for peptide synthesis and enzyme assays, characterization of peptides and reaction products, fluorescence spectral comparison of all substrate and product peptide pairs, correlation of product formation with fluorescence data, calculations for obtaining kinetic parameters from fluorescence data, Hanes plots, and metal competition data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Mg^{2+} concentrations other than 10 mM can be easily used following empirical determination of the percent fluorescence increase under the assay conditions.
(13) Any peptide concentrations below the Mg^{2+} dissociation constant can be used because the fraction of each peptide bound remains constant under these conditions. The absolute fluorescence signal of substrate and product peptides is linear with respect to concentration.

JA0380502