# Monitoring Protein Kinases in Cellular Media with Highly Selective Chimeric Reporters**

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Protein kinases are important regulators of cellular function, and the dynamics of their activities are critical indicators of the health or pathology of living systems.[1,2] In particular, extracellular signal regulated kinases 1 and 2 (ERK1/2) play a pivotal role in the mitogen-activated protein kinase (MAPK) signaling pathway responsible for regulated cell survival and proliferation.[3] The centrality of these enzymes in normal and diseased cell states underscores the need for high-throughput, selective, and sensitive methods that accurately and directly diagnose kinase activities. The benchmark phosphorylation assays for ERK1/2 rely on transfer of radioactive γ-phosphate of $[\gamma\text{-}^{32}P]ATP$ to peptide or protein substrates.[4] While broadly employed, this approach has limitations, including the discontinuous nature of the radioactive assay and the non-native ATP concentrations that are utilized. Alternatively, for cellular imaging, genetically encoded sensors that rely on phosphorylation-based changes in fluorescence resonance energy transfer (FRET) between fluorescent protein pairs[5,6] have been constructed for several kinases, including ERK1/2.[7–10] These sensors are powerful because they can be expressed in cells; however, they cannot be used for high-throughput screening of recombinant enzymes and unfractionated cell lysates owing to the very limited fluorescence changes that accompany phosphorylation. As a complementary approach, probes based on small, organic fluorophores with direct readouts[6,11] can give sensitive and robust signals under physiological conditions and are thus amenable to high-throughput applications. For example, we have incorporated a sulfonamido-oxine (Sox) chromophore into peptides[12,13] to report phosphorylation by chelation-enhanced fluorescence (CHEF; Figure 1a). The weak binding affinity of the unphosphorylated substrate for Mg^{2+} increases significantly upon phosphorylation, resulting in robust (2- to 12-fold) fluorescence enhancements. This versatile peptide-based sensor
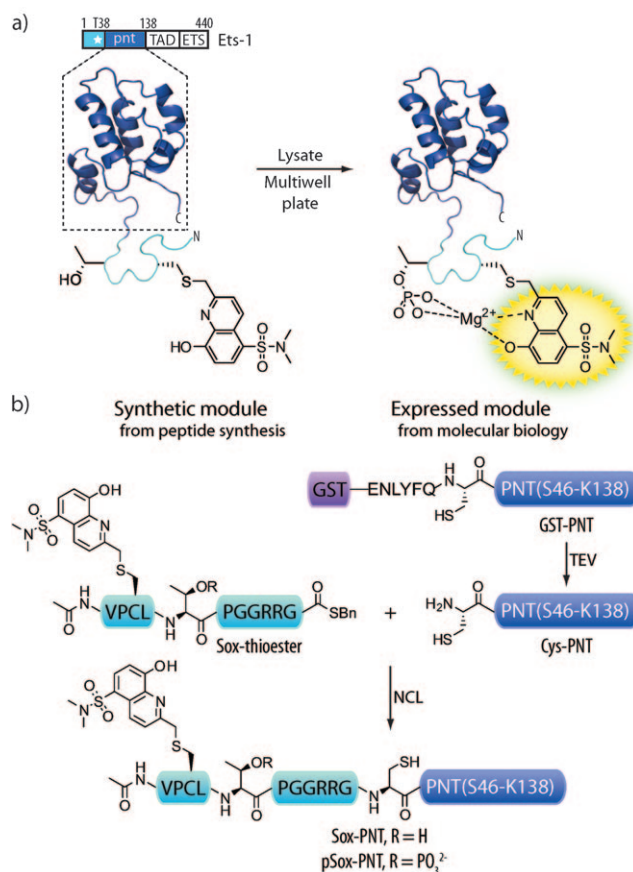
Figure 1. Sox-based chemosensors. a) The ERK1/2 probe utilizes the PNT domain from Ets-1 for specific binding to the enzyme and senses phosphorylation using Sox-dependent CHEF ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 485$ nm). b) The Sox-PNT sensor is synthesized by native chemical ligation (NCL). TEV = tomato etch virus protease; Bn = benzyl.

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design has been applied to monitor the activity of numerous Ser/Thr and Tyr kinases both in vitro[13] and in cell lysates.[12]

With more than 500 different kinases encoded in the human genome, sensor selectivity becomes paramount, particularly when enzymes are studied under conditions that resemble their native environments, such as in unfractionated cell lysates or live cells.[6] While many protein kinases exploit linear recognition motifs comprising four to eight residues that are proximal to the phosphorylation site to drive specificity, a number of physiologically important kinases, including ERK1/2 and other MAPKs, phosphorylate substrates with short and ubiquitous consensus sequences. For these enzymes, specificity is derived from extended recognition elements that include protein–protein interactions

distal to the phosphorylation site.[14] For example, ERK1 and 2 phosphorylate the transcription factor Ets-1 at Thr38 within a short ThrPro (TP) consensus motif.[15] Since this short sequence would be the target of multiple kinases, ERK recognition of Ets-1 depends an adjoining N-terminal pointed (PNT) domain[16] to dock the substrate specifically to ERK1/ 2, which engages the phosphorylation machinery.[17] With this docking-domain strategy, PNT-based substrates demonstrate good affinity for ERK1/2 ($K_M \approx 6$–9 μM),[16] in stark contrast to short peptide substrates derived only from the TP sequence ($K_M > 200$ μM).[16,18] Owing to the size of the PNT domain (11 kDa), these docking-domain interactions cannot be exploited with the types of synthetic peptide-based sensors that have been reported. However, in light of its importance in cellular homeostasis and the prominence of ERK disregulation in cancer, we set out to construct a chimeric sensor for ERK1/2 that combines the advantageous reporting properties of the Sox fluorophore with the outstanding specificity provided by the native protein domain-based recognition (Figure 1a). Most importantly, to ensure facile and high-throughput analysis of ERK1/2 activity, our ultimate requirement is that the probe be highly selective in cell lysates where it would be exposed to hundreds of other active kinases.

Herein we describe the semisynthesis of a chimeric Sox-based ERK1/2 sensor through a key native chemical ligation (NCL) reaction that efficiently conjugates the recombinat PNT domain of Ets-1 to a synthetic ERK1/2 consensus sequence including the Sox sensing module. The extended PNT recognition element confers the ERK1/2 sensor with excellent selectivity, as demonstrated by comparative quantitative analyses with a panel of related recombinant enzymes and in unfractionated lysates from four different cell lines. Most importantly, the docking-domain-based sensor design should be generally applicable to the development of selective sensors for other medically important kinases.

The new sensor was assembled as illustrated in Figure 1, using NCL[19] to ligate the synthetic Sox-containing peptide thioester with the expressed PNT domain, comprising Ets-1 residues 46–138 (Figure 1b). The peptide thioester was synthesized using Fmoc-based solid-phase peptide synthesis (SPPS; Fmoc = fluorenylmethyloxycarbonyl) on highly acid-labile TGT resin, with subsequent off-bead thioesterification of the protected peptide.[20] The Sox chromophore was introduced as the amino acid C-Sox.[13] An optimized phosphorylation motif based on the ERK2 phosphorylation sequence within the myelin basic protein (MBP;[18] TPGGRR) was used in place of the phosphorylated region of Ets-1 (TPSSKE). When the Sox chromophore was incorporated into these short peptides, preliminary studies indicated that the MBP-based sequence had better fluorescent properties (Table S1 in the Supporting Information). The distance between the TP recognition sequence and the PNT domain in the wild-type protein was preserved in the sensor (Table S2 in the Supporting Information). This design introduced residue replacements in the unstructured N-terminal region of Ets-1, thereby minimizing perturbations to the overall secondary structure. Additionally, the C-terminal residue, Met44, was changed to Gly to eliminate the possibility of epimerization during thioesterification and to

increase ligation efficiency.[21] The expressed C-terminal fragment of the sensor, GST-PNT, was proteolyzed to reveal Cys-PNT. After ligation of Cys-PNT to the peptide thioester in nondenaturing conditions, the ERK1/2 probe Sox-PNT was isolated in good yield (24 %; the accurate mass of the isolated material was based on the molar absorptivity ε_{max} of the Sox chromophore; see the Supporting Information). The corresponding phosphoprotein (pThr38) pSox-PNT was constructed using analogous methods.

Initial spectroscopic studies with Sox-PNT and pSox-PNT revealed a robust threefold enhancement in fluorescence upon phosphorylation (Figures S2 and S3 in the Supporting Information). Moreover, similar to Sox-based peptide sensors,[13] Sox-PNT was found to have an excellent Z′ factor value (0.81), which is a statistical quality parameter used to evaluate and validate performance of assays, with useful ranges being 0.5–1.[22] Subsequent in vitro assays determined Sox-PNT to be an efficient substrate for ERK2 when compared with the corresponding Sox peptide (Ac-VP-CSox-LTPGGRRG-OH; Figure 2a and Figure S3 in the
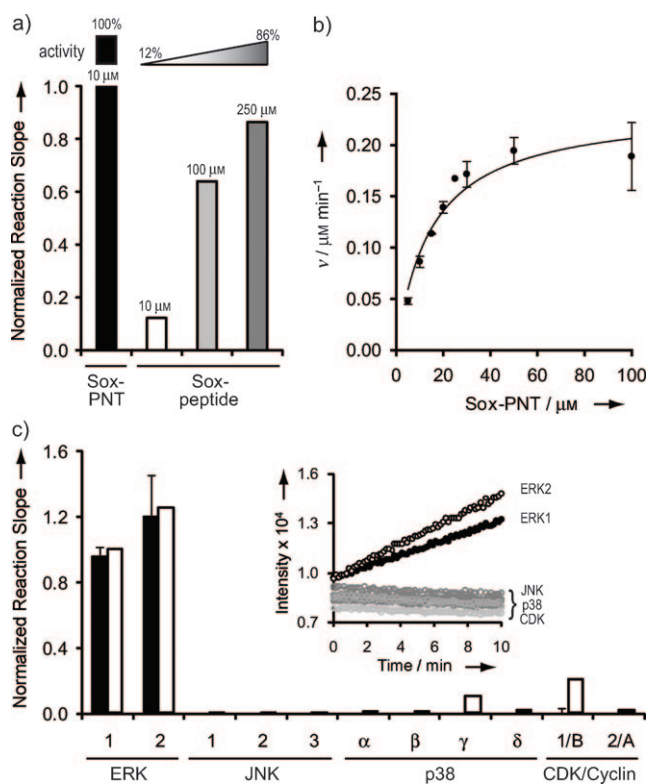
Figure 2. In vitro characterization of Sox-PNT. a) The efficiency of phosphorylation by recombinant ERK2 (11 ng) of the Sox-PNT probe was compared to that of the Sox peptide under identical conditions. b) The kinetic parameters for Sox-PNT were obtained with ERK2 (10 ng) from a direct fit of ν vs. [S] plots using the Briggs–Haldane equation. Plotted values indicate the mean standard error of measurement ($\pm$ s.e.m.) for triplicate measurements. c) Promiscuity of Sox-PNT (5 μM) was tested with a panel of related kinases at 15 nM (black bars) and 150 nM (white bars) of each enzyme. Inset: a representative plot of the change in the fluorescence signal over time obtained with 15 nM enzyme in the fluorescence plate reader. Plotted values for 15 nM enzyme indicate the mean $\pm$ s.e.m. for triplicate measurements.

Supporting Information). Furthermore, Sox-PNT demonstrated a similar K_M value (14.9 μM) and a slightly lower catalytic efficiency ($k_{cat}/K_M = 47\ \text{min}^{-1}\,\mu\text{M}^{-1}$) compared to the wild-type PNT ($K_M = 9\ \mu\text{M}$, $k_{cat}/K_M = 132\ \text{min}^{-1}\,\mu\text{M}^{-1}$),[23] thus indicating that use of the MBP-derived TP sequence had minimal effect relative to the native protein (Figure 2b). In contrast, the MBPtide (APRTPGGRR), the basis of the Sox-PNT phosphorylation sequence, was reported to have substantially poorer kinetic parameters ($K_M = 2\ \text{mM}$, $k_{cat}/K_M = 0.11\ \text{min}^{-1}\,\mu\text{M}^{-1}$)[18] for ERK2, underscoring the importance of the PNT domain in substrate kinetics. Finally, Sox-PNT exhibited high selectivity for ERK1/2 when compared to related kinases from the JNK, p38, and CDK families (Figure 2c).

To demonstrate that the preference of Sox-PNT for ERK1/2 can translate to complex media, the probe was exposed to a panel of unfractionated cell lysates that contained varying levels of active ERK1/2. The activity of cellular ERK1/2 is linked to its phosphorylation state, which is modulated by the epidermal growth factor (EGF) signaling pathway (Figure 3a). Briefly, EGF interacts with EGF receptors (EGFRs), which leads to activation of MEK1/2, which in turn phosphorylates and activates ERK1/2. This event can be regulated either by the upstream MEK1/2 inhibitor U0126 or by the direct ERK1/2 inhibitor PEA-15.[24] Summarized in Figure 3b are the results of ERK1/2 activity analyses on the crude lysates from four mammalian cell lines, which reveal the selectivity of the sensor in these complex media. In all cases, untreated lysates showed relatively low basal activity, while displaying slight variation among different cell lines, as expected. Upon EGF stimulation, there was a four- to tenfold increase in activity.[25] To demonstrate that Sox-PNT was specifically monitoring ERK1/2 activity, cells were exposed to the inhibitor U0126 and subsequently EGF-stimulated and lysed. Under these conditions, the ERK1/2 activity returned to nearly basal levels. Western blot analysis was used to demonstrate that both stimulated and U0126
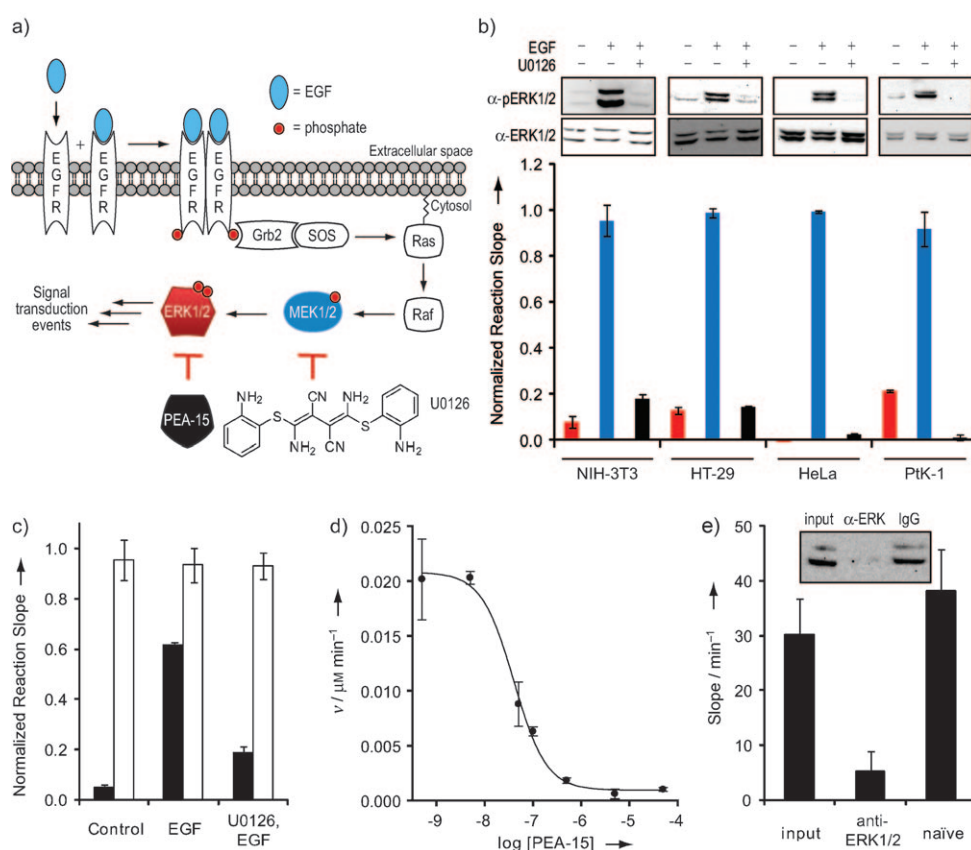
Figure 3. Specificity of the Sox-PNT sensor toward ERK1/2 in unfractionated cell lysates. a) The EGF signalling pathway results in stimulation of ERK1/2 activity. U0126, an inhibitor of an upstream kinase (MEK1/2) and PEA-15, a direct inhibitor of ERK1/2, can regulate the activity of ERK1/2. b) Sox-PNT (5 μM) was used to measure enzyme activity in 40 μg untreated lysates (red bars), EGF-stimulated lysates (blue bars), or U0126-treated and then EGF-stimulated lysates (black bars). Inset: western blot for pERK1/2 (top) and ERK1/2 (bottom). c) The ability of Sox-PNT (black bars, 5 μM) to report the different phosphorylation states of ERK1/2 in HeLa lysates (40 μg) was directly compared to the Sox peptide (white bars, 5 μM). d) To obtain the IC$_{50}$ value for inhibition of ERK1/2 by PEA-15, EGF-stimulated NIH-3T3 lysates (40 μg) were treated with various concentrations of PEA-15, and enzyme activity was measured with Sox-PNT (5 μM). e) Kinase activity was measured with Sox-PNT (5 μM) from an EGF-stimulated HeLa lysate (40 μg) before (input) and after immunodepletion of this lysate with anti-ERK1/2 or naïve rabbit IgG. Inset: western blot for ERK1/2 in the measured samples. The top band in all western blots is ERK1 (44 kDa) and the bottom band ERK2 (42 kDa). Plotted values indicate the mean $\pm$ s.e.m. for triplicate measurements.

inhibitor-treated cells expressed ERK1/2 (Figure 3b), however, only EGF-stimulated samples showed enhanced levels of activated ERK1/2, as evidenced by analysis with the phospho-ERK1/2-specific antibody. In contrast, the Sox peptide, lacking the PNT docking domain, showed promiscuous activity and signaled phosphorylation that could not be correlated with ERK1/2 activity (Figure 3c).

Further evidence that Sox-PNT is selectively modified by ERK1/2 was obtained with PEA-15, a direct protein inhibitor of ERK1/2. Titration of PEA-15 into EGF-stimulated NIH-3T3 lysates resulted in a dose-dependent response with a half inhibitory concentration of 40 nM and a K_i value (30 nM) that reflected the reported K_i values (20 nM; Figure 3d and the Supporting Information).[24]

To directly correlate the observed fluorescent signal to the presence of ERK, we exposed our probe to ERK1/2-depleted lysates. Indeed, immunodepletion of ERK1/2 from EGF-

stimulated HeLa lysate reduced activity by sevenfold compared to the input lysate or the sample that had been depleted with naïve rabbit immunoglobulin G (IgG, Figure 3e). This finding indicates that the Sox-PNT signal is predominantly due to the ERK1/2-mediated phosphorylation. Slight residual activity in anti-ERK1/2 lysate can be attributed to incomplete removal of the kinase, which is highly dependent on the efficiency of antibody and kinase binding. Immunodepletion was confirmed by western blot analysis with the immunodepleting antibody (Figure 3e, inset). Having validated the selectivity of Sox-PNT for ERK1/2, the sensor can be used to measure active ERK1/2 (13 ng) in EGF-stimulated lysate (40 µg; Figure S5 in the Supporting Information), which will be an important tool for quantifying ERK1/2 levels in tissue samples.

The MAPK signaling pathways are composed of numerous kinases intricately regulated by stress responses and extracellular signals. Deconvoluting the specific functions of individual enzymes has been challenging, partly owing to the difficulty of creating probes that exclusively target a kinase of interest. Herein we have presented a selective ERK1/2 activity chemosensor that comprises both a chemical sensing motif and recombinant enzyme docking domain. Thereby, the Sox-based kinase-sensing strategy has been extended beyond the realm of enzymes that recognize linear peptide substrates. Quantitative studies with the probe indicate that the PNT domain confers exquisite selectivity toward ERK1/2, which was impossible to achieve with simple peptide probes. Moreover, the docking-domain approach now allows us to target a wider set of kinases (such as other members of the MAPK family, JNK and p38) that have been elusive to date owing to their complex substrate recognition mechanisms. The chimeric protein probe also offers distinct advantages for solution-based analyses that can be carried out with simple equipment. The reliable semisynthesis of multimilligram (6 mg) quantities of Sox-PNT allows at least 5000 assays to be performed in 384-well plates. Furthermore, Sox-based sensors exhibit large dynamic ranges with excellent Z' factor values,[13] which are a measure of the fidelity of assay data.[22] Currently, in light of the efficient semisynthesis, excellent selectivity, and robustness in high-throughput analysis, the Sox-PNT sensor can be broadly applied for quantifying ERK1/2 activities in applications ranging from drug discovery to diagnostics.

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