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A p38α-Selective Chemosensor for use in Unfractionated Cell Lysates

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Methods for assessing kinase activities have relied on the transfer of a radiolabeled γ-phosphoryl moiety from ATP to substrate and have been very useful for in vitro studies of kinases. However, beyond being discontinuous, this assay is incompatible with unfractionated cell lysates since ATP is a common substrate for most kinases. This has prompted the development of protein-based fluorescence resonance energy transfer (FRET) sensors for probing kinase activity (1-3). While useful, these FRET-based sensors produce modest changes in fluorescence upon phosphorylation. Alternatively, the development of methods based on small organic fluorophores has provided sensitive probes for interrogating biological functions (4, 5). Recently our laboratory has introduced a direct kinase assay strategy based on chelation-enhanced fluorescence of a cysteine derivative of a sulfonamido-oxine fluorophore (6), which we term CSox (Figure 1, panel a). Placed (−)2 or (+)2 relative to the phosphorylation site in an optimized kinase substrate, the CSox amino acid provides a readout of protein-based fluorescence resonance energy transfer (FRET) sensors for probing kinase activity (1-3).

In the case of MAPKs the development of selective probes has proved more challenging due to the minimal local consensus phosphorylation sequence, S/T-P. This class of enzymes (including the ERK, JNK, and p38 family members) derives specificity through the use of extended protein or peptide docking domains that are distant to the phosphorylation site (16, 17). These domains allow for the target of a substrate to a particular kinase and can therefore be viewed as unique address elements. Due to the limited structural information concerning p38α substrates, we chose to employ a strategy in which a known docking peptide sequence (18, 19) (Figure 1, panel b) would be linked to a CSox-based phosphorylation site via a flexible linker (16) (Figure 1, panel c). Initial phosphorylation reactions indicated that this sensor, MEF2A-CSox, could act as a substrate for purified p38α (Supplementary Figure S1). Phosphorylation reactions containing differing amounts of MEF2A-CSox demonstrated a K_M and V_max for p38α of 1.3 μM and 1.1 μmol mg⁻¹ min⁻¹, respectively.

Abstract
Recent efforts have identified the p38α Ser/Thr kinase as a potential target for the treatment of inflammatory diseases as well as non-small cell lung carcinoma. Despite the significance of p38α, no direct activity probe compatible with cell lysate analysis exists. Instead, proxies for kinase activation, such as phosphospecific antibodies, which do not distinguish between p38 isoforms, are often used. Our laboratory has recently developed a sulfonamido-oxine (Sox) fluorophore that undergoes a significant increase in fluorescence in response to phosphorylation at a proximal residue, allowing for real-time activity measurements. Herein we report the rational design of a p38α-selective chemosensor using this approach. We have validated the selectivity of this sensor using specific inhibitors and immunodepletions and show that p38α activity can be monitored in crude lysates from a variety of cell lines, allowing for the potential use of this sensor in both clinical and basic science research applications.

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(Figure 2, panel a). We then assessed the specificity of MEF2A-CSox by exposing it to a panel of related kinases (Figure 2, panel b). MEF2A-CSox was selectively phosphorylated by p38α and showed minimal background activity in the presence of the closely related p38β isoform (8%) and the remaining kinase panel. Importantly, this difference in selectivity for p38α over p38β translated into a 17-fold enhancement in catalytic efficiency for p38α (Supplementary Figure S2). Encouraged by these in vitro studies, we investigated the ability of MEF2A-CSox to report p38α activation in un-fractionated cell lysates.

Several studies have demonstrated p38α activation in response to inflammatory cytokines or cellular stress (20). With this in mind, we treated HeLa cells with increasing amounts of the cytokine TNFα (Supplementary Figure S3). These initial experiments demonstrated that MEF2A-CSox was capable of reporting p38α activation despite appreciable signal due to phosphorylation by off-target kinases. A recent survey of kinase inhibitors (21) indicated that the broad spectrum inhibitor staurosporine is not effective against p38α, which was confirmed using recombinant enzyme (Supplementary Figure S4). Consequently we hypothesized that, in this case, staurosporine may be used to reduce the off-target kinase activities allowing for discrimination of the p38α signal. Indeed, the addition of 1 μM staurosporine to assays using sorbitol-stimulated lysates demonstrated that a portion of off-target kinases could be suppressed by using this promiscuous inhibitor (Figure 3, panel a), and therefore staurosporine was added to all subsequent lysate assays. A comparison between TNFα and sorbitol stimulation indicated an increase of 68% in the rate of MEF2A-CSox phosphorylation in cells stimulated by osmotic shock (Figure 3, panel b), and consequently these lysates were used to optimize assay conditions. Optimal signal-to-noise for sorbitol-stimulated lysates was obtained using 10 μg of total protein (Supplementary Figure S5), which provided a clearly discernible enhancement in the rate of phosphorylation of MEF2A-CSox (Figure 3, panel c). Using small molecule inhibitors of p38α, specifically SB203580, which is ATP competitive (22-24), and BIRB796, a slow-binding allosteric inhibitor (25), the origin of the increase in the rate of MEF2A-CSox phosphorylation upon osmotic shock was investigated. The addition of 1 μM of each of these compounds, which completely abolishes activity of recombinant kinase (Supplementary Figure S6), reduced the rate of phosphorylation of MEF2A-CSox in sorbitol-stimulated HeLa lysates to levels observed for serum starved cells (Figure 3, panel d). Appropriate control measurements established that this effect was not due to the inhibitor solvent (DMSO) (Supplementary Figure S7).

We sought to further verify the selectivity of MEF2A-CSox for p38α through a series of inhibition studies, immunodepletion experiments, and analyses across different cell lines.
Accordingly, the phosphorylation of MEF2A-CSox was determined in the presence of varying concentrations of SB203580 and BIRB796 (Figure 4, panels a and b, respectively). A concentration-dependent decrease in the rate of phosphorylation of MEF2A-CSox was observed in the presence of SB203580, yielding a $K_i$ of 7.5 nM, which reflects the reported $K_i$ of 21 nM (24). A similar dose-dependent response was observed for BIRB796 that was in good correlation with previously reported values (25). Importantly, these experiments indicate that the remaining background activity due to off-target kinases could be essentially eliminated through background subtraction of parallel reactions containing 1 μM SB203580 (see also Figure 3, panel d). Accordingly, to further verify the specificity of MEF2A-CSox, p38α immunodepletion studies were performed in which the activity remaining after the addition of SB203580 was used for background subtraction. These depletions clearly demonstrate that the increase in the rate of phosphorylation of MEF2A-CSox upon stimulation by osmotic shock is predominantly due to p38α (Figure 4, panel c). Moreover, similar depletions for the related kinase ERK5 (26) demonstrated no appreciable loss of signal (Supplementary Figure S8). In combination with the known selectivity profiles for the inhibitors used herein (21) (Supplementary Table S1), these results indicate that MEF2A-CSox is a p38α-selective activity probe and that off-target signal can be virtually eliminated using SB203580.

Finally, we investigated the ability of MEF2A-CSox to report the activation of p38α in a variety of cell lines isolated from different tissues and species. Indeed MEF2A-CSox was capable of reporting the activation of p38α in HeLa (human), Cos7 (simian), and NIH-3T3 (rodent) cells (Figure 4, panel d). The activity in each lysate correlated with Western blot analyses indicating that MEF2A-CSox can be used in a variety of mammalian systems and tissues to directly interrogate p38α activity levels.

We have designed and validated the first isoform-selective p38 activity probe compatible with unfractionated cell lysates. This probe provides isoform-specific activity information that cannot be obtained through the use of currently available phosphospecific antibodies. Because the synthesis described herein produces 2.4 mg of MEF2A-CSox, sufficient material for 7,000 assays in 96-well plate format or 28,000 assays in 384-well format (7), this sensor could easily be utilized to rapidly screen compound libraries to identify p38α inhibitors. Furthermore, we envision that this sensor will be useful for detailing the changes in kinase signaling pathways during cellular transformations such as differentiation and cancer development (15).

**Methods**

**General Reagents and Methods.** Low metals grade chemicals were obtained from Sigma and Alfa Aesar. Lysates were normalized for total protein content using the Bio-Rad protein assay (500−0006) with BSA as a standard. Fluorescence emission was acquired at 485 nm using 360 nm excitation on either a HTS 7000 BioAssay Reader (Perkin-Elmer) or Spectramax Gemini XS (Molecular Devices, 455 nm cutoff) plate reader. All fluorescence assays were performed at 30 °C in 96-well plates (Corning, 3992).

**Synthesis of MEF2A-CSox.** MEF2A-CSox was synthesized using standard Fmoc-based solid-phase peptide synthesis methods as described previously (6). The linker in MEF2A-CSox was installed by coupling three Fmoc-protected AOO linkers (Novabiochem, 851037) to the growing peptide chain on 100 mg of PAL-PEG-PS resin (0.19 mmol g$^{-1}$ substitution, Applied Biosystems). The Sox fluorophore was...
incorporated via on-resin alkylation of a selectively deprotected cysteine residue (6). The resulting peptide was acetyl-capped at the N-terminus and included a C-terminal amide derived from the resin. Purification was carried out by standard reverse phase HPLC. Characterization was performed using ESI-MS, and concentrations were determined by the absorbance of the peptide at an extinction coefficient of 8,427 M$^{-1}$ cm$^{-1}$.

MEF2A-CSox Reactions Containing Recombinant Enzyme. Reactions were carried out using 15 nM of the indicated enzyme (Invitrogen) and 1 μM MEF2A-CSox in a buffer containing 50 mM Tris-HCl (pH = 7.5 at 25 °C), 10 mM MgCl$_2$, 1 mM EGTA, 2 mM DTT, 0.01% Triton X-100, and 1 mM ATP in a final volume of 120 μL. Well-to-well path length variation was corrected by normalizing to starting intensities.

**Determination of Kinetic Parameters of MEF2A-CSox with p38α and β.** Reactions were performed as above using 1 ng of recombinant human p38α or β (Invitrogen) with increasing concentrations of substrate. Initial reaction slopes were then converted to rates as described previously (27).

**Recombinant Kinase Panel Assays.** Reactions were conducted as described above with 1 μM MEF2A-CSox and 15 nM of the indicated kinase (Invitrogen).

**Preparation of Cell Lysates.** HeLa cells were propagated in 90% DMEM supplemented with 10% heat-inactivated FBS, 50 μM 1,2-dithiothreitol, and 50 μg mL$^{-1}$ streptomycin. Cos7 and NIH-3T3 cells were propagated in 90% DMEM supplemented with 10% FBS, 50 μM 1,2-dithiothreitol, and 50 μg mL$^{-1}$ streptomycin. Prior to stimulation, cells were starved overnight (14 h) by the addition of DMEM supplemented with 2 mM l-Glu, 50 μM 1,2-dithiothreitol, and 50 μg mL$^{-1}$ streptomycin. Cells were stimulated by the addition of the indicated amount of TNFa (Cell Signaling) for 10 min or sorbitol to 300 mM for 1 h. Cells were then washed with ice-cold PBS and lysed on ice in 50 mM Tris (pH = 7.5 at 25 °C), 150 mM NaCl, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 30 mM NaF, 1% Triton X-100, 2 mM EGTA, 100 μM Na$_2$VO$_3$, 1 mM DTT, protease inhibitor cocktail II (10 μL mL$^{-1}$, Calbiochem, 539134), and phosphatase inhibitor cocktail 1 (10 μL mL$^{-1}$, Sigma, P22825). Lysates were clarified by centrifugation, and supernatants were flash frozen in liquid nitrogen and stored at ~80 °C.

**Immunodepletions.** Immunodepletions were conducted as described previously (8). Briefly, sorbitol-stimulated HeLa lysates were aliquoted into separate samples (350 μg each) at 4 °C. Depletions were conducted using a rabbit anti-p38α (1 μg) antibody (Cell Signaling, 9218) along with a naive rabbit IgG control (1 μg, GE Life Sciences). Antibody-bound complexes were precipitated by the addition of Protein A agarose conjugated beads (GE Life Sciences). Input (untreated) samples were used to determine the amount of activity lost due to handling, while a separate sample was treated with Protein A beads alone to determine the amount of activity lost due to nonspecific binding to the resin. Lysates were flash frozen and stored at ~80 °C. ERK5 depletions were conducted in a similar manner using the appropriate antibody (Cell Signaling, 3372). The rate of phosphorylation by off-target kinases was background-subtracted using the activity remaining after the addition of 1 μM SB203580 to the input lysates.

**MEF2A-CSox Lysate Assays.** Assays were typically conducted using 1 μM MEF2A-CSox, 10 μg of total protein from cell lysates, and 1 μM 1,2-dithiothreitol, unless otherwise indicated. Reactions were prepared in bulk in a buffer consisting of 50 mM Tris-HCl (pH = 7.5 at 25 °C), 10 mM MgCl$_2$, 1 mM EGTA, 2 mM DTT, and 0.01% Brij 35 P with the indicated concentration of p38α inhibitor and aliquoted into 96-well plates. After addition of lysate, reactions (final volume 120 μL) were incubated at 30 °C, and fluorescence emission was monitored. Data were corrected for lag times (typically 5–10 min, after which fluorescence increases were linear with respect to time for at least 1 h) as well as variations in well-to-well path lengths. For titrations with SB203580, the calculated IC$_{50}$ was converted to a K$\text{a}$ value using the Cheng–Prussof equation with the concentration of ATP in the assay and the reported K$_{D}$ of p38α.
for ATP (24). Slopes for assays containing the slow-binding inhibitor BIRB796 (25) were determined after a 10 min incubation of the entire solution at 30 °C. After this incubation fluorescence increases were linear with respect to time for at least 1 h.

Slopes of phosphorylation reactions for assays containing lysates from different cell lines (Figure 4, panel d) were background corrected using the activity remaining in each individual lysate after the addition of 1 μM SB203580.

Western Blot Analysis. Lysates (20 μg total protein unless noted) were separated by SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. Blots were probed with primary antibodies for total p38 (Cell Signaling, 9215), phospho-p38 (Cell Signaling, 32460). Blots were visualized by enhanced chemiluminescence (Pierce, 105, 409–427).

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Supporting Information is presented following the References.

References


Supplementary Information

A p38α Selective Chemosensor for use in Unfractionated Cell Lysates

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Supplementary Figure S1. MEF2A-CSox is a substrate for p38α. Initial phosphorylation reactions with and without the addition of kinase (15 nM) indicated that MEF2A-CSox (1 µM) was a substrate for p38α.
**Supplementary Figure S2.** Kinetic parameters of MEF2A-CSox for p38β. 

**a)** A direct fit of a velocity versus MEF2A-CSox concentration plot using the Briggs-Haldane equation (1 ng p38β).

**b)** Kinetic parameters of MEF2A-CSox for the indicated kinases, demonstrating that MEF2A-CSox is a more efficient substrate for p38α. Catalytic efficiency was determined using relative $k_{cat}/K_M$ values.
Supplementary Figure S3. MEF2A-CSox phosphorylation varies with TNFα stimulation. 

Fluorescence slopes of reactions conducted without staurosporine using 1 μM substrate and 5 μg of HeLa lysates derived from cells stimulated for 10 min with the indicated amount of TNFα. Assays were also performed with lysates from untreated (serum starved) cells. 

b) The relationship between MEF2A-CSox phosphorylation and the amount of TNFα used for stimulation. 

c) A western blot of the indicated lysates with the corresponding antibodies, demonstrating increased phosphorylation of p38 with increasing amounts of TNFα.
**Supplementary Figure S4.** Staurosporine does not significantly alter p38α activity (1).

Reactions were performed using 1 nM recombinant p38α and 1 µM substrate.
Supplementary Figure S5. The effect of the amount of lysate used in the assay on the rate of phosphorylation of MEF2A-CSox. **a**) Reactions conducted with 1 μM substrate and the indicated amount of serum starved and sorbitol-stimulated HeLa lysates. **b**) The fold change in reaction slope between sorbitol-stimulated and serum starved lysates with respect to the amount of lysate used in the assay. The assay is most sensitive when 10 μg of lysate is used. Presumably the sensitivity of the assay decreases with increasing concentrations of lysate due to the increasing amounts of off-target kinases. All reactions contained 1 μM staurosporine.
Supplementary Figure S6. The addition of 1 µM SB203580 or BIRB796 completely abolishes recombinant p38α activity. Reactions were conducted with 1 µM substrate and 1 nM recombinant enzyme. Slopes for assays containing the slow-binding inhibitor BIRB796 were determined after incubation of the entire reaction at 30 °C for 30 min prior to data collection, after which fluorescence increases were linear with respect to time.
Supplementary Figure S7. The inhibitor solvent, DMSO, does not influence the phosphorylation of MEF2A-CSox. Assays were conducted using 1 µM MEF2A-CSox and 10 µg of sorbitol-stimulated HeLa lysates with the indicated inhibitor. All reactions contained 1 µM staurosporine.
**Supplementary Figure S8.** The related ERK5 kinase does not significantly contribute to the phosphorylation of MEF2A-CSox (1 µM) in sorbitol-stimulated HeLa cell lysates. 

**a)** Western blot analysis of sorbitol-stimulated lysates demonstrating activation of ERK5.

**b)** Immunodepletion of p38α leads to a dramatic decrease in MEF2A-CSox phosphorylation (left) while depletion of ERK5 demonstrated no significant loss of signal (right). Values were background subtracted using the activity remaining in the input lysates after addition of 1 µM SB203580. Western blots of the respective lysates demonstrating depletion of the target kinase are shown in the insets of each panel. Note that p38β levels remain unchanged, indicating that the remaining MEF2A-CSox activity is likely due to residual p38α (~30% remaining by gel densitometry). All reactions contained 1 µM staurosporine.
Supplementary Table S1. Reported dissociation constants of SB203580, BIRB796, and staurosporine for the indicated kinase are given (1).

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K₀ in nM
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