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Temporal Analysis of PP2A Phosphatase Activity During Insulin Stimulation Using a Direct Activity Probe

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Abstract

Protein serine/threonine phosphatases (PSPs) are ubiquitously expressed in mammalian cells. In particular, PP2A accounts for up to 1% of the total protein within cells. Despite clear evidence for the role of PP2A in cellular signaling, there is a lack of information concerning the magnitude and temporal dynamics of PP2A catalytic activity during insulin stimulation. Herein, we describe the development of a direct, fluorescent activity probe capable of reporting on global changes in PP2A enzymatic activity in unfractionated cell lysates. Utilizing this new probe, we profiled the magnitude as well as temporal dynamics of PP2A activity during insulin stimulation of liver hepatocytes. These results provide direct evidence for the rapid response of PP2A catalytic activity to extracellular stimulation, as well as insight into the complex regulation of phosphorylation levels by opposing kinase and phosphatase activities within the cell. This study provides a new tool for investigating the chemical biology of PSPs.

Graphical abstract

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Supporting Information

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Supporting methods as well as supporting figures and tables (PDF)

PSPs have been referred to as the “ugly ducklings” of cell signaling.¹ This is due, in part, to the relatively complex nature of cellular substrate recognition by PSPs. Compared to the >400 protein serine/threonine kinases (PSKs),² the human genome encodes only ~30 PSP catalytic domains.³ This observation, coupled with the indiscriminate substrate selectivity of PSP catalytic domains *in vitro*,⁴ has fostered the “ugly duckling” stereotype of PSPs. Consequently, there is a lack of technologies for the study of PSP signaling, relative to the elegant set of chemical biology tools available for dissecting PSK signaling.^{5–10} Nonetheless, continuing efforts toward determining the structures of PSP enzymes has shed light on the complex mechanisms of PSP regulation.³ In the case of PP2A, specificity in the cellular environment is derived through interaction with regulatory domains and subunits.⁴ The diversity of PSP regulatory domains and subunits is thought to result in the potential formation of hundreds of different holoenzyme complexes within the cell. Thus, while PSP catalytic domains appear to be unspecific, it is becoming clear that PSP signaling in the cellular context is highly regulated and involves a diverse array of regulatory domains, subunits, and post-translational modifications that can alter phosphatase activity.^{11,12} These findings have reinvigorated interest in the role of PSPs as bonafide signaling nodes in human disease.^{13,14} For example, PP2A has well-defined roles in insulin signaling.^{15,16} However, compared to PSKs, relatively little is known about the magnitude and temporal dynamics of PSP signaling. Therefore, there is a critical need for the development and application of chemical tools for analyzing PSP signaling in biologically relevant contexts. As an initial step toward expanding the chemical biology toolset for studying PSP signaling, we hypothesized that global PSP activity could be monitored in unfractionated cell lysates using peptide-based substrates by repurposing a platform originally developed for the detection of kinase activity.

Toward the above goal, we chose to employ the phosphorylation-sensitive sulfonamidooxine (Sox) fluorophore, which allows for the detection of proximal phosphate groups through chelation-enhanced fluorescence (CHEF) in unfractionated cell lysates.¹⁷ First generation Sox-based sensors were dependent upon replacement of the N- or C-terminal peptide substrate sequence with a β -turn motif in order to efficiently bind Mg^{2+} . However, second generation probes utilize a cysteine derivate of Sox, termed CSox, in order to allow for efficient Mg^{2+} binding.¹⁸ By employing this second generation design approach, robust kinase activity probes can be obtained by single amino acid substitutions.^{19–26} We have recently demonstrated that CSox can be repurposed for the analysis of endogenous protein tyrosine phosphatase activity by monitoring a decrease in fluorescence upon dephosphorylation.²⁷ Herein, we significantly extend this approach to afford a selective chemosensor capable of monitoring global PSP activity in unfractionated cell lysates (Figure 1a). Utilizing this new sensor, we probe the temporal dynamics of PP2A activity during insulin stimulation of liver hepatocytes.

As a proof-of-concept for detecting PSP activity with CSox-based probes, we initially set out to develop a selective substrate for monitoring PP2B (calcineurin) activity by employing a well-characterized PP2B targeting domain derived from NFAT1²⁸ (Figure S1a). Indeed, we observed dephosphorylation of this sensor construct by phosphatases in unfractionated HeLa cell lysates. However, the observed activity was not modulated by the well-established PP2B activators calcium or calmodulin (Figure S1b and c).²⁹ In addition, dephosphorylation was

not inhibited by a known PP2B autoinhibitory peptide³⁰ (Figure S1d), indicating that the probe was being dephosphorylated by an off-target PSP under these conditions. Intrigued by this result, we decided to modify our initial design in order to make a more general PSP sensor. The dephosphorylation site of the preliminary PP2B sensor was based on a commonly used calcineurin substrate peptide, which is derived from the RII subunit of cAMP-dependent protein kinase A (PKA).³¹ However, it is known that this peptide can also be dephosphorylated by PP2A and PP1.³² Suspecting that one or both of these activities may be responsible for the dephosphorylation observed in HeLa cell lysates, we synthesized a truncated version of this peptide, termed PSPTide, and installed the Sox fluorophore proximal to the site of dephosphorylation in order to potentially interrogate PP2A and/or PP1 activities (Figure 1b). Additionally, we synthesized a nonphosphorylated version of PSPTide to act as a positive control. Both peptides were synthesized using standard Fmoc-based solid phase peptide synthesis techniques, and CSox was installed as previously described.¹⁸ The peptides were then purified by reverse-phase HPLC to >90% purity (Figure S2) and their identities confirmed by mass spectrometry (Table S1). Since chelation with Mg^{2+} is critical for signal generation in Sox-based sensors, we determined the affinity of PSPTide and its corresponding nonphosphorylated peptide for Mg^{2+} . As expected, we observed a 5.9-fold decrease in the affinity of the nonphosphorylated control peptide for Mg^{2+} relative to PSPTide (Figure 2a and b). On the basis of these measurements, we determined the optimal concentration of Mg^{2+} needed to discriminate between the phosphorylated and dephosphorylated peptides. A robust 2.9-fold increase in fluorescence between PSPTide and the nonphosphorylated control peptide was observed using 5.5 mM Mg^{2+} (Figure 2c), providing candidate conditions for interrogating PSP activity in cell lysates. As an initial test of this reporter construct, we incubated PSPTide in a buffered solution (pH = 7.5) containing the predetermined optimal concentration of Mg^{2+} (5.5 mM) and 10 μ g of total protein obtained from HeLa cell lysates. We were pleased to observe dephosphorylation of PSPTide in the presence of lysate and no significant dephosphorylation in reactions incubated without lysate (Figure 2d). To confirm that PSPTide was not acting as a substrate for PP2B, we assayed our sensor in HeLa cell lysates in the presence of Ca^{2+} (Figure S3a) or recombinant calmodulin (Figure S3b). No significant increase in the dephosphorylation of PSPTide was observed in these experiments.

Building upon previous work demonstrating the potential of this peptide sequence to act as a substrate for PP2A and PP1 as well as the well-established ability to discriminate between PP2A and PP1 activities using inhibitors,^{33,34} we set out to identify the PSPs responsible for the dephosphorylation of PSPTide. Accordingly, we obtained a panel of known inhibitors with differing selectivity toward PP2A and PP1: okadaic acid (OA), calyculin A, and fostriecin.³⁴ Initially, we assayed HeLa cell lysates with increasing concentrations of OA, an inhibitor of both PP2A and PP1 (Figure S4).^{35,36} These experiments yielded two important insights into the potential targets for PSPTide. First, we were able to significantly reduce the observed phosphatase activity with OA, indicating that the activity was at least partially due to PP2A, PP1, or both enzymes. Second, even at micromolar concentrations of OA, off-target dephosphorylation of PSPTide was observed (accounting for ~40% of the total PSPTide dephosphorylation). In order to determine the identity of the enzyme(s) responsible for this remaining off-target activity, we performed assays in HeLa cell lysates containing calyculin

A (a potent PP1 and PP2A inhibitor)³⁷ in the presence or absence of the broad spectrum PP2C alpha inhibitor sanguinarine (Figure S5a).³⁸ In addition, analogous experiments were performed with the dual specificity phosphatase inhibitor orthovanadate (Figure S5b).³⁹ In either case, we did not observe any significant decrease in activity, thus ruling out these two phosphatase families as the source of off-target activity. Furthermore, assaying HeLa cell lysates in the presence of Phosphatase Inhibitor Cocktail III (Sigma) which contains (-)-*p*-bromolevamisole oxalate, a known inhibitor of L-isoforms of alkaline phosphatase,⁴⁰ produced no significant modulation in activity (Figure S5c). Thus, the source of off-target dephosphorylation of PSPTide remains unknown at this time. Nonetheless, due to the selectivity profile of existing inhibitors,^{33,34,37} we could attribute the majority of PSPTide dephosphorylation to PP2A and/or PP1. In order to better characterize the potency of known inhibitors of PP2A and PP1 in our assay format, we determined the IC₅₀ of both the dual PP2A and PP1 inhibitor, calyculin A, as well as the PP2A selective inhibitor, fostriecin (Figure 3a and b). Interestingly, we determined the IC₅₀ of fostriecin for PSPTide dephosphorylation under our assay conditions to be 34 nM. Comparing these results with the experimentally determined IC₅₀ value for calyculin A (1.7 nM) demonstrated that the remaining unknown activity in this assay (Figure S4) was likely not due to PP1 since fostriecin has a relatively weak affinity for PP1 compared to PP2A (~10 000-fold more potent for PP2A).³⁴ This hypothesis was confirmed by immunodepletion of PP1 from HeLa cell lysates, which did not influence the dephosphorylation of PSPTide when assayed with 100 nM fostriecin (Figure S6). Therefore, the activity of PP2A in this assay system can be determined by subtracting the activity resulting from inhibition by calyculin A from the total activity of untreated samples (Figure 3c). However, given the cross reactivity of calyculin A with PP4, PP5, and possibly PP6,³⁴ it is important to definitively verify that the remaining activity in this assay after background subtraction is due to PP2A by orthogonal methods. To address this point, we immunodepleted PP2A catalytic subunits (PP2AC) from HeLa lysates and assayed the resulting lysates with PSPTide using the proposed background subtraction method (Figure 3d). Gratifyingly, an 82% decrease in PSPTide dephosphorylation was observed in PP2A depleted lysates. This decrease in PSPTide dephosphorylation directly correlated to the amount of depleted PP2AC as assessed by Western blot (Figure 3d, inset). The selectivity of this assay format was also confirmed by siRNA knockdown of PP2AC isoforms in HeLa cells (Figure S7). Consequently, PSPTide provides a selective measure of relative PP2A catalytic activity in unfractionated cell lysates. Using this approach, we assessed the relative changes in basal PP2A activity across a panel of human carcinoma cell lines. These experiments demonstrated significant modulation in PP2A activity depending on cell type (Figure 4a). Thus, PSPTide provides a means to rapidly assess basal levels of global PP2A activity across different tissue types. Our laboratory is currently pursuing the use of the PSPTide probe to profile global perturbations in PSP activity during disease development and progression.

Pleased that we were able to selectively monitor endogenous PP2A activity in unfractionated cell lysates, we next wanted to determine the magnitude and temporal dynamics of PP2A activity as a result of initiation of a signaling event. Accordingly, we incubated serum starved HepG2 cells with 100 nM insulin for 2, 5, and 10 min and subsequently prepared lysates. Upon assaying these lysates, we observed significant changes in PP2A activity over

time (Figure 4b). Previous work has clearly established that phosphorylation at Y307 on the catalytic domain of PP2A by insulin receptor kinase leads to repression of catalytic activity.⁴¹ This insulin-induced deactivation of PP2A serves to relieve its negative repression of Akt, leading to a productive insulin response.^{15,16,42,43} As expected, we observed a global decrease in PP2A activity upon insulin stimulation, followed by a gradual restoration of enzymatic function. The temporal dynamics of this decrease in PP2A activity is negatively correlated with an increase in Akt phosphorylation as well as phosphorylation of PP2A at Y307 (Figure 4b, inset). Thus, these results provide direct insight into the magnitude as well as temporal dynamics of PP2A enzymatic activity under biologically relevant stimuli.

In conclusion, we have repurposed a methodology for detecting PSK activity in order to provide a new tool to study the chemical biology of PSPs. The availability of a direct activity assay for monitoring global endogenous PP2A activity in unfractionated cell lysates represents a significant step forward to better understand the role of this enzyme in human disease. This technology could be further applied to high-throughput screening of small molecule inhibitors with recombinant phosphatases. Ongoing work in our laboratory is focused on improving the resolution of this assay for specific PSP holoenzymes. Taken together with our previous studies, this work demonstrates the broad applicability of Sox-based probes for monitoring the activity of both protein tyrosine phosphatases as well as PSPs. Coupled with the existing battery of kinase activity probes,^{19–26} one can now envision the investigation of reversible phosphorylation networks over time in disease models.

METHODS

Methods for assays containing endogenous PP2A are described below. Refer to the Supporting Information for details pertaining to general reagents and procedures, synthesis and characterization of sensors, and control experiments.

Carcinoma Cell Culture and Lysate Assay

Each cell line was cultured, and lysates were prepared as described for HeLa cells (see Supporting Information). Total protein (10 μ g) for each cell lysate was assayed with PSPTide (10 μ M) in PSPTide Assay Buffer with either no inhibitor or calyculin A (500 nM) in triplicate. Cell lines were purchased from ATCC (HCT116, CCL-247; HepG2, HB-8065).

HepG2 Cell Culture and Lysate Assay

HepG2 cells were cultured in 15 cm plastic dishes under the same conditions as described above. After reaching 90% confluency, the cells were cultured for 12 h in serum free media (DMEM, Pen-Strep, Anti-Anti, and 2 mM L-glutamine). The cells were then stimulated with 100 nM insulin (Sigma, I9278) for the indicated times. Cells were then lysed, and lysates were prepared as described above. Total protein (10 μ g) from each cell lysate sample was assayed with PSPTide (10 μ M) in PSPTide Assay Buffer with either no inhibitor or calyculin A (500 nM) in triplicate. Data are shown relative to the activity of PP2A from nonstimulated cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A CSox-based PSP activity probe. (a) The CSox residue is placed proximal to the site of phosphorylation on a peptide substrate. In the presence of a phosphate group and Mg^{2+} , CSox undergoes CHEF when irradiated with 360 nm light. Following dephosphorylation by a PSP, the affinity of the peptide for Mg^{2+} is reduced and fluorescence emission at 485 nm decreases. (b) The PSPTide sequence with the site of dephosphorylation highlighted in red and CSox in blue.

Figure 2.

Characterization of PSPTide. Mg^{2+} K_D of PSPTide (a) and the positive control, nonphosphorylated peptide (b). Error bars represent the standard deviation of triplicate experiments. (c) Fold fluorescence increase of PSPTide compared to the nonphosphorylated peptide at 0.5, 1.0, and 1.5 times the K_D of PSPTide for Mg^{2+} . (d) Incubation of PSPTide (10 μM) with 10 μg of total protein from HeLa cell lysates (40 μL reaction volume).

Figure 3.

Selectivity for PP2A. The IC_{50} of calyculin A (a) or fostriecin (b) in HeLa cell lysates (10 μg). (c) PP2A catalytic activity can be resolved through background subtraction with calyculin A. Activity remaining after incubation with calyculin A is due to unknown, off-target activities. (d) PP2AC alpha and beta isoforms were immunodepleted from HeLa cell lysates and assayed (10 μg total protein) with PSPtide (10 μM) using background subtraction with calyculin A (500 nM). The inset shows a Western blot for PP2AC alpha and beta isoforms in the indicated samples. Error bars represent the standard deviation of triplicate experiments; reactions were carried out in 40 μL . ** $p < 0.01$.

Figure 4.

Analysis of endogenous PP2A activity. (a) Basal PP2A activity in a panel of human carcinoma cell line lysates (10 μ g total protein) assessed with PSPtide (10 μ M). The inset shows a Western blot of beta-actin in each sample, demonstrating normalization of protein content. (b) HepG2 cells were stimulated with 100 nM insulin for 2, 5, and 10 min and lysed. Cell lysates (10 μ g total protein) were then assayed for PP2A activity with 10 μ M PSPtide. The inset shows a Western blot of pY307 PP2A, pT308 Akt, and pan Akt. Error bars represent the standard deviation of triplicate experiments; reactions were carried out in 40 μ L. Assays were background subtracted with 500 nM calyculin A. * $p < 0.05$, ** $p < 0.01$.