Published in final edited form as:

Chemistry. 2018 June 04; 24(31): 7810–7824. doi:10.1002/chem.201705194.

Interrogating Protein Phosphatases with Chemical Activity Probes

Garrett R. Casey and Prof. Cliff I. Stains

Stains Department of Chemistry University of Nebraska–Lincoln Lincoln, NE 68588 (USA)

Abstract

Protein phosphatases, while long overlooked, have recently become appreciated as drivers of both normal- and disease-associated signaling events. As a result, the spotlight is now turning towards this enzyme family and efforts geared towards the development of modern chemical tools for studying these enzymes are well underway. This Minireview focuses on the evolution of chemical activity probes, both optical and covalent, for the study of protein phosphatases. Small-molecule probes, global monitoring of phosphatase activity through the use of covalent modifiers, and targeted fluorescence-based activity probes are discussed. We conclude with an overview of open questions in the field and highlight the potential impact of chemical tools for studying protein phosphatases.

Illuminating Protein Phosphatases

Protein phosphatases are becoming recognized as critical signaling enzymes in both normal and disease physiology. Chemical reporters of enzymatic activity are poised to provide insights into the chemical biology of these important signaling enzymes.

The activities of protein phosphatases and protein kinases are responsible for maintaining the phosphoproteome. Although protein kinases have long enjoyed the spotlight, protein phosphatases have more recently been recognized as important signaling enzymes in both normal and disease physiology. This Minireview aims to highlight advances and opportunities for the development of chemical activity probes for protein phosphatases.

Correspondence to: Cliff I. Stains.

The ORCID number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/chem.201705194>.

Conflict of interest

The authors declare no conflict of interest.

Keywords

biosensors; fluorescent probes; phosphatase; sensors; signal transduction

1. Introduction

Protein phosphorylation is one of the most common post-translational modifications (PTMs), with estimates that up to 13,000 phosphorylated proteins and 230,000 phosphorylation sites may exist in the human proteome.^[1] As a consequence, this PTM plays a critical role in aspects of nearly every cellular process. Central to maintenance of the phosphoproteome are the complementary enzymatic activities of protein phosphatases (PPs)^[2] and protein kinases (PKs).^[3] In the case of phosphorylation, PPs act as the erasers and PKs function as writers (Figure 1). PKs have certainly taken center stage in terms of disease relevance and consequently drug development initiatives, with an estimated 25 % of drug development efforts focused on PKs and over 30 FDA-approved compounds with several more in clinical trials.^[4] However, the arguably overlooked erasers of protein phosphorylation are beginning to garner attention as the importance of PPs in cellular signaling and their potential as drug targets has come into focus.^[5]

The history and difficulties associated with PP discovery, recently presented in two riveting accounts,^[6] likely contributes to the persistent view of PPs as second-class signaling enzymes. Interestingly, the first evidence for a PP appears in the literature 10 years prior to the discovery of the first PK, in 1945 seminal work from the Cori lab described a PR (prosthetic group removing) enzyme that was capable of converting active phosphorylase a into inactive phosphorylase b.^[7] It was not until ten years later, when Fischer and Krebs discovered what they termed phosphorylase b kinase,^[8] that the true identity of the PR enzyme as a PP was revealed. Subsequent work on PKs advanced at a relatively more rapid rate compared to PPs, with the discovery of the first multi-step PK activation pathway and eventually the first FDA-approved PK inhibitor, Imatinib.^[9] There are several underlying

reasons for the disparity between advances in understanding PK versus PP chemical biology, some of which are discussed briefly below.

Perhaps most importantly, PPs did not arise from a common ancestor and are instead an example of a class of enzymes that converged on the same enzymatic function using different protein scaffolds as well as regulatory mechanisms for controlling the activity of the resulting enzymes.^[2c, 6, 10] Thus, while PKs share a common catalytic domain and mechanism, PPs are classified based on substrate specificity and catalytic mechanism into protein tyrosine phosphatase (PTP) and protein serine/threonine phosphatase (PSP) families. PTPs constitute the largest family of phosphatase genes and are defined by the conserved catalytic motif CX₅R. Interestingly, the number of PTPs encoded in the human genome (105) is on par with the number of protein tyrosine kinases (90),^[2b] indicating a possible parity in signaling fidelity between these enzyme families. Conversely, PSPs are divided into two major classes, the phosphoprotein phosphatases (PPPs) and metal-dependent protein phosphatases (PPMs). PSPs present a different challenge, compared to PTPs, since the human genome only encodes $\approx$ 30 PSP catalytic domains compared to >400 protein serine/threonine kinase domains.^[2c] Coupled with the lack of substrate specificity displayed by PSP catalytic domains in vitro, PSPs have long been viewed as troublesome housekeeping enzymes that interfere with PK studies. However, more recent efforts have uncovered the complexity of PSP signaling which relies on a vast array of regulatory domains, subunits, and PTMs.^[11] Thus it is now becoming clear that the cell harbours several hundred distinct PSP holoenzyme complexes. Taken together, this work implies that dephosphorylation may be as regulated and specific in space and time as phosphorylation.^[12] In this context, it is worth noting the PKs are not monospecific enzymes and it is now becoming clear that PK activity is also controlled through the use of scaffolding proteins such that not all possible substrates encounter all possible PKs in a given space or time interval.^[13] Readers are directed to several excellent reviews for more in-depth discussions of PP structure, biology, and resources.^[2c, 6b, 11, 14]

With the field now primed with an increased understanding of PP structure and regulation, the stage is set for chemical biologists to assist in uncovering the roles of PPs in cellular signaling through the development and application of chemical tools. This too is another area where PPs have lagged behind their PK counterparts. Partially due to issues discussed above, as well as the inherent analytical difficulty in detecting removal of a phosphate, a negative signal, that must be present before a PP can act. This minireview aims to highlight advances and opportunities for the development of chemical tools for the analysis of PP activity. Classical approaches, such as radiometric assays, have been highlighted elsewhere.^[15] First, we will highlight small-molecule reporters of PP activity, followed by a discussion of probes for global analysis of PP activity, and a review of targeted probes for monitoring PP activity. We conclude with perspectives for future opportunities for chemical biologists in this rapidly developing area.

2. Small-Molecule Reporters

Small-molecule-based reporter assays for PPs represent an attractive approach for monitoring PP activity. While these sensors lack selectivity in the context of complex

mixtures such as biological samples, they have clear utility in in vitro biochemical studies as well as high-throughput screening (HTS) efforts. Moreover, these scaffolds can provide inspiration for targeted approaches. Below, we discuss colorimetric, fluorescent, and luminescent probes.

2.1 Colorimetric probes

Arguably the most widely used small-molecule substrate for PPs is *p*-nitrophenylphosphate (pNPP, **1**, Figure 2), which provides a rapid colorimetric assay for the determination of enzymatic activity.^[15c, 16] The hydrolysis of pNPP by PPs results in the release of an inorganic phosphate and formation of *p*-nitro-phenol. This product is readily converted to colored *p*-nitro-phenolate in alkaline conditions, necessitating a discontinuous assay format for PPs that operate at neutral pH. The generic nature of pNPP continues to enable HTS efforts to identify PP inhibitors,^[5g] as well as PP substrates.^[17]

Another popular phosphatase activity reporter is the Malachite Green (MG) assay (**2**, Figure 2).^[15c, 16a, 18] Unlike pNPP, this assay format provides a general readout of free phosphate in solution. Under acidic conditions, free phosphate and MG form a green, phosphomolybdate-MG complex, allowing for quantification down to picomolar amounts of phosphate. Although the MG assay is simple and sensitive, detection is discontinuous and care must be taken to avoid sources of off-target phosphate in the assay.

Lastly, BCIP (5-bromo-4-chloro-3-indolyl-phosphate) is commonly used in conjunction with NBT (nitro blue tetrazolium) to produce a blue/purple precipitate following dephosphorylation by a PP (Figure 2).^[19] When BCIP (**3a**) is dephosphorylated, the resulting intermediate dimerizes to form a purple precipitate while NBT (**3b**) is concomitantly reduced, forming a complementary blue precipitate. This colorimetric assay is often used to detect alkaline phosphatase (ALP) activity in ELISA-based assays.

2.2 Initial fluorogenic probe designs

To address issues associated with assay sensitivity, researchers turned to fluorogenic PP substrates such as 4-methylumbelliferone phosphate (4-MUP, **4**, Figure 3).^[20] 4-MUP, which is initially non-fluorescent, is converted to the highly fluorescent product (4-MU) by dephosphorylation under alkaline pH conditions. Since the pK_a of the hydroxyl group at position 7 of 4-MU is 7.9, this probe has limited use at lower pHs (such as pH 4–6, the optimal range of acid phosphatases). To modulate the pK_a of 4-MU, Haugland and co-workers installed fluorine atoms at the 6- and 8-positions, yielding the PP substrate 6,8-difluoromethylumbelliferyl phosphate (DiFMUP, **5**, Figure 3).^[21] Importantly, the resulting dephosphorylation product of DiFMUP displays a pK_a of 4.7, providing a working PP assay over a pH range of 5–8. Coupled with the improved affinity of DiFMUP ($K_M = 5 \mu M$ for PTP1B) compared to pNPP ($K_M = 380 \mu M$ for PTP1B) for PPs, DiFMUP has become a staple for small-molecule PP assays and continues to prove advantageous in various applications.^[5d, 16b, 22] Furthermore, 4-MUP and DiFMUP can provide continuous readouts of PP activity in solution, greatly simplifying mechanistic studies of PPs.

FDP (3,6-fluorescein diphosphate (**6**, Figure 3) incorporates many of the desirable properties of the above-mentioned reporters.^[23] FDP can be used to monitor phosphatase activity over a broad pH range from 5–9, using absorbance or fluorescence, and can be utilized in both continuous and discontinuous formats. FDP contains two identical phosphate groups that can be acted upon by PPs. Hydrolysis of a single phosphate group leads to the formation of fluorescein monophosphate (FMP), resulting in an increase in absorbance at 445 nm. Removal of the remaining phosphate results in formation of fluorescein and a shift in absorbance to 490 nm.

Red-shifted reporters of PP activity have also been described. For example, dephosphorylation of DDAO-P (**7**, Figure 3) results in a red fluorescent product, with emission at 660 nm using 645 nm excitation.^[24] However, one limitation of DDAO-P is that it is weakly fluorescent prior to dephosphorylation which can complicate assay analysis. Nevertheless, DDAO-P has a relatively high turnover rate, good solubility, broad range of pH operation, and is suitable for HTS applications.

Numerous fluorogenic reporters that take advantage of the optimal pH range of ALP activity have been described.^[25] These reporters are particularly attractive for cell microscopy as well as flow cytometry applications. Unlike immunohistochemistry, fluorescence signal in these assays arises from not only the presence of enzyme but also its activity. As an example, the commercially available enzyme-labeled fluorescence-97 (ELF-97, **8**, Figure 3) substrate developed by Singer and coworkers, is water-soluble and non-fluorescent in its phosphorylated state.^[26] Upon dephosphorylation by alkaline phosphatase, ELF-97 forms a fluorescent, insoluble precipitate. Since precipitation occurs at the site of phosphatase activity, fluorescence can be correlated to the location of activity within the cell. In addition, ELF-97 and similar sensors can be used in flow cytometry to sort cells with varying ALP activities. However, ELF-97 is not cell permeable, which necessitates the use of permeabilized cells.

2.3 Next-generation fluorogenic probes

Compared to colorimetric sensors, early fluorogenic probes provide improved sensitivity and broader working pH ranges. However, challenges associated with PP selectivity, cell permeability, and detection wavelengths hindered applications in complex biological samples. Next generation fluorescent probes have been developed to address these issues and, in certain cases, allow for the detection of PP activity in heterogeneous samples.

Building upon the early probe designs described in Section 2.2, recent efforts have focused on increasing sensitivity and cell permeability by incorporating new functionality. For example, Nagano and co-workers have developed a series of ratiometric, FRET-based probes that incorporate a coumarin donor linked through a rigid cyclohexane moiety to a 3',6'-O-protected fluorescein acceptor (**9**, Figure 4).^[27] The authors demonstrated the capability of two PTPs, PTP1B and CD45, to act on **9** in vitro with significantly improved kinetics compared to pNPP. Furthermore, it was shown that a derivatized probe containing acetoxymethyl groups appended to the phosphate and hydroxyl functionalities of **9** was more lipophilic and thus cell permeable, enabling the assessment of intracellular PTP activity in human umbilical vein endothelial cells. Selectivity for PTP activity was demonstrated using

sodium orthovanadate, a cell permeable PTP inhibitor. This strategy represents an improvement over previous probe designs, affording a sensor that can function in living cells and provide a ratiometric readout of PTP activity. The addition of acetoxymethyl moieties to improve the cell permeability of **9** represents an important step forward in the analysis of cellular PP activity. This approach could likely be applied to several sensors discussed herein.

Following this work, Nagano and co-workers developed a series of fluorescein derivatives known as Tokyo Green, by installing methyl and methoxy groups on the benzene ring of fluorescein.^[28] This new fluorophore scaffold was subsequently used for the creation of two PP reporters, TG-Phos and TG-mPhos (**10a** and **10b**, Figure 4).^[29] These two turn-on reporters were shown to be efficient substrates for ALP. However, while both **10 a** and **10b** showed comparable affinity for ALP ($K_M = 0.96$ and $0.77 \mu\text{M}$, respectively), **10 a** could also act as a substrate for PTP1B and PP1a while **10 b** was found to be selective for ALP in vitro among a panel of enzymes.^[29b]

2.3.1 ALP-targeted fluorogenic probes—Several turn-on (**11**,^[30] **12**,^[31] **13**,^[32] and **14**,^[33] Figure 4) as well as ratiometric (**15**,^[34] **16**,^[35] **17**,^[36] and **18**,^[37] Figure 4) probes for ALP have been described. For example, **11**, was used to monitor endogenous ALP activity in real-time in HeLa cells. Probe **12**, which utilizes excited-state intramolecular proton transfer (ESIPT), can be used in similar applications. Alternatively, a series of probes, **13 a–d**, have validated the use of aggregation-induced emission (AIE) as a viable reporting method for ALP activity. Interestingly, **14** is a latent turn-on probe that features an self-immolative moiety for fluorescence activation. Probe **15** was used to demonstrate the potential of small molecule PP probes in pharmacology studies. In this work, drug-induced organ damage was initiated by acetaminophen in zebra fish larvae and **15** was used to discover spatially increased ALP activity. The intramolecular-charge transfer (ICT)-based probes **16** and **18** were used to image ALP activity in HeLa cells, expanding the range of reporting strategies available for PP probes. In addition, **17** utilizes both ESIPT and AIE to allow for visualization of ALP activity in living cells. Although clearly powerful, the off-target reactivity of these ALP probes against the phosphatome remains unclear and may complicate the analysis of fluorescent readouts in heterogenous biological samples containing numerous PPs.

2.3.2 Progress towards selective fluorogenic probes—Although clear progress has been made in the development of ALP probes, relatively less attention has been focused on the remaining PPs. Nonetheless, examples of selective probes for other PPs have been described.

For example, Kim and co-workers utilized the 2-(2'-hydroxy-phenyl)benzothiazole (HBT) fluorophore as a scaffold for the development of PTP probes, capitalizing on ESIPT observed in this fluorophore.^[38] Upon excitation, the enol form of HBT is rapidly converted to the keto form, resulting in a large emission shift. Installation of a phosphate on HBT inhibits the ESIPT process (**19**, Figure 4). Following enzymatic dephosphorylation, the potential for ESIPT is restored. Interestingly, this reporter was screened against a panel of 26 PTPs and remarkable selectivity for MKP-6 was observed. These results indicate that small-

molecule substrates can display varying degrees of selectivity for PPs, pointing to the need for careful characterization before these probes are employed in heterogeneous samples.

Following up on the MKP-6 reporter **19**, Kim and co-workers further investigated a iminocoumarin-based scaffold (**20**, Figure 4).^[39] The resulting probe, 9-(dicyanovinyl)julolidine (DCVJ), is dark until dephosphorylation-induced production of an iminocoumarin product. The authors compared the selectivity of **20** and DiFMUP against a panel of 12 classical PTPs as well as 12 dual-specificity phosphatases (DUSPs). DiFMUP showed little selectivity among the PTPs or DUSPs. However, **20** was selective for six of the DUSPs tested (CDC14B, DUSP13A, 13B, 14, 15, and 16) with less than 20 % relative reactivity for the classical PTP, PTPRR.^[39] Ultimately, this novel iminocoumarin probe has been demonstrated to be a useful tool for selectively monitoring DUSP activity over other classes of PPs.

Another small-molecule reporter selective for monitoring DUSP activity is the Seoul-Fluor-based reporter, **21** (Figure 4).^[40] Enzymatic cleavage of the P–O bond results in a free phenol and a reduction of fluorescence through a photoinduced electron transfer (PET) mechanism. Subsequent deprotonation under basic conditions leads to a turn-off signal. To test the selectivity of **21**, a screen against 35 DUSPs and 30 classical PTP's was conducted. Initial screening at pH 8.0 demonstrated reactivity with DUSP-14, DUSP-13b, and vaccinia H1-related phosphatase (VHR, also known as DUSP-3) at enzyme concentrations of 1 μ M. However, at lower enzyme concentration (0.1 μ M), the selectivity of **21** was enhanced for VHR over DUSP's 14 and 13b. Furthermore, it was determined that while each of the three DUSP's had comparable K_M values, the k_{cat} values were significantly different, indicating that **21** was turned over more efficiently by VHR under optimal assay conditions.

Barrios et. al have also developed a pair of class selective small-molecule reporters (**22a**, **22b**, Figure 4) for PTPs based on the resorufin scaffold.^[41] Addition of the phosphate group quenches resorufin fluorescence, which is restored upon dephosphorylation by a PTP. Interestingly, both probes display comparable catalytic efficiencies across a panel of PTPs, including PTP1B, TCPTP, CD45, VHR, and YopH. Consequently, these probes were used to provide a generic readout of PTP activity in mammalian cells. In particular, the red-shifted emission of these probes provides significant advantages for cellular imaging studies. The colorimetric change upon dephosphorylation of these probes was also leveraged to visualize the presence of pathogenic bacteria possessing PTP activity.

An alternate approach for the generation of PP probes has been described by Yao and co-workers.^[42] Specifically, attachment of phosphate to the two-photon fluorescent centrosymmetric dye, 2-hydroxy-4,6-bis(4-hydroxystyryl)pyrimidine (**23**, Figure 4), was shown to quench fluorescence. Further addition of a photolabile *ortho*-nitrobenzyl caging group on the phosphate moieties provides spatial and temporal control over the activation of **23**. Subsequent appendage to a cell penetrating peptide (CPP) with preferential affinity to specific organelles, allowed for the visualization of localized PP activity in living cells and animal tissues using two-photon excitation at 800 nm. Further investigation of selectivity elements in this probe design could allow for spatial analysis of individual PP activities in biological systems.

2.4 Luminescence-based probes

A subset of small-molecule probes utilizing chemiluminescence to report on ALP activity have been described. These probes provide an important analytical advance, given the decreased background of luminescence-based assays compared to fluorescence-based approaches. Indeed, the detection limit of these probes often surpasses that of fluorescence-based ALP sensors.

For example, a variety of adamantyl-1,2-dioxetane derivatives (**24**, Figure 5) have been developed and are commercially available.^[43] In general, these probes are activated by enzymatic cleavage of the phosphate group in the presence of ALP. Following dephosphorylation, **24** becomes destabilized and the 1,2-dioxetane ring decomposes to afford two carbonyl products. The resulting methyl-2-oxybenzoate anion (Figure 5) emits between 470 and 477 nm and allows for the detection of ALP activity in the femtomolar range in solution.

Complementary efforts have focused on utilizing firefly luciferase and its substrate, D-luciferin, as a probe for PP activity. Three general probe designs based on D-luciferin analogues have been developed. The first of these probes was D-luciferin-*O*-phosphate, **25** (Figure 5), which contains a phosphate group appended to the D-luciferin scaffold in order to block its interaction with luciferase.^[44] When acted upon by ALP, the phosphate group is removed and in the presence of luciferase, ATP, and Mg^{2+} , the resulting D-luciferin is converted to its emissive form (550 nm). Importantly, the light produced in this assay is proportional to ALP activity. The K_M of ALP for **25** was reported to be 43 μM with a limit of detection of 10^{-19} mol.

Zhou and co-workers have also reported two novel D-luciferin derivatives with self-immolative functionalities for detecting ALP.^[45] The first of these reporters, **26** (Figure 5), implements a trimethyl lock^[46] within a phenol propionic moiety linked to aminoluciferin. The phosphate group prevents spontaneous decomposition of the probe. However, removal of the phosphate by ALP results in a spontaneous intramolecular cyclization to form aminoluciferin. The resulting luminescence signal at 594 nm is proportional to ALP activity. Using an alternative quinone methide-based strategy, **27** (Figure 5) undergoes spontaneous decomposition following dephosphorylation by ALP to afford D-luciferin. Interestingly, **27** displayed improved binding compared to **25**, with a $K_M = 340$ nM, as well as an improved limit of detection of 10^{-22} mol.

2.5 Summary

Small-molecule reporters are clearly useful tools for monitoring PP activity in purified samples and, in certain cases, complex environments such as cells. However, significant challenges remain with respect to selective analysis of PP activity in heterogeneous samples. Thus, the field has begun to press forward towards the realization of reporters capable of global and/or targeted analysis of PP activity in living systems. These approaches utilize covalent labeling of active site residues as a proxy for activity (Section 3) or protein- or peptide-based recognition elements to enable targeted analysis of PP activity (Section 4).

3. Global Analysis of PP Activity

The Cravatt laboratory has developed an elegant approach for globally profiling the activity of enzymes termed, activity-based protein profiling or ABPP.^[47] In general, activity-based probes (ABPs) are designed to label an enzyme class/family through covalent modification of nucleophilic residues within or adjacent to an enzyme active site. Thus, the extent of labeling can be used as a proxy for enzyme activity. This approach is compatible with complex biological samples such as cell lysates and labeling can be analyzed using several outputs including mass spectrometry and in-gel fluorescence (Figure 6a). Importantly, this method provides a powerful readout of the functional state of a target enzyme class under normal or disease conditions.^[48] ABPs consist of three fundamental elements: 1) the reactive “warhead” (Figure 6b), 2) a visualization tag or affinity handle (Figure 6c), and 3) a linker connecting the reactive moiety to the tag or handle. The reactive warhead, is generally an electrophilic group chosen to selectively react with a nucleophile in the target enzyme class. Linkers can consist of a flexible spacer (e.g. alkyl chain, PEG) or can provide additional selectivity if peptides or natural products are employed. This modular design approach has been utilized to afford ABPs that target serine hydrolases,^[47] cysteine proteases,^[49] and protein kinases^[50] among others.

3.1 PSP-targeted ABPs

In the context of protein phosphatases, ABPs have been largely based upon covalent inhibitors or pTyr mimics that selectively label PTPs, PSPs, or both. The majority of ABPs designed for PPs target residues that are either essential for catalysis or are adjacent to the active site (examples of labeling mechanisms in the context of PTPs are shown in Figure 7). As an example of labeling active site adjacent residues in PSPs, the natural product toxin microcystin-LR, a known PSP inhibitor, was chosen as the scaffold for the design of an ABP termed AX7574 (**28**, Figure 8).^[51] AX7574, which irreversibly modifies PSPs (PP1, PP2A, PP4, and PP6) through reaction with a non-catalytic cysteine with the methylene present in AX7574, can be used to monitor PSP activity in cell lysates.

3.2 Initial ABPs for PTPs

PTPs have received relatively more attention in the context of ABP development due to the conserved active site cysteine nucleophile in these enzymes. The first example of a class-selective ABP for PTPs was a FMPP-based probe developed by Lo et al. (**29**, Figure 8).^[52] Probe **29** contains a pTyr mimic with a proximal fluorine atom to allow for labeling of PTPs. Following dephosphorylation by a PTP, a subsequent 1,6-elimination of fluorine occurs, forming a highly electrophilic quinone methide. This reactive intermediate is then captured by an adjacent nucleophile, forming a covalent linkage with the target enzyme (Figure 7a). Attachment to a reporter such as dansyl or biotin, allows for visualization of PTP labeling. This probe was found to be selective for PTP1B compared to trypsin, albumin, ovalbumin, β -galactosidase, carbonic anhydrase, and phosphorylase b.

A similar pair of probes, utilizing the quinone methide trapping strategy, were developed by Yao and co-workers (**30**, **31**, Figure 8).^[53] In particular, probe **31** employed 2-difluoromethyl-phenyl phosphate (DFFP), which provides a latent *ortho* quinone methide

(Figure 7a). The reactivity of **30** and **31** were further tested against a small panel of enzymes consisting of proteases, lipases, and phosphatases, demonstrating selectivity for ALP and PTPs. Shen et al. further extended the use of the DFFP-containing design by introducing a small peptide substrate sequence for PTP1B as well as the nonhydrolyzable pTyr mimic known as F₂PmP (**32**, Figure 8).^[54] The resulting ABP was shown to be more selective for PTP1B compared to PTP4A3. Furthermore, Jurkat cell lysates treated with **32** produced strong labeling of a protein whose molecular weight corresponded to PTP1B, demonstrating the potential to use this probe in heterogeneous mixtures. However, off-target labeling was observed via western blotting, potentially due to nonspecific labeling by the reactive quinone methide.

Zhang and co-workers sought to further improve selectivity for PTPs by implementing an α -bromobenzylphosphonate (BBP, Figure 6b) warhead.^[55] This pTyr mimic acts as an irreversible mechanism-based inhibitor for PTPs (Figure 7b). The initial ABP design involved conjugation of BBP to a biotin affinity handle (**33 a**, **33 b**, Figure 8). These probes demonstrated labeling across a panel of 11 PTPs. The selectivity of this design was assessed using cross-reactivity studies with acid, alkaline, and serine/threonine phosphatase families as well as a diverse set of proteins including chymotrypsin, trypsin, BSA, lysozyme, papain, and G-3P dehydrogenase, many of which contain a reactive cysteine residue. Impressively, BBP-based probes only labeled PTPs in this panel. With these selective probes in-hand, a global analysis of PTP activity in a normal versus breast cancer cell line was performed. Interestingly, labeling profiles clearly indicated differences in catalytic activity of four PTPs, supporting the hypothesis of altered PTP activity in cancer. Expanding on this design, Zhang and co-workers developed a fluorescent analogue of their ABP containing a rhodamine fluorophore (**33c**, Figure 8).^[56] This new probe retained selectivity for PTPs while improving sensitivity by over 1000-fold. In-gel fluorescence scanning of various cancer cell lysates (breast, lung, liver, colon, ovary, and cervix) treated with **33c** demonstrated dramatic changes in global PTP activity across the panel. Importantly, H₂O₂-mediated inactivation of PTPs can be monitored using this approach, allowing for the study of H₂O₂-mediated PTP signaling events.

Despite the powerful insights provided by the BBP-based probes, these warheads are susceptible to solvolysis above pH 7.0 and display limited membrane permeability.^[57] The phenylvinyl sulfonate and sulfone (Figures 6b and 7c) chemotypes have been developed as ABPs to address these issues (**34**, Figure 8).^[57] The neutral character of **34** facilitates cell permeability and labeling in intact cells. Incorporation of an azide component allows for two-step labeling via “click chemistry” after cell lysis followed by analysis of tagged PTPs. This new ABP class provides a tool for the investigation of PTP activity in living cells.

3.3 Next-generation ABPs for PTPs

Seeking to develop a targeted ABP for PTPs, Yao and co-workers employed a novel pTyr mimic termed 2FMPT (Figures 6b and 7a) as the ABP warhead, flanked by N'- and C'-terminal peptide recognition elements.^[58] The incorporation of peptide-based recognition elements allows for target-specific profiling of PTPs in cell lysates. For example, a library of 10 distinct probe sequences were screened against SHP1, SHP2, PTP1B, TCPTP, and

LMWPTP. Although different peptide sequences showed varying degrees of cross-reactivity between PTPs, **35** (Figure 8) demonstrated excellent selectivity for PTP1B. Nonetheless, in-gel fluorescence analysis of mammalian cell lysates showed potential off-target labeling of unknown enzymes, reinforcing the difficulty in selectively targeting PTPs with peptide sequences.

Quenched activity-based probes (qABPs) have been developed by Bogoy and co-workers as fluorescence “turn-on” reporters of protease activity.^[59] Unlike their traditional fluorescent ABP counterparts, which are always “on”, qABPs incorporate a mechanism-based activation moiety that results in an increase in fluorescence in real-time upon cleavage by an enzyme. Building on this concept, Yao and co-workers have designed a series of modular qABP’s utilizing latent quinone methide chemistry for use in live-cell imaging.^[60] As shown in Figure 8, the general probe scaffold (**36**) includes a caged pTyr “warhead”, a fluorescence reporter, and a dabcyI quencher. These probes are modular and can be further elaborated with biotin for affinity purification/labeling or with CPPs to facilitate cellular uptake as desired. Importantly, the *ortho*-nitrobenzyl cage, allows for temporally controlled release of the probe in living cells. Enzymatic removal of the phosphate group results in a 1,6-elimination of the dabcyI quencher, leading to an increase or “turn-on” in fluorescence of the appended fluorophore. The resulting quinone methide is reactive to nucleophilic attack, allowing for fluorescent labeling of PTP active sites (Figure 7d). A next-generation probe developed by the Yao group (**37**, Figure 8) leveraged a unique pTyr mimic in the form of a caged phosphocoumarin analogue.^[61] This reporter is dark prior to decaging, dephosphorylation by a PTP, and subsequent PTP labeling through formation of an *ortho*-quinone (Figure 7e). Probe **37** was appended to a non-arginine rich CPP sequence to improve cellular uptake. Varying CPP sequences allowed for differential cellular localization of the probe, enabling analysis of PTP activity in different cellular compartments.

3.4 Summary

ABPP clearly represents a powerful and general technique for globally assessing the relative amounts of catalytically competent enzymes using covalent labeling as a proxy for enzymatic activity. While early ABP designs for PPs suffered from various limitations (e.g. off-target reactivity, stability, and cell permeability), more recent ABPs utilize innovative approaches to overcome these obstacles. Importantly, these tools can provide insight into endogenous PP activity upon treatment with biologically relevant stimuli as well as parse signaling states in normal versus diseased samples. A remaining challenge for the field is the lack of ABPs for dissecting PSP activity in living systems. Such tools could enrich our understanding of the role of this enzyme class in cellular signaling events. While ABPP is a useful tool for profiling global PP activity, the incorporation of recognition elements can also allow for targeted analysis of PP activity. As a complementary approach, fluorescence-based probes have been developed in order to assess endogenous PP activity in real-time. These reporters provide a direct readout of catalytic activity using an easily observable signal.

4. Targeted Fluorescence-Based Activity Sensors

4.1 Introduction

Targeted activity sensors provide a selective readout of one PP, allowing for analysis of its role in cell signaling and disease pathology. The key design feature, and arguably the most challenging aspect in constructing a targeted PP probe, is the identification of a selective recognition element for the PP of interest. Despite difficulties with design, when available, targeted activity probes can provide valuable information concerning biologically-relevant PP signaling events.

4.2 Selective probes for PPs

The first targeted PP activity sensors were described by Barrios and co-workers.^[62] Using a phosphorylated coumaryl amino acid (pCAP, **38**, Figure 9) as pTyr mimic, a number of peptide-based probes were designed for monitoring PTP activity in vitro. Dephosphorylation of pCAP by a PTP leads to a strong increase in fluorescence ($\lambda_{\text{ex}} = 334 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$). The resulting pCAP-based sensors have been employed to monitor PTP activity in living cells, in campaigns to identify PTP inhibitors, and in efforts to develop optimal substrate sequences for PTPs.^[63]

The Zhang laboratory has developed genetically encodable PP activity probes by leveraging conformational changes upon dephosphorylation of a protein-based substrate in order to modulate FRET efficiency between appended fluorescent proteins. The first example was the construction of a probe for the Ca^{2+} and calmodulin-dependent PSP calcineurin, also known as PP2B.^[64] This sensor, termed calcineurin activity reporter (CaNAR, **39**, Figure 9), was composed of the N-terminal 297 amino acid region of nuclear factor of activated T-cells (NFAT) a native substrate of PP2B. As shown in Figure 9, the NFAT substrate sequence is “sandwiched” between cyan and yellow fluorescent proteins (CFP and YFP), which serve as the FRET donor and acceptor, respectively. The N-terminal domain of NFAT contains multiple serine-rich sites that are constitutively hyperphosphorylated by PKs. In the phosphorylated state, the negative charges of the phosphorylated residues interact with the positive charges within a pendant regulatory domain. Thus, dephosphorylation produces a conformational change in the probe, leading to an increase in FRET. CaNAR was capable of monitoring drug-induced dephosphorylation catalyzed by PP2B in living HeLa cells. The genetically encodable nature of these probes allows for straightforward cellular delivery and potential localization to subcellular compartments.

The Lawrence lab constructed a targeted sensor based on the autophosphorylation sequence ($\text{N}'\text{-ARDI-pY-RASYRKG-C}'$) of anaplastic lymphoma kinase (ALK).^[65] The serine residue was replaced by an unnatural amino acid, 2,3-diaminopropionic acid (Dap), appended to a pyrene fluorophore. This new probe (**40**, Figure 9) operates through a tyrosine-induced dynamic quenching mechanism. The phosphorylated sensor exhibits strong fluorescence ($\lambda_{\text{ex}} = 343 \text{ nm}$, $\lambda_{\text{em}} = 378 \text{ nm}$), while de-phosphorylation by a PP such as YOP results in a decrease in fluorescence. It is interesting to note that this approach was originally developed to detect protein tyrosine kinase activity.^[66] Indeed, the idea of repurposing strategies to detect PK activity may afford a means of generating targeted probes for PPs.

Building upon the idea of repurposing kinase activity assays for the detection of PP activity, our laboratory has recently demonstrated that the phosphorylation-sensitive sulfonamido-oxine (Sox, $\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 485 \text{ nm}$) fluorophore, originally reported by Imperiali and co-workers for use as a kinase activity sensor,^[67] can be repurposed for the detection of PP activity.^[68] In this strategy, phosphorylated Sox-containing peptides are synthesized and produce fluorescence through chelation-enhanced fluorescence (CHEF) with magnesium. Upon dephosphorylation, fluorescence is reduced at a rate that is proportional to PP activity (**41**, Figure 9). Our first proof-of-principle probe termed, PTP1Btide-pS3 (N'-ARDI-pY-R-CSox-FFRKG-C'), was based on the autophosphorylation sequence of ALK, a known substrate for PTP1B.^[69] The detection limit of **41** for PTP1B ($K_M = 25.1 \mu\text{M}$, $k_{\text{cat}} = 4.80 \text{ s}^{-1}$) was determined to be 12 pg vs. 3 ng for pNPP, demonstrating the improved parameters achievable by addition of secondary recognition elements. Interestingly, the PTP1Btide-pS3 probe was found to be remarkably selective for PTP1B, displaying weak activity against TCPTP and essentially no activity against SHP1, SHP2, or PTP-PEST. Indeed, analysis of kinetic parameters indicated that PTP1Btide-pS3 was an 8-fold more efficient substrate for PTP1B compared to TCPTP ($K_M = 4.6 \mu\text{M}$, $k_{\text{cat}} = 0.11 \text{ s}^{-1}$). The PTP1Btide-pS3 probe contains residues that are well-known to be disfavoured in highly efficient PTP1B substrates, thus our results indicate that selectivity in the context of peptide substrates for PTPs may be achieved by utilizing nonoptimized substrates that suppresses the activity of off-target enzymes while preserving turnover by the target PTP. To address the selectivity of PTP1Btide-pS3 in the cellular phosphatome, lysates were generated from MEF PTP1B^{+/+} and MEF PTP1B^{-/-} cell lines.^[70] As expected, MEF PTP1B^{+/+} lysates generated higher dephosphorylation activity than MEF PTP1B^{-/-} lysates, however an appreciable amount of unidentified off-target activity was observed in the MEF PTP1B^{-/-} lysates. To overcome this issue and enable further biological studies, an antibody-based pull-down approach was used to enrich PTP1B from lysates. Utilizing this strategy, the temporal dynamics of PTP1B activity upon insulin stimulation of HepG2 cells was investigated. These experiments demonstrated a rapid reduction (33 %) in the activity of PTP1B 2 min after insulin stimulation, correlating with previous literature data.^[71] Building upon these experiments, we investigated potential perturbations in PTP1B activity associated with non-alcoholic fatty liver disease (NAFLD), a metabolic disorder that afflicts 20–30 % of the US population and increases patient risk for development of more aggressive disease states such as liver cancer.^[72] Homogenates from tissue samples of a rat model of NAFLD were obtained, and demonstrated a 90 % increase in PTP1B catalytic activity that was not fully accounted for by the 58 % increase in expression of PTP1B in NAFLD animals. These experiments provide direct evidence for increased PTP1B activity in an animal model of NAFLD and support the use of direct activity assays to profile PTP activity as opposed to indirect proxies such as western blotting. Current efforts are focused on the development of more selective assays to allow for the analysis of PTP activity in unfractionated cell lysates.

To investigate the generality of our approach, we recently designed a sensor capable of reporting on PSP activity (**42**, Figure 9).^[73] This probe, termed PSPTide (Ac-DRRV-pS-V-CSox-NH₂), is based off of the RII subunit of cAMP-dependent protein kinase A (PKA). Validation experiments using a panel of inhibitors with varying selectivity profiles indicated that the majority of the dephosphorylation of PSPTide in unfractionated cell lysates

could be attributed to PP2A. Background subtraction of off-target activity using calyculin A allowed for resolution of PP2A activity in unfractionated lysates, which was verified using both siRNA knockdown and immunodepletions. With this validated assay in-hand, basal levels of PP2A activity in a small panel of human carcinoma cell lines was assessed, demonstrating a significant modulation of PP2A activity across tumor types. Lastly, the temporal dynamics of PP2A activity during insulin stimulation of HepG2 cells was investigated. These experiments were used to correlate direct measurements of PP2A activity with phosphorylation levels at Y307 of PP2A (a negative regulator of PP2A catalytic activity)^[74] and T308 of Akt, which serves to active this downstream mediator of insulin signaling.^[75] Insulin stimulation led to a rapid global decrease (within 2 min) in PP2A activity followed by a gradual return to basal level activity over 10 min. This decrease in PP2A activity was correlated with increased phosphorylation of Y307 in PP2A and T308 in Akt. These experiments provide insight into the carefully tuned dynamics of protein phosphorylation which are maintained by both PP and PKs. Ongoing work is focused on improving assay selectivity for PSP holoenzyme complexes.

4.3 Summary

To date, four different reporter strategies have been utilized to afford selective PP sensors. Perhaps the largest obstacle hindering progress in this area is the discovery of recognition elements to drive probe selectivity. Nevertheless, the limited examples of targeted PP activity sensors point to the promise of uncovering the temporal and spatial dynamics of PP signaling in biologically relevant samples. Thus, we expect that continued work in this area will expand the field's appreciation for the fundamental role of PPs in cell signaling.

5. Outlook

Although the study of PPs has arguably been more challenging than PKs, it is now clear that significant advances have been made in terms of the development of chemical tools to study PPs. The stage is now set for the utilization of these new tools to address fundamental questions concerning PP chemical biology. Such as, what extracellular factors do PPs respond to? What are the intensities and temporal dynamics of PP activities during stimulation and how does this correlate to PK activities? How do localized PP activities change during stimulation? As the role of PPs in cell signaling becomes more defined, several critical hurdles for sensor design remain. For example, are there general design principles that allow for the construction of targeted probes for PPs? How could one target individual PSP holoenzymes in living cells? How can PP activities in individual cellular compartments be monitored? Thus, while PPs have lived in the shadows of their PK counterparts, we predict a bright future ahead for PPs and look forward to the continuing contributions of chemical biology in this emerging area of cell signaling.

Acknowledgments

We thank members of the Stains lab for helpful discussions and proofreading. This work was funded by the NIH (R35GM119751). The content of this work is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

References

1. Vlastaridis P, Kyriakidou P, Chaliotis A, Van de Peer Y, Oliver SG, Amoutzias GD. *GigaScience*. 2017; 6:1–11.
2. a) Alonso A, Pulido R. *FEBS J*. 2016; 283:1404–1429. [PubMed: 26573778] b) Alonso A, Sasin J, Bottini N, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, Mustelin T. *Cell*. 2004; 117:699–711. [PubMed: 15186772] c) Chen MJ, Dixon JE, Manning G. *Sci Signaling*. 2017; 10:eaag1796.
3. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. *Science*. 2002; 298:1912–1934. [PubMed: 12471243]
4. Cohen P. *Nat Rev Drug Discovery*. 2002; 1:309–315. [PubMed: 12120282]
5. a) Chen YNP, LaMarche MJ, Chan H, Fekkes P, Garcia-Fortanet J, Acker MG, Antonakos B, Chen C, Chen Z, Cooke VG, Dobson JR, Deng Z, Fei F, Firestone B, Fodor M, Fridrich C, Gao H, Grunenfelder D, Hao HX, Jacob J, Ho S, Hsiao K, Kang ZB, Karki R, Kato M, Larrow J, Bonte LR, Lenoir F, Liu G, Liu S, Majumdar D, Meyer MJ, Palermo M, Perez L, Pu M, Price E, Quinn C, Shakya S, Shultz MD, Slisz J, Venkatesan K, Wang P, Warmuth M, Williams S, Yang G, Yuan J, Zhang JH, Zhu P, Ramsey T, Keen NJ, Sellers WR, Stams T, Fortin PD. *Nature*. 2016; 535:148–152. [PubMed: 27362227] b) Garcia Fortanet J, Chen C, Chen Y-NP, Chen Z, Deng Z, Firestone B, Fekkes P, Fodor M, Fortin PD, Fridrich C, Grunenfelder D, Ho S, Kang ZB, Karki R, Kato M, Keen N, LaBonte LR, Larrow J, Lenoir F, Liu G, Liu S, Lombardo F, Majumdar D, Meyer MJ, Palermo M, Perez L, Pu M, Ramsey T, Sellers WR, Shultz MD, Stams T, Towler C, Wang P, Williams SL, Zhang J-H, LaMarche MJ. *J Med Chem*. 2016; 59:7773–7782. [PubMed: 27347692] c) He Y, Liu S, Menon A, Stanford S, Oppong E, Gunawan AM, Wu L, Wu DJ, Barrios AM, Bottini N, Cato AC, Zhang ZYY. *J Med Chem*. 2013; 56:4990–5008. [PubMed: 23713581] d) Karver MR, Krishnamurthy D, Kulkarni RA, Bottini N, Barrios AM. *J Med Chem*. 2009; 52:6912–6918. [PubMed: 19888762] e) Krishnan N, Koveal D, Miller DH, Xue B, Akshinthala S, Kragelj J, Jensen M, Gauss CM, Page R, Blackledge M, Muthuswamy SK, Peti W, Tonks NK. *Nat Chem Biol*. 2014; 10:558–566. [PubMed: 24845231] f) Kulkarni RA, Stanford SM, Vellore NA, Krishnamurthy D, Bliss MR, Baron R, Bottini N, Barrios AM. *ChemMedChem*. 2013; 8:1561–1568. [PubMed: 23873737] g) Stanford SM, Krishnamurthy D, Falk MD, Messina R, Debnath B, Li S, Liu T, Kazemi R, Dahl R, He Y, Yu X, Chan AC, Zhang ZYY, Barrios AM, Woods VL, Neamati N, Bottini N. *J Med Chem*. 2011; 54:1640–1654. [PubMed: 21341673] h) De Munter S, Kçhn M, Bollen M. *ACS Chem Biol*. 2013; 8:36–45. [PubMed: 23214403] i) Sakoff J, McCluskey A. *Curr Pharm Des*. 2004; 10:1139–1159. [PubMed: 15078146]
6. a) Brautigan DL. *FEBS J*. 2013; 280:324–325. [PubMed: 22519956] b) Tonks NK. *FEBS J*. 2013; 280:346–378. [PubMed: 23176256]
7. Cori GT, Cori CF. *J Biol Chem*. 1945; 158:321–332.
8. a) Fischer EH, Krebs EG. *J Biol Chem*. 1955; 216:121–132. [PubMed: 13252012] b) Krebs EG, Kent AB, Fischer EH. *J Biol Chem*. 1958; 231:73–83. [PubMed: 13538949]
9. a) Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. *New Engl J Med*. 2001; 344:1031–1037. [PubMed: 11287972] b) Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. *Nat Med*. 1996; 2:561–566. [PubMed: 8616716] c) Roskoski R. *Pharmacol Res*. 2015; 100:1–23. [PubMed: 26207888]
10. Li X, Wilmanns M, Thornton J, Kçhn M. *Sci Signaling*. 2013; 6:rs10.
11. Virshup DM, Shenolikar S. *Mol Cell*. 2009; 33:537–545. [PubMed: 19285938]
12. Yudushkin IA, Schleifenbaum A, Kinkhabwala A, Neel BG, Schultz C, Bastiaens PIH. *Science*. 2007; 315:115–119. [PubMed: 17204654]
13. a) Tobias IS, Newton AC. *J Biol Chem*. 2016; 291:13809–13822. [PubMed: 27143478] b) Langeberg LK, Scott JD. *Nat Rev Mol Cell Biol*. 2015; 16:232–244. [PubMed: 25785716]
14. a) Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK, Møller NP. *Mol Cell Biol*. 2001; 21:7117–7136. [PubMed: 11585896] b) Fahs S, Lujan P, Kçhn M. *ACS Chem Biol*. 2016; 11:2944–2961. [PubMed: 27700050]
15. a) Burridge K, Nelson A. *Anal Biochem*. 1995; 232:56–64. [PubMed: 8600832] b) Killilea SD, Cheng Q, Wang ZX. *Methods Mol Biol*. 1998; 93:23–33. [PubMed: 9664524] c) Lorenz U. *Curr*

- Protoc Immunol. 2011; Chapter 11(Unit 11.7)d) Ludlow, JW., editor. Mol Biol. Humana Press; Totowa, N. J: 1998. Protein phosphatase protocols in Methods; p. 1-336.
16. a) McAvoy T, Nairn AC. Curr Protoc Mol Biol. 2010; 92(Unit 18)b) Montalibet J, Skorey KI, Kennedy BP. Methods. 2005; 35:2–8. [PubMed: 15588980]
17. a) Selner NG, Luechapanichkul R, Chen X, Neel BG, Zhang ZY, Knapp S, Bell CE, Pei D. Biochemistry. 2014; 53:397–412. [PubMed: 24359314] b) Zhang ZY, Maclean D, McNamara DJ, Sawyer TK, Dixon JE. Biochemistry. 1994; 33:2285–2290. [PubMed: 7509638]
18. a) Feng J, Chen Y, Pu J, Yang X, Zhang C, Zhu S, Zhao Y, Yuan Y, Yuan H, Liao F. Anal Biochem. 2011; 409:144–149. [PubMed: 20971056] b) Geladopoulos TP, Sotiroidis TG, Evangelopoulos AE. Anal Biochem. 1991; 192:112–116. [PubMed: 1646572] c) Sherwood AR, Paasch BC, Worby CA, Gentry MS. Anal Biochem. 2013; 435:54–56. [PubMed: 23201267]
19. a) Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC. Anal Biochem. 1984; 136:175–179. [PubMed: 6424501] b) Green, FJ. The Sigma – Aldrich handbook of stains, dyes, and indicators. Aldrich Chemical Co; Milwaukee, Wis: 1990. c) McGadey J. Histochemie. 1970; 23:180–184. [PubMed: 5507043]
20. Fernley HN, Walker PG. Biochem J. 1965; 97:95–103. [PubMed: 16749130]
21. Gee KR, Sun WC, Bhalgat MK, Upson RH, Klaubert DH, Latham KA, Haugland RP. Anal Biochem. 1999; 273:41–48. [PubMed: 10452797]
22. a) Karver CE, Ahmed VF, Barrios AM. Bioorg Med Chem Lett. 2011; 21:285–287. [PubMed: 21129966] b) Karver MR, Krishnamurthy D, Bottini N, Barrios AM. J Inorg Biochem. 2010; 104:268–273. [PubMed: 20083307]
23. a) Huang Z. J Biomol Screening. 1999; 4:327–334.b) Rotman B, Zderic JA, Edelstein M. Proc Natl Acad Sci USA. 1963; 50:1–6.c) Wang Q, Scheigetz J, Gilbert M, Snider J, Ramachandran C. Biochim Biophys Acta, Protein Struct Mol Enzymol. 1999; 1431:14–23.
24. a) Angenendt P, Lehrach H, Kreutzberger J, Glöckler J. Proteomics. 2005; 5:420–425. [PubMed: 15700241] b) Moore KJ. J Biomol Screening. 1999; 4:335–353.c) Leira F, Vieites JM, Vieytes MR, Botana LM. Toxicon. 2000; 38:1833–1844. [PubMed: 10858520]
25. a) Kamalia N, McCulloch CA, Tenenbaum HC, Limeback H. J Histochem Cytochem. 1992; 40:1059–1065. [PubMed: 1376742] b) Murray GI, Ewen SW. J Histochem Cytochem. 1992; 40:1971–1974. [PubMed: 1360482] c) Speel EJ, Schutte B, Wiegant J, Ramaekers FC, Hopman AH. J Histochem Cytochem. 1992; 40:1299–1308. [PubMed: 1506667] d) Ziomek CA, Lepire ML, Torres I. J Histochem Cytochem. 1990; 38:437–442. [PubMed: 1689343]
26. a) Singer VL, Paragas VB, Larison KD, Wells KS, Fox CJ, Haugland RP. Am Biotechnol Lab. 1994; 12:55–56. 58. [PubMed: 7765427] b) Telford WG, Cox WG, Singer VL. Cytometry. 2001; 43:117–125. [PubMed: 11169576] c) Telford WG, Cox WG, Stiner D, Singer VL, Doty SB. Cytometry. 1999; 37:314–319. [PubMed: 10547617]
27. Takakusa H, Kikuchi K, Urano Y, Kojima H, Nagano T. Chem Eur J. 2003; 9:1479–1485. [PubMed: 12658644]
28. Urano Y, Kamiya M, Kanda K, Ueno T, Hirose K, Nagano T. J Am Chem Soc. 2005; 127:4888–4894. [PubMed: 15796553]
29. a) Kamiya M, Urano Y, Ebata N, Yamamoto M, Kosuge J, Nagano T. Angew Chem Int Ed. 2005; 44:5439–5441. Angew Chem. 2005; 117:5575–5577.b) Kawaguchi M, Hanaoka K, Komatsu T, Terai T, Nagano T. Bioorg Med Chem Lett. 2011; 21:5088–5091. [PubMed: 21482108]
30. Kim T-I, Kim H, Choi Y, Kim Y. Chem Commun. 2011; 47:9825–9827.
31. Hu Q, Zeng F, Yu C, Wu S. Sens Actuators B Chem. 2015; 220:720–726.
32. a) Gu X, Zhang G, Wang Z, Liu W, Xiao L, Zhang D. Analyst. 2013; 138:2427–2431. [PubMed: 23462951] b) Liang J, Kwok RTK, Shi H, Tang BZ, Liu B. ACS Appl Mater Interfaces. 2013; 5:8784–8789. [PubMed: 23957823] c) Liu H, Lv Z, Ding K, Liu X, Yuan L, Chen H, Li X. J Mater Chem B. 2013; 1:5550–5556.d) Song Z, Hong Y, Kwok RTK, Lam JWY, Liu B, Tang BZ. J Mater Chem B. 2014; 2:1717–1723.
33. Zhang H, Xu C, Liu J, Li X, Guo L, Li X. Chem Commun. 2015; 51:5–8.
34. Hou X, Yu Q, Zeng F, Ye J, Wu S. J Mater Chem B. 2015; 3:1042–1048.
35. Zhou X, Jiang Y, Zhao X, Zhu Y. Molecules. 2016; 21:1619.

36. Song Z, Kwok RT, Zhao E, He Z, Hong Y, Lam JW, Liu B, Tang BZ. ACS Appl Mater Interfaces. 2014; 6:17245–17254. [PubMed: 25208827]
37. Lu Z, Wu J, Liu W, Zhang G, Wang P. RSC Adv. 2016; 6:32046–32051.
38. Kim T-I, Kang HJ, Han G, Chung SJ, Kim Y. Chem Commun. 2009; 2:5895–5897.
39. Kim TI, Jeong MS, Chung SJ, Kim Y. Chem Eur J. 2010; 16:5297–5300. [PubMed: 20373316]
40. Jeong MS, Kim E, Kang HJ, Choi EJ, Cho AR, Chung SJ, Park SB. Chem Commun. 2012; 48:6553–6555.
41. Biswas S, McCullough BS, Ma ES, LaJoie D, Russell CW, Garrett Brown D, Round JL, Ullman KS, Mulvey MA, Barrios AM. Chem Commun. 2017; 53:2233–2236.
42. Li L, Ge J, Wu H, Xu QH, Yao SQ. J Am Chem Soc. 2012; 134:12157–12167. [PubMed: 22734946]
43. a) Bronstein I, Voyta JC, Edwards B. Anal Biochem. 1989; 180:95–98. [PubMed: 2817349] b) Bronstein I, Voyta JC, Thorpe GH, Kricka LJ, Armstrong G. Clin Chem. 1989; 35:1441–1446. [PubMed: 2667800] c) Janda I, Jaensch H, Braun J, Wood WG. J Clin Chem Clin Biochem. 1990; 28:475–480. [PubMed: 2172439] d) Kobos RK, Blue BA, Robertson CW, Kielhorn LA. Anal Biochem. 1995; 224:128–133. [PubMed: 7710059]
44. a) Geiger R, Miska W. J Clin Chem Clin Biochem. 1987; 25:31–38. [PubMed: 3549963] b) Miska W, Geiger R. J Clin Chem Clin Biochem. 1987; 25:23–30. [PubMed: 3549962] c) Miska W, Geiger R. J Biolumin Chemilumin. 1989; 4:119–128. [PubMed: 2678909]
45. Zhou W, Andrews C, Liu J, Shultz JW, Valley MP, Cali JJ, Hawkins EM, Klaubert DH, Bulleit RF, Wood KV. ChemBioChem. 2008; 9:714–718. [PubMed: 18247448]
46. Levine MN, Raines RT. Chem Sci. 2012; 3:2412–2420. [PubMed: 23181187]
47. Liu Y, Patricelli MP, Cravatt BF. Proc Natl Acad Sci USA. 1999; 96:14694–14699. [PubMed: 10611275]
48. a) Cravatt BF, Wright AT, Kozarich JW. Annu Rev Biochem. 2008; 77:383–414. [PubMed: 18366325] b) Jeffery DA, Bogoy M. Curr Opin Biotechnol. 2003; 14:87–95. [PubMed: 12566007] c) Krishnamurthy D, Barrios AM. Curr Opin Chem Biol. 2009; 13:375–381. [PubMed: 19716756] d) Niphakis MJ, Cravatt BF. Annu Rev Biochem. 2014; 83:341–377. [PubMed: 24905785] e) Speers AE, Cravatt BF. ChemBioChem. 2004; 5:41–47. [PubMed: 14695510] f) Barglow KT, Cravatt BF. Chem Biol. 2004; 11:1523–1531. [PubMed: 15556003] g) Liang F, Kumar S, Zhang ZY. Mol Biosyst. 2007; 3:308–316. [PubMed: 17460790]
49. a) Greenbaum D, Medzihradszky KF, Burlingame A, Bogoy M. Chem Biol. 2000; 7:569–581. [PubMed: 11048948] b) Kato D, Boatright KM, Berger AB, Nazif T, Blum G, Ryan C, Chehade KA, Salvesen GS, Bogoy M. Nat Chem Biol. 2005; 1:33–38. [PubMed: 16407991]
50. a) Liu Y, Shreder KR, Gai W, Corral S, Ferris DK, Rosenblum JS. Chem Biol. 2005; 12:99–107. [PubMed: 15664519] b) Patricelli MP, Szardenings AK, Liyanage M, Nomanbhoy TK, Wu M, Weissig H, Aban A, Chun D, Tanner S, Kozarich JW. Biochemistry. 2007; 46:350–358. [PubMed: 17209545]
51. Shreder KR, Liu Y, Nomanbhoy T, Fuller SR, Wong MS, Gai WZ, Wu J, Leventhal PS, Lill JR, Corral S. Bioconjugate Chem. 2004; 15:790–798.
52. Lo LC, Pang TL, Kuo CH, Chiang YL, Wang HY, Lin JJ. J Proteome Res. 2002; 1:35–40. [PubMed: 12643524]
53. Zhu Q, Huang X, Chen GYJ, Yao SQ. Tetrahedron Lett. 2003; 44:2669–2672.
54. Shen K, Qi L, Ravula M, Klimaszewski K. Bioorg Med Chem Lett. 2009; 19:3264–3267. [PubMed: 19423347]
55. Kumar S, Zhou B, Liang F, Wang W-Q, Huang Z, Zhang Z-Y. Proc Natl Acad Sci USA. 2004; 101:7943–7948. [PubMed: 15148367]
56. Kumar S, Zhou B, Liang F, Yang H, Wang WQ, Zhang ZY. J Proteome Res. 2006; 5:1898–1905. [PubMed: 16889411]
57. Liu S, Zhou B, Yang H, He Y, Jiang ZX, Kumar S, Wu L, Zhang ZY. J Am Chem Soc. 2008; 130:8251–8260. [PubMed: 18528979]
58. Kalesh KA, Tan LP, Lu K, Gao L, Wang J, Yao SQ. Chem Commun. 2010; 46:589–591.

59. Blum G, Mullins SR, Keren K, Fonovic M, Jedeszko C, Rice MJ, Sloane BF, Bogoy M. *Nat Chem Biol.* 2005; 1:203–209. [PubMed: 16408036]
60. Hu M, Li L, Wu H, Su Y, Yang PY, Uttamchandani M, Xu QH, Yao SQ. *J Am Chem Soc.* 2011; 133:12009–12020. [PubMed: 21732629]
61. Ge J, Li L, Yao SQ. *Chem Commun.* 2011; 47:10939–10941.
62. Mitra S, Barrios AM. *Bioorg Med Chem Lett.* 2005; 15:5142–5145. [PubMed: 16203147]
63. a) Mitra S, Barrios AM. *Anal Biochem.* 2007; 370:249–251. [PubMed: 17662953] b) Mitra S, Barrios AM. *ChemBioChem.* 2008; 9:1216–1219. [PubMed: 18412190] c) Stanford SM, Panchal RG, Walker LM, Wu DJ, Falk MD, Mitra S, Damle SS, Ruble D, Kaltcheva T, Zhang S, Zhang ZY, Bavari S, Barrios AM, Bottini N, Weiss A. *Proc Natl Acad Sci USA.* 2012; 109:13972–13977. [PubMed: 22891353] d) Stanford SM, Krishnamurthy D, Kulkarni RA, Karver CE, Bruenger E, Walker LM, Ma CT, Chung TDY, Sergienko E, Bottini N, Barrios AM. *Methods.* 2014; 65:165–174. [PubMed: 23886911]
64. a) Mehta S, Zhang J. *Methods Mol Biol.* 2014; 1071:139–149. [PubMed: 24052386] b) Newman RH, Zhang J. *Mol Biosyst.* 2008; 4:496–501. [PubMed: 18493642]
65. Wakata A, Cahill SM, Blumenstein M, Gunby RH, Jockusch S, Marti AA, Cimbro B, Gambacorti-Passerini C, Donella-Deana A, Pinna LA, Turro NJ, Lawrence DS. *Org Lett.* 2008; 10:301–304. [PubMed: 18085786]
66. Wang Q, Cahill SM, Blumenstein M, Lawrence DS. *J Am Chem Soc.* 2006; 128:1808–1809. [PubMed: 16464077]
67. a) Shults MD, Janes KA, Lauffenburger DA, Imperiali B. *Nat Methods.* 2005; 2:277–283. [PubMed: 15782220] b) Shults MD, Imperiali B. *J Am Chem Soc.* 2003; 125:14248–14249. [PubMed: 14624552]
68. Beck JR, Lawrence A, Tung AS, Harris EN, Stains CI. *ACS Chem Biol.* 2016; 11:284–290. [PubMed: 26580981]
69. Bouterin MC, Mazot P, Faure C, Doly S, Gervasi N, Tremblay ML, Vigny M. *Cell Signal.* 2013; 25:1505–1513. [PubMed: 23499906]
70. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP. *Science.* 1999; 283:1544–1548. [PubMed: 10066179]
71. Mahadev K, Zilbering A, Zhu L, Goldstein BJ. *J Biol Chem.* 2001; 276:21938–21942. [PubMed: 11297536]
72. a) White DL, Kanwal F, El-Serag HB. *Clin Gastroenterol Hepatol.* 2012; 10:1342–1359e1342. [PubMed: 23041539] b) Masarone M, Federico A, Abenavoli L, Loguercio C, Persico M. *Rev Recent Clin Trials.* 2014; 9:126–133. [PubMed: 25514916] c) Abd El-Kader SM, El-Den Ashmawy EM. *World J Hepatol.* 2015; 7:846–858. [PubMed: 25937862]
73. Beck JR, Truong T, Stains CI. *ACS Chem Biol.* 2016; 11:3284–3288. [PubMed: 27805358]
74. Chen J, Martin BL, Brautigan DL. *Science.* 1992; 257:1261–1264. [PubMed: 1325671]
75. Andjelkovic M, Jakubowicz T, Cron P, Ming XF, Han JW, Hemmings BA. *Proc Natl Acad Sci USA.* 1996; 93:5699–5704. [PubMed: 8650155]

Biographies

Garrett Casey received his BS in chemistry and forensic science from Friends University. He is currently pursuing his PhD degree in the laboratory of Cliff Stains in the Department of Chemistry at the University of Nebraska–Lincoln. His research interests are in the design and application of bioprobes for cell signaling events as well as the development of methods for directly controlling cell signaling.

Cliff Stains obtained his PhD degree with Indraneel Ghosh (University of Arizona) followed by postdoctoral studies with Barbara Imperiali (MIT). In 2011, he was appointed as an Assistant Professor in the Department of Chemistry at the University of Nebraska–Lincoln. His research program is focused on developing and applying chemical tools to investigate cell signaling in both normal and disease settings.

Figure 1.

Protein phosphatases catalyze the removal of a phosphoryl group from a protein substrate while protein kinases use ATP as a source of phosphate for addition of a phosphoryl group to a target residue on a protein. Fine tuning the catalytic activities of these enzymes, in part through additional post-translational modifications to the enzymes themselves, allows the cell to rapidly respond to external stimuli by shifting the balance of the phosphoproteome.

Figure 2.
Colorimetric reporters of PP enzymatic activity.

Figure 3.
Fluorogenic probes for PP activity.

Figure 4. Next generation fluorogenic probes for PP activity. ESIPT is excited-state intramolecular proton transfer and ICT is intramolecular charge transfer.

Figure 5.
Luminescence-based activity probes for PP activity.

Figure 6.

Activity-based protein profiling for PPs. a) General schematic of an ABPP reacting with a proteome. A reactive warhead is used to selectively label a target enzyme class. Labeling is subsequently detected using the covalently linked reporter tag or handle via in-gel fluorescence or mass spectrometry. b) Reactive warheads for labeling PPs. c) Representative reporter tags or handles for fluorescence detection or affinity purification.

Figure 7.

Labeling mechanisms for selected ABPs with PTPs. a) Quinone methide-based ABP labeling. b) Proposed labeling mechanisms of BBP-based ABPs. c) Labeling by vinyl phenyl sulfonate-based probes. d) Quinone methide-based labeling involving the simultaneous release of a quencher (Q). e) A self-immobilizing fluorescent tag for PTPs.

Figure 8.
Examples of ABPs used for labeling PPs.

Figure 9.

Examples of targeted, fluorescence-based activity probes for PPs. CFP and YFP are cyan fluorescent protein and yellow fluorescent protein, respectively. CSox is a cysteine residue alkylated with the Sox fluorophore.