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Activity-Based Protein Profiling of Protein Tyrosine Phosphatases

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Summary

The ability to accurately monitor the dynamics involved with the activity and state of a specific protein population in a complex biological system represents one of the major technological challenges in studying systems biology. Over the past several years a number of groups have attempted to spearhead this new frontier of systems biology by developing enzyme family-specific activity-based chemical probes linked to appropriate reporter groups that by nature only target and subsequently tag the active form of these enzymes. In this work, we will highlight the methods used to characterize activity-based probes as to their utility in biological contexts. Specifically, we will address activity-based protein profiling of the protein tyrosine phosphatases, a highly conserved enzyme family responsible for the phospho-tyrosine hydrolysis reaction, a ubiquitous reaction that is absolutely essential to the regulation of a myriad of cellular processes.

Keywords

Activity-based protein profiling (ABPP); Activity-based probes (ABPs); Protein tyrosine phosphatases (PTPs); Biotin probe; Fluorescent probe; Systems biology

1. Introduction

A more detailed look into cellular processes as it pertains to global alterations to protein function in a complex web of interactions is the paradigm of “systems biology.” To date, being able to understand a specific protein population with regard to its distinguishable traits in a dynamic setting such as subgroups that are being positively or negatively regulated (e.g., inactive vs. active states) rather than just the mere observation of natural abundance remains a formidable task both technologically and methodologically.

Activity-based protein profiling (ABPP) represents a relatively new concept in the era of further defining the proteome on a “systems-wide” scale as more than just quantifiable differences in protein expression levels, a methodology currently employed solely by mass spectrometry (MS). ABPP attempts to address, for the first time, protein function in a dynamic setting, thus allowing for a specific protein population to be characterized as to both its natural abundance and its activity; two dimensions that do not necessarily correlate with each other biologically.

Over the past several years a number of groups have attempted to spearhead this new frontier of “system's biology” by developing enzyme family-specific activity-based chemical probes (ABPs) linked to specific reporter groups that by nature only target and subsequently tag the active form of these enzymes both in vitro and in vivo. The enzyme families that have been studied using this ABPP approach are the serine hydrolases (1–3), metalloproteinases (4), protein tyrosine phosphatases (5,6), 26S proteasome (7), and protein and lipid kinases (8).

Protein tyrosine phosphatases (PTPs) represent one of two extremely critical and evolutionarily conserved enzyme families responsible for the reversible tyrosine phospho-transfer reactions that have defined the life processes of multicellular organisms for over 600 million years. PTPs catalyze the dephosphorylation reaction of phospho-tyrosine residues on substrate proteins and

thus represent important signaling enzymes that serve as key regulatory components in various signal transduction pathways (9,10). Defective or inappropriate regulation of PTP activity leads to aberrant tyrosine phosphorylation, which contributes to the development of many human diseases (11).

The sequencing of the human genome has revealed more than 100 PTPs (12) of which little is known about their physiological role and dynamic regulation despite having a very detailed understanding of the mechanism by which they carry out the phospho-tyrosine hydrolysis reaction (13). Knowing the details of this catalytic mechanism provides a major victory toward the development of chemical probes that specifically target and irreversibly modify the nucleophilic cysteine residue in the PTP signature motif ((H/V)C(X)₅R(S/T)).

All ABPs must undergo rigorous testing as to their utility in covalently labeling the active forms of their respective targets both in a simplistic environment (e.g., probe with purified target protein) and a more complex one (e.g., a cell lysate or in vivo). In this work, the general methodology that remains consistent to all these endeavors will be highlighted as it pertains to the characterization and experimental utility of both the α -bromobenzylphosphonate (BBP)-based biotin- and fluorophore-conjugated PTP probes (**I** and **II** respectively; Fig. 1) (5,6).

2. Materials

2.1. Kinetic Characterization of the α -Bromobenzylphosphonate PTP Probes I and II

1. *PTP Probes I and II*. Prepare as 100 mM stocks in 100% DMSO, store at -20°C .
2. *Yersinia PTP (YopH)*. Expressed and purified from *Escherichia coli* (14).
3. *Labeling buffer*. 50 mM sodium succinate, 1 mM EDTA, 1 mM dithiothreitol (DTT), 150 mM NaCl, pH 6.0 (see **Note 1**).
4. *PTP assay buffer*. 50 mM sodium succinate, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, pH 6.0, supplemented with 20 mM pNPP (200 μL total volume).

2.2. Specificity of the α -Bromobenzylphosphonate PTP Probes I and II

1. *Labeling reaction buffer for the PTP enzymes*. 50 mM sodium succinate buffer (pH 6.0) containing 1 mM EDTA and 1 mM dithiothreitol (DTT). Adjust the ionic strength to 150 mM with NaCl (see **Note 1**).
2. *PTP enzymes*. PTP1B, SHP2, HePTP, YopH, and FAP-1 (classical cytosolic PTPs), PTP α and DEP-1 (receptor-like PTP), VHR, PRL-3, and Cdc14 (dual-specificity phosphatases), and LMW-PTP.
3. Labeling reaction buffers for all non-PTP enzymes:
 - a. *Potato and prostatic acid phosphatases*, 100 mM sodium acetate, pH 5.0, 1 mM EDTA.
 - b. *Protein phosphatase 1 (PPI) and λ -phosphatase*, 50 mM 3, 3-dimethylglutarate, pH 7.0, 2 mM MnCl_2 .
 - c. *Alkaline phosphatase*, 50 mM Tris-HCl, pH 9.0, 1 mM MgCl_2 .
 - d. Papain, lysozyme, Grb2-Src homology 2 (SH2), Shc-phosphotyrosine-binding (PTB), and *SNT1-PTB*, 50 mM 3,3-dimethylglutarate, pH 7.0, 1 mM EDTA.
 - e. *Glyceraldehyde-3-phosphate dehydrogenase*, 15 mM sodium pyrophosphate, pH 8.5, 7.5 mM NAD, 1 mM DTT.

- f. *Calpain*, 100 mM imidazole, pH 7.3, 10 mM CaCl₂, 1 mM DTT.
 - g. *Trypsin*, 100 mM Tris-HCl, pH 8.5, 1 mM EDTA.
 - h. *Chymotrypsin*, 50 mM Tris-HCl, pH 7.8, 50 mM CaCl₂.
 - i. *Protein phosphatase 2B*, 50 mM 3,3-dimethylglutarate, pH 7.0, 0.3 μM calmodulin, 2 mM MnCl₂.
 - j. *Src kinase*, 100 mM Tris-HCl, pH 7.0, 1 mM EDTA.
 - k. *Thermolysin*, 50 mM HEPES, pH 7.0, 5 mM CaCl₂.
4. *Quenching solution*. SDS-loading buffer (sample buffer) (62.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 10 mM DTT, 0.2% bromophenol blue).
 5. SDS-PAGE buffers and reagents as highlighted in the SDS-PAGE materials section.
 6. (For use if using the biotin-conjugated PTP probe **I**) – Western blotting reagents as highlighted in **Subheading 2.8**.
 7. (For use if using the fluorophore-conjugated PTP probe **II**) – Typhoon 9400 scanner (Amersham Biosciences, Inc.) in fluorescence mode (green: 532 nm; red: 633 nm) for detection of the fluorescent probes. ImageQuant 3.3 (Molecular Dynamics) for fluorescence quantification.
 8. (For use if using the fluorophore-conjugated PTP probe **II**) – Silver staining reagents as described in **Subheading 2.7**.

2.3. Sensitivity of the α-Bromobenzylphosphonate PTP Probes I and II

1. *PTP Probes I and II*. Prepare as 100 mM stocks in 100% DMSO and store at –20°C.
2. *Yersinia PTP (YopH)*. Expressed and purified from *E. coli* (14).
3. *Labeling buffer*. 50 mM sodium succinate, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, pH 6.0 (see **Note 1**).
4. *Quenching solution*. SDS-loading buffer (sample buffer); 62.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% Glycerol, 10 mM DTT, 0.2% bromophenol blue.

2.4. Effect of H₂O₂ on PTP Activity Monitored by the α-Bromobenzylphosphonate PTP Probes I and II both in vitro and in vivo

1. *Labeling buffer*. 50 mM sodium succinate, 1 mM EDTA, 1 mM DTT, and 150 mM NaCl, pH 6.0 (see **Note 1**).
2. Hydrogen peroxide (H₂O₂) (Sigma).
3. *PTP1B*. Expressed and purified from *E. coli* (15).
4. Human-colon-carcinoma-derived HCT-116 cell culture.
5. SDS-PAGE, gel staining, cell culture, lysis, and protein assay reagents and buffers as described in their respective materials sections. Fluorescence scanning as described previously. Note: supplement lysis buffer with 2 mM PTP probe.

2.5. PTP Profiling in Cancer Cells Monitored the α-Bromobenzylphosphonate PTP Probes I and II

1. MOA231 (lung), MDA-MB-435S (breast), MCF-7 (breast), HepG2 (liver), SKOV3 (ovary), HeLa (cervix), and HCT-116 (colon) cell cultures.

2. *Labeling buffer*. 50 mM sodium succinate, 1 mM EDTA, 1 mM DTT, and 150 mM NaCl, pH 6.0 (*see Note 1*).
3. Cell culture, lysis, SDS-PAGE, and staining reagents and buffers as described in their respective materials sections. Fluorescence scanning as described previously.

2.6. Cell Culture, Lysis, and Protein Assay

1. Dulbecco's Modified Eagle's minimum essential Medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin (50 units/mL), streptomycin (50 µg/mL), and L-glutamine (2 mM).
2. Dulbecco's Phosphate-Buffered Saline (DPBS; 10×) (Gibco/BRL, Bethesda, MD) made 1× before using as cell wash-solution.
3. *Cell lysis buffer*. 50 mM MES, pH 6.0, 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 10% Glycerol, 5 mM EDTA, supplemented with fresh 10 µg/mL leupeptin, 5 µg/mL aprotinin, 1 mM PMSF, and 1 mM DTT.
4. Teflon cell scrapers (Fisher).
5. Bradford Protein Assay (Amersham Biosciences).

2.7. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. *Running Buffer* (5×). 125 mM Tris, 960 mM glycine, 0.5% SDS. Store at room temperature.
2. *Thirty percent (30%) acrylamide/bis solution*. 37.5:1 with 2.6% C (Fisher).
3. *N,N,N',N'*-Tetramethyl-ethylenediamine (TEMED, Fisher).
4. *Ammonium persulfate (APS)*. Prepare a fresh 10% solution in water. Immediately freeze down small single-use aliquots at −20°C (do not store these aliquots for long).
5. *Resolving gels*. 10 and 12.5% SDS-PAGE gels.
6. *SDS-loading buffer (sample buffer)*. 62.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 10 mM DTT, 0.2% bromophenol blue.
7. EZ-Run Protein Standard (Prestained molecular weight marker) (Fisher).
8. GelCode Blue Stain Reagent (Pierce).
9. SilverQuest (Silver Staining Kit) (Invitrogen).

2.8. Western Blotting

1. *Transfer buffer* (10×). 30 g Tris, 144 g Glycine, 10 g SDS. Dilute to 1× before use.
2. NitroBind, Cast, Pure Nitrocellulose 0.22 µm (GE Water and Process Technologies).
3. Whatman Chromatography Paper (Schleicher & Schuell).
4. *TBS-T*. Tris-buffered saline with 1% Tween 20 (TBS-T).
5. *Blocking buffer*. 5% nonfat dry milk in TBST.
6. Primary antibody dilution buffer (for antibiotin western blot). TBST-containing nonfat dry milk (5%). Primary antibody (antibiotin-horseradish peroxidase (HRP) conjugate (Cell Signaling Technology, Beverly, MA)).
7. HRP substrate (ECL Western Blotting Detection Reagents) (Amersham Biosciences).

3. Method

In this work, we will highlight the characterization and utility of both the α -Bromobenzylphosphonate (BBP)-based biotin- and fluorophore-conjugated protein tyrosine phosphatase (PTP) probes (5,6). (BBP) has been shown to be a quiescent affinity inactivator of the *Yersinia* PTP YopH (16). Figure 1 illustrates both the biotin- (**I**) and fluorophore- (**II**) conjugated PTP probes. The biotin conjugate allows for target protein enrichment from a much more complex matrix via affinity-based purification methodology (an approach that, theoretically, is much better suited for later protein identification methodologies like MS). Contrary to this asset of target protein enrichment via affinity-based purification is that detection and identification can only be done after affinity purification, gel electrophoresis, and western blot (all approaches that act to decrease protein yield in preparation for direct visualization or MS). These inherent liabilities make this approach far less sensitive toward visualization when compared to its fluorophore-based counterpart. The fluorescent probe allows for “direct” in-gel visualization of target protein conjugate and, through a number of studies, has proven to be far more sensitive than the biotin-based approach. However, the fluorophore-based approach toward protein isolation for subsequent MS analysis, mechanistically, is more difficult than the biotin-based approach (from a complex matrix-type setting) because target protein must be cut from a gel among a myriad of total cellular protein run at the same time. Both probes are ideal when used to attain goals that base their achievement off of the assets inherent to each probe's specific chemistry. The methodology used to characterize these probes in a biological setting will be described here.

3.1. Kinetic Characterization of the α -Bromobenzylphosphonate PTP Probes I and II

The *Yersinia* PTP YopH provides an excellent model to explore the in vitro kinetic mechanism of probe-induced enzyme inactivation as well as relevant kinetic constants associated with the probe's concentration-dependent inhibitory nature against PTP activity toward “synthetic” or “biological” substrates. Previous work in ABPP has shown that ABPs display differential probe reactivity toward members of the same enzyme family. It is crucial that the probe display inactivating properties that are “general” to the entire family and not “specific” toward any particular member of the group. Para-nitrophenyl phosphate (*p*NPP) is a widely used molecule in the assessment of PTPase activity. The rate of hydrolysis of this molecule directly correlates with the competence of the enzyme for catalytic activity. We have previously shown that both probes **I** and **II** display very similar inactivating kinetics toward the standard PTP YopH (a necessary observation showing that the probe conjugates are relatively inert toward reaction progress) (5,6).

1. Initiate the labeling/inactivation reaction by adding a 5- μ L aliquot of 1 μ M YopH stock (kept on ice) to a 45- μ L solution containing appropriately diluted probe (0.2, 0.5, 1.0, 1.5, 2.0, and 3.0 mM) (make sure to keep the final DMSO concentration at 5% for all concentrations to minimize experimental error) at 25°C.
2. At appropriate time intervals, remove aliquots of 2 μ L from the reaction and subsequently add them to 200 μ L of the PTP assay buffer highlighted in the **Subheading 2** at 30°C.
3. After 3 min, quench each *p*NPP reaction by adding 50 μ L of 5N NaOH. Transfer 200 μ L of the quenched reaction mixture to a 96-well plate and measure the absorbance at 405 nm (A_t), which reflects the residual activity of YopH in the labeling reaction at time point taken.
4. Obtain the pseudofirst-order inactivation constants (k_{obs}) at various concentrations of the probe by fitting the data to Eq. 1: where A_0 , A_t , and A_∞ are activities of the enzyme at incubation time zero, t , and infinity, respectively.

$$\frac{A_t}{A_0} = \frac{A_{\infty}}{A_0} - \left(\frac{A_0 - A_{\infty}}{A_0} \right) e^{-k_{\text{obs}} \cdot t}. \quad (1)$$

5. Perform a nonlinear regression fit of k_{obs} vs. probe concentration to Eq. 2. This will yield the maximal inactivation constant k_i and dissociation constant K_i .

$$k_{\text{obs}} = \frac{k_i \times [I]}{K_i + [I]}. \quad (2)$$

3.2. Specificity of the α -Bromobenzylphosphonate PTP Probes I and II

A measure of the utility of an ABP is its ability to discriminate successfully not only between PTP and non-PTP enzymes, but also between a PTP enzyme that is in an active conformation (one exposing the active site for catalysis) and a PTP enzyme that is, by any means, in an inactive state. Furthermore, the probe must engage the active site chemistry in such a way that the product of the reaction is a fully inactive and covalently bound enzyme. The PTP family of enzymes share a highly conserved signature motif ((H/V)C(X)₅R(S/T), with the cysteine residue being the active site nucleophile responsible for the initiation of the phospho-tyrosine hydrolysis reaction. The specificity of the probe relies on its recognition of this particular active site architecture. A very important test is that it distinguishes not only between PTP and non-PTP phosphatases, but also between the PTP signature motif cysteine residue and other non-PTP enzymes that also initiate catalysis via a nucleophilic reaction involving a cysteine residue.

1. Incubate the PTP probes (1 mM for the biotin-based probe **I**) (0.2 mM for the fluorophore-based probe **II**) with the pre-equilibrated PTPs (or non-PTP enzymes) (25 μ M for the biotin-based probe **I**) (1 μ M for the fluorophore-based probe **II**) at 25°C for 15 min. All enzymes and appropriate buffers are described in **Subheading 2**.
2. After the incubation time period, prepare the enzyme samples to be resolved in a gel matrix using SDS-PAGE (appropriate buffers are highlighted in **Subheading 2**). First, quench the labeling reaction by adding one volume of the quenching solution (*see Subheading 2.2.*).
3. *For use if using the biotin-conjugated PTP probe I* – Divide each sample into halves (one-half goes to one 12.5% SDS-PAGE gel for later staining and the other half to another 12.5% SDS-PAGE gel for later Western blot). Load 3 μ g protein per lane.
4. After electrophoresis perform a western blot by transferring the proteins from one gel to a nitrocellulose membrane overnight at 4°C (*see Chapter “Immunoblotting 2DE Membranes”*). Use the appropriate transfer, blocking and washing buffers as detailed in **Subheading 2.8**. Treat the membrane for 2 h with antibiotin-horseradish peroxidase (HRP) conjugate at a dilution of (1:1,000) in TBS-T-containing 5% nonfat dry milk at 25°C. Treat the antibiotin-HRP-treated blot with HRP substrate for 1 min. Expose the chemiluminescent membrane blot to X-ray film in a dark room.
5. Stain the other gel with Coomassie blue for protein visualization following the appropriate manufacturer's recommendations.
6. *(For use if using the fluorophore-conjugated PTP probe II)* – Immediately scan the gel (after the gel run is completed) containing the fluorescently labeled enzymes using a Typhoon 9400 scanner (Amersham Biosciences) in fluorescent mode, with green (532 nm) and red (633 nm) lasers with preset optimum emission filters. Quantify the scanned images with ImageQuant 3.3 (Molecular Dynamics) (*see also Chapter “Troubleshooting Image Analysis in 2DE”*).

7. After fluorescence scanning, silver stain the gel (according to the manufacturer's instructions) to directly visualize equal protein loading between the lanes.

3.3. Sensitivity of the α -Bromobenzylphosphonate PTP Probes I and II

It has been shown in a number of studies, including our own (6), that the fluorophore-based approach is far more sensitive (in the order of 1,000-fold more sensitive) than the affinity-based biotin approach. This phenomenon is due to a number of reasons including the physical and chemical nature of fluorescence as well as the biotin method inherently being deleterious to the preservation of target protein (i.e., many “sample-losing” steps prior to analysis). Though target protein visualization is naturally a positive aspect to ABPP, it is the relative quantitative information gained from both approaches that is most critical to subsequent protein identification methods such as MS (which requires a certain protein amount to be present, relatively speaking, to obtain sufficient fragmentation (structural) information on sample peptides for proper identification via database similarity searches to take place). Setting up a comparison of probe sensitivities toward a standard PTP enzyme will be described here.

1. Using the *Yersinia* PTP (YopH) as a standard protein (*see Subheading 2.1*), incubate the enzyme (25 μ M) with both the biotin- and fluorophore-conjugated probes (2 mM) for 60 min in the labeling buffer (*see Subheading 2.1*) at 25°C. Set up a serial dilution of the YopH enzyme concentration so that the following “gram” amounts can be loaded into each successive lane: 200, 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 ng.
2. Quench the labeling reactions by adding an equal volume of the quenching solution highlighted in **Subheading 2**. Heat the resulting mixture at 75°C for 5 min.
3. Load the entire sample for all experimental groups. Resolve the proteins by using 12.5% SDS-PAGE gels.
4. Immediately scan the gel containing the fluorescently labeled YopH enzymes using a Typhoon 9400 scanner (Amersham Biosciences) in fluorescent mode, with green (532 nm) and red (633 nm) lasers with preset optimum emission filters. Quantify the scanned images with ImageQuant 3.3 (Molecular Dynamics).
5. The enzymes tagged with the biotin-conjugated probe will have to be visualized by Western blotting using anti-biotin antibody. Set up a western blot of this gel by following the western blotting procedure described in **Subheading 3.2**.

3.4. Effect of H₂O₂ on PTP Activity Monitored by the α -Bromobenzylphosphonate PTP Probes I and II Both in vitro and in vivo

It has been experimentally shown that physiologically, hydrogen peroxide (H₂O₂) acts as a second messenger. One of its physiological targets is the PTP family of enzymes, where it acts as a “transient” negative regulatory modification to PTP activity through direct engagement of the nucleophilic cysteine residue of the PTP signature motif ((H/V)C(X)₅R(S/T)). Hydrogen peroxide treatment, in vitro, of a standard PTP provides a unique opportunity to observe the probes selectivity toward only the active form of the PTP. In vivo, H₂O₂ treatment of cultured cells can provide insights into the inhibition properties of this molecule to specific PTP enzymes of interest as H₂O₂ and the PTP probe act as competitive inhibitors.

1. To carry out the in vitro labeling experiment, incubate (1 μ M) PTP1B and H₂O₂ (1 mM) in a 20- μ L reaction at 25°C in the labeling buffer highlighted in **Subheading 2.4**. Follow the protocol in **Subheading 3.1** to kinetically characterize this reaction in the presence and absence of H₂O₂.

2. To establish an understanding of the probe's selectivity for active PTP in a complex protein mixture, set the probe against a whole cell lysate generated from a Human-colon-carcinoma-derived HCT-116 cell line treated with and without H₂O₂.
3. First, establish a cell culture of Human-colon-carcinoma-derived HCT-116 cells (reagents as described in the cell culture section of the materials). Upon reaching a suitable confluency, treat the HCT-116 cells with or without H₂O₂ (1 mM) for 5 min.
4. After the 5-min incubation period, immediately pour off the cell medium and wash the cell bed with ice-cold 1×PBS (twice). Collect the cells in a sufficient volume (1 mL) (for the size of the plates the cells are grown on) of ice-cold PBS (1×) by scraping them from the dish with a sterilized Teflon cell scraper. Pipette the volume of PBS (1×) and cell suspension in an appropriate collection vessel (suitable for high-speed centrifugation). Centrifuge this cell suspension at 1,000 × *g* at 4°C for 10 min. After this procedure the cell pellet will be clearly visible.
5. Aspirate the PBS (1×) from the cell pellet.
6. Resuspend the cell pellet in the following lysis buffer. 50 mM MES (pH 6.0), 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 10% glycerol, 5 mM EDTA, 10 µg/mL leupeptin, and 5 µg/mL aprotinin, 1 mM PMSF containing 2 mM fluorescent probes (predetermine the volume of lysis buffer to be used).
7. Let the probes incubate with the cell lysate proteins for 30 min on ice. Perform a control experiment at the same time with the cells not treated with H₂O₂.
8. After completion of the labeling reaction, clear the two cell lysates (control and experimental) of a nonprotein cellular debris by centrifugation at 13,000 × *g* for 15 min in a prechilled 4°C centrifuge.
9. Pipette the supernatants into new collection tubes and discard the pellets. Determine the relative protein concentrations by Bradford assay.
10. After establishing a relative protein concentration for each of the experimental groups, use these concentrations to load a comparative amount of each of the experimental group's lysate proteins to a 10% SDS-PAGE gel (load 50 µg total protein per lane). Also, load a prestained molecular weight marker (8 µL) for sample molecular weight determinations.
11. Visualize the fluorescent probes using the method described earlier. After fluorescence scanning, silver stain the gel (according to the manufacturer's instructions) to directly visualize equal protein loading between the two lanes.
12. The enzymes tagged with the biotin-conjugated probe will have to be visualized by Western blotting using antibiotin antibody. Set up a western blot of this gel by following the western blotting procedure (*see Subheading 3.2*).

3.5. PTP Profiling in Cancer Cells Monitored with the α -Bromobenzylphosphonate PTP Probes I and II

Protein tyrosine phosphatases play extremely critical roles in the regulation of a myriad of cellular process. Multicellular organisms use reversible tyrosine phosphorylation to alter protein structure and function during intracellular signal transduction. Cancer has been described as a disease of malfunctioning cells, and in many cases it is the “deregulation” of the reversible phospho-tyrosine protein states that underlies the transformation process. The PTP ABPs offer a unique approach to gaining possible experimental insight into the underlying molecular mechanisms that are contributing to the “transformed” state of various authentic cancer cell lines if PTPs are involved.

1. Establish MOA231 (lung), MDA-MB-435S (breast), MCF-7 (breast), HepG2 (liver), SKOV3 (ovary), HeLa (cervix), and HCT-116 (colon) cell cultures in the growth medium described in (**Subheading 2.5**) under a humidified atmosphere of 5% CO₂.
2. When the cells reach a suitable confluency perform the collection and lysate protocol as described in **Subheading 3.4**. A difference from this protocol is that the lysis buffer does not contain ABP and the labeling reaction is to be done after cell debris removal as previously described. Incubate the cells on ice in lysis buffer for 30 min.
3. After a relative protein concentration has been determined for each of the cell lysates, incubate 50 µg of protein from each lysate with 1 mM of the fluorescent probe (1 mM biotin-based probe can be used) for 1 h at 25°C in the labeling buffer described in (**Subheading 2.1**).
4. Perform **steps 10 and 11** from the previous section (**Subheading 3.4**).
5. The enzymes tagged with the biotin-conjugated probe will have to be visualized by Western blotting using anti-biotin antibody. Set up a western blot of this gel by following the western blotting procedure described in **Subheading 3.2**.

4. Notes

1. The α -bromobenzylphosphonate-based probes undergo significant solvolysis in solution with a pH value >7 (5). In pH 6.0 buffer and at room temperature, the probes are stable for at least 1 h (unpublished data). Store the probes at –20°C in DMSO. Dilute to the desired concentration in the pH 6.0 buffer before experiments.
2. For detection limit of the probe labeling, a serial dilution of the probe-labeled YopH was made in 2×SDS-loading buffer containing 0.01 mg/mL BSA as a carrier protein.

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Fig. 1.
Structures of the biotinylated PTP α -Bromobenzylphosphonate probe **I** and the rhodamine-conjugated PTP α -Bromobenzylphosphonate probe **II**