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Selective activation of SHP2 activity by cisplatin revealed by a novel chemical probe-based assay

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ABSTRACT

Src homology-2 (SH2) domain-containing phosphatase 2 (SHP2) is known to participate in several different signaling pathways to mediate cell growth, survival, migration, and differentiation. However, due to the lack of proper analytical tools, it is unclear whether the phosphatase activity of SHP2 is activated in most studies. We have previously developed an activity-based probe **LCL2** that formed covalent linkage with catalytically active protein tyrosine phosphatases (PTPs). Here, by combining **LCL2** with a SHP2 specific antibody, we established an assay system that enables the direct monitoring of SHP2 activity upon cisplatin treatment of cancer cells. The protocol is advantageous over conventional colorimetric or in-gel PTP assays as it is specific and does not require the use of radioisotope reagents. Using this assay, we found SHP2 activity was selectively activated by cisplatin. Moreover, the activation of SHP2 appeared to be specific for cisplatin as other DNA damage agents failed to activate the activity. Although the role of SHP2 activation by cisplatin treatments is still unclear to us, our results provide the first direct evidence for the activation of SHP2 during cisplatin treatments. More importantly, the concept of using activity-based probe in conjunction with target-specific antibodies could be extended to other enzyme classes.

Introduction

Reversible protein tyrosine phosphorylation–dephosphorylation is one of the main mechanisms that living cells use in the transduction of cellular signals and regulation of biological activities [1–3]. The tyrosine phosphorylation status of the proteins is tightly tuned by the actions of two enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The physiological functions of PTPs are usually addressed using knockdown/knockout systems and congenital dysfunction models. However, posttranslational protein modification through phosphorylation, translocation, or protein–protein interactions were also known to contribute greatly to the modulation of PTP activities [4,5]. Thus, analyzing the PTP activity during signal transduction is an important issue that needs to be addressed in various cellular processes. Currently, there is no direct method to measure the activity of a specific PTP in cells. Although analytical methods utilizing inactive PTP mutants or PTP inhibitors could correlate the phosphatase activities with certain cellular processes, these methods do not provide direct measurements of the PTP activity. The activity of a specific PTP in cells cannot be easily determined because it is usually complicated by the presence of other unrelated phosphatases. Thus, although phosphorylation status or translocation of PTPs has been implicated to the alteration of PTP activity, it is commonly not determined in most studies.

SHP2 is known to participate in several different signaling pathways to mediate cell growth, survival, migration, and differentiation [5]. For example, association of Gab2 and/or Gab3 with SHP2 is required for mononuclear phagocyte development mediated by macrophage colony-stimulating factor [6,7]. It was also shown that transfection of PC12 cells with a dominant-negative SHP2 phosphatase inhibited the production of neurites induced by NGF [8]. However, the question of whether SHP2 activity is activated during these cellular processes was not clearly addressed. Most of the analyses toward addressing the role of SHP2 in signaling pathways were limited to indirect methods. Moreover, since the protein levels of SHP2 are not changed during most of the signaling transductions, the involvement of SHP2 in cellular processes could not be identified through conventional immunoblotting, genomic, or proteomic analyses.

In the past few years, small molecular weight compounds have been used for analyzing biological systems such as enzyme inhibitors/activators, substrates, molecular probes, and protein modifiers [9]. This chemistry-driven approach offers an excellent opportunity to answer biological questions that cannot be easily addressed by traditional biochemical or genetic approaches. For example, activity-based probes have been developed and were

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shown to be a powerful tool to profile various enzymatic activities in complex proteomes [10,11]. Based on the properties of the reporter group utilized by the activity probes, the labeled enzymes could then be monitored or enriched. Previous, we have synthesized and characterized an activity-based probe **LCL2** for PTPs (Fig. 1A, [12]). **LCL2** belongs to a general type probe for PTPs, carrying a phenylphosphate recognition group and a biotin reporter group. Once activated, it generates a reactive intermediate which forms a covalent linkage with the target PTPs nearby. The labeled PTPs could then be characterized and manipulated by taking advantage of the biotin reporter. MALDI-TOF MS experiments indicated that Cys residues are the major modification sites for **LCL2** [13]. Because the labeling event requires catalytically active PTPs, this provides an opportunity to use the labeling with **LCL2** as a measurement for phosphatase activity. In this report we established an assay by combining **LCL2** with antibody against SHP2 to enable the selective detection of SHP2 activity from cells. We demonstrated that its phosphatase activity is selectively activated upon treatment with cisplatin but not with 5-fluorouracil (5-FU), etopoxide, or 5-azacytidine. The results provide the direct evidence for the activation of SHP2 upon cisplatin treatments. More significantly, the established assay could also be applied to determine other PTP activities during different cellular processes.

Materials and methods

Reagent and cell line. Cisplatin (P4394), 5-FU (F6627), 5-azacytideine (A2385), etoposide (E1383), *p*-NPP (*p*-nitrophenyl phosphate, N4645), sodium orthovanadate (S6508), protein A-agarose (P3391), and anti-biotin antibody (B7653) were purchased from Sigma–Aldrich (USA). **LCL2** was synthesized according to previous report [12]. Non-small lung cancer cells H1299 were grown in RPMI 1640 media supplemented with 10% fetal bovine serum in humidified atmosphere with 5% CO_2 at 37 °C. Monoclonal antibody against SHP2 (sc7384, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A) and PTP1B (FG6–1G, Calbiochem, Merck Biosicences) were purchased from local distributors. Polyclonal antibodies (AF1366) against PTP1B were obtained from R&D System.

Cloning, expression, and purification of SHP2 and PTP1B. The cDNA encoding the catalytic domain of human SHP2 was amplified from a cDNA clone (obtained from Dr. T.-C. Meng, Academia Sinica, Taipei) and then expressed in *Escherichia coli* BL21(DE3) as 6-His tagged recombinant proteins. The Ni Sepharose™ (GE-Amersham Biosciences Ltd.) was added to isolate the recombinant SHP2. Purified proteins were dialyzed against storage buffer (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol), aliquoted, and frozen by dry ice–ethanol bath. Isolation of recombinant PTP1B was described previously.[12].

p-NPP assay. Purified PTP1B or SHP2 was added into a 200 μL reaction mixture containing 50 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl, and 5 mM *p*-NPP and incubated at 30 °C for 30 min. Hydrolysis of *p*-NPP was determined by reading the absorbance at 405 nm with a spectrophotometer.

***LCL2** labeling of purified PTPs*. Purified PTPs were incubated with 0.5 mM of **LCL2** in reaction mixture containing 50 mM Tris pH 7.5, 1 mM EDTA, and 150 mM NaCl at 25 °C for 20 min. The reaction products were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then subjected to immunoblotting analysis using monoclonal antibody against biotin. The blots were visualized by ECL chemiluminescence reagents (Perkin–Elmer). The imagines were quantified using Vilber Lourmat quantification tool.

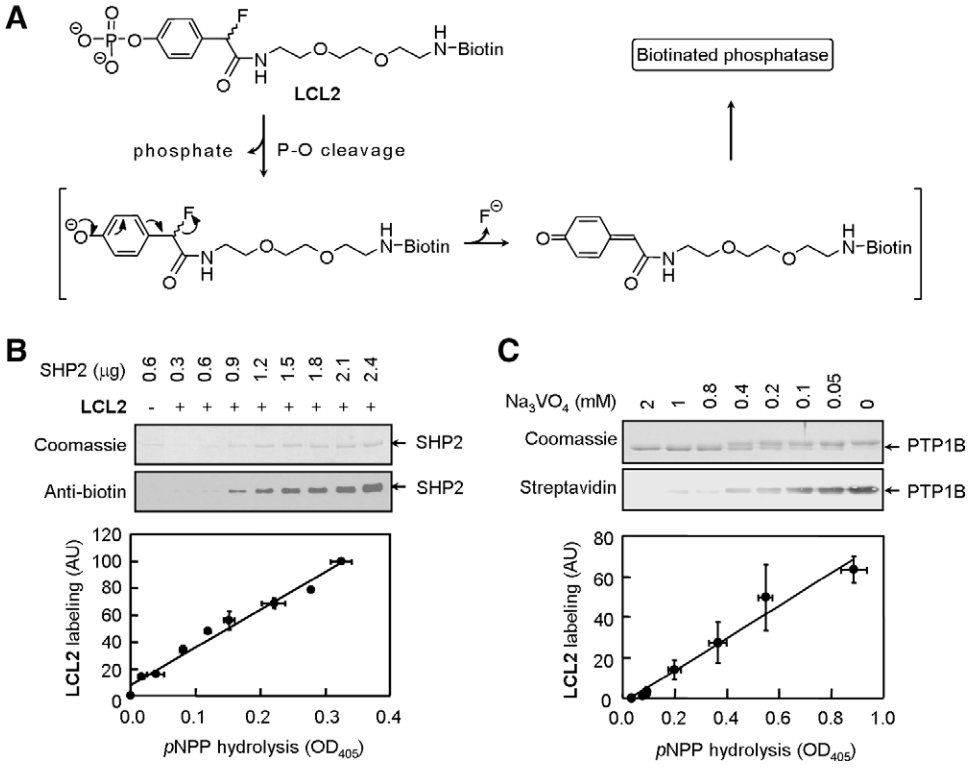

Fig. 1. Correlation between **LCL2** labeling and phosphatase activity. (A) Structure of activity probe **LCL2** and its labeling mechanism for protein tyrosine phosphatase. (B) Protein dependent labeling of phosphatase by **LCL2**. Indicated amounts of the purified SHP2 were incubated with **LCL2** at 25 °C for 20 min. The reaction mixtures were separated by 10% SDS–polyacrylamide gel and then subjected to Coomassie blue staining or immunoblotting analysis using anti-biotin antibody. The phosphatase activity was also estimated using *p*-NPP assay. The level of **LCL2** labeling was quantified using a densitometer and plotted against the activity of phosphatase. The result is obtained from the average of three experiments. The error bars indicate 1 standard deviation. (C) Activity dependent labeling of phosphatase by **LCL2**. Three micrograms of PTP1B were mixed with indicated amount of orthovanadate and 0.5 mM **LCL2** at 25 °C for 20 min. Analysis of the reaction products were similar to that of (B) with the exception that HRP-conjugated streptavidin is used in immunoblotting analysis.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Approximately 2×10^3 cells were plated onto each well of a 96-well plate and incubated in 5% CO_2 at 37 °C for 24 h. To assess the *in vitro* cytotoxicity, indicated amount of cisplatin, 5-FU, 5-azacytidine, or etoposide were freshly dissolved in DMSO, added to the cells at 37 °C and the cell were incubated for another 48 h. Afterwards, MTT (USB) was added to each well to the final concentration of 0.5 mg/mL and incubated at 37 °C for 4 h. A 100 μL solution of lysis buffer containing 20% SDS and 50% *N,N*-dimethylformamide was added to each well and incubated at 37 °C for another 16 h. The absorbance at 570 nm was measured using an ELISA reader.

Immunoprecipitations. To prepare total cell extracts, H1299 cells were washed with cold PBS and then lysed in Tris buffer (Tris–HCl 50 mM, NaCl 150 mM, EDTA 1 mM, DTT 1 mM, TritonX-100 1%) at 0 °C for 30 min. The total lysates were mixed by vortexing for 2 min and then centrifuged at 20,000*g* for 10 min. The supernatants were adjusted to 1 μg/μL protein concentration with the Tris buffer and then subjected to **LCL2** labeling. In a typical reaction, 500 μg of cell extract were incubated with 0.5 mM **LCL2** in Tris buffer at 25 °C for 40 min. Antibodies (3 μg) against specific PTP were then added into the reaction mixtures at 4 °C for 2 h to immunoprecipitate PTP proteins. The immuno-complexes were precipitated with protein A-agarose (0.003 g/reaction) at 4 °C for 2 h, washed twice with Tris buffer, and then separated by SDS–PAGE. Detection of immunoprecipitation efficiency and the extent of **LCL2** labeling were conducted using immunoblotting analysis as described earlier.

Results

*Correlation between the extent of **LCL2** labeling and phosphatase activity*

We have previously shown that **LCL2** selectively labeled PTPs in an activity dependent manner [12]. To evaluate whether the extent of **LCL2** labeling correlates with the activity of phosphatase, we conducted two sets of labeling experiments on purified PTPs. The extent of **LCL2** labeling was determined through quantification of the immunoblotting signals using anti-biotin antibody. In a parallel experiment, the phosphatase activity was measured by a colorimetric assay using *p*-NPP as substrate [14]. We monitored the levels of **LCL2** labeling on varying amounts of purified recombinant SHP2. A linear relationship between the extent of **LCL2** labeling and the phosphatase activity was observed (Fig. 1B). The second set of labeling study was carried out with purified PTP1B. Equal amounts of purified PTP1B were first treated with varying concentrations of orthovanadate, a general phosphatase inhibitor, to attenuate the phosphatase activity and thereafter subjected to **LCL2** labeling. As shown in Fig. 1C (top gel), the **LCL2** labeling decreased with decreasing residual PTP1B activity. A linear correlation was apparent for these two measurements. Thus, both sets of experiments confirmed that the levels of **LCL2** labeling could reflect the phosphatase activity of PTPs. More significantly, the labeling of **LCL2** to PTPs could be used as an indicator for phosphatase activity.

Cisplatin selectively activates SHP2 phosphatase in cancer cells

The key issue for a successful detection of specific PTP activity in cells is to discriminate the signal of the target PTP from the unwanted interferences. This was achieved in this study by utilizing an antibody against specific PTP to filter the signals. In the newly devised protocol (Fig. 2A), the cell extracts were prepared and incubated with **LCL2** to accomplish labeling of active PTPs. After the labeling event, the target PTP (both labeled and unlabeled) was then separated from the other PTPs and possibly some non-PTP proteins through immunoprecipitation with a target-specific antibody. Immunoblotting analyses were then conducted using antibodies against PTP and biotin to determine the precipitation efficiency and to quantify **LCL2**-labeled target PTP.

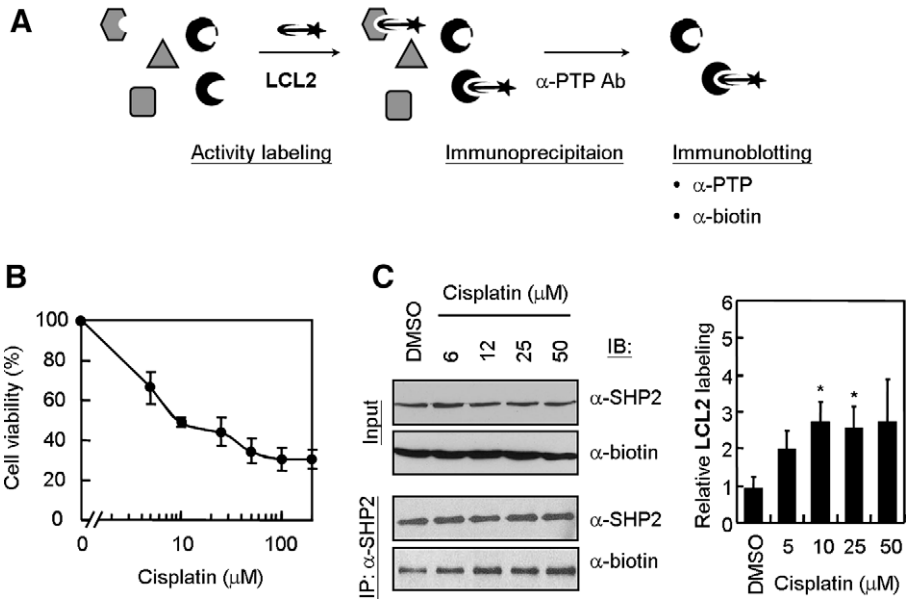

Fig. 2. Detecting the activation of SHP2 activity by cisplatin using the activity probe-based PTP assay. (A) Schematic presentation of activity probe-based PTP assay using **LCL2**. To analyze PTP activity from cell extracts, the cell extracts are first incubated with **LCL2** to label active PTPs. Antibodies against targeting PTPs are then added to immunoprecipitate PTP. Finally, immunoblotting analyses are used to detect the extent of immunoprecipitated and **LCL2**-labeled PTPs, respectively. (B) Viability of cisplatin-treated H1299 cells. H1299 cells were seeded and then treated with varying amounts of cisplatin. Forty-eight hours after treatments, MTT was added into the cells. The survival of H1299 cells was then determined by taking the absorbance at 570 nm. The result is obtained from the average of four repeat. (C) Cisplatin activates SHP2 activity. Cell lysate prepared from cisplatin-treated H1299 cells were collected and then labeled by 0.5 mM of **LCL2** at 25 °C for 20 min. The reaction mixtures were then immunoprecipitated with anti-SHP2 antibody followed by anti-SHP2 and anti-biotin immunoblotting analyses (left). Quantification of the immunoblotting results is presented (right). The relative values of **LCL2** labeling are determined from normalizing the **LCL2**–biotin level with SHP2 level in each experiment and taking no cisplatin treatment as 1. The error bars indicate 1 standard deviation from 3 independent experiments. An asterisk indicates $p < 0.05$.

Here we apply a cell system of which the SHP2 activity was monitored upon cisplatin treatments in the analysis. Cisplatin treatments at 50 μM for 48 h led to 70% cell death whilst higher doses of cisplatin did not appear to further reduce the survival of H1299 cells (Fig. 2B). Total cell extracts from the cisplatin-treated cells were prepared, labeled with **LCL2**, immunoprecipitated by monoclonal antibody against SHP2 and then analyzed by immunoblots using antibodies against SHP2 and biotin, respectively. As shown in Fig. 2C (left, top two blots), cisplatin treatments did not alter the level of SHP2 protein (Input). However, the extent of **LCL2**-labeled SHP2 increased upon treatment with cisplatin (Fig. 2C, bottom two blots). Quantification of the results also indicated that the **LCL2**-labeled SHP2 were increased ~3-fold upon cisplatin treatments (Fig. 2C, right panel). Thus, the phosphatase assay we established by combining the activity probe and a specific antibody is capable of determining the alteration of phosphatase activity in a cellular process.

To test if the activation of SHP2 by cisplatin treatment is selective, the activities of PTP1B and TCPTP were also analyzed. Applying the same protocol, but with different antibodies, the activities of SHP2, PTP1B and TCPTP were simultaneously determined. The overall protein levels of these three PTPs did not appear to be altered by 50 μM cisplatin (Fig. 3). However, the **LCL2**-labeled SHP2 steadily increased until 1.5 h and only decreased slightly after 3 h of cisplatin treatment. During this time, there was no apparent alteration in the concentrations of **LCL2**-labeled PTP1B and TCPTP (Fig. 3). Thus, cisplatin appeared to selectively activate SHP2 activity in cancer cells. It is also important to note that the activity assay protocol could be extended to detect the activities of other PTPs.

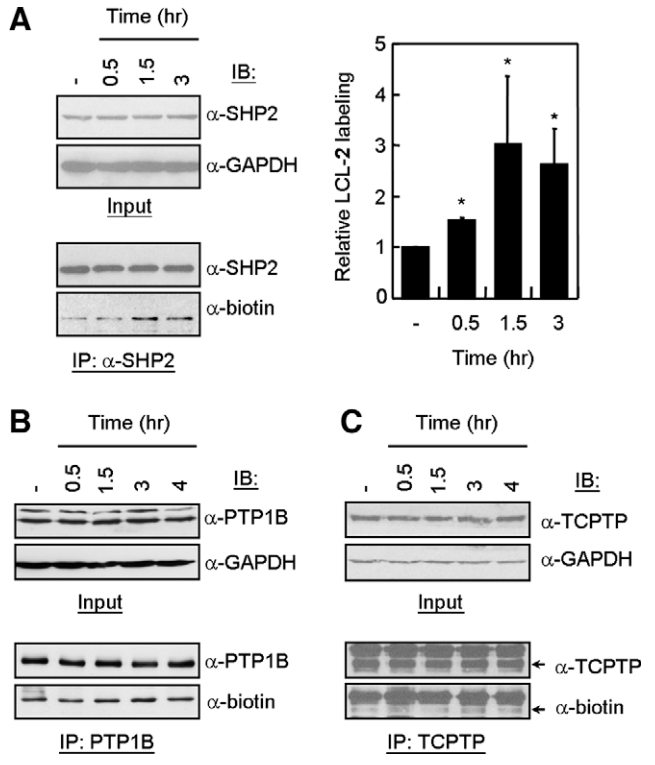

Fig. 3. Selective activation of SHP2 activity by cisplatin. Cell extracts were prepared from H1299 cells that were treated with 50 mM cisplatin for 0.5, 1.5, or 3 h. 500 μg of extracts were incubated with 0.5 mM of **LCL2** at 25 °C for 20 min. The reaction mixtures were then immunoprecipitated by anti-SHP2 (A), anti-PTP1B (B), or anti-TCPTP (C) antibody and then analyzed by immunoblottings. Quantifications of the relative SHP2 activities are presented. An asterisk indicates $p < 0.05$.

Specific activation of SHP2 by cisplatin in cancer cells

With the PTP activity assay protocol established, we proceeded to determine if the SHP2 activity is activated by other anti-tumor agents including 5-FU, etopoxide, and 5-azacytidine. These three drugs affect tumor growth through distinctly different mechanisms. H1299 cells were first treated with different doses of 5-FU, etopoxide, and 5-azacytidine to determine its sensitivity to these drugs (Fig. 4, left panels). It appeared that these drugs showed similar toxicity effects on H1299 cells, having IC_{50} values in the range of ~5–20 μM. We next evaluated the effects of 50 μM drugs on SHP2 activity. As showed in Fig. 4, the **LCL2**-labeled SHP2 seemed unchanged after treating with 5-FU, etopoxide, or 5-azacytidine. Prolonged treatments of these drugs up to 24 h did not show any significant change in the SHP2 activity of H1299 cells either (data not shown), indicating that activation of SHP2 activity was specific to cisplatin treatments.

Discussion and conclusion

Currently, there are two common assays for the detection of phosphatase activity – the colorimetric assay using *p*-NPP as substrate and the in-gel assay using radioisotope-labeled phosphopeptide substrates. Both assays have their merits, but neither of them provides a complete solution to determine the activity of

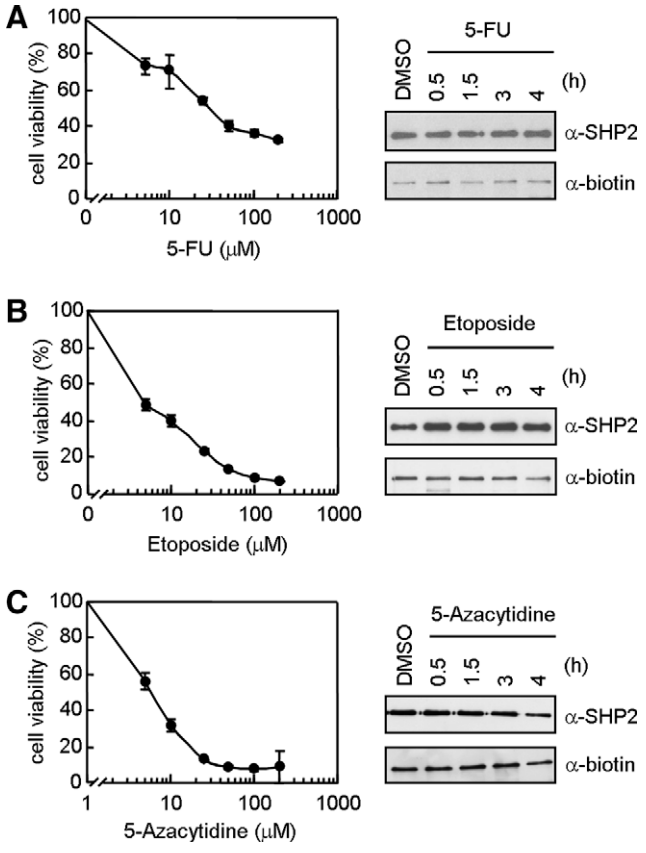

Fig. 4. SHP2 activity is not activated by other anti-tumor drugs. H1299 cells were seeded and then treated with varying amounts of (A) 5-FU, (B) etoposide, and (C) 5-azacytidine. The survivals of H1299 cells were then determined after 48 h of treatments (left panels). The results are obtained from average of four experiments. Cell extracts were also prepared from H1299 cells that were treated with 50 mM of (A) 5-FU, (B) etoposide, and (C) 5-azacytidine for 0.5, 1.5, 3, or 4 h. 500 μg of extracts were incubated with 0.5 mM of **LCL2** at 25 °C for 20 min. The reaction mixtures were then immunoprecipitated by anti-SHP2 antibody and then analyzed by immunoblottings (right panels).

PTPs. The colorimetric assay is easy to operate and requires only a spectrophotometer and a commercially available reagent *p*-NPP [14,15]. However, *p*-NPP is also a general substrate for the great majority of phosphatases. Measurement of its hydrolysis represents the collective action of the phosphatases present in the assay mixture. Therefore, additional steps are required to remove the interfering proteins when the analysis is applied into cell extracts, implying that a partial purification of the target phosphatases should be performed prior to the activity assay. The problem could be partly solved by the in-gel assay, which first separates protein sample with a SDS–polyacrylamide gel and then uses an isotope-labeled substrate to detect the PTP activity [15]. It is sensitive and provides a gel-based format to present PTP activity. However, because the peptide substrate used in the assay may not be compatible with all PTPs and a denaturation/renaturation step is required, the in-gel assay is limited to PTPs that can be renatured to gain their activities. Moreover, the use of radioisotope in the in-gel assay, although sensitive, might be hazardous to the environment. Thus, it is a highly demanding task for the PTP research community to develop a more convenient detection method for individual PTP activity from cells.

In this study, by taking advantage of activity-based probes, we have developed a new assay to detect PTP activity using simple immunoprecipitation and immunoblotting procedures. Through the combination of a universal PTP activity probe and a specific antibody, the phosphatase activity of a specific PTP could be determined and quantified. This newly devised protocol carries the following features: first, although the phenylphosphate moiety of **LCL2** does not discriminate specific PTP, it could serve as a general recognition unit for all PTPs. Second, we have established a cassette-like synthesis strategy that enables the production of a large quantity of **LCL2**. The source of the key reagent is therefore not a concern. Third, the assay does not involve radioisotopes or the denaturing/renaturing of PTPs. Fourth, the success for the assay depends on the availability of a specific antibody for the target PTP. Fortunately, there are more than 20 different commercially available PTP antibodies that could be easily obtained. We believed that our established assay could be used to more precisely define the function of PTP in a cellular process. The general concept of combining a class-selective activity probe with specific antibodies can also be applied to analyze the activity of other enzyme families.

Cisplatin is a potent compound used in anticancer chemotherapies. It forms intrastrand/interstrand DNA adducts that induces apoptosis through signal transduction pathways via ATR, p53, p73 and MAPK [16,17]. Using the current method, we have shown that the SHP2 activity could be activated by cisplatin treatment. The activation appears to be specific to cisplatin since other anti-tumor agents did not activate the SHP2 activity. We also showed that the activity of PTP1B and TCPTP were not activated by cisplatin treatment. Our results agree with prior studies that demonstrated that SHP2 is induced by cisplatin [18]. Although the exact role of SHP2 activation in cisplatin-treated cancer cells is unclear, SHP2 could affect the signaling downstream of cisplatin by regulating the phosphorylation status of p73 and c-abl proteins [18,19] and might also modulate cisplatin-induced apoptosis through regulating members of MAPK pathway [20–23]. Our method could become a powerful tool for elucidating the molecular mechanism of SHP2 in cisplatin-induced cellular effects. The method could also be extended to understand the functional roles of other PTPs.

Acknowledgments

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