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Design and Synthesis of Class-Selective Activity Probes for Protein Tyrosine Phosphatases

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Two mechanism-based activity probes, adopting a cassette-like design, for protein tyrosine phosphatases (PTPs) were synthesized. Both probes carry a phosphate group that serves as the recognition head for the target PTPs but differ in their reporter groups; probe LCL-**1** uses a dansyl fluorophore, while LCL-**2** has a biotin reporter group. LCL-**1** and LCL-**2** are specifically activated by phosphatase, leading to its covalent labeling, as exemplified with PTP-1B. However, they show no activation with other classes of hydrolases, including trypsin and β-galactosidase. LCL-**1** and LCL-**2** thus represent the first example of class-selective probes for phosphatases.

Keywords: activity probe • labeling • protein tyrosine phosphatase • PTP1B • proteomics • quinone methide • signaling

Introduction

Total genomic DNA sequencing has revealed the genomic structures and configurations of many organisms. However, these genomic sequences provide only static information that does not describe the dynamic processes in living cells that are mainly carried out by proteins. For example, the expression of genes in different tissues at different times is varied. It is thus important to locate and identify the protein products on a timely basis in order to understand their functions. Even though the microarray technique has attempted to address such a question, it is limited to the analysis of gene expression at the transcriptional level. On the other hand, recent advances in proteomics studies has served this purpose well.[1−4] Huge arrays of protein products can now be separated with two-dimensional gel electrophoresis. By comparing the difference, proteins of interest could be identified and characterized. However, the presence of a large variety of proteins within cells makes it is difficult to obtain clear solutions from a simple proteomic analysis. This problem would, nevertheless, be greatly simplified if the analysis could be confined to certain functionally related protein families, such as the family of protein tyrosine phosphatases (PTPs), and studied one at a time with the assistance of activity probes toward this enzyme family.

Activity probes are substrates of enzymes that are capable of turning on the trapping mechanism during the action of enzymatic hydrolysis, leading to covalent modifications of the enzymes. The concept of activity probes originates from the suicide substrates of enzyme,[5] but with a different purpose. A suicide substrate is designed to link to an essential residue in the active site of an enzyme to block its enzymatic activity, whereas an activity probe is required for labeling purposes and functions independently from the sites of reaction. The labeled enzymes could then be monitored on the basis of the properties of the reporter groups. This approach has been found to be useful in a variety of applications, including screening of phage-displayed libraries for catalytic antibodies and for mutant β-lactamases.[6,7]

Reversible protein phosphorylation−dephosphorylation is one of the main mechanisms that living cells use in the signal transducing systems and in the regulation of their biological activities.[8,9] The phosphorylation status of the target proteins is exquisitely tuned by the actions of two enzymes, protein kinases (PKs) and phosphatases.[10,11] The former is responsible for adding a phosphate group to a serine, threonine, or tyrosine residue, while the latter serves to remove the phosphate group from these amino acid residues. These two enzyme families are thus important targets in medicinal chemistry and biochemical research. In addition, protein tyrosine phosphatase 1B has been implicated as a negative regulator of the insulin receptor-mediated signaling pathway.[12−14] Due to their importance, increasing efforts have been devoted to the studies of PTP-1B inhibitors.[15−19] Here, we report the design and synthesis of two class-selective activity probes, LCL-**1** and **2**. Using PTP-1B as a model target, we have demonstrated that it could hydrolyze these two activity probes, resulting in selective labeling of PTP-1B.

Experimental Procedures

All reagents and starting materials were obtained from commercial suppliers (Acros, Aldrich, and Merck) and were used without further purification. ^{1}H and ^{13}C NMR were recorded using a Bruker Avance 400 spectrometer. ^{1}H NMR

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spectra in $CDCl_3$ were referenced to residual $CHCl_3$ at 7.24 ppm and ^{13}C NMR spectra to the central peak of $CDCl_3$ at 77.0 ppm. High-resolution mass spectra were recorded on a JEOL-102A mass spectrometer. Analytical TLC (silica gel, 60F-54, Merck) and spots were visualized under UV light and/or phosphomolybdic acid−ethanol. Column chromatography was performed with Kiesegel 60 (70−230 mesh) silica gel (Merck). β-Galactosidase (from *E. coli*) and trypsin (from porcine pancreas) were purchased from Sigma. Both trypsin and β-galactosidase possess active hydrolytic activities. A mixture of proteins was obtained from Amersham Pharmacia.

Synthesis of Probes LCL-1 and 2. Compound numbers are shown in bold.

Phosphoric Acid Diallyl Ester 4-[[2-[2-[2-[5-(Dimethylamino)naphthalene-1-sulfonylamino]ethoxy]ethoxy]ethylcarbamoyl]fluoromethyl]phenyl Ester (6). To a cooled solution of the TFA salt **5** (112.1 mg, 0.195 mmol) and triethylamine (110 μL, 0.78 mmol) in CH_2Cl_2 (1 mL) was added dansyl chloride (55.0 mg, 0.205 mmol) in CH_2Cl_2 (3 mL). The reaction was allowed to warm to room temperature and stirred for 13 h. It was then concentrated and subjected to silica gel column chromatography for purification (CH_2Cl_2/MeOH = 98/2 → 94/6). Compound **6** was obtained as an oil (108.3 mg, 80%). R_f = 0.50 (MeOH/$CHCl_3$ = 1/9). IR (KBr): 3310, 2952, 2782, 1673, 1500, 1268, 1215, 1142, 1016, 943, 791 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.49 (d, J = 8.4 Hz, 1 H), 8.28 (d, J = 8.4 Hz, 1 H), 8.20 (d, J = 7.5 Hz, 1 H), 7.49−7.46 (m, 2 H), 7.41 (d, J = 8.1 Hz, 2 H), 7.19 (d, J = 8.1 Hz, 2 H), 7.13 (d, J = 7.5 Hz, 1 H), 7.06 (br, 1 H, NH), 5.96−5.89 (m, 2 H, =CH), 5.69 (d, J = 48.2 Hz, 1 H, CHF), 5.64 (br, 1 H), 5.35−5.20 (m, 4 H, $=CH_2$), 4.61−4.57 (m, 4 H, allylic), 3.50 (m, 4 H), 3.42 (m, 2 H), 3.35 (m, 4 H), 3.05 (m, 2 H), 2.83 (s, 6 H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.3 (d, J = 21.8 Hz), 151.8, 151.2, 134.9, 131.9, 131.7 (d, J = 16.6 Hz), 130.3, 129.8, 129.6, 129.3, 128.3, 128.2, 128.2, 123.1, 120.1, 118.8, 115.1, 91.0 (d, J = 187.2 Hz), 70.1, 69.4, 69.2, 68.9, 68.8, 45.3, 42.9, 38.8. ^{19}F NMR (400 MHz): δ −178.1 (d, J = 48.2 Hz). ^{31}P NMR (400 MHz): δ −5.94 (d, J = 1.9 Hz). FAB−HRMS for $C_{32}H_{42}FN_3O_9PS$ (M^+ + 1) calcd 694.2363, found 694.2341.

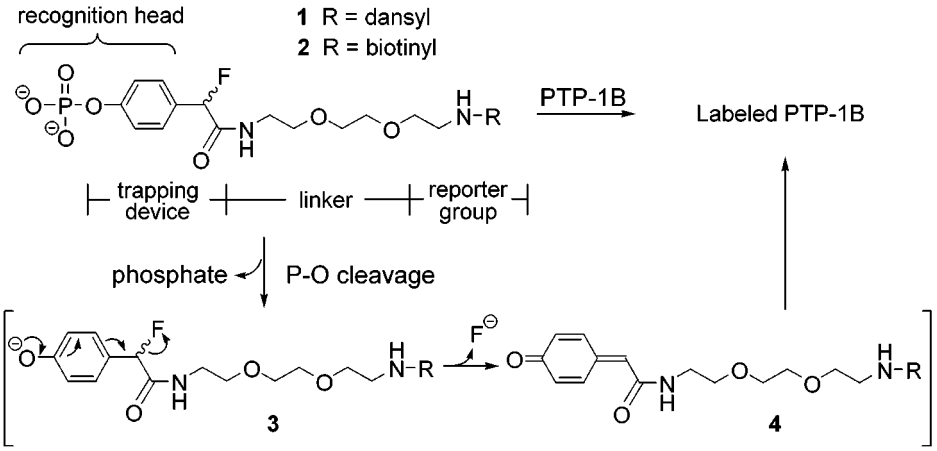
Phosphoric Acid Diallyl Ester 4-[Fluoro[2-[2-[2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-4-yl)pentanoylamino]ethoxy]-ethoxy]ethylcarbamoyl]methyl]phenyl Ester (7). Biotinyl-OSu (52.6 mg, 0.154 mmol) dissolved in warm DMF (1 mL) was added to a solution of the TFA salt **5** (82.0 mg, 0.14 mmol) and triethylamine (79 μL, 0.56 mmol) in DMF (1 mL). The reaction mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure to give a syrup. The desired product **7** was purified by silica gel column chromatography eluted with CH_2Cl_2/MeOH (95/5 → 80/20). Compound **7** (79.4 mg) was obtained in 80% yield. R_f = 0.15 (MeOH/$CHCl_3$ = 1/9). ^1H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, J = 8.4 Hz, 2 H), 7.38 (br, 1 H, NH), 7.18 (d, J = 8.4 Hz, 2 H), 6.83 (br, 1 H, NH), 6.42 (br, 1 H, NH), 5.95−5.85 (m, 3 H), 5.72 (d, J = 48.2 Hz, 1 H, CHF), 5.32 (dd, J = 17.1, 1.3 Hz, 2 H), 5.21 (dd, J = 11.1, 1.3 Hz, 2 H), 4.60−4.51 (m, 4 H), 4.42 (m, 1 H), 4.24 (m, 1 H), 3.60−3.45 (m, 10 H), 3.38−3.34 (m, 2 H), 3.06 (m, 1 H), 2.84 (m, 1 H), 2.66 (m, 1 H), 2.14 (m, 2 H), 1.80−1.52 (m, 4 H), 1.40−1.30 (m, 2 H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.4, 168.4, 164.2, 151.1, 132.0, 131.8, 128.3, 120.1, 118.7, 90.0 (d, J = 186.6 Hz), 69.9, 69.8, 69.2, 68.8, 68.8, 61.6, 60.1, 55.6, 40.3, 38.9, 38.8, 35.6, 28.1, 27.9, 25.5. FAB−HRMS for $C_{30}H_{45}FN_4O_9PS$ (M^+ + 1) calcd 687.2628, found 687.2635.

Triethylammonium Phosphate Mono-4-[[2-[2-[2-[5-(dimethylamino)naphthalene-1-sulfonylamino]ethoxy]ethoxy]-ethylcarbamoyl]fluoromethyl]phenyl Ester (LCL-1). To a cooled solution of compound **6** (109.0 mg, 0.157 mmol) in 4.5 mL of 1,4-dioxane/THF/H_2O (1/1/1) was sequentially added triethylamine (150 μL), HCOOH (150 μL), PPh_3 (14.0 mg), and $Pd(PPh_3)_4$ (15.0 mg). The reaction mixture was allowed to warm to room temperature and stirred for 17 h. The organic solvents were removed under reduced pressure. Water (10 mL) was added to the residue, and it was extracted three times with diethyl ether (10 mL). The aqueous layer was concentrated to dryness. The residue was subjected to silica gel column chromatography eluted with CH_2Cl_2/MeOH/TEA (85/15/5 → 15/85/5) to offer LCL-**1** (90.9 mg) in 81% yield. R_f = 0.15 ($CHCl_3$/MeOH/TEA = 85/15/1). ^1H NMR (400 MHz, $CDCl_3$): δ 8.45 (d, J = 8.7 Hz, 1 H), 8.29 (d, J = 8.7 Hz, 1 H), 8.16 (d, J = 7.5 Hz, 1 H), 7.51−7.41 (m, 2 H), 7.24 (br, 4 H), 7.18 (br, 1 H, NH), 7.11 (d, J = 7.5 Hz, 1 H), 6.37 (br, 1 H, NH), 5.62 (d, J = 48.5 Hz, 1 H, CHF), 3.50−3.40 (m, 4 H), 3.35−3.25 (m, 6 H), 3.08−3.00 (m, 2 H), 2.90−2.76 (m, 12 H), 1.15−1.03 (m, 9 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.9 (d, J = 22.9 Hz), 154.8, 151.7, 135.4, 130.0, 129.7, 129.6, 128.8, 128.2, 128.1, 123.1, 120.6, 19.1, 115.1, 91.5 (d, J = 184.5 Hz), 70.0, 69.9, 69.3, 69.3, 45.3, 45.3, 42.7, 38.8, 8.6. ^{19}F NMR (400 MHz): δ −169.4. ^{31}P NMR (400 MHz): δ −2.79. FAB−HRMS for $C_{26}H_{34}FN_3O_9PS$ (M^+ + 1) calcd 614.1737, found 614.1688.

Triethylammonium Phosphate Mono-4-[fluoro-[2-[2-[2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-4-yl)pentanoylamino]-ethoxyethoxyethylcarbamoyl]methyl]phenyl Ester (LCL-2). To a cooled solution of compound **7** (136.0 mg, 0.198 mmol) in 5 mL of CH_2Cl_2 was added TMSI (85 μL, 0.594 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 1 h. It was quenched with 10% triethylamine in H_2O (5 mL) and extracted three times with $CHCl_3$ (5 mL). The aqueous layer was separated and concentrated under reduced pressure. It was further subjected to lyophilization to give a solid. The desired product was purified by silica gel column chromatography eluted with CH_2Cl_2/MeOH/TEA (85/15/5 → 15/85/5) to offer LCL-**2** (86.9 mg) in 62% yield. R_f = 0.10 ($CHCl_3$/MeOH/TEA = 80/20/1). ^1H NMR (400 MHz, D_2O): δ 7.49 (d, J = 8.2 Hz, 2 H), 7.32 (d, J = 8.2 Hz, 2 H), 5.96 (d, J = 47.5 Hz, 1 H, CHF), 4.62 (m, 1 H), 4.46 (m, 1 H), 3.78−3.68 (m, 8 H), 3.60−3.55 (m, 2 H), 3.46−3.42 (m, 2 H), 3.38 (m, 1 H), 3.21 (q, J = 7.3 Hz, 6 H), 3.04 (dd, J = 13.1, 4.8 Hz, 1 H), 2.81 (d, J = 13.1 Hz, 1 H), 2.36−2.30 (m, 2 H), 1.84−1.58 (m, 4 H), 1.50−1.40 (m, 2 H), 1.30 (t, J = 7.3 Hz, 9 H). ^{13}C NMR (100 MHz, D_2O+CD_3OD): δ 177.7, 172.2 (d, J = 23.8 Hz), 166.2, 154.5, 130.9 (d, J = 19.5 Hz), 130.0, 121.7, 91.9 (d, J = 182.9 Hz), 70.5, 70.5, 69.8, 69.6, 63.1, 61.2, 56.3, 47.6, 40.7, 39.8, 39.7, 36.4,28.9, 28.6, 26.1, 9.2. ^{19}F NMR (400 MHz): δ −171.7 (d, J = 52 Hz). ^{31}P NMR (400 MHz): δ −3.90. FAB−HRMS for $C_{24}H_{37}FN_4O_9PS$ (M^+ + 1) calcd 607.2003, found 607.1984.

Cloning, Expression, and Purification of PTP-1B. The cDNA encoding the catalytic domain of human PTP-1B (1−321) was obtained by RT-PCR using mRNA prepared from human lung cancer cell line H1299. The primers used were PTP1w (5′-AACCATGGAGATGGAAAAGGAGTT-3′) and PTP321c (5′-AAGTC-GACATTGTGTGGCTCCAGGATTCG-3′). The PCR product was subcloned directly into pGEM-T easy vector (Promega) to obtain plasmid pGEM-PTP1B. The PTP-1B sequence was confirmed by DNA sequencing. To express PTP-1B in *E. coli*, the coding region of PTP-1B was cut from pGEM-PTP1B with *Nco*I and *Spe*I and ligated to the *Nco*I and *Spe*I digested pET6H.[20] The resulting plasmid, pET6H−PTP1B, expressed PTP-1B with 6xHis tagged at the N-terminus. *E. coli* BL21(DE3)-

Scheme 1. Structures of the Activity Probes LCL-**1** and -**2** and Their Mechanism-Based Labeling of Protein Tyrosine Phosphatase 1B



pLysS (Novagen)[21] was used as the host for PTP-1B purification. To purify 6xHis-tagged PTP-1B, 1 L cultures of *E. coli* harboring pET6H−PTP1B were grown to OD_{600} to 0.5 and induced with the addition of 1 mM IPTG. The cells were grown at 30 °C for another 4 h before being harvested by centrifugation. Cells were resuspended in 10 mL of sonication buffer (50 mM NaH_2PO_4 pH 7.8, 300 mM NaCl, 5 mM BME, 1 × protease inhibitors (Calbiochem)) and sonicated to release the cell contents. The sonicated cells were centrifuged at 13000*g* for 15 min at 4 °C to obtain total cell free extracts. Ni−NTA-agarose (Qiagen) (0.5 mL) was added to the total cell free extracts and incubated at 4 °C for 1 h. The resin was washed and eluted with 3 mL of buffer containing 50 mM NaH_2PO_4 pH 8.0, 250 mM imidazole, and 20% glycerol. Purified protein was 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. The yield of PTP-1B was ∼1.5 mg from 1 L of *E. coli* culture. At acetate buffer (100 mM sodium acetate pH 7.5, 50 mM NaCl, 1 mM EDTA) and room temperature, 1 μg of the purified PTP-1B hydrolyzed 2.3 μmol of *p*-nitrophenyl phosphate per minute.

^{19}F NMR Time-Course Study of Incubation of LCL-1 with PTP-1B. The experiment was performed in a NMR tube. PTP-1B (1.6 μg) was mixed with 1.2 mM of LCL-**1** in 0.8 mL of 50 mM phosphate buffer (pH 7.0) at room temperature. The reaction was then monitored at 30 min intervals with ^{19}F NMR.

Labeling of PTP-1B with LCL-2. Purified PTP-1B was incubated with 1 mM of LCL-**2** in reaction buffer containing 50 mM Tris pH 8.0, 1 mM EDTA, and 50 mM NaCl at 4 °C for 30 min. The reaction products were separated by 10% SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 10% nonfat dry milk, washed with TTBS (0.05% Tween-20, 20 mM Tris pH 7.6, 137 mM NaCl), and then treated with a streptavidin−horseradish peroxidase conjugate (Amersham-Pharmacia, 1:2000 dilution) in TTBS with 1% nonfat dry milk for 1 h at 25 °C. Visualization of bound streptavidin−horseradish peroxidase conjugate was achieved by treating the membrane with ECL chemiluminescence reagents (Amersham-Pharmacia) and exposed to film for 0.5−30 min before development. Labeling experiments of LCL-**2** with proteins other than PTP-1B, such as trypsin, β-galactosidase, and mixture of proteins, were similarly conducted.

Results and Discussion

An activity probe consists of four major components: a recognition head, a trapping device, a linker, and a reporter group.[22] The recognition head defines the specificity of the target biocatalysts. In tyrosine phosphatases, it consists of a phenyl phosphate group that is connected to a trapping device derived from *p*-hydroxymandelic acid, which takes advantage of the quinone methide chemistry. The effectiveness of this trapping device has previously been examined and confirmed in the case of phosphotriesterase by analyzing the covalently labeled phosphotriesterase with LC-MS.[22] Although some suicide substrates for phosphatases have been reported using similar quinone methide chemistry, these studies did not include a reporter group in their designs.[23,24]

In this study, activity probes (LCL-**1** and -**2**) carrying two different reporter groups were prepared. Probe LCL-**1** has a fluorescent dansyl group, whereas LCL-**2** carries a biotin as its reporter group. The mechanism-based labeling process of PTP-1B is depicted in Scheme 1. Intermediate **3** is first formed when the designated P−OAr bond in LCL-**1** and -**2** is cleaved by PTP-1B. It quickly undergoes 1,6-elimination of the fluoride, hence resulting in the formation of the highly electrophilic quinone methide **4**. The quinone methide **4** in turn could alkylate the nearby nucleophiles on the phosphatases, resulting in covalent labeling of the enzyme. Probes LCL-**1** and **2** provides two different modes of sample visualization. The fluorescent dansyl group in LCL-**1** is good for direct visualization while the biotin group in LCL-**2** is not only useful for highly sensitive secondary detection but also offers the advantage of labeled sample enrichment.[25]

The synthesis of probes LCL-**1** and **2** begins with compound **5**, a key precursor prepared previously with a cassette-like design (Scheme 2).[26] Compound **5** contains the basic skeleton of the activity probes for tyrosine phosphatases. Its amino tail is ready to attach a suitable reporter group, whereas the phosphate head is temporarily protected as a diallyl ester. For the LCL-**1** series, TFA salt **5** was reacted with dansyl chloride in the presence of triethylamine to give the protected product **6** (yield 80%). The diallyl protecting group of the phosphate head was removed by treatment with $(PPh_3)_4Pd/Ph_3P$/TEA/HCOOH to give LCL-**1** as its triethylammonium salt (yield 81%). In the LCL-**2** series, TFA salt **5** was treated with commercially available biotinyl *N*-hydoxysuccinimide (biotinyl-OSu) to give the protected product **7** (yield 80%). The diallyl protecting

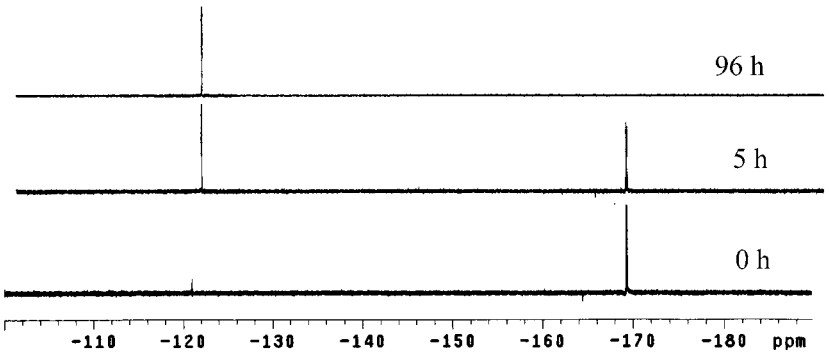
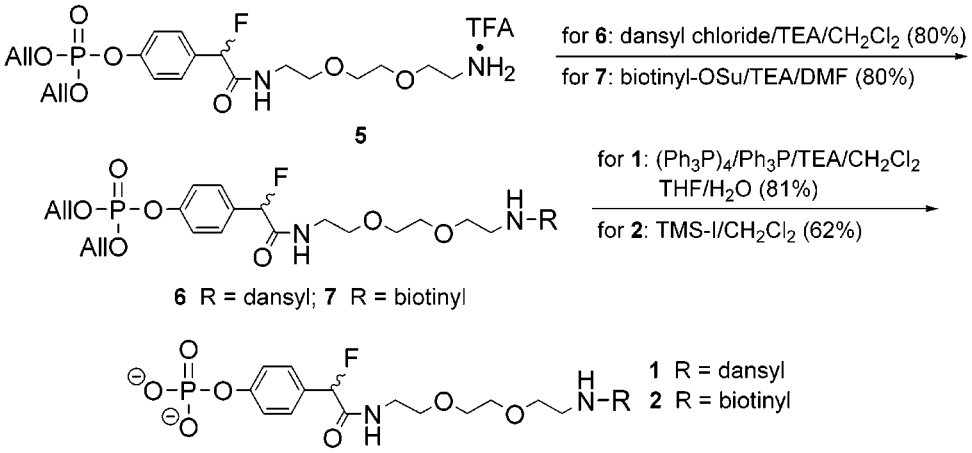


Figure 1. Hydrolysis of LCL-**1** by PTP-1B. Time course study of LCL-**1** incubated with PTP-1B as monitored by ^{19}F NMR at 0, 5, and 96 h (bottom to top): the doublet at −169 ppm is the signal for the starting material; the singlet at −122 ppm represents the inorganic fluoride.

Scheme 2. Synthesis of the Activity Probes LCL-**1** and -**2** for Protein Tyrosine Phosphatases



group was readily removed by treatment with TMSI/CH_2Cl_2 to give LCL-**2**, which was isolated as its triethylammonium salt (yield 62%).

The phosphatase used in this study was a 39 kDa protein with 339 amino acid residues and the catalytic domain of PTP-1B. It was treated with LCL-**1** to determine if the later compound was a possible substrate of PTP-1B. In a typical reaction, probe LCL-**1** (11.6 mM) was incubated with PTP-1B (0.05 μM) in a phosphate buffer (0.79 mL, 50 mM, pH 7.0) at room temperature and the incubation mixture was monitored by ^{19}F NMR, which provides a quantitative determination of the inorganic fluoride released from intermediate **3**. As shown in Figure 1, the doublet at −169 ppm is assigned to LCL-**1** and the singlet at −122 ppm represents the inorganic fluoride. The amount of inorganic fluoride was observed to increase with time (Figure 1, bottom to top) and after 96 h, all the starting material was consumed and only the signal for the inorganic fluoride remained (Figure 1). As a comparison, less than 5% of LCL-**1** was hydrolyzed in the absence of PTP-1B after 96 h. This result indicates that the phenyl phosphate moiety of LCL-**1** mimics the phosphorotyrosine residue of the natural substrate and the long tail carrying the dansyl reporter group does not prevent it from entering the active site.

We next examined the labeling of PTP-1B with LCL-**2**. Two parallel labeling experiments were performed and analyzed (Figure 2). The gel on the left was stained with Coomassie blue that showed the relative amount of loaded proteins. The gel on the right was visualized with streptavidin-conjugated peroxidase chemiluminescence after transferring the reaction

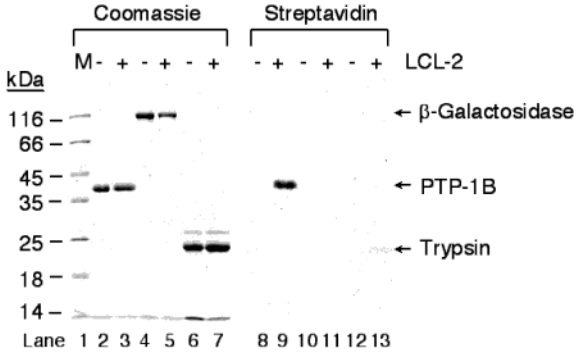


Figure 2. Specific coupling of LCL-**2** to PTP-1B. PTP-1B (2 μg) (lanes 2, 3, 8, and 9), β-galactosidase (2 μg) (lanes 4, 5, 10, and 11), or trypsin (2 μg) (lanes 6, 7, 12, and 13) was mixed with 1 mM of LCL-**2** in buffer containing 50 mM Tris pH 8.0, 1 mM EDTA, and 50 mM NaCl at 4 °C for 30 min. The reaction mixtures were separated by 10% SDS−polyacrylamide gel and subjected to western blotting analysis using streptavidin−horseradish peroxidase conjugate. Bound streptavidin was visualized by chemiluminescence using an ECL kit (Amersham-Pharmacia). The Coomassie blue-stained (left) and the ECL-developed (right) gels were presented.

products onto a nitrocellulose membrane. Lanes 2, 3, 8, and 9 show the labeling results of PTP-1B. In lanes 2 and 3, intense protein bands of PTP-1B were observed, whereas only one band at 39 kDa appeared in the presence of LCL-**2** (lane 9). No labeling was observed in the absence of LCL-**2** (lane 8). To evaluate the selectivity of the activity probes, we also compared

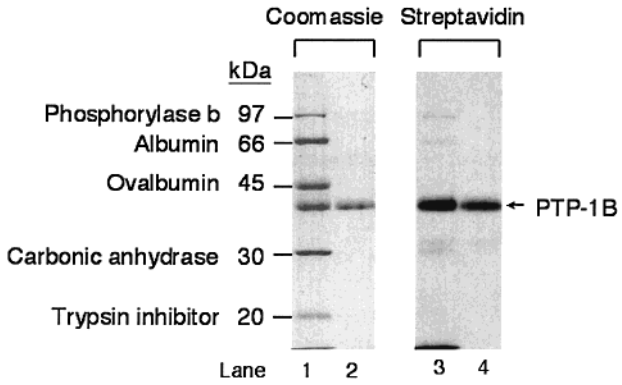


Figure 3. Lack of cross-coupling effect by LCL-**2**. PTP-1B (2 μg) was mixed with (lanes 1 and 3) or without (lanes 2 and 4) 0.85 μg of phosphorylase b, 1.1 μg of BSA, 1.85 μg of ovalbumin, 1.05 μg of carbonic anhydrase, 1.0 μg of trypsin inhibitor, and 1.45 μg of α-lactalbumin. The protein mixture was then incubated with 1 mM of LCL-**2** at 4 °C for 30 min and was analyzed as in Figure 2.

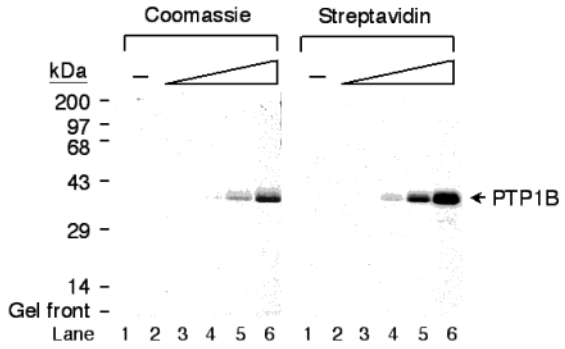


Figure 4. Labeling sensitivity of LCL-**2** to PTP-1B. Varying amounts of PTP-1B were mixed with 1 mM of LCL-**2** at 4 °C for 30 min, and the mixture was analyzed as in Figure 2. The amounts of PTP-1B in lanes 1−6 were 0, 8, 31, 125, 500, and 2000 ng, respectively.

the reactivity of LCL-**2** toward other hydrolases, including trypsin (lanes 4, 5, 10, and 11) and β-galactosidase (lanes 6, 7, 12, and 13). The results obtained show that LCL-**2** did not undergo significant labeling with these two hydrolases (lanes 11 and 13). While it remains to be tested whether these probes also label serine/threonine phosphatases, the result clearly indicated that the labeling is indeed phosphatase-specific.

LCL-**2** does not appear to be an effective substrate for PTP-1B as submilli- to millimolar level of LCL-**2** was required for the labeling reaction (data not shown). To evaluate if such concentration levels of substrate would pose problems when labeling a mixture of proteins, for example during proteomic research, we assessed the effect of cross-labeling of LCL-**2** in the presence of other proteins. Two sets of samples were analyzed (Figure 3). The first sample contained PTP-1B and a mixture of proteins (lanes 1 and 3), and the other sample contained only PTP-1B (lanes 2 and 4). LCL-**2** was incubated with both samples at 4 °C for 30 min, and the mixtures were subjected to 10% SDS−PAGE analysis. From Figure 3, it can be seen that the gel visualized with streptavidin-conjugated peroxidase chemiluminescence showed only a single band of PTP-1B at 39 kDa (lanes 3 and 4). This result indicates that (i) the labeling was specific for phosphatase, hence eliminating the possibility of cross-labeling by bystander proteins during the incubation, and (ii) the presence of other nonrelated proteins did not appear to affect the labeling efficiency of LCL-**2**. A similar labeling experiment was carried out using LCL-**1**. The result is in agreement with that obtained with LCL-**2**. Only one fluorescent band (39 kDa) was visualized under UV irradiation (365 nm), although its signal intensity is weaker than that of Coomassie blue staining. In addition, it was also observed that LCL-**1** could selectively labeled PTP-1B in over-expressed *E. coli* extracts (data not shown). Thus, even though high concentration of activity probe is required for the labeling reaction, we have demonstrated that our activity probes provided sufficient selectivity toward phosphatase.

Since most of the phosphatases involved in the signaling pathways are present only in low abundance, we had to evaluate the sensitivity of the activity probes in detecting cellular phosphatases. Figure 4 shows the results obtained by labeling LCL-**2** with various amounts of PTP-1B. It can be seen that the detecting limit of PTP-1B by LCL-**2** was ~30 ng (~0.8 pmol), a value that is within the range needed to obtain protein-sequence information. Hence, our activity probes is sufficiently sensitive for the analysis of PTPs from cells. In addition, the combination of streptavidin-conjugated agarose beads with the biotin moiety of LCL-**2** could also serve as an enrichment device to enhance the abundance of the phosphatases for further analysis.

The great structural diversity of the PTP family is attributed to the noncatalytic sequences added to the N or C terminus of the catalytic domain. These noncatalytic segments play a regulatory role which includes the ligand binding for receptor PTPs and the transfer of cytoplasmic PTPs to certain subcellular locations.[27,28] Since the structure of the catalytic domain of all PTPs is highly conserved and contains a unique signature motif, $(H/V)C(X)_5R(S/T)$,[29] we envision that the activity probes we prepared in this study could serve as a general probe for the PTP family. However, we should not exclude the possibility that other phosphatases that have a broad range of substrate specificity, such as dual-specificity phosphatases, might also be labeled. However this possibility does not prevent the compounds reported here from acting as a class-selective activity probes for phosphatases, due to its mechanism-based nature.

Conclusion

In conclusion, we have adopted a cassette-like design to construct two activity probes for PTPs. These two probes were activated by PTP-1B, leading to its labeling. Hydrolysis of these two activity probes by PTP-1B appeared to be specific, rendering their selective labeling to PTP-1B. While the fluorescence on LCL-**1** could serve as a tool in direct visualization of the labeled proteins, the biotin reporter group would serve as a sensitive detection unit and potentially could be applied as an enrichment device for labeled phosphatases. Together, the activity probes designed and synthesized in this study represent the first example of class-selective probes for phosphatases. They will provide powerful tools in identifying new members of the important PTP family and in surveying the activity of phosphatases in various cells. This approach together with other class-selective probes will accelerate the search for new disease related targets through proteomics studies.[30]

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