J|A|C|S
COMMUNICATIONS

Published on Web 04/07/2007

Substrate Profiling of Protein Tyrosine Phosphatase PTP1B by Screening a Combinatorial Peptide Library

Mathieu Garaud and Dehua Pei*

Department of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

Received February 22, 2007; E-mail: pei.3@osu.edu

Reversible phosphorylation of proteins on tyrosyl residues is one of the key events that mediate the execution and regulation of many cellular processes. A proper level of phosphorylation is critical for these processes and is controlled by the opposing actions of protein tyrosine kinases, which catalyze the transfer of a phosphoryl group from ATP to the *p*-hydroxyl of tyrosine, and protein tyrosine phosphatases (PTPs), which hydrolyze the phosphotyrosine (pY) back to tyrosine and inorganic phosphate. The human genome encode ~100 PTPs, which share a highly conserved ~250-amino acid catalytic domain and a common catalytic mechanism.[1] In contrast to their well-established catalytic mechanism, the physiological substrates and function of these PTPs remain poorly defined. PTPs appear to have exquisite substrate specificity in vivo, which is at least partially determined by the intrinsic sequence specificity of the catalytic domain.[2] A number of approaches have been employed to define the sequence specificity of PTPs. Initial studies involved kinetic assay of individually synthesized pY peptides.[3] This approach is inherently limited because PTPs can interact with 4−5 residues on either side of pY.[4] In later studies, combinatorial peptide libraries were directly screened against PTPs of interest. However, owing to the lack of stable association between an active PTP and a pY peptide, library screening was typically based on binding instead of catalysis, using either a catalytically impaired PTP mutant or a nonhydrolyzable pY analogue.[5,6] As a result, none of the previous studies have led to comprehensive specificity data on any PTP. Here we report a novel combinatorial library method for the rapid determination of the substrate specificity of PTPs and its application to PTP1B, the prototypical PTP and a target for antidiabetic drug design.[7]

The main challenge in screening pY peptide libraries against a PTP is how to differentiate a small amount of reaction product (i.e., Y peptides) from a large excess of unreacted pY peptides. Our strategy is to selectively oxidize the tyrosine side chain into an orthoquinone by the enzymatic action of tyrosinase (Figure 1).[8] To test this strategy, a pY library containing five random residues, Fmoc-XXXXXpYAALNBBRM-resin [library A, where X is norleucine (Nle) or any of the 17 proteinogenic amino acids except for Met, Cys, and Tyr; B = β-alanine; theoretical diversity = 1.9×10^6], was synthesized on 90-μm TentaGel resin by the split-pool method.[9] Limited treatment of the library with a small amount of PTP should dephosphorylate those beads that carry the best substrates of the PTP. The exposed Tyr side chain is then oxidized into orthoquinone by treatment with an excess amount of tyrosinase in the presence of atmospheric O_2. The resulting orthoquinone is selectively derivatized with biotin hydrazide. Subsequently, the library is incubated with streptavidin-alkaline phosphatase, which is recruited to any biotinylated beads. Upon the addition of 5-bromo-4-chloro-3-indolyl phosphate, the dephosphorylated (and thus biotinylated) beads should become turquoise colored.[10] The positive beads are manually removed from the library, treated with piperidine to remove the N-terminal Fmoc group, and individually sequenced by partial Edman degradation/mass spectrometry (PED/MS).[11]

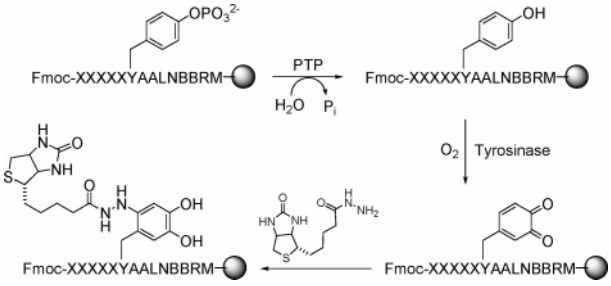

Figure 1. Reactions involved in pY library screening against PTPs.

To test the effectiveness of tyrosinase-mediated derivatization reaction, peptide RALYDNVPE was treated with mushroom tyrosinase and biotin hydrazide and analyzed by HPLC. The untreated peptide had a retention time of 18.8 min; after treatment, the peak at 18.8 min disappeared and a new peak appeared at 16.5 min (Figure S1 in Supporting Information). MS analysis of the reaction product revealed the addition of a single biotin hydrazide molecule to the peptide (Figure S2 in Supporting Information). In contrast, a pY peptide was not affected by the treatment. Next, a pY peptide library TAXXpYXXXLNBBRM (library B, where X is Nle or any of 18 proteinogenic amino acids except for Met and Cys) was subjected to tyrosinase treatment and the screening procedure, resulting in ~20% colored beads. Twenty colored and 20 colorless beads were randomly selected from the library and sequenced. While all 20 colored beads contained at least one Tyr in their sequences, none of the colorless beads contained any Tyr (Tables S1 and S2 in Supporting Information). Finally, when library C (YXXXXXYBBRM) was subjected to the screening procedure, all library beads became colored. These results demonstrate that the oxidation/derivatization reaction is highly selective for Tyr and efficient and that pY is completely resistant to modification under the experimental conditions.

To assess the substrate specificity of PTP1B, a total of 150 mg of library A (~429000 beads) was screened against PTP1B at pH 7.4 in three 50-mg batches. The most colored beads, which should represent the most preferred substrates of PTP1B, were removed from the library and individually sequenced by PED/MS to give 12, 46, and 25 complete sequences, respectively (Tables S3−S5 in Supporting Information). The selected sequences clearly fall into two different classes, with consensus sequences of XXXEWpY (class I) and [RK][AGST][LIV]XXpY (class II), where X can be any amino acid (Figure 2). The same types of sequences were selected from all three experiments, indicating that the library screening method is highly reproducible. In addition to the intensely colored beads, PTP1B treatment also produced medium (~5% of all beads) and lightly colored beads (~30% of all beads). Fifty beads from each color category were randomly selected for sequencing (Tables S6 and S7 in Supporting Information). These

10.1021/ja071275i CCC: $37.00

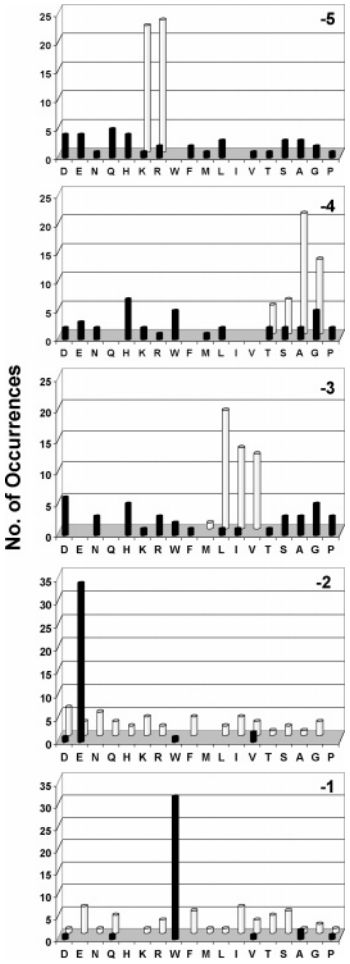

Figure 2. Sequence specificity of PTP1B. Displayed are the amino acids identified at each position from −5 to −1 relative to pY (position 0). Number of occurrences on the *y*-axis represents the number of selected sequences that contain a particular amino acid at a certain position: (closed bar) class I substrates, (open bar) class II substrates, (M) norleucine.

Table 1. Catalytic Constants of PTP1B against Selected Peptides

peptide	bead color	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
KAVFIpYAA	intense	16 ± 2	23 ± 6	700000
RTINEpYAA	intense	13 ± 1	17 ± 2	765000
RTIEWpYAA	intense	7.8 ± 0.1	26 ± 1	300000
QEDEPpYAA	intense	8.3 ± 0.6	20 ± 5	420000
EHTGHpYAA	medium	14 ± 2	290 ± 70	47000
DHVTQpYAA	light		>200	550
RLKQQpYAA	colorless		>200	138

sequences reveal that PTP1B accepts a wide variety of peptides as substrates (albeit less efficiently), but has a general preference for acidic residues N-terminal to pY (Figure S4 in Supporting Information). To identify peptides that are resistant to PTP1B action, library A (10 mg) was treated exhaustively with PTP1B, and 50 of the colorless beads (~40% of all beads) were randomly selected and sequenced (Table S8 in Supporting Information). None of the resistant peptides contained Trp at pY-1 position, and only five peptides had a single acidic residue.

Seven representative peptides from different color categories were individually synthesized, purified, and assayed against PTP1B in solution (pH 7.4). All four peptides derived from intensely colored beads are excellent substrates of PTP1B, with k_{cat}/K_M values in the range of $3-8 \times 10^5$ M^{-1} s^{-1} (Table 1). There is a general correlation between the color intensity of a bead and the kinetic constants (k_{cat}/K_M) of the peptide it carries. Thus, PTP1B recognizes two distinct classes of substrates; the class I substrates have been reported in the literature,[3,5,6] but the class II peptides have not previously been recognized.

A dozen or so proteins have been reported as PTP1B substrates in vivo.[12] The pY sites recognized by PTP1B have been identified in a few of these proteins. The majority of the pY sites including those in insulin receptor,[13] JAK2,[14] and Tyk2 kinases[14] contain (E/D)(Y/pY)pY motifs and belong to class I substrates. STAT5a, a transcription factor involved in cytokine receptor signaling, contains a class II motif, KAVDGpY^{694}VKP.[15] These data suggest that both classes of substrates selected from the peptide library are physiologically relevant.

In summary, a novel combinatorial library method has been developed to systematically assess the substrate specificity of PTPs. Application of the method to PTP1B has revealed that its active site can recognize at least two distinct classes of substrates, one of which was previously unknown. Sequence specificity data of this type should be useful for predicting the protein substrates of PTPs and guiding the design of specific PTP inhibitors.

Acknowledgment. This work was supported by the National Institutes of Health (Grant GM062820).

Supporting Information Available: Experimental details, peptide sequences, HPLC and MS analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Zhang, Z.-Y. *CRC Crit. Rev. Biochem. Mol. Biol.* **1998**, *33*, 1.

(2) (a) Tonks, N. K.; Neel, B. G. *Curr. Opin. Cell Biol.* **2001**, *13*, 182. (b) Tiganis, T.; Bennett, A. M. *Biochem. J.* **2007**, *402*, 1.

(3) For examples see: (a) Cho, H.; Krishnaraj, R.; Itoh, M.; Kitas, E.; Bannwarth, W.; Saito, H.; Walsh, C. T. *Protein Sci.* **1993**, *2*, 977. (b) Zhang, Z.-Y.; Maclean, D.; McNamara, D. J.; Sawyer, T. K.; Dixon, J. E. *Biochemistry* **1994**, *33*, 2285. (c) Harder, K. W.; Owen, P.; Wong, L. K. H.; Aebersold, R.; Clark-Lewis, I.; Jirik, F. R. *Biochem. J.* **1993**, *298*, 395. (d) Dechert, U.; Affolter, M.; Harder, K. W.; Matthews, J.; Owen, P.; Clark-Lewis, I.; Thomas, M. L.; Aebersold, R.; Jirik, F. R. *Eur. J. Biochem.* **1995**, *231*, 673.

(4) (a) Jia, Z.; Barford, D.; Flint, A. J.; Tonks, N. K. *Science* **1995**, *268*, 1754. (b) Yang, J.; Cheng, Z.; Niu, T.; Liang, X.; Zhao, Z. J.; Zhou, G. W. *J. Biol. Chem.* **2000**, *275*, 4066.

(5) (a) Pellegriini, M. C.; Liang, H.; Mandiyan, S.; Wang, K.; Yuryev, A.; Vlattas, I.; Sytwu, T.; Li, Y.-C.; Wennogle, L. P. *Biochemistry* **1998**, *37*, 15598. (b) Huyer, G.; Kelly, J.; Moffat, J.; Zamboni, R.; Jia, Z.; Gresser, M. J.; Ramachandra, C. *Anal. Biochem.* **1998**, *258*, 19. (c) Walchli, S.; Espanel, X.; Harrenga, A.; Rossi, M.; Cesareni, G.; van Huijsduiinen, R. H. *J. Biol. Chem.* **2004**, *279*, 311.

(6) Other library methods applied to PTP include: (a) Vetter, S. W.; Keng, Y.-F.; Lawrence, D. S.; Zhang, Z.-Y. *J. Biol. Chem.* **2000**, *275*, 2265. (b) Wang, P.; Fu, H.; Snavley, D. F.; Freitas, M. A.; Pei, D. *Biochemistry* **2002**, *41*, 10700. (c) Cheung, Y. W.; Abell, C.; Balasubramanian, S. *J. Am. Chem. Soc.* **1997**, *119*, 9568.

(7) Elchebly, M.; Payette, P.; Michaliszyn, E.; Cromlish, W.; Collins, S.; Loy, A. L.; Normandin, D.; Cheng, A.; Himms-Hagen, J.; Chan, C. C.; Ramachandran, C.; Gresser, M. J.; Tremblay, M. L.; Kennedy, B. P. *Science* **1999**, *283*, 1544.

(8) Chen, T.; Vazquez-Duhalt, R.; Wu, C.-F.; Bentley, W. E.; Payne, G. F. *Biomacromol.* **2001**, *2*, 456.

(9) (a) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. Nature **1991**, 354, 82. (b) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84.

(10) Sweeney, M. C.; Wavreille, A.-S.; Park, J.; Butchar, J. P.; Tridandapani, S.; Pei, D. *Biochemistry* **2005**, *44*, 14932.

(11) Thakkar, A.; Wavreille, A.-S.; Pei, D. *Anal. Chem.* **2006**, *78*, 5935.

(12) Bourdeau, A.; Dube, N.; Tremblay, M. L. *Curr. Opin. Cell Biol.* **2005**, *17*, 203.

(13) Bandyopadhyay, D.; Kusari, A.; Kenner, K. A.; Liu, F.; Chernoff, J.; Gustafson, T. A.; Kusari, J. *J. Biol. Chem.* **1997**, *272*, 1639.

(14) Myers, M. P.; Andersen, J. A.; Cheng, A.; Tremblay, M. L.; Horvath, C. M.; Parisien, J.-P.; Salmeen, A.; Barford, D.; Tonks, N. K. *J. Biol. Chem.* **2001**, *276*, 47771.

(15) Akoi, N.; Matsuda, T. *J. Biol. Chem.* **2000**, *275*, 39718.

JA071275I