Microarrays

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A Microarray Strategy for Mapping the Substrate Specificity of Protein Tyrosine Phosphatase**

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Reversible phosphorylation of tyrosine residues in proteins is involved in regulating numerous cellular events, such as cell growth, cell differentiation, cell-cycle regulation, cellular signal transduction, cell adhesion, and the immune response.[1] Phosphorylation is controlled by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Although PTKs have been studied extensively, knowledge about PTP substrate specificities is still limited.[2] The corresponding data could be used to create artificial substrates for kinetic studies,[3] to design inhibitors[4] leading to drug candidates and tools to investigate PTP pathways in cells, and might even give hints for the identification of natural substrates.[5] Several peptide-based approaches have been reported for mapping PTP substrate specificity.[6]

Solution-phase methods are usually employed for the detection of the substrates,[7] but they often do not allow for the rapid screening of multiple substrate candidates in parallel. Microarray techniques offer the opportunity for the fast and efficient screening of a large number of phosphopeptides, as demonstrated for kinase substrate mapping.[8] Up to now however, microarrays have been applied in only a very few cases for substrate mapping of PTPs.[9]

We report here the construction of phosphotyrosine (pTyr) peptide microarrays and their application in the mapping of the substrate specificity of two prototypical PTPs: PTPμ and PTP1B. Our methodology consists of five steps (**A**–**E** in Figure 1). First, a library of individual pTyr peptides is synthesized on the solid phase (**A**). Individual peptides are subsequently immobilized onto glass surfaces in a spatially addressable manner (**B**, **C**). For this covalent immobilization, we[10] made use of the chemoselective Staudinger ligation between an azide and a phosphine previously reported by the research groups of Raines and Bertozzi.[11] The pTyr peptide microarrays are then incubated with the PTP of interest (**D**), washed, treated with a fluorescently labeled anti-pTyr antibody[12] and washed again (**E**). In general, preferred substrates of the PTP display no or lower fluorescence signals relative to a control array, whereas no change in the signal intensity indicates unsuitable substrates.

A library of a tetrapeptide, a hexapeptide, and 46 pentapeptides was synthesized that covered a variety of different types of amino acids in different positions (see the Supporting Information, p. 1). The peptides were prepared following a strategy recently reported by us[10] which employs a resin equipped with a sulfamylbutyryl linker.[12] Release from the resin introduces a hexylchain-linked azide that allows immobilization without any further activation step.

The performance of the pTyr peptide microarray was tested using PTP1B, a well-studied PTP in regard to substrate specificity.[3,6] The amino acid sequence of peptide **1**[19] (Figure 2) is a known substrate for PTP1B ($K_m = 3.2$ μM). Since PTP1B, in general, prefers acidic side chains,[13] peptide **2**[19] (Figure 2) was selected from the library to determine the preference of substrate **1** over **2** to be dephosphorylated. Peptides **1** and **2** were spotted onto phosphane-modified glass slides (1 mM, 250 pL/spot).[12] The phosphanes were attached to dendrimer-coated glass slides that are known to enhance surface coverage.[14] To minimize interactions of the PTP as well as the peptides with the surface we introduced two aminohexyl linkers, that is, one linker between the dendrimer and the phosphane and another one between the peptides and the azide.[12] Incubation of the arrays with various concentrations of PTP1B and subsequent treatment with a conjugate

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

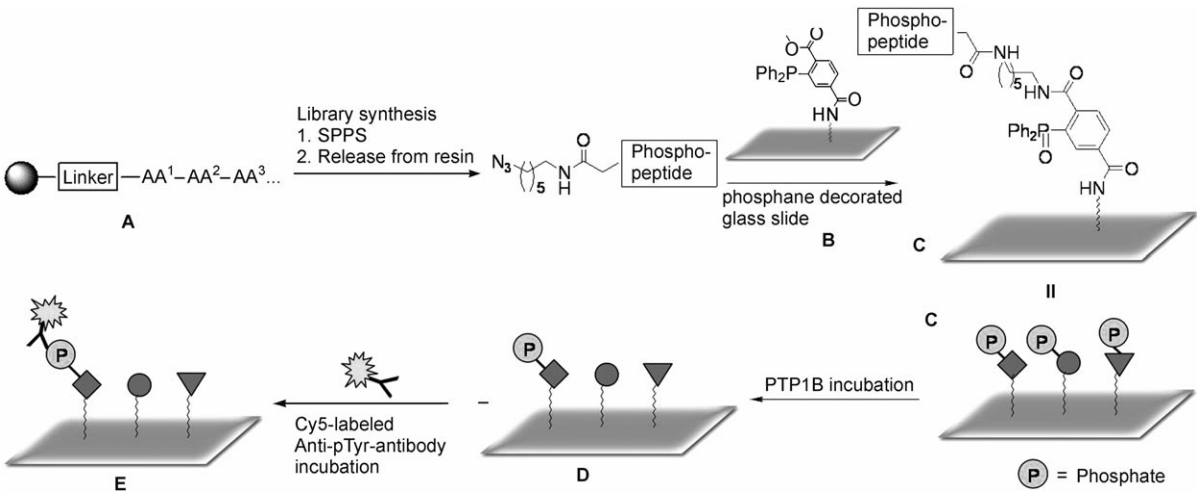


Figure 1. Concept of the construction and use of pTyr peptide microarrays (see text for details).

of the anti-pTyr antibody and biotin-streptavidin-Cy5 (50 nM) showed that both peptides are dephosphorylated by PTP1B (Figure 2). They both exhibit a decrease in the signal intensity as the PTP concentration increases; however, in the case of peptide **1**, the decrease in signal intensity was larger than in the case of **2**. Therefore, **1** is dephosphorylated faster than **2**, which is in agreement with the literature,[13] thereby showing the standard applicability of our microarray method.

We applied the microarray to the identification of peptides that are substrates of PTPμ. PTPμ is a prototypical transmembrane receptor-like phosphotyrosine phosphatase that mediates homophilic cell–cell adhesion.[15a] It binds and dephosphorylates catenin-p120^{ctn}, and the peptides DGDFEEIPEEpYLQ and EGPWLEEEEEApYGWMDF were shown to be substrates of PTPμ.[15b,c] However, little is still known about its intracellular substrates and signaling properties.[15b] The identification of preferred peptide substrates for PTPμ may provide tools that help to elucidate its biological role. The 48 pTyr peptides were spotted into two areas onto glass slides to allow incubation with PTPμ-D1 (the in vitro catalytically active domain D1 of PTPμ) and with buffer solution as a control in parallel (Figure 3). Each individual area contains the pTyr peptides in duplicate. Cy5 azide is cospotted as a standardization factor. For the analysis, the resulting intensities in every area were normalized to the Cy5 signal present in the corresponding area. Subsequently, the normalized signal intensities of the PTP-incubated area were compared to those from the buffer-incubated area, and the relative decrease used for further analysis.[12] This calculation was carried out for two similar microarrays, so that each pTyr peptide was tested four times. The average values from the dephosphorylation experiment on the microarray are listed in Table 1 of the Supporting Table 1.

To define a trend in regard to the properties of the amino acids preferred by PTPμ, the relative frequency of each amino acid in a given position of the peptide substrates that showed a decrease in signal intensity of 65% or more was compared with the structures of the 48 peptides.[12] The results are represented graphically in Figure 4.[16] In general, hydrophobic residues are preferred at positions −2, −1, and +1. Aromatic residues are somewhat favored at position +1, and polar residues occurred at higher frequency at position +2. Remarkably, phosphopeptides having pTyr as the third amino acid were generally better substrates than those having pTyr as the second amino acid. Similar observations concerning the role of N-terminal-flanking amino acids in increasing the rate of dephosphorylation have been made for other PTPs.[3] This finding could reflect that a positive N-terminal charge at position −1 reduces recognition by PTPμ-D1 to a greater extent than a positive charge at position −2. To ensure that our results are not falsified by this effect, we recalculated the frequency of each amino acid on the basis of the 26 peptides having pTyr as the third amino acid.[12] The resulting trend remained the same.

To demonstrate that the results obtained from the microarray analysis correspond to enzymatic activity in solution, all 48 peptides were tested using phosphomolybdate colorimetry.[12,17] Although the two assays used different detection methods, good agreement was found between the results, with only a few exceptions. In the case of PTPμ, from the 15 peptides that showed a signal decrease of 65% or more in the microarray,[12] we found 10 peptides among the most active peptides in the solution-phase assay.[12] The longer PTP incubation time probably resulted in the microarray detecting a higher number of preferred substrates. Remarkably, and importantly, however, no false negatives appeared in the microarray format.

A similar study was carried out for PTP1B, and this also showed good agreement between the microarray and solution-assay data. From the 13 peptides that showed a signal decrease of 65% or more in the PTP1B microarray,[12] 10 peptides were detected among the most active peptides in the solution assay.[12] Notably, peptide **2** was again detected as an inferior substrate to **1**, which confirms the results from the experiment described above. Although we observed some differences in absolute substrate ranking, the results demonstrate that the pTyr peptide microarrays can be reliably applied to a qualitative assessment of PTP substrate specificity and can investigate a large number of compounds in parallel.

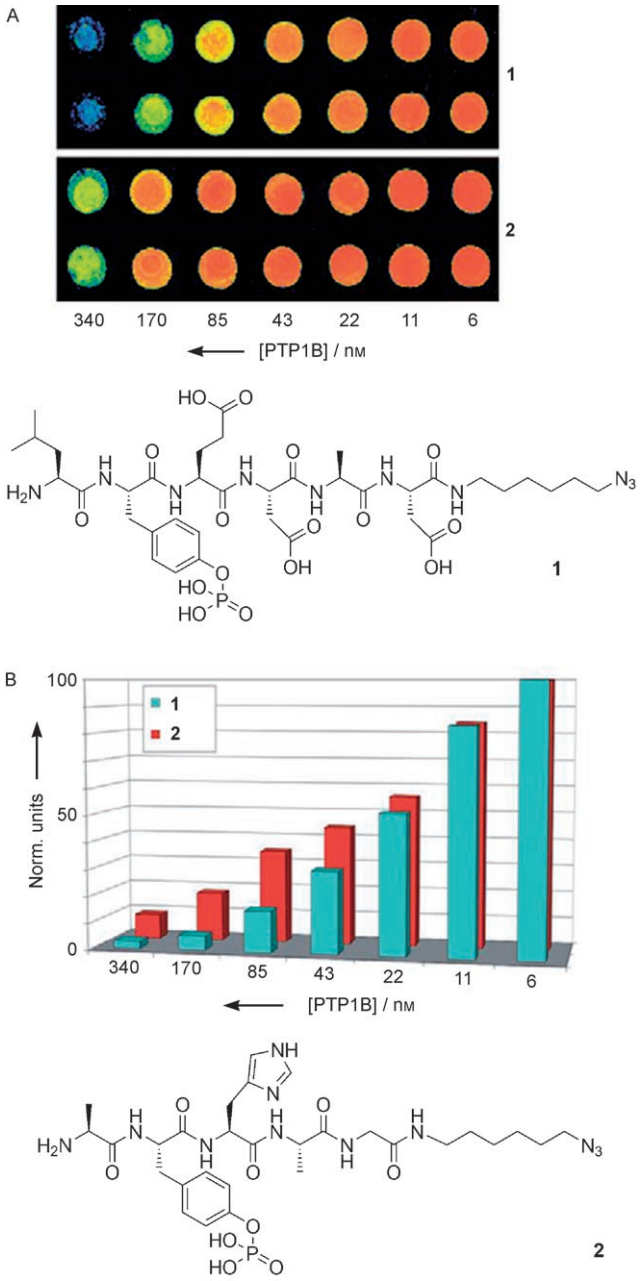


Figure 2. Proof of concept using PTP1B. A) Microarray data; the color change from red to blue (**1**) or green (**2**) shows the decrease in the signal as the concentration increases. B) Histogram of signal intensities.

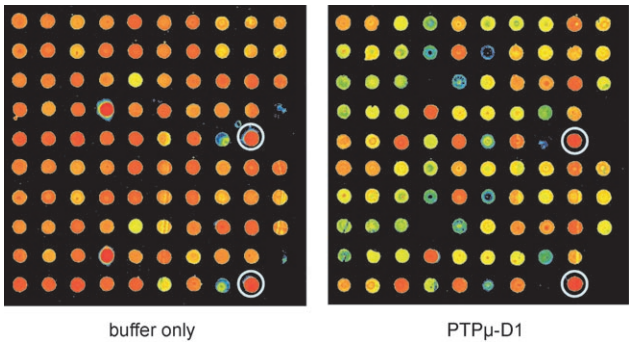


Figure 3. Fluorescence read-out of the Cy5-labeled anti-pTyr-antibody-biotin-streptavidin conjugate (50 nM) bound to immobilized pTyr peptides (1 mM, 250 pL/spot). The slides were incubated (1 h, 37 °C) with PTPμ-D1 (50 μg mL^{-1}) and with buffer solution as a control. The standardization factor of Cy5 is indicated in the circle. All compounds are spotted in duplicate.

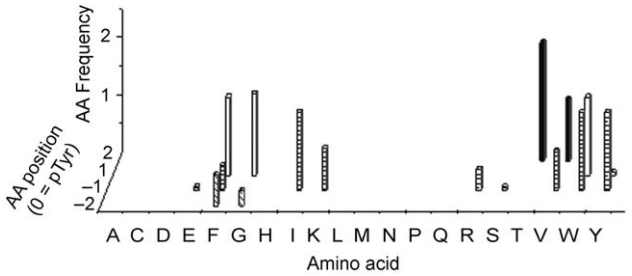
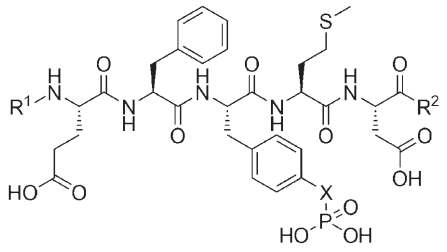


Figure 4. Preference plot for amino acids (AA) at different positions (+2 to −2) in the pTyr peptides based on the microarray data (bars: filled +2, open +1, horizontal stripes −1, diagonal stripes −2). The relative frequency of amino acids in a given position is plotted for those PTPμ substrates that showed a decrease in their signal of 65% or more.



3 X = O, R^1 = H, R^2 = $NH(CH_2)_6N_3$
5 X = O, R^1 = Ac, R^2 = NH_2
4 X = CH_2, R^1 = Ac, R^2 = NH_2

We performed kinetic studies on peptide **3**[19] with PTPμ in solution by phosphomolybdate colorimetry. This peptide was dephosphorylated to yield 379 nmol phosphate min^{-1} mg enzyme, with a K_m value of (53 ± 8) μM and a k_{cat} value of 1.67 s^{-1} at pH 7.5 and 25 °C, which results in a specificity constant of $k_{spec} = 31.5 \times 10^3\,M^{-1}\,s^{-1}$.[18] These data show that substrates that were identified with the microarray can be used for kinetic studies with PTPμ.

Although PTPs usually display broad substrate specificity, deciphering substrate specificity of PTPs may facilitate the discovery of inhibitors. Therefore, peptide **4**[19] was synthesized with a nonhydrolyzable phosphonomethyl-phenylalanine (Pmp) amino acid that replaces the pTyr residue. This phosphonate-based surrogate peptide **4** was then tested in inhibition studies of PTPμ with the hydrolyzable peptide **5**[19] as substrate.[18] We found an IC_{50} value of (50 ± 3) μM. A Pmp-inhibitor analogue of PTP1B substrate **1** with an IC_{50} value of 200 μM has previously been reported.[4]

To gain insight into the binding mode of peptides **4** and **5** to PTPμ, molecular docking studies were performed.[12] The

results showed that the phosphate fits well in the binding pocket confined at the sulfur atom of the cysteine residue in the active site. Detailed analysis of the top 100 orientations identified two sets of preferred conformations (Figure 5A,B)

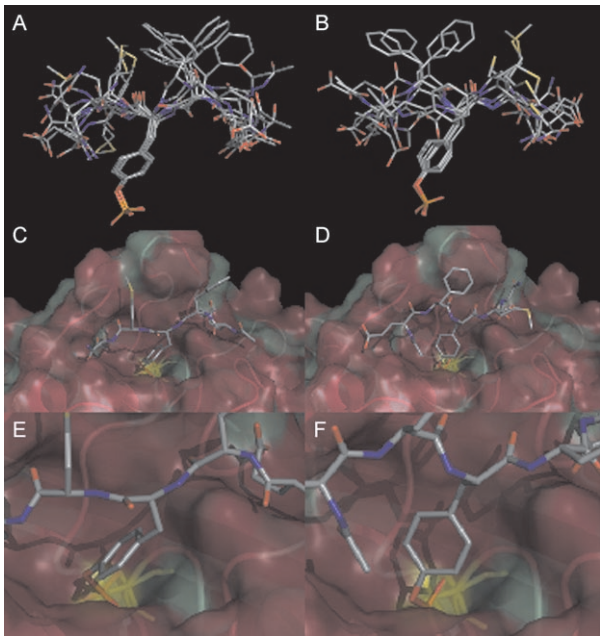


Figure 5. Molecular docking of peptide **5** into the active site of PTPμ. All hydrogen atoms have been omitted for clarity. The protein surface is colored according to apolar (green) and polar (red) properties (see text for details).

that were comparable for the two peptides. These conformations differ in the orientation of the tyrosine phosphate (Figure 5E,F) in the binding pocket, while the peptide chain is rotated by 180°. One representative for each of the two orientations in the binding site of PTPμ is shown in Figure 5C,D.

In conclusion, we have developed an efficient method for preparing phosphopeptide microarrays by employing Staudinger ligation for immobilization, and demonstrated its application for mapping the in vitro substrate specificity of PTPs. Comparison of microarray results with data from solution-phase assays showed clearly that substrate specificity data collected from the microarray are qualitatively reliable. The knowledge gained on substrate specificity was then used to design an inhibitor of PTPμ.

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[1] A. Alonso, J. Sasin, N. Bottini, I. Friedberg, I. Friedberg, A. Osterman, A. Godzik, T. Hunter, J. Dixon, T. Mustelin, *Cell* **2004**, *117*, 699.
[2] L. Bialy, H. Waldmann, *Angew. Chem.* **2005**, *117*, 3880; *Angew. Chem. Int. Ed.* **2005**, *44*, 3814.
[3] Z.-Y. Zhang, A. M. Thieme-Sefler, D. Maclean, D. J. McNamara, E. M. Dobrusin, T. K. Sawyer, J. E. Dixon, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 4446.
[4] T. R. Burke, H. K. Kole, P. P. Roller, *Biochem. Biophys. Res. Commun.* **1994**, *204*, 129.
[5] A.-S. Wavreille, D. Pei, *ACS Chem. Biol.* **2007**, *2*, 109.
[6] a) S. W. Vetter, Y.-F. Keng, D. S. Lawrence, Z.-Y. Zhang, *J. Biol. Chem.* **2000**, *275*, 2265; b) M. Garaud, D. Pei, *J. Am. Chem. Soc.* **2007**, *129*, 5366; c) S. Wälchli, X. Espanel, A. Harrenga, M. Rossi, G. Cesareni, R. Hooft van Huijsduijnen, *J. Biol. Chem.* **2003**, *279*, 311.
[7] J. Montalibet, K. I. Skorey, B. P. Kennedy, *Methods* **2005**, *35*, 2.
[8] M. Schutkowski, U. Reinecke, U. Reimer, *ChemBioChem* **2005**, *6*, 513.
[9] a) X. Espanel, S. Wälchli, T. Rückle, A. Harrenga, M. Huguenin-Reggiani, R. Hooft van Huijsduijnen, *J. Biol. Chem.* **2003**, *278*, 15162; b) X. Espanel, M. Huguenin-Reggiani, R. Hooft van Huijsduijnen, *Protein Sci.* **2002**, *11*, 2326; c) X. Espanel, R. Hooft van Huijsduijnen, *Methods* **2005**, *35*, 64; d) C. Pasquali, M.-L. Curchod, S. Wälchli, X. Espanel, M. Guerrier, F. Arigoni, G. Strous, R. Hooft van Huijsduijnen, *Mol. Endocrinol.* **2003**, *17*, 2228.
[10] a) M. Köhn, R. Wacker, C. Peters, H. Schröder, L. Soulère, R. Breinbauer, C. M. Niemeyer, H. Waldmann, *Angew. Chem.* **2003**, *115*, 6010; *Angew. Chem. Int. Ed.* **2003**, *42*, 5830; b) A. Watzke, M. Köhn, M. Gutierrez-Rodriguez, R. Wacker, H. Schröder, R. Breinbauer, J. Kuhlmann, K. Alexandrov, C. M. Niemeyer, R. S. Goody, H. Waldmann, *Angew. Chem.* **2006**, *118*, 1436; *Angew. Chem. Int. Ed.* **2006**, *45*, 1408.
[11] a) M. B. Soellner, K. A. Dickson, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2003**, *125*, 11790; b) H. C. Hang, C. Yu, M. R. Pratt, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 6.
[12] See the Supporting Information for a more detailed description.
[13] Z. Jia, D. Bradford, A. J. Flint, N. K. Tonks, *Science* **1995**, *268*, 1754.
[14] a) R. Benters, C. M. Niemeyer, D. Wöhrle, *ChemBioChem* **2001**, *2*, 686; b) R. Benters, C. M. Niemeyer, D. Drutschmann, D. Blohm, D. Wöhrle, *Nucleic Acids Res.* **2002**, *30*, E10.
[15] a) S. E. Ensslen, S. M. Brady-Kalnay, *Mol. Cell. Neurosci.* **2004**, *25*, 558; b) G. C. M. Zondag, A. B. Reynolds, W. H. Moolenaar, *J. Biol. Chem.* **2000**, *275*, 11264; c) A. R. Aricescu, T. A. Fulga, V. Cismasiu, R. S. Goody, S. E. Szedlacsek, *Biochem. Biophys. Res. Commun.* **2001**, *280*, 319.
[16] For a preference plot for disfavored amino acids, see reference [12].
[17] K. W. Harder, P. Owen, L. K. Wong, R. Aebersold, I. Clark-Lewis, F. R. Jirik, *Biochem. J.* **1994**, *298*, 395.
[18] **5** gave similar results: $K_m = (8 \pm 1)$ μM, $k_{cat} = 0.995\ s^{-1}$, and $k_{spec} = 12 \times 10^3\ M^{-1}\,s^{-1}$.
[19] Compounds **1**, **2**, **3**, **4**, and **5** correspond to compounds **31**, **50**, **6**, **53**, and **52**, respectively, in the Supporting information.

