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Analysis of Protein Tyrosine Kinase Specificity Using Positional Scanning Peptide Microarrays

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Summary

Protein tyrosine kinases phosphorylate their substrates within the context of specific consensus sequences surrounding the site of modification. We describe a peptide microarray approach to rapidly determine tyrosine kinase phosphorylation site motifs. This method uses a peptide library that systematically substitutes each of the amino acid residues at multiple positions surrounding a central tyrosine residue. Peptide substrates are synthesized as biotin conjugates for immobilization on avidin-coated slides. Following incubation of the slide with protein kinase and radiolabeled ATP, the relative extent of phosphorylation of each of the peptides is quantified by phosphor imaging. This method allows small quantities of kinase to be analyzed rapidly in parallel, facilitating analysis of large numbers of kinases.

Keywords

Protein tyrosine kinases; Peptide microarrays; Enzyme specificity; Substrate profiling; Kinase assay; Signal transduction

1. Introduction

Protein tyrosine kinases (PTKs) are key enzymes in the initiation and propagation of cellular signaling pathways in multicellular eukaryotes (1). Because PTKs often relay cell growth and survival signals, their deregulation frequently contributes to the development of human cancer (2). Accordingly, PTKs have emerged as important drug targets in oncology (3). PTKs function by catalyzing the phosphorylation of specific tyrosine residues in protein substrates. The rate of phosphorylation by a PTK is influenced by the amino acid sequence surrounding the phosphorylated Tyr residue (4). Each PTK has a specific preferred consensus phosphorylation site sequence, or phosphorylation motif. Knowing which sequences are preferred by a kinase facilitates the production of model peptide substrates that can be used in small molecule screening *in vitro* or in cultured cells to identify new kinase inhibitors (5, 6). In addition, bioinformatics tools can be used to identify potential

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PTK substrates by scanning protein sequence databases for sites conforming to the phosphorylation site motif (7, 8).

Approaches to determine consensus motifs phosphorylated by protein kinases typically involve using peptide libraries, in which phosphorylated sequences are selected from a large pool of potential peptide substrates. One approach has been to phosphorylate a complex peptide mixture in solution with the kinase of interest, isolate the phosphopeptides from the pool, and sequence the phosphorylated products using either Edman degradation or mass spectrometry (4, 9). Alternatively, one can examine in parallel the relative phosphorylation rates of a series of peptides either in solution or immobilized on a solid support (10, 11). Peptide microarrays offer the capacity to analyze large numbers of sequences and require small quantities of enzyme, thus facilitating analysis of multiple kinases (12, 13). Here we describe analysis of a positional scanning peptide library (PSPL) in a microarray format (14). Microarrays utilizing PSPLs are unusual in that each spot corresponds to a peptide mixture rather than an individual peptide sequence. The PSPL we describe consists of 189 peptide mixtures of identical length, each having a single Tyr residue at a fixed position. Multiple positions flanking the central Tyr residue are composed of equimolar mixtures of 18 of the 20 amino acid residues. Each microarray spot corresponds to a distinct peptide mixture in which a single residue is fixed at one of 9 positions surrounding the Tyr residue (Figure 1). Microarrays are incubated with the PTK of interest using radiolabeled ATP as a substrate. After washing to remove unincorporated radiolabel, phosphorylation of each peptide mixture on the array is quantified by phosphor imaging. The relative rate of phosphorylation of each component of the PSPL reveals which amino acid residues are preferred or deselected by the PTK at each position within the peptide.

2. Materials

2.1 Microarray components

1. Chicken avidin (see **Note 1**).
2. Binding buffer: 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 10% glycerol, 0.1% Triton X-100.
3. TBST: 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.1% Triton X-100.
4. Humidified chamber: A sealed box with a piece of water saturated paper towel at the bottom.
5. High-density biotin coated glass slides (2.5×7.5 cm, Microsurfaces, Inc.)
6. Distilled, deionized water (ddH₂O).
7. Nitrogen gas.
8. The PSPL (Figure 1, synthesized by Anaspec, Inc.) is a set of 189 peptide mixtures having the general sequence G-A-X-X-X-X-Y-X-X-X-G-A-K-K(biotin) in

¹To avoid potential phosphorylation of avidin itself during the microarray procedure, we have used a bacterially-expressed mutant (Y33F) in which the sole Tyr residue is changed to Phe (15). However, because the Tyr residue is buried within the avidin structure, it is unlikely to be accessible to kinases. Wild-type avidin, either native or recombinant, should be sufficient for use in this procedure.

which $\times$ represents an equimolar mixture of the 18 amino acids (excluding Tyr and Cys) and K(biotin) is ϵ -(*N*-biotin-6-aminohexanoyl)-lysine. In each peptide mixture, one of the $\times$ positions is replaced with one of the 19 unmodified amino acids (excluding Cys), phosphothreonine (pThr), or phosphotyrosine (pTyr) (see **Note 2**).

9. Dimethyl sulfoxide (DMSO).
10. Spotting buffer: 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 15% glycerol, 0.01% Triton X-100 (see **Note 3**).
11. Tabletop centrifuge with microplate rotor.
12. Contact printing microarray robot (for example GeneMachines Omnigrid robotic printer) equipped with low volume delivery pins (see **Note 4**).
13. TBST-biotin buffer: To 1.22 g biotin and 100 ml ddH₂O, add 1M NaOH dropwise with stirring until biotin dissolves completely. Add 700 ml ddH₂O water and 100 ml 10x TBST (100 mM Tris-HCl, pH 7.5, 1.4 M NaCl, 1% Triton X-100). Adjust the pH to 8.0 and add ddH₂O to a final volume of 1L. See **Note 5**.

2.2 Kinase assay components

1. Purified kinase(s) to be assayed (see **Note 6**).
2. Kinase reaction buffer. If the optimal buffer for the kinase is not known, the following buffer works for most protein kinases: 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.5% Triton X-100 and 0.05 μ Ci/ μ l [γ -³³P]ATP (3000 Ci/mmol). See **Note 7**.

²An advantage of using the PSPL is that it provides an indication of the relative rate of phosphorylation of peptides having every possible amino acid at each position within the peptide. However, the PSPL is costly to synthesize, and because it consists of random peptide mixtures some highly specific PTKs may provide an insufficiently low signal. An alternative is to use sets of peptides with defined sequence. Several companies offer either pre-made sets of biotinylated PTK substrates (JPT Peptide Technologies) or economical parallel synthesis of custom peptides (Sigma-Aldrich, Thermo Scientific). We have found that the presence of Cys residues in peptides results in spuriously high signals on the microarray (**14**), perhaps due to immobilization of kinase to the array surface by disulfide formation. We therefore recommend excluding Cys if using a set of custom peptides.

³Glycerol (15%) is included in the spotting buffer to maintain hydration of the peptide spots during the incubation period after arraying. A "ring effect" is a common problem in protein microarrays that can hamper accurate quantitative analysis. This problem can be eliminated by adding detergent (0.01% Triton X-100) to the spotting buffer (**16**).

⁴The avidin-coated microarray surface used in this protocol is extremely hydrophilic relative to standard slides used for DNA microarray fabrication. As a result, the use of standard microarray pins will cause spots to merge together due to excess spreading. It is therefore important to use low volume delivery pins. We used Stealth 3 Micro Spotting Pins (SMP3, ArrayIt), which are rated to deliver 0.7 nl fluid.

⁵TBST-biotin buffer can be stored at 4 °C for up to 2 months and re-used at least 20 times.

⁶A major advantage of the microarray method over other methods for characterizing PTK substrate specificity is that very small quantities of kinase are required for analysis (typically 10 ng or less). This facilitates the use of low-yield mammalian expression systems that have a generally high success rate for producing active kinases compared with bacterial expression. We have typically expressed kinases as either GST or FLAG epitope tag fusion proteins by transient transfection in HEK293T cells and purified by one-step affinity chromatography (**14**, **17**). While mammalian and insect cell expression systems are useful for producing kinases in their active form, a potential problem is that preparations may be contaminated with endogenous kinases that can phosphorylate peptides on the microarray. For this reason, we highly recommend that an inactive mutant of the kinase be generated and analyzed alongside the WT kinase. If the preparation is free of contaminating kinase activity, the mutant protein should produce no signal on the microarray.

⁷It is best to use a buffer known to provide optimal activity for a particular PTK. The most common buffer components influencing kinase activity are monovalent and divalent salts. Some kinases are inhibited by physiological concentrations of monovalent cations (Na⁺ or K⁺), so their salts are often omitted from kinase assay buffers. Kinases require a divalent metal ion cofactor (**18**). While the physiological cofactor is Mg²⁺, some kinases (including many PTKs) are more active in the presence of Mn²⁺. If the suggested buffer does not provide sufficient activity, substituting MnCl₂ for MgCl₂ is recommended.

3. Quenching buffer: 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.1% SDS
4. Humidified chamber: We use a P1000 pipet tip box containing 50 – 100 ml water with the insert turned upside-down so that slides can be placed a few cm above the surface of the water.
5. Standing incubator, set to 30 °C.
6. PAP pen (hydrophobic marker) for marking slides (Abcam).
7. Phosphor storage screen and cassette (GE Healthcare).
8. Phosphor imager instrument (for example Bio Rad Molecular Imager FX Pro Plus).

3. Methods

3.1 Preparation of avidin coated slides for microarray printing

1. Warm biotin-derivatized slides to room temperature (see **Note 8**).
2. Dilute avidin in binding buffer to 50 µg/ml and keep on ice.
3. Apply approximately 200 µl of the diluted avidin solution to each slide to cover the entire surface.
4. Place slides on a piece of parafilm in the humidified chamber. Close the box and incubate for 30 min at room temperature.
5. Wash slides thoroughly with washing buffer, and then rinse with ddH₂O.
6. Blow-dry slides with nitrogen gas. Slides may be stored at 4 °C for up to 4 weeks until printing.

3.2 Preparation of peptide stock solutions

1. Dissolve each peptide in DMSO to achieve a concentration of ~65 µg/ml.
2. Dilute an aliquot of each peptide stock 500-fold in 20 mM HEPES, pH 7.4, and measure the absorbance at 280 nm of duplicate samples.
3. Based on the peptide molar absorptivity, calculate the true peptide concentration in the original DMSO solution (see **Note 9**).
4. Add the appropriate volume of DMSO to each stock to bring the peptide concentration to 10 mM (see **Note 10**).

⁸Slides are generally stored at –80 °C as per manufacturers' instructions. Condensation forming on slides during warming to room temperature does not affect subsequent steps.

⁹Because “dry” peptides can have substantial and variable residual solvent content, weight does not provide a faithful measure of the amount of peptide present. The best way to conveniently determine true peptide concentration is to measure UV absorbance arising from Trp and Tyr residues in the peptide sequence ($\epsilon_{280} = 5690 \text{ M}^{-1} \text{ cm}^{-1}$ for Trp and $1280 \text{ M}^{-1} \text{ cm}^{-1}$ for Tyr) (**19**). Assuming that the mixture positions are 5.5% Tyr and 5.5% Trp, the molar absorptivity is $4350 \text{ M}^{-1} \text{ cm}^{-1}$ for most peptide mixtures in the PSPL, $5550 \text{ M}^{-1} \text{ cm}^{-1}$ for peptides containing Tyr at the fixed positions, and $10040 \text{ M}^{-1} \text{ cm}^{-1}$ for peptides containing Trp at the fixed positions.

¹⁰Peptide DMSO stocks can be stored at –20 °C for several years.

5. Dilute peptides from 10 mM DMSO stock solutions to a final concentration of 1 mM in spotting buffer and aliquot to a round bottom 384-well plate (10 μ L/well). See **Note 11**.

3.3 Microarray printing

1. Remove the aqueous peptide stock plate from the freezer and allow to warm to room temperature. Mix the plate well by manual shaking, and spin the plate in a centrifuge at $2000 \times g$ for 5 min.
2. Mount the peptide stock plate and avidin-coated slides into the plate holder and the slide holder of the microarrayer, respectively.
3. Spot peptides in the humidified chamber of the microarrayer. Peptides should be spotted in duplicate with an inter-spot distance of 0.5 mm, so that each set of 396 (2×189) peptides occupies an 18 mm $\times$ 8 mm area. Up to four duplicate sets of the PSPL can be printed on each slide.
4. Transfer slides to a 50 mL conical tube. Seal the tube and incubate for 1 hour.
5. Remove excess peptide by washing once with 50 ml TBST-Biotin and then three times with 50 ml TBST, 5 minutes per wash.
6. Rinse slides briefly with ddH₂O and then blow dry under a stream of nitrogen. Printed slides should be stored at 4 °C and used within a few weeks.

3.3 Peptide microarray analysis of protein tyrosine kinases

1. Equilibrate the humidified chamber to 30 °C by placing in the standing incubator for 2 hours.
2. Partition the slide by circumscribing each duplicate set of peptides with a PAP pen.
3. Add purified kinase (1–10 ng) in 20 μ l reaction buffer to each duplicate peptide set, place the slide in the pre-warmed humidified chamber. Seal the chamber with parafilm and incubate slides at 30 °C for 30 min.
4. Wash the slide three times with 50 ml quenching buffer and two times with 50 ml 2 M NaCl, 10 minutes per wash.
5. Give the slide a final brief rinse with ddH₂O and dry under a stream of nitrogen. Cover the slide with plastic wrap and expose to a storage phosphor screen.
6. Scan the phosphor screen on a phosphor imager with a resolution of no larger than 50 μ m. Quantify the extent of radiolabel incorporation into each spot using software accompanying the instrument. These raw spot intensity values should be exported into a spreadsheet for subsequent data analysis.
7. Average the data for duplicate spots on the array that corresponding to the same peptide. Normalize the data by dividing each signal by the average value for all peptides with the same fixed position. In this way, the average value at each

¹¹Diluted aqueous peptide stocks can be sealed and stored at –20 °C for at least 8 months.

position is 1, with positively selected residues have a value greater than 1 and negatively selected residues having a value less than 1.

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Fig. 1.

Overview of procedure for PTK analysis with peptide microarrays. The sequence of peptide mixtures in the PSPL is shown at left. Positions marked X are composed of identical degenerate mixtures of amino acid residues. Positions marked Z consist of a single residue, either one of 19 unmodified amino acids (excluding Cys), pThr or pTyr. Representative data for the PTK EphA3 is shown at the bottom right.