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Antibody-Based Fluorescence Detection of Kinase Activity on a Peptide Array

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Abstract—Peptide-based microarrays allow for high-throughput identification of protein kinase substrates. However, current methods of detecting kinase activity require the use of radioisotopes. We have developed a novel fluorescence-based approach for quantitative detection of peptide phosphorylation on chip using fluorescently-labeled anti-phosphoserine and anti-phosphotyrosine antibodies. This method is sensitive, specific and extremely fast, presenting obvious advantages and may find wider uses in high-throughput kinase screenings.

Introduction

Phosphorylation of proteins by kinases is one of the most important mechanisms for regulation of cell functions.[1,2] It has been estimated that more than one third of all proteins can be modified by phosphorylation in mammalian cells, and that more than 1% of the genes in the human genome encode protein kinases.[3] Few kinases to date, however, have yet been identified and fully characterized. One of the most important features of protein kinases is their substrate specificity, which to a large extent is determined by the primary sequence around the phosphorylation site of their targeting proteins. As a result, a number of methods have been developed to identify potential kinase substrates, including combinatorial synthesis of peptide libraries on membrane using the SPOT™ technology,[4] one-bead-one-compound peptide libraries,[5] positional-scanning combinatorial libraries[6] and peptide libraries using affinity-column selection.[7] More recently, peptide-based microarrays have also been developed.[8,9] Compared to the SPOT™ technology, the much higher density of spots allowed in a peptide array makes it possible for simultaneous screenings of tens of thousands of kinase substrates on a 3 inch × 1 inch glass surface. Together with the possibility of linking peptides with addressable elements in a high-density DNA microarray,[10] peptide arrays may one day enable high-throughput screenings of other enzymes.

Fluorescently-labeled antibodies have been reported, in a microarray format, for detection of protein–protein, protein–peptide and protein–small molecule interactions,[8,9,11] as well as cell assay.[9] The use of poly- and monoclonal antibodies directed against phosphoamino acids have been widely used to detect phosphorylated proteins in gel electrophoresis. This method is extremely sensitive since antibodies can detect as little as a few fmol of phosphorylated epitopes.[12] In addition, due to the highly specific nature of antibody–antigen recognition, little or no cross-reactivity of one phosphoamino acid antibody (e.g., anti-phosphotyrosine) to other phosphoamino acids or non-phosphorylated amino acids was observed.[13] However, all peptide arrays developed to date for kinase assay require the use of radioactive ^{32}P for detection of substrate phosphorylation, presenting a potential risk to human health. Furthermore, the long exposure time (usually hours to days) needed for sensitive detection of ^{32}P upon substrate phosphorylation does not lend itself to high-throughput applications. In order for peptide arrays to gain wider popularity for kinase screenings, it is imperative to develop an alternative detection method that poses less health risks than ^{32}P, yet provides similar sensitivity for efficient detection of kinase activity in a microarray format.

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By using fluorescently-labeled antibodies against phosphorylated amino acids, we have successfully developed a fluorescence-based method capable of quantitative detection of kinase activity in a peptide array. Due to its sensitivity, high specificity and the short incubation time needed to detect phosphorylation, this antibody-based approach presents obvious advantages over all existing methods which use radioactive ^{32}P, and may find wider uses in high-throughput kinase screenings.

Results and Discussion

The p60 and PKA substrates (YIYGSFK and ALRRASLG respectively) containing appropriate N-terminal residues, were synthesized according to standard Fmoc-chemistry on Rink amide resin with a Pioneer™ automatic peptide synthesizer (Applied Biosystems, USA). *O*-Benzothiazol-1-yl-*N*,*N*,*N*′,*N*′-etramethyluronium tetrafluoroborate (TBTU), 1-hydroxybenzotriazole (HOBt) and *N*,*N*-Diisopropylethylamine (DIEA) coupling chemistry was used. The phosphorylated substrates were synthesized with Fmoc-Tyr(PO(Obzl)OH)–OH and Fmoc-Ser(PO(OBzl)OH)–OH. An extended cycle (4-h coupling) was used to couple the phosphorylated serine and tyrosine to the peptides. At the end of synthesis, peptides were cleaved off the resin by using reagent R (90% TFA, 5% thioanisole, 3% ethanedithiol, 2% anisole) and precipitated with cold ether. Further purification of peptides was done by HPLC on a Waters™ 600 Station equipped with a Phenomenex™ C_{18} semi-preparative column. The identity of peptides was confirmed by Mass Spectrometry. The derivatization of glass slides with glyoxyxlic acid was done based on previously published protocols[9] and our own.[14] First, glass slides (Fisher Scientific, USA) were cleaned in a piranha solution, followed by treatment with a solution of 2% aminopropyltriethoxysilane in 95% ethanol for 1 h. After curing at 150 °C for 1 h to generate an amine-containing surface, slides were reacted with a solution of 10 mM protected glyoxylic acid (1 M glyoxylic acid, 0.1 mM HCl, 5 M ethylene glycol in DMF, 100 °C, 30 min), 50 mM TBTU and 50 mM HOBt in DMF for 2 h, then washed with DMF (2×), DCM (2×). The acetal protecting group was deprotected by placing the slides in a 10 mM HCl solution for 2 h, washed with H_2O and air died. The N-terminally cysteine-containing peptides were dissolved in PBS, pH 7.4, and arrayed using an ESI SMA™ arrayer (Ontario, Canada), with a spacing of 220 μm between the spots. After a 3-h incubation the slides were washed with PBS and distilled water, air dried and blocked for 3 h with a 1% BSA solution. For kinase assay, the slides were incubated for various periods of time with the respective kinase solutions: (1) p60 assay (25 mM Tris, pH 7.4, 35 mM $MgCl_2$, 7 mM $MnCl_2$, 0.5 mM EGTA, 100 μM ATP, 2U p60); and (2) PKA assay (25 mM Tris, pH 7.4, 15 mM $MgCl_2$, 1 mM DTT, 2 mM EGTA, 100 μm ATP, 2U PKA). The slides were subsequently washed with H_2O, dried and incubated with the FITC-labeled anti-phosphoamino acids. FITC-labeled antibodies were obtained by derivatizing the antibodies with fluorescein-NHS (Molecular Probes, USA) in 0.1 M $NaHCO_3$ (pH 9) following the recommended protocol, and purified with a NAP5 column (Amersham, UK). The slides were washed with H_2O, scanned and the fluorescence intensity of the spots were measured with an ArrayWoRx™ scanner (Applied Precision, USA).

In order to determine whether FITC-labeled anti-phosphoamino acids can be used to detect phosphorylation of kinase substrates in a peptide array, we first tested their detection against phosphorylated amino acids and peptides. Both phosphorylated and non-phosphorylated tyrosines were first spotted on an amine-containing glass slide, followed by detection using FITC-labeled anti-phosphotyrosine and anti-phosphoserine. Only the FITC-labeled anti-phosphotyrosine was able to detect the phosphotyrosine immobilized on the slide (Fig. 1a). Neither binding of anti-phosphoserine to phosphotyrosine, nor that of anti-phosphotyrosine to non-phosphorylated tyrosine, was observed (data not shown). We also attempted to detect the protected phosphotyrosine with both FITC-labeled antibodies and no binding was observed (data not shown). Next, we synthesized both the phosphorylated and the non-phosphorylated peptide substrate of p60 tyrosine kinase (CGG-YIYGSFK), with an additional CGG N-terminal linker for immobilization purpose. Both peptides were spotted onto glass slides functionalized with glyoxylic acid,[9] followed by blocking with BSA and probed for phosphorylation by incubation with FITC-labeled anti-phosphotyrosine and anti-phosphoserine for an hour. Only anti-phosphotyrosine was able to detect the phosphorylated p60 substrate (Fig. 1b), confirming the high specificity of this antibody-based detection of tyrosine phosphorylation.

We next determined whether the fluorescently labeled antibodies can quantitatively detect phosphorylated peptides on chip. Varied ratios of phosphorylated/non-phosphorylated GGC p60 peptides, with a combined constant concentration, were arrayed onto thioester-functionalized slides,[14] incubated for 3 h, and probed with the FITC-labeled anti-phosphotyrosine antibody for 1 h (Fig. 2). It was observed that the intensity of the spots increased as the ratio of phosphorylated to non-phosphorylated p60 increased. A graph of fluorescence intensity versus phosphorylated p60 substrate shows a

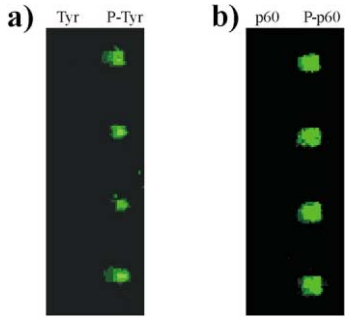

Figure 1. Fluorescence-based detection of phosphorylated amino acids and peptides on a glass slide using FITC-labeled anti-phosphotyrosine, and anti-phosphoserine (data not shown). (a) Tyrosine (left panel) and phospho-tyrosine (right panel), and (b) p60 peptide (left panel) and phosphorylated p60 peptide were probed with FITC-labeled anti-phosphotyrosine.

linear correlation for a p60 ratio ranging from 0 to 100%, demonstrating the feasibility for on-chip quantitation of phosphorylated peptides using this method.

Having demonstrated the usage of FITC-labeled anti-phosphoamino acids for quantitative detection of phosphorylated amino acids and peptides in a microarray, we next investigated their ability to detect on-chip kinase activity. A peptide substrate (CGG-ALR-RASLG) of PKA, a serine protein kinase, was synthesized with an additional CGG linker. Together with the non-phosphorylated substrate of p60 tyrosine kinase, both peptides were spotted onto the same slides functionalized with glyoxylic acid. Following blocking with BSA, the slides were first incubated for an h with the corresponding kinase (p60 tyrosine kinase and PKA kinase for Fig. 3a and b, respectively), then detected with FITC-labeled anti-phosphotyrosine and anti-phosphoserine. Only anti-phosphotyrosine was able to detect the tyrosine kinase activity of p60. Similarly, only anti-phosphoserine was able to detect the serine kinase activity of PKA. The complete absence of cross-detection between the two FITC-labeled antibodies demonstrates the high specificity of the antibodies against their corresponding phosphorylated amino acids/peptides.

In order to further demonstrate the utility of our fluorescence-based method for real-time detection of kinase activity in a microarray format, we next investigate both concentration- and time-dependent detection of peptide phosphorylation on chip. Decreasing concentrations (3, 1, 0.3 and 0.1 mM) of GGC p60 substrate in PBS, pH 7.4, were arrayed onto a thioester-containing glass slides[14] and incubated with p60 kinase for increasing periods of time (1, 5 and 12 h). The slides were incubated with the FITC-labeled anti-phosphotyrosine for 1 h, washed, dried, scanned and the fluorescence intensity of the spots was measured (Fig. 4). The concentration-dependent kinase activity was confirmed by plotting the observed fluorescence intensity over the differing concentrations of the peptide spotted on the slide following the same incubation time (5 h) with the corresponding kinase (Fig. 4b). It was found that the fluorescence intensity was directly proportional to the concentration of the substrate, showing the feasibility for determination of concentration-dependent kinase activity. Using this antibody-based fluorescence detection, kinase phosphorylation was readily detected even with 0.1 mM (which corresponds to $\sim$0.1 pmol with a spot size of 1 nL) of the peptide substrate. No further work was deliberately done to determine the lowest detection limit. However, by increasing the scanning time or changing the dye used to label the antibody, a 10- to 100-fold lower detection limit should be obtainable. The time-dependent kinase activity was also determined by plotting the observed fluorescence intensity obtained from the phosphorylated peptide having the same spotting concentration (3 mM) but differing incubation time with the kinase (Fig. 4c). The result indicated that the fluorescence intensity as a result of peptide phosphorylation correlates well with kinase incubation time. This, together with our previous findings that the amount of phosphorylated substrates is linearly proportional to the fluorescence intensity detected using the FITC-labeled antibodies (Fig. 2), indicating the feasibility of time-dependent quantitation

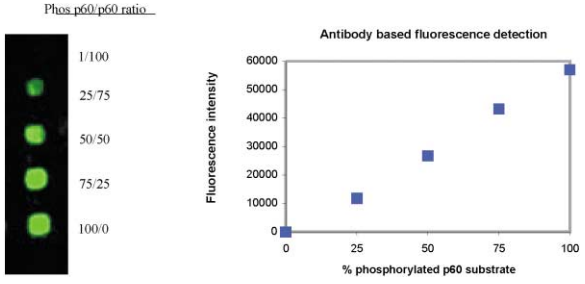

Figure 2. Fluorescence intensity versus amount of phosphorylated substrate. Varied ratios of phosphorylated/non-phosphorylated GGC p60 peptides, with a combined constant concentration, were arrayed onto thioester-functionalized slides,[14] incubated for 3 h, and probed with the FITC-labeled anti-phosphotyrosine antibody for 1 h.

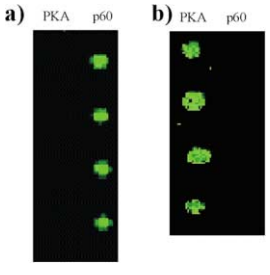

Figure 3. Detection of kinase activity with FITC-labeled antibodies. PKA (left panel) and p60 (right panel) peptides were spotted onto a glyoxylic acid-functionalized slide, phosphorylated with the corresponding kinases (p60 kinase in a; PKA kinase in b) and probed with (a) FITC-labeled anti-phosphotyrosine, and (b) FITC-labeled anti-phospohserine.

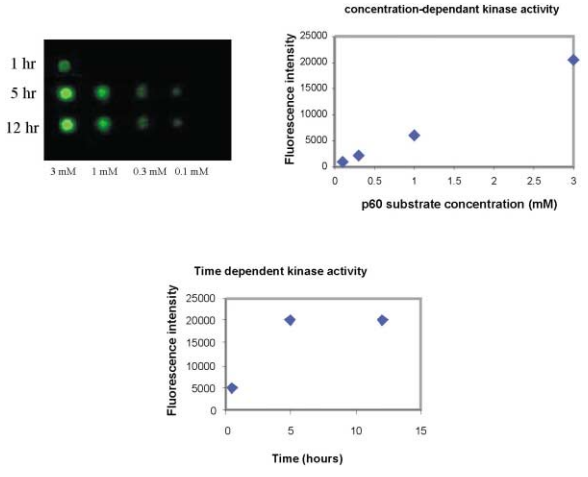

Figure 4. Antibody-based fluorescence measurement of kinase activity: Decreasing concentrations (3, 1, 0.3 and 0.1 mM) of GGC p60 substrate in PBS, pH 7.4, were arrayed on thioester slides and incubated with the p60 kinase for increasing period (1, 5 and 12 h). The slides were incubated with the FITC-labeled anti-phosphotyrosine for 1 h, washed, dried, scanned and the fluorescence intensity of the spots measured. The fluorescence intensity is directly proportional to the phosphorylated substrate concentration, and the concentration-dependent kinase activity can be determined rapidly using FITC-labeled antibody (graph 1). The time-dependent kinase activity can also be determined by direct measurement of the fluorescence intensity (graph 2).

of kinase activity on-chip. Furthermore, in sharp contrast with existing radioactive ^{32}P detection methods, this antibody-based fluorescence detection method is not only safe and highly sensitive, but also requires much shorter time (1 h or less) for detection of phosphorylated amino acids and peptides on chip. It is also noted that, while our manuscript was under review, another group has also independently confirmed the feasibility of using ^{32}P-based methods for quantitative detection of kinase activity in a microarray format.[15]

Conclusion

Antibody-based fluorescence detection was developed as an efficient, sensitive and selective method for quantitative detection of kinase activity in a microarray format, eliminating the usage of radioactive ^{32}P which poses a potential risk to human health. As a result, this detection method should be compatible with most fluorescence-based microarray applications, making it the method of choice for future high-throughput kinase screenings.

Acknowledgements

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