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Novel and efficient detection of cAMP-dependent protein kinase for the development of a protein biochip

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Protein microarrays have recently become a powerful research tool for proteomics and clinical investigations. A novel and efficient detection of cAMP-dependent protein kinase for the development of a protein biochip has been established. The phosphorylation of a substrate by [γ-^{33}P]ATP and [γ-^{32}P]ATP was performed on a glass chip, and a facile detection of a kinase assay using ^{32}P and ^{33}P on a glass chip was accomplished by a radio-TLC imaging scanner. Since the radio-TLC detection method takes shorter detection time (2 min) than those required for X-ray film or bioimage analyzer (6–12 h), the latter can be replaced. The detection by the radio-TLC was proven to be rapid and suitable for a kinase assay using radioisotopes for developing a biochip.

Keywords: radioisotope detection; phosphorylation detection; biomolecule interactions; biochip; radio-TLC detection

Introduction

Microarrays function as a powerful tool for proteomics and medical assays. Microarrays can be used to screen thousands of binding events in a parallel and high-throughput manner, and are of major importance in the detection of a disease and drug discovery. There are generally two kinds of detection strategies for protein microarrays, namely, label-free methods and labeled probe methods. Labeled probe methods are those that employ the use of fluorescence, electrochemiluminescence, chemiluminescence and a radioactivity detection.[1,2] Enzyme-linked immunosorbent assay (ELISA) has usually been used in immunology to detect the presence of an antibody or an antigen in a sample. It has been used as a diagnostic appliance in academic research, clinical diagnosis and plant pathology as well as a quality control check in various industries. Microarray scanner for ELISA has been widely used in the acquisition and analysis of expression data from DNA microarrays, protein microarrays, carbohydrate arrays, tissue arrays and cell arrays.[3]

We previously reported on a feasibility study using [γ-^{32}P]ATP for determining the activity of a cAMP-dependent protein kinase (PKA) assay on a glass chip for developing a biochip.[4] In general, kinases and phosphatases are of considerable interest to researchers involved in drug discovery and development because of their role in a wide variety of diseases. Radioactive labeling is mainly performed using ^{32}P incorporated into adenosine triphosphate (ATP) for detecting the phosphorylation between a kinase and its substrate. The use of radioisotope (RI) is conventionally regarded as one of the most sensitive detection methods.[5] However, it takes a long time to implement a data analysis for a signal intensity. A glass chip is exposed to an X-ray film or imaging plate and then developed or analyzed by a bioimage analyzer. In other words, it takes a long time to expose a glass chip for an analysis of the data. Therefore, this work was performed to investigate the feasibility of using a convenient radio-TLC detection for the phosphorylation assay using RIs on a microarray.

Results and discussion

Phosphorylation of a protein-fused substrate by a kinase and its detection using RI

In this study, we present a tool for the detection of the phosphorylation of a substrate catalyzed by PKA by using an RI detection technique on microarrays. Phosphorylation of an *E. coli* malic enzyme–substrate fusion protein by [γ-^{33}P]ATP or [γ-^{32}P]ATP, catalyzed by PKA, is illustrated in Figure 1. It occurs through the addition of a phosphate group to the serine amino acid of the substrate (LRRASLG) via a transfer of a phosphate ion from a [γ-^{33}P]ATP or a [γ-^{32}P]ATP molecule catalyzed by PKA. The signal intensities are determined by a radio-TLC imaging scanner, X-ray film or a phosphoimage analyzer. The shape of the signal intensity is presented as a spot on the X-ray film or phosphoimage analyzer on the microarrays. Radio-TLC imaging scan for the phosphorylation assay is a rapid detection method. The signal intensity is presented both as quantitative data and also as a graph by the radio-TLC.

A radio-TLC imaging scanner has become the best standard in the industry for analyzing the radiochemical purity of RI-labeled molecules. It directly provides a digital counting of most RIs and

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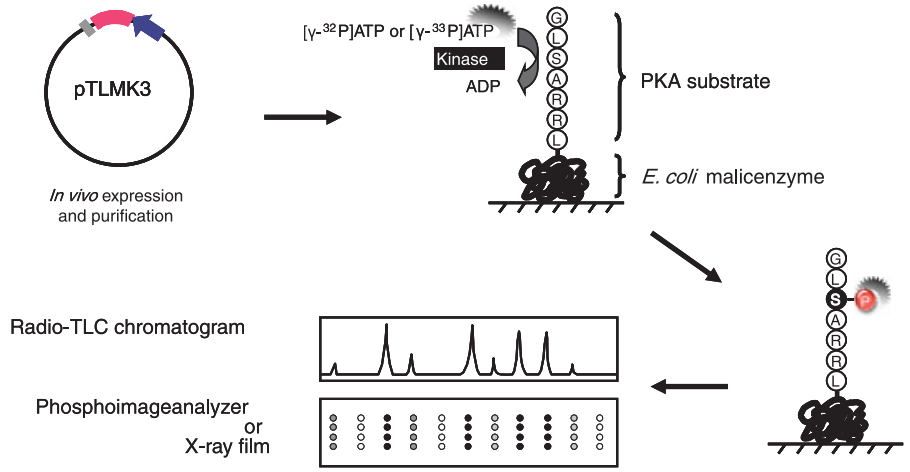

Figure 1. Scheme of the recombinant protein substrate for the kinase assay on a radioactive isotope. The gene, protein, peptide and transferred radiophosphorus are shown in different symbols. This figure is available in color online at www.interscience.wiley.com/journal/jlcr.

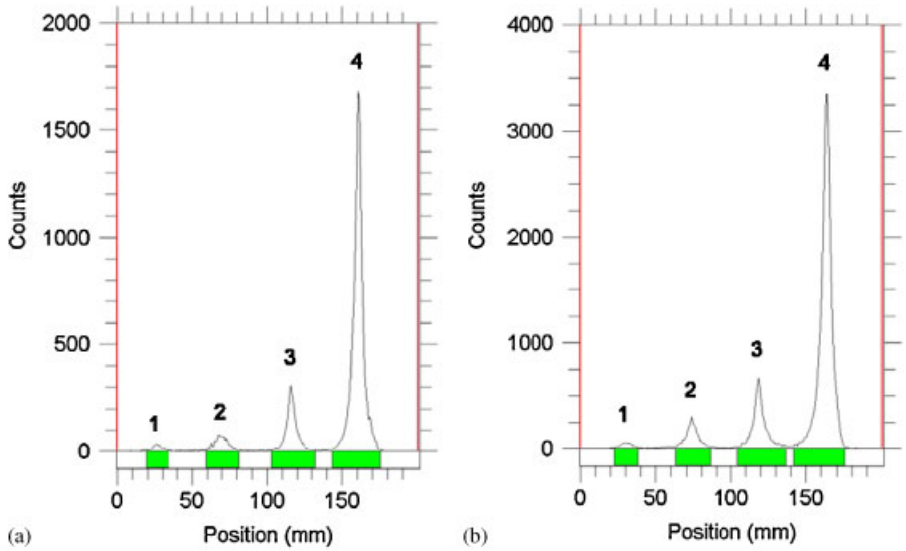

Figure 2. Radioisotope intensities obtained with varying concentrations of [γ-^{33}P]ATP (a) and [γ-^{32}P]ATP (b) by using radio-TLC detection. 1: 0.5 nCi (18.5 Bq), 2: 1.0 nCi (37.0 Bq), 3: 5.0 nCi (185 Bq), 4: 10.0 nCi (370 Bq) of [γ-^{33}P]ATP or [γ-^{32}P]ATP. This figure is available in color online at www.interscience.wiley.com/journal/jlcr.

guarantees quantitative accuracy and reproducibility. This system uses a gas-filled proportional counter, which can detect all beta- and gamma-emitting RIs, and the counting result can be imaged in less than 1 min.

Utility detection of radiolabeled phosphate by using radio-TLC on a glass chip

Detection of a radiolabeled phosphate was measured using radiolabeled ATP molecules on a glass chip. Various concentrations of [γ-^{33}P]ATP or [γ-^{32}P]ATP ranging from 0.5 nCi (18.5 Bq) to 10 nCi (370 Bq) were spotted onto the slide chips and then they were immobilized by an incubator in a humid chamber for 1 h at 30°C. The slide chips were measured by radio-TLC and the signal intensity is presented as graphs in Figure 2. It was found from the graphs that the signal intensities for [γ-^{33}P]ATP and [γ-^{32}P]ATP were continually increased up to 10.0 nCi (370 Bq) of ^{33}P and ^{32}P. The data in Table 1 show the quantitative signal intensity measured with various radioactivities of ^{33}P and ^{32}P, calculated from the graph obtained from the radio-TLC. The signal intensities of 10 nCi (370 Bq) for ^{33}P and ^{32}P are each 3204.3 ± 91.3 and 7716.4 ± 132.4 CPM per one spot of radiophosphorus, respectively. The signal intensity of [γ-^{32}P]ATP was detected as two times higher when compared with [γ-^{33}P]ATP. Detection of the signal intensity on the X-ray film is shown in Figure 3. It was found that the signal intensities of [γ-^{33}P]ATP and [γ-^{32}P]ATP were continually increased up to 10.0 nCi (370 Bq) of ^{33}P and ^{32}P. The signal intensity of [γ-^{33}P]ATP was obtained as clear round spots on an X-ray film, but it had low values when compared with [γ-^{32}P]ATP.

Feasibility studies for the detection of a kinase assay using RIs on a glass chip by a radio-TLC imaging scanner

We present here a strategy for the detection of the radiophosphorylation of a substrate catalyzed by PKA by using radio-TLC on microarrays. Varying concentrations of purified *E. coli* malic–kemptide fusion protein were spotted and immobilized on aldehyde-terminated glass slides, and these were then incubated in a reaction mixture that contained a kinase mixture

Table 1. Quantitative data of the signal intensity of the radiophosphorus detected by a radio-TLC imaging scanner

Type of radioisotope[a]	No.	Radioactivity (nCi)	Mean CPM[b]
^{33}P	1	0.5 (18.5 Bq)	77.3 ± 4.9
	2	1.0 (37.0 Bq)	251.9 ± 7.6
	3	5.0 (185 Bq)	596.5 ± 40.5
	4	10.0 (370 Bq)	3204.3 ± 91.3
^{32}P	1	0.5 (18.5 Bq)	146.2 ± 7.8
	2	1.0 (37.0 Bq)	677.5 ± 51.2
	3	5.0 (185 Bq)	1503.6 ± 83.8
	4	10.0 (370 Bq)	7716.4 ± 132.4

[a]Radioabeled biomolecule is an adenosine triphosphate.
[b]Counts per minute were made for two spots and the values are presented as mean ± SD for one spot.

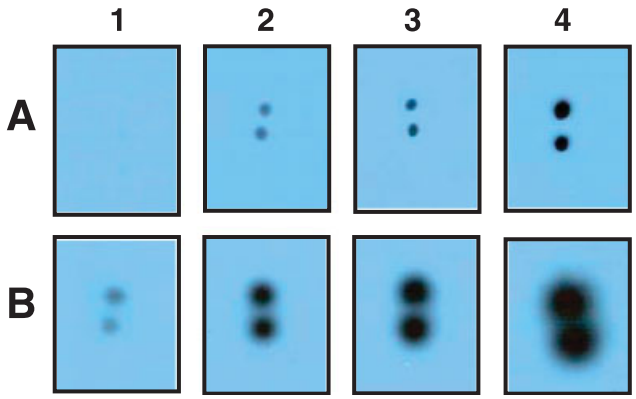

Figure 3. Radioisotope intensities obtained with varying concentrations of [γ-^{33}P]ATP (A) and [γ-^{32}P]ATP (B) by using an X-ray film. 1: 0.5 nCi (18.5 Bq), 2: 1.0 nCi (37.0 Bq), 3: 5.0 nCi (185 Bq), 4: 10.0 nCi (370 Bq) of [γ-^{33}P]ATP or [γ-^{32}P]ATP. This figure is available in color online at www.interscience.wiley.com/journal/jlcr.

with ^{33}P and ^{32}P. The glass slides were then washed and scanned by radio-TLC, and the signal intensity is presented as graphs (Figure 4). Specific signals for the reaction of PKA were detected only for the *E. coli* malic–kemptide fusion protein, but not for the bovine serum albumin (BSA), which was used as a negative control. It was found that the PKA activities could be detected for the specific protein-fused substrate spots phosphorylated by RIs. It was also found from the graphs that the signal intensities for the phosphorylation using [γ-^{33}P]ATP and [γ-^{32}P]ATP were continually increased up to 10.0 ng for the *E. coli* malic–kemptide fusion protein. The data in Table 2 show the quantitative signal intensity measured with various concentrations of a substrate calculated from the graph obtained from the radio-TLC. The signal intensities of 10 ng of fusion protein for the phosphorylation using ^{33}P and ^{32}P are each 356.9 ± 51.6 and 374.8 ± 40.9 CPM per one spot of a substrate, respectively. The signal intensity of the substrate from the phosphorylation using [γ-^{33}P]ATP was found to be higher than [γ-^{32}P]ATP. There is an element of reason regarding the quantitative signal intensity measured with various radioactivities of ^{33}P and ^{32}P in Table 1. In the case of the detection by the X-ray film, it was found that the signal intensities of [γ-^{33}P]ATP and [γ-^{32}P]ATP were continually increased up to 10.0 ng of the fusion protein for the phosphorylation using ^{33}P and ^{32}P (Figure 5). The signal intensity of [γ-^{32}P]ATP was much more clearer, but the phosphorylation using [γ-^{33}P]ATP revealed brilliant spots.

Experimental

Chemicals and plasmid

cAMP-dependent PKA was purchased from Promega (Madison, WI, USA). The aldehyde-terminated slides were purchased from Nuricell Inc. (Seoul, Korea). [γ-^{32}P]ATP and [γ-^{33}P]ATP were purchased from IZOTOP (Budapest, Hungary). Unless specified, all other reagents were obtained from Sigma (St Louis, MO, USA). Plasmid ptlmk3 was a gift from the Korea Advanced Institute of Science and Technology (Daejeon, Korea).

Strains, expression and purification of the recombinant fusion proteins

DNA encoding kemptide peptide fused to the *E. coli sfcA* gene (encoding malic enzyme) was obtained by an overlap PCR amplification using plasmid pEDOb5 carrying the genomic DNA from *E. coli* W3110 as a template.[6] For the preparation of *E. coli* malic enzyme–kemptide fusion protein from the cells, the organism was grown in LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) containing a final concentration of 50 μg/mL ampicillin at 37°C and was expressed in 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation and disrupted by sonication in an ice-cold PBS buffer (200 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 1 mM Na_2HPO_4, pH 7.5). The crude recombinant fusion proteins with His-tag at their C-terminals were purified by an Ni-chelating resin (GE Healthcare, Uppsala, Sweden) using a gradient from 0 to 500 mM imidazole in a 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl. The eluted recombinant fusion protein was dialyzed against PBS buffer (pH 7.5). This recombinant fusion protein could be directly spotted and immobilized on a slide glass without any further purification process. Protein concentration was measured by the method of Bradford (Bio-Rad protein assay, Bio-Rad) using BSA as a standard.[7]

Assay and determination of the PKA activity using RIs

Assay of the PKA activity was performed according to our previous report with a slight modification.[4] The *E. coli* malic enzyme–kemptide fusion protein was diluted to 0.1 mg/mL with PBS buffer containing 1% glycerol and then spotted onto a chip (the aldehyde-terminated slides). For the immobilization of a substrate, prepared slides were incubated in a humid chamber for 1 h at 30°C. The slides were then washed with PBS buffer and

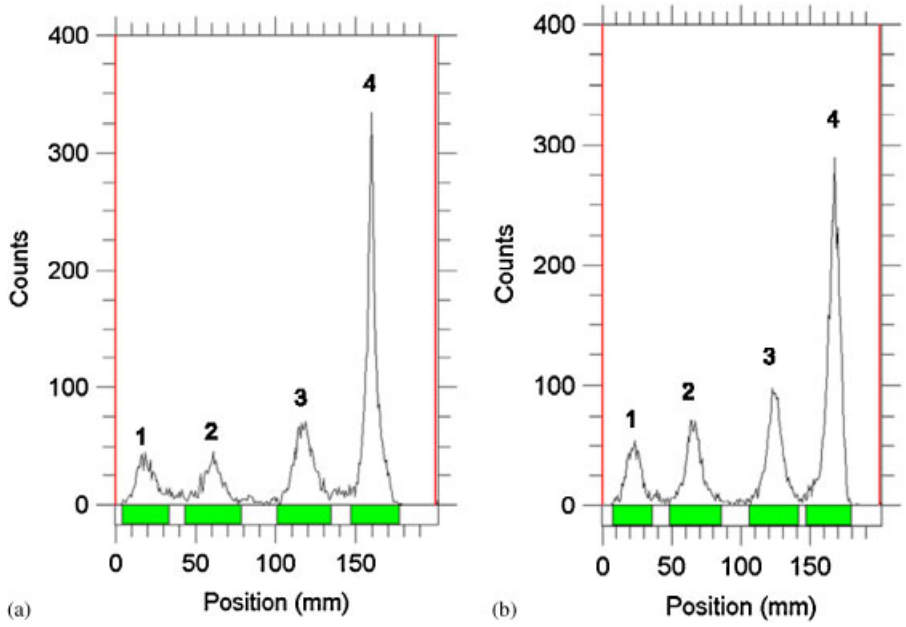

Figure 4. Signal intensities according to varying concentrations of the malic–kemptide fusion protein with [γ-^{33}P]ATP (a) and [γ-^{32P}]ATP (b) by using radio-TLC detection. 1: BSA, 2: 0.1 ng, 3: 1.0 ng, 4: 10.0 ng of *E. coli* malic–kemptide fusion protein. This figure is available in color online at www.interscience.wiley.com/journal/jlcr.

Table 2. Quantitative data of the signal intensity of the substrates phosphorylated by a radio-TLC imaging scanner

Type of radioisotope[a]	No.	Concentration of substrate (ng)	Mean CPM[b]
^{33}P	1	None (BSA)	85.75 ± 10.4
	2	0.1	100.5 ± 17.1
	3	1.0	132.5 ± 20.9
	4	10.0	356.9 ± 51.6
^{32}P	1	None (BSA)	99.4 ± 3.7
	2	0.1	141.0 ± 5.5
	3	1.0	180.8 ± 8.3
	4	10.0	374.8 ± 40.9

[a]Radioabeled biomolecule is a phosphorylated substrate.
[b]Counts per minute were made for four spots and the values are expressed as mean ± SD for one spot.

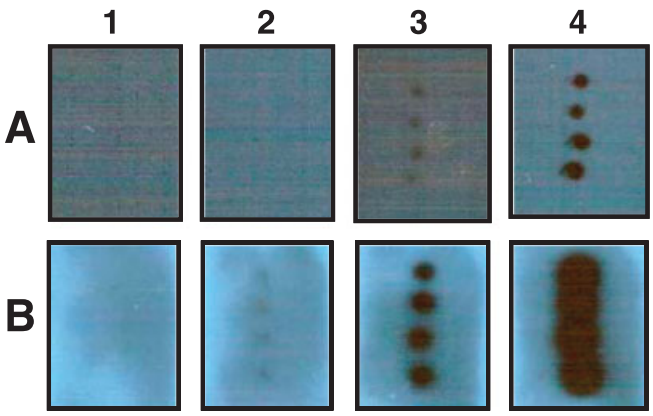

Figure 5. Signal intensities of the phosphorylation of varying concentrations of malic–kemptide fusion protein by [γ-^{33}P]ATP (A) and [γ-^{32}P]ATP (B) detected by using an X-ray film. 1: BSA, 2: 0.1 ng, 3: 1.0 ng, 4: 10.0 ng of *E. coli* malic enzyme–kemptide fusion protein. This figure is available in color online at www.interscience.wiley.com/journal/jlcr.

kinase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) in sequence. The slides were incubated with a reaction mixture for 1 h at 37°C. The reaction mixture composed of the kinase buffers was supplemented with an ATP mixture (100 μM ATP, 0.1 μCi/mL (3.7 kBq/mL) [γ-^{32}P]ATP or [γ-^{33}P]ATP) and 10 unit/mL of PKA. Upon the completion of the reaction, the slides were washed three times for 30 s. The glass chips were analyzed by an AR-2000 radio-TLC Imaging Scanner (Bioscan, Inc., Edmonds, WA, USA), and then they were exposed to an X-ray film (Fuji) and developed.

Conclusion

Kinases are of eminent interest to researchers involved in drug discovery and development. PKA is a ubiquitous serine PKA present in a variety of tissues.[8] Protein microarrays offer an ideal system for a rapid and parallel identification of substrates for a PKA. To investigate this application, we previously reported on a suitable strategy for the detection of the phosphorylation of a

kemptide catalyzed by PKA by using an RI detection technique when developing a protein biochip.[4] The use of RIs in a protein microarray is preferred to other detection methods because it is highly sensitive, thus facilitating the detection of even minor quantities of protein. RIs have many uses in biomedical applications, for example, in radiology, radiotherapy, nuclear medicine and RI scanning.[9] Radioactive labeling is mainly performed using different RIs such as ^{33}P and ^{32}P into ATP for the phosphorylation. We reported here on the degree of incorporation of ^{33}P and ^{32}P into a substrate measured by radio-TLC. In the examined substrate concentration range, the signal intensity was continually increased up to a high concentration of *E. coli* malic–kemptide fusion protein as determined by the radio-TLC. The detection by the radio-TLC was proven to be suitable for a kinase assay using RIs on a glass chip. ^{33}P and ^{32}P have different characterizations in their half-lifes and energies (^{33}P: $E_{\beta\,max} = 249\,keV$, $t_{1/2} = 25.34\,d$; ^{32}P: $E_{\beta\,max} = 1710.3\,keV$, $t_{1/2} = 14.26\,d$). In the case of half-life, ^{33}P has a longer time when compared with ^{32}P. However, in the case of energy, it is the reverse. Hence, we concluded that the selection of ^{33}P and ^{32}P in developing a protein biochip for a convenient detection of the activity of a kinase depends on the applications.

It should also be noted that the radio-TLC detection method takes much shorter detection time (2 min) compared with the conventional detection methods such as using an X-ray film or a bioimage analyzer (6–12 h) for analyzing the RI biochip. The use of this detection method facilitates a rapid data analysis with a high sensitivity. Therefore, we anticipate its application as a routine detector for a biochip using an RI detection technique.

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