Journal of
Proteomics & Bioinformatics

Kinoshita et al., J Proteomics Bioinform 2013, S6
DOI: 10.4172/jpb.S6-008

Review Article Open Access

Phos-tag-Based Microarray Techniques Advance Phosphoproteomics

Eiji Kinoshita*, Emiko Kinoshita-Kikuta and Tohru Koike

Department of Functional Molecular Science, Institute of Biomedical & Health Sciences, Hiroshima University, Kasumi 1-2-3, Hiroshima 734-8553, Japan

Abstract

The reversible phosphorylation of proteins is a dominant post-translational modification that regulates many important cellular processes, including signal transduction, gene expression, and cell cycle progression. Changes in the phosphorylation status of certain proteins have been implicated in a wide range of human diseases. Effective analytical techniques for quantitative determination of levels of protein phosphorylation are therefore essential tools for studies on the proteome, particularly in relation to disease diagnosis and drug discovery. Large-scale identification of phosphoproteins is now possible as a result of dramatic advancements in methods of shotgun proteomics based on mass spectrometry. However, these techniques require expensive instrumentation and involve complicated procedures for sample preparation through enrichment of phosphopeptides by enzymatic digestion. With regard to the analysis of the phosphoproteome, microarray techniques involving peptide or protein arrays, have demonstrated considerable potential as cost-effective, high-throughput, and convenient approaches for defining activities relating to signal transduction by means of phosphorylation reactions. We recently developed a phosphate-affinity probe that is suitable for monitoring changes in levels of phosphorylation of proteins. This affinity probe is known as biotin-pendant Phos-tag {Phos-tag Biotin; Phos-tag = 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex}. We have demonstrated the utility of Phos-tag Biotin as a novel phosphate-affinity probe in a range of techniques, including microarray-based methods, for determining the phosphorylation status of large numbers of peptides and proteins. Here, we review some of the more advanced applications of Phos-tag Biotin. We discuss the impact of Phos-tag-based microarray techniques in relation to the detection of protein phosphorylation multiplexes, and we compare these techniques with conventional probing procedures on microarrays.

Keywords: Phos-tag; Biotin; Peptide microarrays; Antibody microarrays; Tissue microaarays; Protein phosphorylation; Phosphoproteomics

Introduction

The phosphorylation of proteins is a common type of Post-Translational Modification (PTM) that is present in all biological species, and it affects key properties of proteins involved in numerous cellular events [1]. In living cells, the continuous and dynamic phosphorylation and dephosphorylation of proteins at specific amino acid residues are controlled by complex signaling networks [2,3], resulting in the production of a variety of phosphoproteins with various states of phosphorylation [4]. This reversible PTM is catalyzed by the opposing activities of large families of protein kinases (the kinome) and protein phosphatases (the phosphatome). For example, the human genome encodes over 500 protein kinases [5] and about 150 protein phosphatases [6]. These numbers reflect the importance and complexity of protein phosphorylation. Abnormal phosphorylation resulting from an imbalance in the enzymatic reactions of the kinome and the phosphatome has been shown to be a key factor in the etiology of many diseases, including cancers [7] and neurodegenerative disorders [8]. As a result, there has been considerable progress in the development of selective inhibitors of kinases and phosphatases as potential drugs. Some of these inhibitors have already been approved for use in treatment of cancers in humans. Typical examples of such drugs include imatinib mesylate (Gleevec or Glivec; Novartis, Basel, Switzerland) and gefitinib (Iressa; AstraZeneca, London, UK). Imatinib mesylate was designed to inhibit a fusion protein of the breakpoint cluster region and Abelson murine leukemia viral homolog 1 (Bcr-Abl), which has the constitutive activity of a tyrosine kinase [9]. Gefitinib was developed as a potent inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase, and it selectively inhibits EGF-stimulated tumor cell growth [10]. Furthermore, dysregulation of the activities of several protein kinases in human brain neurons can lead to hyperphosphorylation of the microtubule-associated protein Tau, a classic hallmark of the neurodegenerative disorder Alzheimer's disease. The sites and stoichiometry of phosphorylation of the Tau protein are correlated with the pathological characteristics of the disease.

Methods for determining the phosphorylation states of certain proteins are therefore very important in relation to understanding the molecular origins of various diseases and, potentially, for developing tools for therapeutic intervention. One method that is widely used in identifying particular phosphorylation event is the incorporation of a radioisotope label such as ^{32}P or ^{33}P into the phosphorylated protein. The phosphorylation status of the target protein can then be identified and quantified by measuring its radioactivity. A non-radioisotope method that uses polyclonal and monoclonal antibodies is also well established for the detection of protein phosphorylation. The readout from an antibody that recognizes a phosphopeptide or phosphoprotein is measured as a fluorescence, luminescence, or polarization signal; this method can be applied in conjunction with many of the standard biochemical analytical techniques, such as enzyme-linked immunosorbent assay, immunoblotting, or immunocytochemistry. A number of high-throughput techniques have recently been developed for simultaneously identifying a number of phosphorylation events. Among these high-throughput methods, microarray techniques have demonstrated considerable potential as inexpensive and convenient approaches to phosphoproteomics. In

***Corresponding author:** E. Kinoshita, Department of Functional Molecular Science, Institute of Biomedical & Health Sciences, Hiroshima University, Kasumi 1-2-3, Hiroshima 734-8553, Japan, E-mail: kinoeiji@hiroshima-u.ac.jp

Received May 20, 2013; **Accepted** June 25, 2013; **Published** June 28, 2013

Citation: Kinoshita E, Kinoshita-Kikuta E, Koike T (2013) Phos-tag-Based Microarray Techniques Advance Phosphoproteomics. J Proteomics Bioinform S6: 008. doi:10.4172/jpb.S6-008

particular, microarrays consisting of series of peptides that are specific substrates of various protein kinases can be used as powerful tools for the analysis of activities of the cellular kinome [11,12]. A number of such peptide microarrays, including PepChip (Pepscan Therapeutics, Lelystad, Netherlands), PepStar (JPT Peptide Technologies GmbH, Berlin, Germany), PamChip (PamGene, Cambridge, MA, USA), and CelluSpots (Intavis Bioanalytical Instruments AG, Cologne, Germany), are commercially available and these have been successfully used in analyses of the kinome of cultured cell lines and of tissue samples. Levels of phosphorylation of the various substrates on the microarrays have been determined mainly by conventional radioisotope- or antibody-based probing procedures.

Since 2003, we have been involved in developing a technology known as Phos-tag to permit the analysis of phosphorylated biomolecules (Phos-tag consortium, http://www.phos-tag.com/english/index.html). Our Phos-tag technology utilizes Phos-tag, a novel phosphate-binding tag molecule that binds to anionic substituents, especially phosphate monoester dianions (R-OPO_3^{2-}), at neutral pH [13]. The Phos-tag technology has made contributions to the development of several procedures for research on the phosphoproteome, including an immobilized metal-affinity chromatography technique for the separation and enrichment of phosphopeptides and phosphoproteins [14-18] and a phosphate-affinity electrophoresis technique for the detection of changes in the mobilities of phosphoproteins in comparison with those of their non-phosphorylated counterparts [19-23]. These techniques use several derivatives of the original Phos-tag molecule. Among these derivatives, biotin-pendant Phos-tag (Phos-tag Biotin) has been developed as a novel phosphate-affinity probe that has various applications in determining the phosphorylation status of a wide range of peptides and proteins [19,24-27] (Figure 1). Recently, we demonstrated some useful improvements in techniques for the detection of phosphopeptides and phosphoproteins through the use of a newly synthesized biotinylated derivative of Phos-tag that contains a dodeca (ethylene glycol) spacer (Phos-tag Biotin BTL-111, Figure 3) [28,29]. This improved affinity probe permits a wider range of applications in the specific detection of protein phosphorylation, including its use in microarray techniques. These advanced techniques are expected to permit more sensitive screening to provide information that might be capable of resolving complex kinase/phosphatase-dependent intracellular signaling networks, leading to improved disease diagnosis and drug discovery. In this review, we discuss the impact of Phos-tag-based microarray techniques on the development of phosphoproteomics.

Phos-tag Chemistry

The chemical design of selective host molecules for various phosphate anions or, in other words, the development of phosphate-capture molecules has attracted a great deal of interest among researchers. We found that macrocyclic polyamine complexes of zinc(II) can act as a useful family of molecules that are capable of capturing phosphates (K_d=10^{-3} to 10^{-7} M) under physiological conditions [30-35]. Our original molecular design was based on the fact that phosphomonoester dianions act as substrates or inhibitors of zinc-containing enzymes, such as alkaline phosphatase, carbonic anhydrase, or carboxypeptidases, through reversible coordination to the zinc(II) ions present in the enzymes [36]. From the results of studies on relations of various macrocyclic polyamine zinc(II) complexes with such model enzymes, we were able to develop a hypothesis that selective binding of phosphomonoester dianions might be feasible by using a complex containing two zinc(II) ions separated by a distance of 3 to 4 Å.

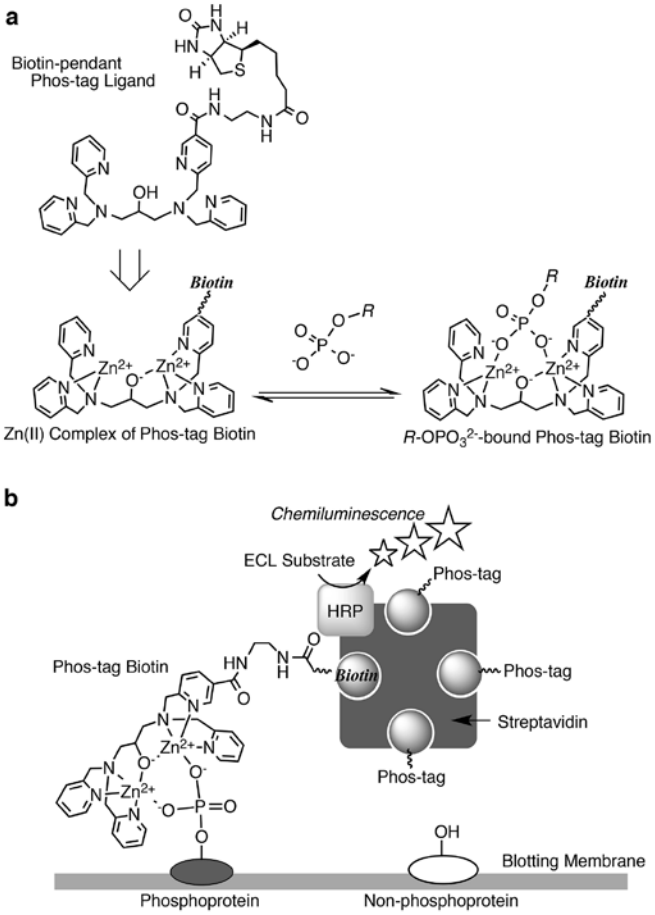

Figure 1: (a) Structure of biotin-pendant Phos-tag ligand (BTL-104) and scheme for reversible capture of a phosphomonoester dianion (R-OPO_3^{2-}) by the Zn(II) complex of Phos-tag Biotin. (b) Schematic representation of ECL detection of phosphoproteins on a protein-blotting membrane by using Phos-tag Biotin. The membrane was probed by using the complex of Phos-tag Biotin with HRP–SA, and then the Phos-tag-bound phosphoproteins were detected by an ECL system.

In 2004, we reported that a binuclear zinc(II) complex of 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olate can act as a novel phosphate-capture molecule [13]. The dizinc(II) complex forms a stable 1:1 complex with a phosphomonoester dianion in aqueous solution. The X-ray crystal structure of the 1:1 binuclear zinc(II) complex of the *p*-nitrophenyl phosphate dianion showed that each phosphate oxygen anion binds to a zinc(II) atom at the fifth coordination site, and that the two zinc(II) ions are separated by a distance of 3.6 Å. Therefore, binuclear zinc(II) complexes with a vacancy on each of the two zinc(II) ions are suitable for binding to phosphomonoester dianions as bridging ligands. In aqueous solution of neutral pH, the binuclear zinc(II) complex binds strongly to the phenyl phosphate dianion (K_d=2.5 × 10^{-8} M). The anion-selectivity indexes of phenyl phosphate dianion against sulfate, acetate, chloride, and diphenyl phosphate monoanions in aqueous solution of pH 7 at 25°C are 5.2×10^3, 1.6×10^4, 8.0×10^5, and $>2\times10^6$, respectively. In addition, the formation of a 1:1 adduct of the binuclear zinc(II) complex with an inorganic phosphate dianion [$HOP(O)(O^-)_2$] was clearly detected by means of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) from the characteristic mass shift and the change

in total charge on the phosphate (from –2 to +1) as a result of binding between the dizinc(II) complex and $HOP(O)(O^-)_2$ [37]. These findings led to the development of a simple, rapid, and sensitive procedure for analysis of phosphorylated compounds, such as phospholipids, by MALDI-TOF MS with the dizinc(II) complex as an MS probe [38-40]. As a result, we named the binuclear zinc(II) complex 'Phos-tag' as an abbreviation of 'phosphate-binding tag molecule'.

Phos-tag Biotin as a Novel Phosphate-Affinity Probe

In 2006, we reported the use of the dizinc(II) complex of Phos-tag Biotin (BTL-104) as a novel phosphate-affinity probe for the analysis of phosphoproteins on a protein-blotting membrane (Figure 1) [19]. Western blotting is a widely accepted technique for the detection of proteins on a protein-blotting membrane after separation by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For this reason, we tested the use of Phos-tag Biotin in this analytical technique and we found that the 4:1 complex of Phos-tag Biotin with horseradish peroxidase-conjugated streptavidin (HRP–SA) can be used as a probing reagent for Enhanced Chemiluminescence (ECL) in detection of phosphoproteins on blotting membranes (Figure 1b). By utilizing this principle, we developed a phosphate-affinity probing procedure for use in Western blotting.

Figure 2a shows results of a typical analysis of an array of four standard non-phosphorylated proteins together with four phosphoproteins and their dephosphorylated derivatives on a polyvinylidene difluoride (PVDF) membrane. The four standard phosphoproteins spotted on the PVDF membrane were detected by using the ECL system in conjunction with the BTL-104-bound HRP–SA complex, whereas no ECL signal was detected from the spots of the corresponding dephosphorylated proteins or from the standard non-phosphorylated proteins. Furthermore, when Phos-tag Biotin lacking zinc ions (biotin-pendant Phos-tag ligand; Figure 1a) was used, no ECL signal was detected from the spots for the phosphoproteins (data not shown). Therefore, the phosphate-affinity ECL signals were produced by the probing reagent through the interaction between the zinc(II) ions and phosphomonoester dianion. This demonstrated that the use of Phos-tag Biotin in this technique permits selective detection of phosphoproteins on a protein-blotting membrane. In addition, the probing procedure does not require any blocking treatment of the blotting membrane, which is advantageous in terms of saving time in Western blotting analyses. We extended the phosphate-affinity detection to an analysis of the phosphorylation status of A431 human epidermoid carcinoma cells before and after stimulation with EGF (Figure 2b). Two-dimensional electrophoresis followed by Western blotting using Phos-tag Biotin showed that the ECL signal intensities and numbers of protein spots in the sample after EGF stimulation (lower panel) increased markedly in comparison with those in the non-stimulated sample (upper panel). The Phos-tag-based technique is therefore useful for evaluating the phosphorylation status of serine, threonine, and tyrosine residues in whole proteins from complex biological samples, such as cell lysates. Furthermore, the technique can improve the chances of identifying new phosphoproteins.

Our next aim was to develop more advanced applications that would permit the specific recognition of phosphopeptides and phosphoproteins by using several newly synthesized Phos-tag derivatives, including a bisbiotinylated Phos-tag (BTL-108), a tetrakisbiotinylated Phos-tag (BTL-109), and a monobiotinylated Phos-tag with a dodeca(ethylene glycol) spacer (BTL-111), as well as the existing derivative Phos-tag Biotin BTL-104 [28]. Among these

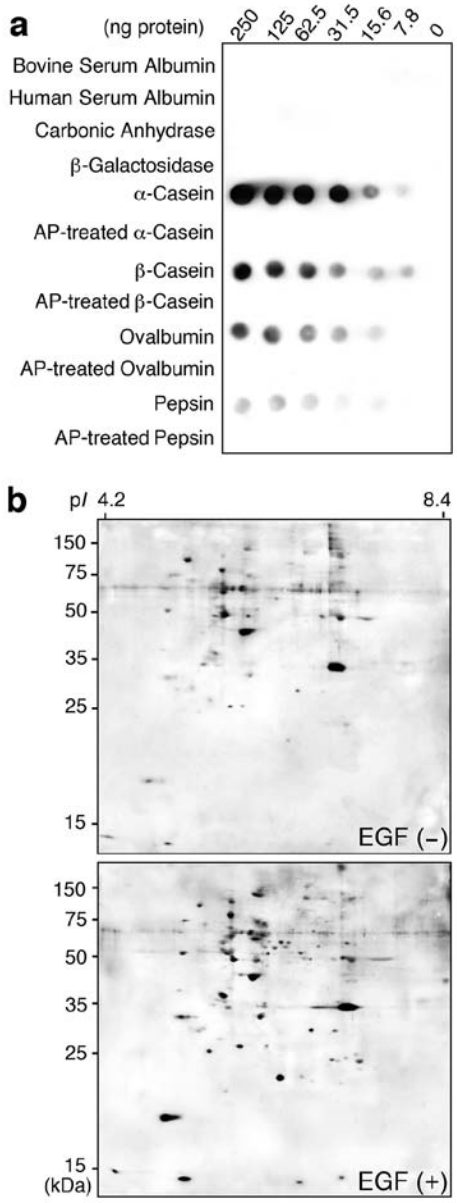

Figure 2: ECL detection of phosphoproteins on protein-blotting membranes by using the complex of Phos-tag Biotin (BTL-104) with HRP–SA. (a) Dot-blotting analysis of four non-phosphorylated proteins (bovine serum albumin, human serum albumin, carbonic anhydrase, and β-galactosidase), four phosphorylated proteins (α-casein, β-casein, ovalbumin, and pepsin), and the corresponding proteins dephosphorylated by treatment with alkaline phosphatase (AP-treated). (b) Electroblotting analysis after two-dimensional electrophoresis of A431 cell lysates before (–, upper panel) and after EGF stimulation (+, lower panel). All ECL images were obtained by using the complex of Phos-tag Biotin (BTL-104) with HRP–SA, an ECL Advance Kit (as an ECL substrate reagent, GE Healthcare Bio-Sciences, Piscataway, NJ, USA), and an LAS 3000 image analyzer (Fujifilm, Tokyo, Japan). [Reprinted with permission from Ref. 19 ©(2006) The American Society for Biochemistry and Molecular Biology].

derivatives, Phos-tag Biotin BTL-111 complexed with HRP–SA showed the best performance among the ECL systems in Western blotting (Figure 3a). To evaluate whether the BTL-111 derivative might be similarly useful in other applications, we examined its application in the analysis of a phosphoprotein by using quartz-crystal microbalance (QCM), and we compared the sensitivity of detection achievable by

this technique with those achievable by the corresponding technique using the BTL-104 derivative. We prepared a QCM sensor chip coated with immobilized NeutrAvidin and we converted this into a novel phosphate-affinity chip by binding the Phos-tag derivative to the NeutrAvidin fixed on the surface of the electrode. Figure 3b shows typical comparative data obtained by using the BTL-111 and BTL-104 chips in analyses of β-casein, a standard phosphoprotein. As in the Western blotting analysis, BTL-111 permitted more sensitive detection of the phosphoprotein than did BTL-104. QCM measurements using the chip with the immobilized BTL-111 also showed a significant advantage in terms of higher sensitivity. These results confirmed that the length of the spacer between the Phos-tag and the biotin moieties affects access to phosphorylated targets, and that a long hydrophilic spacer is effective in analytical applications (Figure 3c). The both derivatives of BTL-111 and BTL-104 are commercially available from Wako Pure Chemical Industries, Ltd., Osaka, Japan (http://www.wako-chem.co.jp/english/labchem/product/life/Phos-tag/Biotin.htm).

Peptide Microarrays

As discussed above, proteins kinases are widely recognized as valuable targets for disease diagnosis and drug discovery. We therefore attempted to utilize the advantages of BTL-111 in a high-throughput assay for monitoring the activities of intracellular protein kinases by using the commercially available peptide microarray CelluSpots. Each

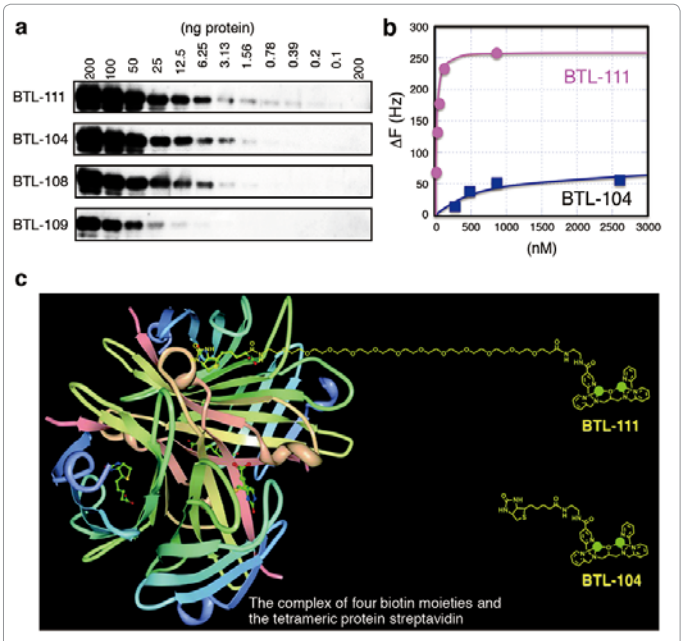

Figure 3: Development of a novel phosphate-affinity probe. (a) Western blotting analysis of β-casein (200–0.1 ng) and dephosphorylated β-casein (200 ng, rightmost lane) by using complexes of various Phos-tag Biotin derivatives and HRR–SA. All the ECL images were obtained by using Lumigen TMA-6 (as an ECL substrate reagent, Lumigen, Southfield, MI, USA) and the LAS 3000 image analyzer. (b) QCM measurements of binding of β-casein to the surface of phosphate-affinity sensor chips coated with BTL-111 or BTL-104 by using an Affinix QNμ (Initium, Tokyo, Japan). (c) Superimposed images of the complex of four biotin moieties, the tetrameric protein streptavidin, and the newly synthesized derivative BTL-111. For reference, the structure of the derivative BTL-104 is also shown. The introduction of the long hydrophilic spacer [dodeca(ethylene glycol)] increases the flexibility of the phosphate-binding moiety of Phos-tag, resulting in a greater sensitivity in the detection of phosphoproteins and phosphopeptides. [Reprinted with permission from Ref. 28 ©(2012) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim].

CelluSpots array consists of 384 peptide spots printed in duplicate on a glass slide (76×26 mm); these peptide spots including four control spots consisting of two types of phosphopeptide, one with a phosphorylated serine/threonine residue (spot #M22) and one with a phosphorylated tyrosine residue (spot #O2). The phosphopeptides were detected by using the same ECL system that we used in the Western blotting analysis described above. First, we probed the peptide microarray before the kinase reaction by using HRP–SA alone (without Phos-tag Biotin); this resulted in the detection of many false-positive ECL signals (left-hand panel of Figure 4a). Because the CelluSpots are microarrays of cellulose-conjugated peptides spotted on a planar surface of the glass slide to permit formation of a three-dimensional layer, it is likely that non-specific interactions between the cellulose and HRP–SA were responsible for the false-positive signals. To eliminate this problem, we incorporated a blocking treatment with a solution containing 10 mM Tris–HCl (pH 7.5), 0.10 M NaCl, 0.10% (v/v) Tween 20, and 10% (w/v) bovine serum albumin (BSA). We then compared the potency of BTL-111 and BTL-104 with respect to their specific detection of control phosphopeptides on the array. The comparative results showed that BTL-111 permitted specific ECL detection of the controls (spots #M22 and #O2 in center panel of Figure 4a), whereas BTL-104 did not do so (right-hand panel of Figure 4a). The results of probing with the BTL-104-bound HRP–SA complex showed that the presence of the blocking protein (BSA) on the array surface interfered with access to the target by the phosphate-binding moiety of BTL-104. We therefore confirmed that the presence of the long spacer in BTL-111 is crucial for capturing phosphorylated targets without steric hindrance in this microarray application (Figure 3c).

Next, we examined the profiling of tyrosine kinase activities involved in the EGF-signaling pathway of A431 cells as a typical example of the application of the technique (Figure 4b). Samples of cell lysate before and after stimulation with EGF were individually subjected to the kinase reaction on tyrosine kinase substrate arrays (YKS-I, Intavis Bioanalytical Instruments AG). Two different images corresponding to EGF-stimulated and non-stimulated lysates were obtained from the ECL system with BTL-111, and these were subsequently superimposed (right-hand panel). The ECL signals from the control spots #M22 and #O2 were used to normalize the two ECL images. The images obtained by detection using the lysates before and after EGF stimulation are represented by green and magenta colors, respectively. When these two images were superimposed, overlapping spots appeared white in the resulting image. In agreement with the results of many other studies on EGF signaling, we confirmed the presence of significant increases in kinase activities of Fyn, Src, and Syk by using the peptide microarray system with BTL-111. High-throughput kinase inhibition profiling is also possible. We examined the influence of a kinase-specific inhibitor, Src kinase inhibitor I, on activities of tyrosine kinases in EGF-stimulated A431 cells. The images obtained by detection using the lysates treated with or without Src kinase inhibitor I are represented by green and magenta colors, respectively (Figure 4c). Green and magenta colors indicate increases and decreases in kinase activities after treatment with the inhibitor, respectively. These results suggest that the peptide microarray system using Phos-tag Biotin is likely to be useful for high-throughput screening of intracellular protein kinase activities and that it has potential applications in disease diagnosis and drug discovery.

Antibody Microarrays

The antibody microarray, a specific form of the protein microarray,

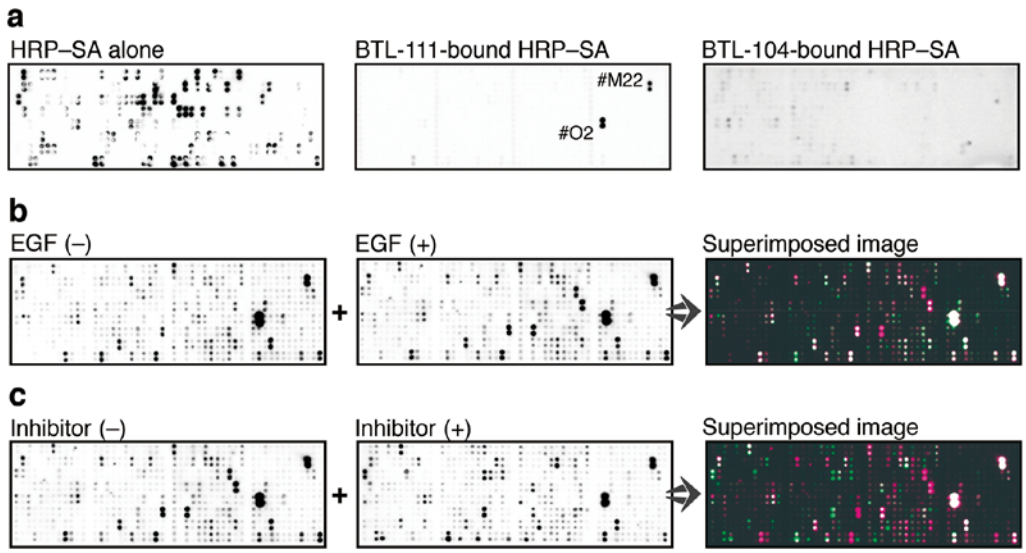

Figure 4: High-throughput profiling of intracellular protein kinase activities by using a peptide microarray system. (a) Comparative data obtained by probing with HRP–SA only (left), with the complex of BTL-111 and HRP–SA (center), or with the complex of BTL-104 and HRP–SA (right). The locations of control spots of phosphopeptides (spots #M22 and #O2) are shown in the center panel. (b) Profiling of Tyr kinase activities involved in the EGF-signaling pathway of A431 cells. The images of detections using the lysates before (control, –) and after (+) EGF stimulation are shown in the left and center panels, respectively. These two images were superimposed (right panel). (c) Profiling of Tyr kinase activities involved in the treatment of EGF-stimulated A431 cells with Src kinase inhibitor I. The images from lysate samples before (control, –) and after (+) treatment with the inhibitor are shown in the left and center panels, respectively. These two images were superimposed (right panel). All the ECL images were obtained by using the Lumigen TMA-6 and LAS 3000 image analyzer. [Reprinted with permission from Ref. 28 ©(2012) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim].

is potentially of considerable value in a wide variety of biological studies ranging from basic research to clinical proteomic studies [41]. A collection of capture antibodies is spotted as an array on a solid surface to permit the detection of multiple antigens. This technique is often used for detecting protein expression in cell or tissue samples in basic research or for detecting particular biomarkers from serum or urine in diagnostic applications. By the use of a cocktail of several site-specific anti-phosphoprotein antibodies, this technique can also be applied in phosphoproteomics as a tool for determining the sites of phosphorylation of several proteins [42]. We recently demonstrated an antibody microarray-based procedure for performing a sandwich assay that uses Phos-tag Biotin BTL-111 in conjunction with the same ECL system described above [29]. In the procedure, we used the commercially available antibody microarray Proteome Profiler (R&D Systems, Minneapolis, MN, USA). This microarray consists of 52 individual capture antibodies spotted in duplicate on a nitrocellulose membrane; each capture antibody is a phosphorylation-independent antibody. Because many false-positive ECL signals were detected by probing with HRP–SA alone (left-hand panel of Figure 5a), a blocking treatment was required in the probing procedure, as in the case of the peptide microarray system described above. The blocking treatment significantly improved the results, and almost no false-positive ECL signals were detected other than the reference spots at three corners (right-hand panel of Figure 5a). We then examined the phosphoproteins present in two lysate samples of Raw 264.7 cells. The first sample was treated with lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA), whereas the second sample was left untreated (Figure 5b). In agreement with the results of many other studies on LPS/PMA signaling, we confirmed the presence of significant increases in levels of phosphorylation of several proteins, including Akt2, HSP27, and p38β by using the antibody microarray system with BTL-111 (Figure 5c). The antibody microarray system using Phos-tag Biotin BTL-111 therefore permits simultaneous detection of the phosphorylation status of multiple proteins in a lysate sample.

Tissue Microarrays

The tissue microarray technique provides a massive acceleration of studies that attempt to correlate molecular *in situ* findings with pathological information [43]. In the proteomic analysis, 'Human Protein Atlas' using the antibody-based tissue microarray technique has been proposed as a powerful tool for pathology-based biomedical research, including protein science and biomarker discovery [44]. By using the Phos-tag-based affinity probe, similarly, this microarray technique would permit molecular profiling of phosphorylation events across a large number of arrays of tissues in various disease states or in response to various agents. Figure 6 shows the results of phosphorylation profiling of tissue array samples (Evaluation slide, SuperBioChips Laboratories, Seoul, Korea) probed with the complex of Phos-tag Biotin and HRP–SA (unpublished data). We used diaminobenzidine (DAB, biphenyl-3,3',4,4'-tetramine) as a detection substrate. Although more detailed researches are necessary and more development work is required, the Phos-tag-based probing procedure with tissue microarrays is expected to provide 'Human Phosphorylation Atlas' as a tool in the field of medicine.

Other Array Formats

In this section, we describe applications of Phos-tag Biotin in two other array formats for the detection of phosphorylation of peptides and proteins. The first application is based on a peptide array technique with a surface plasmon resonance (SPR) imaging system, and it permits a highly sensitive and quantitative detection of on-chip phosphorylation of peptide multiplexes for profiling of protein kinase activities. The

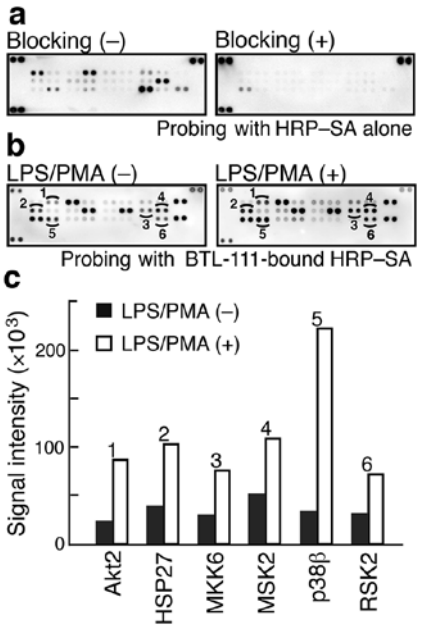

Figure 5: Phosphorylation profiling of intracellular proteins by using an antibody microarray system. (a) Comparative data obtained by probing with HRP–SA only before (left, –) and after (right, +) adding a blocking treatment. (b) Profiling of multiplex protein phosphorylation involved in the LPS/PMA-signaling pathway of Raw 264.7 cells. Images from lysates before (control, –) and after (+) LPS/PMA treatment are shown in the left-hand and right-hand panels, respectively. (c) Densitometric data from the images shown in b. The reported values of signal intensity are means in duplicate. Proteins having more signal intensities with values in excess of 20,000 and which also increased by more than a factor of two after LPS/PMA treatment are shown. All the ECL images were obtained by using the Lumigen TMA-6 and LAS 3000 image analyzer, and the densitometric analysis was performed by using the Multi Gauge software attached to the analyzer. [Reprinted with permission from Ref. 29 © (2013) Elsevier Inc.].

second application is based on the Bio-Plex suspension array technique with a flow-based microplate fluorescence reader system. By using this technique, we demonstrated profiling of phosphorylation of a target protein in an intracellular signal transduction.

We previously developed a procedure for on-chip detection of the phosphorylation status of peptide arrays by using an SPR imaging system in conjunction with Phos-tag Biotin [24]. SPR, which relies on detecting changes in the refractive index on a gold surface, is insufficiently sensitive to permit on-chip detection of increases in molecular weight resulting from phosphorylation alone. Therefore, for SPR assays it is essential to use detection reagents that bind specifically to phosphate groups. Conventionally, anti-phosphoprotein antibodies have been used in the SPR imaging systems. Antibodies, whose epitopes are phosphorylated on serine, threonine, or tyrosine residues, are commercially available and can be used in conjunction with SPR to detect phosphorylation. However, multiple antibodies are required for a peptide array on which a number of kinase substrates are immobilized. Therefore, Phos-tag Biotin, whose binding potency is almost independent on the nature of the amino acid residue, is very useful in the array format for SPR. The array is subjected to an on-chip kinase reaction and then exposed to a solution containing Phos-tag Biotin. The interactions between Phos-tag Biotin and SA are subsequently analyzed by SPR imaging. Furthermore, the SPR signals from SA can be enhanced by using an anti-SA antibody, because the anti-SA antibody is sufficiently large to affect the refractive index near the gold surface. Several later reports have confirmed the utility of the SPR imaging system using Phos-tag Biotin in determining the phosphorylation status of a wide range of peptides [45-47].

The Bio-Plex suspension array technique has been used in cell-signaling studies for the quantitative detection of levels of phosphorylation of protein multiplexes [48]. We recently developed a procedure for performing sandwich assays in the Bio-Plex array format (96-well plate format; Bio-Rad Laboratories, Hercules, CA, USA) in conjunction with Phos-tag Biotin [29]. Phos-tag Biotin was used instead of a biotinylated detection antibody specific for phosphorylated sites on the target protein. A complex between Phos-tag Biotin and phycoerythrin-conjugated SA was prepared to permit detection of the Bio-Plex phosphoproteins. Samples of the lysate from A431 cells before and after EGF stimulation were individually tested with the Bio-Plex suspension array and analyzed by using internally dyed beads coupled with capture antibodies against extracellular-signal-regulating kinases 1 and 2 and the EGF receptor. The procedure using Phos-tag Biotin permitted quantitative detection of time-dependent changes in the phosphorylation levels of the target proteins in the EGF-signaling pathway. Our established procedure offers several significant advantages, including simultaneous detection of phosphorylation of protein multiplexes in a lysate sample, quantitative detection of the total level of phosphorylation in a target protein, and the acquisition of additional information on protein phosphorylation by combining the results from our Phos-tag-based technique with those obtained by the use of site-specific anti-phosphoprotein antibodies. We believe that these advantages might be helpful in resolving the nature of intracellular signaling networks.

Future Prospects

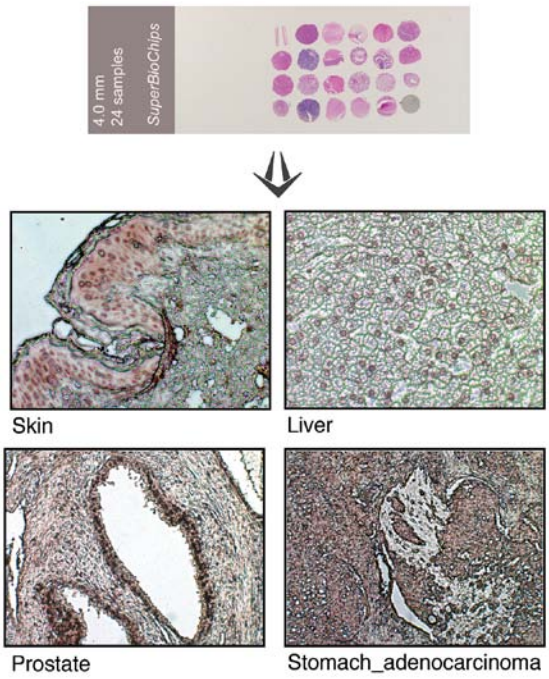

Figure 6: Phosphorylation profiling of tissue array samples probed by using the complex of Phos-tag Biotin with HRP–SA. The visualization as a brown color indicates the existence of phosphorylated biomolecules in the tissue array samples.

The utility and potential of Phos-tag-based microarray techniques are demonstrated by the exploratory data reviewed above. Peptide microarrays hold great potential for performing initial screenings in intracellular kinome analyses. Antibody microarrays have been shown to be useful in targeted phosphoproteomics and they do not require any of the sample pretreatment operations such as protein fractionation or phosphoprotein/peptide enrichment that are needed in MS-based approaches. The use of tissue microarrays might be a practical strategy in the field of medicine for deriving an atlas of the dynamic phosphorylation in various stages of disease or in various phases of clinical trials of a drug. Because the binding of Phos-tag Biotin is barely affected by the nature of the phosphorylated amino acid residue, it is very useful as a phosphate-affinity probe in array-type assays for the detection of the entire level of phosphorylation throughout a given system.

Two major approaches have been widely used for monitoring the phosphorylation status of peptides or proteins in arrays. One approach involves autoradiography on microarrays with radioactive compounds of [γ-$^{32/33}$P]-labeled adenosine triphosphate (ATP) or [$^{32/33}$P]-labeled orthophosphate. This metabolic radiolabeling approach has been used successfully in many laboratories to analyze phosphopeptides or phosphoproteins. However, the approach is limited to specimens that are amenable to labeling, and it entails problems of safety and of waste disposal. The other approach involves immunoassays with antibodies against phosphorylated amino acids. Unfortunately, the lack of specificity of some antibodies can be problematic in some cases. Anti-phosphotyrosine monoclonal antibodies are widely used because they react selectively with a variety of proteins containing phosphorylated tyrosine residues. In contrast, antibodies against phosphoserine or phosphothreonine residues are less popular because of their lower affinities and specificities. To achieve precise characterization of a phosphorylation event, it is desirable to raise a high-quality antibody against a phosphorylated residue; however, raising this type of antibody is costly, time consuming, and frequently unsuccessful. A more global and quantitative estimation of protein phosphorylation might be achieved using the newer phosphospecific Pro-Q Diamond fluorostain [49]. However, it would be a challenge to implement this staining technique to detect a specific phosphorylation event in an array format. Although fluorescence-based probing procedures offer considerable advantages in accurate quantitative measurements, the non-specific adsorption of fluorochromes, which typically contain several combined aromatic groups, or plane or cyclic moieties with several π bonds, onto the microarray surface can often cause background noise. To overcome this difficulty, blocking materials such as hydrophilic proteins [50,51] or cell membrane-mimicking polymers [52] have been adapted for use with array formats as surface matrices, and improvements of the signal-to-noise ratio in the phosphorylation detection have been achieved for the case in which a complex of Phos-tag Biotin with fluorochrome-conjugated SA is used. Therefore, progress in microarray techniques to permit more accurate quantitative measurements of phosphorylation events continues as part of the development of the next generation of techniques for phosphoproteomics.

Conclusions

The phosphorylation status of a particular protein is determined by the equilibrium between the opposing activities of protein kinases and phosphatases. Perturbations in this equilibrium can have fundamental effects on many cellular events and are involved in many human diseases. Therefore, the development of more specific and more efficient methods for detecting phosphorylation of proteins has attracted great interest in relation to phosphoproteomic studies in various biological and medical fields. We believe that considerable progress in phosphoproteomics could be achieved by combining our Phos-tag technology with various existing methodologies, including microarray techniques.

Acknowledgments

We would like to thank Professor Koichi Honke (Kochi University) for his advice regarding the Proteome Profiler antibody microarray technique, Dr. Tomomitsu Ozeki (ULVAC) for his technical help with the QCM measurements, and Mr. Takeshi Kumagai (Bio-Rad Laboratories) for his technical help with the Bio-Plex suspension array technique. This work was supported in part by JSPS KAKENHI Grants and a MEXT KAKENHI Grant.

References

1. Hunter T (2000) Signaling--2000 and beyond. Cell 100: 113-127.
2. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, et al. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127: 635-648.
3. Ubersax JA, Ferrell JE Jr (2007) Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol 8: 530-541.
4. Dinasarapu AR, Saunders B, Ozerlat I, Azam K, Subramaniam S (2011) Signaling gateway molecule pages--a data model perspective. Bioinformatics 27: 1736-1738.
5. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. Science 298: 1912-1934.
6. Kerk D, Templeton G, Moorhead GB (2008) Evolutionary radiation pattern of novel protein phosphatases revealed by analysis of protein data from the completely sequenced genomes of humans, green algae, and higher plants. Plant Physiol 146: 351-367.
7. Brognard J, Hunter T (2011) Protein kinase signaling networks in cancer. Curr Opin Genet Dev 21: 4-11.
8. Martin L, Latypova X, Terro F (2011) Post-translational modifications of tau protein: implications for Alzheimer's disease. Neurochem Int 58: 458-471.
9. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, et al. (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 2: 561-566.
10. Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG (2000) Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. Clin Cancer Res 6: 4885-4992.
11. Schutkowski M, Reineke U, Reimer U (2005) Peptide arrays for kinase profiling. Chembiochem 6: 513-521.
12. Arsenault R, Griebel P, Napper S (2011) Peptide arrays for kinome analysis: new opportunities and remaining challenges. Proteomics 11: 4595-4609.
13. Kinoshita E, Takahashi M, Takeda H, Shiro M, Koike T (2004) Recognition of phosphate monoester dianion by an alkoxide-bridged dinuclear zinc(II) complex. Dalton Trans : 1189-1193.
14. Kinoshita E, Yamada A, Takeda H, Kinoshita-Kikuta E, Koike T (2005) Novel immobilized zinc(II) affinity chromatography for phosphopeptides and phosphorylated proteins. J Sep Sci 28: 155-162.
15. Kinoshita-Kikuta E, Kinoshita E, Yamada A, Endo M, Koike T (2006) Enrichment of phosphorylated proteins from cell lysate using a novel phosphate-affinity chromatography at physiological pH. Proteomics 6: 5088-5095.
16. Kinoshita-Kikuta E, Kinoshita E, Koike T (2009) Phos-tag beads as an immunoblotting enhancer for selective detection of phosphoproteins in cell lysates. Anal Biochem 389: 83-85.
17. Kinoshita-Kikuta E, Yamada A, Inoue C, Kinoshita E, Koike T (2011) A novel phosphate-affinity bead with immobilized Phos-tag for separation and enrichment of phosphopeptides and phosphoproteins. J Integr OMICS 1: 157-169.
18. Tsunehiro M, Meki Y, Matsuoka K, Kinoshita-Kikuta E, Kinoshita E, et al. (2013) A Phos-tag-based magnetic-bead method for rapid and selective separation of

phosphorylated biomolecules. J Chromatogr B Analyt Technol Biomed Life Sci 925: 86-94.

19. Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 5: 749-757.

20. Kinoshita-Kikuta E, Aoki Y, Kinoshita E, Koike T (2007) Label-free kinase profiling using phosphate affinity polyacrylamide gel electrophoresis. Mol Cell Proteomics 6: 356-366.

21. Kinoshita E, Kinoshita-Kikuta E, Koike T (2009) Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. Nat Protoc 4: 1513-1521.

22. Kinoshita E, Kinoshita-Kikuta E (2011) Improved Phos-tag SDS-PAGE under neutral pH conditions for advanced protein phosphorylation profiling. Proteomics 11: 319-323.

23. Kinoshita E, Kinoshita-Kikuta E, Koike T (2012) Phos-tag SDS-PAGE systems for phosphorylation profiling of proteins with a wide range of molecular masses under neutral pH conditions. Proteomics 12: 192-202.

24. Inamori K, Kyo M, Nishiya Y, Inoue Y, Sonoda T, et al. (2005) Detection and quantification of on-chip phosphorylated peptides by surface plasmon resonance imaging techniques using a phosphate capture molecule. Anal Chem 77: 3979-3985.

25. Shigaki S, Yamaji T, Han X, Yamanouchi G, Sonoda T, et al. (2007) A peptide microarray for the detection of protein kinase activity in cell lysate. Anal Sci 23: 271-275.

26. Nakanishi T, Ando E, Furuta M, Kinoshita E, Kinoshita-Kikuta E, et al. (2007) Identification on membrane and characterization of phosphoproteins using an alkoxide-bridged dinuclear metal complex as a phosphate-binding tag molecule. J Biomol Tech 18: 278-286.

27. Horinouch T, Higa T, Aoyagi H, Nishiya T, Terada K, et al. (2012) Adenylate cyclase/cAMP/protein kinase A signaling pathway inhibits endothelin type A receptor-operated Ca^{2+} entry mediated via transient receptor potential canonical 6 channels. J Pharmacol Exp Ther 340: 143-151.

28. Kinoshita E, Kinoshita-Kikuta E, Sugiyama Y, Fukada Y, Ozeki T, et al. (2012) Highly sensitive detection of protein phosphorylation by using improved Phos-tag Biotin. Proteomics 12: 932-937.

29. Kinoshita E, Kinoshita-Kikuta E, Koike T (2013) Sandwich assay for phosphorylation of protein multiplexes by using antibodies and Phos-tag. Anal Biochem 438: 104-106.

30. Koike T, Kimura E (1991) Roles of zinc(II) ion in phosphatases: A model study with zinc(II)–macrocyclic polyamine complexes. J Am Chem Soc 113: 8935-8941.

31. Fujioka H, Koike T, Yamada N, Kimura E (1996) A new bis(zinc(II)-cyclen) complex as a novel chelator for barbiturates and phosphates. Heterocycles 42: 775–787.

32. Koike T, Inoue M, Kimura E, Shiro M (1996) Novel properties of cooperative dinuclear zinc(II) ions: The selective recognition of phosphomonoesters and their P–O ester bond cleavage by a new dinuclear zinc(II) cryptate. J Am Chem Soc 118: 3091-3099.

33. Kimura E, Aoki S, Koike T, Shiro M (1997) A tris(ZnII-1,4,7,10-tetraazacyclododecane) complex as a new receptor for phosphate dianions in aqueous solution. J Am Chem Soc 119: 3068-3076.

34. Kimura E, Koike T (1998) Dynamic anion recognition by macrocyclic polyamines in neutral pH aqueous solution: Development from static anion complexes to an enolate complex. Chem Commun .

35. Kinoshita E, Ishikawa K, Kinoshita-Kikuta E, Ohtani K, Shiro M, et al. (2005) An alkoxide-bridged dinuclear zinc(II) hexaazacryptate: A novel phosphate capture molecule in aqueous solution. Bull Chem Soc Jpn 78: 125-131.

36. Vallee BL (1986) A synopsis of zinc biology and pathology. In: Bertini I, Luchinat C, Maret W, Zeppezauer M (Eds) Zinc enzymes. Birkhäuser, Boston.

37. Takeda H, Kawasaki A, Takahashi M, Yamada A, Koike, T (2003) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of phosphorylated compounds using a novel phosphate capture molecule. Rapid Commun Mass Spectrom 17: 2075-2081.

38. Hirano K, Matsui H, Tanaka T, Matsuura F, Satouchi K, et al. (2004) Production of 1,2-didocosahexaenoyl phosphatidylcholine by bonito muscle lysophosphatidylcholine/transacylase. J Biochem 136: 477-483.

39. Tanaka T, Tsutsui H, Hirano K, Koike T, Tokumura A, et al. (2004) Quantitative analysis of lysophosphatidic acid by time-of-flight mass spectrometry using a phosphate-capture molecule. J Lipid Res 45: 2145-2150.

40. Urikura M, Morishige J, Tanaka T, Satouchi K (2012) Phosphatidic acid production in the processing of cabbage leaves. J Agric Food Chem 60: 11359-11365.

41. Haab BB (2003) Methods and applications of antibody microarrays in cancer research. Proteomics 3: 2116-2122.

42. Zhang H, Pelech S (2012) Using protein microarrays to study phosphorylation-mediated signal transduction. Semin Cell Dev Biol 23: 872-882.

43. Bubendorf L, Nocito A, Moch H, Sauter G (2001) Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol 195: 72-79.

44. Pontén F, Jirström K, Uhlen M (2008) The Human Protein Atlas--a tool for pathology. J Pathol 216: 387-393.

45. Inamori K, Kyo M, Matsukawa K, Inoue Y, Sonoda T, et al. (2008) Optimal surface chemistry for peptide immobilization in on-chip phosphorylation analysis. Anal Chem 80: 643-650.

46. Mori T, Inamori K, Inoue Y, Han X, Yamanouchi G, et al. (2008) Evaluation of protein kinase activities of cell lysates using peptide microarrays based on surface plasmon resonance imaging. Anal Biochem 375: 223-231.

47. Inamori K, Kyo M, Matsukawa K, Inoue Y, Sonoda T, et al. (2009) Establishment of screening system toward discovery of kinase inhibitors using label-free on-chip phosphorylation assays. Biosystems 97: 179-185.

48. Houser B (2012) Bio-Rad's Bio-Plex® suspension array system, xMAP technology overview. Arch Physiol Biochem 118: 192-196.

49. Steinberg TH, Agnew BJ, Gee KR, Leung WY, Goodman T, et al. (2003) Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology. Proteomics 3: 1128-1144.

50. Han X, Sonoda T, Mori T, Yamanouchi G, Yamaji T, et al. (2010) Protein kinase substrate profiling with a high-density peptide microarray. Comb Chem High Throughput Screen 13: 777-789.

51. Han X, Katayama Y (2010) A peptide microarray for detecting protein kinase activity in cell lysates. Methods Mol Biol 669: 183-194.

52. Ikeda H, Kamimoto J, Yamamoto T, Hata A, Otsubo Y, et al. (2013) A peptide microarray fabricated on a non-fouling phosphatidylcholine-polymer-coated surface for a high-fidelity analysis of a cellular kinome. Curr Medicin Chem.

This article was originally published in a special issue, **Microarray Proteomics** handled by Editor(s). Dr. Qiangwei Xia, University of Wisconsin-Madison, USA