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Establishment of screening system toward discovery of kinase inhibitors using label-free on-chip phosphorylation assays

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ABSTRACT

We describe a label-free method for the kinase inhibition assay toward discovery of kinase inhibitors. The surface plasmon resonance (SPR) imaging analysis using zinc(II) compound was adopted on the on-chip phosphorylation analysis. In this study, following three subjects were focused: (1) to monitor the inhibition of three inhibitors supporting by their specific inhibition mechanisms, (2) to quantify the inhibitory activities, and (3) to prove the reliability of the obtained 50% inhibition concentration (IC_{50}) value. First, the inhibitory activities of Amide 5-24, H-89 and Gö6983 on PKA and PKCδ were determined, and specific inhibitions for two kinases could be observed quantitatively. Second, the inhibition curves of Amide 5-24, Amide 14-22 and H-89 were obtained, and the results supported the two previous reports: (1) the inhibition efficiency of Amide 5-24 was much higher than that of Amide 14-22, and (2) the inhibitory activity of H-89 followed ATP-binding site blocking mechanism. Last, the obtained IC_{50} values by the SPR imaging were almost corresponded to those by the solution assay, although on-chip phosphorylation efficiency was low (approximately 12%). In conclusion, validation of the on-chip phosphorylation analysis for kinase inhibitors was achieved. This label-free method might be applied for discovery of kinase inhibitors.

1. Introduction

In recent years, protein kinase inhibitors have been noticed as one of the target-based drugs. To block proliferation, metastasis or invasion of cancer cells, the specific inhibitor of particular protein kinase, e.g. Gefitinib (Paez et al., 2004), Imatinib Mesilate (Schindler et al., 2000; Gorre et al., 2001) have been already developed as therapeutic products of cancer (Levitzki, 2003; Traxler, 2003; Fabian et al., 2005). In addition, many peptides or small molecules have also been designed based on the various approaches (Hah et al., 2006; Reuveni et al., 2002; Toledo et al., 1999; Marsilje et al., 2000).

As a means of the screening or evaluation of such inhibitors, high throughput assays for protein kinase activity have become much important. These methods can be roughly classified into solution assays with microwell plates and array analyses on solid support. As solution assays, enzyme-linked immunosorbent assay (ELISA) (Mahoney et al., 1999), fluorescence resonance energy transfer (FRET) (Sills et al., 2002), and the fluorescence "superquenching" technology (Chen et al., 1999; Jones et al., 2001; Lu et al., 2002; Rininsland et al., 2004) had been developed, and used for the kinase assay. Recently, mass spectrometry analysis was also proposed (Sion-Usakiewicz et al., 2005; Min et al., 2004).

To achieve the higher throughput assay, the array analysis has been widely used. Fluorescence (MacBeath and Schreiber, 2000; Chen et al., 2003; Uttamchandani et al., 2003; Pal et al., 2006) or radiosiotope (RI) using [γ-^{32}P/^{33}P] ATP (Hutti et al., 2004; Falsey et al., 2001; Houseman et al., 2002) is used for detection in the conventional array analysis. In addition, a sol–gel microarray (Rupcich et al., 2005) and a microarray with attachment of gold nanoparticles (Sun et al., 2005; Wang et al., 2005) had been proposed. As one of their applications, the inhibition assays using the peptide arrays are one of the most important and promising output (Shigaki et al., 2007; Han et al., 2008). There are three advantages in the array analysis for the phosphorylation inhibition assay: to have a higher potential for raising the immobilized peptide density, to be applied with the small quantity of samples and reagents, and to obtain the phosphorylation patterns for many kinds of substrate at the same time (Shigaki et al., 2007; Han et al., 2008). In particular, the application of the kinase assay for the cell-based drug discovery is promising. Many kinds of protein kinase are expressed in the cell lysate, and their activities should be reflected by the phosphorylation patterns on the array.

This study was aimed at the establishment and validation of the label-free kinase inhibition assay using peptide array with sur-

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face plasmon resonance (SPR) imaging techniques (Nelson et al., 1999, 2001; Brockman et al., 2000), because SPR imaging array has an important advantage that does not require RI- or fluorescence-labeling for detection. We propose here that it is the high-security method without troublesome procedures for the on-chip phosphorylation assay. In the previous paper (Inamori et al., 2005), we reported the detection and quantification system of the phosphorylated peptides on the gold chip by SPR imaging techniques using zinc(II) compound as the phosphate capture molecule for the detection of phosphorylated site (Kinoshita et al., 2004). Furthermore, we achieved the detection of the change of kinase activities in cell lysate by the chemical stimulation, and successfully monitored the kinase inhibitory activities of stimulating chemicals by SPR imaging analysis (Mori et al., 2008). We also applied the SPR imaging to the quantification for the inhibitory activity of kinase inhibitors. Thus, the peptide microarray based on SPR imaging has potential for applicable to cell-based drug discovery, and biochemical studies to investigate signal transduction pathways.

In this study, following three subjects were focused for the establishment and validation the kinase inhibition assay using SPR imaging toward discovery of kinase inhibitors. The first subject was to verify the evaluation of three kinase inhibitors on the chips. Especially, we investigated the obtained inhibition curves for two kinds of inhibitor from the aspect of the inhibition mechanisms, i.e. competition with substrate or blocking of the ATP-binding site. The second subject was to quantify the inhibitory activity of the kinase inhibitor by SPR imaging analysis. The last subject was to verify whether the obtained 50% inhibition concentration (IC_{50}) values were appropriate by the comparison with the solution assay.

2. Experimental

2.1. Materials

Sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxalate (SSMCC, Pierce), thiol terminated methoxy poly (ethylene glycol) MW 5000 (PEG-thiol, NOF), cAMP-dependent protein kinase catalytic subunit (PKA, Promega), Protein Kinase C Delta Isozyme (PKCδ, Sigma–Aldrich), adenosine 5′-triphosphate disodium salt hydrate (ATP, Sigma–Aldrich), [γ-^{33}P]ATP (GE Healthcare), phosphatidylserine (PS, Wako Pure Chemical Industries), 1,2-diacylglycerol (DAG, Calbiochem), streptavidin (SA, Invitrogen), anti-streptavidin antibody (anti-SA, Vector), *N*-(5-(2-(+)-biotinaminoethylcarbamoyl)pyridin-2-ylmethyl)-*N*,*N*′,*N*′-tris(pyridin-2-ylmethyl)-1,3-diaminopropan-2-ol (Phos-tag biotin, Nard Institute, Japan) were used without further purification. The other reagents and solvents used were of analytical quality. All aqueous solutions were prepared using deionized and distilled water.

2.2. Peptide Preparation

All peptides used in this study and shown in Table 1 were synthesized using a peptide synthesizer and purified by HPLC as described in our previous paper (Inamori et al., 2004, 2008). Kemptide peptide (Kemp et al., 1977), to which cysteine and two glycine residues were attached at the C-terminus, was used as a PKA substrate (probe 1). We prepared the negative control of Kemptide (probe 2), on which the serine is substituted with alanine, and the positive control (probe 3), which has a phosphoserine residue. The substrate of cSrc kinase (probe 4) (Houseman et al., 2002) and the negative control of probe 4 (probe 5), which has a phosphotyrosine residue, were also prepared. As the PKCδ substrate, probe 6 (New et al., 1998) was synthesized. The negative control of probe 6 (probe 7), on which both serine and threonine are substituted with alanine, and the positive control (probe 8), which has a phosphoserine and a phosphothreonine residue, were also prepared.

Table 1
Peptide sequences of surface-immobilized probes.

Probe	Peptide sequence	Specifications
1	CGGLRRAS̲LG-NH_2	
2	CGGLRRAA̲LG-NH_2	Ala-substituted
3	CGGLRRAS̲(PO_3)LG-NH_2	Ser-phosphorylated
4	CGIY̲GEFKKK-NH_2	
5	CGIY̲(PO_3) GEFKKK-NH_2	Tyr-phosphorylated
6	CGGLRRT̲LS̲VA-NH_2	
7	CGGLRRA̲LA̲VA-NH_2	Ala-substituted
8	CGGLRRT̲(PO_3)LS̲(PO_3)VA-NH_2	Ser- and Thr-phosphorylated

2.3. Fabrication of Peptide Arrays

The covalently immobilized peptide array was obtained by an established procedure, which was reported in our previous paper (Inamori et al., 2004, 2008). Briefly, a patterned amino-modified Au-coated chip (Toyobo) was allowed to react with 1 mM SSMCC to create a maleimido-modified surface. Next, 1 mg/mL of cysteine-terminated peptide substrates were delivered on the maleimide surface using an automated spotter (Toyobo). The unreacted maleimido groups on the peptide array were blocked by reacting them with 10 mg/mL PEG-thiol.

2.4. On-chip Phosphorylation by Protein Kinases

The peptide arrays were reacted for 1 h at 30 °C with 400 μL of 0.2 unit/μL PKA solution in a reaction buffer (50 mM Tris–HCl, 50 mM $MgCl_2$ [pH 7.4]) containing 10 μM ATP, or with 300 μL of 0.8 unit/μL PKCδ solution in the reaction buffer (50 mM MOPS buffer, 50 mM $MgCl_2$, 8 μg/mL PS, 800 μg/mL DAG [pH 7.4]) containing 10 μM ATP. For the autoradiography assay, 1.85×10^{17} Bq/mol [γ-^{33}P] ATP was used. After the reactions, the arrays were rinsed with PBS and MilliQ water, and then dried in an airflow.

In the inhibition assay, the inhibitors were added into the kinase reaction solution with various concentrations. We used the protein kinase A inhibitor 5-24 (Amide 5-24, Calbiochem), the protein kinase A inhibitor 14-22 (Amide 14-22, Calbiochem), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide 2HCl (H-89, Biomol) as the PKA inhibitor, and 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Gö6983, Calbiochem) as the PKC inhibitor. Amide 5-24 and Amide 14-22 were used as peptide inhibitors, and H-89, Gö6983 was used as a small molecule inhibitor.

Fig. 1 indicates a comparison of the amino acid sequences for the peptide inhibitors, Amide 5-24 (Cheng et al., 1986; Glass et al., 1989a; Baude et al., 1994; Narayama et al., 1997) and Amide 14-22 (Knighton et al., 1991; Reed et al., 1989). Amide 5-24 has a much longer amino acid sequence than Amide 14-22, and it contains the whole sequence of Amide 14-22. The amino acid sequence 18–22 (RRNAI) was reported as the consensus sequence, and the amino acid sequence 5–17 (TTYADFIASGRTG) was reported as the high affinity-binding region (Knighton et al., 1991). H-89 is selective and widely used as an inhibitor of PKA (Chijiwa et al., 1990; Lochner and Moolman, 2006; Davis et al., 2000). The inhibition mechanism of H-89 is based on the blocking of the ATP-binding site of PKA (Lochner and Moolman, 2006). Meanwhile, Gö6983 is reported as a potent inhibitor of PKC (Stempka et al., 1997).

2.5. SPR Imaging Analysis

SPR imaging analysis for on-chip phosphorylation was carried out as reported in our previous paper (Inamori et al., 2004, 2008). Briefly, 2 μg/mL Phos-tag biotin solution, which was dissolved in the buffer (10 mM HEPES–NaOH, 0.2 M sodium nitrate, 1 mM zinc nitrate, 0.005% (w/v) Tween20, 10% ethanol [pH 7.4]), was added dropwise on the peptide array and incubated for 30 min at room temperature. The array was placed on the SPR instrument (Toyobo) and exposed to 1 μg/mL SA solution in the running buffer (10 mM HEPES–NaOH [pH 7.4]) for 10 min, and then rinsed with the running buffer for 5 min. For the quantification of phosphorylation, 1 μg/mL anti-SA solution in the running buffer was injected subsequently for 20 min into the SPR imaging instrument.

2.6. Autoradiography Experiment

Autoradiography analysis for on-chip phosphorylation was performed according to our previous paper (Inamori et al., 2004, 2008). Briefly, the array was reacted with [γ-^{33}P] ATP and exposed to SG Imaging Plates (Fuji Film) for 30 min. The plates were observed with an imaging analyzer (BAS-1800 II: Fuji Film). Using an analysis program (Fuji Film), the quantity of ^{33}P uptake as the relative amount of radiation was shown by the PSL (photo-stimulated luminescence) value, which is calculated by Image Gauge (Fuji Film).

2.7. Inhibition Analysis for PKA Phosphorylation in Solution by Superquenching-based Universal Assay

We used the QTL (quencher–tether–ligand) LightSpeed PKA kinase activity assay (QTL Biosystems) for the PKA inhibition assay in solution. This system is based on the fluorescence "superquenching" technology. The QTL sensor is comprised of highly fluorescent conjugated polyelectrolytes collocated with phosphate coordinating metal ions on the surface of microspheres (Chen et al., 1999; Jones et al., 2001; Lu et al., 2002; Rininsland et al., 2004). In the PKA reaction, the quencher-labeled Kemptide (LRRASLG) (Kemp et al., 1977) was used as the substrate. The phosphorylated product binds to the QTL sensor resulting in superquenching of the polymer fluorescence. The decrease in fluorescence is a measure of kinase enzymatic activity.