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Sequential phosphorylation analysis using dye-tethered peptides and microfluidic isoelectric focusing electrophoresis

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ABSTRACT

We report a simple method for analyzing sequential phosphorylation by protein kinases using fluorescent peptide substrates and microfluidic isoelectric focusing (μIEF) electrophoresis. When a dye-labeled peptide substrate was sequentially phosphorylated by two consecutive protein kinases (mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3)), its differently phosphorylated forms were easily separated and visualized by fluorescent focusing zones in the μIEF channel based on a change in the isoelectric point (p*I*) by phosphorylation. As a result, ratiometric and quantitative analysis of the fluorescent focusing regions shifted by phosphorylation enabled the analysis of phosphorylation efficiency and the relevant inhibition of protein kinases (MAPK and GSK3) with high simplicity and selectivity. Furthermore, the GSK3 activity in the cell lysates was elucidated by μIEF electrophoresis in combination with immunoprecipitation. Our results suggest that this method has great potential for analyzing the sequential phosphorylation of multiple protein kinases that are implicated in cellular signaling pathways.

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1. Introduction

Phosphorylation cascades involving multiple protein kinases play a central role in signal transduction, protein regulation, and metabolism in living cells (Cohen, 2002; Hunter, 1995; Schenk and Snaar-Jagalska, 1999; Walsh and MacDonald, 2011). The analysis of these phosphorylation cascades, therefore, will provide new insights into their physiological functions in many biological events. While general methods for probing protein kinase activity have adopted [gamma-^{32}P]ATP-labeled substrate in electrophoresis or non-radioisotopic immunoblotting to differentiate between phosphorylated and unphosphorylated substrates (Hastie et al., 2006; Peck, 2006; Yamamoto et al., 2006; Zhu et al., 2000), they are not only based on long processing times, but they are limited to the rapid identification of a cascade event involving multiple kinases or the determination of the quantitative amount of phosphorylation. Fluorogenic or fluorescence resonance energy transfer-based methods using peptide substrates have been alternatively used for monitoring various phosphorylation events, which are based on the fluorescent change from substrate phosphorylation using dye-labeled antibodies or chemicals (Ghadiali et al., 2010; Harvey et al., 2008; Kupcho et al., 2003; Riddle et al., 2006; Shiosaki et al., 2013), but these are mostly used for single kinase reactions. In addition, mass spectrometric analysis has been implemented as a standard means for identifying the degree of phosphorylation (Kim et al., 2007; Mann et al., 2002; Salih, 2005), which a time-consuming and expensive process.

To circumvent these issues, increasing attention has been paid to isoelectric focusing (IEF) technique because it is capable of easily separating diverse phosphorylated proteins according to their isoelectric point (p*I*) based on the negatively accumulating increase in net charge by sequential addition of phosphate group to the protein (Anderson and Peck, 2008). Although capillary-type IEF and two-dimensional IEF gel electrophoresis have been reported to be useful for separating phosphorylated proteins (Kinoshita et al., 2009; O'Neill et al., 2006), fewer efforts have been

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made to analyze phosphorylation cascade, especially in a rapid and quantitative manner. Given a common substrate for multiple kinases, the IEF technique would allow for the phosphorylation cascades of the substrate to be easily resolved during multiple kinase reactions.

Here we report a simple method for analyzing sequential phosphorylation using fluorescent peptide substrates and microfluidic isoelectric focusing (μIEF) electrophoresis. Despite recent advances in μIEF techniques for biological analyses (Das et al., 2007; Sommer and Hatch, 2009; Wen et al., 2010), many attempts have focused on the easy separation of complex biomolecules according to the average p*I*s. To the best of our knowledge, there have been no attempts in integrating the μIEF technique for the simultaneous detection of cascade phosphorylation. To demonstrate the sequential phosphorylation event, we chose two consecutive protein kinases: p42 mitogen-activated protein kinase (MAPK), also known as extracellular signal-regulated kinase 2 (ERK2), and glycogen synthase kinase 3 (GSK3). Since MAPK is considered to be one of the priming kinases for GSK3 in a process by which target substrates are sequentially phosphorylated (Eldar-Finkelman, 2002), we examined the phosphorylation cascade using an annotated peptide substrate from heat shock transcription factor 1 (HSF1), which is consecutively controlled by two protein kinases (Chu et al., 1996; He et al., 1998). Upon sequential phosphorylation, a dye-labeled peptide substrate gave rise to a mono- or di-phosphorylated form, allowing for easy separation and rapid detection in a polydimethylsiloxane (PDMS) μIEF channel equipped with an imaging analyzer. Moreover, the activity and inhibition of the protein kinases were quantitatively investigated by comparing two or three separated lines of the peptides on the μIEF. In contrast to conventional IEF electrophoresis, a microfluidic-based format enabled more reliable analyses with rapid assay time and much smaller reaction volumes (a few microliters).

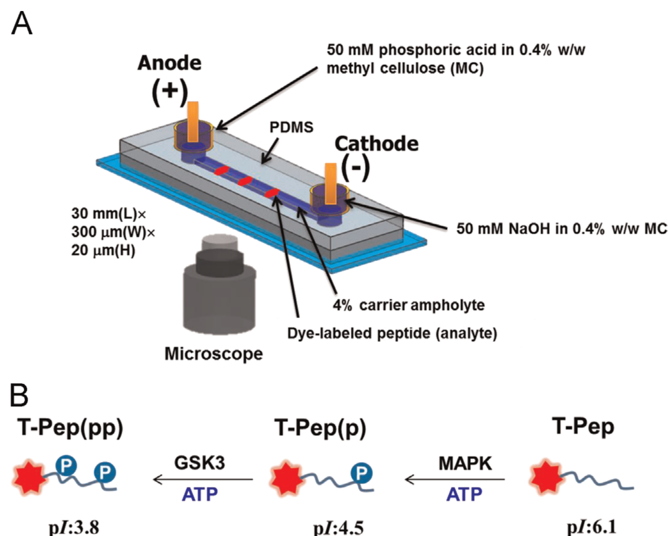
2. Materials and methods

2.1. Materials

P42 mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3) was purchased from New England Biolabs. Anti-human GSK3 beta polyclonal antibody, a protein G magnetic bead and a C18 pipette-tip column were purchased from Thermo Fisher Scientific Inc. (USA). RIPA buffer was purchased from Cell Signaling Technology. Methylcellulose (viscosity 400 cP, MC), lithium chloride (99%, LiCl), adenosine 5′-triphosphate (ATP) disodium salt hydrate (99%, 5'-ATP-Na_2), α-cyano-4-hydroxycinnamic acid (98%, CHCA) and trifluoroacetic acid (99%, TFA) were purchased from Sigma-Aldrich. Acetonitrile (ACN, HPLC grade) was purchased from Merck. Ampholyte (Pharmalyte, broad range pH 3–10) was purchased from GE Healthcare Life Sciences. FR180204 was purchased from Santa Cruz Biotechnology Inc. (USA). Polydimethylsiloxane (PDMS) was purchased from Dow Corning. Isopropyl alcohol (99.5%, IPA) was purchased from Daejung. SU-8, a photoresist (PR), and SU-8 developer (PGMEA) was purchased from MicroChemicals. Tetramethyl-6-carboxyrhodamine (TAMRA)-labeled peptides (T-Pep, TAMRA-KEEPPSPPQSPR; T-Pep(p), TAMRA-KEEPPSPPQpSPR; T-Pep(pp), TAMRA-KEEPPpSPPQpSPR) were synthesized from Peptron, Inc. (Korea). All other chemicals were of analytical grade and were used as received.

2.2. Fabrication of a microfluidic isoelectric focusing (μIEF) device

A microfluidic chip was fabricated by bonding polydimethylsiloxane (PDMS) substrate on a flat PDMS substrate. A PDMS substrate was prepared by using a standard molding technique and soft lithography. To make a PDMS mold, a PR material was uniformly spin-coated at a thickness of 20 μm on a 4 in. Si wafer that was sequentially cleaned with acetone, IPA, and deionized (DI) water. The coated wafer was soft-baked for 3.5 min at 95 °C. Then it was exposed to 365 nm UV light (MDA-400M, Midas) through a film-type photo mask having a pattern of straight channel. In order to enhance the crosslinking of PR, a post-exposure-baking of the wafer was performed for 4.5 min at 95 °C. The wafer was developed for 3.5 min in a solution of PGMEA. The wafer was washed with IPA to remove unexposed PR and dried with nitrogen gas. To fabricate the PDMS substrate, a mixture of PDMS (DC-184A) and curing agent (DC-184B) at a ratio of 10 to 1 was poured into the fabricated mold and degassed for 30 min and cured for 2 h at 70 °C in a vacuum oven (JSVO-60T, JSR). This PDMS substrate was bonded with a flat PDMS substrate after exposure to oxygen plasma (Plasma prep 300, Nanotech). Finally, the PDMS channel surface was coated with MC to make it hydrophilic for the purpose of reducing electroosmotic flow (EOF). The microfluidic chip had a straight channel with a length of 3 cm, a width of 300 μm and a height of 20 μm as shown in Scheme 1A. The reservoirs were attached on the inlet and outlet for the injection of electrolytes.



Scheme 1. (A) Schematic of IEF electrophoresis in a PDMS microfluidic device equipped with fluorescent microscopy and imaging analysis. (B) Illustration of sequential phosphorylation of TAMRA-labeled peptide substrate (T-Pep) by two consecutive protein kinases (MAPK and GSK3), leading to the reduction in its p*I* value.

2.3. μIEF-based analysis of sequential phosphorylation by protein kinases

Typically, protein kinase reactions were performed in a tube prior to μIEF analysis. For the single kinase reaction, appropriate TAMRA-labeled peptide substrate (1 μL at 100 μM), MAPK or GSK3 (1 μL at 25 U), ATP (0.4 μL at 10 mM), and 10 × kinase reaction buffer (2 μL) were mixed at a final volume of 20 μL in standard reaction buffer (20 mM Tris–HCl, pH 7.4). For the sequential protein kinase reaction, the equivalent mixture (each 1 μL at 25 U) of MAPK and GSK3 was added to the same reaction solution. All kinase reactions were typically run for 90 min at 30 °C, followed by a thermal inactivation process for 20 min at 60 °C in order to load the aliquot into the PDMS microfluidic channel under the same condition. As a control, three peptides (T-Pep, T-Pep(p), and T-Pep (pp)) were mixed at a 1:1:1 molar ratio (each 1 μL at 100 μM) at a

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