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Mass-tag technology for monitoring of protein kinase activity using mass spectrometry

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Abstract—Monitoring of intracellular protein kinase activity is very important for fields involving diagnosis and drug screening. However, current methods, such as radiometry using ^{32}P, or ELISA, are laborious and time-consuming. We have developed high-throughput assay system of protein kinase activity using mass-tagged substrate peptide probes and mass spectrometry. This assay system can easily evaluate target kinase activity and will potentially be able to simultaneously profile many protein kinase activities.

Cells possess an intracellular signal transduction system with which they can precisely respond to their outer environment. The system consists of many chemical reaction cascades, and many proteins are associated with these reactions. Intracellular signals are primarily transduced by protein activation (or inactivation) caused by the conformation changes in proteins. Protein phosphorylation is one of the most versatile reactions in cells that can cause protein-conformational change. This reaction is catalyzed by protein kinases and controls various cellular functions, including gene expression,[1] cellular proliferation,[2] and cell death.[3] Therefore, if the enzymatic activity of a protein kinase rises abnormally, this rise can be directly related to various diseases.[4–6] It will therefore be useful to develop an assay system for protein kinases activities in cellular samples for fields involving diagnosis, evaluation of gene-functions, and drug development. Radiometry[7] using ^{32}P, or ELISA[8–10] well known as such an assay system. Although these assays are quite sensitive, they are laborious and time-consuming. Therefore, the development of high-throughput assay systems is required to profile all the protein kinase activity of many samples.

We report herein the use of a mass-tag strategy in an assay system for protein kinase activity using mass spectrometry. Figure 1(a) shows the basic concept of this detection system. Two cellular samples representing two different states are homogenated, respectively, and two types of peptide probes are separately added to each cell lysate. These probes possess the same substrate peptide sequences for the target protein kinase, while one of these peptide probes is isotopically heavier than the other due to the incorporation of deuterium into acetyl groups in the peptide probe. Therefore, two peptides can be distinguished in the mass spectrum. The m/z values of these probes increase by $+80$ with phosphorylation by the target protein kinase, so that protein kinase activities between different cellular states can be compared directly by calculating the phosphorylated ratio in each probe using the peak intensity in the mass spectrum after the both probe solutions were combined.

According to this concept, the peptide probes, PKA-H_6 and PKA-D_6, which possessed the substrate sequence of protein kinase A (PKA) and the His-Tag sequence, R_2-KHHHHHGLRRASLKC-NH_2 ($R = CH_3CO–$ or $CD_3CO–$), were designed and synthesized. In these probes, two normal acetyl groups or deuterated acetyl groups were incorporated into the lysine residue at the amino-terminus. Thus, the difference between the mass numbers of these probes is 6. We chose protein kinase A

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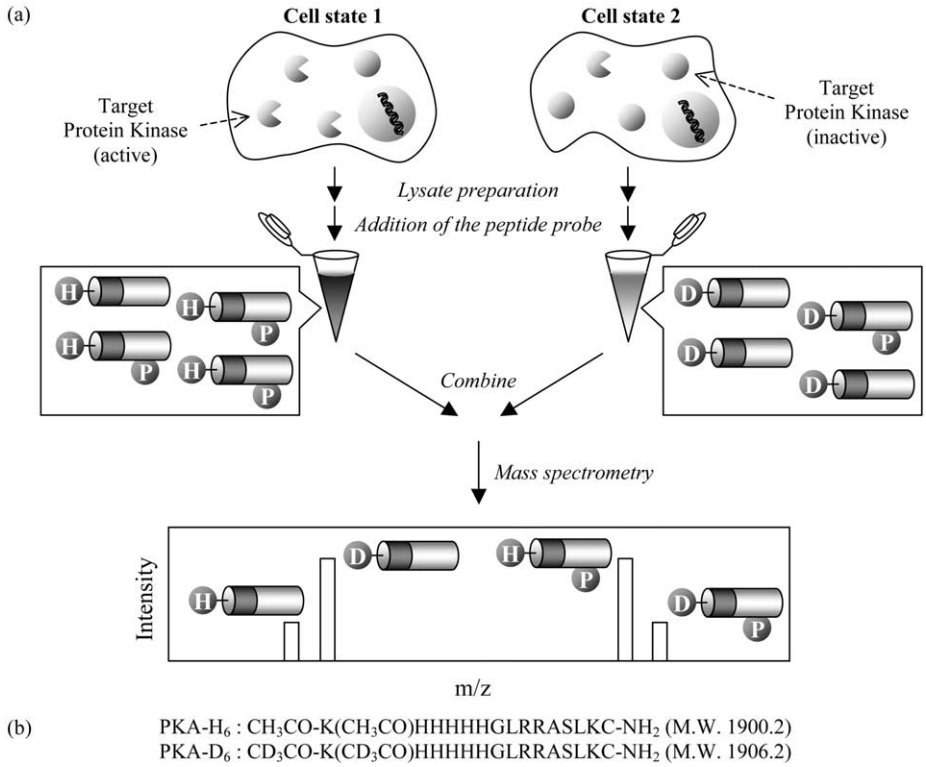

Figure 1. (a) Schematic outline of the protein kinase activity assay system using mass spectrometry. (b) Amino acid sequences of PKA substrate peptide probes.

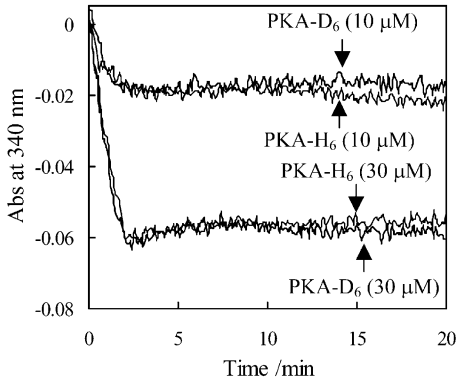

Figure 2. The time-dependent decrease in the absorbance at 340 nm based on the oxidation of NADH, which was led by the phosphorylation of the peptide probes by PKA in the coupled enzyme assay. The experiment was performed with 10 or 30 μM of each peptide probe in PBS(−) (pH 7.2) containing 0.2 mM ATP, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, 4 units of pyruvate kinase, and 60 units of PKA C-subunit at 24 °C.

(PKA) as the first target protein kinase because the enzyme is one of the most important protein kinases and controls many cellular functions such as gene expression,[11] hormone secretion,[12] and cell differentiation.[13] Meanwhile, the His-Tag sequence was introduced in peptide probes to purify them from the cell lysate in case pre-purification of the probes was required. Acetylated peptide probes were synthesized with automatic peptide synthesizer by Fmoc chemistry using corresponding Fmoc-amino acids and Fmoc-Lys(Fmoc)-OH as the N-terminal amino acid [Fig. 1(b)]. In the case of deuterated peptide synthesis, acetic anhydride-d_6 was used as the N-terminal acetylation reagent.

We first investigated whether deuterium labeling of the peptide influenced the kinetics of phosphorylation with PKA. The phosphorylation of these probes was monitored spectrophotometrically with a coupled enzyme assay reported by Cook et al.[14] In this assay, the production of ADP, which is derived from ATP as a byproduct of the phosphorylation, finally brings about an oxidation of NADH using pyruvate kinase and lactate dehydrogenase. The phosphorylation can therefore be monitored as the decrease in absorbance at 340 nm. As shown in Figure 2, the profile of the absorbance-decrease accompanying phosphorylation of the heavy probe (PKA-D_6) showed good agreement with that of the light probe (PKA-H_6), meaning that neither of the peptides differed in their ability to function as a substrate to PKA.

For the next experiment, we examined whether the mass spectra arising from both the peptide probes could be differentiated from each other. Phosphorylated or non-phosphorylated peptide probe solution was prepared separately and mixed together, then analyzed by MALDI-TOF MS. As a result, four peaks were distinctly observed. The following experiment was then performed to confirm that the phosphorylation processes of the peptide probes could be monitored by mass spectrometry. Two sample solutions of PBS(−) containing 4 U/mL PKA, 0.2 mM ATP, and 10 mM $MgCl_2$ were prepared. PKA-H_6 and PKA-D_6 were added separately to each sample to a final concentration of 2

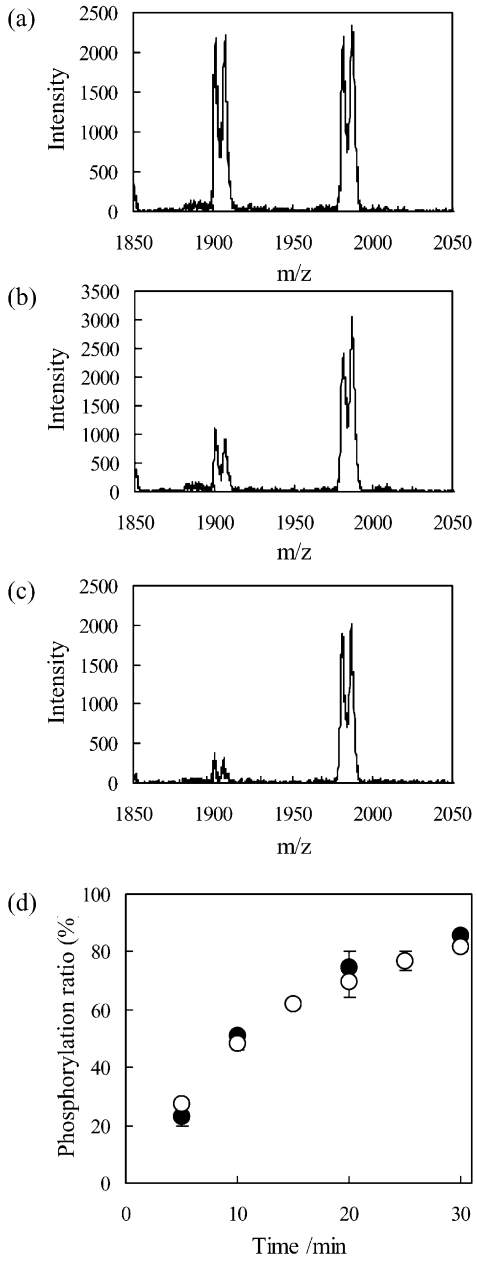

Figure 3. Mass spectra (a) 10 min, (b) 20 min, and (c) 30 min after adding the PKA C-subunit. (d) The time-dependent increase in the phosphorylation ratio calculated from the intensities of the mass peaks (open ciecle: PKA-H_6, closed circle: PKA-D_6).

μM, and the sample was then incubated at 37 °C. Next, 1 μL of each probe solution was taken in every 5 min after addition of the peptide probe, and the two solutions were combined, followed by analysis with MALDI-TOF MS. Figure 3 shows the typical mass spectra and time-dependence of the phosphorylation ratio calculated from the intensities of these mass peaks. The intensities of the mass peaks arising from nonphosphorylated probes (m/z: 1900.99 for PKA-H_6 and 1906.03 for PKA-D_6) gradually decreased with the reaction time, and those from phosphorylated probes (m/z: 1980.43 and 1986.44 for PKA-H_6 and PKA-D_6, respectively) increased at the same time. The time course of the phosphorylations showed good coincidence in the

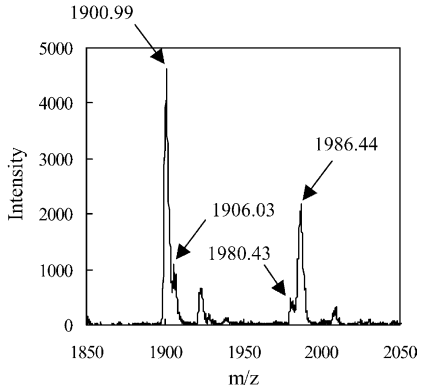

Figure 4. The mass spectrum from the mixture of two samples that contained different PKA activities. The concentration of PKA in PKA-H6 and PKA-D6 solutions were 30 U and 6 U, respectively. Each solution was combined to measure MALDI-TOF MS, after 3 h incubation at 37 °C.

two probes. These results suggested that the phosphorylation processes with PKA could be evaluated by the assay system using mass spectrometry.

For the last experiment, we investigated whether the difference in the PKA activities of the two samples, which contained different amounts of activated PKA, could be evaluated using this assay system. Thus, PKA-H_6 or PKA-D_6 (each 100 μM at a final concentration) was added to the PBS(−) containing 0.2 mM ATP and 10 mM $MgCl_2$ and activated PKA. The PKA activity in each probe solution was 6 U/mL and 30 U/mL for PKA-H_6 and PKA-D_6, respectively. After incubating for 3 h at 37 °C, these solutions were combined and analyzed by MALDI-TOF MS. The obtained mass spectrum (Fig. 4) shows that PKA-D_6, which was added to the sample with higher PKA activity, was more phosphorylated than the PKA-H_6. The peak height of the nonphosphorylated PKA-H_6 was nearly 5 times higher than that of nonphosphorylated PKA-D_6. On the other hand, the peak height of phosphorylated PKA-D_6 was approximately 5 times higher. These results reflect the differences in PKA activity between the two sample solutions quite well. Thus, this assay system was found to be potentially useful for evaluating PKA activity.

We are now attempting to apply this system to the measurement of PKA activity in cellar lysates. Moreover, the mass-tag strategy reported here can easily be applied to the probe peptides for monitoring other protein kinase activities. In this assay system, dozens of peptide probes can be detected simultaneously if each probe is designed to have a distinct mass number. As such, this system will potentially be able to simultaneously profile many protein kinase activities.

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