A new method for evaluation of intracellular protein kinase signals using mass spectrometry

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Abstract

Recently, comprehensive analysis in genome or proteome have attracted a lot of interest to many researchers in pharmacology, because of its useful information, such as expression profile of DNA, RNA and protein, to understand physiological events. However, it has not been possible to completely understand the cellular function using such information, because genes and proteins express their functions through extremely complicated interaction. On the other hand, total profile of the intracellular signals is expected to provide more detailed information to understand physiological events because various cellular functions are regulated directly by intracellular signals. We describe here an approach for the convenient and sensitive evaluation of intracellular protein kinase signals using mass spectroscopy. The method is based on a class of new peptide reagents and MALDI-TOF mass spectrometry. Using this system, activity changes in protein kinase A with a dosage of various pharmacological drugs into PC-12 cell were evaluated. These activity changes were found to have good correlation with the results of CREB-regulated gene expression, which was delivered into the cell line. We also evaluated the activity of protein kinase C and Src. This method can easily obtain the profile of many protein kinase activities and be useful for high throughput estimation of intracellular signalings, which is important to drug screening or evaluation of gene function.

Keywords: Protein kinase; Peptide; Mass spectrometry; Proteome analysis

1. Introduction

Current progress in genome sequencing enables us to profile the expression of genes at the mRNA level [1]. Although the approach is powerful in identifying novel genes that relate to various physiological and especially pathological events, the levels of each messenger RNA do not always reflect the level of protein being expressed [2–5]. In the post-genome era, investigating the dynamics of proteins becomes very important, as they are essential to the design of a number of new drugs. Proteome analysis is one of the most promising approaches for the purpose [6] and provides more accurate information about physiological events. For example, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is mainly used in proteome analysis and is useful to profile the protein expression in cellular sample. Disease-related protein identified by 2D-PAGE is utilized as a novel biomarker for diagnosis and early detection of diseases. However, it is not possible to know the function of all proteins in relation to the disease at this stage, because each of the proteins interacts with the others in a very complicated manner. Therefore, the disease that represents the cellular function cannot be understood only from profiling the protein expression and these extremely complex protein interactions must be
elucidated to better understand functional proteome. On the other hand, many enzymes change their activities through such complicated protein interactions and post-translational modifications. These events have been referred to as an intracellular signal transduction system [7]. In other words, the total profile of the intracellular signals can be regarded as proteome that is looked by the side of function.

In this context, a new strategy for the evaluation of intracellular signals that is both fast and inexpensive can be put into use for drug screening. We propose herein a new method for a rapid profiling of intracellular protein kinase activity. Based on this method, we have recently developed a new molecular probing system that can compare the cellular protein kinase activity in two different cellular states using mass spectrometry, which has become the core instrumental technology in recent post-genomic research because of its high sensitivity and accuracy [8–11]. However, mass spectrometry has a disadvantage of low reproducibility among independent measurements. Thus, stable isotope labeling is a promising way of overcoming this problem [12–14]. In this system, we also employed a stable isotope labeling, so that two types of probes, heavy and light isotope labeled, are used. Both probes in this method have a substrate sequence for a target kinase and are acetylated at N-terminus, but one is deutro-acetylated.

The activity of a target kinase can be estimated by mass spectroscopy after both cell lysates included each probe are mixed. The kinase activity in two different samples can be evaluated by a quartet arising from mass difference of probes in mass spectrum, when each probe is phosphorylated (Fig. 1). This method compensates the disadvantage of mass spectrometry (low reproducibility), because each probe in different cellular sample is simultaneously analyzed by mass spectrometer.

We have previously reported that this method allows us to evaluate the enzymatic activity of isolated protein kinase A [15]. In this report, we describe the estimation of that kinase activity in cell lysate with varying dosages of assorted pharmacological drugs. Furthermore, this assay system was extended for the evaluation of other kinase activity.

2. Materials and methods

2.1. Materials

Fmoc-amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), piperidine, and N-methyl pyrrolidone (NMP) were purchased from Calbiochem-Novabiochem AG (Laufelfingen, Switzerland) and

![Fig. 1](image-url)  
(a) Schematic outline of the protein kinase activity assay system using mass spectrometry. (b) Amino acid sequences of PKA, PKC, and Src substrate peptide probes.
Watanabe Chemical Ind., Ltd. (Hiroshima, Japan). DMF for peptide synthesis grade and acetonitrile for HPLC grade were obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). Acetic anhydride was purchased from PerkinElmer Inc. (Wellesley, MA), and acetic anhydride-d₆ from Cambridge Isotope Laboratories, Inc. (Andover, MA). Trifluoroacetic acid (TFA) and 1,2-ethanediol was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), and Triisopropylsilane was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

α-Cyano-4-hydroxycinnamic acid (CHCA) and Protein kinase C zeta isozyme were purchased from Sigma-Aldrich Co. (St. Louis, MO). Forskolin (Fsk) and Protein kinase A inhibitor (PKI) peptide were obtained from Calbiochem-Novabiochem Co. (San Diego, CA). 2.5S murine NGF was purchased from Promega Co. (Madison, WI).

2.2. Probe peptides synthesis

Probe peptides (PKA-H₆: CH₃CO-K(CH₃CO)HHHHHGLRRAKLKC-NH₂, M.W. 1900.2, PKA-D₆: CD₃CO-K(CD₃CO)HHHHHGLRRAKLKC-NH₂, M.W. 1906.2, PKC-H₆: CH₃CO-K(CH₃CO)WHHHHAAKIQASFRGHMARKK-NH₂, M.W. 2883.4, PKC-D₆: CD₃CO-K(CD₃CO)WHHHHAAKIQASFRGHMARKK-NH₂, M.W. 2889.4, Src-H₆: CH₃CO-K(CH₃CO)IYGEFKKKKHMARKK-NH₂, M.W. 1223.5, Src-D₆: CD₃CO-K(CD₃CO)IYGEFKKKKHMARKK-NH₂, M.W. 1229.5) were synthesized with a Pioneer™ Peptide Synthesis System (Applied Biosystems, Foster, CA) on Fmoc chemistry using corresponding Fmoc-amino acids and Fmoc-Lys(Fmoc)-OH as N-terminal amino acid residue. Deuterium labeling of probe peptides was accomplished using acetic anhydride-d₆ when the N-terminal amino group was acetylated during the final steps of the peptide synthesis. For cleavage of the obtained peptide from the resin and deprotection of the peptide, the peptide resin was treated for 2 h with a cleavage solution containing 94% TFA, 2.5% water, 2.5% 1,2-ethanediol, and 1% triisopropylsilane at a concentration of 10–25 g/mL. Then the resin was filtered out, and the filtrate was added to cold diethylether for precipitation of the cleaved peptide. The obtained peptide was purified with reverse-phase column chromatography (YMC-Pack ODS-A column, 15 cm length, 20 cm ID, 5 μm particle size, 12 nm pore size, YMC. Co., Ltd., Kyoto, Japan) using a Perfusion Chromatography System (Applied Biosystems, Foster, CA) on Fmoc chemistry using corresponding Fmoc-amino acids and Fmoc-Lys(Fmoc)-OH as N-terminal amino acid residue. Deuterium labeling of probe peptides was accomplished using acetic anhydride-d₆ when the N-terminal amino group was acetylated during the final steps of the peptide synthesis. 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2.3. Evaluation of protein kinase activity using mass spectrometry

The evaluation of protein kinase activity was performed as previously reported [15]. Briefly, each peptide probe was incubated in different sample solutions at a final concentration of 2 μM (detailed composition of the solution is described in Fig. 2–4). To terminate the phosphorylation reaction, 1 μL of each solution was mixed with 1 μL of CHCA solution (10 mg/mL CHCA in water/acetonitrile (50/50 v/v) including 0.1% TFA) at the appropriate time interval. Each CHCA mixed solution was mixed and the mixture was then deposited onto the plate for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The MS data were obtained using a Voyager™ mass spectrometer (Applied Biosystems, Foster, CA). The phosphorylation ratio was calculated from the peak intensity of the phosphorylated H-Type (or D-Type) probe divided by the total intensity of the mass peak derived from H-Type (or D-Type) probes.

![Fig. 2. The time-dependent increase in the phosphorylation ratio calculated from the intensities of the mass peaks after the addition of PKCz (a) or Src (b). The phosphorylation with PKCz was performed in 20 mM Tris-HCl, pH 7.5, containing 20 μM ATP, 10 mM MgCl₂, 0.5 mM CaCl₂, 7.5 μg/mL phosphatidylcholine, 1.6 μg/mL diacylglycerol, and 2 μM PKC-H₆ (or PKC-D₆). 120 and 600 ng/mL of PKC were used for PKC-H₆ (open diamonds) and PKC-D₆ (closed circles), respectively. The phosphorylation with Src was done in 20 mM Tris-HCl, pH 7.5, containing 0.2 mM ATP, 5 mM MgCl₂, 5 mM MnCl₂, and 2 μM Src-H₆ (or Src-D₆). 36 and 180 U/mL of Src were used for Src-H₆ (open diamonds) and Src-D₆ (closed circles), respectively.](attachment:image.png)
2.4. Preparation of cell lysate

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂, 95% air at 37°C. The cells were cultured up to an 80% confluent state and were then cultured without any treatment or were treated with various pharmacological drugs for 30–60 min. The cells were washed twice with a phosphate-buffered saline (PBS) and collected using a cell scraper. The obtained cell suspension was centrifuged at 800 rpm for 5 min at 4°C. After the supernatant was removed, 1 mL of PBS was added to the cells to count the number of cells. Next, 400 µL of this solution was mixed with protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 3 µg/mL pepstatin A, 5 µg/mL aprotinin) and sonicated for 30 s on ice to homogenize the cells. The solution was centrifuged at 3900 rpm for 7 min at 4°C, and the supernatant was used as the cell lysate.

In another experiment, PC12 cells were obtained from a RIKEN cell bank (Ibaragi, Japan) and subcultured in 96-well plates coated with poly-D-lysine (Sigma) at a density of 5 x 10⁴ cells per well in a Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% FBS, 5% horse serum, and 1% penicillin/streptomycin (Gibco-BRL). Preparation of the lysate was carried out as described above, except that 200 µL of PBS was added and sonicated after removal of the supernatant. Total protein concentrations in the lysate were evaluated instead of determining the cell number. The total protein concentration was determined by using a Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Gaithersburg, MD).

2.5. Luciferase reporter gene assay

The activation of CREB were determined using a pathdetect cis-reporting system (Stratagene, Seattle, WA), following the manufacturer’s protocol. To determine CREB activation, a pCre-Luc reporter vector was co-transfected with a pRL-SV40 vector into PC12 cells. The pCre-Luc reporter plasmid contains a synthetic promoter with five tandem repeats of the CREB binding sites, which control the expression of the firefly luciferase gene. The pRL-SV40 plasmid encodes a reporter gene (Renilla luciferase) driven by the SV40 promoter and serves as an internal control. Forty-eight hours post-transfection, the cells were treated with the indicated reagent(s) for 6 h and harvested for luciferase assay. Activities of firefly luciferase were normalized to those of Renilla luciferase and were utilized to determine the levels of CREB activation.

3. Results and discussion

3.1. Evaluation of PKC and Src activity using isolated enzyme

We have previously reported that this strategy using peptide probes and mass spectrometry can detect differences in the PKA activities of two samples containing different amounts of activated PKA [15]. To determine whether this system is applicable to the evaluation of other kinase activities, we synthesized isotopically light or heavy
substrate peptide probe of PKC or Src, and analyzed the enzymatic activities of the target kinase. Fig. 2 shows the time-dependent increase of the phosphorylation ratio calculated from the peak intensities of unphosphorylated or phosphorylated peptide probes on the mass spectra. The gradual increment of the phosphorylation ratio was observed, with the phosphorylation rate of the D-type probe being more rapid than that of the H-type probe. This result was in agreement with the concentrations of PKC (or Src) in the D-type probe solution being higher than those in the H-type solution. It was thus determined that this assay system could potentially be applied to evaluating the enzymatic activities of various kinases.

3.2. Evaluation of PKA activity in the cell lysate

To confirm that the proposed assay system could detect kinase activity in the cellular sample, the method was applied to lysate of HeLa cells. In this case, the lysate was prepared from cells, which were cultured either without treatment or after treatment with Fsk or PKI for 30 min. Each PKA probe was incubated in the cell lysate, which was either either treated or untreated with pharmacological drugs. Both lysates were then combined to be analyzed by mass spectrometry. The obtained mass spectra and the time course of phosphorylation of each probe are shown in Fig. 3. When the cells were treated with Fsk, phosphorylation of the peptide probe was augmented compared with that in the control lysate. In contrast to this, the probe phosphorylation in the lysate from the PKI-treated cell sample was depressed compared with that in the control lysate. Fsk is an agonist of adenylyl cyclase, so that PKA should be activated continuously, while PKI is an inhibitor of PKA. Thus, these results are consistent with the pharmacological activities of these reagents.

The peptide probes contained oligohistidine sequences, because we expected that these probes must be isolated from cell lysates containing a complex mixture of biosubstances before measurement using mass spectroscopy. However, such a purification procedure was not needed in the experiment. As such, the ‘His-tag’ sequence should not be required to be introduced into the probe. This mass spectrometry assay can therefore be performed with higher resolution because the molecular weight of 685 for His-tag should be decreased from that of the peptide probe. This ability of the assay should be very important in cases requiring simultaneous monitoring of peptide probes.

3.3. Comparison of the results between reporter gene expression assay and PKA activity evaluated with our mass-tag probe system

To examine whether this methodology can be used to evaluate cellular functions, and to confirm the generality of this assay, we further investigated the method using various pharmacological drugs and compared these results with those based on a reporter gene expression using the CREB system. Thus, PC12 cells were stimulated with various drugs. After the prescribed incubation time, the cell sample was homogenized, and the heavy probe (PKA-D\textsubscript{6}) was then added to a drug-stimulated lysate. A similar experiment was performed at the same time using unstimulated PC-12 cells and the light probe (PKA-H\textsubscript{6}). Both lysates containing PKA-D\textsubscript{6} and PKA-H\textsubscript{6} were combined after incubation for the prescribed time for use in the MALDI-TOF mass measurement. The obtained results are shown in Fig. 4. Fsk (10\textmu M) and PACAP (10\textmu M), which are known to activate PKA in PC-12, increased the luciferase
expression by ca. 500 times compared with that in the control cell. In these cases, the phosphorylation rate of D-type peptide probe in the lysate from the stimulated cells was greater than that of the H-type control probe. However, when the cell was stimulated by NGF (100 ng/ml), TPA (1 µM) and EGF (100 ng/ml), that activate various kinases other than PKA for cell growth, both the expression levels of luciferase and the phosphorylation rates of the peptide probes were not changed compared with the control experiment. Therefore, the evaluation of PKA activity using mass spectrometry showed good agreed with the results of CREB-regulated gene expression. These findings indicate that this assay system is potentially available for evaluating the activity changes of various intracellular protein kinases caused by varying dosages of many types of pharmacological compounds or transfected genes.

4. Conclusion

In conclusion, a new method for evaluating intracellular protein kinase activity using mass spectrometry was described. This method could be applied to monitoring of the enzymatic activity of various kinases in cell lysate without purification by the design of corresponding peptide probes. Moreover, the obtained results from mass spectrometry using the PKA probe system were consistent with the results from a reporter gene assay for CREB. Therefore, it was shown that the kinase activity profile evaluated by this method reflect the phenotype of the cell. Additionally, in this method, dozens of peptide probes can be detected simultaneously if each probe is designed to have a distinct mass number. In future, this system should be able to easily relate the profile of many protein kinase activities to cellular functions and the comprehensive profile of many protein kinase activities should become a index that represents various diseases. Therefore, this method will be available for diagnostics, drug screenings, and evaluation of gene functions.

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