Effect of the Addition of Diammonium Citrate to α -Cyano-4-Hydroxycinnamic Acid (CHCA) Matrix for the Detection of Phosphorylated Peptide in Phosphorylation Reactions Using Cell and Tissue Lysates

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The ionization of phosphorylated peptides is usually suppressed by non-phosphorylated peptides when α -cyano-4-hydroxycinnamic acid (CHCA) is used as a matrix for matrix-assisted laser desorption/ionization-time-of-Flight (MALDI-TOF) mass spectrometry analysis. In the present study, we examined the effect of diammonium citrate addition to the CHCA matrix on the detection of phosphorylated peptides. Substrates for protein kinase C (PKC) and c-Src were synthesized and phosphorylated by reaction with cell and tissue lysate samples. The addition of diammonium citrate to the CHCA matrix increased the sensitivity for distinguishing phosphorylated peptides from background noise. However, the effect depended on substrate concentration. (J Am Soc Mass Spectrom 2007, 18, 1925–1931) © 2007 American Society for Mass Spectrometry

Protein phosphorylation is one of the most important post-translational modifications, as it regulates cellular functions through information processing [1–3]. In this context, the determination of phosphorylated peptides using mass spectrometry has attracted much attention in many biological and pharmacological fields. The detection limit for phosphorylated peptides in mass analysis is dependent on several factors such as ionization efficiency, mass, and hydrophobicity. Matrix-assisted laser desorption/ionization (MALDI) is relatively tolerant of salts and buffer components such as sodium chloride and phosphate, particularly when compared with most other forms of mass spectrometric ionization. However, when the concentration of these components is high, crystal formation may be prevented and ionization impeded [4, 5]. Moreover, cell lysates contain many biomolecules such as proteins and lipids, and relatively high concentrations of contaminants including buffers. These components can also prevent crystal formation and interfere with ionization.

For example, previous studies suggested that the ionization of phosphorylated peptides is usually suppressed by unphosphorylated peptides when α -cyano-

4-hydroxycinnamic acid (CHCA) is used as a matrix [6–10]. Several attempts have been made to reduce this effect [6–10]. Among them, the addition of diammonium citrate to the CHCA matrix can increase the peak intensity of the phosphorylated peptides or proteins [6, 10]. This may be a simple and useful method to identify the phosphorylated peptide or protein for certain protein kinases. However, there is very little data concerning the application of diammonium citrate in matrices for the detection of phosphorylated peptides from cell and tissue samples.

In the present study, two substrates for protein kinase C (PKC) α and c-Src were used to test and optimize the effectiveness of diammonium citrate to enhance our ability to detect phosphorylated peptides from cell and tissue samples. PKC, a calcium- and phospholipid-dependent serine/threonine kinase consisting of 11 isozymes [11–13], appears to be involved in the signal transduction response to a variety of hormones and growth factors. Certain isozymes such as PKC α , are considered to play an important role in carcinogenesis and tumor growth [14].

c-Src is a non-receptor tyrosine kinase that is known to increase in a multitude of human tumors, including breast, stomach, colon, and prostate. Activated c-Src can phosphorylate the human epidermal growth factor receptor (HER), which is the prototype of a family that consists of four members (epidermal growth factor

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receptor/HER1, neu/erbB2/HER2, erbB3/HER3, and erbB4/HER4), leading to tumor cell growth and proliferation [3, 15–17].

Thus, the phosphorylation of serine, threonine, and tyrosine residues by such protein kinases has an important influence on diverse biological phenomena, including signal transduction, cell division and motility, apoptosis, and carcinogenesis [3, 11–19].

Experimental

Peptide Synthesis

Substrates for PKC α (FKKQGSFAKKK; MW, 1336.8) and c-Src (CXXEIYGEFD, where X connotes the group [-NH(CH₂CH₂O)₂CH₂CO-]; MW, 1392.5) were synthesized using an automatic peptide synthesizer according to standard Fmoc-chemistry. The latter peptide can be fixed to the slide glass for application as a peptide array. The Cys residue and linkages are known to have no effect on the phosphorylation of the substrate [20, 21]. After trifluoroacetic acid (TFA) cleavage, the peptide was purified on an Inertsil ODS-3 column (250 $\times$ 20 mm, 3.5 μ m, GL Sciences Inc., Tokyo, Japan) using a BioCAD perfusion chromatography system (Ikemoto Scientific Technology Co., Tokyo, Japan) under a linear A-B gradient at a flow rate of 8 mL/min, where eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile (HPLC grade).

Sample Preparation for MALDI Time-of-Flight (TOF) Mass Spectrometry

The α -cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/mL) was prepared in 50% water/acetonitrile and 0.1% TFA. The modified CHCA matrix included diammonium citrate (5 mg/mL). The matrix and sample were mixed in ratios of 1:1, 2:1, 3:1, 4:1, and 5:1. A total volume of 1 μ L of the analyte/matrix mixture was applied on the sample plate and allowed to dry to induce crystallization.

MALDI-TOF Mass Spectrometry

Experiments were conducted using a Voyager DE RP BioSpectrometry Workstation (Applied Biosystems, Framingham, MA) in positive or negative ion reflectron mode. The accelerating voltage was 20 kV with a 100-ns extraction delay time. Typically, 100 laser shots were averaged to improve the signal-to-noise ratio. All spectra were analyzed using Data Explore software (Applied Biosystems). Triplicate samples were prepared and each was analyzed twice. Means of the phosphorylation ratio obtained from three spots selected manually on the crystals were used as results for assay purposes. The phosphorylation ratio is defined as the ratio of ion intensities for the phosphorylated to unphosphorylated material, and this was calculated as described previously [22].

Phosphorylation of Substrates with PKC α and c-Src Enzyme

Phosphorylation of the PKC α substrate was carried out in 10 μ L of buffer [20 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, 0.5 mM CaCl₂, 100 μ M adenosine triphosphate (ATP), 2 μ g/mL diacylglycerol (DAG), and 2 μ g/mL phosphatidylserine (PS)] containing 30 μ M synthetic peptide and 0.1 μ g/mL of PKC α .

For c-Src, 10 μ L of buffer (20 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, and 100 μ M ATP) containing 30 μ M synthetic peptide and 0.1 μ g/mL of c-Src. After incubation for 60 min at 37 $^{\circ}$ C, the sample was analyzed by MALDI-TOF mass spectrometry.

Cell Culture and Lysate Preparation

B16 melanoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μ g/mL), and amphotericin B (0.25 μ g/mL). The cells were kept in a humidified atmosphere containing 5% CO₂ and 95% air at 37 $^{\circ}$ C. The cultured cell was then scraped and lysed in 20 mM Tris-HCl buffer (pH 7.5) by sonication. The sample was centrifuged at 5000 $\times$ g for 60 min at 4 $^{\circ}$ C. The supernatant (200 μ g/mL) was used as a whole cell lysate (membrane and cytosol fractions) for the phosphorylation reaction.

For the detection of total protein concentration, standard working bovine serum albumin (BSA) solutions of 0.125 to 2 mg/mL were prepared by diluting aliquots of the stock solution (10 mg/mL) in water. A 10- μ L volume of the standard BSA solution was mixed with 1 mL of Bio-Rad Protein assay Dye reagent (BIO-RAD Lab., Hercules, CA) and detected by the absorbance at 595 nm. Calibration curves for concentrations (0.125 to 2 mg/mL) versus detector responses (absorbance at 595 nm) were obtained using a linear regression program. The correlation coefficients of absorbance to concentration were >0.998 . A 10- μ L volume of cell extract was mixed with 1 mL of Bio-Rad protein assay dye reagent. The protein concentration was detected using a UV system, and calculated from the corresponding calibration curve.

Tissue Lysate Preparation

In the present study, all animal studies were performed in accordance with the Guidelines for Animal Experiments, set out by the Kyushu University.

Male 5-wk old BALB/c mice, weighing $\sim$ 20 g, were used in this study. The dorsal side was inoculated subcutaneously with 1×10^6 B16 melanoma cells per 100 μ L of Hanks' balanced salt solution (Gibco) per animal. Tumors were allowed to grow to a mean diameter of $\sim$ 0.7 to 1.0 cm.

Tissue lysates for PKC were obtained from excised tumors grown in mice. After the tumors were weighed, they were homogenized in 10 mL of buffer [20 mM

Figure 1. Typical MALDI-TOF mass spectra obtained from phosphorylation of the substrates for PKC α (a) or c-Src (b) with each target enzyme. An increase of +80 Da was identified for the phosphorylated peptide.

Tris-HCl, pH 7.5, 250 mM sucrose, and protease inhibitor (Roche, Tokyo, Japan)]. The homogenate was centrifuged at $1000 \times g$ at 4°C for 10 min and the supernatant was removed. After washing the precipitate with 5 mL of buffer and re-centrifuging, 1 mL of buffer was added and the precipitate further sonicated for 30 s. After sonication of the homogenate, the samples were centrifuged at $5000 \times g$ at 4°C for 15 min, and the resulting supernatant ($200 \mu\text{g}/\text{mL}$) was used as a whole lysate sample for the phosphorylation reactions. Total protein concentration was detected with the aid of a Bio-Rad protein assay dye reagent. The lysate of normal skin tissue was also prepared according to the same method.

For c-Src, brain tissue was homogenized in 10 mL of buffer [20 mM Tris-HCl, pH 7.5, 250 mM sucrose, and protease inhibitor (Roche)]. The homogenate was centrifuged at $100,000 \times g$ at 4°C for 60 min and the supernatant was removed. After washing the precipitate with 5 mL of buffer and re-centrifuging, 1 mL of buffer with 0.2% Triton X-100 was added and the precipitate further sonicated for 30 s. After sonication of the homogenate, the samples were centrifuged at $100,000 \times g$ at 4°C for 30 min, and the supernatant ($200 \mu\text{g}/\text{mL}$) was used for the phosphorylation reaction.

Statistical Analysis

Statistical analysis consisted of significance testing of the differences between means by Student's *t*-test at the following significance levels: $P < 0.1$ and $P < 0.5$.

Results and Discussion

Phosphorylation of Two Substrates by Target Enzyme

In a previous study, we found that the substrate (FKKQGSFAKKK) showed a higher phosphorylation ratio on PKC α [22]. Here, the same substrate was used in the phosphorylation reaction for PKC α . A substrate specific to c-Src (EEIYGEFD) was synthesized according to a study reported by Songyang et al. [23].

Phosphorylation of the two substrates by each target enzyme was identified by an increase in the *m/z* value of +80 Da (Figure 1) [6–10]. In the case of c-Src, no phosphorylated peak was observed using positive ion reflectron mode, which may be a result of the substrate for c-Src containing four anionic amino acid residues (E and D). On the other hand, detection using the negative ion reflectron mode revealed a phosphorylated peak.

Suitable Ratio of Matrix and Sample

In the present study, CHCA was used as a matrix for analysis. The 1:1 volume ratio of CHCA matrix and melanoma cell lysate sample prevented crystal formation and interfered with the ionization. These results may be caused by known or suspected contaminants and biomolecules such as Tris base, proteins, and lipids. Two simple methods for reducing these contaminants involve washing the samples with 5 to 10 μL of either distilled deionized water or 0.1% TFA. This process also serves to dilute the samples [4]. Since the former method can often lead to sample losses, the dilution method was used in this study. When the ratio of

Table 1. Change of phosphorylation ratio after the addition of activator and/or cofactors to the cell reaction lysate

Activator and/or cofactor	Phosphorylation ratio (%) (<i>n</i> = 3)
Mg ²⁺ + ATP	28.5 ± 2.1
Mg ²⁺ + ATP + Ca ²⁺	24.8 ± 3.6
Mg ²⁺ + ATP + PS	27.0 ± 3.6
Mg ²⁺ + ATP + PS + Ca ²⁺	26.2 ± 5.6
Mg ²⁺ + ATP + DAG	45.5 ± 2.3
Mg ²⁺ + ATP + DAG + Ca ²⁺	46.6 ± 1.1
Mg ²⁺ + ATP + DAG + PS	47.9 ± 5.0
Mg ²⁺ + ATP + DAG + PS + Ca ²⁺	57.2 ± 4.8

CHCA to sample was increased more than 2-fold (2:1 to 5:1), the tolerance for the contaminants was enhanced and crystal formation and ionization occurred. A 5:1 ratio of matrix to sample achieved the best invariable peaks of the phosphorylated peptide from the whole range of the plate (data not shown). In the present study, therefore, a 5:1 ratio of matrix to sample was used for detecting phosphorylated peptides in the cell and tissue lysates.

Moreover, MALDI-TOF mass spectrometry can be used to analyze a wide range of peptide concentrations, from 10^{−4} M (100 pmol/μL) to 10^{−8} M (10 fmol/μL) [4]. In the present study, the concentration of peptide (5 μM) in the ratio 5:1 is included in this range.

Phosphorylation of PKC Substrate with Cell Lysate

After phosphorylation of the PKCα substrate with the cell lysate of B16 melanoma, no peaks of the phosphorylated peptide were found. These results may be caused by a loss of indispensable factors for the PKCα phosphorylation reaction during cell lysate preparation. PKCα consists of a single polypeptide chain with an amino-terminal regulatory region and a carboxyl-terminal catalytic region. The regulatory region of PKCα contains an auto-inhibitory domain (pseudo-substrate) and two membrane-targeting domains (C1 and C2). The C1 domain binds to diacylglycerol (DAG), while the C2 domain contains many acidic amino acids that take part in Ca²⁺ binding. The catalytic region contains two domains (C3 and C4). The C3 domain

contains the adenosine triphosphate (ATP)-binding motif while the C4 domain binds to the substrate. For substrate phosphorylation by PKCα, DAG, Ca²⁺, and ATP were found to be indispensable factors [11–13, 19]. A phosphatidylserine (PS)-binding domain is not found in PKCα, but PS shows synergistic activation with DAG and Ca²⁺ during phosphorylation [11–13]. DAG is a basic component of membranes and serves as hydrophobic anchors in PKC and membrane interaction. It is produced in the hormone-sensitive phosphatidylinositol system. Active phospholipase C cleaves plasma membrane lipid phosphatidylinositol-4,5-bisphosphate into DAG and inositol-1,4,5-trisphosphate [11, 24, 25]. On the other hand, PS is an acidic membrane phospholipid and is produced by the displacement of cytidine monophosphate through nucleophilic attack by the hydroxyl group of serine [11, 24, 25].

Therefore, we investigated the indispensable factors for the PKCα phosphorylation reaction. Phosphorylation did not occur in the absence of Mg²⁺ and ATP. The addition of only ATP did not lead to substrate phosphorylation (data not shown). However, the addition of both ATP and Mg²⁺ was sufficient to initiate the PKC phosphorylation reaction affording a peak in the MS spectrum corresponding to the phosphorylated peptide (Table 1). Many enzymes require both Mg²⁺ and ATP for their activity because the true substrate for enzymes is not ATP^{4−}, but an ATP-Mg²⁺ complex [26, 27]. Moreover, the addition of Ca²⁺ and/or PS in the presence of ATP and Mg²⁺ had no effect on the phosphorylation reaction (data not shown). However, the addition of DAG in the presence of ATP and Mg²⁺ accelerated the reaction. The obtained peak intensity was higher (about two times) than that observed for Ca²⁺ and/or PS addition (Table 1). These results are closely related to the DAG function for the PKCα phosphorylation reaction. DAG is a membrane lipid and its binding to the C1 domain of PKCα activates PKCα. However, the PKCα that exists in the cytosol is inactive [11–13]. In the present study, the total cell lysate (membrane and cytosol fractions) was used for the phosphorylation reaction of PKCα. Therefore, the addition of DAG to these lysate samples led to activation of the PKCα present in the cytosol, resulting in an increase in the phosphorylation ratio.

Table 2. Ionization efficiencies of the phosphorylated and non-phosphorylated peptides

Peptide concentration (μM)	Intensity (%) (<i>n</i> = 3)			
	Without diammonium citrate		With diammonium citrate	
	Peptide	Phosphorylated peptide	Peptide	Phosphorylated peptide
7.5	100	60.3 ± 3.8	100	26.7 ± 5.7
15	100	69.3 ± 4.9	100	55.3 ± 10
30	100	76.7 ± 2.1	100	82.4 ± 2.1
60	100	75.3 ± 2.5	100	60.3 ± 6.8
120	100	76.1 ± 2.7	100	48.2 ± 5.8
240	100	76.8 ± 2.1	100	38.7 ± 5.1

Figure 2. Typical MALDI-TOF mass spectra obtained from phosphorylation of the PKC α substrate with melanoma (a) and (b); normal skin tissue lysate (c) and (d); with (b) and (d) or without (a) and (c) diammonium citrate.

Since c-Src had two domains, one for substrate binding and the other for binding the ATP-Mg $^{2+}$ complex [15–17], phosphorylation peaks were also found on addition of ATP and Mg $^{2+}$ (data not shown). These results mean that ATP can be lost easily during lysate preparation.

Addition Effect of Diammonium Citrate to Matrix

To identify changes in the peak intensity with varying peptide or phosphorylated peptide concentration, we synthesized two peptides, FKKQGSFAKKK and the phosphorylated peptide (FKKQGphosphoSF A KKK) containing phosphorylated Ser instead of the serine residue. Two peptide solutions were mixed in a ratio of 1:1 (vol/vol) at concentrations of 7.5, 15, 30, 60, 120, and 240 μ M, then combined with CHCA in the presence or absence of diammonium citrate, and finally analyzed by MALDI-TOF mass spectrometry. In the absence of

diammonium citrate, the intensity (about 76% of the non-phosphorylated peptide peak intensity) of the phosphorylated peptide was similar to that in the range of 30 to 120 μ M. However, decreases in the intensity of the phosphorylated peptide by 15% and 7% were observed for peptide solution concentrations of 7.5 and 15 μ M, respectively, when compared with that in the range of 30 to 120 μ M. In the presence of diammonium citrate (5 mg/mL), the largest peak intensity (>80% of the non-phosphorylated peak) of the phosphorylated peptide was obtained from a peptide solution concentration of 30 μ M, and then the peak intensity decreased gradually with an increase in the peptide concentration. On the other hand, the intensity at 7.5 and 15 μ M averaged 27% and 55%, respectively (Table 2).

Moreover, we examined whether there is a change in the phosphorylation ratio by the CHCA matrix, prepared with and without diammonium citrate, after the phosphorylation reaction of peptide substrates (15, 30,

Figure 3. Typical MALDI-TOF mass spectra obtained from phosphorylation of the c-Src substrate with brain tissue lysate using a CHCA matrix prepared with (b) or without (a) diammonium citrate.

and 60 μM) with the B16 cell lysate. The phosphorylation ratio in concentrations of 15, 30, and 60 μM was 40.7, 39.3, and 15.9%, respectively, in the CHCA matrix with diammonium citrate, but 51.2, 32.2, and 10.8% without diammonium citrate, respectively. With the addition of diammonium citrate, the ratio decreased in concentrations of 15 ($P < 0.1$) and 60 μM ($P < 0.5$), but increased in 30 μM ($P < 0.5$). These results show that if the concentration of the substrate is optimized, the application of diammonium citrate can elevate the detection sensitivity for phosphorylated peptides.

In addition, we examined whether the addition of diammonium citrate to the CHCA matrix affects the detection of the phosphorylated peptide using tissue lysate. The addition of diammonium citrate to CHCA showed an enhanced ionization of the phosphorylated peptide, and this peak intensity was greater than that obtained for CHCA only (Figure 2a–d). Specifically, the addition of diammonium citrate to the CHCA matrix elevated the detection sensitivity for the small peaks associated with the phosphorylated peptide, helping to distinguish them from the background noise (Figure 2c and d). In addition, the phosphorylation of a c-Src substrate for brain tissue lysate samples produced a higher peak in the presence of diammonium citrate, compared when no diammonium citrate was present in the CHCA matrix (Figure 3). Both Figures 2 and 3 showed a high signal-to-noise ratio in the presence of diammonium citrate, leading to an elevated detection sensitivity for phosphorylated peptides. These results suggest that the addition of diammonium citrate to the CHCA matrix may be of benefit for the detection of small phosphorylation peaks if the peptide concentration is sufficient.

The identification of phosphorylated peptides or

proteins for certain protein kinases is essential for the characterization of, or search for, substrate proteins for enzymes. The identification process will also be important in explorations for new drugs [12, 13, 18, 19]. The application of diammonium citrate to MALDI-TOF mass spectrometry technique may be useful for identifying phosphorylated peptides or proteins for certain protein kinases.

Acknowledgments

This work was supported by CREST, Japan Science and Technology Corporation, a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture in Japan, and a grant-in-aid for Scientific Research from the Ministry of Health, Labor, and Welfare.

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