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Direct on-membrane peptide mass fingerprinting with MALDI–MS of tyrosine-phosphorylated proteins detected by immunostaining☆

Tsuyoshi Nakanishi[a], Eiji Ando[a,*], Masaru Furuta[a], Susumu Tsunasawa[a], Osamu Nishimura[a,b]

[a] *Life Science Laboratory, Analytical & Measuring Instruments Division, Shimadzu Corporation, 1 Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto 604-8511, Japan*
[b] *Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan*

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Abstract

We have identified tyrosine-phosphorylated proteins on membrane from A-431 human epidermoid carcinoma cells by using detection with anti-phosphotyrosine antibody followed by PMF analysis. In there, on-membrane digestion for these protein spots was carried out on microscale region using chemical inkjet technology and the resulting tryptic digests were directly analyzed by MALDI–TOF MS. Proteins identified by a database search included phosphoproteins that are known to be markedly phosphorylated on tyrosine sites after the cells are treated with epidermal growth factor (EGF). This procedure is a rapid and easily handled approach that enables both detection and identification of phosphoproteins on a single blot membrane.

Keywords: Phosphorylation; Immunodetection; On-membrane digestion; Protein identification; MALDI–TOF MS

1. Introduction

Post-translational modifications are known to play a significant role in many biological processes, such as signal transduction-mediated cellular events. A number of proteins in all cells become post-translationally modified with a variety of functional groups, which include phosphate, glycan, lipid, sulfate, nitric oxide and ubiquitin, etc. In particular, protein phosphorylation, one of the post-translational modifications, plays a crucial role in eukaryotic signal transduction, DNA transcription, protein synthesis, cell cycle progression and cell metabolism [1,2]. Since the discovery that reversible phosphorylation regulates the activity of glycogen phosphorylase, a wide variety of phosphorylation cascades regulated by protein kinases and phosphatases have been characterized in which specific serine, threonine and tyrosine residues in proteins become phosphorylated or dephosphorylated [3]. The approach to rapidly detect and identify phosphoproteins has important implications because of the crucial role of protein phosphorylation in biological processes. Currently, there are several approaches for detecting phosphoproteins, such as traditional metabolic labeling using the radioactive isotope ^{32}P, chemical modification of phosphate groups followed by MS analysis, and immunostaining or immunoprecipitation using specific phosphoserine, phosphothreonine or phosphotyrosine antibodies [4]. In current proteomics, mass spectrometry is widely used for identification of detected phosphoproteins [5]. Several previous reports have described the use of mass spectrometric analysis to identify proteins detected by Western blotting. These approaches require dual separations of the same sample on gels, or a step in which proteolytic peptides are extracted from the membrane used for immunodetection before MS analysis [6,7].

In this study we carry out direct identification of phosphoproteins from A-431 human epidermoid carcinoma cells, detecting them using an anti-phosphotyrosine antibody and carrying out on-membrane digestion using piezoelectric chemical inkjet printing in combination with MALDI–TOF MS. In general, in order to identify protein by mass spectrometry after detecting them by Western blotting, it is necessary to carry out a

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* Corresponding author. Tel.: +81 75 823 1359; fax: +81 75 823 1368.
E-mail address: e-ando@shimadzu.co.jp (E. Ando).

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time-consuming operation such as extraction of digested peptides from the blot membrane, or preparation of an alternative gel to separate the proteins by 2-dimensional electrophoresis (2-DE) for in-gel digestion. However, this approach utilizes piezoelectric technology, and direct on-membrane analysis enables us to omit the above time-consuming operations and to rapidly identify the proteins visualized with Direct Blue 71 after immunostaining [8]. Furthermore, the microdispensing function of reagents using piezoelectric inkjet technology could be used to improve on-membrane PMF analysis in the microscale region of the protein spots without cross-contamination between proximate proteins [9]. Reagents at sub-nanoliter volume levels can be microdispensed, allowing digestion of only a tiny region within a protein spot. As a result, we could identify proteins from A-431 human epidermoid carcinoma cells by this method. The proteins identified in this manner included proteins, known to be increasingly phosphorylated on specific tyrosine sites after EGF treatment of A-431 cells. Thus, this procedure has the potential to become a powerful tool for direct identification of phosphoproteins on membrane.

2. Experimental

2.1. Materials

Phospho-enriched whole cell lysates, A-431/PE and A-431 + EGF/PE were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylpyrrolidone (PVP-40), Direct Blue 71 and 2,5-dihydroxy benzoic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Trypsin was obtained from Promega (Madison, WI, USA) and the Immobilon-FL PVDF membrane was purchased from Millipore (Bedford, MA, USA). Phospho-tyrosine mouse mAB (P-Tyr-100) was obtained from Cell Signaling Technology (Danvers, MA, USA) and rabbit anti-phosphoserine was obtained from Zymed Laboratories Inc. (S. San Francisco, CA, USA). Alexa Fluor 633 goat anti-mouse IgG (H + L) and Texus Red goat anti-rabbit IgG (H + L) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Instruments

Direct analyses on the PVDF membrane was performed using a MALDI–TOF MS instrument, AXIMA-CFR plus (Shimadzu Corporation, Kyoto, Japan and Kratos Analytical, Manchester, UK), that was operated in a positive ion mode by using an internal calibration method with trypsin autodigests (m/z = 842.51, 2211.10). For on-membrane digestion, the chemical inkjet printer (Shimadzu Corporation, Kyoto, Japan) was used for microdispensing the reagents onto blotted protein spots as we have previously reported [10].

2.3. Preparation of 2-DE blotted membranes using A-431 human epidermoid carcinoma cells lysates

Phospho-enriched whole cell lysates, A-431/PE and A-431 + EGF/PE (200 μg) were purchased from Santa Cruz Biotechnology. The proteins from the cell lysates were recovered by TCA precipitation and then dissolved in 200 μL Protein Extraction Reagent Type 3 solution (from ProteoPrep Sample Extraction Kit, Sigma). Proteins from the cell lysates of A-431 and A-431 + EGF were prepared according to the ProteoPrep sample extraction kit protocol. Solubilized proteins were reduced with 5 mM tributylphosphine for 60 min at room temperature and then alkylated with 15 mM iodoacetamide for 90 min at room temperature. Pharmalyte (pH 3–10) was added to a final concentration of 0.2% and a trace of bromophenol blue (BPB) was also added. The protein solution was centrifuged at 15,000 × g for 20 min at 20 °C and the supernatant was used for rehydration of IPG strips. Amersham IPG strips (pH 3–10, 13 cm) were rehydrated for 8 h with the prepared sample solution (200 μL) and focused on a Protean IEF Cell apparatus (Bio-Rad, Hercules, CA, USA) for 100 kV/h at a maximum of 8 kV. The focused IPG strips were equilibrated for 10 min with equilibration buffer, and SDS-PAGE (10–20%) was then performed for these strips. The proteins separated by 2-dimensional electrophoresis (2-DE) were blotted onto the Immobilon-FL membrane by the semi-dry electroblotting method. Blotting was performed at constant 200 mA for 40 min using three type of blotting buffer (A: 0.3 M Tris, 20% methanol, 0.02% SDS, B: 25 mM Tris, 20% methanol, 0.02% SDS, C: 25 mM Tris, 40 mM ε-amino-*n*-hexanoic acid, 20% methanol, 0.02% SDS) [11]. The membrane was air-dried after rinsing it with water.

2.4. Immunostaining with an anti-phosphotyrosine and an anti-phosphoserine antibodies

Immunostaining with anti-phosphotyrosine and anti-phosphoserine antibodies was performed according to rapid immunodetection, which has an advantage in that blocking is not required, thus saving time [12]. The blot must be thoroughly dry before beginning rapid immunodetection immunostaining and the following step can be carried out. Dried PVDF membranes were rewetted by dipping into 100% methanol for 10 s and then were air-dried on a filter paper for 15 min. Subsequently, the blot was dried in a vacuum chamber for 30 min. The blot was incubated at 37 °C for 30 min and then was air-dried for 2 h. Primary and secondary antibodies were diluted 1:2000 and 1:5000 for phosphoserine, 1:5000 and 1:5000 for phosphotyrosine, respectively, with TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.25% PVP-40. The blot was incubated with each antibody at a ratio of 0.09 ml/cm^2 of membrane surface area for 30 min. The blot was then washed in plastic containers using TBS at a ratio of 0.9 ml/cm^2 of membrane surface area for 5 min twice. After rinsing with water, a fluorescent image of the blot was acquired using the FLA-5000 analyzer (Fujifilm, Tokyo, Japan).

2.5. Direct identification of proteins detected with an anti-phosphotyrosine antibody on membrane

Antibodies on the membrane were removed by washing it in 0.2 M glycine–HCl (pH 2.0) after acquiring the fluorescent image. After washing for 15 min three times, the blot membrane was rinsed with water and then dried at room temperature. The

membrane was stained with 0.008% (w/v) Direct Blue 71 in 40% ethanol, 10% acetic acid for 7 min and washed with 40% ethanol, 10% acetic acid for 5 min twice [8]. After rinsing with water, the blot membrane was subjected to microscale on-membrane digestion using the chemical inkjet printer. Firstly, the blot membrane was adhered to the stainless steel plates for MS analysis using 3 M™ electrically conductive tape 9713 (St. Paul, MN). A visualized image of the adhered blot was acquired with a scanner of the chemical inkjet printer, and the target protein spots were selected on the basis of the scanned images. Subsequently, the reagents for on-membrane digestion were printed onto microscale region of the protein spots [9]. Ten nanoliters of 0.1% (w/v) PVP solution in 60% MeOH was printed to pre-wet the membrane and then 50 nl of trypsin at 40 μg/ml in 10 mM NH_4HCO_3 containing 10% (v/v) 2-propanol was microdispensed to each target position. On-membrane digestion was performed for 16 h at 30 °C in a humidified chamber. After digestion, 100 nl of 5 mg/ml 2,5-DHB in 0.1% trifluoroacetic acid (TFA) containing 25% (v/v) acetonitrile was printed onto each position on the membrane. Then the blot was subjected to the AXIMA-CFR plus instrument for on-membrane MS analysis. Positional information for the printed region was transferred to the mass spectrometer as an output file from the chemical inkjet printer, and MS analysis was performed for the digested region on the basis of this information. On the basis of the obtained MS spectrum, a database search was conducted using the Sprot database with the aid of Mascot software (Matrix Science, MA), which was set at a tolerance of 0.3 Da for the MS analysis, and at one missed cleavage site as fixed parameters.

3. Results and discussion

3.1. Immunostaining of A-431 cell lysates with an anti-phosphotyrosine antibody

Enriched phosphoproteins from A-431 lysates incubated in the presence and absence of EGF were separated with 2-DE and blotted onto a PVDF membrane. The blot membrane was analyzed using a modified rapid immunodetection approach with an anti-phosphotyrosine antibody [10]. Fig. 1(b) shows the result of immunodetection for proteins containing phosphotyrosines from A-431 cell lysates. After removing the antibodies by washing with 0.2 M glycine–HCl (pH 2.0), the blot membrane was visualized with Direct Blue 71. The stained image is shown in Fig. 1(a). The results, which are shown in Fig. 1(b) indicate the presence of phosphotyrosine on a number of proteins and also suggest that phosphorylation of a number of proteins on tyrosine residues is remarkably induced in A-431 cells treated with EGF. In general, it is known that the binding of EGF to EGF receptors results in receptor dimerization, autophosphorylation and activation of various downstream phosphorylation cascades, especially tyrosine kinase activation [13]. Furthermore, the human epidermoid carcinoma cell line A-431 has already been shown to contain an extraordinarily high concentration of membrane receptors for EGF [14]. The results shown in Fig. 1(b) indicate that a number of specific tyrosine sites in A-431 cells are phosphorylated by EGF-treatment.

3.2. Immunostaining of A-431 cell lysates with an anti-phosphoserine antibody

Phosphoproteins from A-431 cells were subsequently detected by Western blotting with an anti-phosphoserine antibody. Fig. 2(b) shows the result of immunostaining. The blot membrane stained with Direct Blue 71 is shown in Fig. 2(a). Protein spots, especially some proteins having a molecular weight of approximately 35–90 kDa, that were observed with an intense signal in Fig. 1(b) could not be detected with anti-phosphoserine antibody. This result suggests that these proteins of molecular weight 35–90 kDa become phosphorylated on specific tyrosine residues upon EGF treatment. On the other hand, most of the signals detected in immunostaining with the anti-phosphoserine antibody are observed as weak signals, as compared with the anti-phosphotyrosine antibody. This may result from low affinity or specificity of the anti-phosphoserine antibody for its antigen in comparison with the anti-phosphotyrosine antibody. These results suggest that the immunodetection using a highly specific antibody is of importance for further detection with MS analysis. We tried direct on-membrane PMF analysis for some protein spots having an intense signal, which had slight differences in the phosphorylation level on serine residues when comparing the spots in the presence and absence of EGF, but most of these proteins could not be identified (data not shown). Most of the proteins detected with immunostaining using the anti-phosphoserine antibody appeared as faint spots on the membrane stained with Direct Blue 71. Therefore, the microscale on-membrane identification for small amounts of proteins might be unsuccessful in some cases because of decreased practical sensitivity when only a tiny part of a protein spot is digested [9].

3.3. On-membrane direct identification of proteins detected with an anti-phosphotyrosine antibody

We carried out microscale on-membrane digestion using the microdispensing function based on piezoelectric inkjet technology for some proteins immunodetected with a highly intense signal using anti-phosphotyrosine (Fig. 3). The resulting tryptic digests were analyzed directly on the membrane by MALDI–TOF MS, and a database search was performed to find a match to the MS spectrum. The results of the database search are shown in Table 1, which lists proteins that were identified with a reliable score. With regard to proteins 2–8, sequentially arranged proteins, which may have multiple isoforms or modifications such as phosphorylation on different amino acids within a single protein, were analyzed in the microscale region of a single protein spot using piezoelectric inkjet printing technology. These proteins (proteins 2–8) that were slightly shifted according to pI were identified as the same protein, and differences derived from isoforms or modifications could not be observed on the MS spectrum (data not shown). Such differences may depend on ion suppression from the negative charge of a phosphate group or incomplete sequence coverage. When we performed direct on-membrane MS analysis in this experiment, decreasing resolution was observed, compared with analysis carried out on the stainless steel plate [9]. Although roughness of

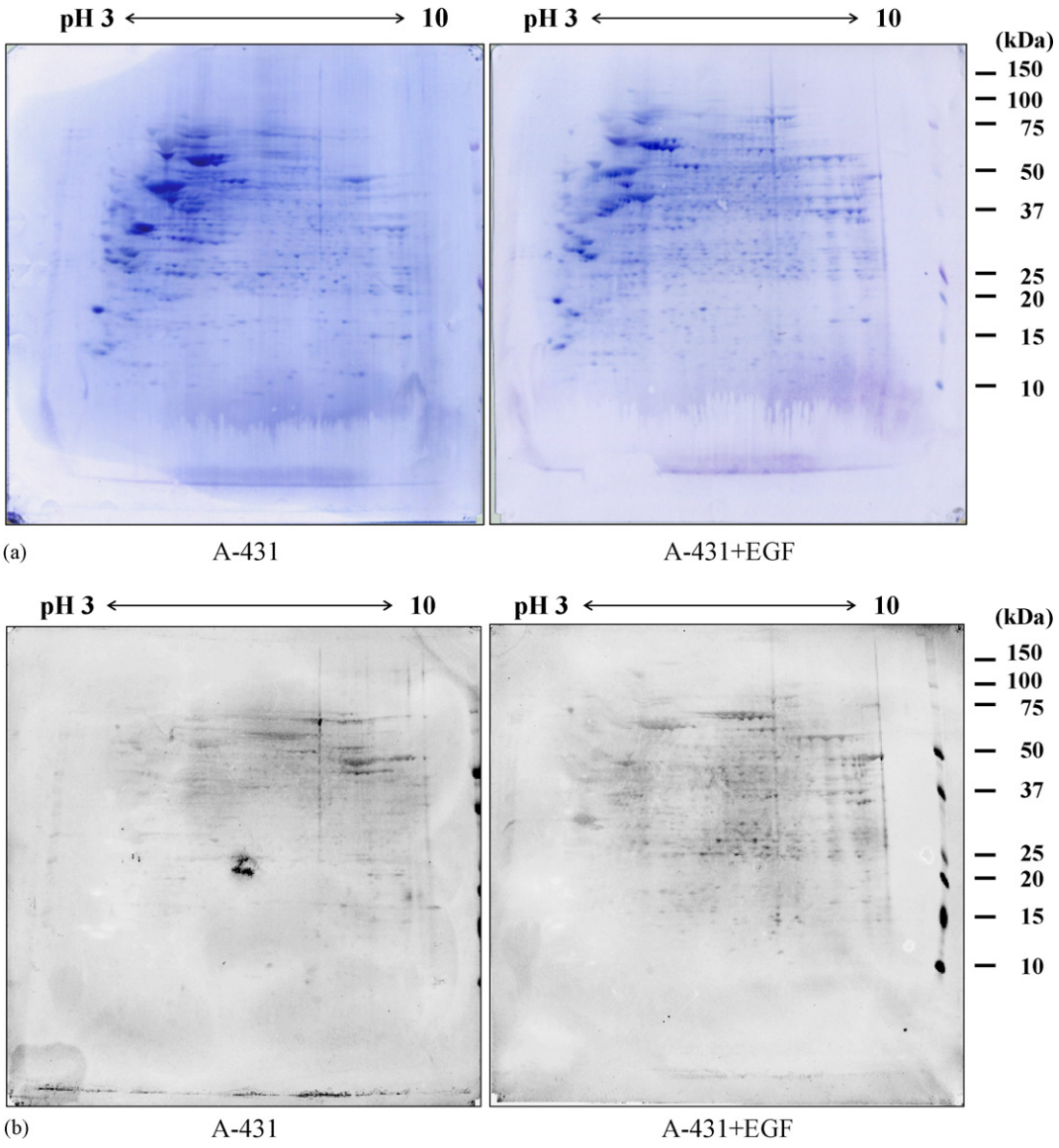

Fig. 1. 2-DE blot image of phospho-enriched whole cell lysates from A-431/A-431 + EGF cell lysates. (a) Image visualized with Direct Blue 71 staining; (b) fluorescent image detected with an anti-phosphotyrosine antibody.

Table 1
Phosphoproteins from EGF-stimulated A-431 cell lysates identified directly on membrane using a database search

	Accession no.	Protein name	M.W.	Score	Matched peptides
Protein 1	P13639	Elongation factor 2	96115	80	12
Protein 2	P26038	Moesin	67761	70	10
Protein 3	P15311	Ezrin (p81)	69339	63	10
Protein 4	P31948	Stress-induced-phosphoprotein 1 (STI1)	63227	75	11
Protein 5	P14618	Pyruvate kinase isozymes M1/M2	58339	118	11
Protein 6	P04406	Lyceraldehyde-3-phosphate dehydrogenase	36070	118	10
Protein 7	P08107	Heat shock 70 kDa protein 1	70294	181	18
Protein 8	P38646	Stress-70 protein, mitochondrial precursor	73920	164	16

The database search was performed using the Sprot database. Typical results of database search (proteins 6) are as follows: amino acid sequences (observed ion *m*/*z* /calculated ion *m*/*z*): VKVGVNGFGR (3–12), 1032.62/1032.60; VGVNGFGR (5–12), 805.43/805.43; AENGKLVINGNPITIFQER (61–79), 2113.13/2113.14; LVINGNPITIFQER (66–79), 1613.97/1613.90; LVINGNPITIFQERDPSK (66–83), 2041.18/2041.11; AGAHLQGGAKR (107–117), 1065.72/1065.59; DGRGALQNIIPASTGAAK (197–214), 1739.97/1739.94; GALQNIIPASTGAAK (200–214), 1411.70/1411.79, LTGMAFRVPTANVSVVDLTCR (227–247), 2323.10/2323.19; VPTANVSVVDLTCR (234–247), 1530.88/1530.80.

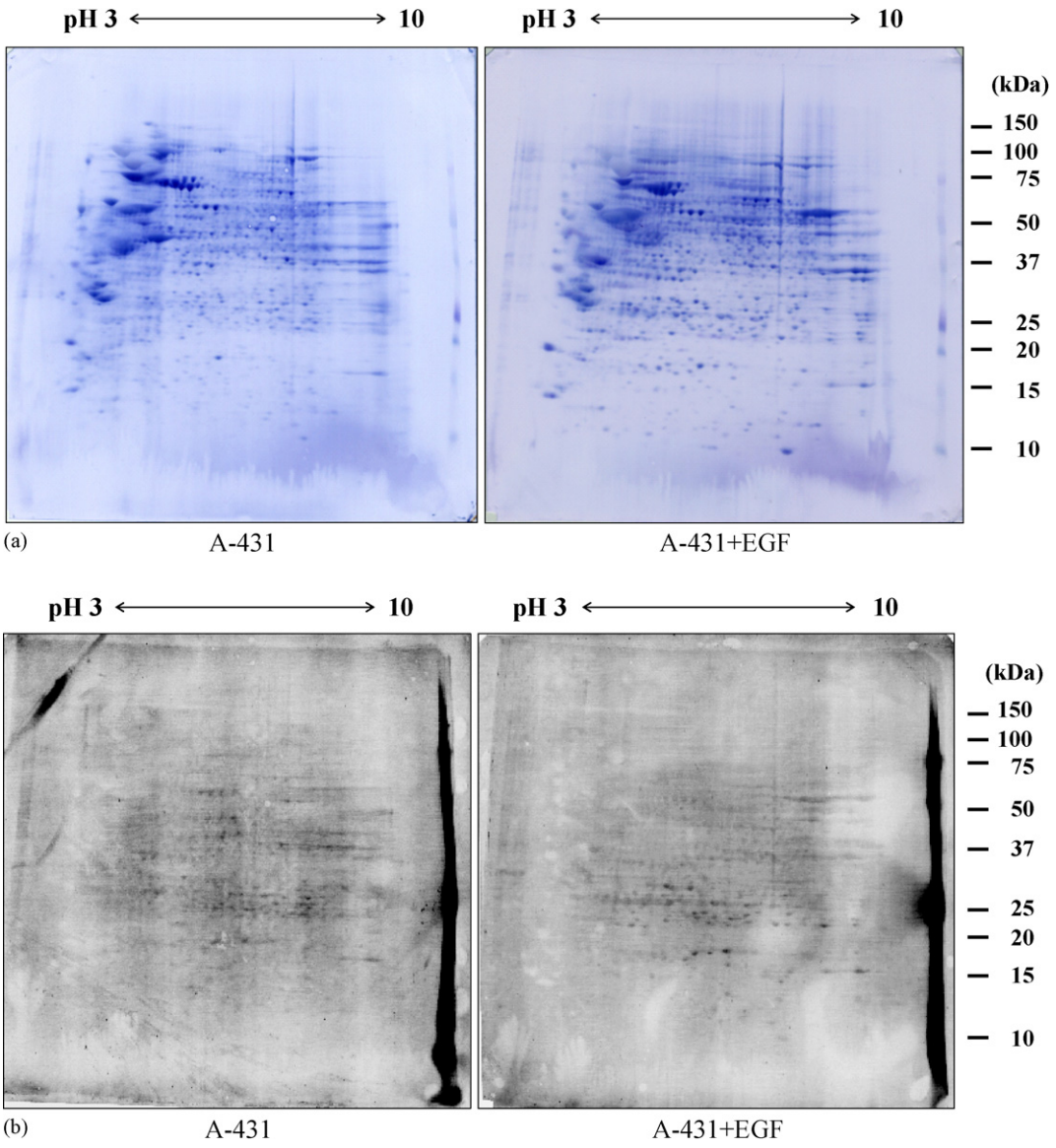

Fig. 2. 2-DE blot image of phospho-enriched whole cell lysates from A-431/A-431 + EGF cell lysates. (a) Image visualized with Direct Blue 71 staining; (b) fluorescent image detected with an anti-phosphoserine antibody.

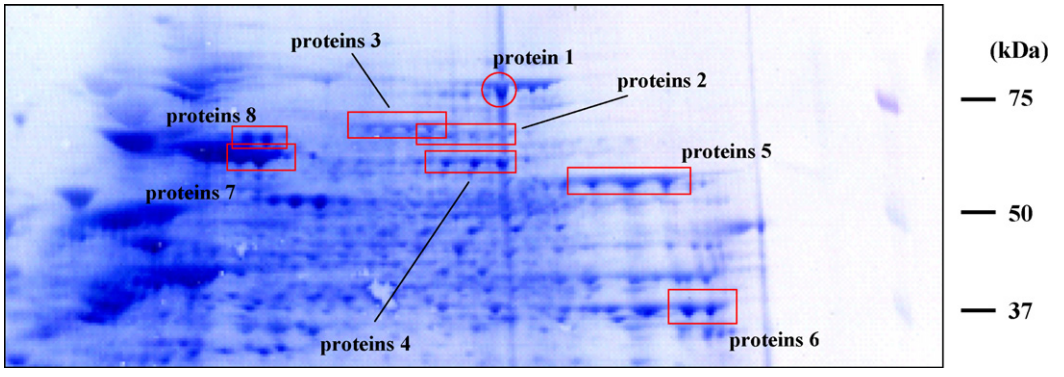

Fig. 3. A series of proteins from EGF-stimulated A-431 cell lysates intensely detected with an anti-phosphotyrosine antibody.

the membrane surface or charging effects on membrane can lead to lower resolution, this resolution was high enough to conduct a database search (peptide tolerance <0.3 Da). In this experiment, adjusting a laser power at low value, which can barely ionize digested peptide fragments, might not cause large decreases in the resolution or mass accuracy.

These identified proteins are known to be phosphoproteins in various species including human [15,16]. In particular, moesin, ezrin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have already been shown to be phosphorylated on specific tyrosine residues in an EGF-dependent manner in A-431 cells [17–19]. Several enzymes related to the glycolytic pathway are phosphorylated by tyrosine kinase in an EGF-dependent manner, and these enzymes were also anticipated to contain phosphotyrosine residues and thus would be detected by this immunostaining. Moesin and ezrin are members of the ezrin/radixin/moesin (ERM) family of cytoskeletal proteins, and they are associated with dynamic membrane-based processes such as the formation and stabilization of filopodia [20]. Both proteins are known to be substrates of a tyrosine kinase in EGF-stimulated A-431 cells. Our results indicate that this immunodetection followed by on-membrane direct MS analysis is a powerful approach for rapid and easy identification of phosphoproteins.

In this study we carried out on-membrane PMF analysis in a microscale region of the protein spot to decrease the difficulty of analyzing either proximate proteins or multiple proteins in a single spot due to the limited resolution of 2-DE. In the 2-DE based approach, in most cases, the major component of a protein mixture can be identified by PMF analysis with MALDI–TOF MS from a single spot. Using our microscale approach we could not identify more than one protein in a single spot by the on-membrane PMF approach. Furthermore, detailed analysis of digested peptide fragments with a tandem mass spectrometry (MS/MS) instrument also becomes important to identify unknown proteins that have not been registered on available databases, and to characterize post-translationally modified proteins. In this experiment we tried on-membrane MS/MS analysis to identify phosphorylated proteins detected by immunostaining, though the exact phosphorylation site of the phosphoproteins could not be identified. To date, MS/MS method is widely used for the characterization of phosphorylation sites. It is still an extremely difficult task to characterize the entire set of phosphorylation sites within phosphoproteins because of troublesome ionization dependent on negative charge of phosphopeptides. However, future refinements of this method should lead to improvements in the on-membrane direct identification approach. Through this approach, we expect that rapid and easy direct identification of phosphoproteins detected by immunostaining become a useful approach in the field of phosphoproteomics.

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