DOI 10.1002/pmic.200600252 *Proteomics* 2006, *6*, 5088–5095

RESEARCH ARTICLE

Enrichment of phosphorylated proteins from cell lysate using a novel phosphate-affinity chromatography at physiological pH

*Emiko Kinoshita-Kikuta[1], Eiji Kinoshita[1, 2], Atsushi Yamada[1], Mika Endo[1] and Tohru Koike[1, 2]**

[1] Department of Functional Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
[2] Frontier Center for Microbiology, Hiroshima University, Hiroshima, Japan

While phosphoproteins have attracted great interest toward the post-genome research (*e.g.* clinical diagnosis and drug design), there have been few procedures for the specific enrichment of native phosphoproteins from cells or tissues. Here, we describe a simple and efficient protocol to enrich phosphoproteins comprehensively from a complex mixture containing solubilized cellular proteins. This method is based on immobilized metal affinity chromatography using a phosphate-binding tag molecule (*i.e.* a dinuclear zinc(II) complex) attached on a highly cross-linked agarose. The binding, washing, and elution processes were all conducted without a detergent or a reducing agent at pH 7.5 and room temperature. An additive, 1.0 M CH_3COONa, was necessary in the binding and washing buffers (0.10 M Tris-CH_3COOH, pH 7.5) to prevent the nonphosphorylated protein from binding. The absorbed phosphoproteins were eluted using a mixed buffer solution (pH 7.5) consisting of 0.10 M Tris-CH_3COOH, 10 mM NaH_2PO_4-NaOH, and 1.0 M NaCl. In this study, we demonstrate a typical example of phosphate-affinity chromatography using an epidermal growth factor-stimulated A431 cell lysate. The total time for the column chromatography (1 mL gel scale) was less than 1 h. The strong enrichment of the phosphoproteins into the elution fraction was evaluated using SDS-PAGE followed by Western blotting analysis.

Received: April 5, 2006
Revised: May 13, 2006
Accepted: June 6, 2006

Keywords:
Cell lysate / IMAC / Phosphate-affinity chromatography / Phosphoprotein / Phosphoproteomics

1 Introduction

Protein phosphorylation is one of the most important PTM that regulate the function, localization, and binding specificity of target proteins [1, 2]. Because phosphorylation, which mainly occurs on serine, threonine, and tyrosine residues in mammalian cells, is a principle for the regulation of life, its perturbation fundamentally affects the numbers of cellular events and is involved in many diseases. Therefore, the specific and efficient enrichment of native phosphoproteins has attracted great interest toward phosphoproteome research in the biological and medical fields.

A number of studies on the enrichment of phosphoproteins from a biological sample, such as cell lysate, have been reported. The most popular method is antibody-immobilized affinity chromatography using anti-phosphorylated amino acid antibodies [3–5]. The antibody-based procedure has an inherent problem with specificity; cur-

Correspondence: Dr. Eiji Kinoshita, Department of Functional Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1–2-3, Hiroshima 734–8551, Japan
E-mail: kinoeiji@hiroshima-u.ac.jp
Fax: +81-82-257-5336

Abbreviations: AP, alkaline phosphatase; **anti-pTyr**, anti-phosphotyrosine; **EGF**, epidermal growth factor; **HRP**, horseradish peroxidase; **HRP–SA**, horseradish peroxidase-conjugated streptavidin; **Phos-tag**, phosphate-binding tag; **RIPA**, radio-immunoprecipitation assay

* Additional corresponding author: Professor Tohru Koike
E-mail: tkoike@hiroshima-u.ac.jp

rently available antibodies against phosphotyrosine are highly specific, while antibodies against phosphothreonine and phosphoserine lag behind. Another method is IMAC using Fe^{3+} or Ga^{3+}. Although this procedure provides a more comprehensive enrichment of phosphoproteins, it has a drawback, namely, lowering of the protein solubility under the acidic condition required in the elution process [6, 7]. To avoid this problem, addition of a detergent (2% SDS) and a reducing agent (0.1 M DTT) in the elution buffer was reported for the IMAC using an agarose-bound iron(III) complex at a high temperature of 95°C [8].

Recently, we have reported that a dinuclear metal complex (*i.e.* 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex) acts as a novel phosphate-binding tag molecule, Phos-tag (commercially available from http://www.phos-tag.com) in an aqueous solution at a neutral pH (*e.g.* K_d = 25 nM for phenyl phosphate dianion) [9]. The Phos-tag has a vacancy on two metal ions that is suitable for the access of a phosphomonoester dianion as a bridging ligand. The resulting 1:1 phosphate-binding complex, $ROPO_3^{2-}$–Phos-tag^{3+}, has a total charge of +1. The anion selectivity indexes of the phenyl phosphate dianion against SO_4^{2-}, CH_3COO^-, Cl^-, and the bisphenyl phosphate monoanion at 25°C are 5.2×10^3, 1.6×10^4, 8.0×10^5, and $>2 \times 10^6$, respectively. These findings have been introduced to the development of procedures for the MALDI-TOF MS of phosphorylated compounds (*e.g.* phosphopeptides and phospholipids) [10–12], the surface plasmon resonance analysis of phosphopeptides [13], the Western blotting analysis of phosphoproteins on a PVDF membrane [14], and the SDS-PAGE separation of a phosphoprotein and the corresponding dephosphorylated protein [14]. Furthermore, we have demonstrated that a novel phosphate affinity chromatography using agarose-bound Phos-tag (Phos-tag agarose) is useful for the retrieval of phosphopeptides in a quantitative and highly selective manner by a spin column method [15]. The technique is also successfully used for the separation of commercially available phosphoproteins, such as ovalbumin, α-casein, and β-casein, from the mixture solution with the corresponding dephosphorylated proteins at a neutral pH.

In this study, we describe an improved method using the same affinity matrix, Phos-tag agarose, for the enrichment of the native phosphoproteins from the complex biological sample. The newly modified technique was achieved using appropriate pH 7.5 buffer solutions for the binding, washing, and elution processes. The adsorption (*i.e.* binding and washing processes) and elution were conducted at room temperature without a detergent or a reducing agent. The procedure leads to a major advantage for gaining the intact information of phosphoproteins. As the first example, we demonstrate the enrichment of phosphoproteins from the lysate of A431 human epidermoid carcinoma cells stimulated by the epidermal growth factor (EGF), thereby identifying candidates for *in vivo* phosphorylation in EGF signaling.

2 Materials and methods

2.1 Materials

Phos-tag agarose (*ca.* 5 μmol/mL-gel phosphate-binding site) [15] and biotin-pendant Phos-tag [13, 14] were obtained from the Phos-tag consortium (http://www.phos-tag.com, Japan). Bovine intestinal mucosa alkaline phosphatase (AP), EGF, and NaCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ECL advance Western blotting detection kit, horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine (anti-pTyr) mAb (clone PY20), HRP-conjugated anti-mouse IgG antibody, HRP-conjugated anti-rabbit IgG antibody, and HRP-conjugated streptavidin (HRP-SA) were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). EGF-unstimulated A431 cell lysate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SYPRO Ruby protein gel stain, RPMI1640 cell culture medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). The anti-phospho-MAP kinase 1/2 (Erk1/2) antibody (clone 12D4), anti-phospho-Shc (Tyr317) antibody, anti-phospho-ErbB-2/HER-2 (Tyr1248) antibody, and EGF-stimulated A431 cell lysate were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Sodium deoxycholate and Na_3VO_4 were purchased from Calbiochem (La Jolla, CA, USA). Sharpline low-range markers for protein molecular weight were purchased from Toyobo (Osaka, Japan). A protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). PVDF membranes (Fluorotrans W) were purchased from Nihon Pall (Tokyo, Japan). The 3MM papers were purchased from Whatman Japan (Tokyo, Japan). Suprec-01 centrifugal filter units were purchased from Takara Bio (Otsu, Japan). Microcon YM-30 and YM-10 centrifugal filter devices were purchased from Millipore (Bedford, MA, USA). Polypropylene columns (1 mL) were purchased from Qiagen (Hilden, Germany). Acrylamide, 6-aminohexanoic acid, ammonium persulfate, aprotinin, bovine serum albumin CH_3COONa, glycerol, glycine, leupeptin, 2-mercaptoethanol, *N,N'*-methylenebisacrylamide, NaF, NP-40, pepstatin, PMSF, SDS, TEMED, Tween 20, and Tris were purchased from Nacalai Tesque (Kyoto, Japan). HCL (12M), CH_3COOH, MeOH, $MgCl_2$, $NaNO_3$, NaOH, and 2-propanol were purchased from Yoneyama Yakuhin Kogyou (Osaka, Japan). Bromophenol blue, EDTA2Na, and NaH_2PO_4 were purchased from Katayama Chemical (Osaka, Japan). An aqueous solution of 0.10 mol/L $Zn(CH_3COO)_2$ was purchased from Kishida Chemical (Osaka, Japan). All reagents and solvents used were of the highest commercial quality and were used without further purification. All aqueous solutions were prepared using deionized and distilled water.

2.2 Apparatus

The pH measurement was conducted with a Horiba F-12 pH meter (Kyoto, Japan) and a combination pH electrode Horiba-6378, which was calibrated using pH standard buffers

(pH 4.01 and 6.86) at 25°C. Fluorescence gel images were acquired on an FLA 5000 laser scanner (Fujifilm, Tokyo, Japan). SYPRO Ruby dye [16] was detected by the 575-nm emission signal on 473-nm excitation. A LAS 3000 image analyzer (Fujifilm, Tokyo, Japan) was used for the observation of the enhanced chemiluminescence (the ECL signal). A micro plate reader MPR-A4i (Tosoh, Tokyo, Japan) was used for the quantification of protein by the Bradford method [17].

2.3 Dephosphorylation of the cell lysate proteins

A mixed solution of 17 μL of 2.0 M Tris, 5.0 μL of 0.10 M $MgCl_2$, and 3.0 μL of an alkaline phosphatase (AP) solution (8 U) was added to a commercially available EGF-unstimulated A431 cell lysate (50 μg proteins in 25 μL of a radioimmunoprecipitation assay (RIPA) lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.25% w/v sodium deoxycholate, 1.0% v/v NP-40, 1.0 mM EDTA, 1.0 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1.0 mM Na_3VO_4, and 1.0 mM NaF). The resulting solution (pH *ca.* 9) was incubated for 12 h at 37°C. The AP-treated lysate (20 μg proteins in 20 μL) was mixed with 50 μL of 1.0 M Tris-CH_3COOH (pH 7.5), and then 17.5 μL of 5.0 M CH_3COONa was added in the solution (20 μg proteins in 87.5 μL at pH *ca.* 7.5). The obtained solution is a sample for the following phosphate-affinity chromatography.

2.4 Phosphate-affinity chromatography with a spin column

Phos-tag agarose (0.1 mL of swelled gel) was placed in a sample reservoir (a Suprec-01 centrifugal filter unit). The filter unit was centrifuged at 2000 × *g* for 20 s to remove the storage buffer (*i.e.* 20 mM Tris-CH_3COOH pH 7.4 containing 20% v/v 2-propanol), and the filtrate was discarded. To form the zinc(II)-bound Phos-tag agarose, a balancing buffer (0.10 mL) consisting of 0.10 M Tris-CH_3COOH pH 7.5, 1.0 M CH_3COONa, and 10 μM $Zn(CH_3COO)_2$ was placed in the sample reservoir and allowed to stand for 5 min at room temperature. The unit was centrifuged at 2000 × *g* for 20 s, and the filtrated buffer was discarded. A binding/washing buffer (0.10 mL) consisting of 0.10 M Tris-CH_3COOH pH 7.5 and 1.0 M CH_3COONa was placed in the sample reservoir, and then the unit was centrifuged at 2000 × *g* for 20 sec. This washing operation was repeated three times. The commercially available EGF-stimulated A431 cell lysate (20 μg proteins in 10 μL of an RIPA buffer) was diluted with 40 μL of the binding/washing buffer. The sample solution (50 μL at pH *ca.* 7.5) was added into the sample reservoir and allowed to incubate for 5 min at room temperature. In the case of the AP-treated lysate, 87.5 μL of the sample solution (see above section) was added into the sample reservoir and allowed to incubate for 5 min at room temperature. Next, the filter unit was centrifuged at 2000 × *g* for 20 s, and the filtrate was collected as a flow-through fraction. The binding/washing buffer (0.10 mL) was added to the sample reservoir, and the unit was centrifuged at 2000 × *g* for 20 s. This washing operation was repeated twice, and the filtrate (0.20 mL) was collected as two washing fractions containing unbound proteins. The flow-through and washing fractions were combined and used for the subsequent analysis. To elute the gel-bound proteins, an elution buffer (0.10 mL) consisting of 0.10 M Tris-CH_3COOH pH 7.5, 1.0 M NaCl, and 10 mM NaH_2PO_4-NaOH pH 7.5 was added to the sample reservoir, and the filter unit was centrifuged at 2000 × *g* for 20 s. The eluting operation was repeated twice, and the filtrate (0.20 mL) was collected as two elution fractions. Finally, to analyze the proteins left in the column, a column-washing buffer (0.10 mL) consisting of 0.10 M Tris-CH_3COOH pH 7.5, 1% w/v SDS, and 10 mM EDTA was added to the sample reservoir. The unit was heated for 5 min at 95°C and then centrifuged at 2000 × *g* for 20 s. The filtrate was collected as a column-washing fraction containing the proteins left in the column. Each fraction was desalted and condensed by using a Microcon YM-10 centrifugal filter unit and then resolved in 20 μL of distilled water. After the resulting solution was divided into two fractions (10 μL), one was analyzed by SDS-PAGE with SYPRO Ruby gel staining, and the other, by SDS-PAGE and Western blotting.

2.5 Preparation of the lysed proteins from cultured cells

A431 human epidermoid carcinoma cell line was supplied by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer at Tohoku University (Japan). The cells were grown in an RPMI1640 medium containing 10% v/v FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells (7×10^6) were placed into the same medium in a 100-mm culture plate. After allowing cell adhesion to the plate, the medium was removed, and the serum-free medium was added. After incubation for 16 h, the cells were stimulated with 5 ng/mL of EGF for 4 h. To terminate the stimulation, the medium was removed, and the remaining cells were rinsed with PBS at room temperature. After removing the saline, the culture plate was placed on ice. The cells were exposed to 0.30 mL of a cold RIPA buffer consisting of 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.25% w/v sodium deoxycholate, 1.0% v/v NP-40, 1.0 mM EDTA, 1.0 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1.0 mM Na_3VO_4, and 1.0 mM NaF. The plate was gently rocked for 15 min on ice, and the adherent cells were then removed from the plate with a cell scraper. The resulting suspension was transferred to a microcentrifuge tube. The plate was washed with 0.20 mL of an RIPA buffer, and the washing solution was combined with the first suspension in a microcentrifuge tube. The mixed sample was incubated for 60 min on ice and centrifuged at 10 000 × *g* for 10 min at 4°C. The supernatant fluid was used as the cell lysate. The concentration of the solubilized proteins was adjusted to 2.0 mg/mL with an appropri-

ate amount of an RIPA buffer. The quantification of protein was performed according to the Bradford method with a Bio-Rad protein assay kit.

2.6 Phosphate-affinity chromatography with an open column

After the bottom filter of a polypropylene column was washed with 20% v/v 2-propanol, Phos-tag agarose (*ca.* 2.0 mL of swelled gel) was placed in the column to make *ca.* 1.0 mL of a compressed gel bed. The gel was washed with 2.0 mL of the binding/washing buffer (0.10 M Tris-CH_3COOH pH 7.5 and 1.0 M CH_3COONa) to remove the storage buffer. To form the zinc(II)-bound Phos-tag agarose, 2.0 mL of the balancing buffer (0.10 M Tris-CH_3COOH pH 7.5, 1.0 M CH_3COONa, and 10 μM $Zn(CH_3COO)_2$) was placed in the column and loaded for 5 min at room temperature. The column was washed with the binding/washing buffer (5.0 mL) to remove excess $Zn(CH_3COO)_2$. The EGF-stimulated cell lysate from the cultured cells (0.50 mg proteins dissolved in 0.25 mL of an RIPA buffer) was diluted with 1.0 mL of the binding/washing buffer. After the sample solution (1.25 mL) was applied to the gel, a flow-through fraction containing unbound proteins was collected. Next, the binding/washing buffer (5.0 mL) was loaded, and a washing fraction was collected. The flow-through and washing fractions were combined and used for the subsequent analysis. To elute the gel-bound proteins, an elution buffer (5.0 mL) consisting of 0.10 M Tris-CH_3COOH pH 7.5, 1.0 M NaCl, and 10 mM NaH_2PO_4-NaOH pH 7.5 was placed in the column. Each fraction was desalted and condensed by using a Microcon YM-10 centrifugal filter unit and then resolved in 0.20 mL of distilled water. The recoveries of proteins in each concentrated fraction were estimated by the Bradford method before SDS-PAGE analysis.

2.7 SDS-PAGE

PAGE conducted according to the Laemmli method [18] was usually performed at 35 mA/gel and room temperature in a 1-mm-thick, 9-cm-wide, and 9-cm-long gel on a PAGE apparatus (model AE6500; Atto, Tokyo, Japan). The gel consisted of 1.8 mL of a stacking gel (4.0% w/v polyacrylamide, 125 mM Tris-HCl pH 6.8, and 0.1% w/v SDS) and 6.3 mL of a separating gel (7.5–12.5% w/v polyacrylamide, 375 mM Tris-HCl pH 8.8, and 0.10% w/v SDS). An acrylamide stock solution was prepared as a mixture of a 29:1 ratio of acrylamide to *N,N'*-methylenebisacrylamide. The electrophoresis running buffer pH 8.4 was 25 mM Tris and 192 mM glycine containing 0.1% w/v SDS. Each sample was prepared by mixing the concentrated fraction and a half volume of an SDS-PAGE loading buffer consisting of 195 mM Tris-HCl pH 6.8, 9.0% w/v SDS, 15% v/v 2-mercaptoethanol, 30% v/v glycerol, and 0.1% w/v bromophenol blue. All samples were heated for 5 min at 95°C before gel loading.

2.8 SYPRO Ruby gel staining

After electrophoresis, the gels were fixed in an aqueous solution containing 10% v/v MeOH and 7% v/v CH_3COOH for 30 min. The fixed gel was stained in a solution of SYPRO Ruby protein gel stain for 2 h and then washed in 10% v/v MeOH and 7% v/v CH_3COOH for 2 h.

2.9 Western blotting

The separated proteins in a polyacrylamide gel were electroblotted to a PVDF membrane for 2 h using a semi-dry blotting system (Nippon Eido NB-1600, Tokyo, Japan) at 2 mA/cm^2 using three kinds of blotting solutions (solutions A, B, and C). After SDS-PAGE, the gel was soaked in solution A (25 mM Tris and 5% v/v MeOH) for 10 min. Three 3MM papers were soaked in solution B (25 mM Tris, 40 mM 6-aminohexanoic acid, and 5% v/v MeOH) and piled on the negative-pole board. The gel, the PVDF membrane, and a 3MM paper were soaked in solution A and piled up in order. Then, two 3MM papers soaked in solution C (0.30 M Tris and 5% v/v MeOH) were piled up. Finally, they were covered with the positive-pole board, and electricity was supplied. For a comprehensive detection of the phosphoprotein, we prepared a complex of biotin-pendant Phos-tag and HRP–SA as described previously [14]. For probing with the prepared Phos-tag-bound HRP–SA, the protein-blotted PVDF membrane was soaked in an aqueous solution containing 10 mM Tris-HCl pH 7.5, 0.10 M NaCl, and 0.1% v/v Tween 20 (TBS-T solution) for 1 h. The membrane was incubated with the Phos-tag-bound HRP–SA solution (1 mL/30 cm^2) in a plastic bag for 30 min and washed twice with TBS-T (2 mL/cm^2) each for 5 min at room temperature. The ECL signal was observed using an appropriate volume of the ECL advance solution. To detect the selected phosphoproteins, we used the corresponding antibodies. Before probing with the antibodies, the blotting membrane was blocked by 1% w/v bovine serum albumin in TBS-T for 1 h. For the detection of the phosphorylated tyrosine residues, the membrane was probed with the HRP-conjugated anti-pTyr mAb (clone PY20) (a commercially available solution was diluted 1:1000 with TBS-T, 1 mL/30 cm^2) in a plastic bag for 1 h and washed twice with TBS-T (2 mL/cm^2) each for 10 min, and the ECL signal was then observed. For phosphorylated MAP kinase 1/2 (Erk 1/2) detection, the membrane was probed with the anti-phospho-MAP kinase 1/2 antibody (clone 12D4) (1.0 μg/mL in TBS-T, 1 mL/30 cm^2) in a plastic bag for 1 h, washed twice with TBS-T (2 mL/cm^2) each for 10 min, probed with HRP-conjugated anti-mouse IgG antibody (a commercially available solution was diluted 1:10 000 with TBS-T, 1 mL/30 cm^2) in a plastic bag for 1 h, and washed twice with TBS-T (2 mL/cm^2) each for 10 min, and the ECL signal was then observed. For the detection of phosphorylated Shc, the membrane was probed with the anti-phospho-Shc antibody (a commercially available solution was diluted 1:1000 with TBS-T, 1 mL/30 cm^2) in a plastic bag for 1 h, washed twice with TBS-T (2 mL/cm^2) each for 10 min, probed with the HRP-conjugated anti-rabbit IgG antibody (a commer-

cially available solution was diluted 1:10 000 with TBS-T, 1 mL/30 cm^2) in a plastic bag for 1 h, and washed twice with TBS-T (2 mL/cm^2) each for 10 min, and the ECL signal was then observed. For the detection of phosphorylated ErbB-2/HER-2, the membrane was probed with anti-phospho-ErbB-2/HER-2 (a commercially available solution was diluted 1:1000 with TBS-T, 1 mL/30 cm^2) in a plastic bag for 1 h, washed twice with TBS-T (2 mL/cm^2) each for 10 min, probed with HRP-conjugated anti-rabbit IgG antibody (a commercially available solution was diluted 1:10000 with TBS-T, 1 mL/30 cm^2) in a plastic bag for 1 h, and washed twice with TBS-T (2 mL/cm^2) each for 10 min, and the ECL signal was then observed.

2.10 Reprobing of the blotting membranes

To eliminate biotin-pendant Phos-tag and HRP–SA from the blotting membrane after ECL analysis, the membrane was incubated with a stripping buffer (5 mL/cm^2) consisting of 62.5 mM Tirs-HCl pH 6.8, 2% w/v SDS, and 0.10 M 2-mercaptoethanol for 20 min at room temperature and washed three times with a TBS-T solution (5 mL/cm^2) each for 1 h at room temperature. The remaining proteins on the membrane were probed with an anti-phosphoprotein antibody.

3 Results

3.1 Separation of phosphoproteins using a spin column method

In order to determine the optimum conditions for the phosphate-affinity chromatography on Phos-tag agarose, we adopted a spin column method (0.1-mL gel scale) using a commercially available A431 cell lysate. Figure 1 shows typical SDS-PAGE results for the flow-through/washing (lane 1), elution (lane 2), and column-washing (lane 3) fractions under the optimum conditions using SYPRO Ruby gel staining and Western blotting. The binding, washing, and elution processes were all conducted without a detergent or a reducing agent at room temperature. The binding/washing buffer pH 7.5 contained 1.0 M CH_3COONa and 0.10 M Tris-CH_3COOH. The elution buffer was a mixed buffer solution consisting of 0.10 M Tris-CH_3COOH pH 7.5, 10 mM NaH_2PO_4-NaOH pH 7.5, and 1.0 M NaCl. The column-washing buffer consisted of 0.10 M Tris-CH_3COOH, 1% w/v SDS, and 10 mM EDTA (*i.e.* an eliminator of Phos-tag-bound zinc(II) ion). The SYPRO Ruby images in Fig. 1a show the distribution of the proteins in the flow-through/washing (lane 1), elution (lane 2), and column-washing fractions (lane 3). The ratio of the gel-bound proteins against total proteins for the EGF-stimulated cell lysate (Fig. 1a, lane 2) is much larger than that for the AP-treatment of the EGF-unstimulated cell lysate (Fig. 1d, lane 2). Few proteins remain in the column after the elution process (see Fig. 1a and d, lane 3). The efficiency of the separation of phosphoproteins was determined by Western blotting using the biotin-pendant

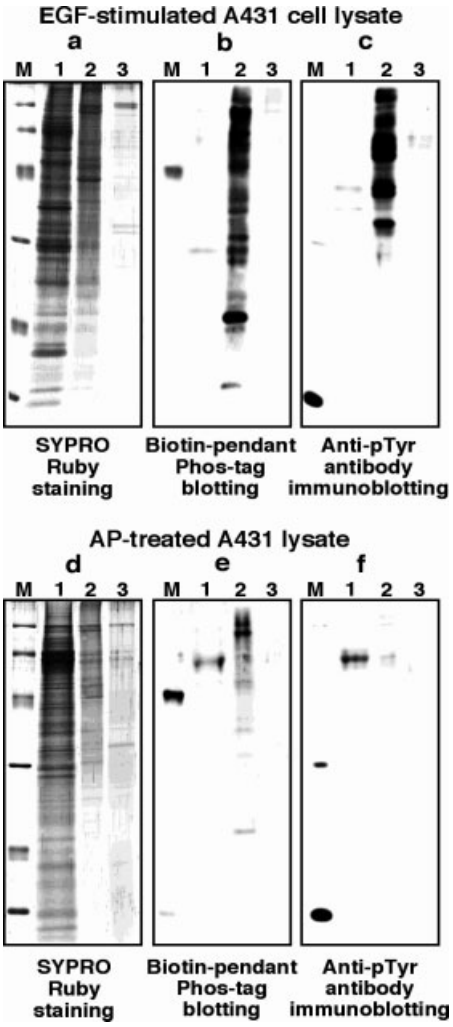

Figure 1. Selective separation of phosphoproteins from an EGF-stimulated A431 cell lysate (upper images) and an AP-treated A431 cell lysate (lower images) using the phosphate-affinity spin column. The SDS-PAGE gels were analyzed by SYPRO Ruby gel staining (a and d), Western blotting with biotin-pendant Phos-tag (b and e), and immunoblotting with an anti-pTyr antibody (c and f). The flow-through/washing, elution, and column-washing fractions were applied in lanes 1, 2, and 3, respectively. The molecular weight standards for 97, 66, 45 (a phosphoprotein, ovalbumin), 29, 20, and 14 kDa are shown from the top in lane M.

Phos-tag complex with HRP–SA [14] (see Fig. 1b). The biotin-pendant Phos-tag method comprehensively detects phosphoproteins as ECL signals (see the single band of a phosphoprotein, ovalbumin, in the molecular weight markers, lane M in Fig. 1b). The strong ECL signals in the elution fraction of the EGF-stimulated cell lysate (lane 2 in Fig. 1b) show that more than 90% of phosphoproteins are separated in the elution fraction. The ECL signals were greatly diminished in the AP-treated cell lysate (see Fig. 1e), indicating that most of the signals in Fig. 1b are phosphoproteins. Furthermore, the efficiency of the separation was evaluated by reprobing of the same blotting

membranes using an HRP-conjugated anti-pTyr antibody (Fig. 1c and f). The ECL signal distribution into each fraction was almost compatible with the result by the biotin-pendant Phos-tag method. Nonspecific ECL signals in lane 1 of Fig. 1e and f could be assigned to an excess amount of AP. In the absence of the zinc(II) ions of Phos-tag agarose (*e.g.* by pretreatment with a zinc(II)-removing buffer consisting of 10 mM EDTA, 0.10 M Tris-CH_3COOH, and 1.0 M CH_3COONa), most of the phosphoproteins were eluted together with nonphosphorylated proteins in the flow-through/washing fraction (data not shown). This fact indicates that the zinc(II)-bound Phos-tag molecule preferentially captures the phosphoproteins in the presence of excess nonphosphorylated proteins. Thus, we could succeed in the efficient separation of phosphoproteins from the mixture of solubilized cellular proteins under the experimental condition. The optimization of the buffers used for the phosphate-affinity chromatography is described below.

Some buffer systems for phosphate-affinity chromatography were examined by the same spin column procedure shown above. We selected a buffer pH of 7.5, which is a physiological pH resulting in less damage to native proteins. What follows is a typical process for the optimized buffer system. First, 0.10 M Tris-CH_3COOH for the binding and washing and a mixed buffer of 0.10 M Tris-CH_3COOH and 10 mM NaH_2PO_4-NaOH for the elution were tested in the absence of neutral salt. The sample applied to the spin column chromatography was the commercially available EGF-stimulated A431 cell lysate. The same SDS-PAGE analysis demonstrated that most of the proteins are retained in Phos-tag agarose after the elution process (see Fig. 2a and d). To prevent the protein from binding strongly to Phos-tag agarose, we added 1.0 M NaCl in the buffers, whose salt was previously reported for Fe(III)-IMAC [19, 20]. In this buffer system, many proteins containing phosphoproteins were observed in the elution fraction (Fig. 2b and e, lane 2). There were few proteins remaining in the column after the elution process (see lane 3). However, the flow-through/washing fraction contained a smaller amount of proteins (see lane 1) than that in the elution fraction (see lane 2), indicating that the buffer system with 1.0 M NaCl failed to prevent the nonspecific binding of nonphosphorylated proteins. Next, we tested a buffer system with 1.0 M CH_3COONa instead of NaCl. The excess amounts of acetate anion should competitively inhibit the nonspecific binding between the carboxyl groups of the proteins and Phos-tag. As expected, a larger amount of protein was obtained in the flow-through/washing fraction (lane 1 in Fig. 2c) than with the NaCl buffer system (lane 1 in Fig. 2b). Most of the gel-unbound proteins were nonphosphorylated proteins, as shown by Western blotting (lane 1 in Fig. 2f). However, some proteins remained in the column after the elution process (lane 3 in Fig. 2c), and a comparatively high molecular weight of the phosphoproteins remained in the column-washing fraction (lane 3 in Fig. 2f). These findings led to the most effective buffer system combining a binding/washing buffer (0.10 M Tris-CH_3COOH and 1.0 M CH_3COONa) and an elution buffer (0.10 M Tris-CH_3COOH, 1.0 M NaCl, and 10 mM NaH_2PO_4-NaOH). The same results were observed in the elution process using 1.0 M $NaNO_3$ instead of 1.0 M NaCl (data not shown). The optimized buffer system was used for a subsequent experiment with an open column.

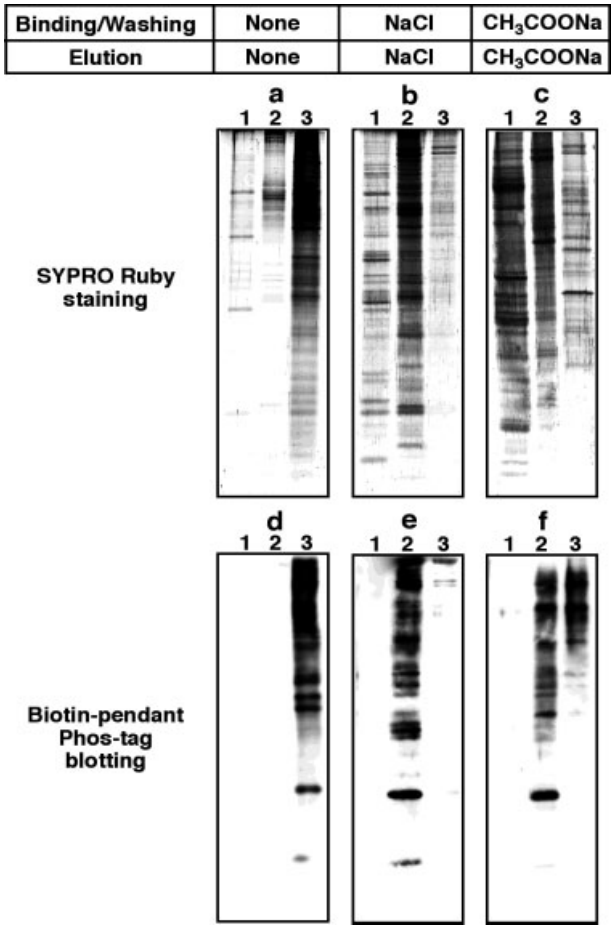

Figure 2. Effect of additional salts on the separation of phosphoproteins in an EGF-stimulated A431 cell lysate using the phosphate-affinity spin column at pH 7.5 (0.10 M Tris-CH_3COOH). No (a and d), 1.0 M NaCl (b and e), and 1.0 M CH_3COONa (c and f) were used in the binding, washing, and elution processes. The flow-through/washing, elution, and column-washing fractions were applied in lanes 1, 2, and 3, respectively. The SDS-PAGE gels were analyzed by SYPRO Ruby gel staining (upper images) and Western blotting with biotin-pendant Phos-tag (lower images).

3.2 Enrichment of phosphoproteins using an open column method

To evaluate the enrichment of phosphoproteins into the elution fraction, the first example of phosphate-affinity chromatography was performed using a comparatively large amount of the cell lysate and an open column of Phos-tag agarose (1 mL). In the case of an 80% confluent A431 cell culture in a 100-mm culture dish, 7×10^6 cells are normally collected, from which 1.3–1.5 mg of the solubilized proteins is obtained. An EGF-stimulated A431 cell lysate (0.50 mg solubilized cellular pro-

teins in 0.25 mL of an RIPA buffer) was prepared from the cultured cells and then diluted with 1.0 mL of the binding/washing buffer. The resulting solution (1.25 mL) was loaded onto the open column. Details of the procedure for affinity column chromatography are described in Section 2. The total time for the phosphate-affinity column chromatography was within 40 min. For SDS-PAGE followed by SYPRO Ruby gel staining and Western blotting (Fig. 3), the cell lysate (0.50, 0.75, and 1.0 μg of proteins for lanes 1, 2, and 3, respectively), the desalted and condensed sample of the elution fraction (0.5, 0.75, and 1.0 μg of proteins for lanes 4, 5, and 6, respectively), and the desalted and condensed samples of the flow-through/washing fraction (2.5 μg of proteins for lane 7) were sequentially applied. The resulting image by SYPRO Ruby gel staining (Fig. 3a) demonstrated that the total amount of proteins for the cell lysate (lanes 1–3) is almost equal to that for the elution fraction (lanes 4–6). Next, we determined the distribution of the phosphoproteins by the Western blotting using the biotin-pendant Phos-tag (Fig. 3b). The stronger ECL signals representing phosphoproteins in the elution fraction (lanes 4–6) were compared with those in the lysate (lanes 1–3). There was a small amount of phosphoprotein in the flow-through/washing fraction (lane 7). Immunoblotting with the antibodies against specific phosphoproteins such as MAPK 1/2 (Fig. 3c), Shc (Fig. 3d), and ErbB-2/HER-2 (Fig. 3e), which are candidates for *in vivo* phosphorylation involved in EGF signaling [21, 22], showed that the corresponding phosphoproteins are strongly enriched in the elution fraction. The phosphorylated MAPK (44 and 42 kDa isoforms, Fig. 3c) and Shc (67 and 52 kDa isoforms, Fig. 3d) were clearly detected even at 0.5 μg of proteins (see lane 4).

Finally, we conducted the quantification of the proteins in the elution fractions of three independent experiments using 0.25 mL of an RIPA buffer containing 0.50 mg protein of the EGF-stimulated A431 cell lysate. The recoveries of the eluted proteins using 1 mL Phos-tag agarose were estimated to be 19, 20, and 22% (*i.e.* 97, 102, and 108 μg, respectively). These yields are consistent with the general contents of phosphoproteins (10–30%) in mammalian cells [23, 24]. When a larger amount of the lysate proteins (*e.g.* 0.60 mg in 0.25 mL of an RIPA buffer) was loaded into the column, some of the phosphoproteins were eluted in the flow-through/washing fraction (data not shown). A similar leak into the flow-through/washing fraction resulted from the use of twice the volume of an RIPA buffer (0.50 mL) containing the solubilized proteins (0.50 mg), which may be attributed to the competitive binding of $HOVO_3^{2-}$ or zinc(II)-elimination by EDTA. Thus, the appropriate capacity of the 1 mL-compressed Phos-tag agarose should be *ca.* 0.50 mg of the proteins lysed in 0.25 mL of an RIPA buffer.

4 Discussion

Ga(III)- and Fe(III)-IMAC are commonly used for the enrichment of phosphopeptides after proteolysis in the protein phosphorylation analysis using MS [25–28]. However, current

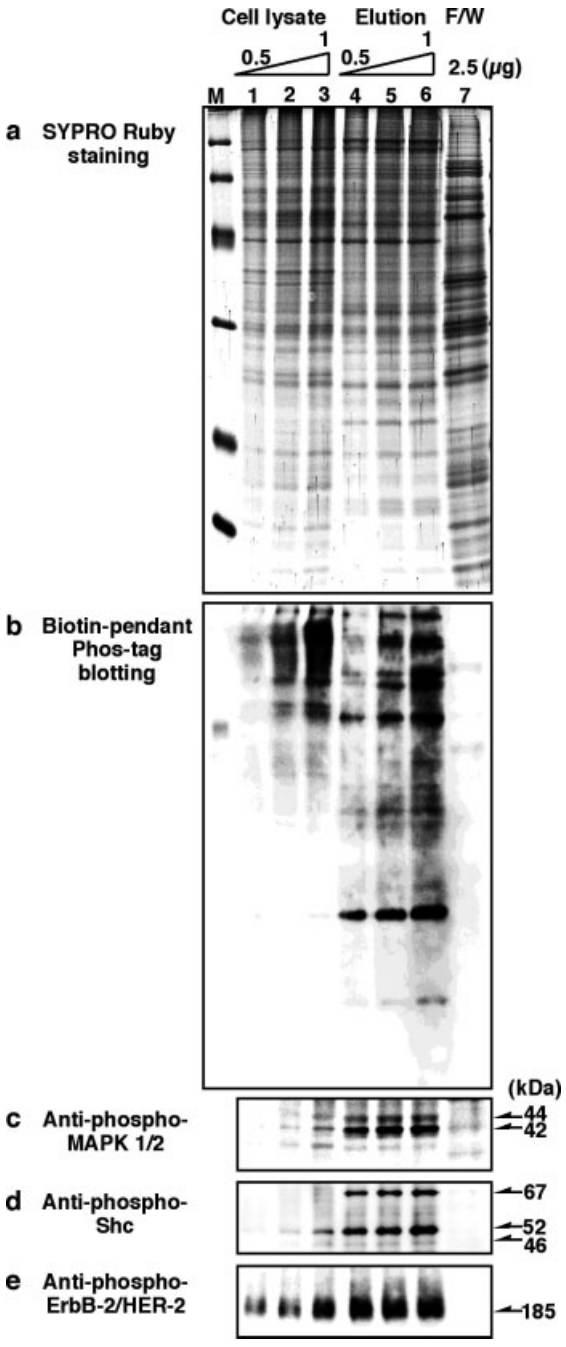

Figure 3. Comparisons of the relative amounts of phosphoproteins from an EGF-stimulated A431 cell lysate before (lanes 1, 2, and 3) and after phosphate-affinity chromatography (lanes 4, 5, 6, and 7). Samples containing 0.50, 0.75, and 1.0 μg proteins in the cell lysate (lanes 1, 2, and 3, respectively) and the elution fraction (lanes 4, 5, and 6, respectively) were used. The flow-through/washing fraction (F/W, 2.5 μg proteins) was used in lane 7. The SDS-PAGE gels were analyzed by SYPRO Ruby gel staining (a), Western blotting with biotin-pendant Phos-tag (b), and immunoblotting with anti-phospho-MAPK 1/2 (c), anti-phospho-Shc (d), and anti-phospho-ErbB-2/HER-2 (e) antibodies. The molecular weight standards for 97, 66, 45 (a phosphoprotein, ovalbumin), 29, 20, and 14 kDa are shown from the top in lane M.

opinion on cellular biology suggests that a more comprehensive study to disclose the role of phosphorylation involved in various cellular events could be achieved by the identification and analysis of phosphoproteins rather than their fragmented phosphopeptides. Recent studies report the enrichment of phosphoproteins from a complex biological sample using affinity chromatography [6–8, 29]. However, they rely on unphysiological conditions, such as acidic or alkaline pH and high temperature in the presence of detergents and/or reducing agents for the adsorption and elution of the phosphoproteins. Affinity chromatography using $Al(OH)_3$ has been shown to be better among them; however, to perform quality enrichment, it was necessary to use an alkaline pH buffer (*ca.* 9) in the eluting process [29].

In this study, we found an improved procedure for immobilized zinc ion affinity chromatography (*i.e.* Zn(II)-IMAC) using a phosphate-affinity gel, Phos-tag agarose, and succeeded in separating and enriching the phosphoproteins comprehensively from a complex mixture of solubilized cellular proteins. The procedure employed pH 7.5 loading buffers for the binding, washing, and elution processes: selective adsorption of the phosphoproteins on the Phos-tag agarose succeeded with a 0.10 M Tris-CH_3COOH buffer containing 1.0 M CH_3COONa, and the efficient elution of the gel-bound phosphoproteins was achieved using a mixed buffer of 0.10 M Tris-CH_3COOH and 10 mM NaH_2PO_4-NaOH containing 1.0 M NaCl. The phosphate-affinity chromatography was conducted without a detergent or a reducing agent at room temperature, leading to a major advantage for gaining intact information of the phosphoproteins. As the first example, we demonstrate a selective separation and strong enrichment of native phosphoproteins from the lysate of A431 human epidermoid carcinoma cells after EGF stimulation, thereby identifying three phosphoproteins, ErbB-2/HER-2 (a membrane receptor protein), Shc (a cytoplasmic protein), and MAPK (a nuclear localization protein) in EGF signaling.

Our Zn(II)-IMAC using Phos-tag agarose provides a simple and efficient procedure for separating native phosphoproteins from the biological sample at a physiological pH less than one hour. While the volume of the Phos-tag agarose gel bed (1.0 mL) is fixed in this report, it can be easily optimized in proportion to the amount of the sample. We believe that phosphoproteomics would progress greatly by combining our phosphate-affinity chromatography and existing methods using 2-DE followed by MS. Since the nondenaturing procedure maintains the protein conformation and activity, the separated and enriched phosphoproteins should be ideal for use in many downstream applications. This enrichment procedure should increase the sensitivity of the detection of phosphoproteins in diverse biological studies.

This work was supported by Grants-in-Aid for Scientific Research (B) (15390013) and for Young Scientists (B) (17790034) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a research grant from the Hiroshima University Fujii Foundation.

5 References

[1] Hunter, T., *Cell* 1995, *80*, 225–236.

[2] Hunter, T., *Cell* 2000, *100*, 113–127.

[3] Pandey, A., Podtelejnikov, A. V., Blagoev, B., Bustelo, X. R. *et al.*, *Proc. Natl. Acad. Sci. USA* 2000, *97*, 179–184.

[4] Lim, Y. P., Diong, L. S., Qi, R., Druker, B. J., Epstein, R. J., *Mol. Cancer Ther.* 2003, *2*, 1369–1377.

[5] Grønborg, M., Kristiansen, T. Z., Stensballe, A., Andersen, J. S. *et al.*, *Mol. Cell. Proteomics* 2002, *1*, 517–527.

[6] Imam-Sghiouar, N., Joubert-Caron, R., Caron, M., *Amino Acids* 2005, *28*, 105–109.

[7] Guerrera, I. C., Predic-Atkinson, J., Kleiner, O., Soskic, V., Godovac-Zimmermann, J., *J. Proteome Res.* 2005, *4*, 1545–1553.

[8] Dubrovska, A., Souchelnytskyi, S., *Proteomics* 2005, *5*, 4678–4683.

[9] Kinoshita, E., Takahashi, M., Takeda, H., Shiro, M., Koike, T., *Dalton Trans.* 2004, 1189–1193.

[10] Takeda, H., Kawasaki, A., Takahashi, M., Yamada, A., Koike, T., *Rapid Commun. Mass Spectrom.* 2003, *17*, 2075–2081.

[11] Hirano, K., Matsui, H., Tanaka, T., Matsuura, F. *et al.*, *J. Biochem.* 2004, *136*, 477–483.

[12] Tanaka, T., Tsutsui, H., Hirano, K., Koike T. *et al.*, *J. Lipid Res.* 2004, *45*, 2145–2150.

[13] Inamori, K., Kyo, M., Nishiya, Y., Inoue, Y. *et al.*, *Anal. Chem.* 2005, *77*, 3979–3985.

[14] Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K., Koike, T., *Mol. Cell. Proteomics* 2006, *5*, 749–757.

[15] Kinoshita, E., Yamada, A., Takeda, H., Kinoshita-Kikuta, E., Koike, T., *J. Sep. Sci.* 2005, *28*, 155–162.

[16] Berggren, K., Chernokalskaya, E., Steinberg, T. H., Kemper, C. *et al.*, *Electrophoresis* 2000, *21*, 2509–2521.

[17] Bradford, M. M., *Anal. Biochem.* 1976, *72*, 248–254.

[18] Laemmli, U. K., *Nature* 1970, *227*, 680–685.

[19] Andersson, L., Porath, J., *Anal. Biochem.* 1986, *154*, 250–254.

[20] Muszyńska, G., Andersson, L., Porath, J., *Biochemistry* 1986, *25*, 6850–6853.

[21] Williams, R., Sanghera, J., Wu, F., Carbonaro-Hall, C. *et al.*, *J. Biol. Chem.* 1993, *268*, 18213–18217.

[22] Wheeler, M., Domin, J., *Mol. Cell. Biol.* 2001, *21*, 6660–6667.

[23] Alberts, B., Bray, D., Lewis, K., Raff, M. *et al.*, *Molecular Biology of the Cell*, Garland Publishing, New York 1994, pp. 195–222.

[24] Mann, M., Ong, S. E., Grønborg, M., Steen, H. *et al.*, *Trends Biotechnol.* 2002, *20*, 261–268.

[25] Posewitz, M. C., Tempst, P., *Anal. Chem.* 1999, *71*, 2883–2892.

[26] Riggs, L., Seeley, E. H., Regnier, F. E., *J. Chromatogr. B* 2005, *817*, 89–96.

[27] Kokubu, M., Ishihama, Y., Sato, T., Nagasu, T., Oda, Y., *Anal. Chem.* 2005, *77*, 5144–5154.

[28] Trinidad, J. C., Specht, C. G., Thalhammer, A., Shoepfer, R., Burlingame, A. L., *Mol. Cell. Proteomics* 2006, *5*, 914–922.

[29] Wolschin, F., Wienkoop, S., Weckwerth, W., *Proteomics* 2005, *5*, 4389–4397.

