

Chapter 2

Phos-tag Affinity Electrophoresis for Protein Kinase Profiling

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Abstract

Protein kinase profiling can provide a basis for understanding the molecular origins of diseases and, potentially, for developing tools for therapeutic intervention. It is therefore very important to develop advanced experimental procedures for convenient and accurate determination of the phosphorylation status of certain substrate proteins in the life sciences. Here, we introduce a method for protein kinase profiling by using a novel type of phosphate-affinity sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The phosphate-affinity site is a polyacrylamide-bound dinuclear metal complex of a phosphate-binding tag molecule known as Phos-tag. The Phos-tag SDS-PAGE method permits detection of changes in the mobility of phosphorylated proteins in comparison with their nonphosphorylated counterparts and thereby allows quantitative analysis of protein kinase reactions without any special apparatus, radioactive isotopes, or chemical labels. If a kinase reaction occurs at one residue of a substrate protein, the monophosphorylated and nonphosphorylated forms can be simultaneously detected as two migration bands on a Phos-tag SDS-PAGE gel. In the case of hyperphosphorylation, the phosphorylated products appear as multiple migration bands, depending on the phosphorylation status in terms of the numbers and the positions of attached phosphate groups. This article discusses applications of label-free kinase activity profiling by the Phos-tag SDS-PAGE method in the analysis of phosphorylated substrates derived from various kinase reactions. The resolving power of the affinity electrophoresis provides detailed information that leads to an overview of the kinase-dependent dynamics of various substrate proteins.

Key words: Affinity electrophoresis, Phosphoproteomics, Phosphorylation, Phos-tag, Protein kinase, SDS-PAGE, Western blotting

1. Introduction

Protein phosphorylation is among the most common post-translational modifications across biological species and it controls a number of key cellular processes by means of changes in the balance between the opposing reversible reactions of specific protein kinases and phosphatases (1). More than 500 protein kinases are predicted to occur in the human proteome alone (2), a number that clearly reflects the

importance of protein phosphorylation. In fact, abnormal protein phosphorylation is closely involved with many human diseases, including cancer and neurodegenerative diseases (3). Many mutations in protein kinase-encoding genes that cause aberrant enzyme activity are associated with various forms of cancer. There has been considerable progress in the development of selective inhibitors for certain kinases as potential drug targets, and some of these inhibitors have been approved for use in humans for the treatment of cancer. Imatinib mesylate (Glivec; Novartis, Basel, Switzerland) and gefitinib (Iressa; AstraZeneca, London, UK) are typical examples of such drugs. The former was designed to inhibit Bcr/Abl tyrosine kinase, which is generated by a chimeric gene resulting from a chromosomal translocation that is characteristic of chronic myeloid leukemia. The latter was developed as a potent inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase, and it selectively inhibits EGF-stimulated tumor cell growth. In human brain neurons, on the other hand, the activities of several protein kinases can be dysregulated, leading to hyperphosphorylation of the microtubule-associated protein Tau, which is a classic hallmark of Alzheimer's disease (4). The sites and stoichiometry of phosphorylation of the Tau protein are correlated with the pathological characteristics of the disease.

Methods for determining the phosphorylation status of certain substrate proteins and for screening for novel inhibitors of certain kinases have therefore become increasingly important. A method conventionally used for defining a particular phosphorylation event is the incorporation of a radioisotope (RI) such as ^{32}P or ^{33}P into a phosphorylated protein. The phosphorylation status of the target protein can then be detected and quantified from its radioactivity. A non-RI method using polyclonal and monoclonal antibodies is also well established for the detection of protein phosphorylation. The readout from the antibody that has recognized a phosphopeptide or phosphoprotein is measured as a fluorescence, luminescence, or polarization signal, and this can be utilized in many analytical procedures, including enzyme-linked immunosorbent assay, immunoblotting, or immunocytochemistry. Because these procedures require specific antibody-based reagents, a lack of availability or specificity of an appropriate antibody can directly affect the performance of the assay in some cases. As an alternative to immunoassays, which are frequently problematic in relation to the antibody-based reagents, chemical labeling of phosphate groups has been used for phosphospecific-site mapping in conjunction with mass spectrometry (MS) (5). Affinity chromatography using the characteristics of metal ions is widely accepted as a technique for comprehensively determining phosphorylation sites. Recently, improvements in the specificity of the chromatography have been accomplished in a number of ways for MS-based studies on the phosphoproteome (6–10). To establish novel therapeutic approaches to human diseases, phosphoproteomic methods for the

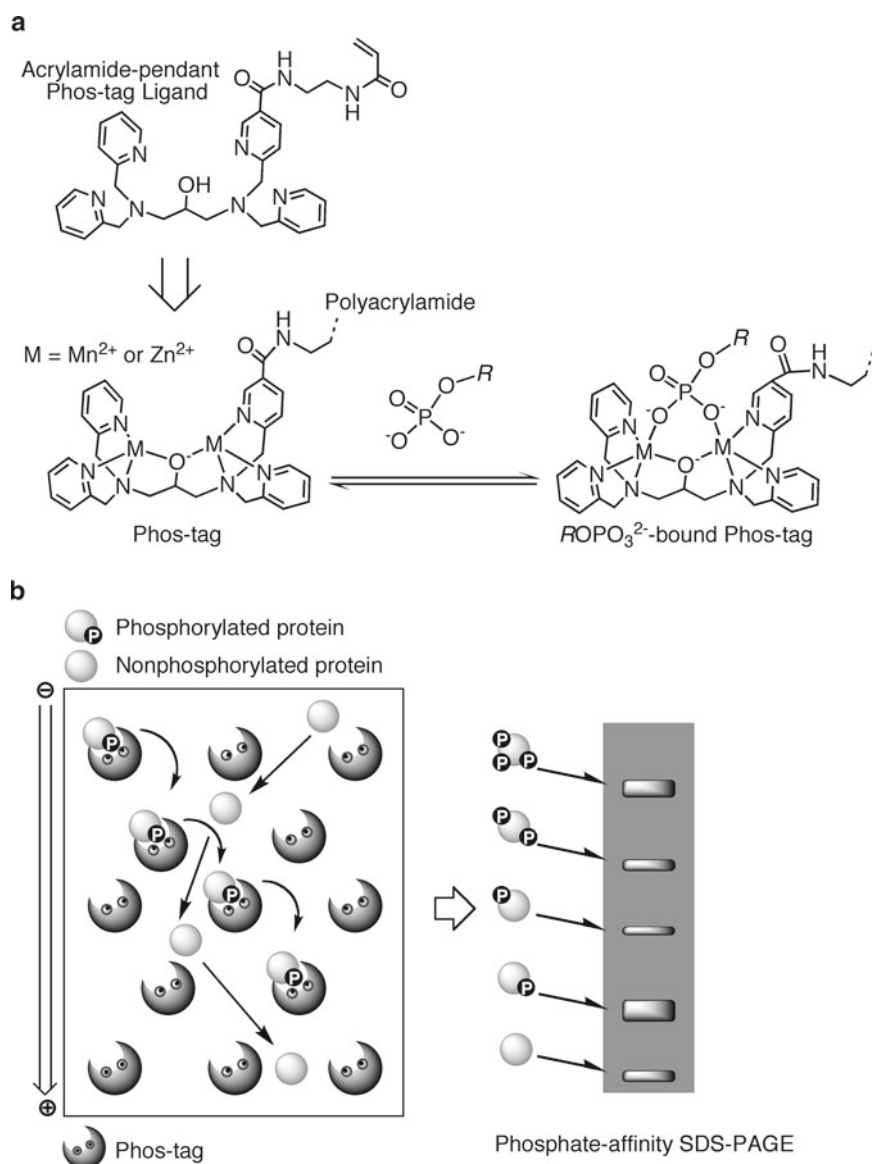


Fig. 1. Structure of acrylamide-pendant Phos-tag ligand and scheme for reversible capture of a phosphomonoester dianion (ROPO_3^{2-}) by Phos-tag (a). Schematic representation of the principle of phosphate-affinity SDS-PAGE (b).

determination of the phosphorylation status of proteins are subject to continual development efforts.

We have developed the Phos-tag technology as a novel approach for the analysis of protein phosphorylation (Phos-tag consortium, <http://www.phos-tag.com/english/index.html>). The Phos-tag technology utilizes a novel phosphate-binding tag molecule, Phos-tag {1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex}, which binds to anionic substituents, especially phosphomonoester dianions, under physiological conditions (Fig. 1a) (11). To date, the technology has contributed to the

development of a range of procedures, including matrix-assisted laser desorption/ionization time-of-flight MS analysis of phosphorylated compounds (12), electrospray ionization MS analysis for online mass tagging of phosphopeptides (13), immobilized metal-affinity chromatography for the separation of phosphopeptides and phosphoproteins (14–17), surface plasmon resonance analysis for reversible peptide phosphorylation (18), Western blotting analysis of phosphoproteins on a protein-blotted membrane (19), and solid-phase-free fluorescence analysis of protein phosphorylation and dephosphorylation in an aqueous solution (20, 21). Furthermore, we have shown that phosphate-affinity sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the tag molecule can be used to detect shifts in the mobility of phosphoproteins in comparison with their nonphosphorylated counterparts (22–27) (Fig. 1b). This Phos-tag affinity electrophoresis (Phos-tag SDS-PAGE) technique offers the following significant advantages (1) no radioactive or chemical labels are required for kinase and phosphatase assays; (2) the time course of the quantitative ratio of phosphorylated to nonphosphorylated proteins can be determined; (3) several phosphorylated forms of a certain protein, depending on the phosphorylation status, can be detected as multiple migration bands; (4) the phosphate-binding specificity is independent of the nature of the phosphorylated amino acid; (5) His- and Asp-phosphorylated proteins involved in a two-component signal-transduction system can be detected simultaneously in their phosphotransfer reactions; (6) different phosphorylated forms of a single protein having identical numbers of phosphate groups can be separated; (7) downstream procedures, such as immunoblotting or MS analysis, can be applied; and (8) the phosphate-affinity procedure is almost identical to normal SDS-PAGE.

Here, we describe applications of label-free kinase activity profiling using the Phos-tag SDS-PAGE methodology, and we demonstrate the resolving power of affinity electrophoresis in separating phosphorylated substrate proteins.

2. Materials (See Note 1)

2.1. Preparation of Tau Proteins Phosphorylated by Various Ser/Thr Kinases

1. Substrate Tau protein: His-tagged recombinant human Tau isoform F (molecular weight \approx 60 kDa), consisting of 441 amino acid residues, purchased from Calbiochem (La Jolla, CA, USA) is used. Store at -80°C .
2. Ser/Thr kinases: Recombinant human glycogen synthase kinase-3 β (GSK-3 β) and recombinant mouse protein kinase A (PKA) catalytic subunit purchased from Calbiochem are used. Recombinant human cyclin-dependent kinase 5 (cdk5)/p35, recombinant mouse mitogen-activated protein kinase 2

(MAPK), recombinant human casein kinase II (CKII), and rat forebrain calcium/calmodulin-dependent protein kinase II (CaMKII) were purchased from Millipore (Billerica, MA, USA). GSK-3 β and cdk5/p35 must be stored at -80°C ; the other proteins can be stored at -20°C .

3. In vitro Ser/Thr kinase reaction: The in vitro phosphorylation assay is carried out by using the recombinant Tau protein (4.1 μg) at 30°C .
4. Kinase reaction buffer for phosphorylation of Tau by GSK-3 β , cdk5/p35, PKA, MAPK, and CKII: The reaction buffer (20 μL) contains 25 mM Tris-HCl (pH 7.5) (e.g., Nacalai Tesque, Kyoto, Japan), 5.0 mM β -glycerol phosphate (e.g., glycerol 2-phosphate disodium salt hydrate, Sigma, St. Luis, MO, USA), 12 mM MgCl_2 (e.g., Nacalai Tesque), 2.0 mM dithiothreitol [(2S,3S)-1,4-disulfanybutane-2,3-diol; e.g., Nacalai Tesque], 0.10 mM Na_3VO_4 (e.g., Nacalai Tesque), 50 μM ATP (e.g., Sigma), 37 kBq [γ - ^{32}P]ATP (e.g., GE Healthcare Bio-Sciences, Piscataway, NJ, USA), and 4.1 μg of Tau. The amounts of the kinases in the buffer are as follows: GSK-3 β 2.0 μg , cdk5/p35 0.10 μg , PKA 2,500 U, MAPK 0.20 μg , and CKII 0.25 μg . The buffer is prepared immediately before use.
5. Kinase reaction buffer for phosphorylation by CaMKII: The reaction buffer (20 μL) contains 20 mM MOPS (pH 7.2) (e.g., Nacalai Tesque), 25 mM β -glycerol phosphate, 15 mM MgCl_2 , 1.0 mM dithiothreitol, 1.0 mM Na_3VO_4 , 1.0 mM CaCl_2 , 20 $\mu\text{g}/\text{mL}$ recombinant bovine calmodulin (Millipore), 50 μM ATP, 37 kBq [γ - ^{32}P]ATP, 4.1 μg of Tau, and 50 ng of CaMKII. The buffer is prepared immediately before use.
6. Sample-loading buffer (3 \times) for stopping the kinase reaction: 195 mM Tris-HCl (pH 6.8), 3.0% (w/v) SDS (e.g., Nacalai Tesque), 15% (v/v) 2-mercaptoethanol (e.g., Nacalai Tesque) (see Note 2), 30% (v/v) glycerol (e.g., Nacalai Tesque), and 0.1% (w/v) bromophenol blue (BPB) (e.g., Nacalai Tesque). Store at -20°C . After incubation for various reaction times (0–300 min), 3.0 μL of the reaction mixture is taken out and added to the sample-loading buffer (1.5 μL).
7. Ser/Thr-phosphorylation sample of Tau: Store at -20°C . An aliquot (1.2 μL) of the resulting solution is used in the Phos-tag SDS-PAGE (164 ng of Tau/lane).

2.2. Preparation of Tau Proteins Phosphorylated by Various Tyr Kinases

1. Substrate Tau protein: see (1) under Sect. 1.
2. Tyr kinases: Recombinant Tyr kinases (ABL, ACK, AXL, EGFR, EPHA1, FES, FGFR1, FYN, INSR, JAK1, LCK, LYN, MET, SRC, TEC, TIE2, TYK2, and YES) purchased from Carina Biosciences (Kobe, Japan) are used. Store at -80°C .

3. In vitro Tyr kinase reaction: The in vitro phosphorylation assay is carried out using the recombinant Tau protein (2.5 μg) at 30°C.
4. Kinase reaction buffer for phosphorylation of Tau with 18 types of Tyr kinase: The reaction buffer (40 μL) contains 60 mM HEPES-NaOH (pH 7.5) (e.g., Nacalai Tesque), 10 mM β -glycerol phosphate, 10 mM MgCl_2 , 1.25 mM dithiothreitol, 0.30 mM Na_3VO_4 , 0.20 mM ATP, 2.5 μg of Tau, and 0.20 μg of each kinase. Prepare immediately before use.
5. Sample-loading buffer (3 \times) for stopping the kinase reaction: see (6) under Sect. 1. After incubation for the appropriate time for each kinase (ABL: 5 min, ACK: 60 min, AXL: 60 min, EGFR: 5 min, EPHA1: 5 min, FES: 60 min, FGFR1: 5 min, FYN: 2 min, INSR: 2 min, JAK1: 60 min, LCK: 2 min, LYN: 2 min, MET: 2 min, SRC: 2 min, TEC: 60 min, TIE2: 60 min, and YES: 5 min), the sample-loading buffer (20 μL) is added to the reaction mixture.
6. Tyr-phosphorylation sample of Tau: Store at -20°C . An aliquot (3.0 μL) of the resulting solution is used in the Phos-tag SDS-PAGE (125 ng of Tau/lane).

2.3. Preparation of Cell Lysate

1. Culture medium for SW480 cells: Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin purchased from Invitrogen (Carlsbad, CA, USA) is used. The cells (10^7 cells) on a 90-mm culture dish (e.g., Sumitomo Bakelite, Tokyo, Japan) are incubated in the medium under a humidified atmosphere of 5% CO_2 and 95% air at 37°C.
2. Cell stimulants: A 100 mM solution of Na_3VO_4 in distilled water is used as a Tyr phosphatase inhibitor and is stored at room temperature. For stimulation with 1.0 mM pervanadate for 30 min, a mixture of 1.0 mM Na_3VO_4 and 3.0 mM H_2O_2 (final concentration) (e.g., Nacalai Tesque) is added to the culture medium. Mix immediately before use. As an adenylyl cyclase activator, a 10 mM solution of forskolin (Enzo Life Sciences, Plymouth Meeting, PA, USA) in dimethyl sulfoxide (e.g., Nacalai Tesque) is prepared and stored at -20°C . For stimulation for 30 min, 10 μM forskolin (final concentration) is added to the culture medium.
3. Washing buffer: Tris-buffered saline (TBS) containing 10 mM Tris-HCl (pH 7.5) and 0.10 M NaCl (e.g., Nacalai Tesque). Store at room temperature. Each culture is washed twice before preparation of the cell lysate.
4. Lysis buffer: a sample-loading buffer (1 \times) consisting of 65 mM Tris-HCl (pH 6.8), 1.0% (w/v) SDS, 5.0% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.03% (w/v) BPB is used. Store at -20°C .

5. Lysate sample: The lysate sample solution is sonicated briefly to reduce its viscosity and then boiled for 5 min. Store at -20°C . An aliquot (10 μL) of the resulting solution is used in the Phos-tag SDS-PAGE.

2.4. Mn^{2+} -Phos-tag SDS-PAGE Using an Alkaline-pH Buffer System (Laemmli's Buffer System)

1. Phos-tag solution: 5.0 mM acrylamide-pendant Phos-tag ligand, which is commercially available from Wako Pure Chemical (Osaka, Japan), in distilled water containing 3% (v/v) methanol (e.g., Nacalai Tesque) (see Note 3). Store at room temperature in the dark.
2. Manganese(II) chloride solution: 10 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (e.g., Nacalai Tesque) in distilled water (see Note 4). Store at room temperature.
3. Thirty percent (w/v) acrylamide/bis solution (29:1 ratio of acrylamide to N,N' -methylenebisacrylamide) (e.g., Nacalai Tesque) (see Note 5). Store at room temperature in the dark.
4. Separating gel buffer (4 \times): 1.5 M Tris-HCl (pH 8.8) and 0.4% (w/v) SDS. Store at room temperature.
5. Stacking gel buffer (4 \times): 0.5 M Tris-HCl (pH 6.8) and 0.4% (w/v) SDS. Store at room temperature.
6. Ammonium persulfate (APS) (e.g., Nacalai Tesque) solution and N,N,N',N' -tetramethylethane-1,2-diamine (TEMED) (e.g., Nacalai Tesque) (see Note 6): 10% (w/v) APS in distilled water. Prepare immediately before use.
7. Electrophoresis running buffer (see Note 7): 25 mM Tris, 192 mM glycine (e.g., Nacalai Tesque), and 0.1% (w/v) SDS. Store at room temperature.
8. Gel staining solutions: Sil-Best Stain for Protein/PAGE purchased from Nacalai Tesque and SYPRO Ruby protein gel stain purchased from Invitrogen are used.

2.5. Zn^{2+} -Phos-tag SDS-PAGE Using a Neutral-pH Buffer System

1. Phos-tag solution: see (1) under Sect. 4.
2. Zinc(II) nitrate solution: 10 mM $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (e.g., Nacalai Tesque) in distilled water (see Note 8). Store at room temperature.
3. Thirty percent (w/v) acrylamide/bis solution (29:1 ratio of acrylamide to N,N' -methylenebisacrylamide): see (3) under Sect. 4.
4. Gel buffer (2.8 \times) (see Note 9): 1.0 M Bis-Tris-HCl (pH 6.8) (e.g., Nacalai Tesque). Store at room temperature.
5. APS solution and TEMED: see (6) under Sect. 4.
6. Electrophoresis running buffer (see Note 10): 100 mM Tris, 100 mM MOPS, and 0.1% (w/v) SDS. Store at room temperature. Sodium bisulfite is dissolved to a concentration of 5 mM in the buffer solution immediately before use (see Note 11).
7. Gel staining solutions: see (8) under Sect. 4.

2.6. Electroblotting

1. Blotting buffer (see Note 12): 25 mM Tris, 192 mM glycine, and 10% (v/v) methanol. Store at room temperature.
2. Disodium ethylenediaminetetraacetate (EDTA) (e.g., Katayama Chemical, Osaka, Japan) solution: 0.5 M EDTA-NaOH (pH 8). Store at room temperature.
3. Blotting buffer containing 1 mM EDTA: Add 0.2 mL of EDTA solution to 100 mL of blotting buffer.
4. TBS-T solution: 10 mM Tris-HCl (pH 7.5), 0.10 M NaCl, and 0.10% (v/v) Tween-20 (e.g., Nacalai Tesque). Store at room temperature.

2.7. Equipment Setup

1. SDS-PAGE equipment: The instructions assume the use of an Atto model AE-6500 mini-slab gel system (1-mm thick, 9-cm wide, and 9-cm long). The setup can be readily adapted to other formats, including large-type gels.
2. Electroblotting equipment: The instructions assume the use of a Nippon Eido model NA-1511C electroblotting wet-tank unit. The setup can be readily adapted to other formats but not to a semi-dry one (see Note 13).

3. Methods

3.1. Phosphate-Binding Tag Molecule, Phos-tag

To capture a phosphorylated compound effectively under physiological conditions, we developed a dinuclear metal complex {1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex}, which was synthesized to mimic the active center of an alkaline phosphatase (11). The dinuclear zinc(II) complex forms stable 1:1 complexes with phosphate monoester dianions (ROPO_3^{2-}) in an aqueous solution (see Fig. 1a). An X-ray crystal structure analysis of the 1:1 dinuclear zinc(II) complex of a 4-nitrophenyl phosphate dianion showed that each phosphate oxygen anion binds to a zinc(II) atom at the fifth coordination site and that the two zinc(II) ions are separated by a distance of 3.6 Å. Thus, the dinuclear zinc(II) complex, which has a vacancy on the two zinc(II) ions, is suitable for access by a phosphate monoester dianion, which forms a bridging ligand. In an aqueous solution at neutral pH, the dinuclear zinc(II) complex binds strongly with a phenyl phosphate dianion ($K_d = 2.5 \times 10^{-8}$ M). The anion selectivity indexes against SO_4^{2-} , CH_3COO^- , Cl^- , and 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. We named this dizinc(II) complex “Phos-tag” as an abbreviation of “phosphate-binding tag molecule.”

3.2. Mn^{2+} -Phos-tag SDS-PAGE Using the Laemmli's Buffer System

We synthesized a Phos-tag ligand with a pendant acrylamide moiety (see Fig. 1a) and we used this as a novel additive (comonomer) for a separating gel in Laemmli's SDS-PAGE method (28), which is a technique widely used for the separation and detection of proteins. The principle underlying the analytical procedure is the change in the mobility of phosphorylated proteins that results from reversible trapping of phosphate moieties by the Phos-tag molecules immobilized in the gel (see Fig. 1b). When we attempted to use polyacrylamide-bound Zn^{2+} -Phos-tag in SDS-PAGE, the expected phosphate-selective shift in mobility was not observed under the general SDS-PAGE conditions used in Laemmli's method. Presumably, the electrophoresis conditions, such as the abundance of SDS anions and the alkaline buffers, are not conducive to selective trapping of phosphate by the zinc(II) complex. We therefore investigated complexes of other metals (Mn^{2+} , Cu^{2+} , Co^{2+} , Fe^{2+} , and Ni^{2+}) as phosphate-trapping molecules under SDS-PAGE conditions. As a result, we found that a polyacrylamide-bound manganese(II) homologue (Mn^{2+} -Phos-tag) can act as a phosphate-affinity site in Laemmli's buffer system (19). In a separating gel containing copolymerized acrylamide-pendant Mn^{2+} -Phos-tag, the degree of migration of a phosphoprotein is less than that of its nonphosphorylated counterpart, because the tag molecules trap the phosphoprotein reversibly during electrophoresis (see Fig. 1b). On this basis, we developed a novel type of phosphate-affinity SDS-PAGE, known as Mn^{2+} -Phos-tag SDS-PAGE, for the separation of phosphoproteins from their corresponding nonphosphorylated analogues (22–26). Subsequent gel staining or Western blotting with phosphorylation-independent antibodies enables the simultaneous detection of phosphorylated and nonphosphorylated proteins. This method is therefore appropriate for quantitative analyses of protein phosphorylation reactions in vitro and in vivo.

3.3. Zn^{2+} -Phos-tag SDS-PAGE Using a Neutral-pH Buffer System

Affinity electrophoresis using a polyacrylamide-bound Mn^{2+} -Phos-tag and Laemmli's buffer system under alkaline pH conditions has been widely used in determining the phosphorylation states of many proteins (29). However, it has some limitations for separation analysis of certain phosphoproteins. For example, it has been reported that no up-shifted band of Tau protein is observed, following treatment by a certain Tyr kinase (23). An additional disadvantage is that a Mn^{2+} -Phos-tag SDS-PAGE gel cast using an alkaline buffer system is unstable and undergoes partial hydrolysis during long-term storage, resulting in changes in the pore size and a poor resolution. To overcome these limitations, we have recently developed an improved Phos-tag SDS-PAGE (Zn^{2+} -Phos-tag SDS-PAGE) method that uses a dizinc(II) complex of acrylamide-pendant Phos-tag in conjunction with a Bis-Tris-buffered neutral-pH gel system (27). Zn^{2+} -Phos-tag SDS-PAGE is performed at 40 mA/

Table 1
Comparison of the buffer system for Zn²⁺–Phos-tag
SDS-PAGE with that for Mn²⁺–Phos-tag SDS-PAGE

| | Zn²⁺–Phos-tag SDS-PAGE | Mn²⁺–Phos-tag SDS-PAGE |
|-----------------------|--|---|
| Running buffer | 100 mM Tris 100 mM MOPS 0.1% (w/v) SDS 5.0 mM sodium bisulfite (pH 7.8, no adjustment) | 25 mM Tris 192 mM glycine 0.1% (w/v) SDS (pH 8.3, no adjustment) |
| Stacking gel buffer | 357 mM Bis-Tris–HCl (pH 6.8) | 125 mM Tris–HCl 0.1% (w/v) SDS (pH 6.8) |
| Separating gel buffer | 357 mM Bis-Tris–HCl (pH 6.8) | 375 mM Tris–HCl 0.1% (w/v) SDS (pH 8.8) |

gel and room temperature using a 1-mm-thick, 9-cm-wide, 9-cm-long gel on a mini-slab PAGE apparatus. The gel consists of 1.8 mL of a stacking gel [4.0% (w/v) polyacrylamide and 357 mM Bis-Tris–HCl, pH 6.8] and 6.3 mL of a separating gel, consisting of an appropriate percentage of polyacrylamide and 357 mM Bis-Tris–HCl buffer (pH 6.8). The acrylamide-pendant Phos-tag ligand (at an appropriate concentration) and two equivalents of Zn(NO₃)₂ are added to the separating gel before polymerization. The running buffer for electrophoresis consists of 100 mM Tris and 100 mM MOPS containing 0.1% (w/v) SDS and 5.0 mM sodium bisulfite. The electrophoresis is continued until the BPB dye reached the bottom of the separating gel. Table 1 shows a comparison between the buffer systems used for Zn²⁺–Phos-tag SDS-PAGE and that used for Mn²⁺–Phos-tag SDS-PAGE.

Here we introduce a procedure for an improved phosphate-affinity electrophoresis by Zn²⁺–Phos-tag SDS-PAGE and we demonstrate its utility by visualization of novel up-shifted bands of the phosphorylated substrates derived from reactions involving various kinases.

3.4. Procedure for Preparation of the Zn²⁺–Phos-tag SDS-PAGE Gel (See Note 14)

1. Clean the glass plates for casting the gels. It is vital that the glass plates (1-mm thick, 9-cm wide, and 9-cm long) are washed thoroughly with a rinsable detergent, and that they are rinsed extensively with distilled water before the gels are cast.
2. Prepare the separating gel solution. As a typical example, a 7.5% (w/v) separating gel solution (~7 mL) (see Note 15) containing 80 μM polyacrylamide-bound Zn²⁺–Phos-tag (see Note 16) is prepared by mixing 1.75 mL of 30% (w/v)

acrylamide/bis solution, 2.5 mL of gel buffer (2.8×), 112 μ L of Phos-tag acrylamide solution, 112 μ L of zinc(II) nitrate solution (two equivalents on Phos-tag), 10 μ L of TEMED, and 2.42 mL of distilled water in a 50-mL centrifuge tube (e.g., Sumitomo Bakelite).

3. Add 100 μ L of APS solution and mix gently.
4. Transfer the separating gel solution to the gap between the glass plates, pour distilled water on top of the separating gel solution, and allow the gel solution to polymerize for about 20 min at room temperature.
5. Prepare the stacking gel solution. In a similar manner to the preparation of the separating gel solution, a 4.0% (w/v) stacking gel solution (~1.8 mL) is prepared by mixing 0.24 mL of 30% (w/v) acrylamide/bis solution, 0.64 mL of gel buffer (2.8×), 2 μ L of TEMED, and 0.87 mL of distilled water in a 50-mL centrifuge tube.
6. Rinse the top of the separating gel with distilled water and remove the residual liquid with a paper towel.
7. Add 50 μ L of APS solution to the stacking gel solution, mix gently, and then pour the solution onto the separating gel.
8. Insert a sample-well comb and allow the gel solution to polymerize for about 20 min.
9. Carefully remove the comb from the stacking gel, and assemble the gel plate and electrophoresis apparatus.

3.5. Procedure for Electrophoresis

1. Fill the electrode chambers with the electrophoresis running buffer (see Note 17).
2. Apply the protein samples mixed with the sample-loading buffer into the wells (see Note 18).
3. Attach the leads to the power supply (e.g., ATTO AE-8750 Power Station 1000XP). Run the gels under a constant-current condition of 40 mA/gel at room temperature until the BPB dye, which contains in the sample-loading buffer, reaches the bottom of the separating gel (80–90 min).

3.6. Procedures for Autoradiography and Gel Staining

1. For autoradiography, remove the fully run gel from the apparatus and dry it using a gel dryer (e.g., an ATTO AE-3750 dryer). Expose an X-ray film to the gel at -80°C for an appropriate time. The X-ray film is then developed to show the radioactive signals on the phosphoprotein bands.
2. For silver staining of the gel by using Sil-Best Stain for Protein/PAGE, remove the gel from the apparatus and stain it according to the manufacturer's instructions to visualize the protein bands.
3. For SYPRO Ruby gel staining, fix the gel in an aqueous solution containing 10% (v/v) methanol and 7.0% (v/v) acetic acid

for 30 min, stain it in a solution of SYPRO Ruby protein gel stain for 3–12 h, and then wash it in 10% (v/v) methanol and 7.0% (v/v) acetic acid for 30 min. Detect the SYPRO Ruby dye-bound proteins from their 575-nm emission signals on excitation at 473 nm using a laser scanner (e.g., an FLA 5000 laser scanner, Fujifilm, Tokyo, Japan).

3.7. Procedure for Electroblothing

1. When the run is complete, remove the gel from the apparatus and soak it in 100 mL of blotting buffer containing 1 mM EDTA for 10 min (see Note 19).
2. Soak the gel in 100 mL of blotting buffer without EDTA for 10 min.
3. Prepare the poly(vinylidene difluoride) (PVDF) membrane (e.g., Fluorotrans W, Nippon Pall, Tokyo, Japan) by cutting it to the same size as the gel and soak it for 30 s in 100% methanol. The membrane is then incubated for 15 min in the blotting buffer.
4. Prepare four pieces of 3MM paper (e.g., Whatman, Maidstone, UK) by cutting them to the same size as the gel.
5. To form a “blotting sandwich” on the electroblotting screen attached to the electroblotting equipment, assemble the gel, PVDF membrane, and 3MM paper as follows. Soak the blotting sponge attached to the electroblotting equipment in the blotting buffer and place it on the electroblotting screen. Then soak two pieces of 3MM paper in the blotting buffer and place them on the sponge, followed sequentially by the gel and PVDF membrane; avoid incorporating air between the various layers. Then place two more sheets of 3MM paper and one sponge soaked in the blotting buffer on the membrane and close the electroblotting screen. Insert the electroblotting screen in the chamber unit of the electroblotting equipment and fill up with the blotting buffer (see Note 20).
6. Subject the gel to constant-voltage conditions (3.5 V/cm) for 16 h (overnight).
7. After blotting, perform the immunoblotting analysis.

4. Typical/ Anticipated Results

4.1. Protein Kinase Profiling Toward Tau by Using Mn²⁺-Phos- tag SDS-PAGE

Through profiling of kinase activity by using Mn²⁺-Phos-tag SDS-PAGE, we characterized six kinds of Ser/Thr kinases that phosphorylated recombinant human Tau protein and might, therefore, be involved in Alzheimer's disease. The products of the individual kinase reactions involving GSK-3 β , cdk5/p35, PKA, MAPK, CKII, and CaMKII were analyzed by normal SDS-PAGE and by

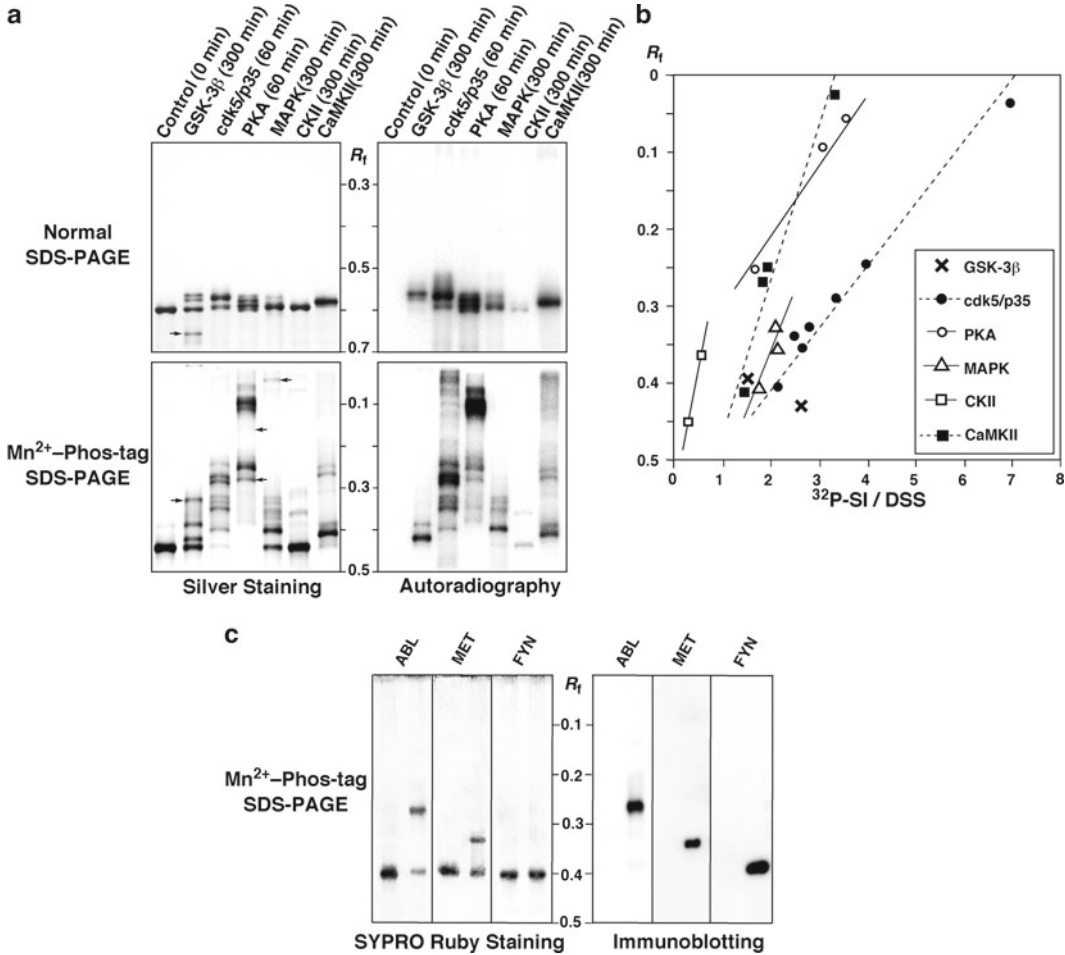


Fig. 2. In vitro Ser/Thr kinase activity profiles in the phosphorylation of Tau protein. **(a)** Normal SDS-PAGE [7.5% (w/v) polyacrylamide] and Mn²⁺-Phos-tag SDS-PAGE [80 μ M polyacrylamide-bound Mn²⁺-Phos-tag and 7.5% (w/v) polyacrylamide] for six kinds of Ser/Thr kinase products of the Tau protein, followed by silver gel staining (*left*) or autoradiography (*right*). **(b)** Plots of the phosphate-incorporation ratios (ratios of the ³²P signal intensity to the density of silver staining for each electrophoresis band in Mn²⁺-Phos-tag SDS-PAGE; ³²P-SI/DSS values) against the R_f values. [Reproduced, with permission, from (22), © 2007, The American Society for Biochemistry and Molecular Biology, Inc.] **(c)** Mn²⁺-Phos-tag SDS-PAGE [80 μ M polyacrylamide-bound Mn²⁺-Phos-tag and 7.5% (w/v) polyacrylamide] for three kinds of Tyr kinase products of the Tau protein, followed by SYPRO Ruby gel staining (*left*) or immunoblotting with an anti-phosphotyrosine antibody (*right*, horseradish peroxidase-conjugated clone PY20, GE Healthcare Biosciences). In each kinase analysis, nonphosphorylated Tau (*left* lane) and the reaction product (*right* lane) were applied. [Reproduced, with permission, from (27) © (2010), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim].

Mn²⁺-Phos-tag SDS-PAGE followed, in both cases, by silver gel staining and autoradiography (Fig. 2a); nonphosphorylated Tau was applied to the leftmost lane in each case as a control. In the normal SDS-PAGE, nonphosphorylated and phosphorylated Tau were observed as migration bands at an R_f value of ~ 0.6 . The R_f value 1.0 is defined as the position of the BPB dye (the bottom of the separating gel). Some bands that were slightly up-shifted

through phosphorylation by these kinases were detected. The faster-migrating band seen in the product lane for GSK-3 β (indicated by an arrow) was assigned to GSK-3 β itself. The corresponding autoradiogram image showed that all the kinase reactions progressed successfully. Although no up-shifted band of Tau from the reaction of CKII was observed on the normal SDS-PAGE gel, the occurrence of phosphorylation was confirmed by means of autoradiography. In contrast to the normal SDS-PAGE, a number of characteristic slower-migrating bands were observed on the Mn²⁺-Phos-tag SDS-PAGE gel. Some faint bands (indicated by arrows) assigned to the commercially available kinases GSK-3 β (R_f =0.32 in the product lane for GSK-3 β), PKA (R_f =0.15 and 0.28 in the product lane for PKA), and MAPK (R_f <0.1 in the product lane for MAPK) were observed. Migration of the non-phosphorylated Tau protein and GSK-3 β was slower than the case in normal SDS-PAGE, possibly because of an electrostatic interaction between cationic Mn²⁺-Phos-tag and anionic SDS-bound proteins. The corresponding autoradiogram image showed that radioactive ³²P was incorporated into the up-shifted proteins. The ³²P signal intensities were different from those for the silver-stained image. These results show that the multiple bands produced by each kinase reaction correspond to Tau proteins phosphorylated in a kinase-specific manner. To determine the relationship between the stoichiometry of phosphate incorporation and the change in mobility (R_f value), the ratios of the ³²P signal intensities to the density of silver staining (³²P-SI/DSS values) of each electrophoresis band of the Mn²⁺-Phos-tag SDS-PAGE gel shown in Fig. 2a were evaluated by densitometry. The ³²P-SI/DSS value is an index of the number of phosphate groups in one molecule of Tau. Plots of the values of ³²P-SI/DSS against the R_f values are shown in Fig. 2b. In each kinase reaction, except for that of GSK-3 β , the R_f value decreased although there was an increase in the ³²P-SI/DSS value. The reverse relationships between the ³²P-SI/DSS values and the R_f values were markedly different for these kinase reactions. These results suggest that the degree of change in mobility of a phosphoprotein might not be related exclusively to the stoichiometry of phosphate incorporation, but that other factors, such as kinase-specific phosphorylation sites, may be involved.

To show that the degree of migration of phosphorylated Tau in Mn²⁺-Phos-tag SDS-PAGE depends on the particular kinase-specific phosphorylation sites, we characterized three distinct monophosphorylated forms of Tau by using Mn²⁺-Phos-tag SDS-PAGE. The three monophosphorylated forms of Tau were specifically phosphorylated in vitro at the Tyr-394, Tyr-197, or Tyr-18 residues by ABL, MET, and FYN, respectively (Fig. 2c). Each monophosphorylated form of Tau was detected as three distinct migration bands. This shows that Mn²⁺-Phos-tag SDS-PAGE is capable of separating substrate proteins that are phosphorylated

at kinase-specific phosphorylation sites. Although no up-shifted band of Tau in the FYN reaction was observed, the phosphorylation of tyrosine was confirmed by immunoblotting analysis using an anti-phosphotyrosine antibody. This demonstrates a limitation of Mn^{2+} -Phos-tag SDS-PAGE in the analysis of protein phosphorylation under the experimental conditions.

4.2. Zn^{2+} -Phos-tag SDS-PAGE for Advanced Protein Kinase Profiling

Each of the three forms of Tau monophosphorylated at the Tyr-394, Tyr-197, or Tyr-18 residue, respectively, was visualized as three distinct migration bands on the Mn^{2+} -Phos-tag SDS-PAGE gel, but no shift was detected for the Tau protein phosphorylated at the Tyr-18 residue by FYN, which is a member of the Src family of kinases. We therefore examined the mobilities of the three monophosphorylated Tau proteins by using the Zn^{2+} -Phos-tag SDS-PAGE method. Figure 3a shows a typical result obtained by using Zn^{2+} -Phos-tag SDS-PAGE, followed, in each case, by staining of the gel with SYPRO Ruby and immunoblotting with an anti-phosphotyrosine antibody. The Zn^{2+} -Phos-tag SDS-PAGE method followed by gel staining permitted the detection of shifts in the mobility of Tyr-phosphorylated Tau in the reactions of all the kinases. Only the up-shifted bands were confirmed by immunoblotting to be Tyr-phosphorylated proteins. The differences in the R_f values of the various monophosphorylated Tau proteins were dependent on the kinase-specific phosphorylation site. In a major improvement, a novel up-shifted band of the Tyr-phosphorylated Tau produced by the reaction of FYN was identified. We also confirmed that the up-shifted Tau bands produced by other Src family kinases (YES, SRC, LCK, and LYNa) all showed identical R_f values to that produced by FYN in Zn^{2+} -Phos-tag SDS-PAGE (Fig. 3b), reflecting an Src family specific phosphorylation at the Tyr-18 residue. Moreover, the utility of Zn^{2+} -Phos-tag SDS-PAGE was demonstrated by visualizing up-shifted bands of Tyr-phosphorylated Tau in reactions of 11 other kinases; these bands could not be detected by using the Mn^{2+} -Phos-tag SDS-PAGE method (Fig. 3b). Improvements in the detection of shifts in the mobility might be due to an increase in affinity for the phosphorylated targets as a result of using the dizinc(II) complex of Phos-tag acrylamide under conditions of neutral pH buffered with Bis-Tris.

4.3. Advanced Resolving Power for the Analysis of Cell Lysates

Finally, we examined the advanced resolving power of Zn^{2+} -Phos-tag SDS-PAGE in the analysis of biologically crude samples. As a typical example, we profiled the hyperphosphorylation status of endogenous β -catenin by using an untreated lysate of human colon adenocarcinoma SW480 cells (Fig. 4). β -Catenin is an 85-kDa protein that binds to the cytoplasmic tail of E-cadherin, a transmembrane adhesion molecule. The binding is regulated by phosphorylation of three critical Tyr residues (Tyr-142, Tyr-489,

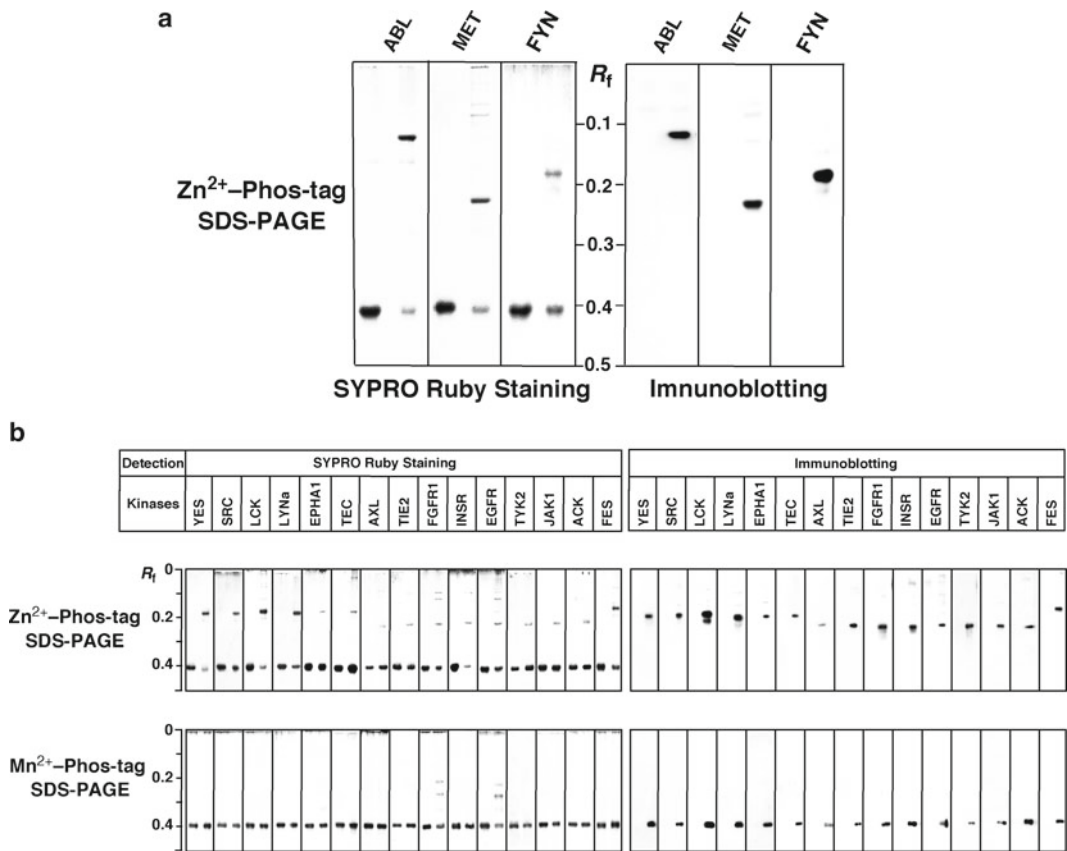


Fig. 3. In vitro Tyr kinase activity profiles in the phosphorylation of Tau protein. (a) Zn²⁺-Phos-tag SDS-PAGE [80 μ M polyacrylamide-bound Zn²⁺-Phos-tag and 7.5% (w/v) polyacrylamide] of three kinds of Tyr kinase products of Tau protein, followed by SYPRO Ruby gel staining (left) or immunoblotting with the anti-phosphotyrosine antibody (right). In each kinase analysis, nonphosphorylated Tau (left lane) and the reaction products (right lane) were applied. (b) Comparison of mobilities of Tau phosphorylated on Tyr moieties by 15 types of Tyr kinase in Zn²⁺-Phos-tag SDS-PAGE and in Mn²⁺-Phos-tag SDS-PAGE. In each kinase analysis, nonphosphorylated Tau (left lane) and the reaction products (right lane) were applied to Phos-tag SDS-PAGE [7.5% (w/v) polyacrylamide containing 80 μ M Phos-tag]. Proteins were stained with SYPRO Ruby or detected by immunoblotting with the anti-phosphotyrosine antibody. [Reproduced, with permission, from (27), © 2010, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim].

and Tyr-654) of β -catenin during developmental and physiological processes (30). β -Catenin is also a key component of the Wnt signaling pathway, which affects cell proliferation and differentiation in many types of cell (31). In Wnt signaling, CKI α phosphorylates β -catenin at the Ser-45 residue. The reaction enhances subsequent phosphorylation by GSK-3 β at the Ser-33, Ser-37, and Tyr-41 residues. β -Catenin is also phosphorylated at the Ser-552 and Ser-675 residues by PKA and AKT, and this phosphorylation induces the transcriptional activity of β -catenin (32, 33). Thus, β -catenin is regulated by sundry protein kinases in vivo, and its various phosphorylation states are closely involved with specific cellular events.

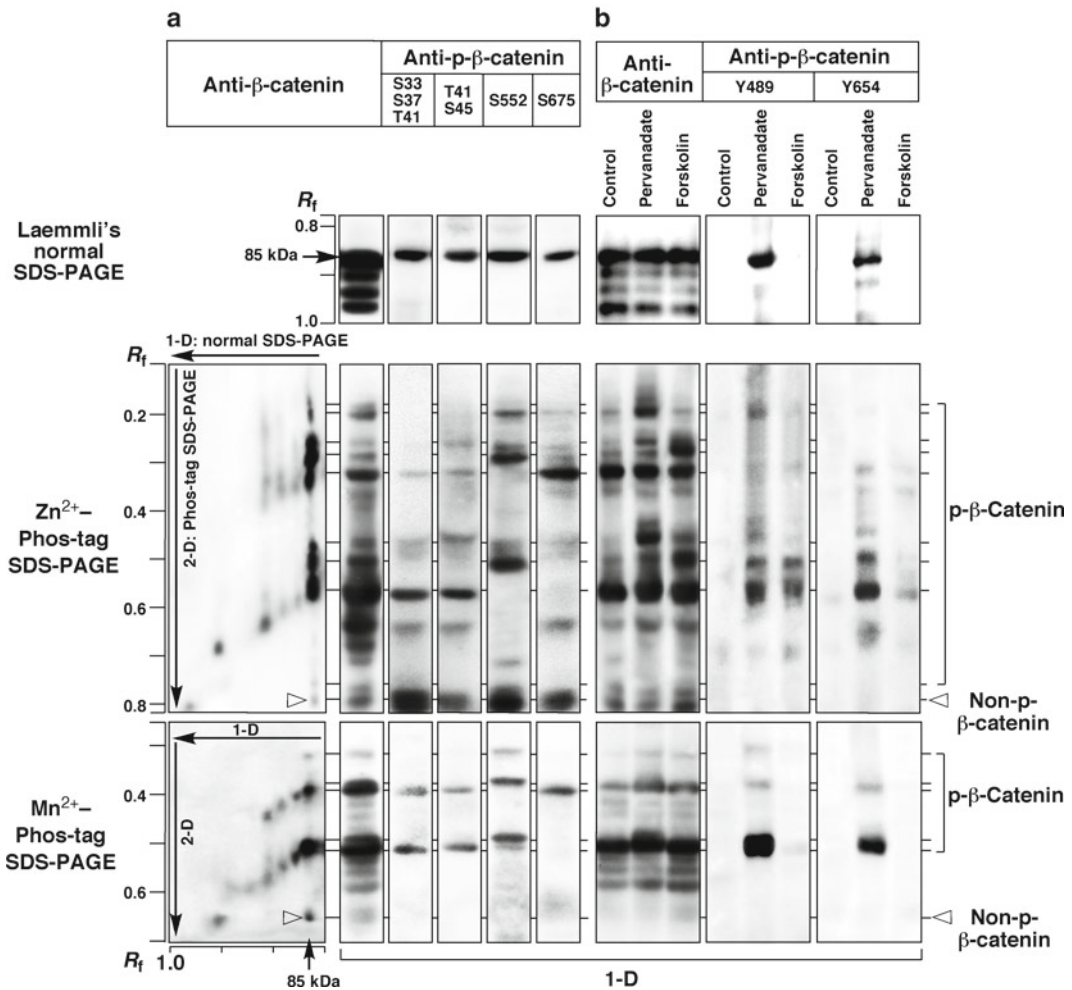


Fig. 4. Profiling of the phosphorylation status of intracellular β -catenin. Laemmli's normal gels using 1DE and 2DE consisted of 5.5% (w/v) and 6.0% (w/v) polyacrylamide, respectively. Each Phos-tag gel consisted of 5.5% (w/v) polyacrylamide containing 25 μ M Phos-tag. β -Catenin was detected by immunoblotting with the antibodies shown above each panel. Anti- β -catenin antibody (against the C-terminal, clone 14) was purchased from BD Bioscience (San Jose, CA, USA). The site-specific antibodies against pS33/S37/T41, pS552, and pS675 were purchased from Cell Signaling Technology (Danvers, MA, USA). The site-specific antibodies against pY489 and pY654 were purchased from ECM Biosciences (Versailles, KY, USA) and Abcam Japan (Tokyo, Japan), respectively. The site-specific antibody against pT41/S45 (clone EP1905Y) was purchased from Millipore. **(a)** The lysate was prepared from cells treated without drug. **(b)** The lysates were prepared from cells treated without drug (control) and with pervanadate (1.0 mM) for 30 min, or with forskolin (10 μ M) for 30 min. The top panels show the results of analyses by Laemmli's normal 1D SDS-PAGE. The center panels show results of analyses by Zn²⁺-Phos-tag SDS-PAGE. The *leftmost panel* shows 2DE coupling of Laemmli's normal SDS-PAGE as the first dimension (1D, *horizontal arrow*) and Zn²⁺-Phos-tag SDS-PAGE as the second dimension (2D, *vertical arrow*). The *bottom panels* show the results of analyses by Mn²⁺-Phos-tag SDS-PAGE. The *leftmost panel* shows 2DE coupling of Laemmli's normal SDS-PAGE (1D, *horizontal arrow*) and Mn²⁺-Phos-tag SDS-PAGE (2D, *vertical arrow*). [Reproduced, with permission, from (27), © 2010, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim].

The 85-kDa β -catenin was detected together with several degraded forms of the protein by immunoblotting of Laemmli's normal gel with anti- β -catenin antibody, and the phosphorylation events at the Ser-33, Ser-37, Thr-41, Ser-45, Ser-552, and Ser-675 residues could be clearly identified by using four kinds of anti-phospho- β -catenin (anti-p- β -catenin) antibodies (Fig. 4a; top panels). Next, we compared the analysis of the same lysate by Zn^{2+} -Phos-tag SDS-PAGE with the corresponding analysis by Mn^{2+} -Phos-tag SDS-PAGE (Fig. 4a; center and bottom panels). To investigate the correlations between the intact and degraded forms, we performed two-dimensional electrophoresis (2DE) with normal SDS-PAGE as the first dimension and Phos-tag SDS-PAGE as the second dimension, followed by immunoblotting with anti- β -catenin antibody (leftmost panels). The 2DE allowed us to distinguish between the intact β -catenin (arrowed at 85 kDa in the first dimension) and degraded forms of the protein (left-side area from the position of 85 kDa). On the 2D Zn^{2+} -Phos-tag SDS-PAGE gel, ten up-shifted bands were identified in the R_f range 0.2–0.8 (indicated by cross lines at the corresponding R_f values). In contrast, only six bands were identified on the Mn^{2+} -Phos-tag SDS-PAGE gel. All the spots identified on the 2D gel correlated with one-dimensional (1D) Phos-tag SDS-PAGE banding images obtained by using site-specific p- β -catenin antibodies (shown in the five right-hand panels).

Furthermore, we demonstrated stimulus-specific phosphorylation profiling of β -catenin by using 1D Zn^{2+} -Phos-tag SDS-PAGE and SW480 lysates after treatment with a tyrosine phosphatase inhibitor (pervanadate) and with an adenylyl cyclase activator (forskolin) as a PKA activator (Fig. 4b). Zn^{2+} -Phos-tag SDS-PAGE followed by immunoblotting with the anti- β -catenin antibody showed characteristic migration patterns of β -catenin produced by individual stimulation. For the pervanadate-treated sample, the signal intensities of the bands at R_f values of 0.20, 0.25, and 0.45 increased, and a novel up-shifted band appeared at an R_f value of 0.43. For the forskolin-treated sample, the signal intensities of the bands at R_f values of 0.25, 0.28, 0.45, and 0.50 increased. These bands had identical R_f values to those detected by anti-p- β -catenin antibodies against the phosphorylated Ser-552 or Ser-675 residue (see Fig. 4a). On the other hand, there was almost no difference in the migration patterns of β -catenin between the lysates analyzed by Laemmli's normal SDS-PAGE and those analyzed by Mn^{2+} -Phos-tag SDS-PAGE. In immunoblotting of the Zn^{2+} -Phos-tag SDS-PAGE gel with p- β -catenin antibodies against the phosphorylated Tyr-489 and Tyr-654 residues, prominent signals of Tyr-phosphorylated β -catenin were detected on the multiple up-shifted bands for the pervanadate-treated sample. Thus, Zn^{2+} -Phos-tag SDS-PAGE permits determination of drug-specific phosphorylation events of β -catenin.

Advanced separation by using Zn^{2+} -Phos-tag SDS-PAGE should permit greater coverage for a larger number of phosphoproteins and should increase the sensitivity of the detection of hierarchical protein phosphorylation and dephosphorylation. This method can therefore assist in mapping low-abundance phosphorylation events, and it should be a useful tool for the profiling of complicated protein kinase-phosphatase networks. Furthermore, it has been demonstrated that Zn^{2+} -Phos-tag SDS-PAGE gels cast in the neutral buffer are stable during long-term storage (at least 3 months) (27). The storage of the gel does not require any special knowhow or equipment. The gel, together with the casting glass plates, is merely wrapped in a Saran wrap to protect it from drying and then stored at room temperature under normal laboratory illumination until required. Therefore, the Zn^{2+} -Phos-tag SDS-PAGE method offers a better shelf life, which makes it particularly appealing for laboratory practice. We can thus present a simple, convenient, and more reliable “in-house” gel system for phosphate-affinity SDS-PAGE.

5. Notes

1. All reagents and solvents used are purchased at the highest commercial quality available and used without further purification. All aqueous solutions are prepared by using deionized and distilled water.
2. 2-Mercaptoethanol is toxic by inhalation, ingestion, and skin contact. When handling this chemical, work in a chemical fume hood, wear gloves and a mask, and use a pipetting aid.
3. The oily product, acrylamide-pendant Phos-tag ligand (10 mg), is placed in a plastic tube and completely dissolved in methanol (0.10 mL). The solution is diluted with distilled water (3.2 mL) by pipetting. Methanol is an inhalation toxin that causes depression of the central nervous system; when handling this chemical, work in a chemical fume hood, wear gloves, and use a pipetting aid.
4. Do not use any other salts such as $\text{Mn}(\text{NO}_3)_2$ or $\text{Mn}(\text{OCOCH}_3)_2$.
5. Because acrylamide monomer is a neurotoxin and a suspected human carcinogen and teratogen, exposure to this substance should be carefully avoided. When weighing powdered acrylamide, work in a chemical fume hood, wear gloves, eye protection, and a mask. Acrylamide is unstable and can polymerize violently on heating to its melting point (84.5°C). It is incompatible with acids, bases, oxidizing agents, reducing agents, iron and its salts, copper, aluminum, brass, and free-radical initiators.

6. TEMED is stored in a desiccator at room temperature. Buy small bottles as its quality may degrade, and gels will take longer to polymerize after opening of the container.
7. Do not adjust the pH with acid or base.
8. Zinc(II) chloride solution (10 mM ZnCl_2 in distilled water) is a suitable substitute.
9. This buffer is used for the separating and stacking gels.
10. Do not adjust the pH with acid or base.
11. Sulfite ion (SO_3^{2-}) is a reducing reagent that diminishes O_2 levels in the electrophoresis running buffer solution and inhibits the oxidation of reduced proteins in the gel. Buffer solution in which sodium bisulfite has been dissolved should be promptly used for electrophoresis. Do not store buffer solution containing sodium bisulfite.
12. Do not adjust the pH with acid or base.
13. Although the semi-dry method is generally the most efficient method for protein blotting in terms of time and consumption of buffer reagents, it is not suitable for electroblotting from the Phos-tag SDS-PAGE gel. The transfer efficiency of proteins is lower in the semi-dry method than that in the wet-tank method.
14. Except for the buffer system, the procedure for Mn^{2+} -Phos-tag SDS-PAGE is almost identical. For details of the procedure see (26). Various contaminants (e.g., EDTA, inorganic salts, or surfactants) in the sample protein solutions often cause disruption (waving and/or tailing) of the electrophoresis bands in Phos-tag SDS-PAGE. To minimize this disruption, it is recommended that the sample is desalted before loading. For example, dialysis filtration can be used to decrease the amounts of the small-molecule substances in the sample. Furthermore, to avoid distortion of bands, do not apply commercially available pre-stained molecular-weight protein markers to Phos-tag SDS-PAGE.
15. Because the optimal percentage of polyacrylamide depends on the molecular weight of the target protein, an appropriate value should be determined for each target.
16. In Phos-tag SDS-PAGE, the R_f values (the degrees of electrophoresis migration) of both phosphorylated and nonphosphorylated proteins are generally smaller than those in normal SDS-PAGE. The optimal concentration of Zn^{2+} -Phos-tag (e.g., 5–100 μM) to achieve sufficient separation between the phosphorylated and nonphosphorylated proteins should be determined for each target. It is recommended that tests would be conducted using low concentrations of 5–25 μM Zn^{2+} -Phos-tag for complex samples, such as cell lysates, that contain various phosphorylated and nonphosphorylated proteins.

17. Take care not to produce bubbles at the bottom surface of the gel set. When bubbles are observed, they should be carefully and completely removed.
18. The same sample-loading buffer is used for both the Mn^{2+} -Phos-tag SDS-PAGE and Zn^{2+} -Phos-tag SDS-PAGE methods.
19. Zn^{2+} -Phos-tag in the gel causes inefficient electroblotting. This can be ameliorated by treatment with EDTA to chelate the zinc ions.
20. The use of wet-tank equipment is strongly recommended for optimal efficiency of protein transfer from the Zn^{2+} -Phos-tag SDS-PAGE gel. The efficiency of transfer from the gel is much higher in the wet-tank method than in the semi-dry method.

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References

1. Hunter T (2000) Signaling: 2000 and beyond. *Cell* 100:113–127
2. Johnson SA, Hunter T (2005) Kinomics: methods for deciphering the kinome. *Nat Methods* 2:17–25
3. Cohen P (2002) Protein kinase: the major drug targets of the twenty-first century? *Nat Rev Drug Discov* 1:309–315
4. Lee VMY, Goedert M, Trojanowski JQ (2001) Neurodegenerative tauopathies. *Annu Rev Neurosci* 24:1121–1159
5. Oda Y, Nagasu T, Chait BT (2001) Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat Biotechnol* 19:379–382
6. Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* 20:301–305
7. Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jørgensen TJD (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics* 4:873–886
8. Kokubu M, Ishihama Y, Sato T, Nagasu T, Oda Y (2005) Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Anal Chem* 77:5144–5154
9. Kweon HK, Håkansson K (2006) Selective zirconium dioxide-based enrichment of

- phosphorylated peptides for mass spectrometric analysis. *Anal Chem* 78:1743–1749
10. Sugiyama N, Masuda T, Shinoda K, Nakamura A, Tomita M, Ishihama Y (2007) Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. *Mol Cell Proteomics* 6:1103–1109
 11. Kinoshita E, Takahashi M, Takeda H, Shiro M, Koike T (2004) Recognition of phosphate monoester dianion by an alkoxide-bridged dinuclear zinc(II) complex. *Dalton Trans*: 1189–1193
 12. Takeda H, Kawasaki A, Takahashi M, Yamada A, Koike T (2003) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of phosphorylated compounds using a novel phosphate capture molecule. *Rapid Commun Mass Spectrom* 17:2075–2081
 13. Prudent M, Rossier JS, Lion N, Girault HH (2008) Microfabricated dual sprayer for on-line mass tagging of phosphopeptides. *Anal Chem* 80:2531–2538
 14. Kinoshita E, Yamada A, Takeda H, Kinoshita-Kikuta E, Koike T (2005) Novel immobilized zinc(II) affinity chromatography for phosphopeptides and phosphorylated proteins. *J Sep Sci* 28:155–162
 15. Kinoshita-Kikuta E, Kinoshita E, Yamada A, Endo M, Koike T (2006) Enrichment of phosphorylated proteins from cell lysate using a novel phosphate-affinity chromatography at physiological pH. *Proteomics* 6:5088–5095
 16. Kinoshita-Kikuta E, Kinoshita E, Koike T (2009) Phos-tag beads as an immunoblotting enhancer for selective detection of phosphoproteins in cell lysates. *Anal Biochem* 389:83–85
 17. Kinoshita-Kikuta E, Yamada A, Inoue C, Kinoshita E, Koike T (2011) A novel phosphate-affinity bead with immobilized Phos-tag for separation and enrichment of phosphopeptides and phosphoproteins. *J Integr OMICS* 1:157–169
 18. Inamori K, Kyo M, Nishiya Y, Inoue Y, Sonoda T, Kinoshita E, Koike T, Katayama Y (2005) Detection and quantification of on-chip phosphorylated peptides by surface plasmon resonance imaging techniques using a phosphate capture molecule. *Anal Chem* 77:3979–3985
 19. Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics* 5:749–757
 20. Takiyama K, Kinoshita E, Kinoshita-Kikuta E, Fujioka Y, Kubo Y, Koike T (2009) A Phos-tag-based fluorescence resonance energy transfer system for the analysis of the dephosphorylation of phosphopeptides. *Anal Biochem* 388:235–241
 21. Somura M, Takiyama K, Kinoshita-Kikuta E, Kinoshita E, Koike T (2011) A Phos-tag-based fluorescence resonance energy transfer system for the analysis of the kinase reaction of a substrate peptide. *Anal Methods* 3:1303–1309
 22. Kinoshita-Kikuta E, Aoki Y, Kinoshita E, Koike T (2007) Label-free kinase profiling using phosphate affinity polyacrylamide gel electrophoresis. *Mol Cell Proteomics* 6: 356–366
 23. Kinoshita E, Kinoshita-Kikuta E, Matsubara M, Yamada S, Nakamura H, Shiro Y, Aoki Y, Okita K, Koike T (2008) Separation of phosphoprotein isotypes having the same number of phosphate groups using phosphate-affinity SDS-PAGE. *Proteomics* 8:2994–3003
 24. Kinoshita E, Kinoshita-Kikuta E, Matsubara M, Aoki Y, Ohie S, Mouri Y, Koike T (2009) Two-dimensional phosphate-affinity gel electrophoresis for the analysis of phosphoprotein isotypes. *Electrophoresis* 30:550–559
 25. Kinoshita E, Kinoshita-Kikuta E, Ujihara H, Koike T (2009) Mobility shift detection of phosphorylation on large proteins using a Phos-tag SDS-PAGE gel strengthened with agarose. *Proteomics* 9:4098–4101
 26. Kinoshita E, Kinoshita-Kikuta E, Koike T (2009) Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. *Nat Protoc* 4:1513–1521
 27. Kinoshita E, Kinoshita-Kikuta E (2011) Improved Phos-tag SDS-PAGE under neutral pH conditions for advanced protein phosphorylation profiling. *Proteomics* 11:319–323
 28. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
 29. Kinoshita E, Kinoshita-Kikuta E, Koike T (2009) Phosphate-affinity gel electrophoresis using a Phos-tag molecule for phosphoproteome study. *Curr Proteomics* 6:104–121
 30. Lilien J, Balsamo J (2005) The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of β -catenin. *Curr Opin Cell Biol* 17:459–465
 31. Kikuchi A (2003) Tumor formation by genetic mutations in the components of the Wnt signaling pathway. *Cancer Sci* 94:225–229
 32. Taurin S, Sandbo N, Qin Y, Browning D, Dulin NO (2006) Phosphorylation of β -catenin by cyclic AMP-dependent protein kinase. *J Biol Chem* 281:9971–9976
 33. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T, Lu Z (2007) Phosphorylation of β -catenin by AKT promotes β -catenin transcriptional activity. *J Biol Chem* 282:11221–11229

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