Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE

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We provide a standard phosphate-affinity SDS-PAGE (Mn^{2+}–Phos-tag SDS-PAGE) protocol, in which Phos-tag is used to analyze large phosphoproteins with molecular masses of more than 200 kDa. A previous protocol required a long electrophoresis time of 12 h for separation of phosphoisotypes of large proteins (~150 kDa). This protocol, which uses a 3% (wt/vol) polyacrylamide gel strengthened with 0.5% (wt/vol) agarose, permits the separation of protein phosphoisotypes larger than 200 kDa within 2 h. In subsequent immunoblotting, phosphoisotypes of high-molecular-mass proteins, such as mammalian target of rapamycin (289 kDa), ataxia telangiectasia-mutated kinase (350 kDa) and p53-binding protein 1 (213 kDa), can be clearly detected as up-shifted migration bands on the improved Mn^{2+}–Phos-tag SDS-PAGE gel. The procedure from the beginning of gel preparation to the end of electrophoresis requires about 4 h in this protocol.

INTRODUCTION

Reversible phosphorylation is a key signaling mechanism for modulating the functional properties of proteins involved in gene expression, cell adhesion, cell cycle, cell proliferation or cell differentiation[1]. There are more than 500 protein kinase genes within the human genome[2,3], a number that clearly reflects the importance of protein phosphorylation. Abnormal protein phosphorylation is closely involved with many human diseases, including cancer, diabetes mellitus, neurodegeneration and immune/inflammatory disorders[4]. Experimental procedures for the determination of the phosphorylation status of certain proteins are therefore very important in relation to studies on a diverse range of physiological and pathological processes.

Separation of a phosphorylated protein and its nonphosphorylated counterpart by using gel-based electrophoresis can facilitate an understanding of the phosphorylation status. The SDS-PAGE procedure[5], in which proteins are separated by electrophoresis on a polyacrylamide gel, is often used to identify phosphorylated proteins because phosphoproteins show different extents of electrophoretic migration compared with their nonphosphorylated counterparts. Uniform binding of SDS to the phosphoprotein can be disrupted by the presence on the phosphoprotein of a negatively charged phosphate group that can decrease the charge density on the phosphoprotein compared with that on its nonphosphorylated counterpart. If there is a sufficient difference in charge density between the phosphorylated and nonphosphorylated forms, the phosphorylated protein will show a retarded migration and will appear at a position corresponding to a higher molecular weight on the gel compared with its nonphosphorylated counterpart. This observation of a shift in mobility has sometimes been used as an index of protein phosphorylation in certain biological events; however, the shift in mobility on phosphorylation depends on protein-specific structural characteristics, and the number of phosphoproteins that can be analyzed by conventional SDS-PAGE is limited.

There are two principal approaches to the detection of protein phosphorylation by combinations of the conventional SDS-PAGE and other techniques. One approach involves autoradiography studies after electrophoresis, using radioactive compounds of [γ-^{32}P]-labeled ATP and [^{32}P]-labeled orthophosphate. A metabolic radiolabeling protocol has been successfully used in many laboratories to identify phosphoproteins; however, this approach is limited to specimens that are amenable to labeling, and it raises certain problems with regard to safety and waste disposal. The second approach involves immunoblotting with antibodies against phosphorylated amino acids. Unfortunately, the lack of specificity of some antibodies can be a problem in some cases. Anti-phosphotyrosine monoclonal antibodies are widely used because they react selectively with various proteins containing phosphorylated tyrosine residues. In contrast, monoclonal antibodies against phosphoserine or phosphothreonine residues are unpopular because their affinity and specificity are less than optimal. To achieve a precise characterization of signaling events, it is desirable to raise a high-quality antibody against a phosphorylated residue; however, raising this type of antibody is costly, time-consuming and often unsuccessful. A more global quantification of phosphoproteins has been achieved newly using a phospho-specific Pro-Q Diamond gel/blot stain[6,7] (Invitrogen) or a phospho-specific Phos-tag gel/blot fluorostain[8–12] (Perkin-Elmer). In addition, an immobilized metal affinity gel-based electrophoresis has been developed recently as a useful tool for separation of phosphoproteins from the nonphosphorylated counterparts[13,14]. Presumably, it would be a challenge to implement these techniques for detection of phosphoprotein isotypes.

Mn^{2+}–Phos-tag SDS-PAGE for mobility shift detection of phosphoprotein isotypes

We previously found that a dinuclear metal complex (i.e., 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex) acts as a phosphate-binding tag molecule in an aqueous solution[15]. We called this zinc complex 'Phos-tag.' The Phos-tag molecule has a vacancy on its two zinc ions that will accept a phosphomonoester dianion as a bridging ligand. In an aqueous solution, the dinuclear zinc(II) complex strongly binds to the phenyl phosphate dianion ($K_d = 2.5 \times 10^{-8}$ M) at a neutral pH.

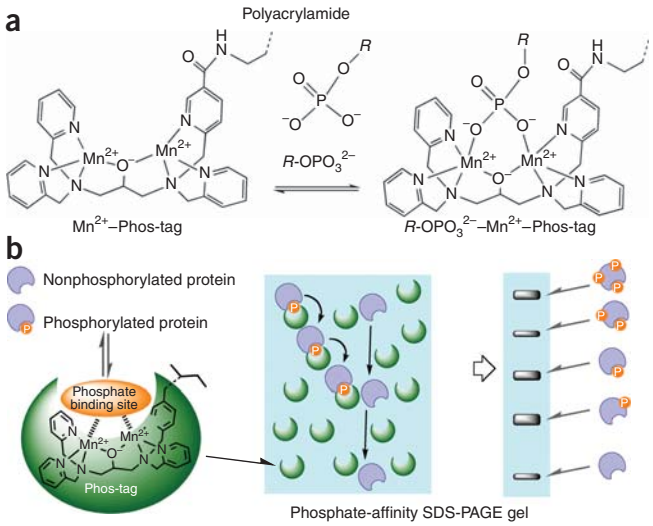

Figure 1 | Phosphate-affinity Mn^{2+}-Phos-tag SDS-PAGE for the mobility-shift detection of phosphoproteins. (**a**) Structure of the polyacrylamide-bound Mn^{2+}–Phos-tag and scheme of the reversible capturing of a phosphomonoester dianion (R-OPO_3^{2-}) by Mn^{2+}–Phos-tag and (**b**) schematic representation for the principle of Mn^{2+}–Phos-tag SDS-PAGE. The polyacrylamide-bound Mn^{2+}–Phos-tag shows preferential trapping of the phosphorylated proteins, which is because of separation of the phosphoproteins from their nonphosphorylated counterparts. In this paper, we focus on the utility of the phosphate-affinity SDS-PAGE for the separation of large phosphoprotein isotypes with molecular masses of more than 200 kDa.

The anion selectivity indexes against SO_4^{2-}, CH_3COO^-, Cl^- and R-OSO_3^- at 25 °C are 5.2×10^3, 1.6×10^4, 8.0×10^5 and $> 2 \times 10^6$, respectively. A manganese(II) homolog of Phos-tag (Mn^{2+}–Phos-tag) can capture a phosphomonoester dianion, such as phosphoserine or phosphotyrosine, at alkaline pH values (~9) (**Fig. 1a**). This finding has led to the development of phosphate-affinity gel electrophoresis for detecting shifts in the mobility of phosphoproteins in comparison with their nonphosphorylated counterparts[16–20]. We used an acrylamide-pendant Mn^{2+}–Phos-tag as a novel additive in a separating gel for normal SDS-PAGE. In a separating gel containing co-polymerized Phos-tag, the degrees of migration of phosphoproteins are less than those of their nonphosphorylated counterparts because the tag molecules trap phosphoproteins reversibly during electrophoresis. On the basis of this principle, we recently established a novel type of gel electrophoresis, Mn^{2+}–Phos-tag SDS-PAGE, for the separation of phosphoproteins from their corresponding nonphosphorylated analogs (**Fig. 1b**). The Mn^{2+}–Phos-tag SDS-PAGE protocol offers the following significant advantages: (i) no radioactive or chemical labels are required for kinase and phosphatase assays; (ii) the time course quantitative ratio of phosphorylated to nonphosphorylated proteins can be determined; (iii) several phosphoprotein isotypes, depending on the phosphorylation status, can be detected as multiple migration bands; (iv) the phosphate-binding specificity is independent of the kind of phosphorylated amino acid; (v) His- and Asp-phosphorylated proteins involved in a two-component signal-transduction system can be detected simultaneously in their phosphotransfer reactions; (vi) separation of phosphoprotein isotypes having the same number of phosphate groups is possible; (vii) a downstream procedure, such as immunoblotting or MS analysis, can be applied; and (viii) the phosphate-affinity procedure is almost identical to the normal. In general, the migration of the nonphosphorylated protein isotype in SDS-PAGE with Mn^{2+}–Phos-tag becomes slower than that in normal SDS-PAGE without Mn^{2+}–Phos-tag, possibly because of an electrostatic interaction between cationic Mn^{2+}–Phos-tag and anionic SDS-bound proteins[16,17].

Previous and currently improved protocols of Mn^{2+}–Phos-tag SDS-PAGE

In an earlier protocol using a general mini-slab PAGE system, the concentrations of Mn^{2+}–Phos-tag were between 20 and 100 μM, and the electrophoresis was carried out at a constant current of 15–35 mA per gel for < 2 h[9,21–33]. We recently found that a lower concentration (5 μM) of Mn^{2+}–Phos-tag with a smaller current of 5 mA per gel for 12 h can dramatically improve the separation of a phosphoprotein having a large molecular mass of 150 kDa from its nonphosphorylated counterpart in a 5% (wt/vol) polyacrylamide slab gel[34,35]. However, even this procedure did not permit the separation of large phosphoproteins with molecular masses of more than 200 kDa. Although a highly porous polyacrylamide gel is generally used for the separation of high-molecular-mass proteins, ~5% (wt/vol) of polyacrylamide is the minimum concentration that permits handling of the gel after electrophoresis. In this protocol introduced, this problem was circumvented by homogeneous addition of 0.5% (wt/vol) agarose to a tender SDS-PAGE gel containing 3% (wt/vol) polyacrylamide and 20 μM Mn^{2+}–Phos-tag[36]. The agarose gels or agarose–polyacrylamide composite gels are usually used for the separation of high-molecular-mass proteins[37–39]. The SeaKem Gold Agarose gel (Lonza, Rockland, ME, USA), especially provided for large DNA separation, has been reported to work best among various types of agarose or polyacryamide gels for detecting giant myofibrillar proteins, such as titin (3,000–4,000 kDa) and nebulin isoforms (600–900 kDa)[38,39]. The improved procedure using a SeaKem Gold Agarose–Polyacrylamide composite gel containing Mn^{2+}–Phos-tag permitted the separation of phosphoprotein isotypes having molecular masses of 200–350 kDa within 2 h. Similarly, a better resolution and/or faster analysis was achievable with the amended procedure for proteins of ~150 to 180 kDa. We show a typical result of the mobility shift detection of the multiple phosphorylation events on epidermal growth factor receptor (~180 kDa) after growth factor-dependent signaling in **Supplementary Figure 1**. This protocol for high-molecular-mass phosphoproteins retains the advantages of the phosphate-affinity SDS-PAGE methodology, as mentioned above.

Applications of the protocol

We herein describe a useful protocol that addresses the observation of differentially phosphorylated forms of high-molecular-mass proteins, such as mammalian target of rapamycin (mTOR, 289 kDa), ataxia telangiectasia-mutated kinase (ATM, 350 kDa) and p53-binding protein 1 (53BP1, 213 kDa), using a strategy of combining polyacrylamide, agarose and the phosphate-binding tag molecule, Phos-tag[36]. This solid protocol of the gel-based electrophoretic separation could also assist in mapping low-abundance phosphorylation events on other large proteins in a cellular signal transduction and should increase the utility for detection of hierarchical phosphorylation and dephosphorylation using the high-quality

site-specific anti-phosphoproteins antibodies in cases where multiple kinase/phosphatase reactions occur. The applications for larger phosphoprotein isotypes on other *in vivo*/*in vitro* kinase/phosphatase systems must give novel information on the relationship between protein phosphorylation and the various biological responses. With regard to the native gels without SDS, we have shown the applications for the analysis of nucleic acids, such as the genotyping of a single-nucleotide polymorphism[40] and the detection of DNA methylation[41], using polyacrylamide slab gels containing the dinuclear zinc(II) complex of Phos-tag. The PAGE using the polyacrylamide-bound Phos-tag enabled the mobility shift detection of the 5′-phosphorylated DNA fragment as a slower migration band.

Limitations of the protocol

The gel including agarose is often melted partially during electroblotting because of the development of heat. The melted gel sticks to the blotting PVDF membrane and is difficult to remove from the membrane. To avoid this problem, we need to use relatively lower constant-voltage conditions (3.5 V cm^{-1}) for 16 h (overnight) using the wet-tank electroblotting procedure. Regarding the detection of protein phosphorylation, the observation of up-shifted phosphoisotypes of a certain protein using this protocol is entirely dependent on immunoreactivity with its antibody. Any phosphorylation that masks an antibody epitope will not be observed by immunodetection. Furthermore, this protocol has limitations, which show no mobility shift for the phosphorylation events of certain proteins under the experimental conditions because the migration degree of the protein phosphoisotypes on the Mn^{2+}–Phos-tag SDS-PAGE gel is because of not only phosphate stoichiometry but also the phosphorylation sites[18,19]. Adversely, this characteristic sometimes permits the separation of phosphoprotein isotypes having the same number of phosphate groups.

MATERIALS

REAGENTS

Deionized and distilled water ▲ CRITICAL All aqueous solutions should be prepared using deionized and distilled water.

- Acrylamide-pendant Phos-tag ligand[16] (order product name; Phos-tag Acrylamide AAL-107, Phos-tag Consortium)
- Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) (Nakalai Tesque, cat. no. 21211-32)
- Acrylamide (Nakalai Tesque, cat. no. 00839-95) ! CAUTION As 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.
- N,N′-Methylenebisacrylamide (Nakalai Tesque, cat. no. 22402-02)
- SeaKem Gold Agarose (Lonza, cat. no. 50152)
- Tris (Nakalai Tesque, cat. no. 35434-05)
- Hydrochloric acid (HCl) (Nakalai Tesque, cat. no. 18321-05) ! CAUTION It is dangerously irritating to the skin, eyes and mucous membranes. When handing this chemical, work in a chemical fume hood, wear gloves, eye protection and a mask.
- Glycine (Nakalai Tesque, cat. no. 17141-95)
- SDS (Nakalai Tesque, cat. no. 08933-05)
- N,N,N′,N′-Tetramethylethylenediamine (TEMED) (Nakalai Tesque, cat. no. 33401-72)
- Ammonium peroxodisulfate (APS) (Nakalai Tesque, cat. no. 02627-34)
- 2-Mercaptoethanol (Nakalai Tesque, cat. no. 21438-82) ! CAUTION It is toxic. When handling this chemical, work in a chemical fume hood, wear gloves, a mask and use a pipetting aid.
- Bromophenol blue (BPB) (Nakalai Tesque, cat. no. 05808-32)
- Glycerol (Nakalai Tesque, cat. no. 17018-25)
- Methanol (Nakalai Tesque, cat. no. 21915-93) ! CAUTION It is an inhalation toxin leading to depression of the central nervous system. When handling this chemical, work in a chemical fume hood, wear gloves and use a pipetting aid.
- Disodium ethylenediaminetetraacetate (EDTA) (Katayama Chemical, Osaka, Japan, cat. no. 09-1420)
- Sodium chloride (NaCl) (Nakalai Tesque, cat. no. 31320-05)
- Polyoxyethylene sorbitan monolaurate (Tween 20) (Nakalai Tesque, cat. no. 28353-85)

EQUIPMENT

- Mini-slab PAGE system (Model AE-6500, Atto, Tokyo, Japan)
- Microwave oven (RE-13-JB6P, Sharp)
- 100-ml flask
- 50-ml centrifuge tube (Sumitomo Bakelite, cat. no. MS-56501)
- Oven (EYELA SLI-220, Tokyo Rikakikai)
- 21G needle
- Filter paper (Advantec, 70-mm circle)
- Power supply (Model AE-8750 Power Station 1000XP, Atto)
- Electroblotting unit (Model NA-1511C, Nippon Eido)
- 3MM paper (Whatman, 40-cm square, cat. no. 3030-909)
- Poly(vinylidene difluoride) (PVDF) membrane (Nippon Pall, FluoroTrans W, cat. no. EH-2222)

REAGENT SETUP

Phos-tag Acrylamide solution 5.0 mM Acrylamide-pendant Phos-tag ligand obtained from the Phos-tag Consortium (order product name; Phos-tag Acrylamide AAL-107) in distilled water. Store at room temperature (< 28 °C) in the dark. ▲ CRITICAL As the Phos-tag ligand is an oily product with a high viscosity, it should be completely dissolved by intensive pipetting. The Phos-tag Acrylamide solution should be used within 3 months of preparation.

Manganese(II) chloride solution 10 mM of $MnCl_2 \cdot 4H_2O$ in distilled water. Store at room temperature. ▲ CRITICAL The aqueous solution of $MnCl_2$ is stable for at least 6 months. Do not use any other salt such as $Mn(NO_3)_2$.

Agarose solution Suspend 1.5% (wt/vol) SeaKem Gold Agarose in 50 ml of distilled water in a 100-ml flask and melt completely in a microwave oven before use. ▲ CRITICAL We have confirmed that some commercially available agaroses with a gel strength of more than 1,000 g cm^{-2} at 1.5% (wt/vol) (e.g., Agarose LO3 TAKARA (> 2,200 g cm^{-2} at 1.5% (wt/vol)) purchased from Takara Bio (cat. no. 5003), Agarose KANTO (900–1,400 g cm^{-2} at 1.5% (wt/vol)) purchased from Kanto Chemical (cat. no. 01097), Agarose KANTO ME (1,400–1,700 g cm^{-2} at 1.5% (wt/vol)) purchased from Kanto Chemical (cat. no. 01088) and Agarose KANTO LE (1,200–1,500 g cm^{-2} at 1.5% (wt/vol)) purchased from Kanto Chemical (cat. no. 01098)) are suitable as substitutes for SeaKem Gold Agarose with a gel strength of > 3,500 g cm^{-2} at 1.5% (wt/vol). However, NuSieve GTG (> 500 g cm^{-2} at 4% (wt/vol)) from Lonza (cat. no. 50081) was not suitable as a substitute when adding it to the SDS-PAGE gel to be a final concentration of 0.5% (wt/vol). NuSieve 3:1 (> 1,400 g cm^{-2} at 4% (wt/vol)) from Lonza (cat. no. 50091) did not have enough strength for subsequent electroblotting handling when adding it to the SDS-PAGE gel to be a final concentration of 0.5% (wt/vol).

30% (wt/vol) acrylamide/bis solution (29:1 ratio of acrylamide to *N,N′*-methylenebisacrylamide) Store at room temperature in the dark and use within 3 months of preparation.

Separating gel buffer 1.5 M Tris-HCl (pH 8.8), 0.4% (wt/vol) SDS. Store at room temperature and use within 3 months of preparation.

Stacking gel buffer 0.5 M Tris-HCl (pH 6.8), 0.4% (wt/vol) SDS. Store at room temperature and use within 3 months of preparation.

APS solution 10% (wt/vol) APS in distilled water. ▲ CRITICAL Prepare just before use.

Electrophoresis running buffer 25 mM Tris, 192 mM glycine, 0.1% (wt/vol) SDS. Store at room temperature and use within 3 months of preparation.

Sample-loading dye solution (3×) 195 mM Tris-HCl (pH 6.8), 3.0% (wt/vol) SDS, 15% (vol/vol) 2-mercaptoethanol, 30% (vol/vol) glycerol and 0.10% (wt/vol) BPB. Store at −20 °C and use within 2 weeks of preparation.

Blotting buffer 25 mM Tris, 192 mM glycine, 10% (vol/vol) methanol. Prepare the buffer before use.

EDTA solution 0.5 M EDTA (pH 8). Store at room temperature and use within 3 months of preparation.

Blotting buffer containing 1 mM EDTA Add 0.2 ml of EDTA solution to 100 ml of blotting buffer. Prepare before use.

TBS-T solution 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.10% (vol/vol) Tween-20. Store at room temperature and use within 3 months of preparation.

EQUIPMENT SETUP

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.

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, except for semi-dry equipment. ▲ CRITICAL Although the semi-dry method is generally the most efficient (time or buffer's reagent consumption) way of protein blotting, it is not suitable for a long-time procedure of electroblotting from the gel containing agarose. In addition, the transfer efficiency of high-molecular-mass proteins is generally lower in the semi-dry method than in the wet-tank method.

PROCEDURE

Gel preparation ● TIMING 1 h

1| Clean the glass plates for casting the gels. It is critical that the glass plates (1-mm thick, 9-cm wide and 9-cm long) should be washed thoroughly clean with a rinsable detergent and rinsed extensively with distilled water before the gels are cast.

2| Prepare separating gel solution with an appropriate percentage of polyacrylamide (**Fig. 2**). As a typical example, a 3% (wt/vol) separating gel solution (~7 ml) containing 20 μM polyacrylamide-bound Mn^{2+}–Phos-tag and 0.5% (wt/vol) agarose is prepared by mixing 0.70 ml of 30% (wt/vol) acrylamide/bis solution, 1.75 ml of separating gel buffer, 28 μl of Phos-tag Acrylamide solution, 28 μl of manganese(II) chloride solution (2 equivalents on Phos-tag), 7 μl of TEMED and 2.05 ml of distilled water in a 50-ml centrifuge tube.

▲ CRITICAL STEP The optimal percentage (wt/vol) of polyacrylamide (e.g., 2.7–3.5%) depends on the molecular weight of the target protein. Refer to **Figure 2**, which shows a typical example of the relationship between the degree of migration (R_f value) and molecular weight of several proteins (200–350 kDa) in 2.7–3.5% (wt/vol) polyacrylamide slab gels without Mn^{2+}–Phos-tag. In Mn^{2+}–Phos-tag SDS-PAGE, the R_f values of both phosphorylated and nonphosphorylated proteins are generally smaller than those in normal SDS-PAGE. The optimal concentration of Mn^{2+}–Phos-tag (e.g., 20–100 μM) to achieve sufficient separation between the phosphorylated and nonphosphorylated proteins should be determined.

3| Prepare the 1.5% (wt/vol) agarose solution. Suspend SeaKem Gold Agarose (0.75 g) in 50 ml of distilled water in a 100-ml flask and then melt the agarose solution completely by using a microwave oven.

▲ CRITICAL STEP To avoid changes in agarose solution concentrations caused by boiling and subsequent evaporative loss of distilled water, the lost volume should be supplied in the agarose solution after complete melting.

4| Add 2.33 ml of the hot agarose solution (> 90 °C) to the separating gel solution and mix gently.

? TROUBLESHOOTING

5| Add 100 μl of APS solution and mix carefully without producing bubbles.

6| Transfer the warm separating gel solution (at least more than gelling temperature of the agarose) to the gap between the glass plates and allow the acrylamide to polymerize for about 20 min. Do not overlay the separating gel solution with any liquid, because an air-protecting liquid cools the top surface of the separating gel solution and makes the top edge of the separating gel ragged.

■ PAUSE POINT After Step 6, the polymerized separating gel will be stable at room temperature within 16 h (overnight). To avoid drying out the gel, it is necessary to introduce a layer of distilled water on top of the polymerized separating gel.

▲ CRITICAL STEP The distilled water should be added to prevent drying out the gel only after the gel has fully cooled/polymerized.

? TROUBLESHOOTING

7| There is an agarose layer (2–3 mm), which contains non-polymerized acrylamide, on the top of the separating gel. Pour 100 μl of distilled water on the separating gel and scrape off the agarose layer by using a 21-G needle. Wipe the top surface of the separating gel with filter paper to remove scraps of agarose and the residual distilled water.

▲ CRITICAL STEP It is important to remove the agarose layer to allow the stacking gel to adhere to the separating gel.

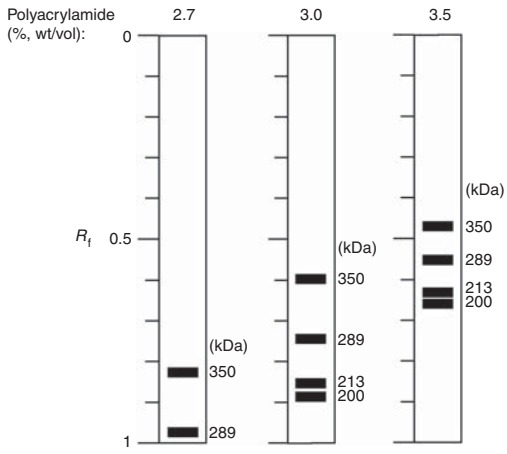

Figure 2 | Schematic representation of the relationship between the degree of migration (R_f value) and molecular weight for several proteins (200–350 kDa) in 2.7–3.5% (wt/vol) polyacrylamide slab gels. The degree of migration of 200-kDa protein was determined by means of gel staining of the myosin heavy chain. The degrees of migration of 350-, 289- and 213-kDa proteins were determined by western blotting analyses of HeLa cell lysates using the antibodies against ATM, mTOR and 53BP1, respectively (see ANTICIPATED RESULTS). The R_f value of 1.0 is defined as the position of the BPB dye.

8| Prepare the stacking gel solution. In a similar manner to the preparation of the separating gel solution, prepare a 3% (wt/vol) stacking gel solution containing 0.5% (wt/vol) agarose by mixing 0.20 ml of 30% (wt/vol) acrylamide/bis solution, 0.50 ml of stacking gel buffer, 2 µl of TEMED and 0.58 ml of distilled water in a 50-ml centrifuge tube.

9| Melt the agarose solution completely using a microwave oven. Remelt the agarose solution prepared in Step 3, which has gelled in the 100-ml flask because of cooling.

10| Add 0.67 ml of the hot agarose solution (>90 °C) to the stacking gel solution to give the required final concentration and mix gently.

11| Add 50 µl of APS solution, mix gently without forming bubbles and then pour the warm stacking gel solution (the temperature should be at least that of the gelling temperature of the agarose) onto the separation gel.

12| Insert a sample-well comb and allow the gel to polymerize for about 20 min.

13| Carefully remove the comb from the stacking gel and assemble the gel plate and electrophoresis apparatus.
? TROUBLESHOOTING

Electrophoresis ● TIMING 2.5–3 h

14| Fill the electrode chambers with the electrophoresis running buffer. Take care not to allow bubbles to form at the bottom surface of the gel set. If bubbles are observed, they should be removed completely.

15| Apply the protein samples mixed with the sample-loading dye solution into the wells.

16| Attach the leads to the power supply. Run the gels under constant-current conditions (15 mA per gel) at room temperature until the BPB dye reaches the bottom of the separating gel (about 2 h).

Electroblotting ● TIMING 17 h

17| 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.
▲ CRITICAL STEP Mn^{2+}–Phos-tag in the gel causes inefficient electroblotting. This can be ameliorated by treatment with EDTA to chelate the manganese ions.

18| Soak the gel in 100 ml of blotting buffer without EDTA for 10 min.

19| Prepare PVDF membrane by cutting it to the size of the gel and soak it for 30 s in 100% methanol. Later wash the membrane for 1 min with distilled water and incubate it for 15 min in the blotting buffer.

20| Prepare four pieces of 3MM paper by cutting them to the size of the gel.

21| Assemble gel, PVDF membrane and 3MM paper to a 'blotting sandwich' on electroblotting screen attached to the electroblotting equipment as follows: soak blotting sponge attached to the electroblotting equipment in the blotting buffer and place on the electroblotting screen. Then soak two pieces of 3MM paper in the blotting buffer and place on the sponge, followed by piling up the gel and PVDF membrane. Avoid the incorporation of air between the different layers. Then place two more 3MM papers 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.
▲ CRITICAL STEP The use of wet-tank equipment is strongly recommended for optimal efficiency of protein transfer from the Mn^{2+}–Phos-tag SDS-PAGE gel. The efficiency of high-molecular-mass proteins from the gel is much higher in the wet-tank method than in the semi-dry method.

22| Transfer the gel under constant-voltage conditions (3.5 V cm^{-1}) for 16 h (overnight).

23| After blotting, soak the PVDF membrane in TBS-T solution and then carry out the immunoblotting analysis.
? TROUBLESHOOTING

● TIMING
Steps 1–13, Gel preparation: 1 h
Steps 14–16, Electrophoresis: 2.5–3 h
Steps 17–23, Electroblotting: 17 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
4	The separating gel solution hardens before it can be poured into the gel-casting system	Agarose gelling	After the agarose is melted in the microwave oven, the solution should be mixed and poured into the casting system as quickly as possible. If necessary, preheat the casting system and plastic pipette tips in an oven at 40–45 °C
6	The top edge of the separation gel becomes ragged	Foamy separating-gel solution	The separating gel solution should be mixed carefully, avoiding the formation of bubbles
		Pouring an air-protecting liquid on top of the separating gel solution before polymerization occurs	Do not add any liquid until the separating gel has polymerized
13	When the clip and spacer are removed, the polymerized gel shrinks or slips down between the glass plates	Polyacrylamide gel is not sufficiently strengthened by agarose	Make sure that the final agarose concentration recommended is 0.5% (wt/vol)
23	The partial gel sticks to the PVDF membrane and hardly separates from the membrane	Agarose–polyacrylamide composite gel is partially melted during electroblotting because of the generation of heat	Do not set the voltage conditions to higher than 3.5 V cm^{-1}. Even if an electroblotting unit with a cooling apparatus is used, the higher voltage conditions using the cooling function for this step are not recommended

ANTICIPATED RESULTS

Separation of the phosphoisotypes of mTOR

A highly conserved Ser/Thr protein kinase, mTOR, controls cell growth and metabolism in response to nutrients and growth factors[42–45]. When sufficient nutrients are available, mTOR is phosphorylated at the Ser-2448 residue by the PI3 kinase/Akt signaling pathway and then is autophosphorylated at the Ser-2481 residue[46,47]. As mTOR is a large protein with a molecular mass of more than 200 kDa, it was impossible to detect shifts in the mobility on its phosphorylation events by the previous protocol for the phosphate-affinity Mn^{2+}–Phos-tag SDS-PAGE.

For demonstration of mobility shifting of phosphorylated isotypes of mTOR from the nonphosphorylated counterpart using this improved protocol, a set of cellular lysates was prepared from HeLa cells (10^7 cells) as follows. After incubation in a fetal bovine serum (FBS)-free medium for 16 h, the cells were treated with 0 (control) or 10% (vol/vol) FBS for 30 min. Each of the induced and control cultures was washed twice with a TBS solution consisting of 10 mM Tris-HCl (pH 7.5) and 100 mM NaCl and lyzed in 0.5 ml of a sample-loading dye solution (1×) containing 1.0% (wt/vol) SDS and 5% (wt/vol) 2-mercaptoethanol.

We first carried out normal SDS-PAGE analysis using a 3% (wt/vol) polyacrylamide gel strengthened with 0.5% (wt/vol) agarose and the set of lysates (20 μg proteins per lane) (**Fig. 3a**, no Mn^{2+}–Phos-tag). Successive immunoblotting with the anti-mTOR antibody showed that mTOR appeared as a single migration band at an R_f value of 0.74. The same samples were also analyzed separately using the anti-phospho-mTOR (anti-p-mTOR) antibody against the phosphorylated Ser-2448 residue (pS2448) and anti-p-mTOR antibody against the phosphorylated Ser-2481 residue (pS2481). An increase in the phosphorylation level on the FBS stimulation was detected by both immunoblotting analyses with the anti-p-mTOR antibodies.

Next, we subjected the same samples to Mn^{2+}–Phos-tag SDS-PAGE using a 3% (wt/vol) polyacrylamide gel containing 0.5% (wt/vol) agarose and 20 μM polyacrylamide-bound Mn^{2+}–Phos-tag (**Fig. 3a**, Mn^{2+}–Phos-tag). By subsequent immunoblotting with the anti-mTOR antibody, additional multiple up-shifted bands were observed for the FBS-treated sample. Immunoblotting with the anti-p-mTOR antibodies showed that only up-shifted bands were phosphorylated isotypes of mTOR. Furthermore, by treatments with wortmannin and rapamycin, which are well known to inhibit the phosphorylation of mTOR, shifts in the mobility upon addition of FBS were suppressed dose dependently (**Fig. 3b**). We showed the detection of shifts in the mobility of the

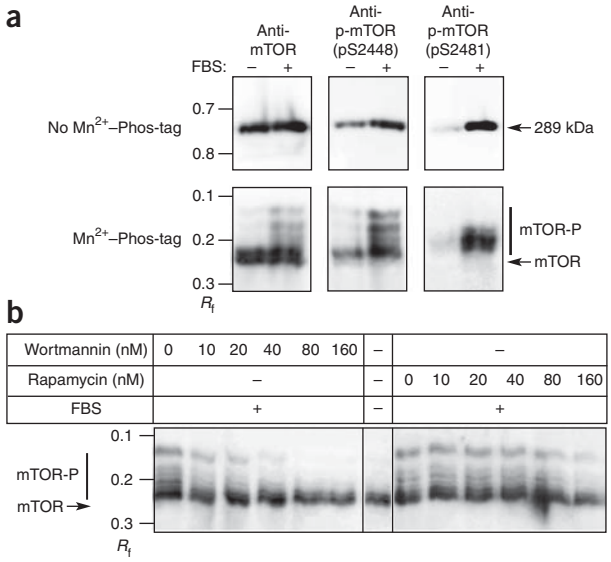

Figure 3 | Separation and detection of the phosphoisotypes of mTOR. Phosphorylation of mTOR in HeLa cells treated with FBS (**a**) and the phosphorylation inhibition assays with wortmanin and rapamycin (**b**). The HeLa cells (10^7 cells) (supplied from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer at Tohoku University, Japan) were incubated in an FBS-free Dulbecco's modified Eagle medium (DMEM) for 16 h and then treated with 0% (−) or 10% (vol/vol) FBS (+) for 30 min. DMEM and FBS were obtained from Invitrogen (Carlsbad, CA, USA). For the mTOR phosphorylation inhibition assays with wortmaninn and rapamycin, cells were pre-incubated with inhibitors for 10 min and then treated with 10% (vol/vol) FBS for 30 min without washing out the inhibitors. (**a**) The lysates were subjected to SDS-PAGE on 3% (wt/vol) polyacrylamide gel strengthened with 0.5% agarose containing 0 μM (no Mn^{2+}–Phos-tag) or 20 μM Mn^{2+}–Phos-tag, followed by immunoblotting with the anti-mTOR antibody (rabbit monoclonal, clone 7C10), anti-p-mTOR antibody against pS2448 and anti-p-mTOR antibody against pS2481. Figures are reproduced from Kinoshita *et al.*[36] with permission of the publisher, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. In the inhibition assays, the lysates were subjected to SDS-PAGE on 3% (wt/vol) polyacrylamide gel strengthened with 0.5% agarose containing 20 μM Mn^{2+}–Phos-tag, followed by immunoblotting with the same anti-mTOR antibody described above (**b**). These primary antibodies and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The PVDF membrane was blocked by using 1% (wt/vol) bovine serum albumin (BSA, Nakalai Tesque, Kyoto, Japan). The detection of the electrophoresis migration band was performed using the ECL Advance Western blotting detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and an LAS 3000 image analyzer (Fujifilm, Tokyo, Japan).

phosphorylated isotypes of mTOR from the nonphosphorylated counterpart on the agarose-polyacrylamide composite gel containing Mn^{2+}–Phos-tag.

Separation of the phosphoisotypes of DNA damage signaling-related proteins, ATM and 53BP1

In response to endogenous or exogenous DNA damage, a specific signaling pathway is activated to maintain genetic stability[48,49]. Some particular proteins at the DNA damage-signaling checkpoint are phosphorylated in response to UV irradiation or DNA-damaging reagents. Among these proteins, ATM (350 kDa) and 53BP1 (213 kDa) were too large to observe the mobility shifting of the phosphoisotypes in the previous protocol for Mn^{2+}–Phos-tag SDS-PAGE. It has been reported that DNA damage induces a rapid autophosphorylation at the Ser-1981 residue, which initiates ATM kinase activity[48]. Sequentially, 53BP1 is phosphorylated by ATM kinase at the Ser-25, Ser-29 and Ser-1778 residues[49,50].

For demonstration of the separation of phosphorylated and nonphosphorylated ATM and 53BP1 by using the improved protocol, a set of cellular lysates was prepared from HeLa cells (10^7 cells) as follows. After incubation in an FBS-free medium for 16 h, the cells were treated with 0 μM (control) or 2 μM actinomycin D, which inhibits transcription of RNA polymerase through the binding to double-stranded DNA by intercalation/groove binding mechanism, for 5 h. Each of the induced and control cultures was washed twice with a TBS solution and lyzed in 0.5 ml of a sample-loading dye solution (1×).

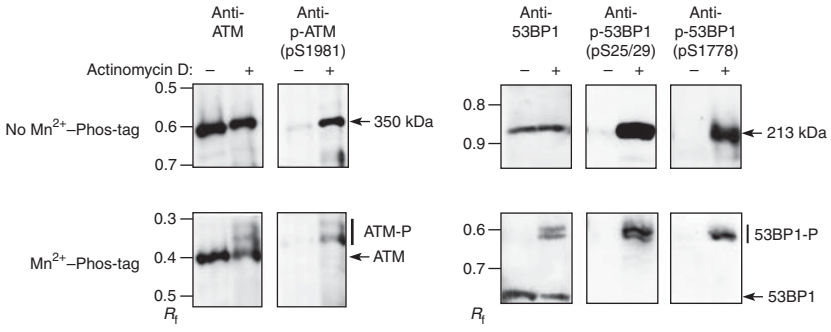

Figure 4 | Phosphorylation of DNA damage signaling-related proteins in HeLa cells treated with actinomycin D. The HeLa cells (10^7 cells) were incubated in an FBS-free DMEM for 16 h and then treated with 0 μM (−) or 2 μM actinomycin D for 5 h (+). (The actinomycin D was obtained from Sigma, St. Louis, MO, USA.) The lysates were subjected to SDS-PAGE on 3% (wt/vol) polyacrylamide gel strengthened with 0.5% agarose containing 0 μM (no Mn^{2+}–Phos-tag) or 20 μM Mn^{2+}–Phos-tag, followed by immunoblotting with the anti-ATM antibody (mouse monoclonal, clone 2C1), anti-p-ATM antibody against pS1981 (mouse monoclonal, clone 10H11.E12), anti-53BP1 antibody, anti-p-53BP1 antibody against pS25/29 and anti-p-53BP1 antibody against pS1778. Anti-p-ATM antibody against pS1981 was obtained from Rockland Immunochemicals (Rockland, Gilbertsville, PA, USA). An anti-ATM antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other primary antibodies and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary and secondary antibodies were probed by using PhosphoBlocker blocking reagent (Cell Biolabs, San Diego, CA, USA) for the anti-p-53BP1 antibody against pS25/29 or Canget Signal for the anti-53BP1 and anti-p-53BP1 antibodies against pS1778. For other antibodies, the blocking procedure was performed using 1% (wt/vol) BSA (Nakalai Tesque, Kyoto, Japan). The electrophoresis migration band was detected using the ECL Advance Western blotting detection kit and an LAS 3000 image analyzer. Figures are reproduced from Kinoshita *et al.*[36] with permission of the publisher, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

We first performed normal SDS-PAGE analysis using a 3% (wt/vol) polyacrylamide gel strengthened with 0.5% (wt/vol) agarose and the set of lysates (20 μg proteins per lane) (**Fig. 4**, no Mn^{2+}–Phos-tag). Successive immunoblotting with the anti-ATM or anti-53BP1 antibody showed that ATM and 53BP1 appeared as a single band at an R_f value of 0.6 or 0.87, respectively. The same samples were also analyzed separately using the anti-p-ATM antibody against pS1981, anti-p-53BP1 antibody against pS25/29 or anti-p-53BP1 antibody against pS1778. We confirmed that each site was phosphorylated in response to actinomycin D.

Next, we subjected the same samples to Mn^{2+}–Phos-tag SDS-PAGE using a 3% (wt/vol) polyacrylamide gel containing 0.5% (wt/vol) agarose and 20 μM of polyacrylamide-bound Mn^{2+}–Phos-tag (**Fig. 4** Mn^{2+}–Phos-tag). Regarding immunoblotting analysis with the anti-ATM antibody, a single band at an R_f value of 0.4 was observed for the control sample, and two additional up-shifted bands were observed for the actinomycin D-treated sample. Immunoblotting with the anti-p-ATM antibody against pS1981 showed that only up-shifted bands were phosphorylated isotypes of ATM. As for immunoblotting analysis with the anti-53BP1 antibody, a single band at an R_f value of 0.78 was observed for the control sample, and two additional up-shifted bands at 0.62 and 0.6 were detected for the treated sample. Immunoblotting with the anti-p-53BP1 antibody against pS25/29 showed that only two bands retarded were 53BP1 isotypes phosphorylated at both the Ser-25 and Ser-29 residues. Furthermore, immunoblotting with the anti-p-53BP1 antibody against pS1778 showed that the phosphoisotype observed at an R_f value of 0.62 was also phosphorylated at the Ser-1778 residue. Thus, the phosphorylated proteins were separated from the nonphosphorylated counterparts and detected on the agarose–polyacrylamide composite gel containing Mn^{2+}–Phos-tag.

In this study, we carried out the analyses using the agarose–polyacrylamide composite gels without Mn^{2+}–Phos-tag complexes as control electrophoresis experiments. As another control, the agarose–polyacrylamide gel containing the same concentration of only Phos-tag ligand (no complex with Mn^{2+}) or only $MnCl_2$ is also suitable for the mobility shift detection of phosphoprotein isotypes. We have confirmed that the electrophoresis running feature is identical when using a normal SDS-PAGE gel without Mn^{2+}–Phos-tag complexes.

Note: Supplementary information is available via the HTML version of this article.

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