Notes & Tips

Phos-tag beads as an immunoblotting enhancer for selective detection of phosphoproteins in cell lysates

Emiko Kinoshita-Kikuta, Eiji Kinoshita*, and Tohru Koike

Department of Functional Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Hiroshima 734-8553, Japan

Short title: Phos-tag as an immunoblotting enhancer

* Corresponding author. Fax: +81 82 257 5336.

E-mail address: kinoeiji@hiroshima-u.ac.jp (E. Kinoshita)

[1] *Abbreviations used:* MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; RIPA, radio-immunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

ABSTRACT

The low specificity of anti-phosphoprotein antibodies is often a problem in immunoblotting analyses. We introduce a simple pretreatment procedure for cell lysates to give more-specific detection of phosphoproteins in immunoblotting. Cellular phosphoproteins were preferentially trapped on Phos-tag agarose phosphate-affinity beads in a homemade spin-centrifuge microtube unit, and nonphosphorylated proteins were excluded in the filtrate. The phosphoprotein-bound beads suspended in a sample-loading dye solution were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blotting. We demonstrated improved detection of phosphorylated Shc and mitogen-activated protein kinase isoforms in A431 cell lysates by this new technique.

Keywords: Phos-tag; Immunoblotting enhancer; Anti-phosphoprotein antibody; Phosphoprotein; Phosphorylation; Signal transduction; Western blotting

In signal-transduction research, phosphoproteins are usually detected by using anti-phosphoprotein antibodies [1]. However, low levels of phosphoproteins in various cell lysates frequently escape detection, even by immunoblotting with anti-phosphoprotein antibodies. If high concentrations of the antibody and long exposure times are employed as means of circumventing this problem, a lack of specificity can be a problem. Optimization of the conditions for specific detection of individual phosphoproteins by using anti-phosphoprotein antibodies is generally performed empirically and is time consuming.

In this report, we describe a simple and rapid procedure for pretreatment of cell lysates to permit more-specific detection of cellular phosphoproteins by immunoblotting. The procedure, which uses Phos-tag agarose phosphate-affinity beads [2,4] and a homemade spin-centrifuge microtube unit, aims to achieve efficient separation of phosphoproteins from complex mixtures containing solubilized cellular proteins together with a marked enhancement of specific detection in immunoblotting with anti-phosphoprotein antibodies. As the first practical examples of the application of this procedure, we demonstrate improved immunoblot detection of phosphorylated Shc and mitogen-activated protein kinase (MAPK)[1] isoforms from the lysate of A431 human epidermoid carcinoma cells stimulated by epidermal growth factor (EGF). The EGF-dependent phosphorylation events on Shc and MAPK isoforms in the A431 cell are well established [3−5].

Lysates of A431 cells before and after EGF stimulation were prepared by using a nondenaturing buffer, such as a radio-immunoprecipitation assay (RIPA) buffer consisting of 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.25% (w/v) sodium deoxycholate, 1.0% (v/v) Nonidet P-40, 1.0 mM *N,N,N′,N′*-ethylenediaminetetraacetic acid, 1.0 mM phenylmethanesulfonyl fluoride, 1.0 μg/mL aprotinin, 1.0 μg/mL leupeptin, 1.0 μg/mL pepstatin, 1.0 mM Na_3VO_4, and 1.0 mM NaF, as previously described [4]. The procedure for pretreatment of the lysates is shown schematically in Supplementary Fig. S1. First, a homemade spin-centrifuge microtube unit was prepared. The bottom of a 0.5-mL tube (e.g., PCR tube, Product number; 672201, Greiner Bio-One, Frickenhausen, Germany) was pricked with a 21-G needle to make a small pore. The pricked tube was placed inside a 1.5-mL tube (e.g., Microtube, Product number; 616201, Greiner Bio-One) and then 40 μL of Phos-tag

agarose slurry (zinc(II)-bound form) was added to the spin-centrifuge microtube unit. The Phos-tag agarose beads are commercially available from the Phos-tag Consortium (http://www.phos-tag.com/english/index.html, order name; Phos-tag Agarose AG-503). The unit was centrifuged at 2,000 × *g* for 20 s to remove the storage buffer (20 mM Tris–CH_3COOH [pH 7.4] containing 20% [v/v] 2-propanol), and the filtrate was discarded. A balancing buffer (40 μL) consisting of 0.10 M Tris–CH_3COOH (pH 7.5), 1.0 M CH_3COONa, and 10 μM $Zn(OCOCH_3)_2$ was added to the Phos-tag agarose beads and the mixture was allowed to stand for 5 min at room temperature. The unit was centrifuged at 2,000 × *g* for 20 s, and the filtrated buffer was discarded. A binding/washing buffer (40 μL) consisting of 0.10 M Tris–CH_3COOH (pH 7.5) and 1.0 M CH_3COONa was placed on the beads, and then the unit was centrifuged at 2,000 × *g* for 20 s. This washing operation was repeated 3 times. The lysate (20 μg proteins in 10 μL of an RIPA buffer) was added to the beads and the mixture was allowed to incubate for 5 min at room temperature. When 40 μL of Phos-tag agarose slurry is used, the amount of cellular proteins should not exceed 20 μg. The unit was centrifuged at 2,000 × *g* for 20 s, and the filtrate was collected as a flow-through fraction if required. The binding/washing buffer (40 μL) was added to the beads, and the unit was centrifuged at 2,000 × *g* for 20 s. This washing operation was performed once, and the filtrate was collected as a washing fraction if required. Distilled water (40 μL or more) was added to the beads, and the whole swollen gel slurry was completely transferred to a new 1.5-mL tube. The tube was centrifuged at 2,000 × *g* for 20 s, and the supernatant fluid was discarded. This step is critical for reducing the quantity of salts remaining in the beads, which can cause distortions of protein band patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We confirmed that none of the proteins trapped by the beads was eluted in the discarded supernatant fluid. Finally, 10 μL of a sample-loading dye solution consisting of 65 mM Tris–HCl (pH 6.8), 1.0% (w/v) SDS, 5.0% (v/v) 2-sulfanylethanol, 10% (v/v) glycerol, and 0.03% (w/v) Bromophenol Blue was added to the beads and the tube was heated for 5 min at 95 °C. The whole suspension containing the Phos-tag agarose beads as a trapping fraction was transferred to a sample well of an SDS-PAGE gel. If necessary, more of the sample-loading dye solution was used to ensure complete transfer of the Phos-tag beads.

SDS-PAGE and subsequent Western blotting were performed as described previously [4–6].

Figure 1A shows typical SDS-PAGE results for the flow-through (lane 1), washing (lane 2), and trapping (lane 3) fractions of the EGF-stimulated A431 cell lysate (20 μg proteins solubilized in 10 μL of an RIPA buffer) after pretreatment using the Phos-tag agarose beads. The lysate (20 μg proteins) without the pretreatment was applied in lane 4 as a control. The SDS-PAGE gel was stained by Coomassie Brilliant Blue (left-hand panel), and then the same gel was electroblotted by using a poly(vinylindene fluoride) membrane. The blotted proteins were probed with biotin-pendant Phos-tag complexes with horseradish peroxidase-conjugated streptavidin (right-hand panel), as previously described [6]. The Western blotting method using the biotin-pendant Phos-tag comprehensively detected cellular phosphoproteins as chemiluminescence signals (see lanes 3 and 4 in the right-hand panel). Although the amount of the bead-trapped proteins (lane 3 in the left-hand panel) was smaller than that of the total proteins in the EGF-stimulated cell lysate (lane 4 in the left-hand panel), there was almost no difference in the amount of phosphoproteins between the samples of the trapping fraction (lane 3 in the right-hand panel) and the control lysate (lane 4 in the right-hand panel). These results show that the pretreatment procedure using the Phos-tag agarose beads permits efficient separation of phosphoproteins from a complex mixture containing solubilized cellular proteins. In the case of the EGF-stimulated A431 cell lysate used in this study (2.0 μg protein/μL), when more than 10 μL of the lysate was subjected to the pretreatment procedure using 40 μL of Phos-tag agarose slurry, some phosphoproteins leaked out to the flow-though and/or washing fractions (data not shown).

Next, we examined the utility of the pretreatment procedure using the Phos-tag agarose beads in immunoblotting analyses with anti-phosphorylated Shc and MAPK antibodies for the detection of the EGF-dependent phosphorylation of Shc and MAPK isoforms. Figure 1B shows typical results of immunoblot detection of phosphorylated Shc and MAPK using the A431 cellular lysates (20 μg protein solubilized in 10 μL of an RIPA buffer) before (–) and after (+) EGF stimulation. The locations of three Shc isofrom bands (66, 52, and 46 kDa) and two MAPK1/2 bands (44 and 42 kDa) were determined by reprobing the same blotting membrane with an anti-Shc antibody and an anti-MAPK antibody, respectively (see both

panels in Fig. 1B). As for the Shc detection, in samples that were not subjected to the pretreatment procedure, many false signal bands were observed in both lanes and it was very difficult to identify the EGF-dependent phosphorylation of Shc isoforms (see left-hand panel in the results of the Shc detection). In contrast to these results, immunoblotting of the samples with the pretreatment procedure gave clearer signals (right-hand panel). In particular, in the lysate after EGF stimulation (lane +), strong signals derived from three phosphorylated Shc isoforms were visualized. As for the MAPK detection, furthermore, a similar result was obtained. The results show that the pretreatment procedure using the Phos-tag agarose beads permits a marked enhancement in specific detection in immunoblotting with the anti-phosphoprotein antibody.

In summary, we have demonstrated a novel application of Phos-tag agarose beads for the pretreatment of A431 cell lysate that results in an improved detection of the EGF-dependent phosphorylated Shc and MAPK isoforms in immunoblotting analyses with the anti-phosphoprotein antibodies. This approach was based on the efficient separation of phosphoproteins from a complex mixture containing solubilized cellular proteins. The simple procedure, using the homemade spin-centrifuge microtube unit, results in more-specific immunoblot detection of phosphoproteins in the cell lysate. The total time for the procedure is less than 30 min for 10 lysate samples. Furthermore, it is not necessary to change the general protocol for immunoblotting. Because a constant volume of phosphoprotein-bound beads suspended in a sample-loading dye solution is used in SDS-PAGE, quantitative analysis of samples is possible. Thus, the Phos-tag agarose beads can serve as an immunoblotting enhancer for more-specific detection of phosphoproteins in mapping low-abundance phosphorylation events on certain proteins from samples of interest.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (B) (19390011) and (C) (19590040) from the Japan Society of the Promotion of Science (JSPS), a Grant-in-Aid for Young Scientists (B) (20790036) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), and a research grant from the Iketani Science and Technology Foundation.

References

[1] H. Kaufmann, J.E. Bailey, M. Fussenegger, Use of antibodies for detection of phosphorylated proteins separated by two-dimensional gel electrophoresis. Proteomics 1 (2001) 194–199.

[2] E. Kinoshita, A. Yamada, H. Takeda, E. Kinoshita-Kikuta, T. Koike, Novel immobilized zinc(II) affinity chromatography for phosphopeptides and phosphorylated proteins. J. Sep. Sci. 28 (2005) 155–162.

[3] A. Citri, Y. Yarden, EGF-ERBB signalling: towards the systems level. Nat. Rev. Mol. Cell Biol. 7 (2006) 505–516.

[4] E. Kinoshita-Kikuta, E. Kinoshita, A. Yamada, M. Endo, T. Koike, Enrichment of phosphorylated proteins from cell lysate using a novel phosphate-affinity chromatography at physiological pH. Proteomics 6 (2006) 5088–5095.

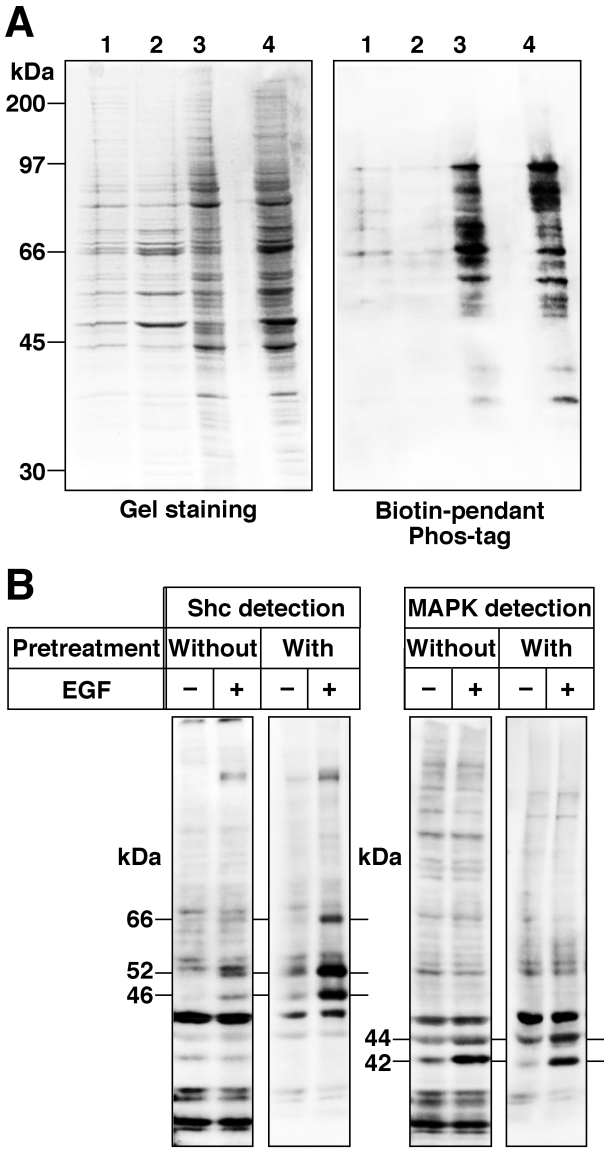
[5] E. Kinoshita-Kikuta, Y. Aoki, E. Kinoshita, T. Koike, Label-free kinase profiling using phosphate affinity polyacrylamide gel electrophoresis. Mol. Cell. Proteomics 6 (2007) 356–366.

[6] E. Kinoshita, E. Kinoshita-Kikuta, K. Takiyama, T. Koike, Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol. Cell. Proteomics 5 (2006) 749–757.

Figure legend

Fig. 1. Evaluation of the utility of the pretreatment procedure using Phos-tag agarose for immunoblotting. (A) The 8% (w/v) polyacrylamide SDS-PAGE gel made by a mini-slab gel-casting system (model AE6500; Atto, Tokyo, Japan) was stained with Coomassie Brilliant Blue (left-hand panel), and subjected to subsequent electroblotting followed by probing with biotin-pendant Phos-tag from the Phos-tag Consortium (order name; Phos-tag Biotin BTL-104) (right-hand panel). The flow-through, washing, and trapping fractions of the EGF-stimulated A431 cell lysate (20 μg proteins solubilized in 10 μL of an RIPA buffer) after pretreatment with Phos-tag agarose were applied in lanes 1, 2, and 3, respectively. The lysate (20 μg proteins) without the pretreatment was applied as a control in lane 4. The A431 cells (10^7 cells) were stimulated with 50 ng/mL EGF for 5 min and lyzed in an RIPA buffer. The concentration of the solubilized proteins was adjusted to 2.0 mg/mL. (B) The A431 cell lysates (20 μg proteins solubilized in 10 μL of an RIPA buffer) with or without pretreatment using Phos-tag agarose were subjected to SDS-PAGE (8% [w/v] polyacrylamide mini-slab gel) followed by immunoblotting with an anti-phosphorylated Shc antibody against the phosphorylated Tyr-317 residue (Millipore, Billerica, MA, USA) (Shc detection panels) and an anti-phosphorylated MAPK1/2 antibody (Millipore, clone 12D4) (MAPK detection panels). The A431 cells (10^7 cells) were stimulated with 0 ng/mL (–) or 50 ng/mL (+) EGF for 5 min and lyzed in an RIPA buffer. The concentration of the solubilized proteins was adjusted to 2.0 mg/mL. The positions of three Shc isoforms (66, 52, and 46 kDa) and two MAPK isoforms (44 and 42 kDa) were determined by using an anti-Shc antibody (Millipore) and an anti-MAPK1/2 antibody (Millipore), respectively.

Fig. 1


A
1 2 3 4
1 2 3 4
kDa
200
97
66
45
30
Gel staining
Biotin-pendant
Phos-tag
B
Shc detection
MAPK detection
Pretreatment
Without
With
Without
With
EGF
– + – +
– + – +
kDa
kDa
66
52
46
44
42

Supplementary Figure S1 Scheme for the pretreatment procedure for cell lysate using Phos-tag agarose to give more-specific phosphoprotein detection in immunoblotting.