[Short Communication]

Identification of two phosphorylated species of β -catenin involved in the ubiquitin-proteasome pathway by using two-dimensional Phos-tag affinity electrophoresis

Emiko Kinoshita-Kikuta, Eiji Kinoshita* and Tohru Koike

Department of Functional Molecular Science, Institute of Biomedical & Health Sciences, Hiroshima University

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SUMMARY

We recently reported a neutral-pH gel system buffered with 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol hydrochloride (Bis-Tris-HCl) for use in Zn^{2+} -Phos-tag sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for advanced profiling of protein phosphorylation. In the current study, we extended the utility of Zn^{2+} -Phos-tag SDS-PAGE with the Bis-Tris-HCl buffer system to a detailed analysis of phosphorylated β -catenin, which is closely involved in the ubiquitin-proteasome pathway. The Phos-tag-based approach, followed by Western blotting with an anti- β -catenin antibody, allowed us to assign nine phosphorylated species of β -catenin produced in complicated signaling pathways of cultured HEK293 and SW480 cells. Two-dimensional image coupling with normal Laemmli's SDS-PAGE as the first dimension gave more detailed information, not only on the phosphorylation of β -catenin, but also on the phosphorylation-dependent polyubiquitination by visualizing multiple ubiquitinated forms derived from two phosphorylated species of β -catenin in lactacystin-treated HEK293 cells. We identified two distinct phosphorylated species of β -catenin that are responsible for polyubiquitination. The first contains phosphorylated residues at S33, S37, T41, and S45, and the second contains these sites and an additional phosphorylated residue at S675. The profiling of double post-translational modifications of β -catenin is consistent with the widely accepted phosphorylation-dependent ubiquitination model in the absence of a Wnt signal.

Key words: β -Catenin, Phosphorylation, Ubiquitination, Two-dimensional Phos-tag affinity electrophoresis

INTRODUCTION

We recently reported an improved Phos-tag affinity electrophoresis technique known as Zn^{2+} -Phos-tag SDS-PAGE, in which a Zn^{2+} -Phos-tag complex is used in conjunction with a Bis-Tris-HCl buffer system for the detection of shifts in the mobility of phosphoproteins¹⁾. This technique showed major improvements over our previous Mn^{2+} -Phos-tag SDS-PAGE²⁾ using polyacrylamide-bound Mn^{2+} -Phos-tag and the conventional Laemmli's buffer system under alkaline pH conditions, as demonstrated by the visualization of many more up-shifted migration bands derived from phosphorylated species of β -catenin, a typical intracellular signaling molecule.

In this study, we extended the utility of Zn^{2+} -Phos-tag SDS-PAGE to further analyses of mobility shifts of β -catenin, the phosphorylation states of which are regulated *in vivo* by various protein kinases. The protein is a key com-

ponent of the Wnt signaling pathway as a transcriptional activator that affects cell proliferation and differentiation in many types of cell³⁾. In the absence of a Wnt signal, β -catenin interacts with Axin, casein kinase I α (CKI α), glycogen synthase kinase-3 β (GSK-3 β), and adenomatous polyposis coli gene product. In the complex, CKI α serves as a priming kinase that phosphorylates the S45 residue of β -catenin and enhances subsequent phosphorylation at the S33, S37, and T41 residues by GSK-3 β . The multi-phosphorylated β -catenin is ubiquitinated and degraded by the proteasome pathway. In this phosphorylation-dependent manner, β -catenin in the cytoplasm is maintained at low levels in quiescent cells. On the other hand, the phosphorylation-dependent ubiquitin-proteasome pathway is frequently disordered in cancer cells, where the level of β -catenin protein increases. In addition to phosphorylation by CKI α and GSK-3 β , β -catenin can also be phosphorylated at the S552 and S675 residues by protein

* Corresponding author: Eiji Kinoshita; Department of Functional Molecular Science, Institute of Biomedical & Health Sciences, Hiroshima University, Kasumi 1-2-3, Hiroshima 734-8553, Japan
E-mail: kinoeiji@hiroshima-u.ac.jp
Fax: +81-82-257-5281

kinases A and B (AKT), respectively, and each phosphorylation reaction induces transcriptional activity of β -catenin without affecting the ubiquitin-proteasome pathway^{4,5}. Thus, β -catenin is regulated by various protein kinases *in vivo*, and its various states of phosphorylation are closely related to specific cellular events.

This short communication describes two-dimensional Phos-tag affinity electrophoresis for profiling of double post-translational modifications of phosphorylation and ubiquitination of β -catenin, and it concludes that the Phos-tag-based strategy might include significant potential for the analysis of phosphorylation-dependent post-translational modifications on various cellular proteins.

MATERIALS AND METHODS

Materials

Phos-tag Acrylamide, which is commercially available from Wako Pure Chemical Industries (Osaka, Japan), was synthesized as described previously². Anti- β -catenin antibodies against the C-terminal (clone 14) and N-terminal regions were purchased from BD Bioscience (San Jose, CA, USA) and ECM Biosciences (Versailles, KY, USA), respectively. The site-specific phospho- β -catenin antibodies against pS33/S37/T41, pS552, and pS675 were purchased from Cell Signaling Technology (Danvers, MA, USA). The site-specific phospho- β -catenin antibody against pT41/S45 (clone EP1905Y) and anti- β -actin antibody (clone EP1123Y) were purchased from Millipore (Danvers, MA, USA).

Preparation of cell extracts

Cultured HEK293 and SW480 cells (10^7 cells) were treated with 0 μ M (–) or 5.0 μ M lactacystin (+) for 5 h. The cells were lysed with 0.50 mL of an SDS-PAGE sample-loading buffer 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 (BPB), and then boiled for 3 min before running electrophoresis.

Normal (Phos-tag-free) SDS-PAGE and Zn^{2+} -Phos-tag SDS-PAGE

Normal SDS-PAGE was performed by using a 1-mm-thick, 9-cm-wide, and 9-cm-long gel on a PAGE apparatus (Atto, model AE-6500, Tokyo, Japan). The separating gel buffer and the stacking gel buffer were 375 mM Tris–HCl (pH 8.8) and 125 mM Tris–HCl (pH 6.8), respectively. The running buffer consisted of 192 mM glycine, 25 mM Tris, and 0.10% (w/v) SDS. For Zn^{2+} -Phos-tag SDS-PAGE using the same PAGE apparatus, the gel consists of a separating gel [5.5% (w/v) polyacrylamide and 357 mM Bis-Tris–HCl, pH 6.8] and a stacking gel [4.0% (w/v) polyacrylamide and 357 mM Bis-Tris–HCl, pH 6.8]. The acrylamide-pendant Phos-tag ligand and two equivalents of ZnCl_2 were added to the separating gel before polymerization. The running buffer consisted of 0.10 M Tris and 0.10 M MOPS contain-

ing 0.10% (w/v) SDS and 5.0 mM sodium bisulfite. Electrophoresis was performed at 40 mA until the BPB dye reached the bottom of the separating gel. Normal SDS-PAGE and Zn^{2+} -Phos-tag SDS-PAGE are followed by immunoblotting analyses with appropriate antibodies.

Two-dimensional Phos-tag affinity electrophoresis

Normal SDS-PAGE as the first dimension was performed as described above. Lysates (10 μ L) of SW480 and HEK293 cells were subjected to normal SDS-PAGE [6.0% (w/v) polyacrylamide]. Electrophoresis was performed at 30 mA until the BPB dye reached the bottom of the separating gel. For two-dimensional electrophoresis (2-DE), each lane containing lysate proteins was independently cut from the first-dimensional SDS-PAGE gel and soaked for 30 min in 10 mL of 50 mM Bis-Tris–HCl (pH 6.8) buffer containing 2.0% (w/v) SDS, 15% (v/v) 2-sulfanylethanol, and 0.10% (w/v) BPB; it was then independently soaked for 10 min in 10 mL of the same buffer without 2-sulfanylethanol. Finally, each lane was separately placed on the top of the second-dimensional Zn^{2+} -Phos-tag SDS-PAGE gel between two glass plates. Zn^{2+} -Phos-tag SDS-PAGE as the second dimension using each lysate was separately performed as described above. Each 2-DE gel was analyzed by immunoblotting with the anti- β -catenin antibody against the C-terminal (clone 14).

RESULTS AND DISCUSSION

First, we examined the phosphorylation and ubiquitination of β -catenin by means of normal SDS-PAGE, followed by immunoblotting with two kinds of anti- β -catenin antibodies against the C-terminal and N-terminal regions, respectively, and four kinds of site-specific anti-phospho- β -catenin antibodies against the phosphorylated S33/S37/T41, T41/S45, S552, and S675 residues, respectively (Fig. 1). Sets of cellular lysates treated in the presence of 5.0 μ M of the proteasome inhibitor lactacystin (+) or in its absence (–) were prepared separately from HEK293 and SW480 cells. By immunoprob- ing with anti- β -catenin antibodies against the C-terminal and N-terminal regions, β -catenin (85 kDa; arrowed) and the multiple ubiquitinated forms of the protein were detected in the lactacystin-treated HEK293 cells in the same way as reported previously⁶. The up-shifted polyubiquitination bands arise through an increase in molecular mass of 8.5 kDa that results from attachment of one molecule of ubiquitin. In the SW480 cells, on the other hand, ubiquitinated forms were not observed, irrespective of any treatment with lactacystin. With respect to the 85-kDa β -catenin band, we confirmed that the enhanced chemiluminescence (ECL) signals for the SW480 cells were stronger than those for the HEK293 cells. Immunoprob- ing analysis of the same lysate samples with an anti- β -actin antibody showed that each lane contained an almost equal quantity of the total lysate

Fig. 1. Profiling of the phosphorylation and ubiquitination of intracellular β -catenin in HEK293 and SW480 cells by means of normal SDS-PAGE. Both lysates (10 μ L) were subjected to normal SDS-PAGE on 5.5% (w/v) polyacrylamide gel for β -catenin, or on 10% (w/v) polyacrylamide gel for β -actin, followed by immunoblotting with the appropriate antibody shown above each panel. The R_f value of 1.0 is defined as the position of the BPB dye.

proteins. These results confirmed previous reports that β -catenin is accumulated in SW480 cells as a result of a disorder in the ubiquitin-proteasome pathway⁶⁾. For both cells, several bands below 85 kDa were detected only on immunoprobings with the C-terminal antibody, suggesting that a system for cleavage from the N-terminal region of β -catenin through the ubiquitin-proteasome-independent pathway is conserved, as described previously⁷⁾. Immunoprobings with anti-phospho- β -catenin antibodies also showed that the level of β -catenin protein in SW480 cells is higher than that in HEK293 cells. Strong ECL signals were specifically detected in SW480 cells, and we were able to confirm the occurrence of all the phosphorylation reactions that we sought. In contrast, all the anti-phospho- β -catenin antibodies used permitted the detection of quite poor ECL signals of the β -catenin band in HEK293 cells, so we could not confirm the presence of phosphorylation reactions at the S33, S37, T41, S45, S552, and S675 residues. The normal SDS-PAGE method, furthermore, did not permit the detection of shifts in the mobility of phosphorylated β -catenin.

To detect phosphorylation of β -catenin as shifts in the mobility, next, we analyzed the same samples by Zn^{2+} -Phos-tag SDS-PAGE with the Bis-Tris-HCl buffer system, followed by immunoblotting with the C-terminal antibody. In both lysate samples, similar banding patterns were detected (left-hand panel, Fig. 2). When referred to our previous data regarding the phosphorylation-dependent shifts in the mobility of β -catenin in SW480 cells¹⁾, we could assign nine bands of phosphorylated species as indicated by cross lines of #1–#9. The fastest-migrating band was assigned to the nonphosphorylated form of the protein (shown by the open arrowhead). We have already demonstrated that all of the up-shifted bands (#1–#9) showed cross-activity with the anti-phospho- β -catenin antibodies we used in Fig. 1¹⁾. Therefore, phosphorylation at the S33, S37, T41, S45, S552, and S675 residues of β -catenin in lactacystin-treated HEK293 cells, which could not be confirmed

by normal SDS-PAGE, were verified by using Zn^{2+} -Phos-tag SDS-PAGE. To determine the phosphorylated species of β -catenin that are responsible for the polyubiquitination, the lactacystin-treated HEK293 lysate sample was analyzed by two-dimensional electrophoresis (2-DE) with normal SDS-PAGE coupled with Zn^{2+} -Phos-tag SDS-PAGE followed by immunoblotting with the C-terminal antibody (center panel, Fig. 2). The 2-DE image of the lactacystin-treated HEK293 lysate gave more-detailed information, not only on the phosphorylation of β -catenin, but also on the phosphorylation-dependent polyubiquitination. All up-shifted spots on the 2-DE gel were correlated with nine bands in the one-dimensional electrophoresis (1-DE) gel. Multiple up-shifted right-diagonal spots in the area to the right-hand side of the 85-kDa position (arrowed) were ubiquitinated forms of β -catenin and they were derived from two phosphorylated species assigned as #5 and #8, indicating that the two species are responsible for polyubiquitination. According to our previous study [1], both #5 and #8 showed cross-activity with the anti-phospho- β -catenin antibodies against phosphorylated S33/S37/T41 and against phosphorylated T41/S45. Moreover, #5 showed highly cross-activity with the anti-phospho- β -catenin antibody against phosphorylated S675, but #8 did not do so at all. In the SW480 cells, on the other hand, ubiquitinated forms were not observed in the 2-DE gel (right-hand panel, Fig. 2) as well as in the 1-DE gel (see Fig. 1), irrespective of any treatment with lactacystin. These results are consistent with the widely accepted phosphorylation-dependent ubiquitination model of β -catenin. Thus, this 2-DE strategy using Zn^{2+} -Phos-tag SDS-PAGE permitted the profiling of double post-translational modifications of phosphorylation and ubiquitination.

CONCLUSION

Zn^{2+} -Phos-tag SDS-PAGE followed by immunoprobings with β -catenin C-terminal antibody allowed us to detect

Fig. 2. Identification of the specific phosphorylated species responsible for polyubiquitination of β -catenin in HEK293 cells.

Lysates (10 μ L) of SW480 (without treatment by lactacystin) and HEK293 (with treatment by lactacystin) were subjected to one-dimensional Zn^{2+} -Phos-tag SDS-PAGE (1-DE) on 5.5% (w/v) polyacrylamide gel containing 25 μ M polyacrylamide-bound Zn^{2+} -Phos-tag (left-hand panel). The two arrows in 1-DE indicate the specific phosphorylated species that are responsible for the polyubiquitination of β -catenin in HEK293 cells. Each 2-DE with normal SDS-PAGE on 6.0% (w/v) polyacrylamide gel as the first dimension and Zn^{2+} -Phos-tag SDS-PAGE on 5.5% (w/v) polyacrylamide gel containing 25 μ M polyacrylamide-bound Phos-tag as the second dimension was independently performed using the same lactacystin-treated HEK293 lysate sample solution (10 μ L) (center panel) or the same lactacystin-untreated SW480 lysate sample solution (10 μ L) (right-hand panel). The R_f value of 1.0 is defined as the position of the BPB dye.

nine phosphorylated species of β -catenin produced in complicated signaling pathways *in vivo*. By using a lactacystin-treated sample of HEK293 lysate, we assigned two specific phosphorylated species of β -catenin containing the phosphorylated S33, S37, T41, and S45 residues that are responsible for polyubiquitination. The 2-DE image gave more detailed information on the dynamics of endogenous β -catenin in the cell, separately visualizing multiple polyubiquitinated forms derived from the two different phosphorylated species, one containing the phosphorylated residues at S33, S37, T41, and S45, and the other containing

these common sites and an additional phosphorylated residue at S675. Therefore, it is worthwhile considering the use of this Phos-tag-based 2-DE strategy for the analysis of phosphorylation-dependent multiple post-translational modifications on various cellular proteins.

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ABBREVIATIONS

SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CKI α , casein kinase I α ; GSK-3 β , glycogen synthase kinase-3 β ; BPB, bromophenol blue; 2-DE, two-dimensional electrophoresis; ECL, enhanced chemiluminescence; 1-DE, one-dimensional electrophoresis

REFERENCES

- 1) Kinoshita E, Kinoshita-Kikuta E. Improved Phos-tag SDS-PAGE under neutral pH conditions for advanced protein phosphorylation profiling. *Proteomics*. 2011;11:319–323.
- 2) Kinoshita E, Kinoshita-Kikuta E, Takiyama K, *et al.* Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics*. 2006;5:749–757.
- 3) Kikuchi A. Tumor formation by genetic mutations in the components of the Wnt signaling pathway. *Cancer Sci*. 2003;94:225–229.
- 4) Taurin S, Sandbo N, Qin Y, *et al.* Phosphorylation of β -catenin by cyclic AMP-dependent protein kinase. *J Biol Chem*. 2006;281:9971–9976.
- 5) Fang D, Hawke D, Zheng Y, *et al.* Phosphorylation of β -catenin by AKT promotes β -catenin transcriptional activity. *J Biol Chem*. 2007;282:11221–11229.
- 6) Hinoi T, Yamamoto H, Kishida M, *et al.* Complex formation of adenomatous polyposis coli gene product and axin facilitates glycogen synthase kinase-3 β -dependent phosphorylation of β -catenin and down-regulates β -catenin. *J Biol Chem*. 2000;275:34399–34406.
- 7) Abe K, Takeichi M. NMDA-receptor activation induces calpain-mediated β -catenin cleavages for triggering gene expression. *Neuron*. 2007;53:387–397.