Phos-tag Western blotting for detecting stoichiometric protein phosphorylation in cells

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Method Article

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Abstract

Introduction

To investigate the physiological roles of protein phosphorylation, it is important to analyze sites and stoichiometry of phosphorylation in cells. The generation of phosphorylation site-specific antibodies is useful to detect targeted phosphorylation sites and visualize their intracellular distribution[1]. However, it is difficult to determine the stoichiometry of phosphorylation by using these antibodies. Phosphate-affinity polyacrylamide gel electrophoresis is useful to detect stoichiometric protein phosphorylation[2]. The phosphate-affinity site is a polyacrylamide-bound dinuclear Mn^{2+} complex \(Mn^{2+}-Phos-tag) that can enhance mobility shifts of phosphorylated forms of many proteins. Phosphorylation levels of cellular proteins of interest can be assessed by subsequent Western blotting.

Reagents

> HBS: 20 mM Hepes-NaOH, pH7.4, and 150 mM NaCl > RIPA buffer: 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1 to1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, PhosSTOP phosphatase inhibitors \(Roche), and Complete EDTA-free protease inhibitors \(Roche) > 5 mM acrylamide-pendant Phos-tag ligand \(AAL-107; NARD Institute, Amagasaki, Japan) in H_2O > 10 mM $MnCl_2$ in H_2O > Transfer buffer A: 300 mM Tris and 5% MeOH > Transfer buffer B: 25 mM Tris and 5% MeOH > Transfer buffer C: 25 mM Tris, 40 mM 6-amino-n-caproic acid, and 5% MeOH

Equipment

> Vertical electrophoresis unit \(BE-S27; Bio Craft, Tokyo, Japan) > Semi-dry electroblotting apparatus \(Trans-Blot SD Cell, Bio-Rad) > Luminescent image analyzer \(LAS-3000, Fujifilm)

Procedure

A. Preparation of whole cell extracts 1. After stimulation, rinse cells twice with ice-cold HBS. 2. Following steps should be done on ice or at 4^{o}c. 3. Add 1.0 ml of RIPA buffer to a 100 mm cell culture dish and incubate for 15 min. 4. Harvest lysed cells with a cell scraper. 5. Collect supernatants after centrifugation at 20,000 g for 15 min. 6. Add Laemmli sample buffer, mix, and heat at 95^{o}c for 10 min. 7. Store samples at -80^{o}c until use. **B. Phosphate-affinity polyacrylamide gel electrophoresis** 1. Prepare the separating gel containing Mn^{2+}-Phos-tag. We routinely use 7.5% polyacrylamide gel with 25 micro M Phos-tag and 50 micro M $MnCl_2$. These conditions may need to be optimized for proteins of interest. 2. Prepare the normal stacking gel. 3. Load samples onto the gel. Note that lanes adjacent to prestained molecular weight markers are strongly disordered during electrophoresis. 4. Run the gel at 5 to 15 mA. Phosphorylation-dependent mobility shifts of some proteins are dramatically improved by lowering the current as reported previously[3], although the bands become fuzzy. **C. Electrophoretic transfer** 1.

Incubate the gel with gentle agitation in transfer buffer B supplemented with 1 mM EDTA for 10 min. 2. Incubate the gel with gentle agitation in transfer buffer B without EDTA for further 10 min. 3. Prepare PVDF membrane by wetting it in MeOH for 30 sec and then soaking it in transfer buffer B for more than 30 min. 4. Electrophoretically transfer proteins in the gel onto the membrane using a semi-dry blotting apparatus at 20 V for 90 min according to the method of Kyhse-Andersen[4]. Briefly, assemble the gel, PVDF membrane, and filter papers in the following order: the anode, two layers of filter paper soaked in transfer buffer A, one layer of filter paper soaked in transfer buffer B, PVDF membrane, the gel, three layers of filter paper soaked in transfer buffer C, and the cathode. **D. Blocking, incubation with antibodies, and detection** 1. Process the membrane according to standard Western blotting procedures.

Critical Steps

Do not wash cells with a phosphate-containing buffer such as PBS. Load all lanes of each gel with only simultaneously prepared samples. Because transfer of phosphorylated proteins from a gel with Mn^{2+}-Phos-tag is difficult, the gel should be first soaked in EDTA-containing transfer buffer B and the original semi-dry transfer method[4] using three transfer buffers should be performed.

Anticipated Results

We have tested over 10 proteins that are known to be phosphorylated in cells by a ligand-dependent manner and have noticed that phosphorylation-dependent mobility shifts of about two-thirds of them are clearly enhanced by adding Mn^{2+}-Phos-tag to acrylamide gels. Some large proteins were reported to be more separated between phosphorylated and unphosphorylated forms by lowering Phos-tag concentrations \(3.5 to 5 micro M)[3]. However, we found that this was ineffective in cases of Nup153 and Nup214 \(see Figure 1).

References

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Figures

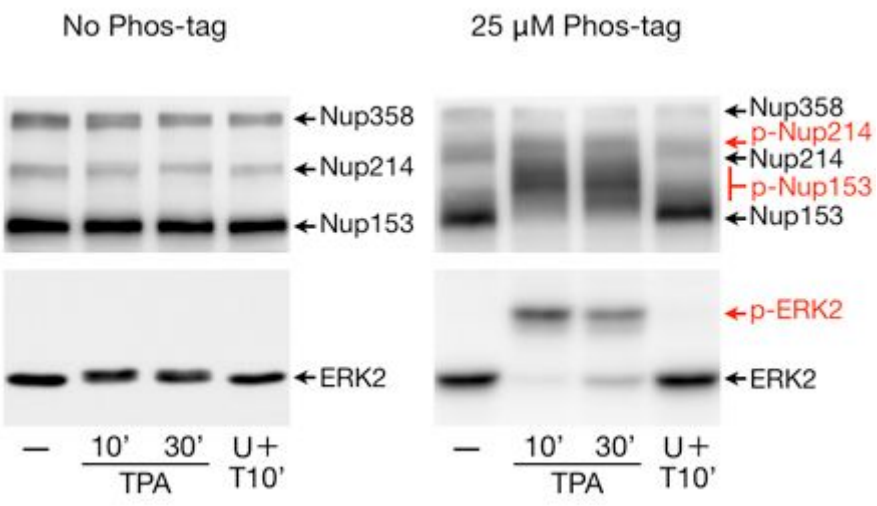

Figure 1

Detection of phosphorylation of nucleoporins and ERK2 by Phos-tag Western blotting. Serum-starved HeLa cells were stimulated with 100 ng/ml TPA for the indicated times with or without a 30-min pretreatment with U0126 (U). The lysates were subjected to SDS-PAGE in the presence (right) or absence (left) of Mn^2+^-Phos-tag followed by immunoblotting with mAb414 (top) and anti-ERK2 mAb (bottom). The mAb414 reacts with FG repeats in several nucleoporins including Nup358, Nup214, and Nup153.