

Chapter 2

Method for the Generation of Antibodies Specific for Site and Posttranslational Modifications

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Abstract

Protein phosphorylation plays critical roles in multiple aspects of cellular events. Site- and phosphorylation state-specific antibodies are indispensable to analyze spatially and temporally distribution of protein phosphorylation in cells. Such information provides some clues of its biological function. Here, we describe a strategy to design a phosphopeptide as an antigen for a site- and phosphorylation state-specific antibody. Importantly, this strategy is also applicable to the production of other types of antibodies, which specifically recognize the site-specific modification, such as acetylation, methylation, and proteolysis. This protocol also focuses on the screening for monoclonal version of a site- and phosphorylation state-specific antibody.

Key words Phosphopeptide, Site- and phosphorylation state-specific antibody, ELISA, Immunoblotting, Immunocytochemistry

1 Introduction

Protein phosphorylation is known to change the affinity of a protein towards its interacting partner, its enzymatic activity, or its subcellular localization. Such functional changes of proteins by phosphorylation are implicated in multiple aspects of cellular events such as signal transduction [1, 2], cell cycle progression/checkpoint [3, 4], cytoskeletal rearrangements [5], etc.

In order to investigate the biological role(s) of protein phosphorylation in cells, it is of great importance to analyze the cellular protein phosphorylation. In the past, labeling of cells with radioactive phosphate was a widely used strategy to monitor *in vivo* phosphorylation of proteins. However, there are several problems related to radiation exposure in this method. Recently, mass spectrometry (MS) analysis is a convenient alternative to the above method because it does not require radioisotopes. However, these strategies have two major difficulties. First, it is impossible to obtain clear images of the spatial and temporal distribution of

protein phosphorylation in cells, since these analyses require cell lysis. Second, in order to analyze the site-specific protein phosphorylation, these methods require many steps, such as the purification of a protein of interest from cells, the fragmentation of the protein by a protease [6], etc.

In 1983, Sternberger's group reported that a subset of their neuron-specific monoclonal antibodies recognized specifically phosphorylated forms of proteins but not non-phosphorylated forms [7]. This study also demonstrated that use of such an antibody in immunocytochemistry could lead to visualization of the intracellular distribution of protein phosphorylation [7]. However, for generating an antibody that can recognize a protein phosphorylated specifically at targeted residue(s), immunizing with a phosphorylated whole protein has little chance of being successful.

To overcome this difficulty, we immunize rabbits or mice with a phosphorylated peptide corresponding to a target phosphorylated residue and its surrounding sequence of amino acids in 1990. This method, which we first established [8–10], has both a greater chance of obtaining a phosphoepitope-specific antibody and the advantage that one can predesign a targeted phosphorylation site(s) [11–14]. By 1994, several companies were able to synthesize a phosphopeptide chemically. This chemical production enables us to use this technology more easily. Our method with a modified peptide has also been applied to the production of antibodies that can specifically recognize the other types of site-specific protein modification, such as acetylation [15] and methylation [16]. In this protocol, we describe not only the strategy to design a phosphopeptide as an antigen but also the screening for monoclonal version of a site- and phosphorylation state-specific antibody.

2 Materials

2.1 Antigen Production

1. Synthetic peptides: we usually design phosphorylated and non-phosphorylated versions of peptides to contain targeted residue(s) [phosphorylation site(s)] and the flanking 5 amino acids at both sides, because 5 or 6 amino acid residues are considered to constitute an antigen epitope recognized by an antibody molecule. In order to conjugate it to a carrier protein such as keyhole limpet hemocyanin (KLH), we usually introduce a Cys (C) residue at the amino-terminal side of the synthetic peptide. As an example, we show synthetic peptides for the production of an antibody against phosphoSer296 or phosphoSer345 on Chk1 (Fig. 1). Some variations are allowable in amino acid length from a phosphorylation site [13, 14]. However, we do not recommend that you use peptides containing over 10 amino acids at either side of a phosphorylation

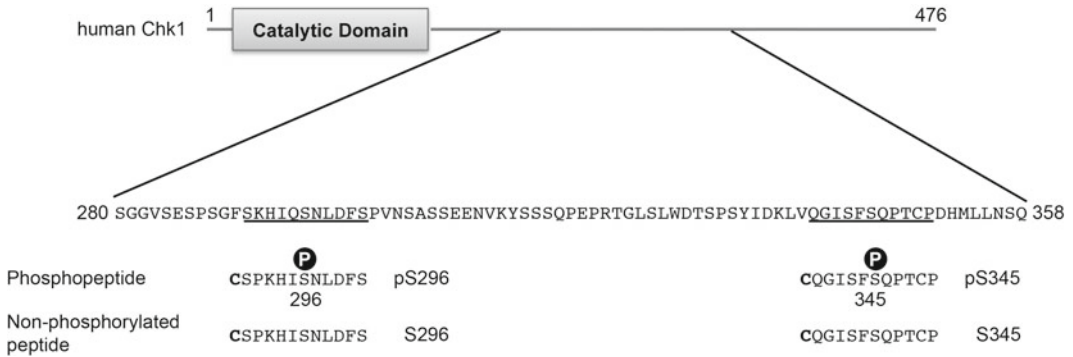


Fig. 1 Design of synthetic peptides for the production of each anti-phospho-specific antibody. We show a set of synthetic peptides in the case of production of an antibody against phosphoSer296 or phosphoSer345 on Chk1. Each peptide sequence corresponding to human Chk1 is underlined. Additional Cys residue in the amino-terminal site is indicated as a *bold letter*. Each phosphate group in phosphoSer is also indicated as P within a *circle*

site, because such peptides may elevate the possibility of the production of antibodies against non-phosphorylated epitope. The above method in the peptide design can be also applicable to the production of an antibody that can specifically recognize the other types of site-specific protein modification, such as acetylation [15] and methylation [16]. Nowadays, many companies can perform not only the synthesis of non-modified peptides but also that of peptides including phosphorylated, acetylated, and/or methylated amino acid(s). We usually order 15 mg non-modified peptide and 25 mg phosphopeptide from Peptide Institute Inc. (Osaka, Japan; *see Note 1*).

2. Phosphopeptide-conjugated carrier protein: Many companies also perform the conjugation of phosphopeptide to carrier protein such as KLH. On ordering peptides, we usually request Peptide Institute to conjugate 5 mg of phosphopeptide to KLH. We also request to leave the peptide-conjugated KLH aqueous, because lyophilized KLH is difficult to be dissolved in the aqueous buffer such as phosphate-buffered saline (PBS). Store in aliquots at -80°C before use.

2.2 ELISA

1. Peptide dilution buffer: $0.1\text{ M Na}_2\text{HPO}_4 \cdot \text{NaH}_2\text{PO}_4$ (pH 7.4).
2. ELISA blocking buffer: $10\text{ mM Na}_2\text{HPO}_4 \cdot \text{NaH}_2\text{PO}_4$ (pH 8.0), 5% (w/v) BSA, 5% (w/v) sucrose, 0.1% NaN_3 .
3. ELISA second antibody solution: HRP-conjugated anti-mouse or -rat IgG (Life Technologies, Gaithersburg, MD) diluted at 1: 1,000 in $10\text{ mM Na}_2\text{HPO}_4 \cdot \text{NaH}_2\text{PO}_4$ (pH 8.0), 100 mM NaCl , 1% (w/v) BSA, 0.1% (w/v) p-hydroxy phenylacetic acid, 0.025% (w/v) thimerosal.

4. ELISA Reaction buffer: 0.4 mg/ml o-phenylenediamine, 5 % (v/v) methanol, 0.01 % (v/v) H₂O₂ (*see Note 2*).
5. 2 N H₂SO₄.
6. ELISA 96-well plates (Nunc-Immuno Plates Maxisorb, Thermo Fisher Scientific, Inc., Roskilde, Denmark).

2.3 Immunoblotting

1. IB transfer buffer: 25 mM Tris, 192 mM Glycine, 20 % (v/v) methanol.
2. Tris buffered saline containing Tween-20 (TBS-T): 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1 % (v/v) Tween-20.
3. IB blocking solution: 5 % (w/v) skim milk in TBS-T.
4. IB second antibody solution: HRP-conjugated appropriate secondary antibody (Life Technologies) diluted at 1: 20,000 in TBS-T.
5. PVDF membrane (Immobilon P, Millipore, Bedford, MA).
6. Extra Thick Blot Paper (Bio-Rad, Hercules, CA).
7. Trans-blot® SD semi-dry transfer cell (Bio-Rad).
8. Can Get Signal™ (Toyobo, Osaka, Japan).
9. Electrochemiluminescence detection liquid (ECL; Life Technologies).
10. FUNA-UV-LINKER (Funakoshi, Tokyo, Japan).

2.4 Immunocytochemistry

1. PHEM: 60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 4 mM MgSO₄ (adjust pH 7.0 with KOH) [17].
2. IF blocking buffer: 5 % heat-inactivated donkey serum in PHEM (*see Note 3*).
3. Mouse monoclonal anti-Chk1 antibody (Clone G4, Santa Cruz Biotechnology, Santa Cruz, CA).
4. IF second antibody solution: Alexa Fluor 488-conjugated donkey anti-mouse or -rat IgG (Invitrogen, Carlsbad, CA) diluted at 1: 1,000 and 0.5 µg/ml of 4', 6-diamidino-2'-phenylindole-dihydrochloride (DAPI; Dojindo Laboratories, Kumamoto, Japan) in IF blocking buffer.

3 Methods

3.1 Immunization and Hybridoma Production

For each immunization, we use 100 µg of the conjugated protein (containing approximately 20 µg of synthetic peptide) per a mouse or a rat. Animal immunization, cell fusion, HAT selection, and limiting dilution for the establishment of monoclones are performed according to ordinary protocols for monoclonal antibody production (*see other Chapters*).

3.2 First Screening for Monoclonal Version of a Site and Phosphorylation State-Specific Antibody by ELISA

1. Dilute phosphorylated or non-phosphorylated version of peptide to 0.3 µg/ml with Peptide dilution buffer (*see Note 4*).
2. Add 50 µl of the peptide solution into each well of ELISA 96-well plates.
3. Incubate for 2 h at room temperature (RT) or overnight at 4 °C.
4. Remove the peptide solution from each well and wash with 100 µl of PBS/well three times.
5. Add 300 µl of ELISA blocking buffer into each well.
6. Incubate for 4 h at 37 °C or overnight at 4 °C.
7. Remove ELISA blocking buffer (*see Note 5*).
8. 5–9 days after cell fusion (*see Note 6*), collect each culture supernatant from each well in the 96-well culture dish. After the collection, add fresh growing medium into each well.
9. Use 2 types of ELISA plates per each supernatant; one is coated with phosphopeptide and the other with non-phosphorylated version of peptide as described above. Add 50 µl of each culture supernatant per well and then incubate for 1 h at 37 °C.
10. Remove the supernatant and then wash with 100 µl of PBS 5 times.
11. Add 100 µl of ELISA second antibody solution into each well and then incubate for 1 h at 37 °C.
12. Remove the supernatant and then wash with 200 µl of PBS 5 times.
13. Add 100 µl of ELISA reaction buffer (*see Note 2*) into each well and incubate for 1 h at RT.
14. Stop the reaction by the adding 100 µl of 2 N H₂SO₄ and measure the absorbance at 492 nm with an ELISA plate reader.
15. Pick up and propagate each desirable monoclonal (*see Note 7*), and then collect additional culture supernatant (0.5–1 ml) for the second and third screening.

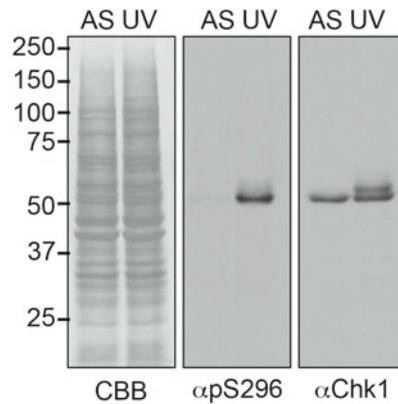
3.3 Second Screening by Immunoblotting

1. Prepare positive and negative control samples for SDS-PAGE. In the case of a monoclonal antibody against Chk1 phosphorylated at Ser296 [18], we prepared lysates of HeLa cells irradiated with (positive control) or without (negative control) 254-nm ultraviolet (UV) light (*see Note 8*).
2. Load a set of positive and negative control samples per each supernatant (*see Notes 9 and 10*). For the cut of transferred membrane, load pre-stained protein marker between sets of samples.
3. After SDS-PAGE, soak the gel in IB transfer buffer for 10 min at RT with rocking.

4. Wet PVDF membrane with methanol and transfer it to IB transfer buffer.
5. Soak an Extra Thick Blot Paper with IB transfer buffer. Place it in the lower electrode (anode) of the Bio-Rad Trans-blot® SD semi-dry transfer cell.
6. Place the PVDF membrane and then the gel on the top.
7. Soak another an Extra Thick Blot Paper with IB transfer buffer, put it on top, and mount the cathode.
8. Transfer for 1 h at RT. Set voltage to 25 V (actual starting voltage is around 6–7 V) and limit current to 1.5–2 mA/cm² gel area (*see Note 11*).
9. Soak the transferred membrane in IB blocking solution for 1 h or over night at RT with rocking.
10. Wash the membranes 3 times in TBS-T at RT with rocking, briefly. Then, cut the membrane on lanes of the pre-stained marker (*see Note 12*).
11. Soak a piece of membrane in each culture supernatant diluted at 1: 100 in TBS-T (*see Note 13*) at RT with rocking.
12. Wash the membrane twice (for 10 min each) in TBS-T at RT with rocking.
13. Soak the membranes in IB second antibody solution (*see Note 13*) for 30 min at RT with rocking.
14. Wash the membranes twice (for 10 min each) in TBS-T at RT with rocking.
15. For chemiluminescent detection, incubate the membranes in electrochemiluminescence detection liquid (equal volume of detection reagents 1 and 2; *see Note 14*) for 1 min.
16. Place the membranes in a film cartridge between two overhead transparency sheets and expose an X-ray film to the membrane (*see Note 15*).
17. Select desirable culture supernatant(s) which immunoreact specifically with band(s) corresponding to the position of a protein of interest in the positive control sample but not in the negative control sample (Fig. 2).
18. Pick up and propagate each desirable monoclonal (*see Note 16*).

3.4 Third Screening by Immunocytochemistry

1. Place the sterilized coverslip in a suitable tissue culture dish. Plate the cell suspension into the above dish (or chamber slide) and culture for at least 24 h (*see Note 17*).
2. To fix cells, transfer coverslips into wells of 3.7 % ice-cold formaldehyde in PBS (*see Note 18*). Incubate cells for 10 min.
3. Wash in ice-cold PBS, twice for 10 min each.
4. Permeabilize cell membrane by removing PBS and applying ice-cold 0.1 % Triton X-100 in PBS (*see Note 19*) for 10 min.



AS: non-treated, asynchronous HeLa cells (Negative Control)
 UV: HeLa cells irradiated with UV light (Positive Control)

Fig. 2 The second screening by immunoblotting in the case of an antibody against phosphoSer296 on Chk1 (α pS296). As a positive control, we treated HeLa cells as follows. The culture medium was removed and the cells were irradiated in uncover tissue culture dishes with 254-nm ultraviolet (UV) light at a dose of 10 J/m². Fresh culture medium was added back and the cells were incubated for an additional 2 h. We used non-treated (asynchronous; AS) HeLa cells as a negative control

5. Wash in ice-cold PBS, twice for 10 min each.
6. Transfer each coverslip into humidified chamber. Block non-specific binding sites by applying 50 μ l of IF blocking buffer per one 13-mm coverslip (*see Note 20*). Incubate for 30–60 min at RT.
7. Transfer coverslips into wells of PBS and then into humidified chamber. Apply 50 μ l of each culture supernatant per one 13-mm coverslip (*see Note 20*). Incubate for 1 h at RT.
8. Transfer coverslips into wells of PBS and then wash in PBS, twice for 10 min each.
9. Transfer each coverslip into humidified chamber and immediately apply 50 μ l of IF second antibody solution per one 13-mm coverslip (*see Note 20*). Incubate for 15 min at RT in the dark.
10. Transfer coverslips into wells of PBS and then wash in the dark, twice for 10 min each.
11. Gently dip the coverslip into deionized water to rinse the PBS salt from the sample.
12. Mount the coverslip and then analyze using fluorescent microscopy.
13. Select culture supernatant(s) showing desired specificity in all the screening. After the selection, we recommend co-staining with an antibody against a protein of interest if possible.

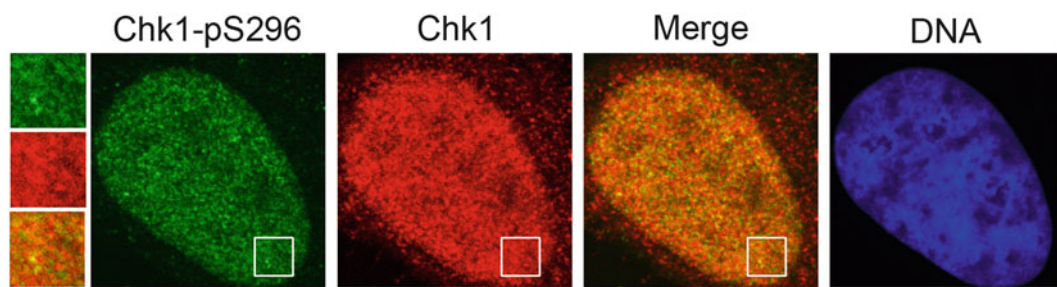


Fig. 3 Immunostaining of UV-irradiated HeLa cells with a rat monoclonal antibody α pS296 (a site and phosphorylation state-specific antibody for Ser296 on Chk1) and a mouse monoclonal anti-Chk1 antibody. HeLa cells were irradiated with UV light as described in the legend of Fig. 2. The immunoreactivity with α pS296 (green) and anti-Chk1 (red) was visualized through the incubation with Alexa Fluor 488-conjugated donkey anti-rat IgG and Alexa Fluor 555-conjugated donkey anti-mouse IgG, respectively. The nucleus was also stained with DAPI. Magnifications of insets are shown in the *left*. We observed no α pS296-immunoreactivity in non-treated cells (not depicted)

The specificity of a phospho-specific antibody is strongly supported by the colocalization of signals of both antibodies (Fig. 3).

14. Store each desirable monoclonal in liquid nitrogen. Collect each culture supernatant enough to carry out any additional assays and then freeze it in aliquots.

4 Notes

1. The usage of highly pure peptide (over 90 % purity) is the key to the production of excellent antibodies.
2. The solution should be freshly prepared.
3. Use the serum from the same organism from which you buy the secondary antibody.
4. The pH of the peptide solution should be 7.0–7.5. If not, use 50 mM $\text{Na}_2\text{CO}_3 \cdot \text{NaHCO}_3$ (pH 9.0) for dilution.
5. If the plates are not immediately used, leave the plates to air-dry for 5–10 min and then store them at 4 °C. In most cases, they can be stored at 4 °C for years.
6. ELISA assay should be performed before hybridomas in any well start to die.
7. Positive clones are defined as their supernatants showing at least 0.3 higher absorbance unit in phosphopeptide-coated plate than in its non-phosphorylated version.

8. Purified protein of interest (e.g., recombinant protein, immunoprecipitated protein) can be alternatively used for the screening. The protein for positive control should be phosphorylated at a target site in vitro or in vivo. Use a non-phosphorylated (dephosphorylated) protein or a protein mutated at a target phosphorylation site to Ala/Val/Phe as a negative control.
9. A protein of interest in the positive and negative control samples should be loaded in the same amounts. The loading amounts should be optimized for each screening. If the specific signal is too weak, increase the loading amounts. However, loading excess amounts may lead to non-specific reaction against other proteins or non-phosphorylated protein of interest.
10. We recommend loading one more set for an antibody against a protein of interest to detect its migrating position on the transferred membrane.
11. The transfer condition should be optimized for each protein. We recommend using an antibody against a protein of interest for this optimization.
12. This procedure should be quick so that the membrane stays moist.
13. Alternatively, dilute the primary and secondary antibodies with the immunoreaction enhancer solution such as Can Get Signal™, instead of TBS-T.
14. The mixture should be enough to cover the membranes completely.
15. We recommend exposing several X-ray films for different periods to determine the optimal exposure time (0.5–5 min exposure is used for most of our assays).
16. If it is difficult to determine desirable clones in the second screening, we recommend performing the third screening (using all ELISA-positive samples) preferentially.
17. Prepare both positive and negative control cells. In the case of a monoclonal antibody against Chk1 phosphorylated at Ser296 [18], we prepared UV-irradiated HeLa cells as a positive control and the non-treated cells as a negative control.
18. Before the third screening, we recommend determining which fixative and permeabilization reagent (see below) are most appropriate for a protein of interest using an antibody against it. Formaldehyde fixation masks some epitopes from recognition of some antibodies. Ice-cold methanol or 10 % trichloroacetic acid (TCA) is an alternative to formaldehyde and tends to preserve epitopes recognized by some antibodies [19]. In the case of methanol fixation, the treatment with permeabilization reagent is not required (see the chapter by Bauer in this book)

19. Various permeabilization reagents (e.g., Triton X-100, Saponin, methanol, acetone, etc.) perforate the cell membrane by different mechanisms.
20. This procedure should be quick so that the coverslips are not dried up. The coverslip should be completely covered with each solution.

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