Video Article

Oligopeptide Competition Assay for Phosphorylation Site Determination

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Abstract

Protein phosphorylation at specific sites determines its conformation and interaction with other molecules. Thus, protein phosphorylation affects biological functions and characteristics of the cell. Currently, the most common method for discovering phosphorylation sites is by liquid chromatography/mass spectrometry (LC/MS) analysis, a rapid and sensitive method. However, relatively labile phosphate moieties are often released from phosphopeptides during the fragmentation step, which often yields false-negative signals. In such cases, a traditional *in vitro* kinase assay using site-directed mutants would be more accurate, but this method is laborious and time-consuming. Therefore, an alternative method using peptide competition may be advantageous. The consensus recognition motif of 5' adenosine monophosphate-activated protein kinase (AMPK) has been established[1] and was validated using a positional scanning peptide library assay[2]. Thus, AMPK phosphorylation sites for a novel substrate could be predicted and confirmed by the peptide competition assays. In this report, we describe the detailed steps and procedures for the *in vitro* oligopeptide-competing kinase assay by illustrating AMPK-mediated nuclear factor erythroid 2-related factor 2 (Nrf2) phosphorylation. To authenticate the phosphorylation site, we carried out a sequential *in vitro* kinase assay using a site-specific mutant. Overall, the peptide competition assay provides a method to screen multiple potential phosphorylation sites and to identify sites for validation by the phosphorylation site mutants.

Video Link

The video component of this article can be found at https://www.jove.com/video/55708/

Introduction

Protein phosphorylation at a specific residue plays a significant role in a wide range of cellular processes. Thus, an understanding of signaling networks requires the identification of specific phosphorylation sites. In addition, the phosphorylation site determines the effect on protein function because individual domains within a protein possess different structures and functions. The activity of nuclear factor erythroid 2-related factor 2 (Nrf2), a key antioxidant transcription factor, is bi-directionally regulated through phosphorylation at different sites. Our studies have focused on the kinases that catalyze the phosphorylation of Nrf2. The stress response of Nrf2 against oxidative challenge occurs rapidly, mainly through phosphorylation at serine 40 and mediated by protein kinase C (PKC)-δ, which activates Nrf2[3,4]. Conversely, Fyn catalyzes the inhibitory phosphorylation of Nrf2 at tyrosine 568 for tight control of the activity[5].

The most common method used to discover phosphorylation sites is liquid chromatography/mass spectrometry (LC/MS) analysis. Rapid and highly sensitive phosphorylation site mapping data can be generated this way; however, it has several technical limitations, often generating false-negative signals. Poor sequence coverage frequently occurs in LC/MS analysis. To identify phosphorylation sites, information on the maximal amino acid coverage of a protein is required[6]. Proteolysis of the protein of interest with several proteases during the digestion step may be of help in improving sequence coverage. Another obstacle for the identification of phosphorylation residues is the facile loss of phosphoric acid that is frequently observed for serine- and threonine-phosphorylated peptides[6,7]. Labile phosphate moieties are often released from phosphopeptides during the fragmentation process[7]. The second option when searching for phosphorylation sites is using a peptide microarray method. It is possible to screen for the kinase target sites using a microarray chip containing peptide fragments derived from a protein of interest. However, due to the equipment requirement for the production and detection of a microarray chip, the peptide microarray method is considered time-consuming and expensive.

To overcome these challenges, an *in vitro* oligopeptide-competing kinase assay can be used for a protein kinase with known recognition motifs. If the consensus recognition motif of a kinase is established, putative phosphorylation sites of a candidate substrate can be predicted, and the authenticity of the sites can be validated. The most convincing method for this procedure is to show the abrogation of phosphorylation in a mutant protein in which the predicted residue is substituted with a non-phosphorylatable amino acid (*i.e.,* serine or threonine to alanine; tyrosine to phenylalanine). However, the production and isolation of mutant proteins is time-consuming. As an alternative at the initial stage of research, the competitive peptide kinase assay is straightforward and convenient. Here, we describe a protocol for an *in vitro* peptide competition assay and for the validation of the phosphorylation site.

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Protocol

1. Safety

CAUTION: This protocol uses [γ-^{32}P]-ATP to assess the activity of AMPK. Phosphorus-32 is a radioactive isotope, largely emitting beta radiation. Since the size of a beta particle is extremely small, it can easily penetrate clothing and skin. Both external and internal exposure to beta radiation may be harmful to human health, including by causing skin burns and tissue damage.

1. Carry out all the steps that require the use of [γ-^{32}P]-ATP using proper protection, such as acrylic shielding.
2. Ensure that all personnel are equipped with electronic personal dosimeters (EPD) to monitor the extent of exposure to harmful radiation during all procedures.
3. Handle open-source radiation only after appropriate training and regulatory approval.
   NOTE: Here, all experiments were conducted after the completion of training on open-source radiation use at the Seoul National University and the approval of the Korean government (nssc.go.kr/nssc/en).
4. Dispose of radioactive waste per local regulations.
   NOTE: Here, directives from the Institute of Environmental Protection & Safety of the Seoul National University were followed. The followings are a list of regulators in the United States and European countries: The United States of America, United States Nuclear Regulatory Commission (http://www.nrc.gov/); United Kingdom, Health and Safety Executive (HSE) (http://www.hse.gov.uk); France, Autorité de sûreté nucléaire (https://www.asn.fr/); and Germany, Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMU) (http://www.bmu.de).

2. *In Vitro* Competitive Kinase Assay

1. **Construction of Competitive Peptides**
   1. Determine the consensus motif phosphorylated by AMPK.
      NOTE: AMPK recognizes the consensus motif, Φ-[X,β]-X-X-Ser/Thr-X-X-X-Φ. In the consensus sequence, Φ represents hydrophobic amino acids (*i.e.,* valine [V], leucine [L], methionine [M], and isoleucine [I]) and β represents basic amino acids (*i.e.,* lysine [K], arginine [R], and histidine [H])[1].
   2. Select serine or threonine residues from target sequences according to the above consensus motif. Use three putative sites of human Nrf2 (#1, 148-157 comprising Ser153; #2, 330-339 comprising Ser335; and #3, 553-562 comprising Ser558)[8].
   3. Commercially synthesize 10-residue peptides that mimic the putative sites. Obtain the synthesized product as a lyophilized powder with a purity greater than 98% for each peptide.
   4. Dissolve the peptides using a kinase buffer to achieve a concentration of 71.667 g/L. If a peptide is not soluble in a kinase buffer, dissolve it in 20% DMSO. If it remains insoluble, a peptide mimicking the putative site may be extended to increase solubility.

2. ***In Vitro* Competitive AMPK Kinase Assay**
   1. Prepare 5× kinase buffer as follows: 100 mM HEPES, pH 7.4; 25 mM $MgCl_2$; 5 mM EGTA; 5 mM DTT; 125 mM β-glycerophosphate (serine/threonine phosphatase inhibitor); 5 mM Na_3VO_4 (tyrosine phosphatase inhibitor); 0.5% (v/v) Protease Inhibitor Cocktail Set III, 1 mM cold ATP; and 500 µM AMP.
      NOTE: AMP is an essential component of the kinase buffer to test AMPK activity. The buffer can be used to measure the activity of both serine/threonine kinases and tyrosine kinases.
   2. Thaw the following recombinant protein and synthetic peptides on ice: AMPK; Nrf2; and oligopeptides #1, #2, and #3.
   3. Prepare a reaction buffer on ice: 0.15 µg of AMPK, 0.4 µg of Nrf2 (~4 pmol), 0.43 mg of oligopeptide (~300 nmol), and 6 µL of 5× kinase buffer. Adjust the final volume to 30 µL with sterile distilled water (DW).
      NOTE: The addition of [γ-^{32}P]-ATP after preparing the reaction buffer may reduce the radioactive signal due to kinase autophosphorylation.

3. **Running the Reactants**
   NOTE: All the processes below must be carried out under protection with an acrylic shield, rack, or block, as well as appropriate personal protective equipment.
   1. Set heating blocks to 30 °C before starting the experiment.
   2. Add 1 µL of [γ-^{32}P]-ATP (1 µCi) to the reaction tubes on ice. Mix the reaction buffer by pipetting up and down several times.
      NOTE: The radioactivity of ^{32}P has a short half-life of approximately 14 days[9]. Adjust the volume by considering the half-life (*e.g.,* after one month from the production of [γ-^{32}P]-ATP, add 4 µL of [γ-^{32}P]-ATP to make 1 µCi).
   3. Incubate the sample in the heating block at 30 °C for 15-30 min. During this step, prepare 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Adjust the percentage of the gel according to the size of the target protein.
   4. Stop the kinase reaction by adding 3 µL of 10× SDS sample buffer (10% (v/v) glycerol, 20% (w/v) SDS, 15.42% (w/v) dithiothreitol, 90% (v/v) 0.5 M Tris-HCl (pH 6.8), and 0.02% (w/v) bromophenol blue). Mix the reaction buffer by pipetting up and down several times.
   5. Run the samples in a 7.5% SDS-PAGE gel at 70 V for 20 min and continuously at 140 V for 1 h.
      NOTE: The bromophenol blue line at the end of the gel contains extremely high radioactive signals. It is recommended to remove the gel below the blue line before proceeding to the next step to reduce background signals.
   6. After SDS-PAGE, gently remove the gel from the caster. Place the gel in a glass tube for the next step.
   7. Fix the gel with fixation buffer for 20 min (50% methanol and 10% glacial acetic acid).
   8. Gently move the gel onto filter paper and cover it with a transparent wrapper. Note that the gel may be easily torn at this step. Dry the gel with a vacuum gel dryer at 80 °C for 1 h.

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4. **Visualization**
    1. Expose the gel to either a phosphor screen or an X-ray film overnight (usually for approximately 16 h).
    NOTE: A phosphor screen can be reused after exposure to the extra-bright light. When an X-ray film is used, expose the gel over two days. X-ray films have lower sensitivities than phosphor screens.
    2. Scan the phosphor screen with a phosphor imager. Export the image as a high-resolution TIFF or bitmap file.
    3. After visualization, move the gel to a glass or plastic container for Coomassie brilliant blue (CBB) staining.
    4. Wash the gel with DW. Discard the DW and stain the gel with the staining reagent for 1 h.
    5. Discard the reagent and destain the gel with DW for 1 h or overnight. Place the gel between transparent plastic films (or the equivalent) to scan with an office scanner.

3. *In Vitro* Kinase Assay Using a Site-specific Mutant

1. **Site-Directed Mutagenesis of pGEX-Nrf2**
    1. Mutagenic Primer Design
    NOTE: Designing proper mutagenic primers is critical for the successful outcome of mutagenesis. The following are some considerations for designing mutagenic primers. Create a primer consisting of 25 to 45 bases, with the desired mutation in the middle of the primer. The primer should have 40-60% GC content and terminate with one or more G or C bases. Ensure that the T_m of the primer is equal to 78 °C or greater according to the formula [T_m= 81.5 + 0.41(% GC) - (675/length of bases) - % mismatch].
    NOTE: Purify the primer because its purity is important for mutation efficiency. There are several websites for designing mutagenic primers.
        1. Open the primer design program for "designing primers for site-directed mutagenesis" (http://www.genomics.agilent.com/primerDesignProgram.jsp).
        2. Paste human Nrf2 DNA sequence (Access No. NM_006164.4) and select the "upload translated" button.
        3. Select the serine 558 residue from the translated amino acid sequence to be changed to alanine. Using the mutagenic primer, substitute the codon for serine from agc to gcc, for alanine.
        4. Consider primer length, T_m, and the GC content.
        NOTE: The mutagenic primer is finally designed as 5'-ctactgaaaaaacaactc **gcc**accttatatctcgaag-3'.

    2. Reaction for Mutant Strand Synthesis using Polymerase Chain Reaction (PCR)
        1. Prepare the PCR reaction mixture on ice: 10× reaction buffer, 50 ng of pGEX-GST-Nrf2, 125 ng of forward primer, 125 ng of reverse primer, 1 µL of dNTP mix (10 mM each), 2.5 U *Pfu* DNA polymerase, and sterile DW to 50 µL.
        2. Run the PCR for pGEX-GST-Nrf2-mutant strand synthesis with the following cycling program: 1 cycle at 95 °C for 30 s; 18 cycles at 95 °C for 30 s, at 55 °C for 1 min, and at 68 °C for 90 s; and 1 cycle at 68 °C for 5 min.
        NOTE: Depending on the plasmid length, the cycling parameters may be changed.
        3. Place it on ice for 2 min to cool the reactants for the next step. Alternatively, store them at 4 °C overnight.

    3. *Dpn I* Digestion and the Selection of a Mutant Plasmid
        1. Add 1 µL of *Dpn I* restriction enzyme (10 U/µL) to each of the reactants after cooling. Mix thoroughly and gently by pipetting and centrifuge for 1 min.
        2. Incubate at 37 °C for 2 h to digest parental pGEX-GST-Nrf2 double-stranded DNA.
        3. Gently thaw *DH5 α* supercompetent cells on ice.
        4. Add 50 µL of the reactant to 150 µL of pre-chilled *DH5 α* supercompetent cells and incubate on ice for at least 30 min.
        5. Transfer the tubes to a heating block preheated to 42 °C and leave them for 90 s. Place them on ice for 2 min.
        6. Add pre-warmed lysogeny broth (LB) to the transformed *DH5 α* cells and incubate at 37 °C for 1 h with shaking at 180 rpm.
        7. Spread the transformed cells on LB-ampicillin agar plates and incubate overnight at 37 °C.
        NOTE: Ampicillin is used for selection since the plasmid GEX-GST-Nrf2 has the ampicillin resistance marker. Use appropriate antibiotics, depending on the resistance marker of the mutagenized plasmid.
        8. Pick a single colony from the plate, transfer it to 5 mL of LB-ampicillin, and incubate overnight it at 37 °C with shaking at 180 rpm. For verification of the mutagenic sequence, evaluate at least three colonies.
        9. Extract DNA using a DNA miniprep kit for DNA sequence analysis; follow the manufacturer's protocol.
        10. Verify the mutagenic DNA sequences using an automatic DNA sequence analyzer according to the manufacturer's protocol. Use at least 200 ng of the DNA construct for the verification of mutant sequences.

2. **Purification of Recombinant GST-Nrf2 Protein**
    1. Expression of Mutagenic GST-Nrf2 Fusion Protein in *Escherichia coli*
        1. Transform the mutagenic pGEX-GST-Nrf2 plasmid into *BL21* cells. Inoculate a single colony in 25 mL of LB broth containing ampicillin (100 µg/mL) and incubate at 37 °C on a rotator overnight.
        2. Inoculate the cultured cells in 100 mL of LB broth containing ampicillin (100 µg/mL) at a 1:100 dilution ratio.
        3. Incubate at 37 °C on a rotator until the absorbance at 600 nm reaches around 0.4-0.8 (approximately 4 h).
        4. Add 100 µL of 1 M IPTG to 100 mL of cultured cells to prepare a final concentration of 1 mM IPTG.
        5. Incubate the cells at 30 °C on a rotator for 6 h or overnight for protein expression.
        6. Centrifuge the cells at 5,000 x g for 20 min and resuspend the cell pellets with 1 mL of bacterial lysis buffer (25 mM HEPES, 5 mM EDTA, 2 mM DTT, 0.1% CHAPS, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, and 1 mM PMSF) on ice.
        7. Lyse the cell pellets using an ultrasonic homogenizer with 2-s intervals for 2 min at 50% of the maximum power output.
        8. Centrifuge the cells at 12,000 × g for 15 min at 4 °C and collect the supernatant.

    2. Purification of Recombinant GST-Nrf2 Protein

1. Prepare 200 µL of 50% (v/v) glutathione-agarose beads and wash with 1 mL PBS (pH 7.3; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). Collect the beads by centrifugation at 500 × g for 1 min. Repeat the washing step three times.
2. Add the cell lysate to the prepared 200 µL of 50% (v/v) glutathione-agarose beads and incubate at 4 °C on an end-over-end rotator for 2 h.
3. Collect the glutathione-agarose beads combined with GST-Nrf2 protein by centrifugation at 500 x g for 1 min. Remove the supernatant using a 1 mL syringe with a 31 G needle.
4. Wash the beads with 1 mL of PBS (pH 7.3), as in step 1.
5. Add 500 µL of elution buffer (50 mM Tris-HCl and 10 mM reduced glutathione, pH 8.0) to the glutathione-agarose beads combined with GST-Nrf2. Incubate at 4 °C on an end-over-end rotator for 30 min.
6. Centrifuge at 500 x g for 1 min and collect the supernatant.
7. Again add 300 µL of elution buffer to the beads and repeat steps 3.2.2.5-3.2.2.6 twice.

3. Confirmation of the Purified GST-Nrf2 Protein
   1. Prepare 5 µL of the sample for visualization of protein quantity with 0.5 µg of standard protein BSA.
   2. Run the samples in a 7.5% SDS-PAGE gel at 70 V for 20 min and then at 140 V for 60 min.
   3. Stain the gel with 0.1% CBB staining solution (0.1 g of CBB R250, 40 mL of 99% methanol, 10 mL of glacial acetic acid, and 50 mL of distillated H_2O) for 2 h and destain (30 mL of methanol, 10 mL of glacial acetic acid, and 60 mL of distilled H_2O) until the bands are clearly shown.
   4. Place the destained gel on filter paper and cover with a transparent wrapper. Dry the gel with a vacuum gel dryer at 80 °C for 1 h.
   5. Determine the quantity of mutant GST-Nrf2 protein with densitometry for the *in vitro* AMPK kinase assay.

3. ***In Vitro* AMPK Activity Assay with a Site-Specific Mutant**
   NOTE: The *in vitro* AMPK kinase assay is performed according to steps 2.2-2.4, using 0.4 µg of purified wildtype GST-Nrf2 or the GST-Nrf2-S558A mutant without peptide inhibitors. Follow all the radiation safety guidelines described in step 1.
   1. Prepare a reaction buffer on ice with 0.15 µg of AMPK, 0.4 µg of mutant or wildtype GST-Nrf2, and 6 µL of 5 kinase buffer. Fill up to 30 µL with sterile DW.
   2. Add 1 µL of [γ-^{32}P]-ATP (1 µCi/µL) to the reaction tubes on ice. Mix the reaction buffer by pipetting up and down several times and centrifuge.
   3. Incubate the samples in a heating block at 30 °C for 30 min.
   4. Stop the kinase reaction by adding 3 µL of 10× SDS sample buffer.
   5. Run in a 7.5% SDS-PAGE gel at 70 V for 20 min and then at 140 V for 1 h.
   6. Fix the gel with the fixation buffer for 20 min, move it onto the filter paper, and cover the gel with a transparent wrapper.
   7. Dry the gel with the vacuum gel dryer at 80 °C for 1 h and then expose it overnight to either a phosphor screen or an X-ray film.
   8. Scan the phosphor screen with a phosphor imager and move the gel to a glass tube for CBB staining after visualization according to step 2.4.

Representative Results

Figure 1 and **Figure 2** demonstrate the outcomes from repeated experiments in the previously reported paper[8]. Three different 10-residue oligopeptides mimicking the putative AMPK target sites (#1, 148-157 comprising Ser153; #2, 330-339 comprising Ser335; and #3, 553-562 comprising Ser558) were synthesized and used as competitors in the *in vitro* kinase assay. The phosphorylation of Nrf2 by AMPK was greatly diminished in the presence of oligopeptide #3 (**Figure 1**). Next, a site-directed mutagenesis assay was performed to validate the result of the peptidomimetic experiment. Given the inhibitory effect of oligopeptide #3 on the AMPK-mediated phosphorylation of Nrf2 (**Figure 1**), we generated a S558A-Nrf2 mutant. The phosphorylation levels were compared between the wildtype Nrf2 and the S558A mutant using the *in vitro* AMPK kinase assay. The single amino acid substitution of Nrf2 (Ser→Ala at 558) prevented AMPK from phosphorylating Nrf2, indicating that AMPK directly phosphorylates Nrf2 at Ser558 (**Figure 2**).

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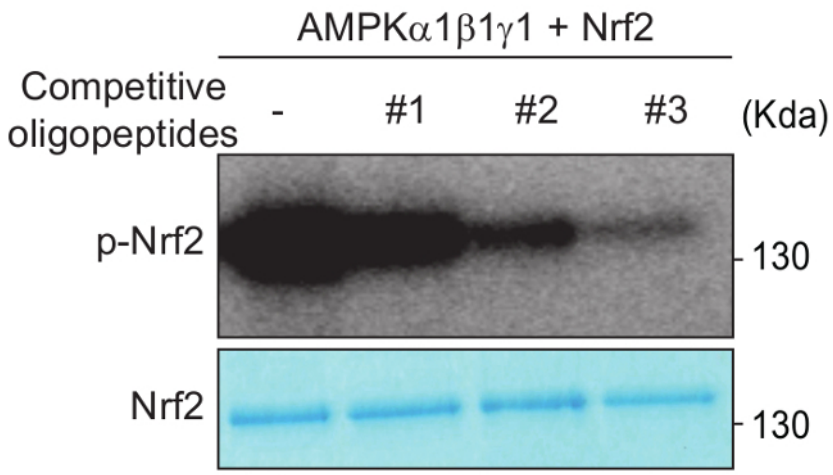

Figure 1: *In vitro* competitive AMPK kinase assay. *In vitro* kinase assays were done in the presence of the indicated oligopeptides (#1, 148-157 comprising Ser153; #2, 330-339 comprising Ser335; and #3, 553-562 comprising Ser558).

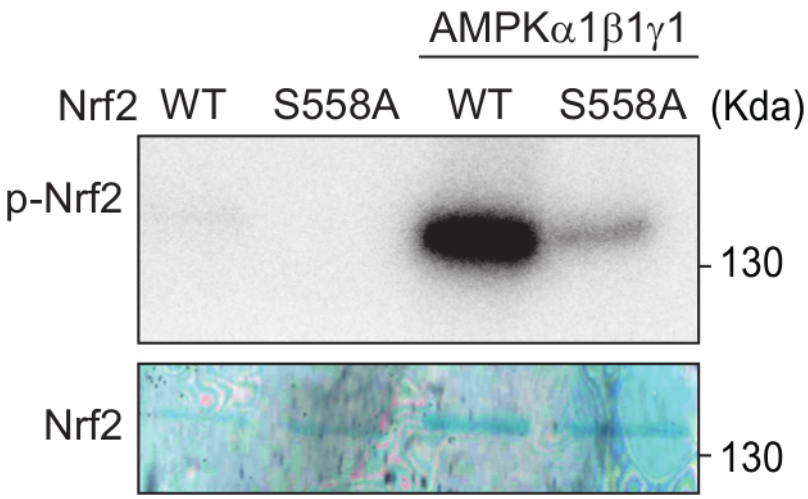

Figure 2: *In vitro* AMPK kinase assay using a site-specific mutant protein. *In vitro* kinase assays were done in the presence of wildtype GST-Nrf2 or its S558A mutant.

Discussion

As a simple and convenient way to assess the authenticity of the predicted phosphorylation sites mediated by AMPK, here we describe an *in vitro* kinase assay that can be used to discover a specific phosphorylation site using competitive peptides and to verify it using a site-specific mutant. The representative data obtained from the *in vitro* competitive AMPK activity assay matched the results from an assay using a site-directed mutant protein, indicating that the peptide competition assay is a useful tool for determining a phosphorylation site. This method was also used in the study identifying the specific site phosphorylated by PKCδ, which phosphorylates Nrf2 at serine 40[3]. Since peptides competitively bind to a protein, the principle may also be applied to an *in vitro* GST pull-down assay to identify a binding motif[10,11]. Moreover, this peptidomimetic method may also be applicable to the identification of other post-translational modifications, such as acetylation at lysine residues.

Using this protocol, it may be difficult to dissolve some hydrophobic peptides. If a peptide is insoluble in a kinase buffer, dissolving it in 20% DMSO may be helpful. To dissolve extremely hydrophobic peptides, first attempt to dissolve it in 100% DMSO and then dilute the solution with a kinase buffer. Sonication may also be of help in dissolving the peptides. If it remains insoluble, a peptide mimicking the putative site should be extended to increase the solubility.

The protocol introduced in this paper describes an "*in vitro* system" in which a purified kinase and substrate are reacted. In the *in vitro* system, small differences in the concentration of competitive peptides will not affect the results unless the concentration is lower than the substrate protein. The result obtained from an *in vitro* experiment includes the inherent possibility of being an artefactual event produced by artificial proximity and the high concentration of reactants. To eliminate this prospect, the interpretation of the data should be accompanied by the outcomes from cell-based experiments. Another possibility for obtaining false-positive results using peptides may be due to alterations in substrate conformation and/or kinase recognition. To exclude this possibility, it is necessary to do confirmation experiments with mutant proteins.

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On the other hand, false-negative data can also be produced from this system. Many kinases act as protein complexes within the cellular context and require co-factors for full functionality. AMPK is a representative protein kinase complex composed of AMPKα, AMPKβ and AMPKγ subunits, and the binding of AMP to the AMPKγ subunit is necessary for the activation of the kinase. Indeed, AMPK did not phosphorylate Nrf2 in an AMP-free kinase buffer (data not shown). Thus, it should be noted that AMP must be dissolved in the kinase reaction buffer for a successful *in vitro* assay.

The use of mass spectrometry for the identification of phosphorylation sites, the most popular method, has several technical limitations, often generating false-negative signals by the facile loss of phosphoric acid during the fragmentation process[7]. A peptide microarray method can be the second option to search for phosphorylation sites. However, this method is considered time-consuming and expensive. As an alternative method for screening putative phosphorylation sites, the competitive peptide method may be a convenient and inexpensive way to gain insight into the nature of a putative phosphorylation site.

Disclosures

The authors have nothing to disclose.

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