Preface

It has become clear that tumors result from excessive cell proliferation and a corresponding reduction in cell death caused by the successive accumulation of mutations in key regulatory target genes over time. During the 1980s, a number of oncogenes were characterized, whereas from the 1990s to the present, the emphasis has shifted to tumor suppressor genes (TSGs). It has become clear that oncogenes and TSGs function in the same pathways, providing positive and negative growth regulatory activities. The signaling pathways controlled by these genes involve virtually every process in cell biology, including nuclear events, cell cycle, cell death, cytoskeletal, cell membrane, angiogenesis, and cell adhesion effects. Mutations in tumor suppressor genes have been identified in familial cancer syndromes, and the same genes in many cases have been found to be mutationally inactivated in sporadically occurring cancers. In their normal state, TSGs control cancer development and progression, as well as contribute to the sensitivity of cancers to a variety of therapeutics. Understanding the classes of TSGs, the biochemical pathways they function in, and how they are regulated provides an essential lesson in cancer biology. We cannot hope to advance our current knowledge and to develop new and more effective therapies without understanding the relevant pathways and how they influence the present approaches to therapy. Moreover, it is important to be able to access not only the powerful tools now available to discover these genes, but also their links to cell biology and growth control.

The scope of this two volume work, *Tumor Suppressor Genes*, *Volume 1: Pathways and Isolation Strategies* and *Volume 2: Regulation, Function, and Medicinal Applications*, is broad in the sense that it covers all the known tumor suppressor pathways and provides key information on the road to their discovery, analysis, and uses in cancer therapeutics. The aim of the first volume, *Pathways and Isolation Strategies*, is to educate the reader about known TSGs and the relevance of the biochemical pathways they regulate to human cancer. The reader has an opportunity in Volume 1 to access state-of-the-art protocols that have been successful in the identification of TSGs in the past, and that can be applied to isolate novel TSGs. With a novel TSG in hand, the reader has an opportunity in Volume 2, *Regulation, Function, and Medicinal Applications*, to explore the cell biology and biochemical function of the encoded protein, as well as its physiological role in vivo. Finally, in Volume 2, the reader is exposed to strategies for the use of information on TSGs to develop diagnostic and therapeutic strategies for cancer.

The two volumes of *Tumor Suppressor Genes* bring together many of the world's experts in the identification and characterization of TSGs. The work is thus intended to become the core reference and compilation of the emerging path-

ways and the growing number of molecules that suppress cancer. Importantly, it should also serve as a wide-ranging source of protocols useful in understanding and characterizing the function of TSGs. One of the challenges facing cancer researchers and clinicians is to bring forward and develop active therapeutics. This book, by example, puts forward highly useful paradigms for rational drug design, based on our dramatic new understanding of molecular pathogenesis.

Tumor Suppressor Genes thus lays down a firm and timely foundation for understanding cancer. In this age of expression profiling and proteomics, there has already been revealed a remarkable complexity and interrelatedness of seemingly diverse processes and signal transduction pathways. For the student, this book provides a reference to the basics concerning the identity of the major TSGs and the signaling pathways they use to inhibit tumors. For the investigator, it provides not only a critical update, but also an extremely useful compendium of newly assembled research protocols, including both classical methods and state-of-the-art techniques. For the translational scientist, the book provides fertile ground for the development of therapeutic strategies based on understanding the mechanisms of action and appreciating the existing preclinical data. One of the criticisms of an effort leading to such a book is that the field is moving very quickly and material is likely to be outdated. However, with many of the world's leading experts providing a comprehensive overview of all tumor suppressing pathways, along with their detailed protocols, we believe we have provided an invaluable resource for continued learning and discovery. Finally, *Tumor Suppressor Genes* provides a bridge to those interested in translational research by giving examples of the rationale for many of the most promising manipulations that may lead to novel therapeutic agents.

Tumor Suppressor Genes is targeted at a broad audience including medical and graduate students, postdoctoral fellows, physician scientists, academics, and principal investigators. The text provides the critical information needed to rapidly gain appreciation of important TSGs in human cancer, as well as modern methods for their discovery, analysis, and clinical application. The reader is enabled to learn the background and then access the literature in which studies designed to define the biology and biochemistry of known TSGs have been performed. Details of protocols with examples of their previous uses allow the researcher to apply current technologies to novel or known genes whose role has not yet been defined.

In summary, *Tumor Suppressor Genes* is a comprehensive compilation of the known tumor suppressing pathways, and the key molecular approaches to their discovery, analysis, and clinical applications. It should be of enduring value to students at all levels of experimental biology and medicine, as well as those clinicians who want to better understand the molecular biology of cancer.

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Generation and Application of Phospho-specific Antibodies for p53 and pRB

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1. Introduction

The functions of many proteins are likely to be regulated by phosphorylation. Thus, antibodies that can recognize specifically phosphorylated sites on proteins have a wide variety of uses for studying the function and regulation of phosphoproteins. We have improved methods for generation of phosphorylation site-specific antibodies and have successfully obtained antibodies for the analysis of most of the phosphorylation sites on p53 and RB proteins.

The RB protein (pRB) was first shown by Taya and colleagues to be phosphorylated by a cyclin-dependent kinase (Cdk) at multiple sites in vitro (reviewed in **ref. *1***). Subsequently a variety of novel Cdks and their inhibitory proteins have been identified, and it is commonly understood now that phosphorylation of pRB by Cdks plays a key role in the regulation of cellular proliferation and in cancer ***(1–3)***. In an attempt to elucidate the physiologic relevance of phosphorylation of pRB, it was decided to generate antibodies to recognize specific phosphorylation sites of pRB using chemically synthesized phosphopeptides as antigens. However, it was not easy to synthesize peptides containing stably phosphorylated serine or threonine. Therefore, we have improved methods for obtaining such peptides.

Taking advantage of this improved methodology, the production of antibodies to specific phosphorylation sites of pRB was initiated. After demonstrating that this approach is successful for pRB, we also embarked upon generation of a series of antibodies specific for specific phosphorylation sites of p53.

We generally synthesize phosphopeptides as shown in **Fig. 1** for immunization of rabbits or mice. Cys is coupled to the N-terminus of most peptides to allow conjugation with KLH for more effective immunization. Because epitopes of antibodies can comprise as few as three or four amino acid residues, it is recommended that, when possible, only three residues be placed C-terminal to phosphoserine/threonine. In our experience we have observed that if there are more residues on this external side of the phosphorylated residue, antibodies directed against unphosphorylated epitopes are pref-

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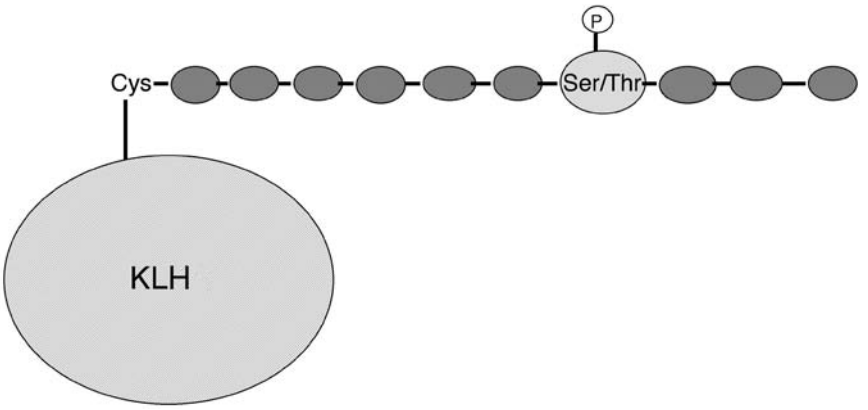

Fig. 1. Schematic presentation of a phosphopeptide antigen.

erentially produced. It is acceptable to include 6 amino acids plus N-terminal Cys on the other (internal) side of the phosphorylated residue.

If the peptide is too hydrophobic this will render it insoluble, so it is better to replace 2 or 3 amino acids on the internal side (Cys side) with 2 or 3 Arg residues. For the purpose of chemical synthesis, internal Met or Cys residues should be avoided. When an inappropriate amino acid residue is located on the N-terminal side, it is recommended to exchange the length of the N-terminal side with that of the C-terminal side, putting the Cys residue for conjugation with KLH at the C-terminus.

1.1. Chemical Synthesis of Phosphopeptides

Establishing methods for generating the most effective phosphopeptides is very important. To synthesize phosphopeptides, there are two basic strategies: (a) The prephosphorylation method, in which the protected phosphorylated amino acid derivatives are used as the building blocks of peptides; or (2) the postphosphorylation method, in which the unprotected hydroxy groups are phosphorylated on the completely assembled peptide chain. In this chapter we focus on the method we have used most extensively, which is the prephosphorylration procedure for the synthesis of desired phosphorylated peptides involving the *t*-butoxycarbonyl (Boc), and the 9-fluorenylmethoxycarbonyl (Fmoc) strategies.

2. Materials

2.1. Recommended Standard Synthesis of Phosphopeptides by the Boc Strategy (4,5)

1. Automated peptide synthesizer: ABI-430A (Applied Biosystems, Foster City, CA, USA).
2. Resin: Boc-amino acid preloaded PAM resin (Applied Biosystems, Foster City, CA, USA)
3. All of the Boc-amino acid derivatives Arg (Mts), Glu (OBzl), Asp (OBzl), Cys (MeOBzl), His (Bom), Lys (Cl-Z), Ser (Bzl), Thr (Bzl), Tyr (Br-Z), Trp (Hoc) were purchased from Peptide Institute (Osaka, Japan), and the phosphorylated, Boc-Ser (ALL2) and Boc-Thr (PO3All2) were synthesized for our own uses ***(6,7)***.

4. Preparative reversed-phase (RP)-HPLC: YMC-Pack ODS-AM (30 × 250 mm, YMC, Kyoto, Japan) using Shimdzu LC-8 A HPLC Apparatus.

2.2. Recommended Standard Synthesis of Phosphopeptides by the Fmoc Strategy (8)

1. Automated peptide synthesizer: ABI-433A (Applied Biosystems, Foster City, CA, USA).
2. Resin: Fmoc-amino acid preloaded *p*-hydroxymethyl-phenoxymethylated resin (Wang resin) (0.25-mmol sacle) (Applied Biosystems, Foster City, CA, USA).
3. All of the Fmoc-amino acid derivatives were purchased from Peptide Institute (Osaka, Japan).
4. Arg (Pbf), Glu (OtBu), Asp (OtBu), Cys (Trt), His (Trt), Lys (Boc), Ser (tBu), Thr (tBu), Tyr (tBu), Trp (Boc), and the phosphorylated Fmoc-amino acids, Fmoc-Ser(PO(OH)OBzl) and Thr(PO(OH)OBzl), were purchased from Watanabe Chemical Industries (Hiroshima, Japan) or Novabiochem (Laufelfingen, Switzerland).
5. Preparative RP-HPLC: YMC-Pack ODS-AM (30 × 250 mm, YMC, Kyoto, Japan) using Shimdzu LC-8 A HPLC apparatus.

3. Methods

3.1. Recommended Standard Synthesis of Phosphopeptides by the Boc Strategy (4,5)

3.1.1. Synthesis of Protected Peptide Resin by the Boc Strategy

The elongation of the desired protected peptide resin was carried out by the 0.5-mmol scale standard protocol of the benzotriazol active ester method in the system software version 1.40. The synthesis starts with the first Boc-amino acid attached to the PAM resin. After deprotection (50% trifluorocetic acid [TFA]/dichloromethane [DCM] (v/v) of the Boc group of first amino function, the next Boc-amino acid was coupled (N,N′-dicyclohexylcarbodiimide [DCC]/N-hydroxybenzotriazole [HOBt]). Consecutive deprotection/coupling steps accomplished chain elongation. The finally obtained protected peptide resin was dried and used for the following deprotection procedure (*see* **Note 1**).

3.1.2. Deprotection and Cleavage Procedure by TFMSA

To the peptide resin (0.3-mmol scale) were added thioanisole (36 mmol, 120 eq), *p*-cresol (36 mmol, 120 eq), trifluoromethanesulfonic acid (TFMSA) (36 mmol, 120 eq), and TFA (30 mL) in a round-bottom flask (500 mL) with stirring at −2−0° (*see* **Note 2**).

After the reaction mixture was stirring for 2.5 h at −2−0°, the reaction mixture was diluted by dry ether (300 mL) at −2−0°. Thus obtained precipitate was filtered off by the aid of a microfilter, and the crude phosphopeptide was extracted by TFA to separate the resin. Dry ether was added again to the TFA solution obtained, and the crude phosphopeptide precipitate was filtered off and dried over NaOH *in vacuo*.

3.1.3. Purification Procedure

To a solution of thus obtained crude compound (0.3 mmol) in water (20 mL) was added dithiothreitol (DTT) (1.5 mmol), and the pH of the solution was adjusted to pH 8–9 with dilute ammonia aqueous to prepare the thiol-free peptide (*see* **Note 3**). After the mixture was allowed to stand at room temperature for 10–20 min, the reduction reac-

tion was stopped by addition of TFA. The mixture was immediately purified by preparative RP-HPLC (CH_3CN/0.1% TFA aq. linear gradient system). The desired fractions were combined and lyophylized to obtain the purified titred phosphopeptide.

Isolated peptide was identified by amino acid analysis and mass spectrometry. The purity of the final product was inspected by analytical RP-HPLC.

3.2. Recommended Standard Synthesis of Phosphopeptides by the Fmoc Strategy (8)

3.2.1. Synthesis of the Protected Peptide Resin by the Fmoc Strategy

The elongation of the desired protected peptide resin was carried out by the 0.25-mmol-scale standard protocol of the 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/diisopropylethylamine (DIPEA) method in the system software. The synthesis starts with the first Fmoc-amino acid atached to the Wang resin (0.25 mmol).

After deprotection (piperidine/dimethyl sulfoxide (DMF), (1/4 v/v) of the Fmoc group of the first amino function, the next Fmoc amino acid was coupled (HBTU/DIPEA). Consecutive deprotection/coupling steps accomplished chain elongation. The finally obtained protected peptide resin was dried and used for the following deprotection procedure.

3.2.2. Deprotection and Cleavage Procedure by TFA

To the peptide resin (0.25 mmol) were added TFA (20 mL), triisopropylsilane (TIS) (0.54 mL), H_2O (0.54 mL), and thiophenol (0.54 mL) (92.5/2.5/2.5/2.5, v/v) in a round-bottom flask (500 mL) with stirring at room temperature.

After the reaction mixture was stirring for 1.5–2 h at room temperature, the reaction mixture was diluted by dry ether (300 mL). The precipitate obtained was filtered off with a microfilter, and the crude phosphopeptide was extracted with 0.1% TFA to separate the resin. The extracted solution was lyophylized to obtain the desired crude phosphopetide. The crude product was purified in the same way as described for the Boc Strategy.

3.3. Generation of Phospho-specific Antibodies

3.3.1. Coupling Peptide to Carrier Protein with MBS

1. Dissolve keyhole limpet hemocyanin (KLH) to a concentration of 16 mg/mL in 1 mL of 10 m*M* sodium phosphate buffer, pH 7.2 (*see* **Note 4**).
2. Prepare 280 mg/mL of *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) in dimethylformamide freshly.
3. Add 10 µL of MBS with stirring to avoid a high local concentration and continue to stir for 30 min at room temperature.
4. Centriguge at 15,000 rpm at 4°C for 5 min. Take a supernatant.
5. Separate the MBS-activated KLH from the free MBS by gel filtration on Sephadex G25 equilibrated with 50 m*M* sodium phosphate buffer, pH 6.0.
6. Pool MBS-activated KLH and divide equally into 10 microcentrifuge tubes. Store at −80°C until use.
7. Thaw MBS-activated KLH in one tube.
8. Add 0.5 vol of 0.2 *M* Na_2HPO_4 (pH should be 7.3–7.5).

9. Dissolve 1 mg of the peptide containing cysteine residue at the N- or C-terminus in 0.5 mL of distilled water, and add to MBS-activated KLH.
10. Stir the reaction for 3 h at room temperature.
11. Divide equally into 10 microcentrifuge tubes. Store at −80°C until use.

3.3.1.1. Deoxidation of Peptide

When free sulfhydryl residue in the peptide seems to be oxidized, the peptide should be deoxidized by the following protocol. (Ellman's reagent can be used to determine if there are free sulfhydryls available on the terminus of the peptide.)

1. Dissolve 1 mg of the peptide containing cysteine residue at the N- or C-terminus in 0.5 mL of 20 m*M* sodium phosphate buffer, pH 8.5.
2. Add $NaBH_4$ to a final concentration of 0.1 *M*.
3. Stand the reaction for 45 min at room temperature.
4. Adjust the final pH to 4.0 by adding 0.1 *N* HCl.
5. Stand the reaction for 5 min at room temperature.
6. Adjust the final pH to 7.5 by adding 0.2 *M* Na_2HPO_4.
7. Add to MBS-activated KLH.
8. Stir the reaction for 3 h at room temperature.
9. Dialyze against phosphate-buffered saline (PBS) at 4°C overnight.
10. Divide equally into 10 microcentrifuge tubes. Store at −80°C until use.

3.3.2. Production of Anti-phosphopeptide Polyclonal Antibody

3.3.2.1. Immunization of Rabbits

Two or more female rabbits (weight ~2.5 kg) were immunized subcutaneously by standard protocol with ~100 μg of phosphopeptide-KLH conjugate emulsified with Freund's complete adjuvant (FCA) 4 times at biweekly intervals, and boosted with phospho-peptide-KLH conjugate in Freund's incomplete adjuvant (*see* **Note 5**). One week after boost, bleed approx. 70 mL from marginal ear vein of rabbits.

3.3.2.2. Ammonium Sulfate Precipitation

1. Incubate rabbit antiserum (~30 mL) for 30 min at 56°C (heat inactivation).
2. Centrifuge at 15,000 rpm at 4°C for 5 min.
3. Transfer the supernatant to an appropriate vessel.
4. Determine the volume of serum and add an equal volume of PBS.
5. Add slowly 0.8 vol of saturated ammonium sulfate solution.
6. Stir gently for 15 min at room temperature.
7. Centrifuge at 15,000 rpm at 4°C for 15 min.
8. Discard the supernatant.
9. Resuspend the precipitate with PBS (2 vol of initial antiserum).
10. Repeat **steps 4–7.**
11. Resuspend the precipitate with PBS (0.5 vol of initial antiserum)
12. Dialyze against 50 vol of PBS using dialyzing tubing at 4°C overnight (3 changes).
13. Centrifuge at 15,000 rpm at 4°C for 15 min for remove any remaining debris.

3.3.2.3. Coupling the Peptide-Containing Cysteine Residue to the Gel

1. Pack 1 mL of iodeacetyl immobilized gel (SulfoLink coupling gel: Pierce, Rockford, IL, USA) into appropriate-sized column (*see* **Note 6**).
2. Equilibrate the column with 6 column vols of 50 m*M* Tris-HCl, pH 8.5, and 5 m*M* EDTA-Na.

3. Dissolve 2 mg of the peptide containing cysteine residue at the N- or C-terminus in 1 mL of 50 m*M* Tris-HCl, pH 8.5, and 5 m*M* EDTA-Na.
4. Apply the peptide solution on the column and rotate the column for 1 h at room temperature or overnight at 40°C.
5. Wash the column with 3 column vols of 50 m*M* Tris-HCl, pH 8.5, and 5 m*M* EDTA-Na.
6. Add 1 mL of 50 m*M* cysteine solution to the column to block any remaining active iodeacetyl groups, and rotate the column for 1 h at room temperature.
7. Wash the column with at least 15 column vols of 1 *M* NaCl and 15 column vols of 0.05% NaN_3.
8. Equilibrate the column with 6 column vols of PBS containing 0.3 *M* NaCl.

3.3.2.4. Affinity Chromatography

1. Equilibrate the phosphopeptide coupled gel in a column with 5 gel vols of PBS containing 0.3 *M* NaCl.
2. Filter the γ-globulin fraction of the antiserum using a Millipore filter (pore size: 0.45 μm).
3. Apply the antibody solution onto the column and pass through the column twice.
4. Wash the column with 10 column vols of PBS containing 0.3 *M* NaCl and 0.1% Triton X-100.
5. Wash the column with 10 column vols of PBS containing 0.3 *M* NaCl.
6. Elute with 0.17 *M* glycine-HCl (pH 2.5) containing 10% glycerol. Collect 2 mL of each eluate in appropriate tubes containing 400 μL of 1 *M* Tris-HCl (pH 8.0) on ice.
7. Identify the immunoglobulin-containing fractions by measuring absorbance at 280 nm (1 OD = 0.7 mg/mL).
8. Dialyze these fractions against PBS at 4°C overnight (3 changes).
9. Determine the antibody concentration by measuring absorbance at 280 nm.
10. Apply the antibody solution onto non-phosphopeptide-coupled gel column equilibrated with PBS containing 0.3 *M* NaCl for absorption of anti-non-phosphopeptide antibody and pass-through the column twice. Collect the pass through fraction.
11. Determine the antibody concentration by measuring absorbance at 280 nm.
12. Confirm the reactivity against phosphopeptide and the absence of reactivity against non-phosphopeptide by ELISA using non-phosphopeptide- and phosphopeptide-coated microplates.

3.3.2.5. ELISA for Measurement of Specificity

1. Coat a 96-well microtiter plate with 50 μL/well of each synthetic peptide at the concentration of 5 μg/mL in 0.1 *M* $NaHCO_3$ (pH 9.0).
2. Incubate the plate at 4°C overnight. Wells are emptied, and uncoated sites on the plates are blocked by the addition of 200 mL of 2% bovine serum albumin (BSA) and 1% sucrose in PBS, followed by incubation for 2 h at room temperature.
3. Wells are emptied and incubated with 50 μL/well of serially diluted anti-phosphopeptide antibody for 1 h at room temperature.
4. Wash microtiter plate 3 times with PBS containing 0.05% Tween-20.
5. Add 50 μL/well of 1:30,000-diluted horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (MBL) or anti-mouse IgG (MBL) in PBS containing 1% BSA.
6. Incubates for 1 h at room temperature.
7. Wash microtiter plate 3 times with PBS containing 0.05% Tween-20.
8. Add 50 μL/well of 1 m*M* tetramethyl benzidine (TMB) in 0.1 *M* sodium acetate, pH 6.0, containing 0.01% H_2O_2 for 15 min at room temperature.
9. The reaction is stopped by adding 50 μL/mL of 2 *N* H_2SO_4, and absorbance at 450 nm is determined using a microplate reader.

3.3.3. Production of Anti-phosphopeptide Mouse Monoclonal Antibody

3.3.3 1. Immunizing Mice

Four or more female Balb/c mice (4–5 wk) were immunized at the footpad with 10–20 μg of phosphopeptide-KLH conjugate emulsified with Freund's complete adjuvant (FCA) twice at 4-d intervals, 3 d after last immunization, boosted with ~20 μg of phospho-peptide-KLH conjugate in Freund's incomplete adjuvant (*see* **Notes 7** and **8**).

3.3.3.2. Producing Hybridomas

Production of hybridoma is according to standard protocol using polyethylene glycol except that the lymphocytes from two leg lymph nodes of immunized mice are fused to myeloma cells. This protocol is very efficient for producing hybridomas that secret anti-phospho specific monoclonal antibody when an appropriate mouse strain was selected.

3.4. Applications

3.4.1. p53

When cells are exposed to a variety of stresses including DNA damage, heat shock, hypoxia, and osmotic shock, p53 protein accumulates rapidly and is also activated as a transcriptional factor, which then leads to growth arrest or apoptosis. We have shown that phosphorylation plays an important role in regulating stabilization and activation of p53 using phospho-specific antibodies.

At least 8 in-vivo phosphorylation sites are known in human p53 ***(9,10)***. We have generated rabbit polyclonal antibodies to recognize each phosphorylation site. In addition, we have also made antibodies for several other potential phosphorylation sites. Although monoclonal antibodies for several sites were obtained, polyclonal antibodies are much stronger and normally they have been used for most of our experiments.

3.4.1.1. Ser-15

We showed that phosphorylation of Ser-15 occurs after DNA damage and is important for its release from its negative regulator MDM2 ***(11,12)***. Phosphorylation of Ser-20 is also involved in this release. Thereafter, Ser-15 was shown to be phosphorylated by ATM ***(13–15)*** or ATR ***(16)***.

3.4.1.2. Ser-20

We showed that Chk1 and/or Chk2 directly phosphorylate this site ***(17)***.

3.4.1.3. Ser-33

Two different kinases have been shown to phosphorylate Ser-33: the cyclin H CDK7/MAT1 complex ***(18)*** and the p38MAP kinase ***(19,20)***.

3.4.1.4. Ser-46

We showed that phosphorylation of this site regulates apoptosis induction by p53 ***(21)***. It was also shown that p53DINP1 is involved in this phosphorylation ***(22)***.

3.4.1.5. Ser-392

This site was found to be specifically phosphorylated after DNA damage by UV but not γ-radiation or etoposide-induced DNA damage ***(23)***.

3.4.2. pRB

There are about 13 in-vivo phosphorylation sites on pRB ***(1)***. In an attempt to elucidate the physiologic function of pRB, we generated all antibodies to recognize each phosphorylation site. Using these antibodies, we showed that Ser-780 is phosphorylated by Cdk4-cyclin D1 but not by Cdk2-cyclin E or by Cdk2-cyclin A ***(24)***. This was the first clear demonstration of different substrate specificities of two cyclin-dependent kinases, Cdk4 and Cdk2. This study also established the presence of a Cdk4-specific phosphorylation site on pRB. Subsequently, we showed that 13 phosphorylated sites can be classified to Cdk4- or Cdk2-specific sites using these antibodies (**ref. *25*** and unpublished data).

In contrast to p53, strong and good monoclonal antibodies were obtained for phosphorylation sites of pRB. Probably, this is at least partially due to the abundance of pRB in cells compared to p53.

4. Notes

1. Caution: DCC is an aggressive allergen. To avoid the chemical hazard, you have to avoid direct contact with the DCC solution, and it is necessary to perform all handling in a well-ventilated hood.
2. Caution: TFMSA is a very strong acid. It must be slowly added with careful handling in a well-ventilated hood.
3. Cys residue is necessary for the conjugation with corresponding carrier protein at the N- or C-terminus position of your peptide sequence. If Cys residue is not in your peptide sequence, this DTT reduction procedure is not necessary. Then, the TFMSA-treated products can be used directly for the purification procedure.
4. Alternatively, Bovine Serum Albumin (BSA) or Ovalbumin (OVA) can be used as a carrier protein.
5. TiterMax Gold (CytRx Corp., Atlanta, GA, USA) or RIBI adjuvant (RIBI ImmunoChem Research, Hamilton, MT, USA) can be substituted for FCA.
6. NHS-activated Sepharose 4 Fast Flow, Thiopropyl Sepharose 6B (Amersham Pharmacia Biotech, UK) can be used for this purpose.
7. If any positive clone could not be obtained using Balb/c mice, another strain, A/J, DBA2, or C57Black, has to be used.
8. TiterMax Gold (CytRx Corp., Atlanta, GA, USA) or RIBI adjuvant (RIBI ImmunoChem Research, Hamilton, MT, USA) can be substituted for FCA.

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