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(54) **METHODS FOR GENERATING PHOSPHORYLATION SITE-SPECIFIC IMMUNOLOGICAL REAGENTS**

METHODEN ZUR HERSTELLUNG IMMUNOLOGISCHER REAGENTIEN POSITIONSSPEZIFISCH FÜR PHOSPHORYLIERUNGEN

PROCEDE POUR GENERER DES REACTIFS IMMUNOLOGIQUES SPECIFIQUES AU SITE DE PHOSPHORYLATION

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- (56) References cited:
WO-A-01/40268 **US-A- 5 580 742**
US-A- 5 599 681 **US-A- 5 814 459**
- **OTAKA A ET AL: "Synthesis and Application of**
N-Boc-L-2-amino-4-(diethylphosphono)-4,4
difluorobutanoic acid for Solid-Phase Synthesis
of Nonhydrolyzable Phosphoserine Peptide
Analogues" TETRAHEDRON LETTERS,
ELSEVIER SCIENCE PUBLISHERS,
AMSTERDAM, NL, vol. 36, no. 6, 6 February 1995
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- HIGASHIMOTO YUICHIRO ET AL: "Human p53 is phosphorylated on serines 6 and 9 in response to DNA damage-inducing agents" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 275, no. 30, 28 July 2000 (2000-07-28), pages 23199-23203, XP002303535 ISSN: 0021-9258

DescriptionBackground of the Invention

[0001] Protein phosphorylation is important in the regulation of a wide variety of cellular processes. Regulation of protein activity by phosphorylation of serine, threonine, and tyrosine residues is highly utilized. Histidine, arginine, and lysine residues on proteins are also phosphorylated by cellular processes, but the significance is unknown due to the difficulty of studying these highly unstable modifications. The detection and quantitation of changes in the phosphorylation state of a protein is of great utility in the study of its functional significance.

[0002] Standard methods for measuring the state of protein phosphorylation typically involve prelabeling of the added phosphate moiety by incorporation of a radioactive isotope of phosphorous (as a phosphate). Such phosphorylation assays suffer from several methodological pitfalls, including health risks and disposal problems associated with the high amounts of [³²P]Pi required for the prelabeling experiments, the bother of working with regulated substances, and a lack of site specificity when several sites are phosphorylated in one protein or peptide moiety. As a result of these drawbacks, immuno-chemical based methods for detecting protein phosphorylation state are increasing in popularity. The degree of sensitivity and selectivity achievable with immuno-chemical methodology makes it an attractive alternative (Matsui et al., *J. Cell. Bio.* 140: 647-657 (1998); Conrad et al., *Hybridoma* 16: 167-173 (1997)).

[0003] Phosphorylation state-dependent monoclonal antibodies specific for a variety of cytoskeletal proteins have been produced and characterized. These antibodies were isolated by immunization protocols in which the specific targeting of phosphorylated epitopes was not the primary objective. More recently, small synthetic phosphopeptides have been used to improve the chance of targeting antibody production to epitopes on the phosphorylation sites (Sakaguchi et al., *Genes and Dev.* 12: 2831-2841 (1998); Matsui et al., *J. Cell Bio.* 140: 647-657 (1998); Chen et al., *FASEB J.* 2: A550 (1988); Czernik et al., *Methods in Enzymology* 201: 264-283 (1991)). While more direct, this method still suffers from the limitation of rapid dephosphorylation of the polypeptide antigen upon immunization which reduces the titer of phospho-specific antibodies. This is particularly a problem when using antigen containing phosphoserine and phosphothreonine, both of which usually are considerably less stable than phosphotyrosine.

Summary of the Invention

[0004] The present invention provides methods for generating antibodies which specifically react to a polypeptide phosphorylated at a particular amino acid. Methods for generating both monoclonal and polyclonal antibodies are provided. The method involves providing a polypeptide which has an incorporated mimetic of the phosphorylated amino acid residue. The mimetic has antigenic determinants also present on the naturally phosphorylated amino acid. The polypeptide antigen is used by standard methods to generate either monoclonal or polyclonal antibodies which cross-react with the natural phosphorylated polypeptide, and specifically recognize a specific phosphorylation state of the polypeptide.

[0005] The mimetics contains a non-hydrolyzable linkage between the carbon atom and the phosphorous atom (of the phosphate group). In a preferred embodiment, this linkage is a CF₂ group. Incorporation of this linkage group into phosphoserine produces the mimetic F₂Pab. F₂Pab is used in place of phosphoserine in a polypeptide sequence derived from p53 to produce antibodies which recognize a specific phosphorylation state of p53. In another embodiment, the CF₂ linkage group is incorporated into phosphothreonine to produce the mimetic F₂Pmb. In another embodiment the CF₂ linkage is incorporated into phosphotyrosine to produce the mimetic F₂Pmp.

Brief Description of the Drawings**[0006]**

Figure 1 represents analogues of phosphoserine and phosphothreonine.

Figure 2 represents analogues of phosphotyrosine.

Figure 3 is a diagrammatic representation of data from an ELISA using the affinity-purified pAbF15 antibody after passage through a (p53(Ac-11-22)Cys) column to deplete antibodies which cross-react with the unphosphorylated peptide.

Figure 4 is a diagrammatic representation of data from an ELISA using the affinity-purified pAbF15 antibody after passage through a (p53(Ac-32-43) (37P)Cys) column to deplete antibodies which cross-react with p53 phosphorylated at serine 37.

Detailed Description of the Invention

[0007] The present invention is based in part on the discovery that phosphopeptide mimetics previously used as phosphatase inhibitors (Burke et al., *Biochem. Biophys. Res. Commun.* 204: 129-134 (1994); Chen et al., *Biochem. Biophys. Res. Commun.* 216: 976-984)) are antigenic. More specifically, a mimetic incorporated into a polypeptide which is then used to inoculate an animal to evoke an immune response, elicits antibodies to antigenic determinants located at least in part on the mimetic. The present invention is also based on the discovery that the antibodies generated against the mimetic cross-react with the natural phosphopeptide. This cross-reactivity indicates that mimetics possess antigenic determinants highly similar to those of the natural phosphopeptide, when presented in the context of the same flanking amino acids. The antibodies generated to the mimetic-peptide have sufficient binding activity and specificity for the natural phosphorylated polypeptide to serve as tools in immuno-diagnostic identification of the phosphoprotein from which the antigenic amino acid sequence was derived. These discoveries have lead to the present invention which provides methods for producing and isolating immunological reagents which identify a particular phosphorylation state of a predetermined protein.

[0008] The chemical structure of a phosphopeptide mimetic appropriate for use in the present invention must closely approximate the natural phosphorylated residue which is mimicked, and must also be chemically stable (e.g. resistant to dephosphorylation by phosphatases). This is achieved with a synthetic molecule that comprises the amino acid atomic structure with a non-hydrolyzable linkage to a phosphate moiety, in lieu of the naturally occurring oxygen bridge. In a preferred embodiment, a CF₂ group links the amino acid to the phosphate. Mimetics of several amino acids which are phosphorylated in nature can be generated by this approach. Mimetics of phosphoserine, phosphothreonine, and phosphotyrosine are generated by placing a CF₂ linkage from the appropriate carbon to the phosphate moiety. In a preferred embodiment, the mimetic molecule L-2-amino-4-(diethylphosphono)-4,4-difluorobutanoic acid (F₂Pab) substitutes for phosphoserine (Otaka et al., *Tetrahedron Letters* 36: 927-930 (1995)), L-2-amino-4-phosphono-4,4-difluoro-3-methylbutanoic acid (F₂Pmb) substitutes for phosphothreonine, and L-2-amino-4-phosphono(difluoromethyl) phenylalanine (F₂Pmp) substitutes for phosphotyrosine (Akamatsu et al., *Bioorg. & Med. Chem.* 5: 157-163 (1997)) in an antigenic polypeptide (Fig. 1 and Fig. 2). In an alternate embodiment, the oxygen bridge of the natural amino acid is replaced with a methylene group (Fig. 1 and 2).

[0009] The synthesis of F₂Pab is described by Otaka et al., *Tetrahedron Lett.* 36: 927-930 (1995). The synthesis of F₂Pmp is described by Smyth et al., *Tetrahedron Lett.* 35: 551 (1994) and Akamatsu et al., *Bioorg. & Med. Chem.* 5: 157-163 (1997). Synthesis of F₂Pmb is accomplished by methods analogous to F₂Pab synthesis, using a backbone precursor that has one additional methyl group.

[0010] To generate immunological reagents specific for a predetermined phosphoprotein, the appropriate mimetic(s) is incorporated into a synthetic peptide which corresponds to the sequence surrounding the phosphorylated residue(s). Methods for this incorporation are known or otherwise available to one of skill in the art. The length of sequence required for the generation of phospho-specific antibodies may depend upon the particular phosphoprotein of study. Generally the presence of sequence corresponding to 3 or 4 residues flanking the phosphoresidue is sufficient to generate the required specificity. In one embodiment, the polypeptide antigen comprises a tandem repeat of this sequence with the appropriate mimetic incorporated. Spacer amino acids can be incorporated between the repeated sequence to facilitate antigen processing and presentation. The polypeptide can be produced by chemical synthesis by one of skill in the art through the application of routine experimentation. As detailed in the Exemplification section which follows, a polypeptide antigen with the amino acid sequence specified by SEQ ID NO: 1, listed below, can be used to generate polyclonal antibodies which recognize p53 phosphorylated at serine 15. Several phosphopeptides corresponding to other natural serine phosphorylation sites of p53 have been successfully used to generate phospho-specific antibodies (Sakaguchi et al., *Genes and Dev.* 12: 2831-2841 (1998)). Experiments presented in the Exemplification section indicate that polypeptide antigens with F₂Pab incorporated in place of the natural phosphoserine, can also be used in the method of the present invention, to generate phosphospecific antibodies. These polypeptide antigens, listed below, are designated by SEQ ID NOS: 2-10.

SEQ ID NO: 1

Pro-Leu-F₂Pab-Gln-Glu-Thr-Ala-Pro-Leu-F₂Pab-Gln-Glu-Thr-Ala-Cys

SEQ ID NO: 2

5 Met-Gly-Glu-Pro-Gln-F₂Pab-Asp-Pro-Ser-Val-Glu-Pro-Cys

10 SEQ ID NO: 3

Pro-Gln-Ser-Asp-Pro-F₂Pab-Val-Glu-Pro-Pro-Leu-Ser-Cys

15 SEQ ID NO: 4

Glu-Pro-Pro-Leu-F₂Pab-Gln-Glu-Thr-Phe-Ser-Asp-Leu-Cys

20 SEQ ID NO: 5

Ser-Gln-Glu-Thr-Phe-F₂Pab-Asp-Leu-Trp-Lys-Leu-Leu-Cys

25 SEQ ID NO: 6

30 Pro-Glu-Asn-Asn-Val-Leu-F₂Pab-Pro-Leu-Pro-Ser-Gln-Ala-Cys

35 SEQ ID NO: 7

Leu-Ser-Pro-Leu-Pro-F₂Pab-Gln-Ala-Met-Asp-Asp-Leu-Cys

40 SEQ ID NO: 8

Asn-Asn-Thr-Ser-Ser-F₂Pab-Pro-Gln-Pro-Lys-Lys-Lys-Cys

45 SEQ ID NO: 9

50 Cys-Lys-Lys-Gly-Gln-Ser-Thr-F₂Pab-Arg-His-Lys-Lys-Leu-Met-Phe

55 SEQ ID NO: 10

Cys-Phe-Lys-The-Glu-Gly-Pro-Asp-F₂Pab-Asp

[0011] The mimetic-containing polypeptide may be further modified to more closely resemble the natural product or, alternatively, to promote antigenicity. Such modifications include, but are not limited to, enzymatic modifications, chemical protection or deprotection, denaturation and chemical coupling. In a preferred embodiment, the polypeptide is coupled to the carrier protein, keyhole limpet hemocyanin. Other carrier proteins can be substituted including for example, bovine serum albumin, ovalbumin and purified protein derivative of tuberculin. Chemical coupling may be achieved, for example using any bifunctional reagent.

[0012] Once produced and appropriately modified, the polypeptide antigen is used to inoculate an animal under conditions which elicit an immune response. Products of the immune response are isolated and screened for components cross-reactive with antigenic determinants otherwise unique to the phosphoprotein. These components are isolated and used as reagents to identify a particular phosphorylation state of the protein from which the polypeptide antigen sequence was derived.

[0013] Immunization of the animal used to produce the immunological reagents is generally performed as a timed series of inoculations. In a preferred embodiment, the natural phosphopeptide-conjugate is used in one or more final inoculations (boosts). This step is intended to increase the number of B cells that make the most useful antibodies without unduly stimulating synthesis of antibodies reactive with the unphosphorylated peptide which results from stimulation of antibody synthesis by the dephosphorylated peptide. These boosts are performed according to conventional techniques, and can be further optimized empirically.

[0014] One aspect of the present invention is a method for generating and isolating polyclonal antibodies which bind specifically to a protein or polypeptide which is phosphorylated at a predetermined amino acid residue, using phosphopeptide mimetics incorporated into polypeptides as antigen. Polyclonal antibodies are prepared by immunizing an animal with immunogen prepared as described above, using conventional techniques (see e.g., Harlow and Lane (Eds.), *Antibodies, A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988)). Briefly, the immunized animal is maintained under conditions whereby antibodies reactive with the immunogen are produced. Blood is collected from the animal upon reaching a desired antibody titer. The serum, containing the antibodies, is separated from the other blood components. Alternatively, antibody containing ascitic fluid can be induced and isolated from the immunized animal. The polyclonal antibody-containing serum or ascitic fluid can optionally be further separated into fractions of particular types of antibodies (e.g., IgG or IgM).

[0015] Because the peptide antigen used contains antigenic determinants common to both the phospho- and unphosphorylated protein, the serum generated by this method usually contains a subset of antibodies which specifically bind the phosphoprotein, and also a subset of antibodies which bind independent of phosphorylation state. Such unwanted binding activities can be cleared or depleted from the antiserum by conventional techniques (Czernik et al., *Methods in Enzymology* 201: 264-283 (1991)). When necessary, monospecific antibodies can be purified from the serum using the antigenic determinant in affinity purification (e.g. by affinity chromatography) or conversely, by depleting the serum of all other antibody activity.

[0016] Another aspect of the present invention is a method for generating and isolating monoclonal antibodies which bind specifically to a predetermined phosphoprotein epitope, using phosphopeptide mimetics incorporated into polypeptides as antigen. Monoclonal antibody secreting hybridomas are produced using the antigen described above in conventional techniques (see e.g., Harlow and Lane (Eds.), *Antibodies, A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988)). In a preferred embodiment, phospho-specific monoclonal antibodies are produced by murine hybridomas formed by fusion of: a) a mouse myeloma or hybridoma which does not secrete antibody with, b) a murine spleen cell which secretes antibodies, obtained from a mouse immunized with a mimetic-containing polypeptide antigen.

[0017] Typically, several mice are immunized with a primary injection of antigen followed by a number of boosting injections. During or after the immunization procedure, sera from the mice are screened to identify mice which have mounted a substantial immune response. For selected mice, the spleen cells are obtained and fusions are performed. Suitable fusion techniques include, for example, the Sendai virus technique (Kohler and Milstein, *Nature* 256: 495 (1975)), or the polyethylene glycol method (Kennet, *"Monoclonal Antibodies, Hybridomas - A New Dimension in Biological Analysis"*, Eds. Kennet, McKern and Bechtol, Plenum Press, NY (1980)).

[0018] The hybridomas that result are then screened for production of antibodies specific for the antigen. Several assays can be used for screening and can be performed with either the phosphomimetic-polypeptide antigen or the naturally phosphorylated-polypeptide antigen. A suitable screening technique is a solid phase radioimmunoassay. A solid phase is prepared by coupling the appropriate antigen to an insoluble matrix. The immunoadsorbent is brought into contact with culture supernatants of hybridomas. After a period of incubation, the solid phase is separated from the supernatants, then contacted with a labeled antibody against murine immunoglobulin. Label associated with the immunoadsorbent indicates the presence of hybridoma products reactive to antigen.

[0019] The monoclonal antibodies can be produced in large quantities by injecting antibody producing hybridomas into the peritoneal cavity of mice and after an appropriate time, harvesting ascitic fluid from the mice. The monoclonal antibodies are then isolated from the fluid. Alternatively, the antibodies can be produced by culturing the hybridomas *in*

vitro and isolating secreted monoclonal antibodies from the culture medium directly.

[0020] Methods of the present invention described above can be used to produce immunological reagents specific for either a known or a suspected protein phosphorylation state. When specific for a known phosphorylation state, the antibodies can be used to closely monitor protein phosphorylation under various conditions (e.g. cell cycle, hormonal stimulation, or stress). Alternatively, phospho-specific antibodies are produced to a suspected naturally occurring phosphorylation state. These immunological reagents can be used to confirm or rule out the physiological occurrence of a suspected phosphorylation state.

[0021] Results presented in the Exemplification section, which follows, indicate that the methods of the present invention, described above, also result in the isolation of antibodies which bind a particular phosphoresidue, independent of flanking amino acid sequences. Such antibodies arise when produced as either monoclonal or polyclonal antibodies. These antibodies exhibit binding to the particular phospho-residue largely independent of the flanking amino acid sequence. The antibodies can be identified and, when necessary, purified by their ability to bind to phosphoresidues of polypeptide sequences unrelated to the initial antigen. Such antibodies provide valuable research tools. One use of these antibodies is in an immuno-based kinase assay which is applicable to a wide range of proteins.

[0022] It will be recognized by one of skill in the art that the teachings of the present invention can be applied to the isolation of phospho-specific antibodies produced by systems other than those described above, through the application of merely routine experimentation. One such example is the use of a mimetic containing antigen to screen a combinatorial library for antibodies that exhibit binding activity. The present disclosure is intended to encompass this and related applications.

Exemplification

Generation of polyclonal antibodies.

[0023] Antibodies specific for p53 phosphorylated at serine 15 were affinity purified from hyperimmune serum by passing the serum over a series of polypeptide linked columns. An initial passage of the serum over a column containing a polypeptide of p53 residues 11-22, phosphorylated at serine 15, enriched for p53 specific antibodies. The bound antibodies were eluted and then cleared of antibodies not specific for phosphoserine by passage over a column containing the unphosphorylated p53 polypeptide. The flow-through was retained and characterized.

[0024] Binding specificity of the resulting antiserum was determined by enzyme-linked immunosorbent assay (ELISA) using the polypeptides p53(1-39), p53(1-39)15P, and p53(25-63)37P. Specific binding to both the phosphorylated polypeptides was detected at antibody dilutions less than 1:320 (Fig. 3), with more binding to p53(1-39)15P, at all concentrations. Binding to non-phosphorylated polypeptide was insignificant at all concentrations. The cross-reactivity of the antiserum to the p53(25-63)37P polypeptide indicated the presence of non-specific phosphoserine antibodies.

[0025] To clear the antiserum of non-specific phosphoserine antibodies the antiserum was passed over a column of p53(Ac-32-43(37P)Cys). The resulting unbound flow-through was tested as above and found to be highly specific for p53 phosphorylated at serine 15 (Fig. 4). Reactivity and specificity for the p53 serine 15 was confirmed by immunoblot analysis using unphosphorylated p53 and p53 that was enzymatically phosphorylated at serine 15 by the protein kinase DNA-PK.

[0026] The generation of antibodies that specifically recognize naturally phosphorylated polypeptides, from peptide antigen with F₂Pab substituted for phosphoserine, demonstrates that F₂Pab functions as an effective phosphoserine mimic when used as antigen. Importantly, these results also indicate that peptides into which F₂Pab is incorporated, when used as antigen, undergo the necessary antigen processing and presentation required for the induction of B-lymphocytes in the production of specific antibodies. The results presented above indicate that F₂Pab incorporated into an antigen can be used to generate antibodies that specifically recognize a particular site of phosphorylation within a peptide, and also antibodies with a broader recognition of phosphoserines in general.

[0027] The successful generation of polyclonal antibodies specific for phosphoserines, using the F₂Pab mimic described here, indicates that immunization of mice or other animals with polypeptides containing F₂Pab, by otherwise standard methods, will also result in the stimulation and clonal expansion of B-lymphocytes to produce antibodies specific for a naturally phosphorylated residue, polypeptide, or protein. Such B-lymphocytes can be isolated and used in the production of hybridomas to generate monoclonal antibodies specific for phosphoproteins.

Methods of the Invention

[0028] Production of Immune Serum. The polypeptide used as antigen is specified by SEQ ID NO: 1. The polypeptide consists of fifteen residues, the sequence corresponding to a seven amino acid sequence of p53 amino acid residues 13-19, being repeated twice and containing the phosphoserine mimic F₂Pab in place of serine 15, the polypeptide having an additional carboxy-terminal cysteine residue in addition to the p53 amino acid sequence. The polypeptide was chem-

ically synthesized using t-Boc chemistry on an Applied Biosystems 430A peptide synthesizer according to manufacturer recommendations, except that Boc-F₂Pab(Et₂)-OH was manually coupled using t-Boc chemistry. The assembly of the peptide chain was by standard methods (Synthetic Peptides, G. Grant (Ed.), W. H. Freeman & Co., New York (1992)). Cleavage of the peptide from the resin and removal of the side-chain protecting groups were carried out using a two-

step deprotection method (Otaka et al., *Tetrahedron Lett.* 36: 927-930 (1995)). The peptides were purified by high pressure liquid chromatography (HPLC) on a Vydac C-8 column with 0.05% TFA/water-acetonitrile.

[0029] The polypeptide was coupled through the carboxy-terminal cysteine residue to keyhole limpet hemocyanin (KLH). The resulting KLH-F₂Pab-peptide conjugate was used to immunize a rabbit by standard procedures. Briefly, the rabbit was injected subcutaneously with 500 µg of the peptide conjugate mixed with adjuvant on days 0, 7, and 14. Complete Freund's adjuvant was used for the first injection, and Incomplete Freund's adjuvant was used for additional injections. Blood was collected on day 21. After day 21, immunization and blood collection was repeated weekly for five weeks.

Generation of Polypeptides for Affinity Chromatography.

[0030] Polypeptides of 13 amino acids corresponding to the human p53 sequence of residues 11 to 22, and having an additional carboxy-terminal cysteine residue, were chemically synthesized by standard methods as non-phosphorylated: (p53(Ac-11-22Cys)); or as phosphorylated at serine 15: (p53(Ac-11-22(15P)Cys)). Polypeptide corresponding to the human p53 sequence of residues 32 to 43 with an added carboxy-terminal cysteine residue, was synthesized as phosphorylated at serine 37: (p53(Ac-32-43(37P)Cys)).

[0031] Peptides were synthesized by the solid phase method with Fmoc chemistry (Synthetic Peptides, G. Grant (Ed.), W. H. Freeman & Co., New York (1992)) using an Applied Biosystems 430A peptide synthesizer (Foster City, CA). Phosphoserine residues were incorporated as Fmoc-Ser(PO(OBzl)OH)-OH (Novabiochem, San Diego, CA) (Wakamiya et al., *Chem. Lett.* 6: 1099-1102 (1994)). Cleavage of the peptide from the resin and removal of the side-chain protecting groups were carried out using reagent K (TFA : phenol : thioanisole : H₂O : EDT = 82.5 : 5 : 5 : 5 : 2.5) for 3 hours at room temperature (King et al., *Int. J. Pept. Protein Res.* 36: 255-266 (1990)). The peptides were purified by HPLC on a Vydac C-8 column (Hesperia, CA) with 0.05 % TFA/water-acetonitrile, or on a pH-stable Vydac C-8 column (Hesperia, CA) with 0.2 % hexafluoroacetone-NH₄OH, pH 7.0/acetonitrile (for 1-39 peptides). The mass of peptides were confirmed by electrospray ionization mass spectrometry on a Finnigan MAT SSQ 7000 (Finnigan MAT, San Jose, CA).

[0032] Generation of Polypeptide Linked Columns. The polypeptides (p53(Ac-11-22Cys)), (p53(Ac-11-22(15P)Cys)), and (p53(Ac-32-43(37P)Cys)) were coupled to Sulfolink (Pierce Chemical Co.) according to the instructions provided by the manufacturer. The resulting Sulfolink conjugates were used to create three columns, each packed with one of the polypeptide - Sulfolink conjugates.

[0033] Affinity Purification. Antibodies specific for p53 phosphorylated at serine 15 were purified from hyperimmune rabbit serum by affinity chromatography using a series of peptide linked columns. The serum was first passed through a (p53(Ac-11-22(15P)Cys)) column. After washing, the adsorbed antibodies were eluted with ImmunoPure IgG Elution Buffer (Pierce Chemical Co.), and were immediately neutralized by adding 1 M Tris buffer (pH 9.5). Eluate was passed over a (p53(Ac-11-22Cys)) column to remove antibodies that bound unphosphorylated p53. The unbound flow-through was collected and then passed over a (p53 Ac-32-43)(37P)Cys column to deplete it of antibodies that bind to phosphoserines irrespective of their position within a polypeptide.

[0034] Antibody Analysis. Affinity purified antibody preparations were characterized for binding to the polypeptides p53(1-39), p53(1-39)15P, or p53(1-39)37P by ELISA assay, as described in the Pierce Chemical Technical Library and in Engvall et al., *Immunochemistry* 8: 871-875 (1971). Plates were coated with the indicated polypeptide by diluting the polypeptide into sodium carbonate-bicarbonate buffer, pH 9.6 (1.59 g Na₂CO₃, 2.93 g NaHCO₃ per liter, pH adjusted with HCL) to 1 µg/ml and 50 or 100 ng (50 or 100 µl) was incubated at 4°C overnight or for about 3 hours at room temperature. The peptide solution was then removed and plates were blocked by incubation with 1 % BSA in PBS for 1 hour at room temperature. Polypeptide-coated wells were incubated with antibody dilutions between 1/10 and 1/1000. Bound antibody was detected by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody, followed by addition of the HRP substrate ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)). HRP oxidation of the ABTS produced a green color product which was used to identify antibody presence. The amount of rabbit IgG bound to the polypeptide-coated plate was determined by measuring the optical density of the mixture at 405 nm (OD₄₀₅). Results were confirmed by separate ELISA assay and spot blots ("western" blots).

[0035] Polypeptides used in the ELISA analysis were synthesized by Fmoc chemistry as described above. Longer peptides were synthesized by a fragment condensation method (Sakamoto et al., *Int. J. Peptide Protein Res.* 48: 429-442 (1996)).

Claims

1. A method for isolating polyclonal antibodies which bind specifically to a polypeptide phosphorylated at a particular amino acid, comprising:

a) providing a synthetic polypeptide comprising a mimetic of the particular amino acid residue wherein the mimetic must be chemically stable, which is achieved with a synthetic molecule comprising the amino acid structure with a non-hydrolyzable linkage to a phosphate moiety, in lieu of the naturally occurring oxygen bridge, the synthetic polypeptide being formulated in a physiologically acceptable carrier;

b) immunizing an animal with the synthetic polypeptide of step a) ;

c) collecting and preparing serum or ascitic fluid from the immunized animal of step b);

d) screening for the presence of antibodies specific for the phosphorylated polypeptide; and

e) when necessary, further enriching for antibodies specific for the phosphorylated polypeptide through conventional methods.

2. The method of Claim 1 wherein antibodies specific for the phosphorylated polypeptide are further enriched by a) affinity purification ; and/or b) depleting co-mingled antibodies which exhibit undesired binding activities.

3. A method for isolating monoclonal antibodies which bind specifically to a polypeptide which is phosphorylated at a particular amino acid residue, comprising:

a) providing a synthetic polypeptide comprising a mimetic of the particular phosphorylated amino acid the synthetic polypeptide being formulated in a physiologically acceptable carrier;

b) immunizing an animal with the synthetic polypeptide of step a) ;

c) collecting B-cells from the animal of step b)

d) generating hybridomas by fusing B-cells from step c) with myeloma cells;

e) screening hybridomas of step d) for the production of antibodies specific for the phosphorylated polypeptide;

f) isolating and propagating hybridomas identified in step e) ; and

g) isolating monoclonal antibodies from the isolated hybridomas of step f).

4. The method of Claim 1 or claim 3, wherein binding of the antibodies to the phosphorylated polypeptide is : a) dependent on the amino acid sequences which flank the phosphorylated amino acid; or b) independent of the sequences which flank the phosphorylated amino acid.

5. The method of Claim 1 or Claim 3 wherein the animal is further immunized with a natural phosphopeptide-conjugate comprised of an appropriate amino acid sequence as a boost.

6. The method of Claim 1 or Claim 3, wherein the synthetic polypeptide of step a) is modified prior to the immunization of step b); and optionally wherein the modification occurs through coupling of the polypeptide to a carrier protein selected from the group consisting of keyhole limpet hemacyanin (KLH), bovine serum albumin (BSA), ovalbumin and purified protein derivative of tuberculin (PPD), using any bifunctional reagent.

7. The method of Claim 1 or Claim 3, wherein the particular phosphorylated amino acid residue is selected from the group consisting of serine, threonine and tyrosine.

8. The method of Claim 1 or Claim 3, wherein the mimetic contains a non-hydrolyzable linkage from a carbon atom to a phosphate group.

9. The method of Claim 8, wherein the non-hydrolyzable linkage comprises a CF₂ group, and optionally wherein a) F₂Pab is a mimetic for phosphoserine ; b) F₂Pmp is a mimetic for phosphotyrosine ; and/or c) F₂Pmb is a mimetic for phosphothreonine.

10. The method of Claim 1 to Claim 3, wherein the synthetic polypeptide of step a) has an amino acid sequence derived from the amino acid sequence of p53, and optionally wherein the polypeptide is as specified in SEQ ID NO: 1; or is selected from the group consisting of SEQ ID NOS: 2, 3, 4, 5, 6, 7, 8, 9, and 10.

Patentansprüche

1. Verfahren zur Isolierung von polyklonalen Antikörpern, die spezifisch an ein Polypeptid binden, das an einer bestimmten Aminosäure phosphoryliert ist, wobei man:

a) ein synthetisches Polypeptid bereitstellt, das ein Mimetikum des bestimmten Aminosäurerestes umfasst, wobei das synthetische Polypeptid in einem physiologisch akzeptablen Träger formuliert ist, und wobei das Mimetikum chemisch stabil sein muss, was mit einem synthetischen Molekül erreicht wird, das die Aminosäurestruktur mit einer nicht hydrolysierbaren Bindung an einen Phosphatrest anstelle der natürlich vorkommenden Sauerstoffbrücke umfasst;

b) ein Tier mit dem synthetischen Polypeptid von Schritt a) immunisiert;

c) das Serum oder die Aszitesflüssigkeit vom immunisierten Tier von Schritt b) sammelt und aufbereitet;

d) auf die Gegenwart von Antikörpern screen, die spezifisch für das phosphorylierte Polypeptid sind; und

e) falls nötig, Antikörper, die spezifisch für das phosphorylierte Polypeptid sind, durch herkömmliche Methoden weiter anreichert.

2. Verfahren von Anspruch 1, wobei Antikörper, die spezifisch für das phosphorylierte Polypeptid sind, weiter angereichert werden durch:

a) Affinitätsaufreinigung; und/oder

b) Depletion von beigemischten Antikörpern, die ungewünschte Bindungsaktivitäten zeigen.

3. Verfahren zur Isolierung von monoklonalen Antikörpern, die spezifisch an ein Polypeptid binden, das an einem bestimmten Aminosäurerest phosphoryliert ist, wobei man:

a) ein synthetisches Polypeptid bereitstellt, das ein Mimetikum der bestimmten phosphorylierten Aminosäure umfasst, wobei das synthetische Polypeptid in einem physiologisch akzeptablen Träger formuliert ist;

b) ein Tier mit dem synthetischen Polypeptid von Schritt a) immunisiert;

c) B-Zellen von dem Tier von Schritt b) sammelt;

d) Hybridomas durch Fusionierung von B-Zellen von Schritt c) mit Myelomzellen generiert;

e) die Hybridomas von Schritt d) auf die Produktion von Antikörpern screen, die spezifisch für das phosphorylierte Polypeptid sind;

f) die in Schritt e) identifizierten Hybridomas isoliert und züchtet; und

g) monoklonale Antikörper aus den isolierten Hybridomas von Schritt f) isoliert.

4. Verfahren von Anspruch 1 oder 3, wobei das Binden der Antikörper an das phosphorylierte Polypeptid:

a) abhängig ist von den Aminosäuresequenzen, die die phosphorylierte Aminosäure flankieren; oder

b) unabhängig ist von den Sequenzen, die die phosphorylierte Aminosäure flankieren.

5. Verfahren von Anspruch 1 oder 3, wobei das Tier ferner mit einem natürlichen Phosphopeptid-Konjugat immunisiert wird, das eine geeigneten Aminosäuresequenz als Verstärkung beinhaltet.

6. Verfahren von Anspruch 1 oder 3, wobei das synthetische Polypeptid von Schritt a) vor der Immunisierung von Schritt b) modifiziert wird; und wobei die Modifizierung gegebenenfalls durch Kopplung des Polypeptids an ein Trägerprotein, das aus der Gruppe ausgewählt wird, die aus Schlüsselloch-Napfschnecken-Hämacyanin (keyhole limpet hemacyanin; KLH), Rinderserumalbumin (bovine-serum albumine; BSA), Ovalbumin und gereinigtem Proteinderivat (purified protein derivate; PPD) von Tuberkulin besteht, mittels irgendeines bifunktionellen Reagenzes erfolgt.

7. Verfahren von Anspruch 1 oder 3, wobei der bestimmte phosphorylierte Aminosäurerest aus der Gruppe ausgewählt wird, die aus Serin, Threonin und Tyrosin besteht.

8. Verfahren von Anspruch 1 oder 3, wobei das Mimetikum eine nicht hydrolysierbare Bindung eines Kohlenstoffatoms an eine Phosphatgruppe enthält.

9. Verfahren von Anspruch 8, wobei die nicht hydrolysierbare Bindung eine CF₂-Gruppe umfasst; und wobei gegebenenfalls:

- a) F₂Pab ein Mimetikum für Phosphoserin ist;
- b) F₂Pmp ein Mimetikum für Phosphotyrosin ist; und/oder
- c) F₂Pmb ein Mimetikum für Phosphothreonin ist.

10. Verfahren von Anspruch 1 oder 3, wobei das synthetische Polypeptid von Schritt a) eine Aminosäuresequenz aufweist, die sich von der Aminosäuresequenz von p53 ableitet; und wobei gegebenenfalls das Polypeptid das in SEQ ID NO: 1 spezifizierte ist; oder aus der Gruppe ausgewählt wird, die aus den SEQ ID Nos: 2, 3, 4, 5, 6, 7, 8, 9 und 10 besteht.

Revendications

1. Procédé pour isoler des anticorps polyclonaux qui se lient spécifiquement à un polypeptide phosphorylé à un acide aminé particulier, comprenant :

- a) fournir un polypeptide synthétique comprenant un mimétique du résidu d'acide aminé particulier, dans lequel le mimétique doit être chimiquement stable, ce qui est obtenu avec une molécule synthétique comprenant la structure d'acide aminé avec une liaison non hydrolysable à un groupe phosphate, au lieu du pont oxygène naturel, le polypeptide synthétique étant formulé dans un véhicule physiologiquement acceptable ;
- b) immuniser un animal avec le polypeptide synthétique de l'étape a) ;
- c) collecter et préparer le sérum ou le liquide ascitique de l'animal immunisé de l'étape b) ;
- d) cribler pour la présence d'anticorps spécifiques du polypeptide phosphorylé ; et
- e) quand cela est nécessaire, encore enrichir en anticorps spécifiques du polypeptide phosphorylé par les procédés classiques.

2. Procédé selon la revendication 1, dans lequel les anticorps spécifiques du polypeptide phosphorylé sont encore enrichis par :

- a) purification d'affinité ; et/ou
- b) épuisement des anticorps en mélange qui présentent des activités de liaison non souhaitées

3. Procédé d'isolement d'anticorps monoclonaux qui se lient spécifiquement à un polypeptide qui est phosphorylé à un résidu d'acide aminé particulier, comprenant :

- a) fournir un polypeptide synthétique comprenant un mimétique de l'acide aminé phosphorylé particulier, le polypeptide synthétique étant formulé dans un véhicule physiologiquement acceptable ;
- b) immuniser un animal avec le polypeptide synthétique de l'étape a) ;
- c) collecter les cellules B de l'animal de l'étape b)
- d) générer des hybridomes en fusionnant les cellules B de l'étape c) avec des cellules de myélome ;
- e) cribler les hybridomes de l'étape d) pour la production d'anticorps spécifiques du polypeptide phosphorylé ;
- f) isoler et propager les hybridomes identifiés dans l'étape e) ; et
- g) isoler les anticorps monoclonaux des hybridomes isolés de l'étape f).

4. Procédé selon la revendication 1 ou la revendication 3, dans lequel la liaison des anticorps au polypeptide phosphorylé est :

- a) dépendante des séquences d'acides aminés qui flanquent l'acide aminé phosphorylé ; ou
- b) indépendante des séquences qui flanquent l'acide aminé phosphorylé.

5. Procédé selon la revendication 1 ou la revendication 3, dans lequel l'animal est en outre immunisé avec un conjugué-phosphopeptide naturel comprenant une séquence d'acides aminés appropriée comme rappel.

6. Procédé selon la revendication 1 ou la revendication 3, dans lequel le polypeptide synthétique de l'étape a) est modifié avant l'immunisation de l'étape b) ; et éventuellement dans lequel la modification se produit par le couplage du polypeptide à une protéine transporteur choisie dans le groupe constitué par l'hémocyanine de patelle (HP), la sérumalbumine bovine (SAB), l'ovalbumine et la protéine purifiée dérivée de la tuberculine (PPD), en utilisant un quelconque réactif bifonctionnel.

7. Procédé selon la revendication 1 ou la revendication 3, dans lequel le résidu d'acide aminé phosphorylé particulier est choisi dans le groupe constitué par la sérine, la thréonine et la tyrosine.

5 8. Procédé selon la revendication 1 ou la revendication 3, dans lequel le mimétique contient une liaison non hydrolysable d'un atome de carbone à un groupe phosphate.

9. Procédé selon la revendication 8, dans lequel la liaison non hydrolysable comprend un groupe CF_2 ; et éventuellement dans lequel :

- 10 a) le F_2Pab est un mimétique de la phosphosérine ;
 b) le F_2Pmp est un mimétique de la phosphotyrosine ; et/ou
 c) le F_2Pmb est un mimétique de la phosphothréonine.

15 10. Procédé selon la revendication 1 ou la revendication 3, dans lequel le polypeptide synthétique de l'étape a) a une séquence d'acides aminés dérivée de la séquence d'acides aminés de p53 ; et éventuellement dans lequel le polypeptide est tel que spécifié dans SEQ ID n° 1 ; ou
 est choisi dans le groupe constitué par les SEQ ID n° 2, 3, 4, 5, 6, 7, 8, 9 et 10.

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Analogues of Phosphoserine and Phosphothreonine

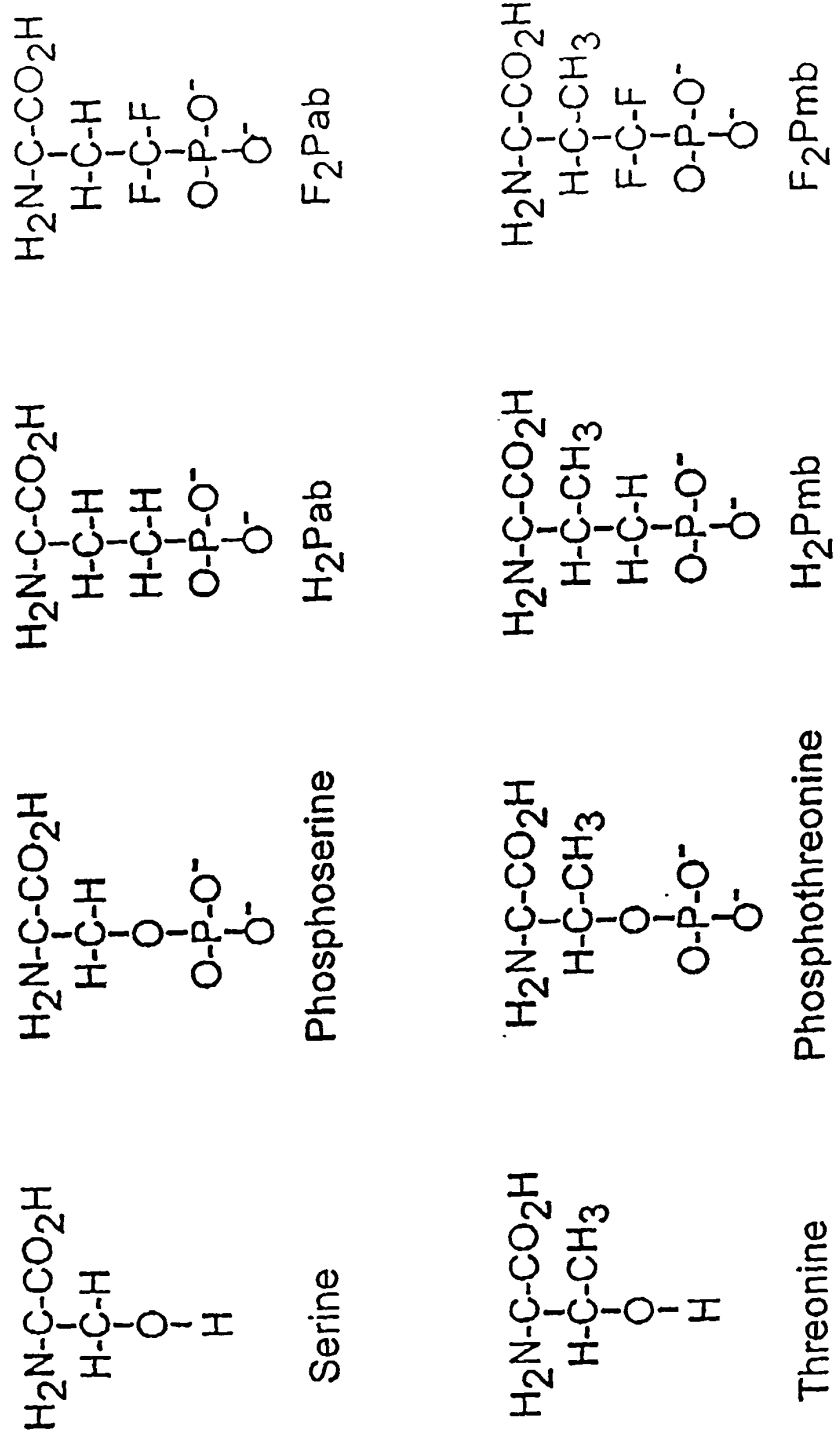


FIG. 1

Analogue of Phosphotyrosine

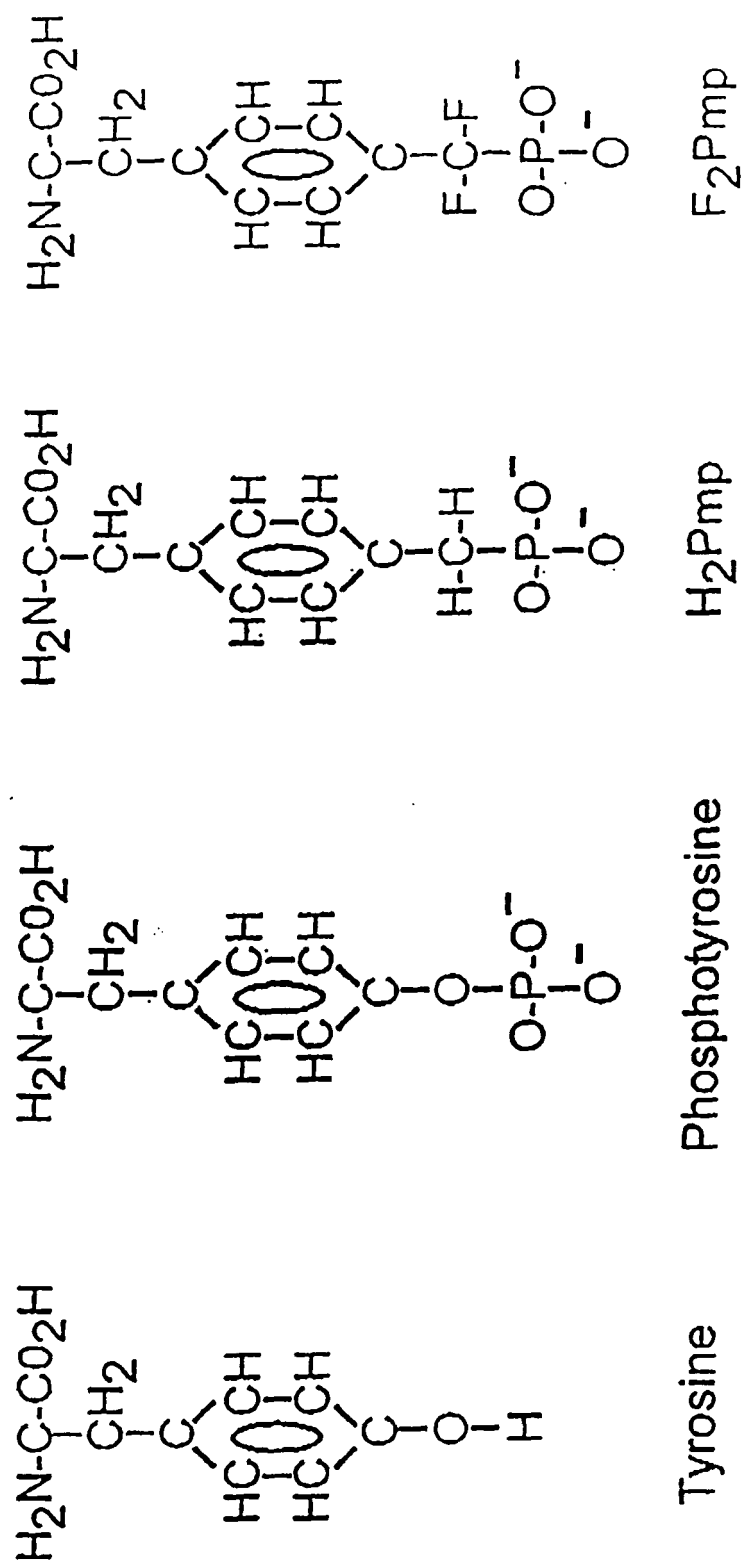


FIG. 2

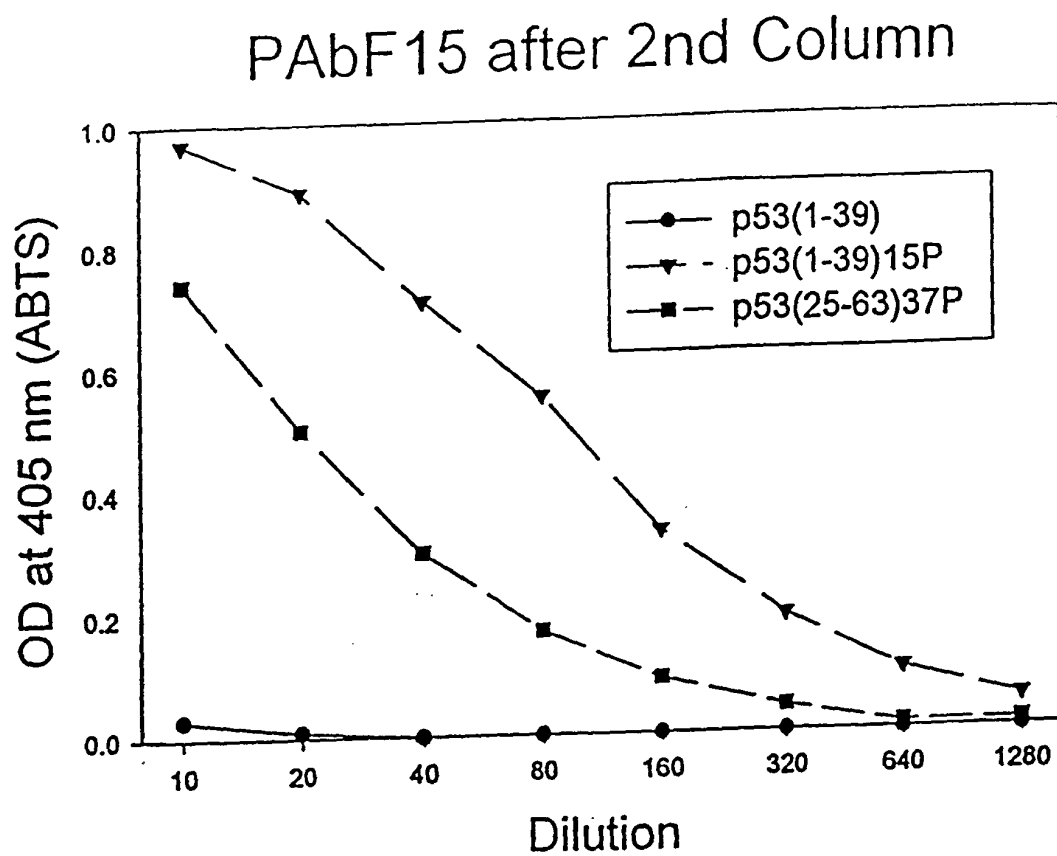


FIG. 3

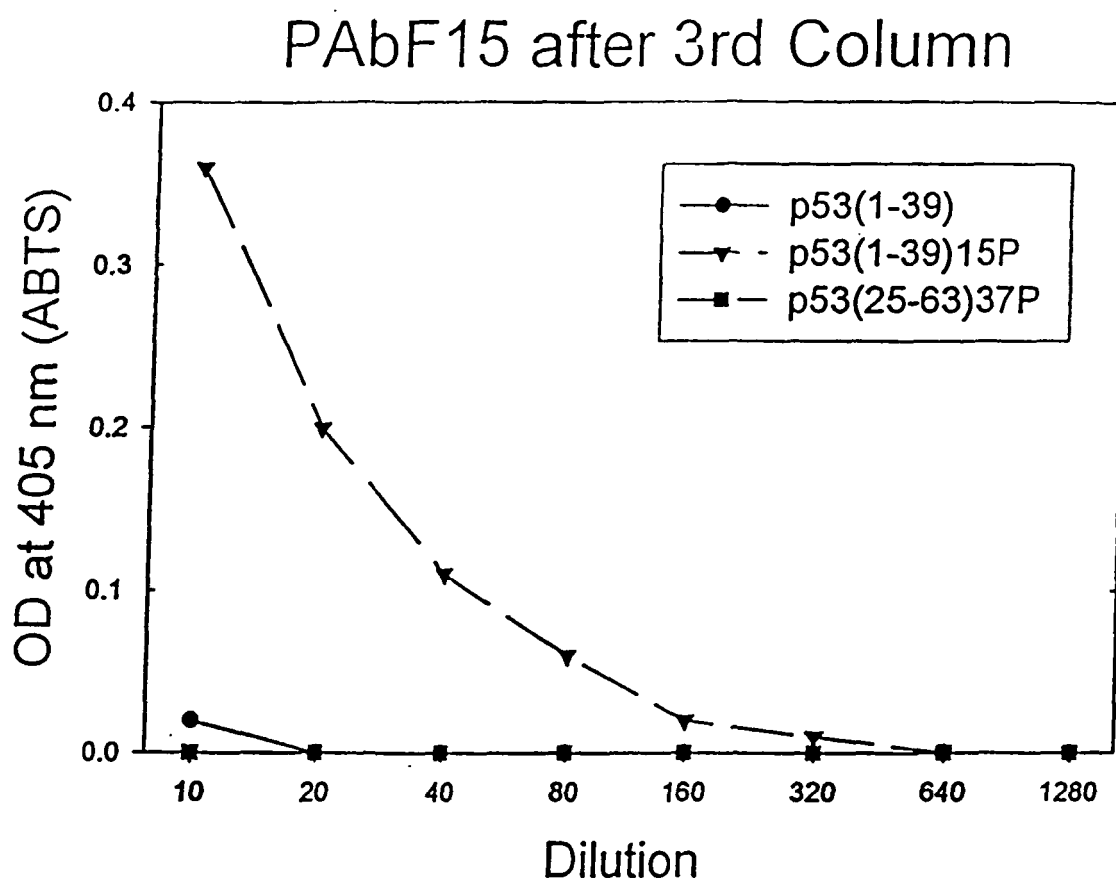


FIG. 4