Monoclonal Antibody Register

New Monoclonal Antibodies Recognizing p53 Protein Phosphorylated by Casein Kinase II at Serine 392

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Background

The p53 tumour suppressor protein is a nuclear phosphoprotein which plays a key role in cell-cycle regulation. The p53 protein protects cells from undergoing tumorigenic alterations by inducing either the cell growth arrest or programmed cell death in response to a variety of cellular stress signals such as DNA damage, hypoxia, heat shock, oncogene activation or metabolic changes (Levine, 1997). One of the critical issues of p53 response to cellular stress or DNA damage is the way of its activation. It has been suggested that post-translational modification involving phosphorylation of the p53 protein is most likely the mechanism through which the activity of the p53 protein may be regulated. p53 is phosphorylated on several serine residues within the N- and C-terminal regions by several cellular kinases. The N-terminal part of p53 is phosphorylated using different protein kinases including casein kinase I (CKI) (Milne et al., 1992), DNA-PK (Lees-Miller et al., 1992), Chk1, Chk2, MAP kinase (Milne et al., 1994) and c-Jun kinase (Milne et al., 1995). There are also at least three phosphorylation sites on the C-terminus of human p53 at amino acids 315, 378 and 392. Serine^{315} of p53 is a target for cdk1 and cdk2 (Price et al., 1995). Serine^{378} is phosphorylated by PKC (Baudier et al., 1992). Serine^{392} is phosphorylated by purified casein kinase II (CKII) *in vitro* (Blaydes and Hupp, 1998). Phosphorylation of the human p53 at Ser^{392} has been shown to enhance p53 sequence-specific DNA binding *in vitro* (Hupp and Lane, 1994), which is responsible for transcriptional activation of the p53 protein. Recently, it has also been shown that phosphorylation of Ser^{392} is important for p53-mediated transcriptional activation *in vivo* (Hao et al., 1996).

Phosphorylation of different proteins in cells can be studied using a range of different methods, but the primary technique for determining phosphate incorporation into the specific sites in target proteins involves labelling of cells with [^{32}P] phosphate followed by phospho-amino-acid analysis or sequencing of the protein of interest (Van der Geer et al., 1993). The main problem associated with this technique is the incubation of cells with a radioactive precursor [^{32}P], which itself can activate growth arrest and stress-responsive signalling pathways, obviously perturbing protein phosphorylation (Yeargin and Haas, 1995; Dover et al., 1994). Preparation of monoclonal antibodies that are specific to either phosphorylated or non-phosphorylated epitopes within the target protein provides a powerful alternative to the above techniques.

Description of the antibodies S-P-1.1, S-P-2.1 and S-P-3.1

Production

The hybridoma cell lines producing S-P-1.1, S-P-2.1 and S-P-3.1 phospho-specific monoclonal antibodies were selected after immunization of BALB/c mice with the phosphopeptide SRHKKLMFKTEGPDS$^{(PO)}{}_3$D coupled to keyhole limpet haemocyanin (KHL). Mouse splenocytes were fused with non-producing myeloma cell line SP2/0 using polyethylene glycol as a fusogen. Dot-blot using the phosphorylated and non-phosphorylated forms of peptide coupled to bovine serum albumin (BSA) was used as a screening method (Fig. 1). Monoclonal antibody Bp53-6.1 (Bartek et al., 1993) was used as a positive control, as this antibody recognizes both forms of the peptide. Hybridoma cell lines were prepared using limiting-dilution cloning and re-cloning. Monoclonal antibodies were characterized more precisely on bacterially expressed p53 protein phosphorylated *in vitro* at Ser^{392} with CK II.

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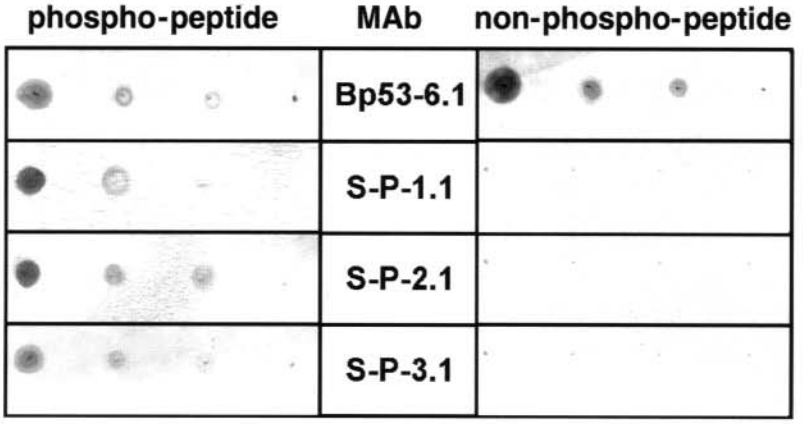

Fig. 1. Dot-blot analysis of phosphorylated and non-phosphorylated peptide SRHKKLM FKTEGPDS$^{(PO_3)}$D coupled to BSA using specified monoclonal antibodies (Bp53-6.1 – control MAb recognizing epitope ^{381}KKLMFKTEGP^{390}, S-P-1.1, S-P-2.1, S-P-3.1 – MAbs recognizing the p53 protein phosphorylated at Ser^{392}).

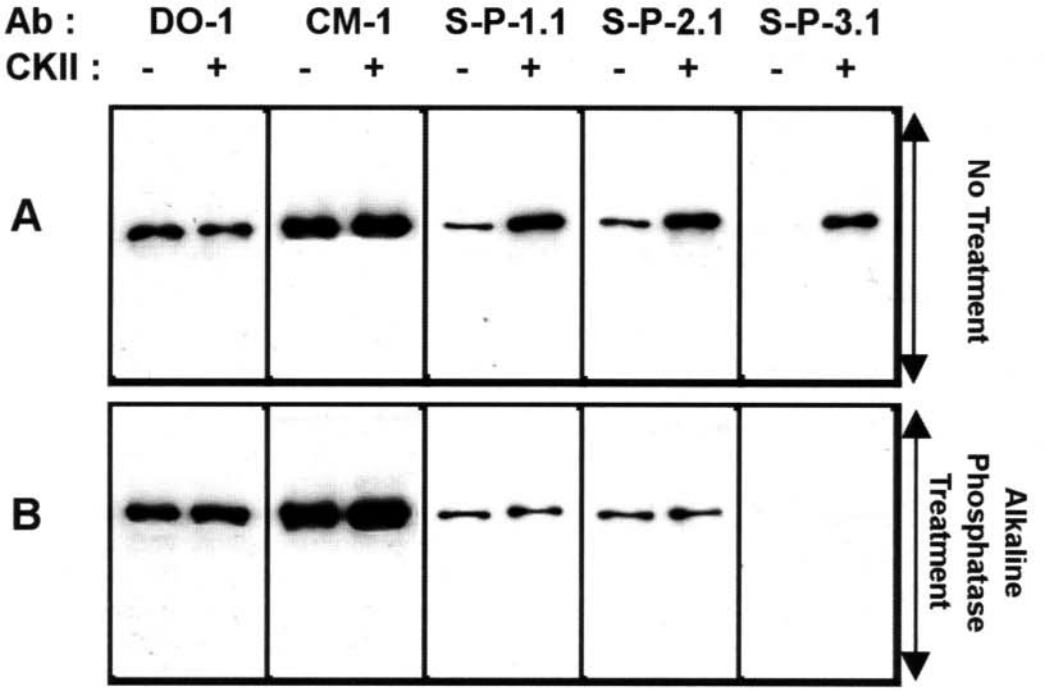

Fig. 2. Western-blot analysis of bacterially expressed p53 protein phosphorylated *in vitro* with casein kinase II at Ser^{392}. The kinase reaction was performed in the reaction mixture containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 100 μg/ml phosphatidylserine, 20 μg/ml dioleoyl-sn-glycerol, 1 mM 3-[(-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS) and 0.2 mM ATP at 30°C for 30 min and the same samples were loaded to both A and B gels. After the blotting the B membranes were incubated with calf intestinal alkaline phosphatase at room temperature for 60 min while gently shaking and then all the membranes were blocked in PBS with 5% low-fat milk and incubated with indicated antibodies for 2.5 h at room temperature. Control polyclonal antibody CM-1 recognized several epitopes of human p53 protein, monoclonal antibody DO-1 recognized the epitope ^{20}SDLWKL^{25}, and monoclonal antibodies S-P-1.1, S-P-2.1, S-P-3.1 recognized the C-terminus of the p53 protein phosphorylated at Ser^{392}.

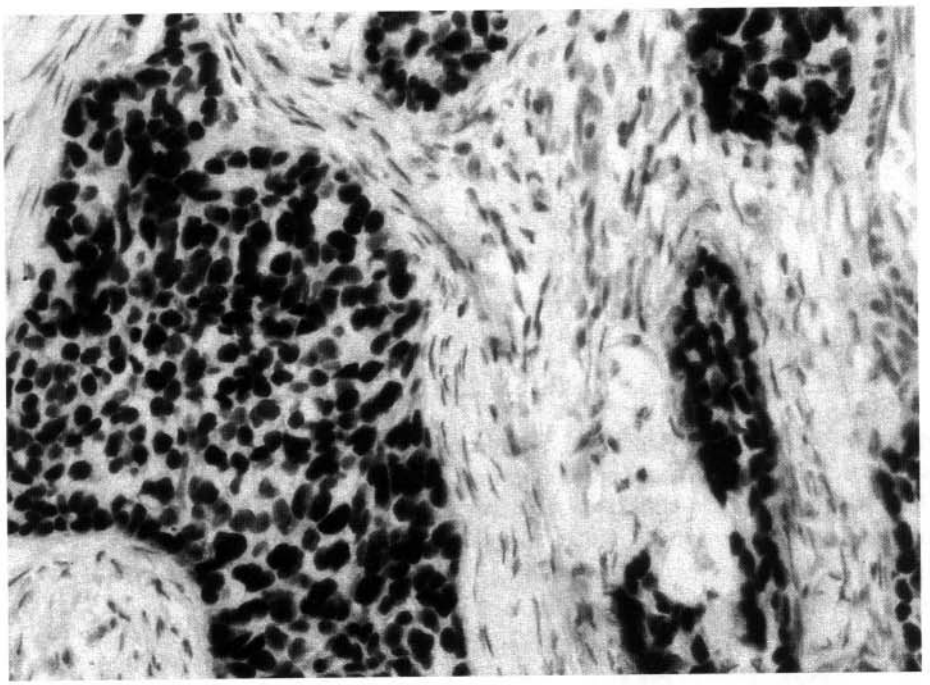

Fig. 3. Detection of p53 posttranslationally modified by phosphorylation at Ser^{392} in ovarian cancer with a phospho-specific antibody S-P-3.1. For antigen retrieval, formaldehyde-fixed tissue was incubated at 97°C in citrate buffer for a period of 30 min. Magnification 200x.

Specificity

First, to test the specificity of MAbs for posttranslationally modified protein at Ser^{392}, p53 over-expressed in baculoviruses and bacteria was analyzed. Western blots using purified p53 protein were performed using S-P-1.1, S-P-2.1 and S-P-3.1 MAbs in denaturing conditions. All three antibodies very strongly recognize the p53 protein over-expressed in baculoviruses; antibodies S-P-1.1 and S-P-2.1 also recognize, with different affinity, the protein over-expressed in bacteria (data not shown). To test the specificity of MAbs on protein posttranslationally modified only on the Ser^{392}, bacterial p53 protein was *in vitro* phosphorylated at this site with CKII. Western blot analysis employing S-P-1.1, S-P-2.1 and S-P-3.1 MAbs detected *in vitro* phosphorylated p53. Antibodies S-P-1.1 and S-P-2.1 did not fail completely to detect non-phosphorylated bacterially expressed p53 (Fig. 2A). To demonstrate the same levels of p53 in each reaction, the non-phosphorylated and phosphorylated p53 were immunoblotted with monoclonal antibodies specific to the C-terminal domain DO-1 (Vojtesek et al., 1992), and polyclonal sera CM-1 (Midgley et al., 1992) recognizing many epitopes on the p53 protein. All three newly developed MAbs recognize well the phosphorylated form of the peptide; two of them (S-P-1.1, S-P-2.1) can also bind, with lower affinity, to non-phosphorylated protein p53 produced in bacteria; the antibody S-P-3.1 binds specifically only to p53 modified at Ser^{392} (Fig. 1). The specificity of the S-P-3.1 antibody to phosphorylated p53 was additionally proved using the experiments with phosphatase treatment (Fig. 2B) in order to remove phosphate groups from different sites of the p53 molecule. After this treatment the antibodies S-P-1.1 and S-P-2.1 still recognized a small proportion of non-phosphorylated p53 protein, whilst S-P-3.1 was not capable of reacting with dephosphorylated p53 at all.

The process of constructing a MAb against a specifically phosporylated epitope does not exclude the possibility of obtaining an antibody reacting with different affinity with both the phospho- and non-phospho-form of an identical protein. Therefore, careful screening of specific hybridomas is of prime importance for eliminating even weak cross-reactivities and for obtaining a pure and site-specific antibody.

Properties

Antibodies S-P-1.1 (IgG1), S-P-2.1 (IgG1) and S-P-3.1 (IgG1) can recognize phosphorylated p53 at Ser^{392} under denaturing and non-denaturing conditions with a different degree of cross-reactivity, the S-P-3.1 being the most specific. All three antibodies can be used for analysis of the cellular p53 status by ELISA, immunoblotting and immunohistochemistry (Fig. 3). The antibody S-P-3.1 seems to be biologically most relevant for experimentation, since it does not display any cross-reactivity with the non-phosphorylated form of p53.

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