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SHORT REPORT

A monoclonal antibody against DNA binding helix of p53 protein

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Three monoclonal antibodies (Mabs) were generated against p53 DNA-binding core domain. When tested by immunoprecipitation, Western blot and immunofluorescence techniques, Mab 9E4, as well as 7D3 and 6B10 reacted with both wild-type and various mutant p53 proteins. The epitopes recognized by Mabs 7D3, 9E4 and 6B10 were located respectively within the amino acid residues 211-220, 281-290 and 291-300 of human p53 protein. The epitope recognized by 9E4 Mab coincides with helix 2, also called p53 DNA binding helix, which allows the direct contact of the protein with its target DNA sequences. This antibody may be useful to study transcription-dependent and transcription-independent activities of wild-type and mutant p53 proteins. *Oncogene* (2001) **20,** 1398–1401.

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Encoded by a tumor suppressor gene, p53 protein is one of the most intensively investigated molecules in the tumor biology field (Levine, 1997). Wild-type p53 protein is a transcription factor that regulates the expression of a large list of genes involved in many different cellular processes such as growth arrest, apoptosis, senescence, DNA repair and tumor metastasis. The best-described functions of wild-type p53 are cell cycle arrest and apoptosis as a response to DNA damage. Cell cycle arrest is mediated by p53-induced transcriptional activation, whereas apoptosis was reported to be induced by both transcription-dependent and transcription-independent pathways (Agarwal *et al*., 1998).

In normal cells, p53 protein is actively degraded by a mechanism involving p53-mdm2 interaction. Following DNA damage or oncogene activation, p53 is stabilized and accumulates in cells (Oren, 1999). Transcriptional activation induced by p53 results from its nuclear localization and binding, as a tetramer, to specific p53-binding pentamers (PuPuPuCA/T) located at the regulatory regions of different p53-responsive genes (Levine, 1997). The central core region of p53 is directly involved in its binding to target DNA motifs. This region is known as an independently folded, compact structural domain. Cho *et al*. (1994) demonstrated that the structure of the p53 core domain contains a β sandwich composed of two antiparallel β sheets, and a loop-β – sheet-α – helix motif that packs tightly against one end of the β sandwich. At this end of the β barrel, there are two long loop regions (L2 and L3) that are stabilized by a tetrahedrally coordinated zinc atom. Although the β barrel comprises a major part of the core domain structure, two loops and one α helix of p53 are directly involved in DNA binding. Protein-DNA interactions are composed of major groove contacts with C-terminal sequence of the α helix H2 (aa 278–286) and loop L1 (aa 112–124) of the loop–sheet–helix motif; minor groove interactions which take place in the A:T-rich region of the DNA and involve Arg^{248} from L3 loop (aa 236–251), and interactions of p53 with the phosphate backbone connecting major and minor groves (Cho *et al*., 1994). The majority of p53 gene alterations are missense mutations leading to the synthesis of mutant proteins. These mutant proteins are unable to bind the target DNA sequences due to the substitutions at key amino acid residues of the DNA binding core domain (Soussi *et al*., 2000).

Monoclonal antibodies directed against linear and conformational epitopes at different domains of p53 protein are highly useful tools to investigate structure-function relationship of wild-type and mutant p53 proteins. Most of these antibodies react with epitopes located at the antigenically dominant N-terminal and C-terminal regions of p53 (Legros *et al*., 1994). The centrally located core region is poorly antigenic, and only a few monoclonal antibodies have been generated to this critical DNA binding domain (Legros *et al*., 1994). We generated three monoclonal antibodies against p53 DNA binding domain, from mice immunized with recombinant full length human p53 protein, by selective screening of antibody-producing hybridomas using a truncated p53 polypeptide lacking both N-terminal and C-terminal regions (a histidine-tagged 237 amino acid polypeptide spanning residues 72–308 of human p53 protein).

Three hybridoma clones (named 6B10, 7D3 and 9E4) producing monoclonal anti-p53 antibodies were selected for further studies. All three Mabs were first

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tested for their ability to recognize human p53 protein using western immunoblotting technique (Figure 1). Both 9E4 and 6B10 recognized wild-type p53 expressed in HepG2 cells, but 7D3 reacted only weakly. All three antibodies also reacted with two different mutant p53 proteins, p53-Y220C and p53-R249S, expressed in Huh-7 and Mahlavu cells, respectively. p53-deleted Hep3B cells were used as a negative control (Hsu *et al*., 1993). Interestingly, 9E4 reacted with three antigens in these p53-deficient cells, as well as three other cell lines tested (Figure 1). These antigens showed a different migration pattern than wild-type or mutant p53 proteins (compare 9E4 with 6B10 in Figure 1), and appear to be unrelated to p53 protein. The apparent molecular weights of two antigens were higher than that of p53. The nature of these antigens recognized by 9E4 is presently unknown. On the other hand, 9E4 antibody reacted only with p53 when tested by immunoprecipitation after ^{35}S-methionine labelling of transfected Saos cells (Figure 2). This suggests that 9E4 recognizes several cross-reacting antigens under denaturing conditions of Western blot assay, but not in their native form. The immunoprecipitation experiments with both 9E4 and 6B10, in comparison with DO7 antibody (Vojtesek *et al*., 1992) also indicated that their immunoreactivities with different p53 proteins were weaker, probably because their respective epitopes are less accessible under non-denaturing conditions of immunoprecipitation assay (Figure 2). Figure 2 shows that all tested p53 mutants are recognized by 9E4, and to a lesser degree by 6B10. Some of these mutants such as p53-R175P retain transcriptional activity and the ability to induce G1 arrest, but have lost apoptotic activity, while others such as p53-R175Y and p53-R175 W have lost both activities (Ryan and Vousden, 1998).

We also tested 9E4, 6B10 and 7D3 antibodies by indirect immunofluorescence after fixation and permeabilization of cells with methanol. A strongly positive nuclear staining was observed with 9E4 and 6B10 in many cell lines expressing different mutant p53 proteins. The 9E4 Mab also reacted strongly with a cytoskeleton-associated antigen in different cell lines tested, including p53-negative Hep3B cells, under these conditions. These observations confirm our hypothesis that antigens cross-reacting with 9E4 antibody are recognized only under denaturating conditions. The immunoreactivity observed by 7D3 was weak in indirect immunofluorescence assay, similarly to Western blot data (data not shown).

The main characteristics of our antibodies are summarized in Table 1. The epitopes recognized by these antibodies were determined by Pepscan ELISA, as previously described (Legros *et al*., 1994). The 7D3 Mab reacted with an epitope located within amino acid residues 211–220 (TFRHSVVVPY) of human p53. This 10 amino-acid fragment carries epitopes for two previously identified antibodies, namely Pab240 that recognizes the residues 213–218 (Stephen and Lane, 1992) and HO13.1 (Legros *et al*., 1994). The 6B10 Mab recognizes amino acids residues 291–300 (KKGEPHHELP), similar to HO7.1 and HO33.8 antibodies described by Legros *et al*. (1994).

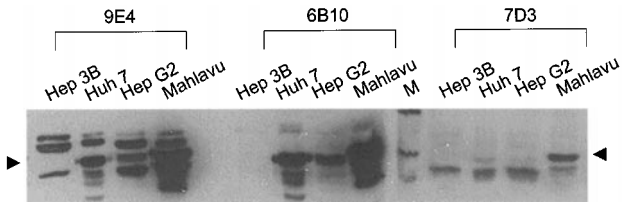

Figure 1 Immunoreactivities of 6B10, 7D3 and 9E4 monoclonal antibodies with wild-type and mutant p53 protein as tested by Western immunoblotting. Cell lysates were prepared from indicated cell lines using a buffer containing 150 mM NaCl, 1 mM EDTA, 1.0% NP-40, 10 mM Tris (pH 8.0), 1.0% sodium deoxycolate and 1 × complete EDTA-free protease inhibitor cocktail (Roche). A total of 30 μg protein was loaded from each lysate and subjected to 10% SDS–PAGE. Transfer of the proteins to PVDF membrane (Millipore) was performed by Bio-Rad semi-dry transfer cell. Membranes were blocked in TBS-T containing 3% dried non-fat milk powder and incubated with the indicated anti-p53 antibodies. Detection was performed with Lumilight-plus kit (Roche). Black arrows indicate p53 protein. Note that Hep 3B cells are p53-negative, while HepG2, Huh-7 and Mahlavu cells express wild-type, mutant p53-Y220C and mutant p53-R249S, respectively (Hsu *et al*., 1993)

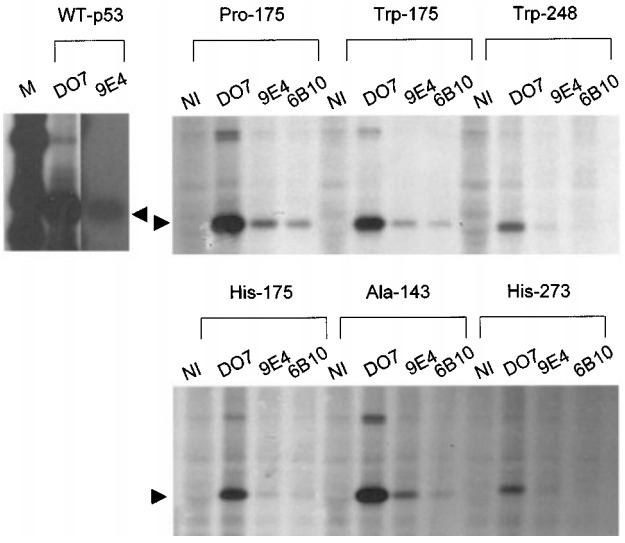

Figure 2 Immunoprecipitations of wild-type and mutant p53 proteins with 9E4 and 6B10 monoclonal antibodies indicate that both 9E4 and 6B10 recognize both wild-type and mutant p53 proteins, although their immunoreactivities are weak in comparison to D07 monoclonal antibody. The experiments for wild-type p53 protein were performed with in-vitro translated human p53. All other experiments were performed with p53 negative SaOs cells following transient transfection with the indicated mutant forms of human p53. Following transfections, cells were metabolically labelled with ^{35}S-methionine, and lysed in a buffer containing 150 mM NaCl, 1 mM EDTA, 1.0% NP-40, 10 mM Tris (pH 8.0), 1.0% sodium deoxycholate, 10 μg/ml leupeptine, 1 μg/ml pepstatin and 10 μg/ml aprotinin. Following centrifugation, supernatants were immunoprecipitated with the indicated antibodies and Protein G agarose, run on SDS–PAGE and subjected to autoradiography

The Mab 9E4 recognizes a new epitope located within amino acids residues 281–290 (DRRTEEENLR). This epitope comprises six (DRRTEE) of the nine amino acid

Table 1 Characteristics of monoclonal antibodies 6B10, 7D3 and 9E4

Monoclonal antibodies	*Ig isotype* (light chain)[a]	*Epitope on human p53* (amino acid residues)[b]	*Related structural motifs* (amino acid residues)
7D3	IgG2a (κ)	TFRHSVVVPY (211–220)	Pab240 epitope[c] (213–218)
9E4	IgG1 (κ)	DRRTEEENLR (281–290)	H2 Helix[d] (278–286)
6B10	IgG1 (κ)	KKGEPHHELP (291–300)	HO7.1 and HO33.8 epitopes[b] (291–300)

[a]Isotypes were determined by 'Mouse-Hybridoma Subtyping Kit' (Boehringer). [b]Epitopes were mapped by Pepscan ELISA assay (Legros *et al*., 1994). [c]Stephen and Lane (1992). [d]Cho *et al*. (1994)

residues (PGR**DRRTEE**) that form the DNA binding H2 α helix motif (H2) of human p53 (residues 278–286). p53 interactions with its target pentamer involve both major and minor groove contacts. Several amino acid residues of H2 motif are involved in these contacts. The Arg^{280} residue, reinforced by Asp^{281}, makes the most critical major groove contact with the invariant C:G base pair of the pentamer consensus. The Asp^{281}, does not participate directly to DNA contacts, but it forms salt bridges with both Arg^{280}, and Arg^{273} which itself binds to a phosphate group in the consensus motif. Finally, Arg^{283} of H2 helix participates to DNA backbone contacts by binding to another phosphate of the consensus motif. A forth residue of H2 helix, Arg^{282}, one of the six mutational hotspots of p53, plays a structural role in the loop–sheet–helix motif, being involved in the packing of H2 helix against the β hairpin and L1 loop (Cho *et al*., 1994). Thus, the epitope recognized by 9E4 harbors several key amino acid residues directly involved in specific binding of p53 to its target DNA sequences.

The positions of epitopes recognized by 7D3 and 9E4 Mabs are shown in Figure 3. We believe that the 9E4 antibody will be a quite useful tool for different studies related to the specific binding of p53 to its target DNA sequences, as well as for the comparison of its transcription-dependent and transcription-independent cellular activities. It is expected that 9E4 antibody will block both specific DNA-binding and transcriptional regulatory activities of p53, when introduced into cells by micro-injection or as an intracellular antibody (Cohen *et al*., 1998; Caron de Fromentel *et al*., 1999). By the same methods, 9E4 may also be useful to test whether certain p53 mutants display any transcriptional activity, either as a repressor or activator, directly or indirectly (Blandino *et al*., 1999). Finally, two recently discovered p53 homologue proteins, namely p63 and p73, are known to display p53-like transcriptional activities. These new proteins have different amino acid sequences in the region homologous to 9E4 antibody epitope on p53 protein. In contrast to the DRRTEEENLR sequence on p53, p63 and p73 have respectively DRKADEDSIR and DRKADEDHYR sequences (underlined residues differ from that of p53) at the same region (Kaghad *et al*., 1997; Osada *et al*., 1998). It is highly unlikely that 9E4 will be able to recognize these corresponding amino acid residues on p63 and p73. Therefore, 9E4 may be used to block specifically any p53-related transcriptional activity, when studying cellular activities of p63 or p73 under experimental conditions. Such studies are under investigation.

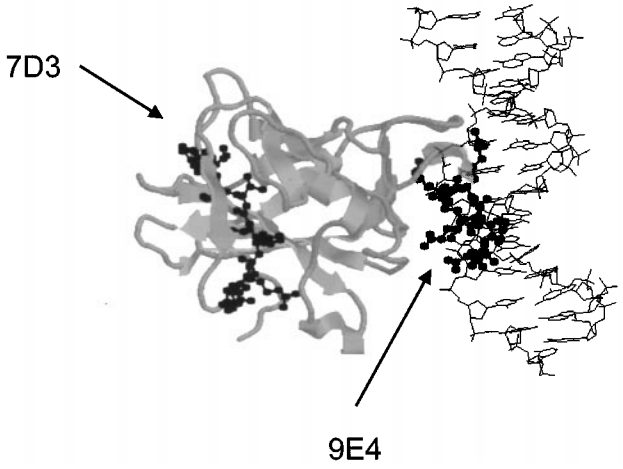

Figure 3 The location of p53 protein epitopes recognized by 7D3 and 9E4 mouse monoclonal antibodies on the p53 core domain. The three dimensional model of the core domain of human p53 and its DNA binding site (Cho *et al*., 1994), illustrating the epitope structures (ball and stick in black) involved in direct interaction with 7D3 and 9E4 monoclonal antibodies

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