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Monoclonal antibodies raised against Xenopus p53 interact with human p73

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The p53 tumor suppressor gene belongs to a multigene family that includes two paralogues, p63 and p73. The structure of the p63 and p73 genes is quite similar, but both have common activities with p53, such as DNA binding and transactivation. Both p53 and p73 bind to mdm2, but only p53 is degraded through the activity of mdm2. p63 neither binds to nor is degraded by mdm2 despite important conservation in the key interacting residues. Using a panel of monoclonal antibodies raised against human and *Xenopus* p53, we have been able to find several antibodies that cross-react strongly with human p73. These antibodies react both with exogenous p73 expressed in mammalian cells and with endogenous p73. Interestingly, all these antibodies react with the same epitope localized in the amino-terminus of p53, but have no cross-reaction with p63. This epitope corresponds to the exact mdm2 binding site to p53. These antibodies inhibit the interaction between either p53 or p73 and mdm2, and may be useful tools for the study of these proteins. Furthermore, our studies suggest that there exist specific spatial requirements for the interaction between p53 or p73 and mdm2.
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Results and discussion

Mutations in the p53 gene are the most common genetic alterations in human cancers (Soussi *et al*., 2000). The p53 tumor suppressor gene is located on chromosome 17p13 and encodes a nuclear 393-amino acid nuclear transcription factor that is implicated in the regulation of normal cell growth and apoptosis (Levine, 1997). Most of the known p53 gene alterations are mis-sense mutations clustered in the evolutionarily highly conserved exons 4–10 (Soussi *et al*., 2000). These mutations result in a biologically inactive p53 protein that stably accumulates in the cell nucleus and can be detected by immunohistochemistry. In the absence of wild-type p53 protein, genetic aberrations are more likely to accumulate, leading to genetic instability and cell transformation.

Recently, two genes referred to as p63 and p73 have been found to encode proteins that share significant amino acid identity in the transactivation domain (30%), the DNA binding domain (60%) and the oligomerization domain (37%), with p53 (Marin *et al*., 1998; Yang and McKeon, 2000). This homology suggests that the products of this gene family may act as transcription factors but their biological functions are likely to be distinct. There is little evidence for p73 and p63 mutations in human cancer, but several reports indicate that the p73 protein accumulates in the nucleus of tumor cells from different types of cancers (Ikawa *et al*., 1999). Furthermore, specific p73 antibodies have been described in the sera of patients with various types of cancer (Tominaga *et al*., 2001).

Monoclonal antibodies (mAbs) directed to p53 protein have been valuable tools for investigating the structure-function relationship of wild-type and mutant p53. We have demonstrated that most of these antibodies react with epitopes localized at the antigenically dominant amino- and carboxy-termini of the p53 protein (Legros *et al*., 1994b; Vojtesek *et al*., 1995). A detailed analysis of mAbs specific for the amino-terminus indicates that critical residues involved in the interaction with the protein correspond to key residues on p53 involved in its interaction with the mdm2 protein (Portefaix *et al*., 2000). Although p73 is not degraded by mdm2, it interacts specifically with mdm2. This observation prompted us to determine whether several monoclonal antibodies specific for the amino-terminus of p53 could cross-react with p73.

Using recombinant p53 and p73α expressed in insect cells, we analysed a panel of monoclonal antibodies specific for human p53 and *Xenopus* p53 (Xp53). As shown in Figure 1a, some of these antibodies X18, X73 or X77 cross-reacted with p73α. It is interesting to note that these three antibodies were obtained via the immunization of mice with Xp53. None of the antibodies specific for human p53 cross-reacted with p73α (Figure 1 and data not shown). All these results were confirmed by BIAcore analysis using a gst-p73α construct (Figure 1b). The epitopes recognized by these three mAbs lie in the amino-terminus of the p53 protein. Recent detailed epitope mapping suggests that X18, X73 and X77 have a similar epitope. The minimum peptide recognized by these three mAbs is $F^{19}SDLW^{23}$ with an essential requirement for residues

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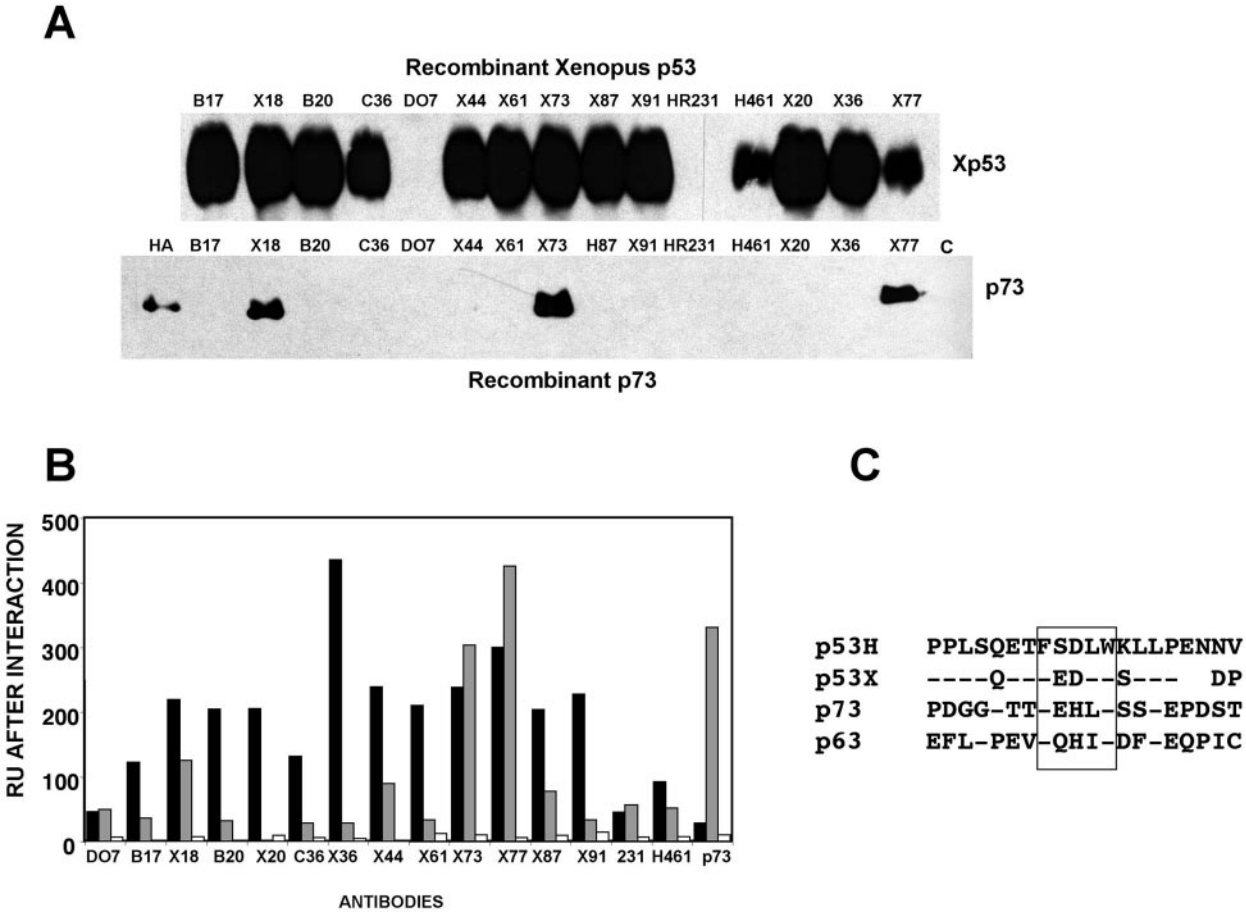

Figure 1 Specific recognition of p73 by p53 monoclonal antibodies. (**a**) Western blot analysis. Xp53 or Ha-tagged-p73α expressed in insect cells via recombinant baculovirus was used for immunoprecipitation with the various mAb. After electrophoresis, proteins were transferred to a membrane that was probed either with X20 (specific for Xp53) or with HA antibody (Babco). Development was performed using chemoluminescence (Pierce) as previously described (Bensaad *et al*., 2001). C; control experiment without antibody; (**b**) BIAcore analysis: the BIAcore 3000 surface plasmon resonance-based biosensor system (SPR) was used to measure the interactions between the different proteins (analyte) and the immobilized antibodies (ligand). Tested mouse antibodies were linked to the CM5 sensor chips via a rabbit antibody directed toward mouse immunoglobulins, yielding resonance values ~1000 resonance units (RU). Recombinant *Xenopus* p53 was produced in insect cells via a recombinant baculovirus. The gst−p73α and gst−p63α protein samples were produced in the *E. coli* bacterial strain and extracted by sonication followed by incubation in a lysis buffer (1 × PBS −1%, Triton X100) with protease inhibitors. Samples were injected at 25°C at a flow rate of 10 μl/min onto the sensor chip surface where the various antibodies had been immobilized (HBS running buffer: 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4). The binding surface was then regenerated by washing with 10 mM glycine-HCl pH 3: *Xenopus* p53, gst−p73α and gst−p63α are shown as black, grey and white bars respectively. (**c**) comparison of the amino-terminus of p53, p63 and p73. The sequence FSDLW corresponds to the epitope of X77 (Portefaix *et al*., 2000). Dashes indicate identical residues compared to human p53. All antibodies with the X prefix were raised against *Xenopus* p53. The specific epitopes of all these mAbs have already been described (Legros *et al*., 1994a,b; Portefaix *et al*., 2000) and are available on line on our web site (http:////p53.curie.fr)

F^{19} and W^{23} (Portefaix *et al*., 2000). As shown in Figure 1c, this region, which corresponds to the mdm2 binding site, is slightly conserved in the various p53 and also in p73 and p63. The two most conserved residues are the F^{19} and W^{23} residues essential for antibody binding. Using BIAcore analysis (Figure 1b) and Western blot (data not shown, but see Figure 3), we also analysed the behavior of these antibodies toward p63. Surprisingly, none of them reacted with the p63 protein despite the sequence conservation. This suggests that the sequence between the F^{19} and the W^{23} residue may make an important contribution to the binding of the antibody.

Different p53 and p73 constructs were transfected in H1299. This cell line has a deletion of both p53 alleles, which is convenient for exogenous p53 expression. The Western blot in Figure 2a shows that the X77 mAb specifically recognized human and *Xenopus* p53. In cells transfected with constructs expressing either p73α or p73β, a specific band with the expected molecular weight was detected. Longer exposure did not enable detection of any endogenous or exogenous bands (data not shown). This interaction is not detected with a monoclonal antibody specific for the carboxy-terminus of Xp53, such as X36 (data not shown). No cross-reaction was detected with p63 α and β (Figure 2a).

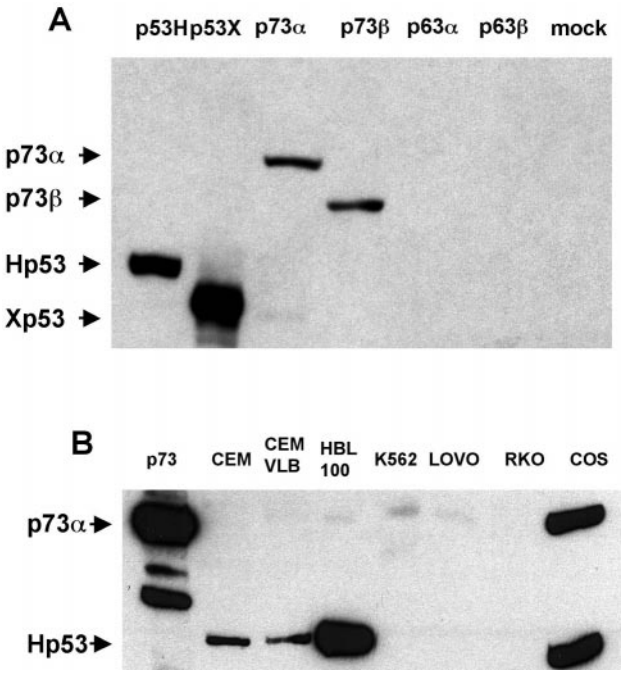

Figure 2 (**a**) H1299 cells (7.10^5 cells) were transfected with 5 μg of expression vector specific for the various p53, p73 and p63 proteins. After 48 h, proteins were extracted with RIPA buffer and used for Western blotting. For cells transfected with human p53 and *Xenopus* p53 expression vector, 4 and 1 μg of protein extract were loaded onto the gel respectively. For the others, 20 μg of extract were used. Western blot was performed with X77 mAb. (**b**) Cell extracts from various cell lines (15 μg) were analysed by Western blot with X77 mAb. p73: positive control with recombinant p73 expressed in insect cells; CEM and CEM–VLB: human T-cell lymphoma; HBL100: human breast fibroblast transformed by SV40; Lovo: human colorectal carcinoma; RKO: human colorectal carcinoma; COS: monkey kidney cells transformed by SV40

Control Western blot with a specific p63 mAb indicates that both proteins were overexpressed after transfection (data not shown).

In order to verify whether X77 is able to recognize endogenous p73, we tested several untransfected cell lines by Western blot. As described by Marin *et al.* (1998), we confirmed the high level of endogenous p73 in COS cells using X77 mAb (Figure 2b). In K562 and Lovo cells, we observed a very faint accumulation of a 73 kDa protein, but its identity remains to be confirmed. Taken together, these observations confirm the specificity of these antibodies toward the p73 protein and their lack of reactivity toward p63.

The specificity of these antibodies was also tested by immunofluorescence (Figure 3). No non-specific labeling was detected with any of these antibodies. X18 and X77 specifically stained the nucleus of cells transfected with human p53, Xp53 and p73α in an indistinguishable pattern. No p73 staining was obtained with X20 or DO7 (data not shown).

As indicated above, the epitopes recognized by X18, X73 and X77 contain the sequence $F^{19}SDLW^{23}$, which is an essential part of the sequence interacting with mdm2 (Kussie *et al.*, 1996), with three residues essential for p53 mdm2 interaction, F^{19}, W^{23} and L^{26}. The three monoclonal antibodies were shown to inhibit the interaction between human p53 and mdm2 (T Soussi unpublished data, see also Figure 4). Using a pull-down assay, we showed that the incubation of labeled p73α with gst–mdm2 led to a specific interaction with the two proteins (Figure 4). Preincubation of the p73 proteins with X77 blocked this interaction. Control experiments with human p53 gave similar results, whereas a monoclonal antibody specific for the carboxy-terminus of p53 (HR231) did not lead to any inhibition. These results indicate that the interaction between these antibodies and p53 or p73α is similar, and requires similar epitopes corresponding to the mdm2 binding site. The finding that these

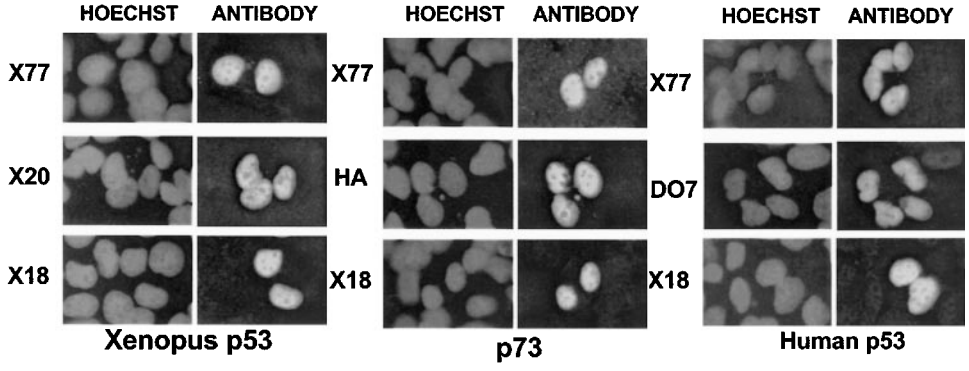

Figure 3 Indirect immunofluorescence analysis of p53 and p73. Saos-2 cells were grown in a glass coverslip in 24-well plates (10^5 cells per well). Each well was transfected using 0.5 μl of lipofectamine 2000 (Life Science) and 500 ng of vector expressing various p53 or p73α. After 36 h, the cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X100 in PBS for 10 min at room temperature. The slides were incubated with blocking solution (2% BSA in PBS) for 10 min and then probed with various monoclonal antibodies for 2 h at 37°C. The antibody-antigen complexes were detected with Alexa-conjugated goat anti-mouse IgG by incubation for 30 min at room temperature. The slides were washed three times in PBS after each incubation. Cells were stained for 10 min at room temperature with Hoescht for DNA visualization. The name of the antibody used for staining is shown on the side of the figure. DNA transfected is indicated under each picture

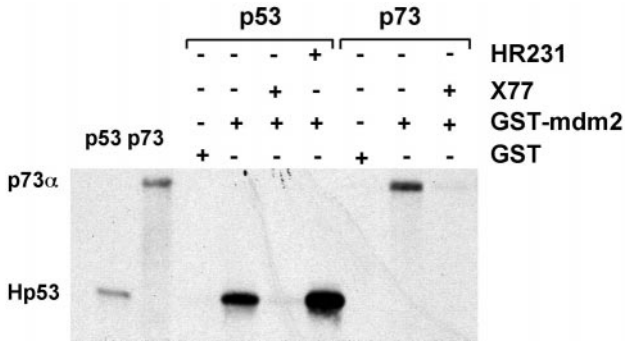

Figure 4 p73 and p53 interaction with mdm 2 is blocked by X77. 50 000 c.p.m. of radiolabeled p53 or p73α obtained by *in vitro* transcription translation of the corresponding cDNA were incubated with gst-mdm2 coupled to glutathione sepharose. After 2 h of incubation at 4°C, bound proteins were washed three times with RIPA buffer, run on an SDS-polyacrylamide gel and detected by fluorography. For blocking experiments, the labeled proteins were pre-incubated with the indicated antibody for 2 h at 4°C before processing the pull-down. Input (2500 c.p.m.) is shown at the left of the gel (p53 and p73)

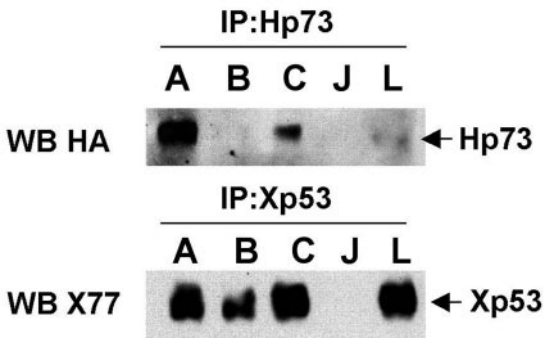

Figure 5 *Xenopus* p53 antibodies cross react with p73. Sera from rabbit immunized with Xp53(A,B,C,L) or patient sera with p53 autoantibodies (J) were used for immunoprecipitation of recombinant Ha-tag p73α (upper panel) or recombinant Xp53 (lower panel). After separation by electrophoresis, Western blot was performed with the indicated antibody

antibodies do not react with p63 is not unexpected. Despite sequence homology, the p63 protein neither interacts with mdm2 nor represses its transcription (Kojima *et al*., 2001; Little and Jochemsen, 2001; Wang *et al*., 2001). As these monoclonal antibodies mimic the interaction of mdm2 with p53, this demonstrates that, despite sequence homology at the three key residues (F^{19}, W^{23} and L^{26}), this interaction has other requirements, either at the sequence level or the structural level, or both.

Among the large panel of mAbs produced toward human and *Xenopus* p53, the three mAbs that cross-react with human p73 were produced by mouse immunization with *Xenopus* p53. These results suggest that the antigenicity of Xp53 is closer to those of human p73 than Hp53. We checked the reactivity of four sera from rabbit immunized with Xp53 toward p73α (Figure 5, lane A,B,C,L). Two of these sera strongly reacted with human p73α (A and C), whereas another was weaker (L) and the remaining one was negative (B). Human sera from a patient with a high level of p53 antibodies did not react with Xp53 (J) nor with p73α. We had previously demonstrated that there was no cross-reaction between human p53 and human p73 antibodies in cancer patient sera (Tominaga *et al*., 2001).

In conclusion, our results indicate that several monoclonal antibodies raised against Xp53 have a strong reaction with the amino-terminus of human p73. In fact, the reactivity of these mAbs to the two proteins was indistinguishable. The localization of their epitopes in the amino-terminus of p73 is different from those already available (Marin *et al*., 1998). The genomic organization of the p73 gene is rather complex. Two separate promoters give rise to the transactivating (TA) and ΔN products which lack the amino-terminus and the epitope for the various mAbs described in the present work. Alternative splicing at the 3′ end yields further p73 isotypes (α, β and γ) (Yang and McKeon, 2000). It is noteworthy that the ΔN product is the major species in several cell models. Therefore, the mAbs might be very useful to distinguish the various p73. Finally, we should be cautious when using mono or polyclonal antibodies reputed to be specific for p53.

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