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Regulation of the specific DNA binding activity of *Xenopus laevis* p53: evidence for conserved regulation through the carboxy-terminus of the protein

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Recombinant human p53 isolated either from *E. coli* or from insect cells is poorly active for binding to DNA but it can be dramatically stimulated by phosphorylation, antibody binding to the carboxy-terminal negative regulatory domain, short peptides derived from this negative regulatory domain or short single strands of DNA. We report here that *Xenopus* p53 has a very similar behavior. Using a new set of monoclonal antibodies directed either to the amino- or the carboxy-terminus of *Xenopus* p53, we demonstrate that the frog protein can be activated by specific carboxy-terminus monoclonal antibodies in order to bind to human p53 DNA response element. In addition, we report that such activation of both humans and frogs protein can also be achieved by small peptides derived from the carboxy-terminus of both p53. Although, the sequence of this region is not conserved in the various p53 species, the presence of conserved basic residues indicates that such activation is charge-dependent. This is confirmed by the finding that small poly-lysine peptides can activate both human and *Xenopus* p53. *In vivo* expression of *Xenopus* p53 indicates that this protein is able to transactivate a wide variety of human p53 response elements as long as the experiments are performed at 32°C since activity at 37°C, a temperature well above the natural temperature of *Xenopus*, is lost. Finally, we demonstrate that human mdm2 is able to down regulate the transcriptional activity of *Xenopus* p53.

Keywords: p53; xenopus; manoclonal antibodies; transactivation

Introduction

The p53 protein plays a crucial role in the cellular response to DNA damage by activating either an apoptotic or growth arrest pathway in proliferating cells. Among the various biochemical activities linked to the p53 protein, its main function seems to be its ability to activate transcription from genes containing two contiguous monomers of the sequences (Pu)3 × C(A/T)(A/T)G(Py)3 × (El-Deiry *et al*., 1992). The transcription domain of p53 is localized in the amino-terminal part of the protein (residues 1–42), whereas the DNA binding domain is localized in the central region of the protein (residues 90–290). The importance of this DNA binding region is emphasized by the observation that more than 7000 described point mutations of the p53 gene are clustered in this region (Béroud and Soussi, 1997). Many of these mutations are correlated with a loss of the wild type function of the p53. Genes with DNA binding sites for p53 include the promoter regions of the p21WAF1 gene (El-Deiry *et al*., 1993), Cyclin G (Okamoto and Beach, 1994; Zauberman *et al*., 1995), the IGF BP3 gene (Buckbinder *et al*., 1995), the Bax gene (Miyashita and Reed, 1995) and the introns of the gadd45 (Kastan *et al*., 1992) and mdm2 genes (Juven *et al*., 1993; Wu *et al*., 1993).

Recently, the importance of the carboxy-terminal region of p53 has been highlighted by characterization of the tetramerization domain and the discovery of a negative regulatory domain that inhibits specific DNA binding (Hupp *et al*., 1992; Shaulsky *et al*., 1990). Activation of sequence-specific DNA binding can be achieved by phosphorylation or N-glycosylation of the p53 carboxy-terminal region and by binding of monoclonal antibody PAb421 or bacterial hsp 70, which both bind to the carboxy-terminal region of the p53 (Hupp *et al*., 1992; Shaw *et al*., 1996). It has been suggested that such activation require conformational change, as small peptides derived from the negative regulatory domain can also stimulate specific DNA binding (Hupp *et al*., 1995). The non specific binding of small oligonucleotides by the carboxy-terminal region of p53 has also been shown to activate the specific DNA binding activity of the central region of p53 (Jayaraman and Prives, 1995). Recently, a cellular protein, ref1, has been shown to be able to convert latent p53 to one that is active for DNA binding (Jayaraman *et al*., 1997).

All these studies were performed on human and murine p53. Starting with vertebrates, more than 20 p53 genes or cDNA have been isolated and sequenced, providing a basis for developing new animal models to study this gene (Soussi and May, 1996) for a review. Indeed, in the case of the cat, chicken and dog, mutations in the p53 gene have been detected in the central region where mutational hot spots for human

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cancer are located. Even more interesting is the identification of the p53 gene in invertebrates such as the squid (Ishioka *et al*., 1995). It should be noted that, although p53 has not been found, in yeast, overexpression of human wild type p53 inhibits cell division in *S. cerevisiae* and *S. pombe*, whereas mutant p53 does not induce a detectable phenotype (Bischoff *et al*., 1992; Nigro *et al*., 1992; Wagner *et al*., 1991). The cloning of *Xenopus laevis* p53 (Xp53) in 1987 has led to the identification of five highly conserved domains, four of them corresponding to the key regions involved in the DNA binding activity of p53 (Soussi *et al*., 1987). Previous studies have demonstrated that Xp53 can bind to SV40 large T antigen (Soussi *et al*., 1989) or to specific DNA binding sites such as the RGC sequence (Wang *et al*., 1995).

In the present work, we demonstrate that regulation of specific DNA binding activity by the carboxy-terminus of the p53 protein was not specific for Hp53 but could be extended to Xp53. Using either new monoclonal antibodies generated toward Xp53 or synthetic peptides, we show that activation of a latent form of Xp53 was a prerequisite for specific DNA binding. By *in vitro* gell shift assay, we observed that Xp53 specifically recognized the various Hp53 DNA binding response elements. *In vivo* expression of Xp53 in mammalian cells showed evidence of a temperature-sensitive transactivation activity of the protein. This activity was specifically inhibited upon overexpression of the mdm2 protein.

Results

Production and characterization of monoclonal antibodies directed toward Xenopus *p53*

A mouse immunized with purified wild type Xp53 was used for hybridoma production, as described by Legros *et al*. (1993, 1994a). The secretion of antibodies was tested using an ELISA with the same protein as antigen. A high number of monoclonal antibodies (mAbs) were obtained, confirming previous results showing the high immunogenicity of p53. Using PEPSCAN ELISA methods (Legros *et al*., 1994a), we mapped the epitope recognized by these mAbs (Table 1). All but three mAbs could be mapped. Similarly to our previous studies on Hp53, most of these mAbs recognized linear epitopes localized in the amino and carboxy-terminus of p53 (Legros *et al*., 1994a). The seven mAbs interacting with the amino-terminus of Xp53 fell into two categories corresponding to two immunodominant regions identified in human p53 (Hp53). They corresponded to mAbs B17 and C36, respectively (Table 1). As these two mAbs were shown to be able to cross-react with Xp53 (Legros *et al*., 1994a), the new mAbs obtained toward Xp53 were also tested on a set of peptides corresponding to Hp53. All these mAbs specific for the amino-terminus of p53 recognized human p53 using the human p53 peptides, but also the entire protein (Figure 2). None of the Xp53 mAbs specific for the carboxy-terminus of p53 showed such cross-reaction, and were found to be specific for Xp53 (Figure 2 and Table 1).

Taken together, these results indicate that the presence of immunodominant epitopes is not specific for Hp53 and can be extended to Xp53. Furthermore, the observation that the two specific amino-terminus sequences are systematically found as major epitopes suggest that they are highly accessible to the immune system.

Xp53 binding to human p53 targets and can be activated by carboxy-terminus mAbs

In previous studies, we showed that Xp53 can bind specifically to sites containing the p53 consensus sequence derived for Hp53, but no experiments were performed on the natural recognition sites (Ridgway *et al*., 1994; Wang *et al*., 1995). Furthermore, no studies

Table 1 Characteristics of the monoclonal antibodies

mAb	*Isotype*	*Antigenic site on Xp53*	*Antigenic site on Hp53*	*Localization*	*Reference*
B17	IgG2a κ	16–25	16–25	Amino-termini	(Legros *et al*., 1994)
C36	IgG1 κ	16–30	16–30	Amino-termini	(Legros *et al*., 1994)
HT216	IgM κ	ND	1–64 (a)	Amino-termini	(Legros *et al*., 1993)
X18	Ig1 κ	16–25	16–25	Amino-termini	This work
X73	Ig1 κ	16–25	16–25	Amino-termini	This work
X77	Ig1 κ	16–25	16–25	Amino-termini	This work
X44	Ig1 κ	16–30	16–30	Amino-termini	This work
X61	Ig1 κ	16–30	16–30	Amino-termini	This work
X87	Ig1 κ	16–30	16–30	Amino-termini	This work
X91	Ig1 κ	16–30	16–30	Amino-termini	This work
X36	Ig1 κ	349–360	–(b)	Carboxy-termini	This work
X10	Ig1 κ	349–360	–(b)	Carboxy-termini	This work
X65	Ig1 κ	349–360	–(b)	Carboxy-termini	This work
X45	Ig1 κ	349–360	–(b)	Carboxy-termini	This work
X86	Ig1 κ	349–360	–(b)	Carboxy-termini	This work
X89	Ig1 κ	349–360	–(b)	Carboxy-termini	This work
X85	Ig1 κ	349–363	–(b)	Carboxy-termini	This work
X29	Ig1 κ	233–363 (c)	–(b)	Carboxy-termini	This work
X57	Ig1 κ	233–363 (c)	–(b)	Carboxy-termini	This work
X20	Ig1 κ	233–363 (c)	–(b)	Carboxy-termini	This work
X62	Ig1 κ	233–363 (c)	–(b)	Carboxy-termini	This work

The localization of the epitopes have been performed by the PEPSCAN methods using a set of overlapping peptides corresponding to Hpp53 or Xp53 or by Western blot on truncated p53 protein. (a) HT216 could not be mapped by the PEPSCAN method; (b) These antibodies do not cross react with Hp53; (c) these antibodies were only mapped by Western blot using truncated p53 express in *E. coli*

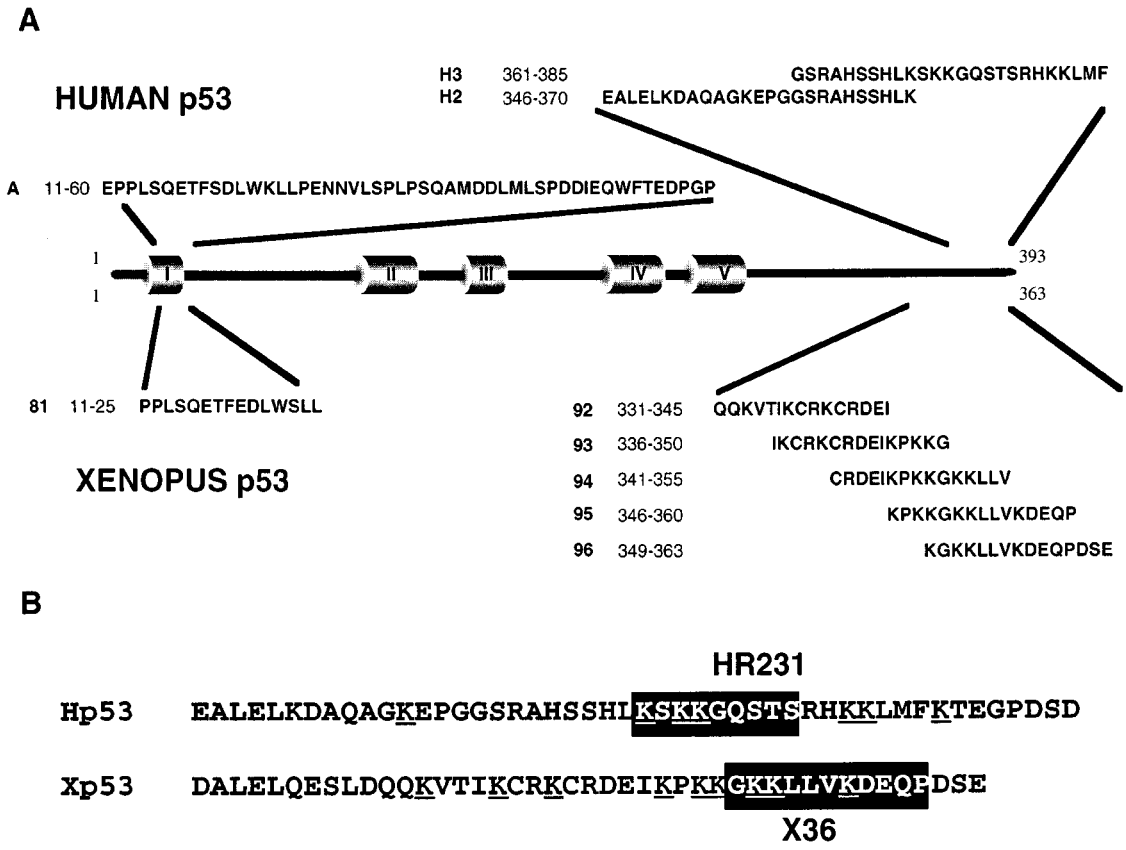

Figure 1 Peptides used in this study. (**a**) Peptides were derived from the amino- and carboxy-termini of Hp53 and Xp53. I – V correspond to the 5 domains conserved throughout evolution. (**b**) sequences of the carboxy-terminus of Hp53 and Xp53. Lysine residues are underlined

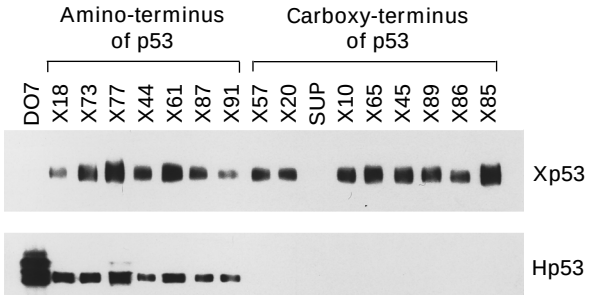

Figure 2 Immunoblotting analysis of anti-p53 mAbs with human and *Xenopus* p53. The protein extract was separated on a single slot 10% polyacrylamide gel, and separated proteins were transferred electrophoretically to nitrocellulose. The nitrocellulose sheet was cut into strips, and the separate strips were incubated with the different antibodies tested. The blot was developed using anti-mouse peroxidase second antibody and luminol (NEN-Dupont de Nemours). Human and *Xenopus* p53 were produced in insect cells infected with recombinant baculovirus. DO7 was specific for human p53 whereas Sup is a negative control with culture medium

were focused on the role of the carboxy-terminus of Xp53 in regulation of this specific DNA binding activity.

We thus assessed the behavior of Xp53 toward various DNA binding sites of human origin as no *Xenopus* p53 response genes have been cloned so far. Hp53 was used as a control in all these experiments. Hp53 produced in insect cells cannot bind strongly to DNA as long as it has not been activated in the carboxy-terminus region (Hupp *et al*., 1992). For activation, we used HR231, a mAb which has an epitope localized in the carboxy-terminus of Hp53. The amino acid residues essential for the recognition of Hp53 were slightly different from PAb421 (JM Portefaix, *et al*., manuscript in preparation). Nevertheless, HR231 was able to specifically activate DNA binding of Hp53 to DNA (Figure 3a). H279, a mAb specific for the amino-terminus of Hp53, was unable to activate DNA binding effectively, whereas it was able to supershift the HR231-p53-DNA complex (Figure 3a). This observation indicates that activation of Hp53 DNA binding activity is not specific for PAb421, but can be extended to other carboxy-terminus mAbs with different recognition sites.

Without any activation, the DNA binding activity of Xp53 was also very weak, whereas it was activated more than 50-fold by X36, a mAb specific for the carboxy-terminus of Xp53 (Figure 3a). This complex was also supershifted by X77, a mAb specific for the amino terminus of Xp53. All the mAbs specific for the carboxy-terminus of Xp53 were able to activate such binding (data not shown), whereas none of the mAbs specific for the amino-terminus were able to activate DNA binding (Figure 3a and data not shown). These results indicate that Xp53, like Hp53, is a highly flexible protein and emphasize the role of the carboxy-terminus, a non-conserved region, in the regulation of p53 function.

Xp53 is able to bind to all sequences we have tested so far including the CONS, WAF1, GADD45 and the Mdm2 sequences (Figure 3b and data not shown). As expected from the high sequence conservation between the two p53 proteins, the binding activity of Xp53

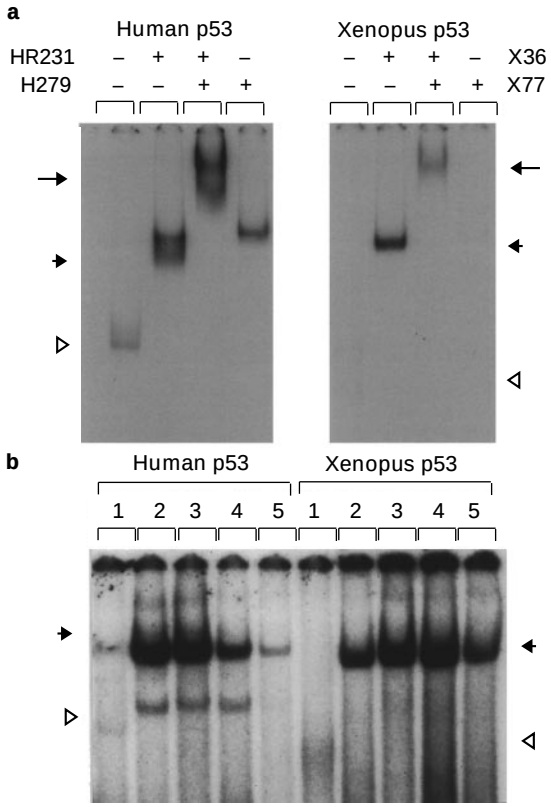

Figure 3 Activation of sequence-specific DNA binding of Hp53 and Xp53. Activation and DNA binding experiments were performed by gel-shift assay as described in Materials and methods. (**a**) Effect of various mAbs on DNA binding activity. Open arrow marks the migration of the p53-DNA complex due to the small fraction of activated p53 present in insect cells (Hupp and Lane, 1994). For Xp53, this band is always very faint and does not appear on the picture. Short black arrow marks the position of p53 complexes bound to one monoclonal antibody (such as HR231 or H279), whereas the long black arrow marks the position of p53 complexes bound to two monoclonal antibodies (such as HR231 and H279). The probe used in this experiment was Waf1. (**b**) DNA binding experiments were performed with various human p53 probes: 1 and 2, WAF1; 3, BB9; 4, GADD45; 5, PCNA. In lane 1, no antibodies were used for p53 activation whereas HR231 and X36 mAbs were used for activation of Hp53 and Xp53, respectively, in lanes 2–5

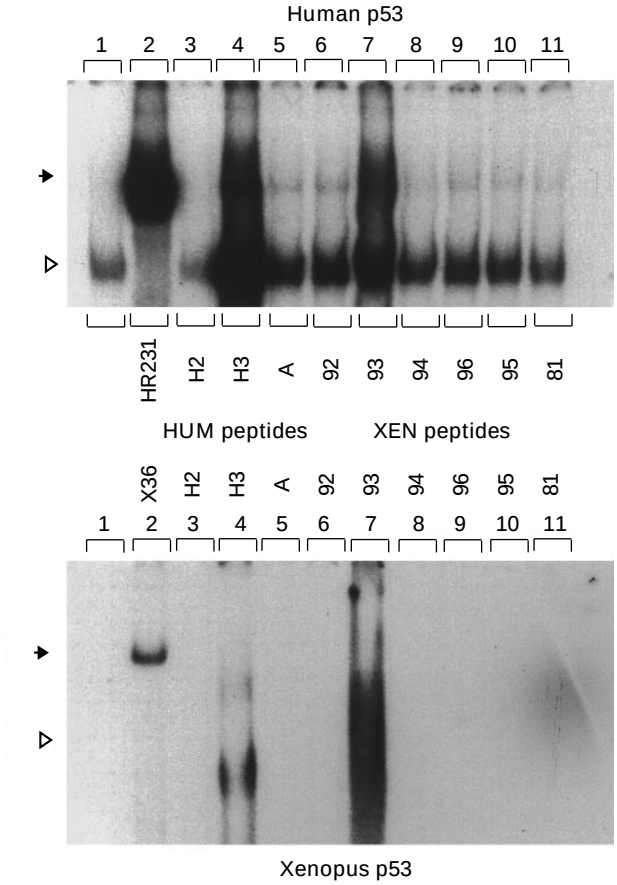

Figure 4 Specific activation of the DNA binding activity of Hp53 and Xp53 by small peptides. Peptides corresponding to various regions of Hp53 and Xp53 are fully described in Materials and methods and in Figure 1. White arrow marks the migration of the p53 DNA complex due to the small fraction of activated p53 present in insect cells. Black arrow marks the position of p53 complexes bound to one monoclonal antibody (such as HR231 or X36). The probe used in this experiment was BB9

toward the DNA sequence of human origin is very efficient, suggesting that frog p53 could substitute for Hp53 *in vivo*.

Activation of the sequence specific DNA binding activity of Xp53 by synthetic peptides

Small peptides derived from the carboxy-terminus of Hp53 can activate the latent sequence specific DNA binding activity of p53, suggesting an allosteric model for activation of Hp53 (Hupp *et al*., 1995). Using a series of peptides derived either from Hp53 or Xp53, we examined activation of specific DNA binding activity. Figure 4 shows that Hp53 can be specifically activated by peptides corresponding to a specific region of the carboxy-terminus of Hp53 (peptide H3) whereas peptides derived from the amino-terminus of Hp53 (peptide H2 or A) are unable to activate DNA binding. Xp53 is also well activated by a peptide corresponding to the carboxy-terminus of Xp53 (peptide X93), whereas surrounding peptides (92 to 95) and the amino-terminus-derived peptide (peptide 81) are inactive. Surprisingly, the activating peptides are able to activate both Xp53 and Hp53 independently of their origin. Hp53 is specifically activated by peptide 93, whereas Xp53 is activated by peptide H3 (Figure 4). The sequence of these activating peptides is somewhat different between the two species, except for conservation of five lysine residues, suggesting that the charge of the peptide is an important feature in activation of p53.

In order to confirm this hypothesis, we tested the behavior of a short poly-lysine peptide. Preliminary experiments indicated that large amounts of such basic peptides induced the formation of high molecular weight complexes which were not able to migrate in the gel (data not shown; see also Figure 5, lanes 11 and 12). Such complexes occurred in the absence of p53 and were probably due to the aggregation of DNA with poly-lysine. Nevertheless, using various amounts of peptides, specific activation of either Hp53 or Xp53 could be detected (Figure 5, lanes 5 and 6). This result indicate that charged residues were an important component in the activation of p53-specific DNA binding by small peptides. These results are in

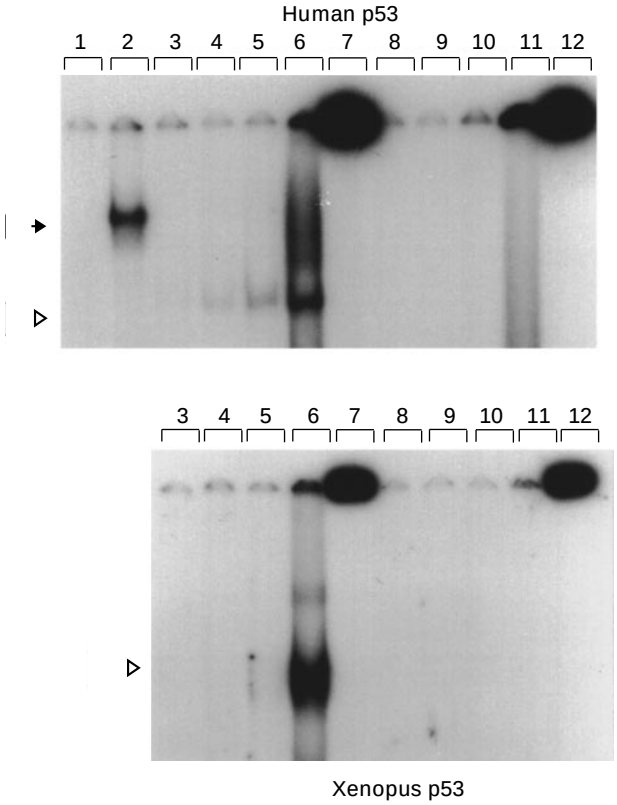

Figure 5 Specific activation of the DNA binding activity of Hp53 and Xp53 by a short poly-Lys peptide. The WAF1 probe was incubated either alone with the peptide (lanes 8–12) or with both the peptide and p53 (lane 3–7). The amount of peptide for each reaction with 2 ng (lanes 3 and 8); 4 ng (lanes 4 and 9); 10 ng (lanes 5 and 10); 20 ng (lanes 6 and 11); or 100 ng (lanes 7 and 12). White arrow marks the migration of the p53-DNA complexes. Black arrow marks the position of p53 complexes bound to monoclonal antibody HR231 (lane 2). Lane 1 corresponds to the latent activity of Hp53 toward DNA without any activating molecules

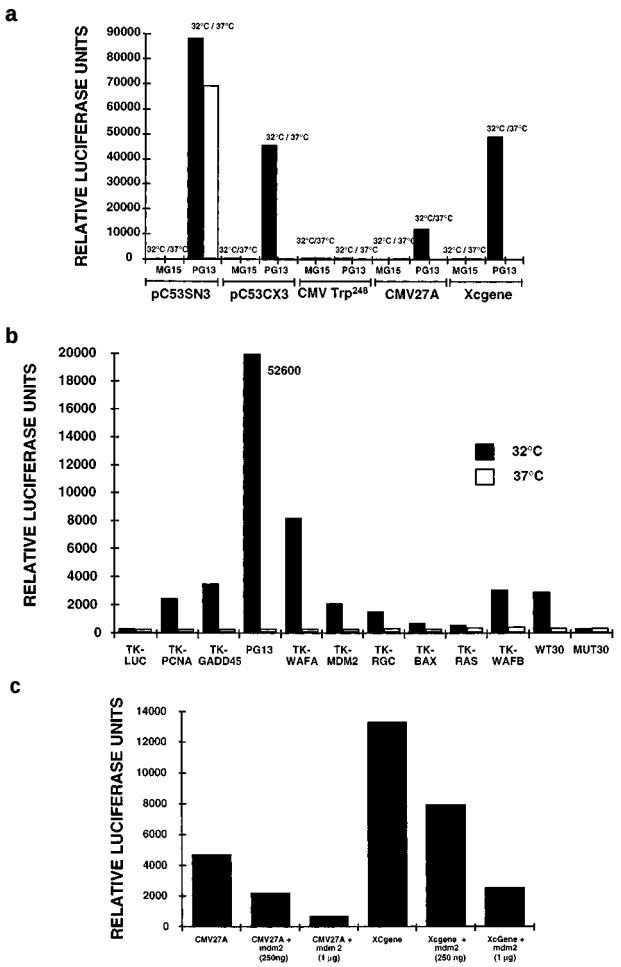

Figure 6 Transactivation activities of Xp53. (**a**) thermosensitivity of Xp53 at 37°C. Duplicate plates of Saos-2 cells were transfected with 250 ng of various p53 expression vectors and 2 μg of reporter plasmids PG-13 (wild type p53 response element) or MG-15 (mutant p53 response element). After transfection, cells were incubated either at 32°C or 37°C for 48 h before harvesting. (**b**) Xp53 transactivates various human p53 response elements only at 32°C. Duplicate pieces of Saos-2 cells were transfected with 250 ng of the Xcgene plasmid and 2 μg of reporter plasmids corresponding to various p53 response elements. Luciferase activity was tested 48 h after transfection. The activity of cells transfected with PG13 went beyond the limit of the scale used in this graph. (**c**) Transactivation activity of Xp53 is down regulated by human mdm-2. The two Xp53 expression vectors were cotransfected either with an empty expression vector or with various amounts of an Hmdm2 expression vector. Cells were incubated at 32°C for 48 h before harvesting

agreement with the recent work by Shaw *et al*. (1996) demonstrating that such Lys residue in the C-terminus are essential for DNA binding activation.

p53 transactivation

Several constructs encoding Xp53 have been constructed and cotransfected in the Saos-2 cell line devoid of endogenous p53. CMV27A expresses Xp53 from a full-length cDNA, whereas the Xcgene expresses a higher amount of Xp53 from a hybrid cDNA-genomic construct (data not shown). The reporter plasmid of pG13-Luc contains the RGC p53 DNA binding sequence linked to a minimal polyoma early promoter. Under normal conditions of cell growth (37°C), no transactivation was observed with Xp53, whereas control experiments with Hp53 were positive (Figure 6a). In a previous work, we demonstrated that Xp53 could be denatured at 37°C, as this temperature is well above the permissive temperature for normal frogs (25°C) (Soussi *et al*., 1989). This was demonstrated by the binding of hsp70 to the incorrectly folded Xp53 at 37°C, whereas no binding could be detected at 32°C (Soussi *et al*., 1989).

Transfection experiments were repeated and the cells were plated at 32°C for 48 h before extraction. As a control, the Hp53 ts mutant Ala^{143} (pC53CX3) was used (Zhang *et al*., 1994). Wild type Hp53 transactivated at both temperatures (Figure 6a), whereas the Trp^{248} mutant did not transactivate at either temperature (Figure 6a). As expected, the thermosensitive Ala^{143} Hp53 mutant was inactivated at 37°C, whereas it had wild type activity at 32°C (Zhang *et al*., 1994). Similarly, Xp53 was only active at 32°C. The higher transactivation capacity of the Xcgene construct was undoubtedly due to the higher amount of Xp53 expressed by this construct. All these transactivation activities were specific, as no activity was found using the MG-15 reporter plasmid that contained a mutant

p53 DNA binding element (Figure 6a). Thus, we tested the behavior of Xp53 to transactivate various reporter plasmids containing a p53 DNA binding element derived from genes targeted by human p53. As shown in Figure 6b, all these promoters were transactivated by Xp53 at 32°C. No activity were detected at 37°C.

The mdm2 protein is a negative regulator of p53 transactivation capacity. The binding of mdm2 to p53 occurs in the highly conserved domain I, localized in the amino-terminus of p53. *In vitro* experiments indicated that Xp53 binds strongly to human mdm2 (K Ory and T Soussi, published results). Recently, it has been shown that the Xmdm2 can bind to a murine p53 peptide corresponding to the amino-terminus of p53 (Kussie *et al*., 1996). Cotransfection experiments of mdm2 and Xp53 indicated that Hmdm2 was able to inhibit Xp53 in a dose dependent manner (Figure 6c). Control experiments with an empty vector indicated that this inhibition was specific for the presence of mdm2 (data not shown).

Discussion

The study of wild type native p53 is hampered by the minute levels of endogenous p53 which can be detected in mammalian cells. Although there are several systems which can overexpress wild type p53 in homologous or heterologous systems, such systems have several drawbacks, including the saturation of the cellular post-translational modification system and the abnormal amplification of p53 activity, such as growth arrest or apoptosis. In a previous report, we found that wild type Xp53 protein accumulated in normal oocytes (Tchang *et al*., 1993). Such accumulation persisted during the early development of the frog and then decreased to reach minute levels similar to those seen in mammalian cells. To our knowledge, *X. leavis* oocytes and eggs are the only biological systems which express sufficient native wild type p53 which can be studied without the drawbacks associated with artificial overexpression, this observation is of interest if we consider the recent model suggesting an important role for p53 during early development and embryogenesis (Hall and Lane, 1997).

In a first step of this study, we generated a new panel of mAbs directed specifically toward Xp53. mAbs directed to the p53 protein have been invaluable tools for both clinical and basic research. In particular, they have been able to distinguish several biological conformations of the protein (Gannon *et al*., 1990; Legros *et al*., 1994b; Ory *et al*., 1994). The recent report that mAbs directed to the carboxy-terminus of Hp53 can activate sequence specific DNA binding of Hp53 emphasizes their usefulness (Abarzua *et al*., 1995; Hupp *et al*., 1992). The fusion experiment described in the present report led to the production of more than 30 hybridoma cell lines which secreted mAbs specific for Xp53, and some of them were chosen for full characterization. Using the PEPSCAN ELISA, the exact localization of the epitope recognized by these mAbs was delineated. We showed that most of these epitopes correspond to linear antigenic determinants which lie in the amino- or carboxy-terminus of Xp53. Such behavior was previously described for Hp53, as most of the mAbs produced in various laboratories recognize similar immunodominant epitopes (Lane *et al*., 1996; Legros *et al*., 1994a). Preferential recognition of these regions by antibodies specific for non-conformational epitopes suggests that these regions are localized at the surface of the p53 protein as unfolded structures and emphasizes the structural homology between the various p53 species. It should be noted that the immuno-dominant region localized in the amino-terminus of p53 corresponds to the mdm2 binding site (Kussie *et al*., 1996).

Using the gel mobility shift assay, we have shown that Xp53 can bind specifically to various human p53 recognition sequences as long as p53 is activated either by a carboxy-terminus mAb or a specific peptide. This result is totally in accordance with the observation that the key p53 residues involved in specific DNA binding are highly conserved through evolution (Cho *et al*., 1994; Soussi *et al*., 1990). Furthermore, Xp53 is able to transactivate human promoters such as WAF-1, Mdm2 or bax, which contain the p53 response element. Although no p53 target has been identified in the frog, we can reasonably postulate that they will contain a consensus sequence similar to those found in humans. The regulation of p53 via mdm2 is also conserved throughout evolution, and it has been shown that Xmdm2 is able to bind the mammalian p53 (Kussie *et al*., 1996; Marechal *et al*., 1997). Recent studies have suggested that down regulation of p53 via mdm2 could be due to p53 degradation (Haupt *et al*., 1997; Kubbutat *et al*., 1997). This question was not addressed in the present study, but it could be of interest to determine whether the interaction between Xp53 and mammalian mdm2 (or Xmdm2 and mammalian p53) leads to the degradation of p53.

The carboxy-terminus of Hp53 has been shown to play an important role in controlling the specific DNA binding function. This activity was shown to be activated by various pathways: phosphorylation (Hupp *et al*., 1992), antibody specific for the carboxy-terminus of the protein (Hupp *et al*., 1992), small peptides which could mimic the carboxy-terminus of the p53 (Hupp *et al*., 1995), short single stranded DNA (Jayaraman and Prives, 1995), deletion of the last 30 amino-acids (Hupp *et al*., 1992) and the interaction with a cellular protein (Jayaraman *et al*., 1997). In the present study, we demonstrate that this feature is not unique to Hp53, but can also be extended to Xp53. Recombinant Xp53 produced in insect cells binds very poorly to DNA as long as it has not been activated by a mAb specific to the carboxy-terminus region of p53. There is no activation with a mAbs directed toward the amino-terminus p53. Such results are totally in agreement with a previous report by Hupp and Lane on Hp53, and emphasize the fundamental function of the carboxy-terminus of p53 in the regulation of its biological function (Hupp *et al*., 1992). The finding that peptides from the carboxy-terminus of Xp53 can activate Hp53 and vice versa sheds new light on this activation process. It was previously suggested that mAbs specific to the carboxy-terminus of p53 could disrupt some interaction between the carboxy-terminus and the DNA binding domain of p53 (Hupp *et al*., 1992). Such a model was confirmed by the finding that peptides corresponding to the epitopes of this monoclonal antibody could also activate specific DNA binding of p53. This activation can be explained by competition of the peptides and the carboxy-

terminus for a binding site on p53 which remains to be defined. Our results indicate that the sequence of these peptides *per se* is not essential, but suggest that the net basic charge is also a key element in this regulation. This is also demonstrated by the observation that a poly-Lys peptide is able to activate specific DNA binding. As shown in Figure 1, the sequences of the carboxy-terminus of Hp53 and Xp53 are very different, except for conservation of many Lys residues that can be found in all p53s sequenced so far (Soussi and May, 1996). Recently, a cellular protein, ref-1, was shown to activate p53 DNA binding. The amino-terminus of ref-1, the p53 activating region, is highly basic and contains a high number of lysine residues, including four Lys-Lys doublets. Thus, it is possible that p53 activation via ref-1 involves a mechanism similar to those observed with the various peptides. The activation of p53 DNA binding by small oligonucleotides could be contradictory to this model. Indeed, it is possible to propose a model that explains all these observations. This model is based on the proposition that the carboxy-terminus of p53 prevents specific DNA binding by masking (through an interaction?) the DNA binding domain localized in the central region of the molecule. Activation of specific DNA binding by short single-strand DNA occurs through the binding of these short oligonucleotides to the carboxy-terminus of p53. Such interactions induces repulsion of the p53 carboxy-terminus complexed to DNA. In the second pathway, the basic peptide binds to the central region of p53 and competes with the carboxy-terminus of p53 for binding. Such interactions release the carboxy-terminus and allow specific DNA binding. Recent evidences from our laboratory indicate that activating p53 peptides bind to the central region of p53 (S Dumon, M de Goer and T Soussi, unpublished results). This model suggests that specific DNA binding of p53 can be activated via different pathways. Although all this work was performed *in vitro*, the complexity of the various p53 pathways after DNA damage suggests that several independent mechanisms could lead to p53 activation *in vivo*.

Materials and methods

Generation of Xp53 monoclonal antibodies and immunological methods

Wild type Xp53 was overexpressed using recombinant baculovirus. Using a selective extraction procedure (T Soussi, unpublished results), the recombinant p53 could be estimated at 50–75% pure, and was used either for immunization or screening.

For immunization, BALB/c mice were injected intraperitoneally using 10 μg of p53. No adjuvants were used in any immunization. Mice were repeatedly immunized using the same procedure every 4–6 weeks. One week after the second or subsequent injection, serum samples were collected and tested for the presence of Xp53 antibodies using either immunoprecipitation or Western blot. For monoclonal antibody production, a mouse immunized with wild type Xp53 was boosted i.v. with 10 μg of Xp53 4 days before fusion. Fusion experiments with the NS1 myeloma have been previously described (Legros *et al*., 1994a). The secretion of antibodies was tested using an ELISA with Xp53 as antigen.

PEPSCAN ELISA and Western blot were performed as previously described (Legros *et al*., 1994a). Peptides consisting of overlapping 15 mer spanning the Xp53 were produced by Cambridge Research Biochemicals (UK). Each peptide overlapped with its neighbour by ten amino acids and was biotinylated at the amino-terminal end. Isotyping of the monoclonal antibodies has already been described (Legros *et al*., 1994a).

Monoclonal antibodies and peptides

For activation of p53 DNA binding experiments, the following peptides were used: Peptide A, Hp53 residues 11–60; peptide H2, Hp53 residues 346–370; peptides H3, Hp53 residues 361–385; peptides 81, Xp53 residues 11–25; peptides 92, Xp53 residues 331–345; peptides 93, Xp53 residues 336–350; peptides 94, Xp53 residues 341–355; peptides 95, Xp53 residues 346–360; peptides 96, Xp53 residues 349–363 (see also Figure 1). All these peptides were purchased from Chiron Mimotopes. The polyLys peptide was synthesized using Fmoc strategy and purified by reverse-phase chromatography using a C18 column.

Monoclonal antibodies HR231 and H279 recognized both wild type and mutant Hp53 via an epitope localized in the carboxy-terminus (HR231) for amino-terminus of p53 (H279) (Legros *et al*., 1993, 1994a). All monoclonal antibodies were used as purified immunoglobulins.

Oligonucleotides and DNA binding assay

The following oligonucleotides were used in this study (only the sequence of the upper strand is given): BB9 5′-TGTCCGGGCATGTCCGGGCATGTCCGGGCATGT-3′ (Halazonetis *et al*., 1993); WAF1-A 5′-CCCGGGATCAG-GAACATGTCCCAACATGTTGAGCTGAGCTC-3′ (El-Deiry *et al*., 1993); PCNA 5′-CCCGGGTCCACATA-TGCCCGGACTTGTTCTGCGGAGCTC-3′ (Shivakumar *et al*., 1995); GADD45, 5′-CCCGGGTACAGAACATG-TCTAAGCATGCTGGGGAC-3′ (Kastan *et al*., 1992). Complementary oligonucleotides were hybridized and end-labelled using the Ready to Go kinase kit (Pharmacia) and ^{32}P-γ-ATP. Probes were stored at 4°C.

DNA binding was performed in a two-step procedure. In the first step, the p53 was activated with either peptides or mAbs in the DNA binding buffer (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM DTT, 4 mM spermidine, 18% glycerol, 0.05% NP40, 11 μg/ml of poly dIdC). This activation reaction was performed in a volume of 20 μl for 30 min at 20°C with either 1 μg of monoclonal antibody or 2 ng of peptide. In the second step, 0.2 ng of labelled DNA probe was added and a second incubation for 30 min at 20°C was performed. Reaction products were loaded onto a 4% polyacrylamide gel containing 0.5 × TBE. Electrophoresis was performed for 2 h. Gels were dried and exposed to X-ray film.

Plasmids

The entire gene encoding the full length Xp53 gene was cloned using the Xp53 cDNA as a probe (T Huet, C Caron de Fromentel, P May and T Soussi, unpublished results). The Xcgene construct contained a hybrid between the Xp53 cDNA 27A previously described and the Xp53 gene (exon 3 to 11) (T Soussi, unpublished results). The expression of p53 was controlled by a CMV promoter. The vectors CMV27A (Xp53 cDNA) and CMVTrp^{248} (Hp53 mutant at codon 248) have already been described (Ory *et al*., 1994; Soussi *et al*., 1989). The plasmid ptk-luc was obtained from D Beach (Okamoto and Beach, 1994). It contained the HSV-tk promoter cloned in front of the Luc gene from pGL2Basic (Promega). For construction of the reporter plasmids with various p53 response elements, phosphorylated double-stranded oligonucleotides were cloned in ptk-Luc (V Lacronique and T Soussi manuscript

in preparation). pCMVneoBam is an expression vector containing the cytomegalovirus constitutive promoter and the neomycin resistance gene under the control of the simian virus 40 promoter. pC53SN3 is derived from pCMVneoBam by insertion of a wild type p53 cDNA, whereas pC53CX3 contains a single point mutation in the p53 cDNA at codon 143 (substitution of valine to alanine). The PG13-Luc reporter plasmid contains the RGC p53 DNA binding sequence linked to a minimal polyoma early promoter, whereas MG15-Luc contains a mutated RGC sequence and is used as a negative control (Kern *et al*., 1991). All these plasmids were kindly provided by B Vogelstein. The mdm2 expression vector was obtained from M Oren.

Transfection experiments

The Saos-2 line was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with glucose (4.5 g/l), L-glutamine and sodium pyruvate. The medium was supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were grown at 37°C in a humidified 5% atmosphere.

For transient transfection, Saos cells were plated in 24-well plates (10^6 cells per 1.5 cm wells). After 24 h, cells were transfected using DMRIE-C (Gibco-BRL, Life Science). A total of 2.5 μg of DNA (2 μg of reporter gene and 0.5 μg of p53 plasmid) and 5 μg of DMRIE-C were used. The reporter gene and 0.5 μg of p53 plasmid) and 5 μg of DMRIE-C were used. The luciferase activity was tested as described previously (Ory *et al*., 1994).

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References

Abarzula P, Losardo JE, Gubler ML and Neri A. (1995). *Cancer Res*., **55,** 3490–3494.
Béroud C and Soussi T. (1997). *Nucleic Acids Res*., **25,** 138.
Bischoff JR, Casso D and Beach D. (1992). *Mol. Cell. Biol*., **12,** 1405–1411.
Buckbinder L, Talbott R, Velascomiguel S, Takenaka I, Faha B, Seizinger BR and Kley N. (1995). *Nature*, **377,** 646–649.
Cho YJ, Gorina S, Jeffrey PD and Pavletich NP. (1994). *Science*, **265,** 346–355.
El-Deiry WS, Kern SE, Pientenpol JA, Kinzler KW and Vogelstein B. (1992). *Nature Genetics*, **1,** 45–49.
El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell*, **75,** 817–825.
Gannon JV, Greaves R, Iggo R and Lane DP. (1990). *EMBO J*., **9,** 1595–1602.
Halazonetis TD, Davis LJ and Kandil AN. (1993). *EMBO J*., **12,** 1021–1028.
Hall PA and Lane DP. (1997). *Curr. Biol*., **7,** R144–R147.
Haupt Y, Maya R, Kazaz A and Oren M. (1997). *Nature*, **387,** 296–299.
Hupp TR and Lane DP. (1994). *Cold Spring Harb Symp Quant Bio3*, **59,** 195–206.
Hupp TR, Meek DW, Midgley CA and Lane DP. (1992). *Cell*, **71,** 875–886.
Hupp TR, Sparks A and Lane DP. (1995). *Cell*, **83,** 237–245.
Ishioka C, Englert C, Winge P, Yan YX, Engelstein M and Friend SH. (1995). *Oncogene*, **10,** 1485–1492.
Jayaraman L, Murthy KGK, Zhu C, Curran T, Xanthoudakis S and Prives C. (1997). *Gene Develop*., **11,** 558–570.
Jayaraman L and Prives C. (1995). *Cell*, **81,** 1021–1029.
Juven T, Barak Y, Zauberman A, George DL and Oren M. (1993). *Oncogene*, **8,** 3411–3416.
Kastan MB, Zhan QM, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace AJ. (1992). *Cell*, **71,** 587–597.
Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C and Vogelstein B. (1991). *Science*, **252,** 1708–1711.
Kubbutat MHG, Jones SN and Vousden KH. (1997). *Nature*, **387,** 299–303.
Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ and Pavletich NP. (1996). *Science*, **274,** 948–953.
Lane DP, Stephen CW, Midgley CA, Sparks A, Hupp TR, Daniels DA, Greaves R, Reid A, Vojtesek B and Picksley SM. (1996). *Oncogene*, **12,** 2461–2466.
Legros Y, Lacabanne V, D'Agay MF, Larsen CJ, Pla M and Soussi T. (1993). *Bull. du Cancer*, **80,** 102–110.
Legros Y, Lafon C and Soussi T. (1994a). *Oncogene*, **9,** 2071–2076.
Legros Y, Meyer A, Ory K and Soussi T. (1994b). *Oncogene*, **9,** 3689–3694.
Marechal V, Elenbaas B, Taneyhill L, Piette J, Mechali M, Nicolas JC, Levine AJ and Moreau J. (1997). *Oncogene*, **14,** 1427–1433.
Miyashita T and Reed JC. (1995). *Cell*, **80,** 293–299.
Nigro JM, Sikorski R, Reed SI and Vogelstein B. (1992). *Mol. Cell. Biol*., **12,** 1357–1365.
Okamoto K and Beach D. (1994). *EMBO J*., **13,** 4816–4822.
Ory K, Legros Y, Auguin C and Soussi T. (1994). *EMBO J*., **13,** 3496–3504.
Ridgway PJ, Soussi T and Braithwaite AW. (1994). *J. Virol*., **68,** 7178–7187.
Shaulsky G, Goldfinger N, Benzeev A and Rotter V. (1990). *Mol. Cell. Biol*., **10,** 6565–6577.
Shaw P, Freeman J, Bovey R and Iggo R. (1996). *Oncogene*, **12,** 921–930.
Shivakumar CV, Brown DR, Deb S and Deb SP. (1995). *Mol. Cell. Biol*., **15,** 6785–6793.
Soussi T, Caron de Fromentel C and May P. (1990). *Oncogene*, **5,** 945–952.
Soussi T, Caron de Fromentel C, Méchali M, May P and Kress M. (1987). *Oncogene*, **1,** 71–78.
Soussi T, Caron de Fromentel C, Stürzbecher HW, Ullrich S, Jenkins J & May P. (1989). *J. Virol*., **63,** 3894–3901.
Soussi T and May P. (1996). *J. Mol. Biol*., **260,** 623–637.
Tchang F, Gusse M, Soussi T and Méchali M. (1993). *Dev. Biol*., **159,** 163–172.
Wagner P, Simanis V, Maimets T, Keenan E, Addison C, Brain R, Grimaldi M, Sturzbecher HW and Jenkins J. (1991). *Oncogene*, **6,** 1539–1547.
Wang Y, Farmer G, Soussi T and Prives C. (1995). *Oncogene*, **10,** 779–784.
Wu XW, Bayle JH, Olson D and Levine AJ. (1993). *Gene Develop*., **7,** 1126–1132.
Zauberman A, Lupo A and Oren M. (1995). *Oncogene*, **10,** 2361–2366.
Zhang W, Guo XY, Hu GY, Liu WB, Shay JW and Deisseroth AB. (1994). *EMBO J*., **13,** 2535–2544.