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Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein

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The p53 tumor suppressor gene product is a transcriptional activator that may be associated with its ability to suppress tumor cell growth. The acidic amino terminus of the p53 protein has been shown to contain this *trans*-activation activity as well as the domains for mdm-2 and adenovirus 5 E1B 55-kD protein binding. An extensive genetic analysis of this amino-terminal p53 domain has been undertaken using site-specific mutagenesis. The results demonstrate that the acidic residues in the amino terminus of p53 may contribute to, but are not critical for, this *trans*-activation activity. Rather, the hydrophobic amino acid residues Leu-22 and Trp-23 of human p53 are both required for *trans*-activation activity, binding to the adenovirus E1B 55-kD protein and the human mdm-2–p53 protein in vitro. In addition, hydrophobic residues Leu-14 and Phe-19 are crucial for the interactions between p53 and human mdm-2 (hdm-2). Hydrophobic residues Trp-23 and Pro-27 are also important for binding to the adenovirus 5 (Ad5) E1B 55-kD protein in vitro. These mutations have no impact on the ability of the p53 protein to bind to a p53-specific DNA element. These results suggest that 2–4 critical hydrophobic residues in the amino-terminal domain of the p53 protein interact with the transcriptional machinery of the cell resulting in transcriptional activation. These very same hydrophobic residues contact the hdm-2 and Ad5 E1B 55-kD oncogene products.

[*Key Words*: p53 protein; *trans*-activation; mdm-2 binding; E1B 55-kD binding]

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Wild-type p53 protein is the product of a tumor suppressor gene that has the ability to suppress oncogenic transformation (Eliyahu et al. 1989; Finlay et al. 1989), negatively regulate cell cycle progression (Baker et al. 1990; Diller et al. 1990; Martinez et al. 1991), and induce apoptosis in certain cell types (Yonish-Rouach et al. 1991; Shaw et al. 1992). Wild-type p53 protein has been shown to possess a transcriptional activation function (Fields and Jang 1990; O'Rourke et al. 1990; Raycroft et al. 1990) that may be linked to its tumor suppression function because many mutant p53 proteins found in human cancers have lost both *trans*-activation activity (Raycroft et al. 1991; Unger et al. 1992) and tumor suppressor activity (Hinds et al. 1990; Chu 1991). The transcriptional activation domain of p53 has been mapped to the amino-terminal residues 1–42 (Unger et al. 1992), whereas the DNA-binding domain of p53 resides in residues 120–290 (of 393 amino acids). The amino-terminal domain is rich in acidic amino acids, and it has been suggested that these negatively charged residues are required for the *trans*-activation function by p53. Inhibition of the *trans*-activation activity of wild-type p53 by several viral and cellular oncoproteins appears to correlate with their transforming ability. Two such oncogene products, mdm-2 (Chen et al. 1993; Oliner et al. 1993) and adenovirus 2 early 1B (Ad2 E1B) 55-kD protein (Kao et al. 1990), have been shown to bind to the amino terminus of p53. The *mdm-2* gene was originally cloned as a cellular oncogene amplified on a mouse double-minute chromosome (Cahilly-Snyder et al. 1987). Overexpression of the *mdm-2* gene in BALB/c-3T3 cells was shown to increase their tumorigenic potential (Fakharzadeh et al. 1991). The *mdm-2* gene product has also been shown to complex with p53 and inhibit p53-mediated *trans*-activation (Momand et al. 1992; Oliner et al. 1993). mdm-2 binds to the first 52 residues of p53 (Chen et al. 1993; Oliner et al. 1993).

The Ad2 and Ad5 E1B 55-kD proteins bind to p53 in transformed cells (Sarnow et al. 1982) and in vitro (Kao et

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al. 1990), and inactivation of p53 function by E1B 55-kD protein contributes to oncogenic transformation by this virus (Barker and Berk 1987; White and Cipriani 1990). It has been shown that the domain in p53 required for in vitro binding to Ad2 E1B 55-kD protein is the amino-terminal 123 residues (Kao et al. 1990). The transforming activity of the adenovirus E1B protein also has been shown to depend on its ability to inhibit *trans*-activation by p53 (Yew and Berk 1992).

The amino-terminal domain of p53 may well mediate enhanced rates of transcription by interacting with the transcriptional machinery of the cell. p53 protein binds to TBP (the TATA-binding protein) (Seto et al. 1992), and this is mediated in part by contacts between TBP and the amino-terminal domain of p53 (Liu et al. 1993; Ragimov et al. 1993; Truant et al. 1993). To identify the amino acid residues in the p53 amino-terminal domain that are critical for these types of interactions in transcriptional activation, an extensive series of site specific mutations were constructed in the amino-terminal domain of p53. Each mutant and combinations of mutations were then tested for the ability to *trans*-activate a test gene. Two distinct p53-responsive elements were measured for their ability to mediate transcriptional activation by these mutant p53 proteins. In addition, these same mutants were tested for their ability to bind to the human mdm-2 (hdm-2) and Ad5 E1B 55-kD oncogene products. The results of this study indicate the following. (1) The acidic residues at the amino terminus of the p53 protein may influence, but are not critical for, the transcriptional activation. (2) Both hydrophobic residues at amino acid position Leu-22 and Trp-23 are critical for transcriptional activation. Mutations in either residue alone have considerably less of an impact on the ability of the protein to activate transcription than the double mutant. This observation helps to explain why p53 mutations in cancerous cells cluster in the DNA-binding domain (to eliminate the transcription factor function) but are rarely if ever observed in the amino-terminal domain (one requires a double mutational event here). (3) The same residues of p53 critical for transcriptional activation (codons Leu-22 and Trp-23) are required for the binding of the oncogene products hdm-2 and Ad5 E1B 55 kD. (4) Additional hydrophobic residues (at codons Leu-14 and Phe-19) are crucial for interactions between p53, and hdm-2 protein and hydrophobic residues at codon Trp-23 and Pro-27 are important for binding to Ad5 E1B 55-kD protein in vitro. (5) The mutations in the *trans*-activational region of p53, residues 22 and 23 and 14–19, produce p53 proteins that are wild type in their ability to bind to p53-specific DNA sequences and the TBP. These data suggest that the p53 *trans*-activation domain residues 22 and 23 may interact with other components of TFIID (TAFs) or the holo-transcriptional machinery in addition to TBP itself (Seto et al. 1992; Liu et al. 1993; Truant et al. 1993).

Clearly, the viral oncogene products target the same residues in the amino-terminus of p53 that are employed to interact with the transcriptional machinery of the cell.

Results

Hydrophobic residues at the amino terminus of the p53 protein are critical for transcriptional trans-*activation*

To analyze in some detail the interactions of the amino-terminal domain of the p53 protein with the transcriptional machinery of the cell, an extensive series of mutations were created in the first 42 amino acid residues of the protein. These were incorporated into p53 cDNAs as single, double, or triple mutations or, in one case, six mutations in one gene. These mutants are listed in Table 1 with their codon number (1–393 codons), the amino acid present in wild-type p53, and the alteration made in specific mutants. In particular, acidic residues in wild-type p53, which were thought to be critical in mediating transcriptional activation, were changed to basic residues (a nonconservative change). These mutant cDNAs were then cloned into an expression vector regulated by the cytomegalovirus (CMV) promoter (see Materials and methods).

Transcriptional activity was measured in Soas-2 cells that have no endogenous p53 gene or protein. Wild-type p53 or mutant cDNA clones were cotransfected with p53 reporter plasmids containing either the p50–2CAT [from the creatine phosphokinase gene (Zambetti et al. 1992)] or the CosX1CAT [from the *mdm-2* gene (Wu et al. 1993)] p53-responsive DNA element. Wild-type p53 activity is set at 100%, and each mutant (average of three to five experiments) is given as a percentage of that activity. As negative controls, the vector alone (PRC/CMV) and the codon 273RH mutant in the DNA-binding domain showed a reduction in the CAT activities to 4–8% (Table 1; Fig. 1). Different p53-responsive elements can give some variation in results (three- or four-fold differences), which is a common observation in the literature using diverse p53 reporter or responsive elements. Because of this, 8- to 10-fold differences are considered significant. On the basis of these criteria, only double mutants at codons 22 and 23 eliminate transcriptional activation by 13- to 20-fold. Each of the single mutants at codons 22 or 23 had much less of an impact on transcriptional activation. None of the mutants containing two acidic to two basic amino acid changes showed a reduction in *trans*-activation of more than three-fold (Table 1). Even a mutant with six substitutions of basic for acidic residues (at codons 7, 11, 17, 21, 41, 42) reduced the transcriptional activity threefold with P50–2CAT or sixfold with CosX1CAT relative to wild type.

The conclusion of this study (Table 1) is that hydrophobic residues Leu-22 and Trp-23 must both be altered to significantly inhibit transcriptional activation by the p53 protein. Altering either one singly leaves 20–74% of the wild-type activity. All of the acidic residues at the amino terminus of p53 have relatively weaker contributions to transcriptional activation than the codon Leu-22 and Trp-23 hydrophobic amino acids.

The loss of transcriptional activity of the mutant at codons 22 and 23 may not be attributable solely to its

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Table 1. *Relative levels of CAT activity, human mdm2 and Ad5 E1B 55-kD protein binding affinity of the amino terminus human p53 mutants*

Position	Change from	Change to	CAT activity relative to wild-type human p53 (%)[a] p50–2CAT	CAT activity relative to wild-type human p53 (%)[a] CoSX1CAT	hdm-2-binding affinity relative to wild-type human p53 (%)[b]	E1B 55-kD binding affinity relative to wild-type human p53(%)[c]
Vector			5	4		
Wild-type p53			100	100	100	100
2,	Glu	Lys	79	72	66	84
3	Glu	Lys				
7,	Asp	His	58	64	81	
11	Glu	Lys				
13	Pro	Thr	56	63	85	141
15	Ser	Gly	300	117	66	
14,	Leu	Gln	56	41	1	61
19	Phe	Ser				
16,	Gln	Leu	219	210	124	41
18	Thr	Ile				
17,	Glu	Lys	40	41	80	228
21	Asp	His				
22	Leu	Gln	17	59	56	49
23	Trp	Ser	22	74	22	3
22,	Leu	Gln	5	8	2	6
23	Trp	Ser				
24	Lys	Thr	127	94	181	9
25,	Leu	Gln	143	34	39	18
26	Leu	His				
27	Pro	Tyr	126	73	779	4
28	Glu	Lys	56	36	162	47
31,	Val	Ser	96	95	101	35
32	Leu	Arg				
48,	Asp	His	215	85		
49	Asp	His				
61,	Asp	His	40	36	150	154
62	Glu	Lys				
17,	Glu	Lys	54	79	22	217
21,	Asp	His				
28	Glu	Lys				
7,	Asp	His	35	18	12	257
11,	Glu	Lys				
17,	Glu	Lys				
21,	Asp	His				
41,	Asp	His				
42	Asp	His				
273	Arg	His	8	6		

[a]Saos-2 cells were cotransfected with reporter and mutant or wild-type human p53 plasmids as described in Materials and methods. CAT activity was determined relative to wild-type p53. Each entry represents the average from three independent experiments. Results of mutant 22–23 are given as average from five independent experiments.
[b]The conditions used to analyze the human p53 and mdm-2 interaction were as described in Materials and methods. Results are given as average from two independent experiments.
[c]The methods used to analyze the interaction between E1B 55K and human p53 are described in Materials and methods. Results are given as average from two independent experiments.

failure to interact with the transcriptional machinery. It remains possible that codon 22–23 mutants failed to bind to DNA in the p53-responsive element. To test this, a Gal4 fusion protein was constructed with the Gal4 DNA-binding domain and the p53 amino terminal 53 residues containing the codon 22–23 mutants. When this was transfected into Saos-2 cells with a Gal4-responsive element regulating a CAT expression vector, the mutant p53 protein at codons 22–23 failed to *trans*-activate the test gene, whereas the wild-type p53 amino terminus does enhance the activity of the test gene (data not shown). These data indicate that the codon 22–23 defect in activating transcription occurs even when the mutant protein is bound to a DNA element.

Next, the p53 wild-type protein, the 14–19 mutant protein, 22–23 mutant protein, and the codon 175RH

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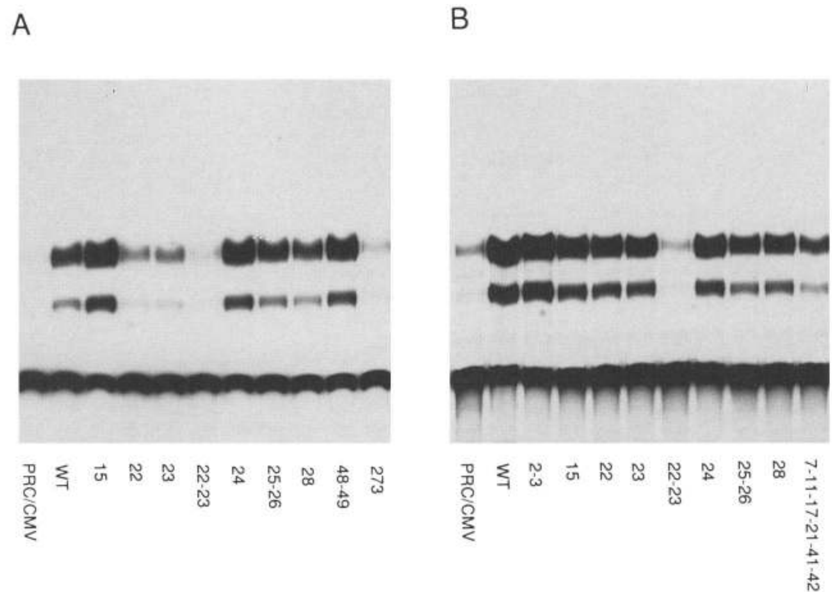

Figure 1. *Trans*-activation by wild-type and mutant p53s. Saos-2 cells were cotransfected with p53 plasmid DNA and p50–2CAT or CosX1CAT and assayed for CAT activity as described in Materials and methods. (*A*) p50–2CAT; (*B*) CosX1CAT.

mutant protein were produced in baculovirus vectors, and the proteins were purified by antibody affinity chromatography as described previously (Momand et al. 1992). These proteins were incubated with a labeled p53-responsive DNA element oligonucleotide, and a gel shift analysis was employed to test for p53 binding to this specific DNA sequence. Figure 2 demonstrates that the p53 wild-type, and 14–19 and 22–23 mutants all bind to this DNA element, whereas a known mutation in the DNA-binding domain, 175RH, fails to bind to this oligonucleotide. The addition of antibody pAb421 directed against p53 both stimulates binding as described previously and supershifts the p53–DNA complex, demonstrating the specificity of these reactions. At approximately equal p53 wild-type and 14–19 or 22–23 mutant protein concentrations, these mutants have a wild-type ability to bind to DNA. The reaction of pAb421 with the DNA-binding mutant (p53–175RH) protein did permit a small level of DNA binding, suggesting the possibility of a conformational change induced by the antibody, to renature (to a wild-type conformation) a small percentage of this protein. These data demonstrate that p53 mutants 22–23 are defective in transcription and bind normally to p53-responsive DNA elements.

The codon 22–23 mutant p53 proteins were then studied to examine the conformation of the p53 protein and its half-life in cells. A drastically shorter half-life of the p53 mutant protein or a radical alteration in conformation resulting from codon 22–23 double mutations could also give rise to the phenotypes seen in Table 1. To measure protein levels and immunoreactivity, p53 mutants were transiently expressed in Saos-2 cells (Fig. 3A) or translated in vitro (Fig. 3B). Both the double-mutant 22–23 p53 protein and wild-type p53 protein were equally recognized by pAb1620 (Fig. 3), an antibody that reacts only with wild-type human p53 (Milner et al. 1987). Both proteins failed to react with pAb240 (Fig. 2), an antibody that recognizes only the mutant forms of p53 (Gannon et al. 1990). The hot spot p53 mutant, 175RH, which was commonly found in human tumors, was included in this experiment as a control. This mutant translated in vitro was recognized strongly by pAb240 but poorly by pAb1620. Also, the double-mutant 22–23 p53 protein was recognized by a conformation-sensitive human-specific antibody, pAb1801, (Fig. 3), whose epitope is located between residues 32 and 79 (Banks et al. 1986). These results suggested that the double-mutant 22–23 p53 protein is maintained in a wild-type-like conformation over much of the protein. The 22–23 double mutation clearly does not alter the conformation of the p53 protein in a major testable way. The stability of mutants 22–23, 22, and 23 proteins were also examined by transiently expressing these mutant p53 proteins in Saos-2 cells and analyzing their half-lives. All three of these mutant proteins were stable over a 4-hr chase period and were expressed at approximately the same level as wild-type p53 protein (Fig. 4). When transiently expressed in vivo in large amounts, the wild-type p53 protein has been shown to have a longer than normal half-life (hours rather than minutes). Thus, the loss of transcriptional activation by the codon 22–23 double mutant protein is not the result of an unstable protein but is more likely the result of a failure of this mutant to interact with the transcriptional machinery of the cell.

Identification of p53 residues required for binding to hmdm-2

Previous studies (Chen et al. 1993; Oliner et al. 1993) have mapped the p53-binding site to the mdm-2 protein in the first amino-terminal 52 amino acids of the p53 protein. The 1–52 amino acid domain is sufficient to bind to the hdm-2 protein. To identify the p53 amino acid contacts with the mdm-2 protein, the mutants con-

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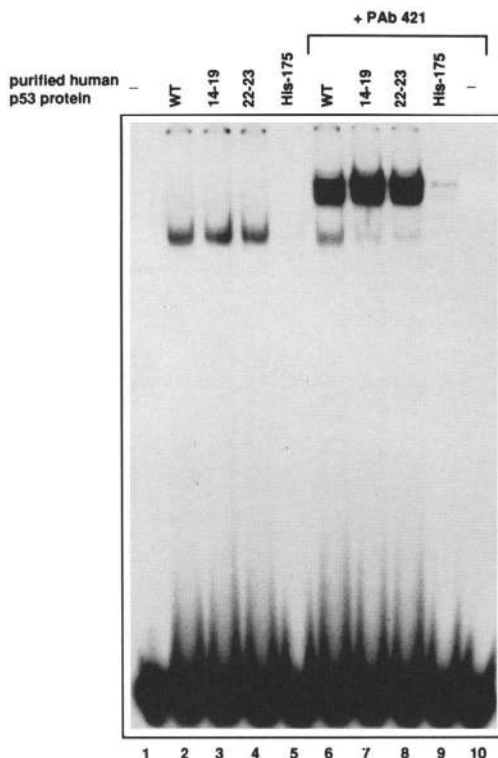

Figure 2. DNA gel shift by p53 wild-type and mutant proteins. The p53 wild-type (WT) or mutant proteins (14–19, 22–23, or His-175) were synthesized by baculovirus vectors and purified. They were incubated with a labeled DNA oligonucleotide (a p53-specific consensus sequence) and run out on the gel to look for protein binding. The oligonucleotide was incubated with no protein added (lane *1*), the p53 WT (lane *2*), 14–19 mutant (lane *3*), 22–23 mutant (lane *4*), and His-175 (lane *5*); p53 WT plus pAb421 antibody gives a supershift (lane *6*), as does the 14–19 mutant plus pAb421 (lane *7*) and 22–23 mutant plus pAb421 (lane *8*). The His-175 mutant fails to bind to DNA (lanes *5,9*), as well as pAb421 antibody alone (lane *10*).

structed and tested in Table 1 were surveyed for binding to the hdm-2 protein. Radiolabeled hdm-2 and mutant p53 proteins were generated by translation of these proteins in vitro. Each mutant p53 was mixed with hdm-2 protein and incubated for 30 min at 30°C. The mixture was then immunoprecipitated with a mdm-2-specific monoclonal antibody 4B2 (Chen et al. 1993; Olson et al. 1993). This antibody recognizes both human and murine mdm-2 proteins. The efficiency of forming an in vitro complex between p53 mutants and mdm-2 proteins was determined by quantitating the amount of mutant and wild-type p53 protein that coimmunoprecipitated with anti-mdm-2 antibody. An example of such an experiment is shown in Figure 5, and the results using the entire panel of mutants are summarized in Table 1. The majority of the mutants retained near wild-type levels of mdm-2-binding efficiency. A few mutants showed moderately reduced binding efficiencies (between 20 and 40% of wild type). However, double mutants Leu-14–Phe-19 and Leu-22–Trp-23 essentially failed to bind mdm-2 in vitro; the binding efficiencies range from undetectable to <5% of wild-type in individual experiments.

Identification of p53 residues required for binding to Ad5 E1B 55-kD protein

Previous experiments have demonstrated that the p53 domain that binds to the Ad2 or Ad5 E1B 55-kD protein resides in the amino-terminal 123 amino acids of p53 (Kao et al. 1990). Furthermore, the transforming ability of this oncogene product correlates with its ability to block transcriptional *trans*-activation by the p53 protein (Yew and Berk 1992). For this reason, the panel of mutants listed in Table 1 were tested for their ability to bind to the Ad5 E1B 55-kD protein. To analyze the p53/55-kD complex, the experimental conditions described by Kao et al. (1990) were used, except that the Saos-2 cells were employed instead of HeLa cells. Wild-type or mutant human p53 cells synthesized in vitro were labeled with [^{35}S]methionine and mixed with the Saos-2 cell extracts infected with wild-type Ad5. The mixture was incubated for 30 min at 30°C, and the labeled p53 proteins were coprecipitated with unlabeled E1B 55-kD protein using an anti-E1B 55-kD protein monoclonal antibody, 2A6 (Sarnow et al. 1982). The quantitative results of E1B 55-kD binding of each p53 mutant are summarized in Table 1, with these data shown in Figure 6. Most of the mutants tested showed levels of E1B 55-kD protein binding close to that of wild-type p53. A few mutants showed a two- to threefold reduction in binding efficiency. However, mutations in a small region from residues 23–27 had the poorest binding affinity to E1B 55-kD protein (Table 1; Fig. 6). In this region, mutants 23 and 27 almost failed to bind to the 55-kD protein in vitro, with binding efficiencies ~4% of the wild-type level. A tyrosine substitution at the residue Pro-27 in the wild-type protein is of some interest because it produced a p53 protein that bound very poorly to E1B 55 kD, had enhanced binding to the hdm-2 protein, and retained near wild-type levels of *trans*-activation activity (Table 1; Figs. 5 and 6). These results indicate that these mutations do not result in dramatic changes in protein conformations, but, rather, the residues involved are critical for protein–protein contacts. It appears likely that residues 23, 24, and 27 play an important role in p53–E1B 55-kD interactions.

The interactions of p53 mutants with TBP

Previous experiments have shown that wild-type p53 protein binds to TBP (Seto et al. 1992) and that the amino terminus of p53 appears to mediate this interaction (Liu et al. 1993; Truant et al. 1993). It was of some interest, therefore, to determine whether p53 mutants at 22–23 or 14–19 failed to bind to TBP. Several assays have been employed to demonstrate p53–TBP interactions, including coimmunoprecipitation of a mixture of these two proteins, a far Western blot procedure with labeled TBP binding to p53 transferred from denaturing gels to filter paper, or the cosedimentation or cochromatography of

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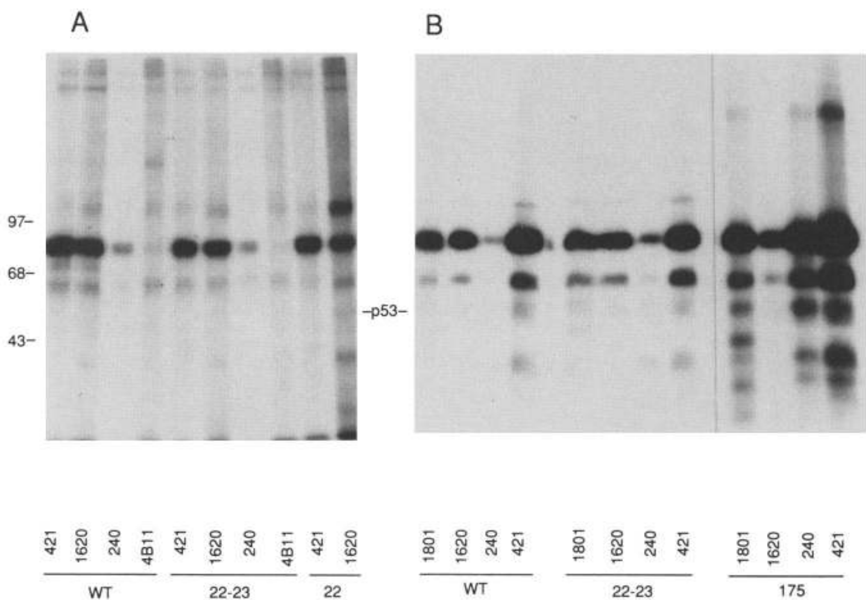

Figure 3. Immunoreactivity of wild-type and mutant p53s. (*A*) Immunoprecipitation of p53 transiently expressed in Saos-2 cells. Wild-type or mutant p53 was transfected into Saos-2 cells with 20 gm of p53 DNA and 20 gm of salmon sperm DNA by calcium phosphate protocol (Graham and van der Eb 1973). Forty-eight hours after transfection, cells were labeled with [^{35}S]methionine and subjected to immunoprecipitations with different anti-p53 monoclonal antibodies and an anti-mdm-2 monoclonal antibody, 4B11, as described in Materials and methods. (*B*) Immunoreactivity of p53 synthesized in vitro. Wild-type or mutant p53 was translated in the reticulocyte lysates and labeled with [^{35}S]methionine. The synthesized mixture was equally aliquoted and incubated with different anti-p53 monoclonal antibodies and protein A–Sepharose for 2–3 hr at 4 °C. The immunoprecipitates were then washed and subjected to 12.5% SDS–polyacrylamide gel electrophoresis and X-ray autoradiography.

p53–TBP in solution (Seto et al. 1992). Purified preparations of wild-type p53, 22–23 or 14–19 mutants or the 175RH mutant in solution with labeled TBP readily formed complexes that were coimmunoprecipitated using p53-specific antibodies (Fig. 7A). Similarly, far Western blots with wild-type and mutant p53 proteins demonstrated binding between TBP and both the wild-type and mutant p53 proteins (Fig. 7B). In some experiments, the p53 22–23 mutant bound less well to TBP (50% reduction) and the 175RH mutant bound better to TBP than wild type, but these variations are not considered significant. When TBP was used in a far Western blot to bind to proteins in a SF9 cell crude lysate (the cells producing baculovirus p53 proteins), four to seven specific bands were identified, demonstrating the specificity of these procedures. Whereas the 14–19 and 22–23 mutant p53 protein produced by baculovirus vectors bind to DNA and to TBP similar to the wild-type protein, both mutant proteins fail to bind to mdm-2 when mixed in vitro or when obtained from coinfected cells (results not shown).

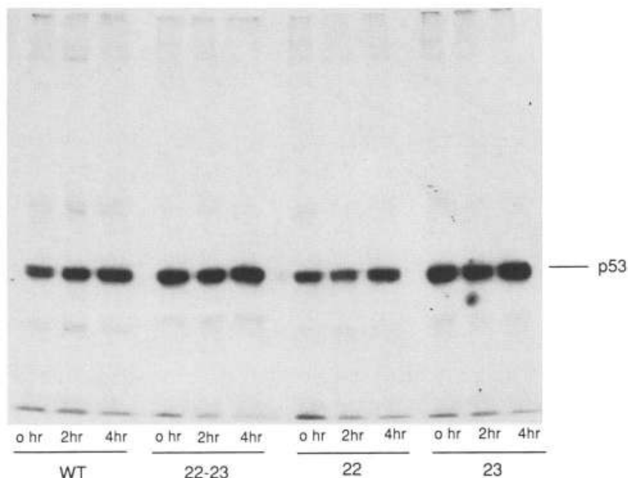

Figure 4. Stability of p53 mutants. Saos-2 cells transfected with wild-type and mutant p53 were pulsed with [^{35}S]methionine for 3 hr and chased for 2 or 4 hr in DMEM supplemented with 10% FBS. Equivalent amounts of TCA-precipitable counts from the different time points were immunoprecipitated with pAb421, and the immunoprecipitated proteins were separated on 12.5% SDS–polyacrylamide gel and X-ray autoradiography.

These data indicate that although p53 residues 22–23 are critical for contacting the transcriptional machinery of the cell, they do not entirely direct their contacts via TBP protein, which apparently binds elsewhere in the p53 amino-terminal domain. It remains possible that p53 protein makes multiple contacts with TBP, TFIID, and the TAFs.

Discussion

The p53 protein has been dissected into three physical and functional domains: (1) the amino-terminal 42 amino acids that act along with an interchangeable, discrete DNA-binding domain (Fields and Jang 1990; Raycroft et al. 1990; Unger et al. 1992) to enhance the rate of transcription of a gene adjacent to a DNA-binding site; (2) a DNA-binding domain localized between amino acid residues 120 and 290 recognizes specific DNA sequences (Bargonetti et al. 1993; Pavletich et al. 1993; Wang et al. 1993); and (3) a carboxy-terminal domain (residues 311–393) that contains the nuclear localization signal and sequences required to form tetrameric p53 proteins. The

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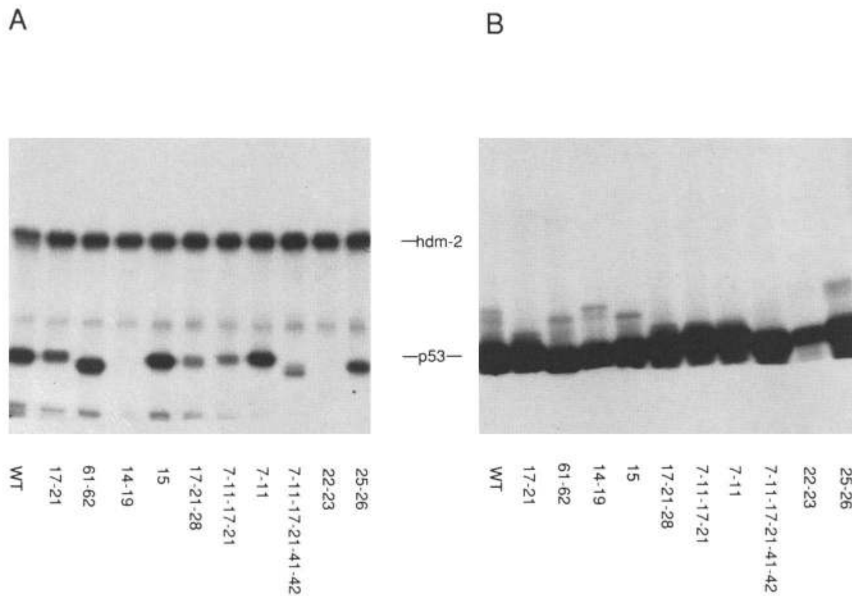

Figure 5. Ability of p53 mutants to complex with hdm-2. Hdm-2 and mutant p53 were generated by in vitro translation in rabbit reticulocyte lysates labeled with [^{35}S]methionine. Each mutant p53 protein was mixed with hdm-2 and incubated for 30 min at 30°C. The mixture was then immunoprecipitated with a mdm-2-specific monoclonal antibody, 4B2, as described in Materials and methods. (*A*) Coprecipitation of p53 mutants with hdm-2 and (*B*) in vitro translation products of p53 mutants. Two microliters of the ^{35}S-labeled in vitro translation products was separated on the SDS–polyacrylamide gel to verify the mutant protein synthesis.

amino-terminal domain is thought to contact the transcriptional machinery of the cell. The p53 protein does interact with TBP using sequences at the amino-terminal domain of p53 (Seto et al. 1992; Liu et al. 1993; Truant et al. 1993). In particular, it had been suggested that the highly acidic amino acid nature of the amino-terminal domain—there are 9 acidic residues among the first 42 residues of human p53—might mediate the interactions between p53 and some basal transcription factors (Mitchell and Tjian 1989; Stringer et al. 1990). To test these ideas, an extensive analysis, using site specific mutagenesis of the first 42 amino acid residues of the human p53 protein, was carried out.

Placing nonconservative genetic alterations in the amino-terminal domain of p53 has resulted in clear loss of function of this protein for its transcription factor phenotype, binding to the hdm-2 protein, and binding to the Ad5 E1B 55-kD protein (see Table 1). There are two ways in which such mutations could result in these loss of functions: (1) By altering the amino acid contacts between two proteins, the function is lost and the mutation identifies such contact points in a protein; and (2) by altering the conformation or stability of the entire p53 protein, a function may be lost, but the real protein contact points are not elucidated. Several lines of evidence favor the former interpretation of the results presented here. (1) Several conformational-sensitive monoclonal antibodies recognize these amino-terminal domain mutant proteins in a "wild-type" or native conformation (pAb1620) and not in a "mutant" or denatured conformation (pAb240). The mutant conformation of p53 produces a protein that fails to act as a transcription factor (Fig. 3). The mutations in the amino-terminal domain do not alter the DNA-binding domain structure as observed

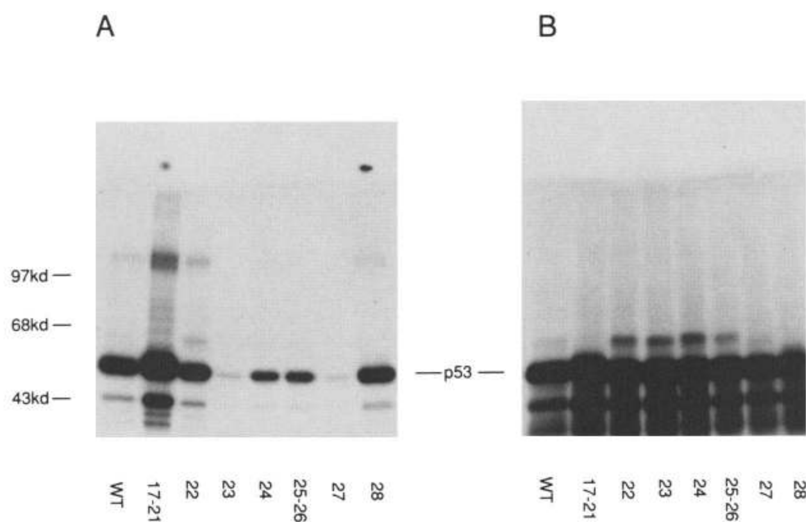

Figure 6. Ability of p53 mutants to bind Ad5 E1B 55-kD protein. Mutant or wild-type p53 synthesized in vitro was labeled with [^{35}S]methionine and mixed with whole Saos-2 cell extracts infected with wild-type Ad5. The mixture was incubated for 30 min at 30°C and precleared with protein A–Sepharose for 15 min at 4°C. The labeled mutant p53 proteins were coprecipitated with unlabeled E1B 55-kD protein by anti-E1B 55-kD protein monoclonal antibody 2A6 (Sarnow et al. 1982). The precipitated proteins were detected by 12.5% SDS–polyacrylamide gel electrophoresis and autoradiography. (*A*) Coprecipitation of p53 mutants with E1B 55-kD protein and (*B*) in vitro translation products of p53 mutants. Two microliters of the ^{35}S-labeled in vitro translation products were run on the SDS–polyacrylamide gel to verify the synthesis of mutant proteins.

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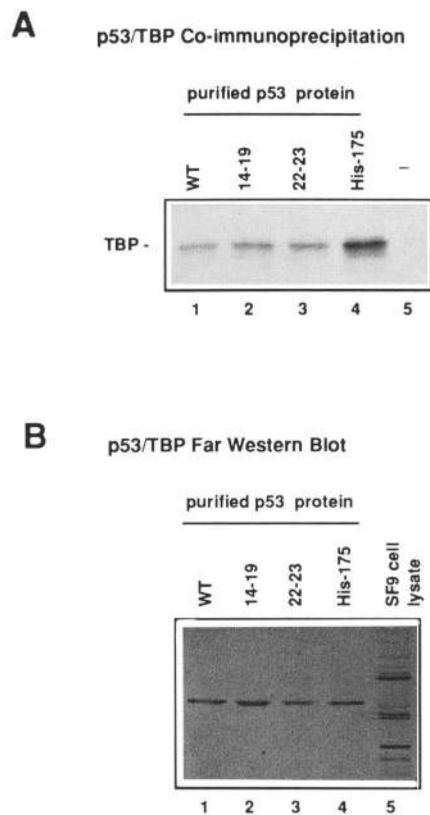

Figure 7. p53 protein–TBP interactions. (*A*) Purified p53 wild-type protein (lane *1*), the 14–19 mutant p53 (lane *2*), the 22–23 mutant p53 (lane *3*), and the His-175 mutant p53 (lane *4*) proteins were mixed in solution with TBP that had been synthesized and labeled in a wheat germ in vitro translation system. The p53-specific monoclonal antibody pAb421 was used to immunoprecipitate p53 that coimmunoprecipitated the labeled TBP in all four cases. (*B*) Purified p53 wild-type (lane *1*), mutant 14–19 (lane *2*), mutant 22–23 (lane *3*), mutant His-175 (lane *4*), or a crude lysate of SF9 cells that are used in baculovirus infections (lane 5) were all run out on an SDS–polyacrylamide gel and subsequently transferred to nitrocellulose paper. Labeled TBP was incubated and tested for binding to p53 (lanes *1–4*). The labeled TBP binds to a subset of specific proteins in SF9 cells (lane *5*).

with these monoclonal antibodies. (2) The mutants at Leu-22–Trp-23, in the amino terminus, that fail to act as a transcription factor are domain autonomous, that is, they fail to function with either the p53 or Gal4 DNA-binding domains. Furthermore, these mutant p53 proteins bind as well as wild-type proteins to p53-specific DNA sequences (Fig. 2). (3) In DNA transfection experiments, the stability of the amino-terminal mutant proteins was similar to the wild-type protein in Saos-2 cells used to test the transcription factor phenotype. (4) Nonconservative mutations in adjacent residues to 22 or 23 (also 21 or 24) have little impact on the transcription phenotype (the 17–21 double mutant is 40% of wild-type, and the 24 single mutant is 94–127% of wild-type levels of transcription), consistent with the idea that amino acid residue–protein contacts are being mapped, not major conformational changes in the p53 protein.

These sharp boundaries for amino acid residue functions plus the otherwise wild-type character of the mutant proteins studied here strongly suggest that protein–protein contacts are being elucidated by these mutants. Clearly then, two critical hydrophobic residues at amino acids Leu-22 and Trp-23 are crucial for p53 communication with the transcriptional machinery of the cell. Most interestingly, the oncogene proteins hdm-2 and Ad5 E1B 55-kD proteins target the very same amino acids for inhibition of p53 function. Binding of p53 to the Ad5 E1B 55-kD protein appears to require residues 23–27 of the protein (see Table 2). Thus, the acidic residues in the p53 amino-terminal domain play less of a role than the critical hydrophobic residues in these protein–protein interactions. Even mutations in six of the acidic amino acids, creating a mutant whose charge went from −9 to +3 (Table 1), left 18–35% of the wild-type p53-mediated transcriptional activity, whereas changing two or three acidic residues at a time had minimal effects.

Even though the wild-type p53 protein appears to bind to TBP (Seto et al. 1992) via its amino-terminal domain (Liu et al. 1993; Truant et al. 1993), the evidence presented here (Fig. 7) indicates that this is not, or is only minimally, mediated by the 22 and 23 or 14–19 residues of p53. The p53 protein may bind both to TBP and to TAFs. One may even require the entire holo-TFIID complex to completely understand the contacts of p53 residues 14–19 or 22–23 with the transcriptional machinery of the cell. It is clear from the results presented here that p53 hydrophobic residues 22 and 23 make critical contacts to promote transcription in genes with p53-responsive elements.

The results presented in Table 1 help to explain a paradox that has existed in the nature and position of p53 mutations detected in human cancers. Figure 8 presents the position and frequency of p53 missense mutations in p53 cDNAs isolated from human cancers. The data base used for this analysis (Levine et al. 1994) is composed of 1447 mutations from 51 different cancer types. Of all these mutations, 85.6% are missense mutations and 92% cluster in the DNA-binding domain of p53 between codons 120 and 290. Even when mutations are found outside of this central domain, they are most often qualitatively different. For example, six mutations at codon

Table 2. *Summary of p53 amino acid residues in the amino-terminal domain involved in p53 protein interactions*

p53 Function	Residues involved
1. Transcriptional activation	Leu-22 and Trp-23
2. Human mdm-2 binding	Leu-14 and Phe-19, Leu-22 and Trp-23
3. Ad5 E1B 55-kD protein	Trp-23 and Leu-22–Trp-23, Lys-24, Pro-27 Leu-25 and Leu-26 to a lesser degree

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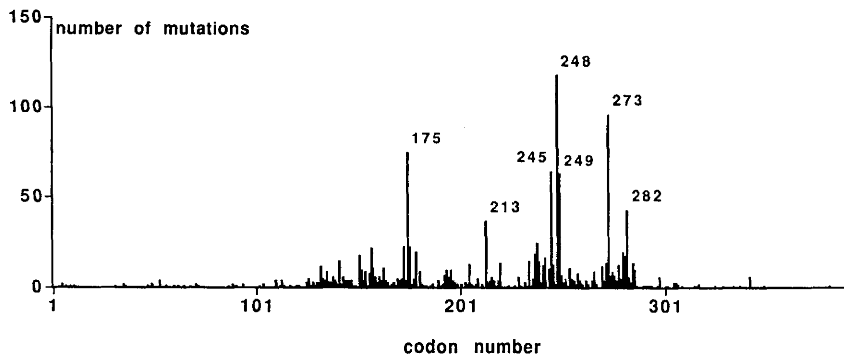

Figure 8. The codon position in the p53 gene of 1447 different mutations that have occurred in 51 different types of human cancers. The graph shows the frequency of different missense mutations as a function of the codon in the p53 gene derived from various types of cancers in humans. The results are from the Princeton University data base of such mutations (Levine et al. 1994).

298 and six at codon 342 are all (12) chain-termination mutations. Codon 53 contains four independent mutations, and three are chain-termination mutants. It is thought that these mutants result in selection against the transcriptional activity of the p53 protein (Farmer et al. 1992; Kern et al. 1992; Zambetti et al. 1992). Why, then, are all of the missense mutations clustered in the DNA-binding domain and few, if any, in the amino-terminal *trans*-activation domain? The answer (Table 1) appears to be that two independent mutations at codons Leu-22 and Trp-23 are required to have the same phenotype as one mutation in the DNA-binding domain (273RH mutant). This distribution of mutations in Figure 8 is consistent with the results presented here and reinforces the evidence that the p53 "loss-of-function" mutation is to select for the loss of a transcription factor. The fact that two oncogenes, one human (*mdm-2*) and one viral (Ad5 E1B 55-kD), target the very same amino acids used in protein contacts for transcriptional activation is also most consistent with the critical role of transcription in the function of the p53 protein as a tumor suppressor gene.

Soussi et al. (1990) have pointed out that the p53 proteins whose cDNAs have been sequenced from rainbow trout to human contain five highly conserved regions of amino acid sequences. Four of them are in the DNA-binding domain (residues 120–143, 172–182, 238–259, 271–290) and one is in the amino-terminal domain between residues 13 and 19. Residues Leu-22 and Trp-23, however, are also identical in all of the p53 proteins in this comparison (Fig. 9) and, so, the conserved region I

```
Xenopus           M----EPSSETGMDPPLSQETFEDLWSLLP----------------DPLQ    30
Trout             M----ADLAEN-VSLPLSQESFEDLWKMNL----------------NLVA    29
Af. Gr. Monkey    M---EEPQSDPSIEPPLSQETFSDLWKLLPENNVLSPLP---SQAVDDLM    44
Rhesus Macaque    M---EEPQSDPSIEPPLSQETFSDLWKLLPENNVLSPLP---SQAVDDLM    44
Human             M---EEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLP---SQAMDDLM    44
Mouse             MTAMEESQSDISLELPLSQETFSGLWKLLPPEDILP-----SPHCMDDLL    45
Rat               ---MEDSQSDMSIELPLSQETFSCLWKLLPPDDILPTTATGSPNSMEDLF    47
Hamster           ---MEEPQSDLSIELPLSQETFSDLWKLLPPNNVLSTLP--SSDSIEELF    45
Chicken           MAEEMEPLLEPT-------EVFMDLWSMLP-------------YSMQQLP    30
                       .   .         * *  **..                  . .
```

Figure 9. The amino-terminal amino acid sequence of p53 from several species. The one-letter amino acid code is used to compare the sequences of p53 proteins from diverse species. Gaps have been employed to maximize the similarities in these sequences. The last residue number is given at *right* for each sequence. Asterisks indicate identity in all sequences. Dots indicate similarities in all sequences.

should probably be extended from amino acid 13 to 23 to more closely reflect the functional significance of this conservation.

Materials and methods

Plasmids and site-specific mutagenesis

pBKS–p53 consists of a Bluescript KS(−) vector (Stratagene) and the entire wild-type human p53 cDNA ligated at their *Eco*RI sites. pRC/CMVp53 consists of a pRC/CMV vector (Invitrogen) and the entire wild-type human p53 cDNA ligated at their *Hin*dIII and *Xba*I sites. Mutations were generated in pBKS–p53 or pRC/CMVp53 by annealing oligonucleotides with one or multiple mismatches to uridine-containing single-stranded DNA template as described previously by Kunkel (1985). The oligonucleotide was extended with T4 DNA polymerase, and the resulting double-stranded DNA was sealed with T4 DNA ligase. The DNA was used to transform *Escherichia coli* JM109 (Invitrogen). Single-stranded or double-stranded DNA was prepared and sequenced by the dideoxy procedure (Sanger et al. 1977) using appropriate primers. p53 mutants that generated in pBKS–p53 were cleaved with *Hin*dIII and *Xba*I recloned in these sites in the expression vector PRC/CMV.

For CAT assays, three reporters were used, p50–2CAT, CosX1CAT, and $pG_4E1BCAT$. p50–2CAT consists of two copies of p53-responsive element from the murine muscle creatine phosphokinase promoter (Zambetti et al. 1992). CosX1CAT contains a p53-responsive element from murine *mdm-2* gene (Wu et al. 1993). $pG_4E1BCAT$ contains four Gal4 DNA-binding sites, the E1B TATA sequence, and the CAT gene (kindly provided by Dr. T. Shenk, Princeton University).

Cell culture and DNA transfection

The human osteogenic sarcoma (Saos-2) cells that do not express endogenous p53 (Masuda et al. 1987) were used for CAT assays and transient expression of p53 protein. Saos-2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal bovine serum (FBS) in a 5% CO_2, 37°C incubator. For CAT assays, the cells grown on a 10-cm^2 tissue-culture dish were cotransfected with 2.5 μg of p53 plasmid DNA and 2.5 μg of reporters plus 15 μg of salmon sperm DNA by calcium phosphate protocol (Graham and van der Eb 1973). Forty-eight hours after transfection, CAT activity was assayed as described previously (Zambetti et al. 1992) and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics).

For transient expression of p53 protein, Saos-2 cells grown on a 15-cm^2 tissue-culture dish were transfected with 20 μg of p53

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DNA and 20 μg of salmon sperm DNA by the calcium phosphate protocol (Graham and van der Eb 1973). Forty-eight hours after transfection, cells were labeled with [^{35}S]methionine and subjected to immunoprecipitation.

Immunoprecipitation and gel electrophoresis

Cells transfected with wild-type or mutant p53 were labeled with [^{35}S]methionine for 3–4 hr. The cells were lysed, and the lysates were precleared with protein A–Sepharose for 15 min at 4°C. The anti-p53 monoclonal antibody pAb240 (Gannon et al. 1990), pAb421 (Harlow et al. 1980), or pAb1620 (Milner et al. 1987) or anti-mdm-2 monoclonal antibody 4B11 (Chen et al. 1993) and protein A–Sepharose were added to cleared supernatant, and the mixture was mixed continuously on a rocking platform for 4 hr to overnight at 4°C. For the determination of the immunoreactivity of p53 synthesized in vitro, wild-type or mutant p53 was precipitated with the anti-p53 monoclonal antibody, pAb1801 (Banks et al. 1986), pAb240, pAb421, or pAb1620. The immunoprecipitates were then washed and subjected to 12.5% SDS–polyacrylamide gel electrophoresis and X-ray autoradiography as described previously (Finlay et al. 1989).

For half-life analyses, Saos-2 cells transfected with wild-type or mutant p53 were labeled with [^{35}S]methionine for 3 hr, washed three times with DMEM, and chased for 2 or 4 hr in DMEM supplemented with 10% FBS. Equivalent amounts of TCA-precipitable counts from the different time points were immunoprecipitated with pAb421, and the immunoprecipitated proteins were separated on a 12.5% SDS–polyacrylamide gel.

In vitro translation and complex formation assays

Radiolabeled hdm-2 and mutant p53 were generated by in vitro translation in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Each mutant p53 protein was mixed with mdm-2 and incubated for 30 min at 30°C. The mixture was then immunoprecipitated with the mdm-2-specific monoclonal antibody 4B2 (Chen et al. 1993). The efficiency of in vitro complex formation between p53 mutants and mdm-2 was determined by quantitation of the coprecipitated mutant p53 in comparison to wild type.

For the p53 E1B 55-kD binding assays, the protocol of Kao et al. (1990) was used. Wild-type or mutant p53 translated in the reticulocyte lysates and labeled with [^{35}S]methionine, as described previously (Chen et al. 1993), was mixed with whole Saos-2 cell extracts infected with wild-type Ad5. The mixture was incubated for 30 min at 30°C and precleared with protein A–Sepharose for 15 min at 4°C. The anti-E1B 55-kD protein monoclonal antibody 2A6 (Sarnow et al. 1982) and protein A–Sepharose were added to cleared supernatant, and the mixture was mixed continuously on a rocking platform for 2 hr at 4°C. The immunoprecipitates were then washed and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

Purification of baculovirus-produced p53 proteins

Baculoviruses expressing human wild-type p53 and the 14–19 and 22–23 mutants were generated by cloning the cDNAs into PVL1393 and cotransfection with baculogold viral DNA (Pharmingen). Viruses were then subjected to one round of plaque purification. The baculovirus for expression of the His-175 mutant p53 has been described previously (Bargonetti et al. 1991). Proteins were expressed in high-five insect cells (Invitrogen) and purified by pAb421 immunoaffinity procedures as described previously (Momand et al. 1992).

Gel electrophoretic mobility-shift assay

The p53 consensus probe 5′-GGGACATGCCCGGGCATGTC-3′ (Funk et al. 1992) was end-labeled, and $\sim 1 \times 10^5$ cpm was added in each reaction. Reactions were performed in electrophoretic mobility-shift assay (EMSA) buffer (20 mM Tris at pH 7.5, 50 mM NaCl, 5% glycerol, 0.1% NP-40, 1 mM $MgCL_2$, 1 mM DTT, 0.1 mg/ml of BSA) in a total volume of 20 μl. Twenty nanograms of each of the purified p53 proteins was incubated with the probe for 15 min at room temperature followed by the addition of 100 ng of poly[d(I-C)] and incubation for an additional 10 min. For antibody supershift assays, 1 μg of purified pAb421 was added at the start of the reaction. Mixtures were loaded onto a 4% polyacrylamide gel containing 0.25× TBE and electrophoresed at room temperature, 200 V for ~2 hr.

p53/TBP protein interaction assays

For coimmunoprecipitation assays, ~400 ng of baculovirus-produced, purified p53 proteins was mixed with 8 μl of ^{35}S-labeled TBP programmed wheat germ lysate (Promega TNT system). Reactions were carried out in 100 μl of buffer containing 20 mM Tris at pH 7.5, 100 mM NaCl, 0.1% NP-40, 1 mM $MgCL_2$, 0.1 mg/ml of BSA, 1 mM DTT, and 1 mM PMSF for 30 min at 30°C. Reactions were then immunoprecipitated with pAb421 hybridoma supernatant and protein A–Sepharose. Immunoprecipitates were washed twice with 800 μl of reaction buffer, analyzed by 9% SDS-PAGE, and exposed to autoradiography. Far Western blot analysis was performed as described previously (Seto et al. 1992). Three hundred nanograms each of the purified p53 proteins and 3 μg of uninfected SF9 cell lysate were separated by 9% SDS-PAGE and transferred to nitrocellulose. Blots were probed with 40 μl of human TBP programmed wheat germ lysate, washed, and exposed to autoradiography.

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