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Function, Oligomerization, and Conformation of Tumor-Associated p53 Proteins With Mutated C-Terminus

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Abstract Mutations that affect the oligomerization domain (OD) of the p53 tumor suppressor may be of particular interest because of the remarkable contradiction between the conservation of the OD and its relative functional resistance to amino acid substitutions, and because of recent hints that cellular protein factors may interact with the OD. Both point to the possibility that this domain fulfills tasks beyond oligomerization. We report that the tumor-associated mutants 330H, 334V, and 337C are defective for homo-oligomerization by three criteria. Accordingly, 330H and 337C failed to bind to a p53 recognition motif in gel-shift assays and to stimulate reporter genes efficiently in transient transfections. 334V retained some activity in both assays despite being oligomerization-defective. The ability of the mutants to induce apoptosis correlated with their performance in the DNA binding and transactivation assays. However, mutants 330H and 337C were able to provoke cell death when overexpressed, which in combination with their failure to transactivate genes suggests competence for the induction of transactivation-independent apoptosis at high protein levels. Although 334V and 337C failed to homo-oligomerize, they were able to hetero-oligomerize with a p53 with wild-type OD, and 334V was able to interfere with transactivation by wt p53. All mutants showed a reduced reactivity with antibody PAb421 and a distinct calpain cleavage pattern indicative of conformational alterations. In conclusion, tumor-associated OD mutants of p53 can be functionally competent to different degrees despite of being oligomerization defective. J. Cell. Biochem. 76:572–584, 2000. © 2000 Wiley-Liss, Inc.

Key words: apoptosis; transactivation; tetramerization; Li-Fraumeni syndrome

p53 is a homotetrameric, multifunctional, and predominantly nuclear transcription factor that becomes activated from a latent state and metabolically stabilized in response to various forms of stress, including DNA damage, nucleotide pool imbalance, and hypoxia. Monomeric human p53 consists of 393 amino acid residues and is subdivided into several functional domains: (1) a transactivation domain between residues 1 and 42 that interacts with elements of the basal transcription machinery and with oncoprotein Mdm2, which subjects p53 to nuclear export and degradation [Haupt et al., 1997; Kubbutat et al., 1997]; (2) a proline-rich domain between residues 61 and 94 that is critical for the mediation of apoptosis, for transcriptional repression of genes that lack p53-response elements, and for the transactivation of a p53-induced gene that functions during apoptosis [Sakamuro et al., 1997]; (3) the core sequence-specific DNA binding domain between residues 102 and 292; and (4) the C-terminal oligomerization domain (OD; 326–355) and regulatory domain (360–393) [reviewed in Levine, 1997]. Of the various functions attributed to p53 (e.g., as a cell cycle inhibitor under stress conditions, a mediator of cell death, a regulator of senescence, and a cofactor in DNA repair), not all depend on the ability of the protein to transactivate genes, but all do seem to require the integrity of the central core DNA binding domain. Consistent with this, most of the many p53 mutations associated with tumors are found in this domain, while less than 1% are detected in other parts of the protein

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[Hollstein et al., 1996]. However, since the core domain has been analyzed more extensively, a bias toward the discovery of core domain mutations is likely.

Although mutations in the C-terminus are relatively infrequent in tumors, they may be of particular interest for different reasons. First, the C-terminus of p53 is the target of regulatory modifications, including phosphorylation, acetylation, O-glycosylation, and the interaction with proteins such as 14–3-3 [Waterman et al., 1998; for review, see Prives, 1998]; thus, mutations in the OD may affect protein interaction and/or conformation and thereby any of these modifications. Second, mutations at codons 337 and 344 have been found to be inherited in families with Li-Fraumeni and Li-Fraumeni-like syndrome that are susceptible to the development of multiple primary neoplasms early in life [Varley et al., 1996; Lomax et al., 1997]. However, the effects of these mutations on tetramer stability and protein function are not entirely clear and may be different in the in vitro and in vivo settings [Waterman et al., 1995; Mateu and Fersht, 1998; Lomax et al., 1998; Davison et al., 1998]. Third, the remarkable overall conservation of the OD stands in some contrast to its relative functional resistance to amino acid substitutions and suggests the biological relevance of this domain that may reach beyond oligomerization [Clore et al., 1994; Mateu and Fersht, 1998]. For instance, the OD could be important for the interaction with cellular protein factors [Wagner et al., 1998; Schneider et al., 1998].

The three-dimensional structure of the OD of p53 has been studied by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [Jeffrey et al., 1995; Lee et al., 1994; Clore et al., 1994, 1995]. p53 is a dimer of dimers; each monomer of the OD consists of a β-strand (326–333), a turn (Gly334) and an α-helix (335–353); two antiparallel α-helices form a dimer, and two dimers are arranged orthogonally to give rise to a four-helix bundle. Substitutions for Leu at position 344 in the center of the α-helix are expected to disrupt the helical structure; a change of codon 344 to Ala has been shown to permit dimer but not tetramer formation [Waterman et al., 1995]. Mutation of Arg337 to Cys removes a salt bridge and hydrophobic interactions that are required to stabilize the dimer. However, there seems to be considerable tolerance for mutations in this part of the protein [Clore et al., 1994; Mateu and Fersht, 1998], and tetramerization may not be necessary for all aspects of tumor suppression [Ishioka et al., 1997]. The aim of this study was to gain a better understanding of the functions of mutants 330H, 334V, and 337C that have been described to occur naturally in tumors, and of which 337C has been associated with Li-Fraumeni-like syndrome [Lomax et al., 1997].

MATERIALS AND METHODS

Cell Culture and Plasmids

Human Hep3B hepatoma cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified 7% CO_2 atmosphere. All p53 sequences were cloned into the unique *Bam*HI site of expression vector pCMV-pA, between the cytomegalovirus (CMV) promoter/enhancer and the SV40 polyadenylation site. The C-terminal mutations and the N-terminal deletion comprising amino acid positions 1–79 were introduced by polymerase chain reaction (PCR) cloning and verified by sequencing. Codon 330 was changed from CTT to CAT (330H), codon 334 from GGG to GTG (334V), codon 335 from CGT to TGT (335C), and codon 337 from CGC to TGC (337C).

Transfections, Reporter Assays, and Cell Death Studies

For protein expression in Hep3B cells, cultures were lipofected with the polycationic liposomal reagent DOSPER, following the manufacturer's recommendations (Boehringer-Mannheim). Reporter assays were performed upon transfection with the $CaPO_4$-coprecipitation method. The precipitate was left on the cultures for 7 h. Chloramphenicol acetyl transferase (CAT) activity in total cell protein extracts was determined at 32 h after transfection by a nonchromatographic CAT assay as described [Roemer and Mueller-Lantzsch, 1996]. For apoptosis studies, Hep3B cells were transiently co-transfected with an effector plasmid and indicator plasmid pCMV-lacZ. To identify transfected cells, the cultures were fixed at 48 h after transfection with 1.25% gluraraldehyde for 20 min. Cells were then stained with the chromogenic substance 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal, Boehringer-Mannheim). The numbers of lacZ-positive surviving cells with

intact morphology was quantified as described [Liu et al., 1996; Theis et al., 1997]. For liquid lacZ enzyme assays, cell extracts were prepared by three cycles of freeze thawing (−70°C/37°C) in 0.25 M Tris pH 7.8. Protein was then incubated at 37°C for 30–60 min in the presence of 1 mM $MgCl_2$, 150 mM β-mercaptoethanol, and 1/5 vol. of a stock solution (4 mg/ml in 0.1 M Na_3PO_4, pH 7.3) of chlorophenol red-β-D-galactopyranoside (Boehringer-Mannheim). Enzyme activity was determined photometrically. Finally, TUNEL assays were carried out as specified by Theis et al. [1997].

In Vitro Transcription/Translation of p53 and DNA Binding Assays

The various p53 genes were PCR-amplified with a 5′-primer designed to contain the promoter for the T7 RNA polymerase, and the amplified sequences were incubated for 90 min at 30°C with a transcription/translation reaction mixture provided with the T7-coupled reticulocyte lysate kit from Promega (Madison, WI). p53 expression was monitored by Western blot analysis with antibody DO-1, and an estimated amount of 10 ng of protein (typically 1/25th of the reaction mixture) was used in the gel-shift assays. For electrophoretic mobility shift assays (EMSA), p53 proteins alone or p53 incubated with antibodies PAb421 or DO-1 at 2 µg/ml, were added to 4 µl binding buffer (230 mM Tris pH7.5, 50 mM NaCl, 4.5% glycerol, 5 mM DTT, 5 mM $MgCl_2$, 0.125% Triton X-100), 1 µg bovine serum albumin (BSA), and 2.5 µg poly dA-dT (Boehringer-Mannheim) in a final volume of 18 µl and incubated for 30 min at room temperature (RT). The samples were then further incubated for 30 min at RT in the presence of 0.5 ng of ^{32}P-labeled oligonucleotide (see legend to Fig. 1). The p53/DNA/antibody com-

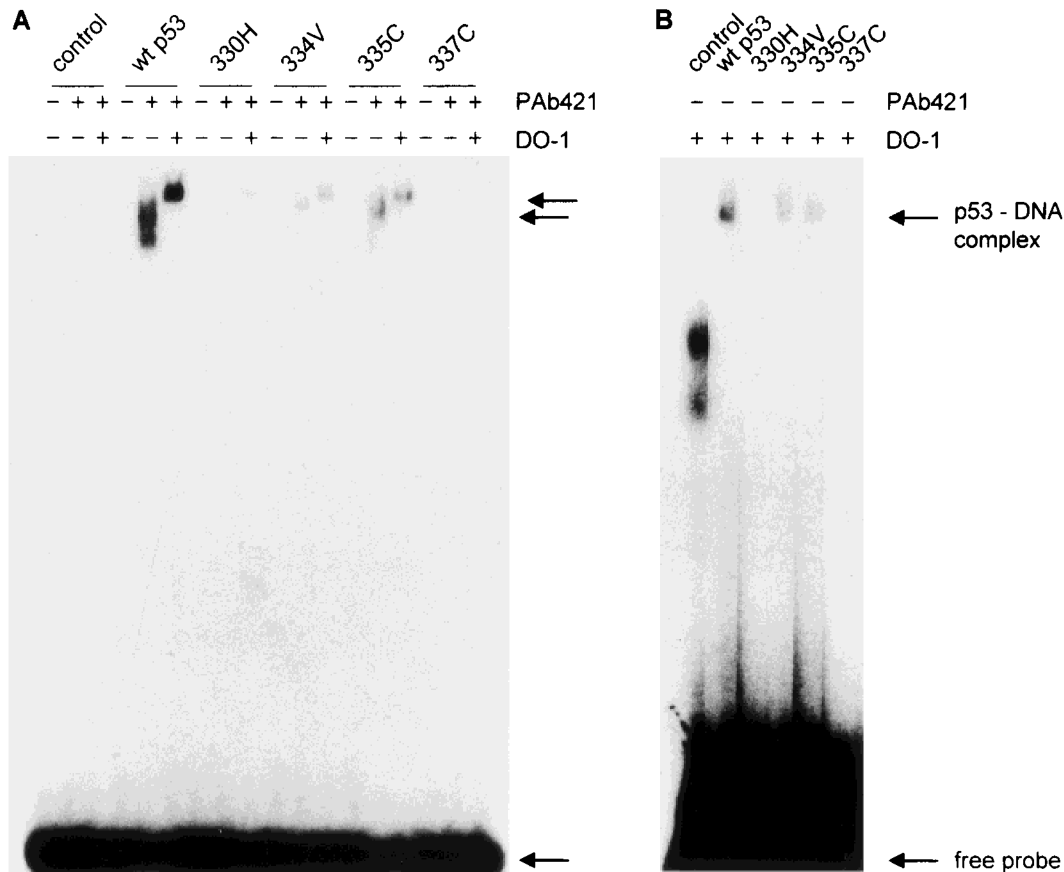


Fig. 1. Expression and DNA binding of wt p53 and the oligomerization-domain mutants. **A:** In vitro-synthesized proteins were incubated with ^{32}P-labeled p53 recognition sequence 5′-GATCCAGACATGCCTAGACATGCCTA GGAATT-3′ (half-sites underlined) in the absence or presence of antibodies PAb421 and Do-1, and the complexes were subjected to electrophoretic mobility shift assay (EMSA) on a 4% polyacrylamide gel. **B:** p53 proteins were incubated with DNA and antibody DO-1 only, and the shifted bands were visualized after a prolonged exposure time of 7 days.

plexes were separated from the free probe on a native 4% polyacrylamide gel following standard procedures.

Western Blotting and Immunoprecipitation

Cell extracts were prepared at the indicated time points after transfection by lysing the cells in a buffer containing 10 mM Tris pH7.6, 140 mM NaCl, 0.5% Nonidet P-40 (NP-40), 0.5 mM PMSF, 2 µg/ml aprotinin and 0.5 µg/ml leupeptin. For Western blot analysis, total cell protein was subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore). p53 protein was detected by enhanced chemiluminescence (Amersham) upon incubation with antibodies DO-1, PAb421, or PAb122 (see text and figure legends for details). For immunoprecipitation, cell lysates were mixed with protein G-Sepharose plus 1 µg of antibody DO-1 and rocked overnight at 4°C. The Sepharose-protein complexes were washed twice with 1 ml lysis buffer. p53 was recovered by resuspension of the complexes in standard Laemmli buffer and analyzed on 10% SDS-polyacrylamide gels.

Oligomerization Analyses and Calpain Cleavage

Whole cell lysates from DOSPER-transfected cultures were incubated with 0.0025% glutaraldehyde for 15 min RT (the optimal glutaraldehyde concentration was determined experimentally). Monomers and multimers were separated on a 7.5% SDS-polyacrylamide gel and detected with antibody DO-1. For zone velocity sedimentation analysis, total extracts from cells transfected with a p53 variant for 24 h were loaded onto 4–20% sucrose gradients that were then centrifuged for 14 h in a Beckman SW41 rotor at 40,000 rpm and 4°C. The gradients were fractionated into 15 samples; p53 was detected after SDS-PAGE by Western blotting with antibody DO-1. To study the cleavage pattern produced by the calcium-activated neutral protease (calpain; Sigma Chemical Co., St. Louis, MO), p53 in cell lysates was immunoprecipitated with DO-1, and the whole precipitate was incubated for 30 min at 37°C with 0.05 U calpain in the presence of 1 mM $CaCl_2$. The cleavage was stopped by the addition of Laemmli loading buffer; the generated fragments were analyzed on 10% SDS-polyacrylamide gels and by immunoblotting with antibody DO-1.

RESULTS

DNA Binding and Transactivation

Most mutations found in the p53 gene are located in the central DNA binding domain. However, an extension of the survey has revealed that some tumor-derived mutations affect the regulatory C-terminal domain comprising amino acid residues 300–393. We have investigated the function of natural mutant L330H associated with hepatocellular and ovarian carcinoma, mutant G334V associated with small cell lung cancer, and mutant R337C associated with breast, esophagal, and hepatocellular carcinoma, as well as with Li-Fraumeni-like syndrome, in human p53-deficient Hep3B hepatoma cells. For comparison, and as controls, we included the artificial mutant R335C constructed by us, as well as wild-type (wt) p53 in the study. For oligomerization analysis, a p53 deleted in the OD (p53-ΔO; Δ327–347) and the conformational mutant R175H were employed as further controls.

In vitro translation of p53 has proved to be a highly useful and reliable system for DNA binding studies [e.g., Hall and Milner, 1995; Hainaut et al., 1995; Chene, 1999]. In a first set of experiments, the genes for wt p53, 330H, 334V, 335C, and 337C were translated in vitro with the T7-coupled reticulocyte lysate transcription/translation kit from Promega. p53 protein was detected by Western blotting with p53 antibody DO-1 (epitope: aa 20–25). To determine the ability of the proteins to bind to p53 recognition motifs, equal quantities of p53 protein were assayed for binding to the sequence 5′-GATC-CAGACATGCCTAGACATGCCTAGGAATT-3′ (half-sites underlined) by EMSA, as detailed under Materials and Methods. DNA binding of wt p53 was observed only after incubation with antibody PAb421 (epitope: aa 372–382); therefore, samples were included that contain PAb421 at 2 µg/ml. As expected, wt p53 bound to its consensus sequence and produced a strong signal. Additional incubation with antibody DO-1 supershifted the p53/DNA/PAb421 complex (Fig. 1A). Mutants 334V and 335C showed weak but detectable specific DNA binding and also produced complexes that were supershifted by DO-1. By contrast, mutants 330H and 337C failed to bind DNA in this assay; no shift of the DNA probe was detected even after long exposure. However, mutant 330H produced a very weak signal when antibody DO-1 was included

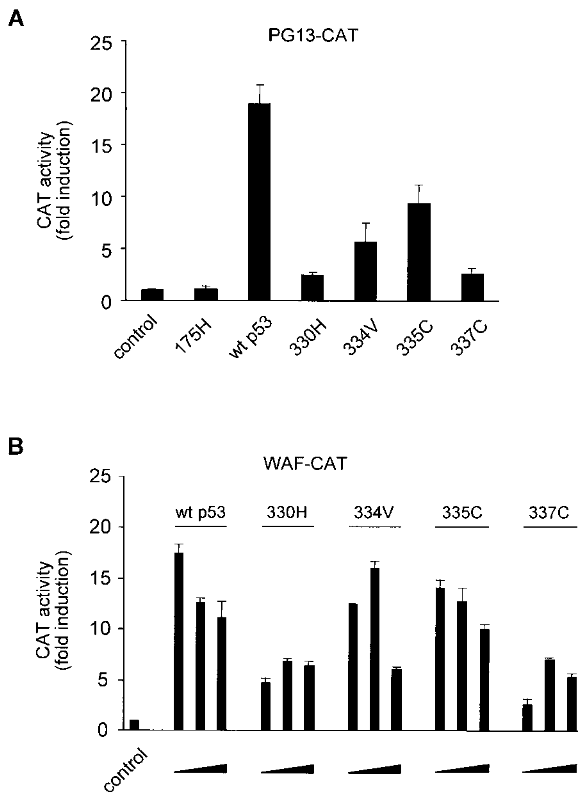


Fig. 2. Transcriptional transactivation by wt and mutant p53. **A:** Hep3B cells were transfected with 4 µg of PG13-CAT reporter vector plus 0.01 µg of empty vector (control) or one of the p53-producing vectors. A plasmid expressing the transactivation-defective mutant 175H was included as a further control. CAT activity was measured at 32 h after transfection. **B:** Cells were transfected as in A, except that reporter plasmid WAF-CAT was used and increasing quantities of effector plasmids were employed (0.01, 0.05, 0.1 µg). Error bars denote standard deviations derived from three transfections.

in addition to PAb421 (Fig. 1A), consistent with previous observations that interactions with the N-terminal domain can support DNA binding by mutant forms of p53 [Hupp et al., 1993]. Incubation with DO-1 alone, in the absence of PAb421, also stimulated DNA binding by wt p53 and to a lesser extent by the mutants 334V and 335C; however, this stimulation was very weak as compared with that obtained by PAb421 and was detectable only after lengthy exposure times (Fig. 1B). Together, these results show that 334V and 335C are impaired, and that 330H and 337C are defective for DNA binding in this assay.

We then addressed whether the p53 mutants are able to stimulate CAT gene expression from (1) PG13-CAT, a plasmid containing upstream of a CAT reporter gene a minimal polyoma virus promoter fused to 13 copies of the p53 binding site from the ribosomal gene cluster (RGB), and (2) WAF-CAT, a reporter consisting of a CAT gene and 2.4 kb of the promoter of the p53-regulated CDKN1A (p21 WAF/Cip1) gene. First, Hep3B cells were transiently co-transfected with 4 µg of PG13-CAT and only 0.01 µg of control vector or one of the p53 expression plasmids. In addition to the empty control vector, a plasmid producing the transactivation-defective mutant 175H was included as a further control. CAT assays at 32 h after transfection indicated that wt p53 stimulated CAT expression from PG13-CAT to levels approximately 20-fold over background. By contrast, 330H and 337C were essentially inactive. Mutants 334V and 335C induced CAT activity to only 25 to 50% of the levels obtained with wt p53 (Fig. 2A). These findings are entirely consistent with the results from the DNA-binding studies outlined in Figure 1. In a second set of experiments, Hep3B cells were transfected with 4 µg of WAF-CAT plus increasing quantities of p53 expression plasmids within the range of 0.01–0.1 µg. Although all mutant proteins appeared to be more active on WAF-CAT than on PG13-CAT, the relative activity of the mutants remained constant. At a quantity of 0.01 µg effector plasmid, 334V and 335C were active as transcription factors, while 330H and 337C were only weakly active. However, at the highest plasmid concentration, all CAT activities dropped off (Fig. 2B). The decreased reporter gene activity at high p53 quantities has been reported before and likely reflects the propensity of p53 to suppress basal promoter activity and the fact that p53 functions as a mediator of apoptosis at high protein levels [Roemer and Mueller-Lantzsch, 1996]. These findings point to the possibility that 334V and 335C retain some apoptotic and gene repressor activity (see below). Notably, at an intermediate plasmid concentration of 0.05 µg the level of CAT activity induced by 334V increased above that stimulated by the wild-type protein. This may be artifactual: mutants impaired for the mediation of cell death and gene repression but with some competence for transactivation may mimic a transactivator even more potent than wt p53 at certain plasmid quantities. Together, the results show that 334V and 335C retain some capabilities to specifically bind to DNA and transactivate reporter genes, while 330H and 337C are strongly impaired.

Apoptosis and Gene Repression

Since Hep3B cells that undergo apoptosis detach from the dishes and are thus lost from the cultures, co-transfection of a p53 plasmid and an indicator plasmid producing the bacterial β-galactosidase (lacZ) permits rapid microscopic identification and quantitation of surviving transfected cells with intact morphology. Furthermore and by contrast to FACS analysis, microcolonies of transfected cells can be identified; it thus becomes possible to distinguish between (1) low numbers of transfected cells caused by proliferation inhibition, and (2) low numbers of transfected cells caused by cell death [Theis et al., 1997; Roemer and Mueller-Lantzsch, 1996; Liu et al., 1996]. We have employed this procedure to study the effects of the mutants on the survival of Hep3B cells. Subconfluent cultures were co-transfected with 1 µg of indicator plasmid pCMV-lacZ and 0.5 µg of control vector or one of the p53-producing plasmids. Cells were stained for lacZ expression at 48 h after transfection. As is shown in Figure 3A, wt p53 induced rapid loss of viable cells whereas mutants 330H and 337C failed to reduce cell viability significantly at the lower plasmid quantity. As in the DNA binding and transactivation assays, mutants 334V and 335C retained some activity as inducers of apoptosis. The fact that 334V and 335C are cytotoxic can explain why these mutants gave rise to less CAT activity in the transactivation assays at high plasmid quantities. In all experiments, 334V was almost as toxic as wt p53, despite its reduced ability to bind to the consensus DNA motif and to transactivate reporter genes at low effector plasmid quantities (Figs. 1A, 2), suggesting that the cytopathic effect of 334V is partly independent of transactivation. At high plasmid quantity (1.5 µg), all mutants were able to induce apoptosis in Hep3B cells, even though 334V and 335C were always the more potent inducers. This finding suggests that all mutants are able to provoke transactivation-independent apoptosis when strongly overproduced. That cell loss was indeed caused by apoptosis was confirmed by the analysis of DNA fragmentation, a hallmark of apoptosis, by terminal transferase-mediated dUTP nick end-labeling (TUNEL) assays (data not shown).

p53 has been documented in the past to repress many regulatory elements placed on plasmids as well as some genes in vivo that lack p53

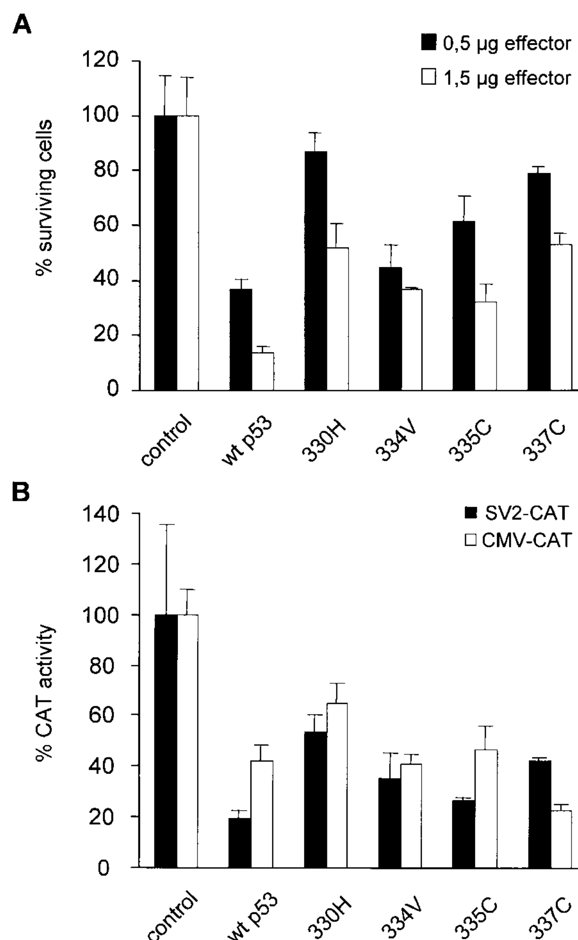


Fig. 3. Apoptosis and gene repression by wt and mutant p53. **A:** Hep3B cells were transfected with 1 µg of pCMV-lacZ indicator plasmid plus either 0.5 or 1.5 µg of empty vector or p53 expression plasmid. Cells were stained for lacZ expression at 48 h after transfection. The numbers of surviving transfected cells with intact morphology were determined as described under Materials and Methods. **B:** Repression of the SV40 and CMV regulatory sequences driving CAT. Hep3B cells that have been reported to be relatively resistant to p53-mediated apoptosis were transfected with 0.5 µg pSV2-CAT or 0.1 µg pCMV-CAT, plus 0.2 µg of control vector or p53 plasmid. CAT activity was determined at 32 h after transfection.

recognition sequences [Gottlieb and Oren, 1996; Murphy et al., 1996; Polyak et al., 1997]. The apparent repression of almost all promoters on plasmids has led to the suggestion that this phenotype may be caused by the titration of a basal transcription factor such as TBP by p53, but has also cautioned some to study in more detail if much of this effect may be simply the consequence of cell viability loss during apoptosis. In particular, the observation that the p53-induced apoptosis agonist Bax, which is not a transcription factor, was alone able to “repress” transcription hints at that possibility [Ryan

and Vousden, 1998]. To address this issue before undertaking repression studies with the mutants, we first transfected Hep3B cells with 1 µg of a CMV-lacZ reporter plasmid plus 0.2 µg of either empty expression vector as control or vector producing wt p53. As expected, the numbers of blue-staining (lacZ-positive) cells on dishes that had received p53 plasmid decreased with time and relative to the control-transfected cultures due to apoptosis. A decrease of approximately 30% was detectable as early as 24 h after the beginning of transfection. However, lacZ enzyme activity initiated from the CMV promoter decreased significantly stronger (by 65%) than the numbers of surviving transfected cells that had received p53 plasmid. In a second experiment, we co-transfected 0.5 µg control plasmid or p53 vector with 0.5 µg of a plasmid producing GFP and luciferase. Although cell loss in the p53-transfected cultures, analyzed by FACscan, was reduced from 39% to 8% in the presence of 100 µM of the pan-caspase inhibitor Z-VAD-FMK (R&D Systems), luciferase activity initiated from the CMV promoter was reduced to less than 40% of the controls, regardless of the presence of Z-VAD-FMK (data not shown). These results indicate a cumulative effect of cell loss and repression and show that repression by p53 is not simply a cell loss artifact.

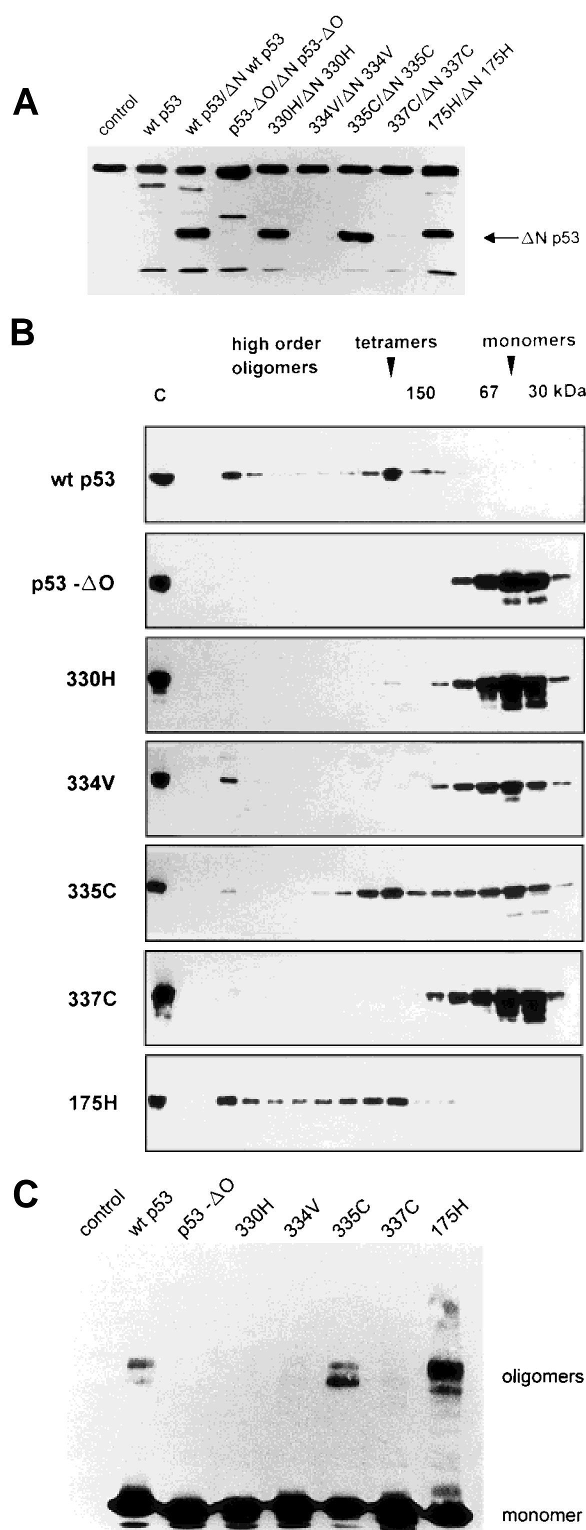
To study repression by the mutants and to minimize the effect of cell death, we resorted to a Hep3B cell subclone previously been shown to be largely refractory to p53-mediated apoptosis. We transfected these cells with 0.2 µg of control vector or p53 expression plasmid plus 0.5 µg of pSV2-CAT or 0.1 µg of pCMV-CAT reporter plasmids known to be subject to repression by p53. We found that the repression profile obtained with pSV2-CAT mirrors precisely the apoptosis profile outlined in Figure 3A: wt p53 and mutants 334V and 335C are highly active, whereas mutants 330H and 337C are less active. Similar results were obtained with pCMV-CAT, except that mutant 337C was more active as a repressor of the CMV promoter despite impairment of this mutant in apoptosis induction (Fig. 3B). The results of the repression assays were confirmed in our Hep3B cells. In summary, p53 mutants 334V and 335C retain much of the ability of wt p53 to induce programmed cell death and concomitantly, to repress promoters placed on plasmids, while 330H is relatively impaired for these functions. 337C is a less effective inducer of apoptosis as well, but it is functional for CMV promoter repression.

Oligomerization

Tetramerization is a prerequisite for proper function of p53; however, some transactivator and suppressor activity has been observed with mutants defective for tetramerization [Crook et al., 1994; Pellegata et al., 1995; Wang et al., 1993]. Since the mutations under study affect the β-strand (330H), the turn (334V), and the α-helix (335C and 337C) of the OD, it was interesting to ask whether they are compromised for oligomerization. We employed three assays to address this issue: (1) co-transfection of a plasmid producing a mutant and a plasmid expressing the same mutant with an N-terminal deletion, tested to see whether antibody DO-1 recognizing the N-terminus was able to co-precipitate the deleted variant; (2) separation of p53 monomers, tetramers, and high-order oligomers in transfected cell extracts via sucrose gradients; and (3) Western blot analysis of p53 molecules after glutaraldehyde crosslinking. These experiments were designed to address the issue of homo-oligomerization (i.e., oligomerization among the same molecules). However, because mutant forms of p53 can exert a dominant-negative effect through hetero-oligomerization with wt p53, and the stability of a dimer consisting of one mutant and one wild-type protein may well be higher than that of two mutant proteins, we also examined hetero-oligomerization by two methods: (1) co-immunoprecipitation with DO-1 of a mutant and the N-terminally deleted wild-type p53; and (2) co-transfection of a mutant and wt p53 plasmid with a reporter plasmid responsive to wt p53.

Hep3B cells were either transfected with empty vector, a plasmid producing wt p53, or co-transfected with vectors expressing the full-length plus an N-terminally deleted form of one of the proteins under study (see Fig. 4A for details). At 24 h after transfection, total cell extracts were prepared; antibody DO-1 that recognizes an epitope between aa 20 and 25 in the N-terminus was used to immunoprecipitate full-length p53. The expression of all forms of p53 was confirmed by Western blot analysis with antibody PAb122 recognizing the C-terminus (data not shown). As shown in Figure 4A, the N-terminally deleted forms of the proteins were co-precipitated with wt p53 and with mutants 330H, 335C, and 175H. By contrast, no

shortened p53 was co-precipitated with p53-ΔO and with mutants 334V and 337C. This indicates that 334V and 337C are defective for oligomerization under these experimental conditions whereas 330H and 335C are intact.



Total extracts from cells transfected to produce the full-length proteins were then subjected to zone velocity sedimentation in 4–20% sucrose gradients. The gradients were fractionated into 15 samples, and p53 was visualized in Western blot analyses with antibody DO-1. As expected, wt p53 and 175H sedimented mostly as tetramers and high-order oligomers. 335C was able to tetramerize, but significant quantities of protein sedimented as monomers and dimers. By contrast, p53-ΔO, 334V, and 337C were completely defective for tetramerization, and 330H generated only a very faint signal in the fraction containing tetramers (Fig. 4B). Furthermore and in contrast to 175H, all OD mutants except 334V failed to aggregate as high-order oligomers. Thus, the results obtained with mutants 334V, 335C, and 337C, and those obtained with the control proteins wt p53, p53-ΔO, and 175H were entirely consistent with the findings from the co-precipitations. 330H gave contradictory results: it oligomerized in the co-precipitation, but not in the sedimentation assays. This finding suggests that the N-terminally deleted form of 330H associates more stably with 330H than the full-length protein does. Rescue of an oligomerization-defective mutant by deletion of the N-terminal 22 amino acid residues has been reported before [Jayaraman et al., 1997]. To further study oligomerization, glutaraldehyde crosslinking was performed.

Total extracts from Hep3B cells transfected to express the full-length proteins were treated with 0.0025% glutaraldehyde, subjected to SDS-PAGE, and immunoblotted with antibody DO-1.

Fig. 4. Oligomerization of wt and mutant p53. **A:** Wt p53, 330H, 335C, and 175H oligomerize with their N-terminally deleted counterparts. Hep3B cells were co-transfected with plasmids expressing one of the depicted p53 proteins (2.5 µg of plasmid producing full-length p53 plus 10 µg of plasmid expressing the ΔN variant). ΔN-p53 are the respective p53 proteins with a deletion of aa 1–79. At 24 h after transfection, total cell lysate was immunoprecipitated with antibody DO-1 (epitope: aa 20–25); the precipitate was then subjected to immunoblot analysis with antibody PAb122 (epitope: aa 372–378). **B:** Zone velocity sedimentation of p53 proteins in 4–20% sucrose gradients. Hep3B cells were transfected with 10 µg of p53 plasmids; total cell extracts were treated as described under Materials and Methods, and p53 was detected with antibody DO-1. **Lane C**, total loaded p53 proteins. **C:** Glutaraldehyde crosslinking of p53 proteins. Extracts from cells transfected with 10 µg of empty vector (control) or p53 plasmids were treated with 0.0025% glutaraldehyde. Monomers and crosslinked oligomers of p53 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected with antibody DO-1.

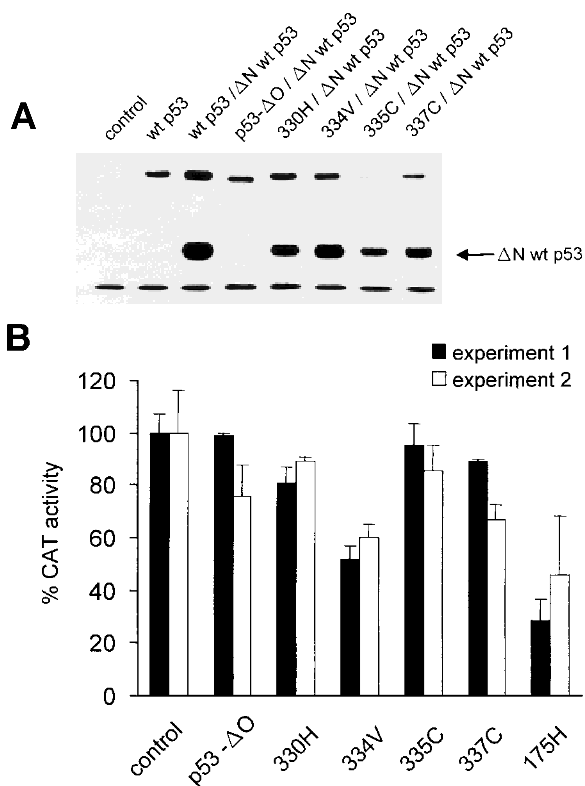


Fig. 5. Hetero-oligomerization of mutant p53 and interference with transactivation. **A:** All tumor-associated mutants oligomerize with an N-terminally deleted p53 with wild-type OD (ΔN wt p53). Hep3B cells were transfected as in Fig. 4A, except that a plasmid that produces ΔN wt p53 was included in the transfections. The immunoblot was stained with antibody PAb421 (epitope: aa 372–382). Note that the signals of the full-length mutant proteins, especially of 335C, appear weak, in keeping with the observation that the PAb421-epitope is partially lost in the mutant proteins (see Fig. 6A). **B:** Effect of mutant p53 on the transactivation by wt p53. Cells were transfected with 4 μg of WAF-CAT, 0.01 μg of wt p53 plasmid, and either empty vector (control) or mutant p53 plasmid at a quantity of 0.1 μg. CAT activity was determined at 32 h after transfection. The combined results from two experiments are presented. The fainter lower bands represent the immunoglobulin light chains.

As before, wt p53, 335C, and 175H generated strong signals indicative of oligomerization, whereas p53-ΔO, 330H, 334V, and 337C failed to do so (Fig. 4C). Combined, we therefore interpret these results to show that in Hep3B cells and under conditions of protein overproduction, the tumor-derived p53 mutants 330H, 334V, and 337C are defective for oligomerization, while the artificial mutant 335C is partially competent.

Two mutated ODs may form less stable aggregates than are achieved with one mutant and one wild-type domain. It was thus possible that the failure of the tumor mutants to tetramerize is (partially) overcome by co-expression with a p53 carrying a wild-type OD, and consequently, that these mutants can form hetero-oligomers with endogenous wt p53. To address this, co-precipitations similar to those outlined in Figure 4A were employed, except that now an N-terminally deleted p53 with a wild-type C-terminus was used. Remarkably, only p53-ΔO deleted in the OD failed to oligomerize in this assay whereas mutants 334V and 337C were now able to bring down the smaller p53, as were the remaining p53 proteins (Fig. 5A). Thus, despite of their defects in homo-oligomerization, 334V and 337C may be able to interfere with endogenous wt p53 in heterozygous cells in vivo.

To address the issue of dominance more directly, we examined whether any of the mutants can interfere with wt p53 stimulating a p53-responsive reporter gene. Several potential difficulties and limitations had to be considered. For instance, the quantities of mutant p53 plasmids in the transfections had to be chosen below the quantities that induce apoptosis (see Fig. 2A) but sufficiently high to see whether there was any interference. Furthermore, some mutants themselves possessed a limited transactivation capability (Fig. 1B). With this in mind, we co-transfected 4 μg of pWAF-CAT and only 0.01 μg of wt p53 plasmid plus 0.1 μg of empty vector into Hep3B cells. We observed stimulation of CAT activity to approximately 16-fold over background. We then replaced the 0.1 μg of empty vector with mutant p53 vector and asked for the effect of these proteins on the induction of CAT activity by wt p53. The results are summarized in Figure 5B. As expected, dominant-negative mutant 175H reduced the CAT activity drastically. By contrast, neither p53-ΔO nor 330H or 335C showed any significant effect, although both 330H and 335C were able to hetero-oligomerize with wt p53 in the co-precipitation study. Notably, mutant 334V that associated with p53 harboring a wild-type C-terminus (Fig. 5A), but not with itself (Fig. 4A), reduced the levels of CAT measurably. Mutant 337C also showed some limited suppressor activity. This reproducible effect may mirror the available quantities of free monomers: 334V and 337C cannot homo-oligomerize; thus, more monomers are available to associate with wt p53. Together, we interpret these data to suggest that mutant 334V, and

possibly 337C, can exert a dominant-negative effect in cells.

Antibody Reactivity and Conformation

The C-terminus is important not only for tetramerization, but also for the regulation of p53, the latter requiring the interaction with several regulatory proteins. For instance, the activation of p53 upon γ-irradiation involves dephosphorylation at position 376 and the subsequent binding of 14–3-3 proteins [Waterman et al., 1998]. The same region of p53 (aa 370–382) harbors the epitope for the activating antibody PAb421 and is target for acetylation by p300, phosphorylation by cycH-cdk7-p36, and O-glycosylation [Prives, 1998]. We obtained a first hint that the regulation via amino acid positions 370–382 might be compromised in the mutants when we studied the reactivity of these proteins with antibodies PAb421 and DO-1. When transfected Hep3B cells were analyzed for protein expression with PAb421, only a fraction of the protein in the lanes with 330H, 334V, 335C, and 337C reacted. When the same blots were probed with DO-1, approximately equal, high levels of p53 were detected (Fig. 6A). This finding suggests that the PAb421-epitope is masked in a fraction of the molecules, possibly through posttranslational modifications. It has been reported recently that phosphorylation at position Ser376 inhibits binding by PAb421 [Waterman et al., 1998]. Unfortunately, we were not able to show binding of the mutants by antibody PAb376P used in that study. PAb376P binds specifically to an epitope with phosphorylated Ser376 (a gift of T.D. Halazonetis, Philadelphia, PA). Further studies to identify modifications are under way.

The calcium-dependent protease calpain that can cleave polypeptides according to their tertiary structure has been used in the past to probe for different conformations of wt, mutant, and phosphorylated p53 [Pariat et al., 1997; Shieh et al., 1997]. To gain information on the structure of the mutants, we transfected cells and immunoprecipitated p53 from whole cell extracts with antibody DO-1. The proteins were then cleaved with calpain, subjected to SDS-PAGE, and visualized with DO-1. wt p53 and even conformational mutant 175H produced similar patterns of cleavage products, whereas 330H, 334V, and 337C gave rise to additional polypeptides of 47 and 49 kDa (Fig. 6B). Remarkably, p53-ΔO that lacks the OD and was as defective for oligomerization as 334V and 337C, failed to generate these additional signals. Mutant 335C not associated with tumors produced a normal pattern like that of wt p53. These observations suggest that the tumor-derived mutants 330H, 334V, and 337C possess an altered tertiary structure.

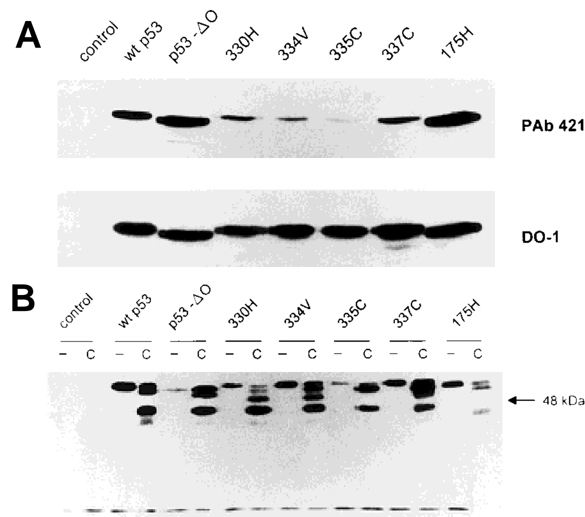


Fig. 6. Distinct antibody reactivities and conformation. **A:** Partial loss of the PAb421 epitope in the oligomerization domain mutants. Lysates from cells transfected with 10 μg of control vector or plasmid producing p53 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblot analysis with antibodies DO-1 and PAb421. **B:** Calpain cleavage pattern of p53 immunoprecipitated with DO-1 from lysates prepared 24 h after transfection with 10 μg of p53-producing plasmids. Precipitates were incubated with 0.05 U calpain for 30 min at 37°C in the presence of 1 mM $CaCl_2$. Bands were visualized with antibody DO-1. Note the appearance of additional fragments of 47–49 kDa in the 330H, 334V, and 337C lanes.

DISCUSSION

Efficient function of the p53 tumor suppressor requires homo-oligomerization [Pietenpol et al., 1994]. It was therefore suspected that tumor-associated p53 proteins with mutations in the OD might be impaired for oligomerization, even though it was conceivable that other important functions map to this domain and might be compromised by mutations that leave oligomerization intact [Wagner et al., 1998; Schneider et al., 1998]. However, structural analysis by NMR has suggested that the three amino acid positions Leu330, Gly334, and Arg337, which had been subject to mutation in the naturally occurring p53 variants studied in this paper, stabilize dimers through hydrophobic (330, 334, and 337), and electrostatic (337)

interactions and may be critical determinants of oligomerization [Clore et al., 1994]. Consistent with this, recent thermodynamic stability studies with purified recombinant OD have shown that single-residue changes at positions Leu330 and Arg337 strongly destabilize oligomers [Mateu and Fersht, 1998], and other studies have documented that in vitro translated 330H and 337C are defective for oligomerization and DNA binding [Rollenhagen and Chene, 1998]. Furthermore, in vitro translated p53 with a mutation of Gly334 to Gln or a substitution of Arg337 by Leu was reported to be compromised for oligomerization, as indicated by the failure to bind to DNA [Waterman et al., 1995]. The results presented conform to these observations: Mutants 330H, 334V, and 337C overproduced in Hep3B cells upon transfection were defective for homo-oligomerization by sedimentation and glutaraldehyde crosslinking analysis. 330H retained the ability to interact with an N-terminally truncated form of itself under conditions of overexpression, suggesting a higher stability of the full-length/deleted protein dimers.

At variance with our findings are the results presented by Lomax et al. [1997, 1998]. These authors report that 337C can tetramerize efficiently and bind to DNA. The variant results likely reflect the different experimental systems. It has recently been shown that 337C may indeed oligomerize, but that the oligomers are unstable at 37°C [Davison et al., 1998; Mateu and Fersht, 1998]. Thus, the extent to which 337C is compromised may vary with temperature, expression level, and even the cell type in which the protein is produced. However, the conflict between the cited and the present study extends to the function of the protein. Lomax et al. [1998] report that the 337C can activate a reporter gene and suppress cell proliferation in Saos-2 cells, whereas we find that the mutant is defective for transactivation in Hep3B cells. This is likely to reflect not only differences in cell type, but also the different quantities of p53 expression plasmid used for transfection. At the higher quantity used by Lomax et al. (1 µg compared with the 0.01 µg used by us), intracellular p53 may rise to levels that permit aggregation even of an oligomerization-defective mutant. Other researchers have also reported that high levels of 337C can transactivate [Rollenhagen and Chene, 1998]; however, in their study, as in ours, but unlike the study by Lomax and colleagues, 337C was defective for oligomerization. We interpret the existing data to show that 337C is defective for oligomerization and function at 37°C but that it can exhibit some intermediate activity in transactivation and growth suppression assays, probably in dependence of expression level and cell type.

Although monomeric p53 retains some ability to bind to DNA and transactivate genes [Crook et al., 1994; Pellegata et al., 1995; Wang et al., 1993], efficient function of p53 requires oligomerization [Pietenpol et al., 1994]. This is mirrored by the DNA binding and transactivation studies. 330H and 337C are defective for oligomerization and DNA binding and are the most impaired of all tested mutants for the stimulation of p53-responsive promoters. Artificial mutant 335C showed some intermediate activity in these assays. Notably, however, 334V bound to DNA and transactivated reporters as efficiently as 335C despite of being incompetent for oligomerization in co-precipitations, sedimentation assays, and crosslinking studies. Mutation 334V distorts the overall conformation of the OD [Clore et al., 1995]; one explanation for our findings may thus be that DNA contact stabilizes the conformation of 334V and thereby supports oligomerization and DNA binding. Altogether, it appeared that the mutant proteins performed better on the p21 WAF/Cip-1 promoter than on the RGB sequences in PG13-CAT, although the relative activity of the mutants remained constant (cf. Fig. 2A,B). Whether certain promoter structures can stabilize p53 tetramers with mutated OD remains to be seen.

Like transactivation, apoptosis induction by the mutants correlated with their ability to bind to DNA when the plasmid quantities used in the transfections were low (0.5 µg). 330H and 337C were defective, whereas 334V and 335C showed intermediate activity when compared with wt p53. However, at high plasmid quantity, all mutants were able to induce cell death in Hep3B cells, even though 334V and 335C were always the more potent apoptosis inducers. This finding may indicate that very high protein levels support some oligomerization and consequently, transactivation of apoptosis genes. It seems more likely though that the mutants become functional for the mediation of transactivation-independent apoptosis at high protein levels. Our results are consistent with previous reports showing that high levels of mutant 337C can provoke apoptosis in Saos-2

cells [Lomax et al., 1998]. Furthermore, our results may explain why 337C was able to suppress colony formation so efficiently in the study by Lomax et al. [1998] in spite of being impaired for oligomerization and DNA binding [Davison et al., 1998; Mateu and Fersht, 1998; and this paper).

Induction of apoptosis has been found to be closely correlated with the repression by p53 of genes that lack p53 binding sites [e.g., Ryan and Vousden, 1998]. A notable exception is a hybrid protein consisting of the VP16 transactivation domain fused to p53 from aa 100–393; the hybrid is defective for apoptosis mediation but intact for gene repression [Attardi et al., 1996]. We report that, much like VP16-p53, 337C is relatively nontoxic but a potent repressor of the CMV promoter, suggesting that both functions are separable not only in artificial hybrid proteins but in naturally occurring mutants as well.

p53 appears to reside in cells in a latent form. DNA binding by p53 can be activated in vitro by various factors, including the interaction of antibody PAb421 with the C-terminal regulatory domain spanning residues 370–382 [Hupp et al., 1992]. In vivo, p53 may be activated through the acetylation of this domain by histone acetylase p300 [Gu and Roeder, 1997] or through the association, for instance, with regulatory 14–3-3 proteins [Waterman et al., 1998]. Activation by both PAb421 antibody and 14–3-3 proteins is inhibited by the phosphorylation of Ser376 of human p53 [Waterman et al., 1998]. We observed that all the OD mutants 330H, 334V, 335C, and 337C, except p53-ΔO, produced only weak signals in immunoblots with PAb421 and suspected that a fraction of these proteins may be modified, but we were not able to find phosphorylation of Ser376 with phosphopeptide-specific antibody PAb376P. The remarkable fact that p53 with a deleted OD is not refractory to PAb421 binding points to the possibility that the mutational changes, but not lack of the OD induce a conformational change that leads to the loss of the PAb421 epitope in a portion of the proteins. Such effects may also explain in part why mutant 334V competent of hetero-oligomerization can act dominant-negatively on wt p53: alterations of the tertiary structure could conserve the negative regulatory effect of the C-terminal domain and could thereby inhibit the DNA binding ability of the hetero-tetramer.

Recent work has shown that p53 can adopt conformations that confer different susceptibilities to degradation by the calcium-activated protease calpain [Pariat et al., 1997]. This has been exploited to study conformational changes. For instance, calpain digestion patterns of p53 suggest that significant changes in the tertiary structure occur in response to phosphorylation at serines 15 and 37 [Shieh et al., 1997]. A similar approach by us revealed that indeed 330H, 334V, and 337C but not p53-ΔO can give rise to a distinct calpain cleavage pattern and suggests that these mutations are associated with conformational alterations that exist independent of oligomerization. Taken together, the tumor mutants 330H, 334V, and 337C were oligomerization-defective, impaired for PAb421 binding, and structurally altered; by contrast, no correlation among these properties were found with 335C and p53-ΔO.

In summary, 330H, 334V, and 337C are defective for homo-oligomerization. Moreover, 330H and 337C are strongly impaired for DNA binding and transactivation and are able to induce significant apoptosis only at high intracellular protein levels. By contrast, 334V possesses some intermediate DNA binding ability and concomitantly, a substantial ability to transactivate genes and induce cell death. 334V and 337C are able to hetero-oligomerize with wt p53 and thus constitute potential dominant-negative mutants. All tumor-derived mutants showed signs of altered tertiary structure and/or posttranslational modification of the C-terminus. In combination with the large body of previous work one must conclude that different tumor-associated OD-mutants, although all defective for oligomerization, can nevertheless be functionally competent to different degrees. This may reflect that the OD is a multifunctional domain, and that specific mutations may affect functions other than oligomerization.

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