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Oligomerization Is Not Essential for Growth Suppression by p53 in p53-Deficient Osteosarcoma Saos-2 Cells

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The carboxy-terminal portion of the p53 protein contains the tetramerization domain, and the introduction of multiple missense mutations in this domain disrupts the formation of p53 tetramers, resulting in the production of dimeric or monomeric forms of p53. It has recently been shown that a single missense or nonsense mutation in this domain affects the functional properties of p53 both in yeast and in mammalian cells. In this study, we tested the oligomerization of p53 with mutations in the oligomerization domain, when expressed in a human osteosarcoma cell line, Saos-2, *in vivo*. We found that single point mutations, including two missense and two nonsense mutations, in the α-helix of the oligomerization domain disrupted the oligomerization of p53, but that p53 still retained its ability to inhibit colony formation of cells to some degree. These results suggest that oligomerization and the carboxy-terminal basic domain are not prerequisite for p53-dependent tumor suppression, and this may explain why few of the tumor-derived p53 mutations that have been examined so far are carboxy-terminal mutations.

DNA damage in mammalian cells induces the p53 response pathway, resulting in the activation of p53 (1), cell-cycle arrest at the G1/S transition (2, 3) and apoptosis (4, 5). Accumulating evidence suggests that one of the important functions of p53 is transcriptional *trans*-activation, through its sequence-specific DNA-binding activity. This idea is strongly supported by the following facts; (i) mutations of the p53 gene are the most frequent genetic events in diverse tumors (6), (ii) the majority of tumor-derived p53 mutations are missense mutations in the central-core domain of the protein (7, 8), and (iii) loss of the ability to suppress neoplastic growth in representative p53 mutations correlates with the inactivation of both its sequence-specific DNA-binding (9, 10) and *trans*-activation (11-16). Although several genes, including *GADD45* (2), *MDM2* (17, 18), *WAF1/CIP1/SDI1* (19-20), *BAX* (21), *cyclin G* (22) and *IGF-BP3* (23) are *trans*-activated by p53 after DNA damage, through their p53 binding sequences, it is still unclear how the genotoxic signals activate the p53 protein, and how activated p53 orchestrates cell-cycle arrest and apoptosis.

The p53 protein contains three separate domains (24); a transcriptional *trans*-activation domain in the amino (N-) terminal portion, a sequence-specific DNA-binding domain in the central core and an oligo(tetra)merization domain in the carboxy (C-) terminal portion. Recent studies have shown that the sequence-specific DNA-binding domain alone can bind specifically to DNA *in vitro* (25-27), which may explain why the majority of tumor-derived p53 mutations map to the sequence-specific DNA- binding domain. However, it has been also suggested that a discrete sequence outside the DNA-binding domain affects p53's ability to bind DNA. For example, the N-terminus of p53 may negatively regulate sequence-specific DNA-binding interactions in the absence of an intact C-terminus (25), and the C-terminal portion of p53 seems to regulate DNA-binding since modification of this region, by either monoclonal-antibody binding near the C-terminus, C-terminal deletion, or phosphorylation of serine 392 by casein kinase II, can activate p53 DNA binding (28). Therefore, the question remains: why have so few N- and C-terminal domain mutations been found in spite of the involvement of these domains in full p53 function? Lin *et al.* (29) demonstrated that the introduction of a single missense mutation in the N-terminal *trans*-activation domain did not prevent transcriptional activation, which may partly answer the question.

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Stürzbecker *et al.* (30) have previously reported two C-terminal mutations, a monomeric mutant (341K343-E348E355K) which contains four mutations in the α-helix, and a dimeric mutant (365A372L379A386L) which contains four mutations in the C-terminal basic region. We have previously reported that a single missense or nonsense mutation in the p53 oligomerization domain disrupts transcriptional *trans*-activation through the sequence-specific DNA-binding, as well as inducing cell-cycle arrest (31). In this study, mutations were analyzed to clarify whether a single mutation in the C-terminal domain can disrupt p53 oligomerization, and to investigate why few tumor-derived mutations have been identified in the C-terminal domain.

MATERIALS AND METHODS

Cell culture and colony formation assay. The human osteosarcoma Saos-2 cell line which express no endogenous p53 was obtained from American Type Culture Collection, and the cells were grown as described previously (32). Colony formation assay was performed as described previously (31).

Plasmids. Plasmids pCMVp53mut (L344P, K351E, K351stop and Q354stop) were originally derived from a yeast p53 vector generated using a yeast functional assay (31). Each C-terminal mutation contains a single point mutation: CTG to CCG, AAG to GAG, AAG to TAG and CAG to TAG, at codons 344, 351, 351 and 354, resulting in the substitutions leucine to proline (L344P), lysine to glutamic acid (K351E), lysine to Amber (K351stop) and glutamine to Amber (Q354stop), respectively. V143A is a tumor-derived mutation with alanine substituted for valine at codon 143. pCMVp53mut (V143A/K351stop) was constructed by introducing the smaller *Bsu*36I fragment of pCMVmut (K351stop) into the larger *Bsu*36I fragment of pC53-SCX3 (33), and expresses a mutant p53 with two amino-acid substitutions, V143A and K351stop. All the p53 expression vectors used in this study were identical to pC53-SN3 (33), except for the indicated point mutations, and they were expressed under the control of the cytomegalovirus immediate-early promoter.

Immunoblotting and immunoprecipitation. Three to ten micrograms of each p53 expression vector was transiently transfected into the Saos-2 cells by the calcium-phosphate precipitation method using the Profection Mammalian Transfection Systems (Promega). After 36 h the cells were washed with PBS(−) and the proteins were extracted with DOC buffer (34) containing 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate and proteinase inhibitors. To detect p53 protein, cell lysates were subjected to SDS/PAGE (10%) followed by immunoblot analysis using the monoclonal antibody pAb1801 (Oncogene Science), as described previously (31). The cell lysates were also subjected to immunoprecipitation with the monoclonal antibody pAb421 (Oncogene Science) or pAb1801 using a previously described method (35). The immunoprecipitated proteins were separated by SDS/PAGE (10% polyacrylamide), followed by immunoblotting analysis using rabbit anti-human-p53 serum (Chemicon International Inc.). p53 proteins were visualized by horseradish peroxidase labeled sheep anti-rabbit antibody using an enhanced chemiluminescence technique (ECL kit, Amersham).

Chemical cross-linking analysis. Ten micrograms of each cell lysate was incubated in the absence or presence of 0.01-0.1% glutaraldehyde (GA) for 20 min at room temperature, before being boiled in a sample loading buffer containing 1% SDS. The reaction mixtures were separated by SDS/PAGE (with a 5-15% polyacrylamide gradient) and were visualized by immunoblotting using pAb1801.

RESULTS AND DISCUSSION

Mutations in the α-Helix of the Oligomerization Domain of p53

Recently, the three-dimensional structure of the tetramerization domain (residues 319 to 360) of human p53 was solved by a multidimensional nuclear magnetic resonance study (36) and the crystal structure of the domain (residues 325 to 356) has also been determined (37). These studies, and an analysis of the predicted secondary structure (30), have shown that the tetramerization domain contains a β-strand from residue 326 to 333, and an α-helix from residue 335 to 354, and a basic region from residue 363 to 386. Fig. 1 shows a schematic representation of the p53 constructs used in this study and their expression in human osteosarcoma Saos-2 cells. Each mutation is a missense (L344P, K351E) or nonsense mutation (K351stop, Q354stop) within the α-helix (Fig. 1A) and was previously generated by random mutagenesis and analyzed in both yeast and mammalian cell assays (31). Note that the levels of p53 expression from both the wild-type p53 and the K351stop vectors, were lower than those of the other constructs, and that the reduced expression was suppressed by the introduction of the V143A (Fig. 1B). One possible explanation for this observation is that the expression of these clones in Saos-2 cells reduces cell viability by a mechanism such as apoptosis, although we did not pursue this issue further.

Effects of Mutations within the α-Helix on the Homo-oligomerization of the Mutant p53 in Vivo

Immunopurified p53 proteins, including human, murine and *X enopus* p53, have been shown by cross-linking analysis to exist as tetramers (38-40). In this study, using similar conditions, human p53 was expressed in p53-null human osteosarcoma Saos-2 cells before being incubated with 0.01% to 0.05% glutaraldehyde (GA), and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS/PAGE) with a 5-15% polyacrylamide gradient. The p53 protein was then visualized by immunoblotting using the monoclonal antibody pAb1801 (Fig. 2A). When incubated with 0.01% GA, wild-type p53 took two forms, the monomer, and a form which migrated between the 83 kD and 137 kD markers (lane 2), suggesting it was a dimer. At the higher GA concentration (0.05%), larger forms of p53, which migrated more slowly than the 137 kD marker, were observed in addition to the monomer and dimer forms (lane 3). Although these larger forms did not appear as sharp bands in the gel, it is likely that they were p53 oligomers, including tetramers which migrated near the 207 kD marker. We repeated this procedure using lysates of cells that expressed p53 mutants, as indicated in Fig. 1. All of the p53 mutants showed a markedly reduced ability to form dimers in the presence of

0.01% GA (lanes 5, 8, 14 and 17). However, both K351E and Q354stop preferentially formed dimers in the presence of 0.05% GA with no larger oligomers being formed (lanes 8, 9, 17 and 18), whereas L344P existed entirely as a monomer (lane 15). Surprisingly, K351stop, which is three amino acids shorter than Q354stop, formed both dimers and larger oligomers including tetramers, although it was less efficient than wild-type p53 (lanes 5 and 6). We also analyzed the V143A and V143A/K351stop, containing a tumor-derived mutation which should denature the p53 sequence-specific DNA-binding domain (41), in the presence of 0.01-0.1% GA. As shown in Fig. 2B, the introduction of the V143A mutation into wild-type and K351stop vectors, did not affect oligomerization. These results indicate that: (i) the cross-linking of wild-type p53 monomers to form dimers, and of dimers to form tetramers is mediated by the C-terminal oligomerization domain as previously reported (30), (ii) single point mutations in the α-helix of the oligomerization domain can disrupt normal oligomer formation, and (iii) a tumor-derived mutation in the sequence-specific DNA-binding domain does not affect oligomerization.

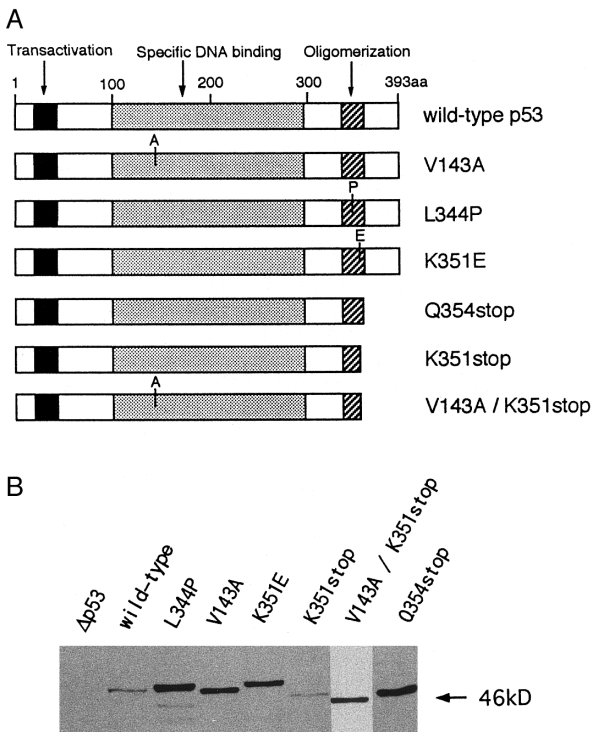

FIG. 1. (A) Schematic representation of the predicted structures of the p53 proteins used in this study. Each C-terminal mutation contains a single point mutation at codons 344,351, 351 or 354, resulting in the substitutions leucine to proline (L344P), lysine to glutamic acid (K351E), lysine to Amber (K351stop) or glutamine to Amber (Q354stop), respectively. V143A is a tumor-derived mutation with alanine substituted for valine at codon 143. V143A/K351stop contains two mutations, V143A and K351stop. Filled boxes represent transcriptional *trans*-activation domain (67); stippled boxes represent sequence-specific DNA-binding domain (25-27); boxes with diagonal lines represent oligomerization domain (26, 30, 68). (B) Expression of p53 proteins. Five micrograms of p53 expression vector, pC53-SN3 (wild-type), pC53-SCX3 (V143A) or pCMVp53mut (L344P, K351E, K351stop, Q354stop, or V143A/K351stop) was transiently transfected into the Saos-2 cells. As a negative control, a vector lacking the p53 sequence was used (Δp53). To detect p53 protein, the cell lysates were subjected to SDS/PAGE (10%) followed by immunoblot analysis using the monoclonal antibody pAb1801. *Note.* The two mutant cDNA clones, V143A and V143A/K351stop, include a polymorphism at codon 72 for an amino-acid substitution of proline to arginine, and migrate slightly faster than wild-type and K351stop, respectively (69).

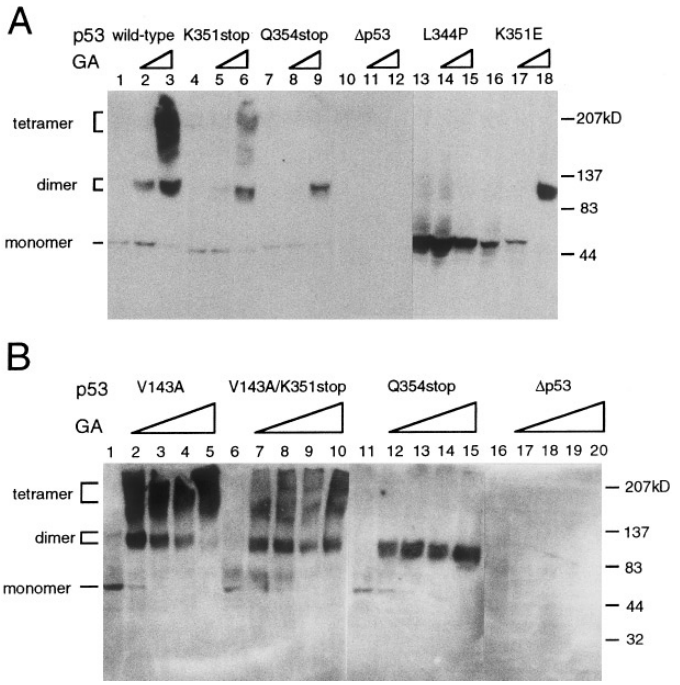

FIG. 2. Oligomerization of mutant p53 proteins *in vivo.* (A) Saos-2 cells were transiently transfected with the indicated p53 constructs (wild-type, lanes 1, 2 and 3; K351stop, lanes 4, 5 and 6; Q354stop, lanes 7, 8 and 9; Δp53, lanes 10, 11 and 12; L344P, lanes 13, 14 and 15; K351E, lanes 16, 17 and 18). Each lysate was incubated in the absence (lanes 1, 4, 7, 10, 13 and 16) or presence of 0.01% (lanes 2, 5, 8, 11, 14 and 17) or 0.05% (lanes 3, 6, 9, 12, 15 and 18) glutaraldehyde (GA) for 20 min at room temperature, before being boiled in a sample loading buffer containing 1% SDS. The reaction mixtures were separated by SDS/PAGE (with a 5-15% polyacrylamide gradient) and were visualized by immunoblotting using pAb1801. (B) Similar experiments were performed using the indicated p53 constructs (V143A, lanes 1, 2, 3, 4 and 5; V143A/K351stop, lanes 6, 7, 8, 9 and 10; Q354stop, lanes 11, 12, 13, 14 and 15;Δp53, lanes 16, 17, 18, 19 and 20) in the absence (lanes 1, 6, 11 and 16) or presence of 0.01% (lanes 2, 7, 12 and 17), 0.025% (lanes 3, 8, 13 and 18), 0.05% (lanes 4, 9, 14 and 19) or 0.1% (lanes 5, 10, 15 and 20) GA.

Hetero-oligomer Formation between Full-Length p53 and Truncated p53

Hetero-oligomers of p53 have been previously reported to form between human wild-type and tumor-

derived mutant p53, and between human and murine p53, when coexpressed in cells and *in vitro* (30, 39, 42, 43). To examine whether two nonsense mutations near the tail of the α-helix, K351stop and Q354stop, interfere with the ability of the mutant p53 to oligomerize with full-length p53, Saos-2 cell lysates expressing p53 were coimmunoprecipitated using the monoclonal antibody pAb421, which recognizes residues 372 to 381 of p53, and then the p53 was visualized by immunoblotting using a rabbit anti-human p53 serum. The results were shown in Fig. 3. Wild-type and mutant V143A p53, both of which express full-length p53, were immunoprecipitated by pAb421 whereas two truncated mutant forms, K351stop and Q354stop, were not immunoprecipitated because they lack the epitope against pAb421. When wild-type p53 was cotransfected with Q354stop or K351stop, it was efficiently coimmunoprecipitated with Q354stop but not with K351stop. Similar results were obtained when V143A p53 was cotransfected with Q354stop or K351stop. Furthermore, when wild-type p53 was coexpressed with Q354stop, the level of wild-type p53 protein increased to the level of Q354stop, and when V143A was coexpressed with K351stop, the level of V143A protein was reduced by the level of wild-type p53. These results suggest possible dominant effects of Q354stop against wild-type p53, and of K351stop against V143A, on p53 protein levels in Saos-2 cells although the mechanism

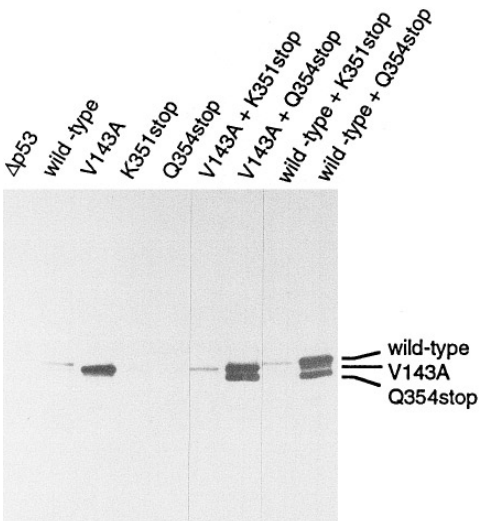

FIG. 3. Hetero-oligomer formation between full-length p53 and truncated p53 *in vivo*. Cell lysates transfected with 10 μg of Δp53 alone or cotransfected with 5 μg each of Δp53 and wild-type p53 (wild-type), Δp53 and V143A (V143A), Δp53 and K351stop (K351stop), Δp53 and Q354stop (Q354stop), V143A and K351stop (V143A+K351stop), V143A and Q354stop (V143A+Q354stop), wild-type p53 and K351stop (wild-type+K351stop) or wild-type p53 and Q354stop (wild-type+Q354stop), were immunoprecipitated with the monoclonal antibody pAb421. The immunoprecipitated proteins were separated by SDS/PAGE (10% polyacrylamide), followed by immunoblotting analysis using rabbit anti-human-p53 serum.

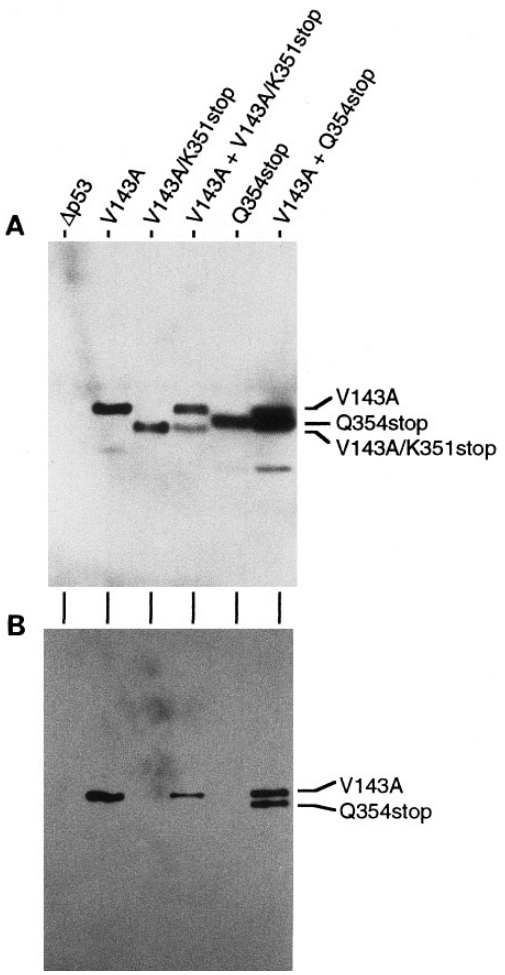

FIG. 4. Hetero-oligomer formation between V143A and V143A/K351stop or Q354stop *in vivo*. Cell lysates transfected with the indicated p53 constructs were immunoprecipitated with the monoclonal antibody pAb1801 (A) or pAb421 (B) and were immunoblotted using rabbit anti-human-p53 serum.

controling p53 protein levels is not known. The steady-state level of the K351stop protein is relatively low (Fig. 1B). Therefore, to determine if that was why K351stop could not be coimmunoprecipitated, a V143A/K351stop vector, which does not inhibit colony formation and therefore expresses a much higher level of p53 protein than the K351stop vector, was tested by immunoprecipitation with full-length V143A protein. As shown in Fig. 4, V143A/K351stop did not interact with full-length p53, whereas Q354stop did. These results indicate that this 43 amino acid deletion from the C-terminus of p53 markedly reduced the ability to interact with the p53 protein with the normal C-terminus, whereas the 40 amino acid deletion retained it. This observation supports a previous report that deletion of 42, but not of 30 residues, from the C-terminal end abolishes hetero-oligomer formation with full-length p53, from vectors co-translated *in vitro* (44).

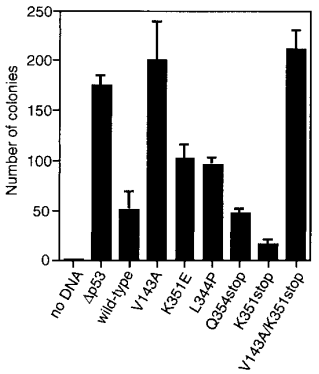

FIG. 5. Effect of the expression of wild-type p53 or C-terminal mutant proteins on colony formation by Saos-2 cells. The indicated p53 expression vectors (3 μg) were transfected into Saos-2 cells and then selected by Geneticin (500 μg/ml) for three weeks. The selected colonies were stained and the numbers were counted using a method described previously (45). The results from three independent experiments are expressed as mean values + the standard deviation.

Inhibition of Colony Formation by p53 Oligomerization Domain Mutants

The p53 clones were tested for their ability to inhibit Saos-2 cell growth when selected by Geneticin (G418). As shown in Fig. 5, the expression of wild-type p53 in Saos-2 cells inhibited colony formation (28% as compared to a negative control vector lacking the p53 sequence Δp53), whereas the expression of a tumor-derived p53 mutation, V143A, failed to do so (114%), which is consistent with previous reports (32, 45, 46). When p53 clones mutated in the oligomerization domain were expressed, each mutation inhibited colony formation to some extent. The dimeric mutant Q354stop, which has full *trans*-activation activity and arrests cells at G1/S, inhibited colony formation to near wild-type levels (27%). Another dimeric mutant, K351E, which has partial effects on both *trans*-activation and cell-cycle arrest, significantly inhibited colony formation, but less efficiently (58%). Interestingly, the monomer-mutant L344P, which has no ability for both *trans*-activation and cell-cycle arrest, showed partial colony formation inhibitory ability (55%). Furthermore, K351stop, which has a limited effect on *trans*-activation and does not cause cell-cycle arrest, showed strong inhibition of colony growth (9.7%). These results indicate that mutations in the α-helix of the oligomerization domain do not abolish the growth suppression ability of p53.

We have characterized the oligomerization of human p53 clones with mutations localized in the putative α-helix of the oligomerization domain. K351E and Q354stop predominantly formed dimers when expressed in p53-null Saos-2 cells. L344P predominantly remained as monomers, and K351stop retained the ability to self-oligomerize, although it was less efficient than wild-type p53. Since none of the mutations completely abolished p53-dependent inhibition of colony formation, it seems that oligomerization of p53 is not necessary for tumor suppression by p53. In particular, K351stop and Q354stop lacked the C-terminal end (containing the basic region), yet they still inhibited colony formation, suggesting that the C-terminal basic region of p53 is not required for p53-dependent tumor suppression. This observation is consistent with previous reports (47, 48). These results may explain why few mutations have been found in the C-terminal portion of p53; from a database of 2572 p53 mutations (8), only 23 mutations map to the C-terminal portion between residues 326 and 393, including nine nonsense mutations at codon 342, nine frameshift mutations and four missense mutations, G334V, R337C, E349D and G356W. It is therefore very interesting to examine the functional defects of these tumor-derived p53 mutations.

Overall, the results described above, together with our previous data (31), strongly suggest that p53-dependent tumor-suppression occurs via an unknown mechanism that is independent of *trans*-activation and G1/S cell-cycle arrest, although we have only tested *trans*-activation activity through on artificial promoter containing the RGC sequence, rather than through a natural p53-responsive promoter such as the *WAF1/CIP1/SDI1* and *BAX* promoters. This hypothesis is supported by recent studies: (i) the N-terminal *trans*-activation domain of p53 is not required for the suppression of transformation by E1A and activation *ras* (49, 50), (ii) transcriptional activation of p53 does not always correlate with transformation suppression (51, 52), (iii) p53-mediated apoptosis can occur in the absence of transcriptional activation of p53-target genes (53-55) and (iv) knockouts of *WAF1/CIP1/SDI1* or *BAX*, genes that are *trans*-activated by p53, does not disrupt p53-dependent apoptosis in normal cells (56, 57). We are currently examining whether the *trans*-activation-independent tumor suppression observed in this study is an outcome of apoptosis.

Accumulating evidence indicates other functional properties of the p53 C-terminus that are related to DNA repair, replication, transcription and translation. The C-terminal domain alone can repress p53-dependent *trans*-activation (58). In addition, the C-terminus can recognize short DNA structures such as DNA ends, short single-stranded DNA and single-stranded gaps, suggesting that p53 itself may recognize damaged DNA structures (59-61). The C-terminus also contains residues that can bind RNA and promote self-annealing of RNA (62), and the ability to bind RNA may be important for translational regulation of p53 (63). Furthermore, the C-terminus can interact with ERCC3, XPB and XPD, which are components of TFIIH (64, 65), as well as with replication protein A (66). It is therefore very interesting to examine whether the C-

terminal mutations used in this study alter these functional properties of p53.

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