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COOH-Terminal Domain of p53 Modulates p53-mediated Transcriptional Transactivation, Cell Growth, and Apoptosis[1]

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ABSTRACT

The tumor suppressor protein p53 contributes to the control of cell cycle checkpoints and stress-induced apoptosis and is frequently mutated in many different types of human cancers. The COOH terminus of p53 modulates the transcriptional and apoptotic activities of the protein. Although COOH-terminal mutants of p53 are uncommon, we proposed that these p53 mutants nevertheless contributed to the selective clonal expansion of the cancer cells. Therefore, we analyzed the tumor-derived p53 COOH-terminal domain (CTD) mutants (352D/H, 356G/W, 342-stop, 360-del, and 387-del) functionally. The results have revealed that all mutants have impaired apoptotic activity when compared with wild-type p53. However, some of these mutants still transcriptionally transactivate *p21$^{Waf1/Cip1}$* and inhibit cell growth. Interestingly, of the tumor-derived CTD mutants, oligomerization-defective mutant 342-stop was the only one that did not exhibit sequence-specific DNA binding or failed to transactivate *p21$^{Waf1/Cip1}$*, *Bax*, and *IGF-BP3* transcriptionally. The failure to inhibit cell growth by this tumor-derived CTD mutant supports the hypothesis that p53 sequence-specific transcriptional transactivity to *p21$^{Waf1/Cip1}$* is correlated with induction of cell cycle arrest and that the p53 transcriptional transactivity requires oligomerization of the p53 protein. These and other data indicate that the CTD of p53 is an important component of p53-mediated apoptosis and cell growth arrest and that inactivation of the apoptotic function, but not the inhibition of growth, is an important step during human tumorigenesis.

INTRODUCTION

Two major functions of p53 are the negative regulation of cell growth and the induction of apoptosis. Cells lacking functional p53 fail to arrest in G_1 following γ-irradiation (1), whereas introduction of wild-type p53 leads to G_1 arrest in either p53-null or p53-mutated cells (2–4). Growth arrest is controlled principally by transcriptional modulation of p53-transactivated genes, such as *p21$^{Waf1/Cip1}$* (an inhibitor of cyclin-dependent-kinase) and *GADD45* (a growth arrest and DNA damage responsive gene; Refs. 5–7). Apoptosis can be triggered by overexpression of transfected wild-type p53 in p53-null tumor cells (8). Thymocytes derived from p53 knock-out (p53 −/−) mice are resistant to radiation- and drug-induced cell death (9). Some tumor-derived p53 mutants display defects in apoptotic activity and still retain growth suppression (10). These data indicate that the distinct functions of p53 are mediated via specific pathways.

p53 has multiple, unique, functional domains. These include the NH_2-terminal transactivation domain, the sequence-specific DNA-binding domain that is necessary for the transcription transactivation, and the CTD.[3] The CTD of p53 has drawn considerable attention in recent years based on its multiple functions (11–16). The COOH terminus of p53, comprising amino acids 311–393, can be subdivided into at least two structural determinants: the oligomerization domain (amino acids 319–360) and an adjacent basic region (amino acids 363–393), which also has been referred to as an apoptotic domain (17); a transcriptional regulatory domain (18); or a DNA damage recognition domain (14, 19). The oligomerization domain is required for the p53 tertiary structure, which regulates transcriptional transactivity by altering the conformation of the protein (20, 21). Modifications of the negative transcriptional regulatory domain of p53 CTD by phosphorylation, acetylation, deletion, or antibody binding have been shown to play important roles in the regulation of p53 sequence-specific binding (12, 16, 22–25). Wild-type p53 can transactivate genes transcriptionally through binding to specific DNA sequences in the promoter regions of genes involved in cell cycle control and apoptosis, *e.g.*, *p21$^{Waf1/Cip1}$*, *Bax*, and *IGF-BP3* (5, 26, 27). The COOH terminus seems to exert a negative effect on the DNA-binding activity of the core p53 domain (22, 28). Deletion of the last 30 amino acids was shown to abolish this repressor activity (22, 28). Furthermore, it was found that an alternative COOH-terminal spliced p53 exhibited an enhanced DNA-binding activity (29).

p53 mediates apoptosis by both transcription-dependent and transcription-independent pathways (7, 17, 26, 30–32). The contribution of sequence-specific transcriptional activation to the induction of apoptosis is less clear than the induction of cell cycle arrest. The CTD is involved in the apoptotic function of p53 by protein-protein interactions (17). The CTD of p53 can bind directly to XPB and XPD, inducing apoptosis (17, 33). The hepatitis B virus X protein binds to CTD and inhibits p53-mediated apoptosis (34). The CTD of p53 can bind single strands of DNA and DNA ends that are likely to be present in irradiated cells, implicating that CTD is responsible for sensing these types of DNA damage (17, 35).

We have reported previously that overexpression of wild-type p53 in primary human fibroblasts induced apoptosis and that deletion of the last 40 amino acids at the CTD abolished p53-mediated apoptosis completely (17). These data indicate that the CTD may contribute directly to apoptosis. Analysis of the p53 mutation spectrum (36, 37) indicates that ~5–15% of the p53 mutations occur in the CTD during human tumorigenesis. Most of the somatic mutations in the p53 CTD are either nonsense mutations or lead to frameshifts, resulting in a truncated protein (Fig. 1*A*). To further examine the biological functions of the p53 CTD, we investigated tumor-derived p53 CTD mutants that were presumably selected during the clonal evolution of human cancers. The results indicate that the CTD of p53 regulates transcriptional activity and sequence-specific DNA binding and modulates the apoptotic process. The CTD of p53 also is involved in G_1 arrest and cell growth inhibition, is closely correlated with transcriptional activation of *p21$^{Waf1/Cip1}$*, and can be independent of p53-mediated apoptosis.

MATERIALS AND METHODS

p53 Mutagenesis and Vectors. Five tumor-derived p53 CTD mutants: 352D/H, 356G/W, 342-stop (342R/Stop), 360-del (360 1-bp deletion), and 387-del (387 19-bp deletion; Refs. 38–42) were constructed by site-directed mutagenesis. The mutations were verified by DNA sequencing. Wild-type p53

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[3] The abbreviations used are: CTD, COOH-terminal domain; LFS, Li-Fraumeni syndrome; EMSA, electrophoretic mobility shift assay; TFIID, transcription factor IID.

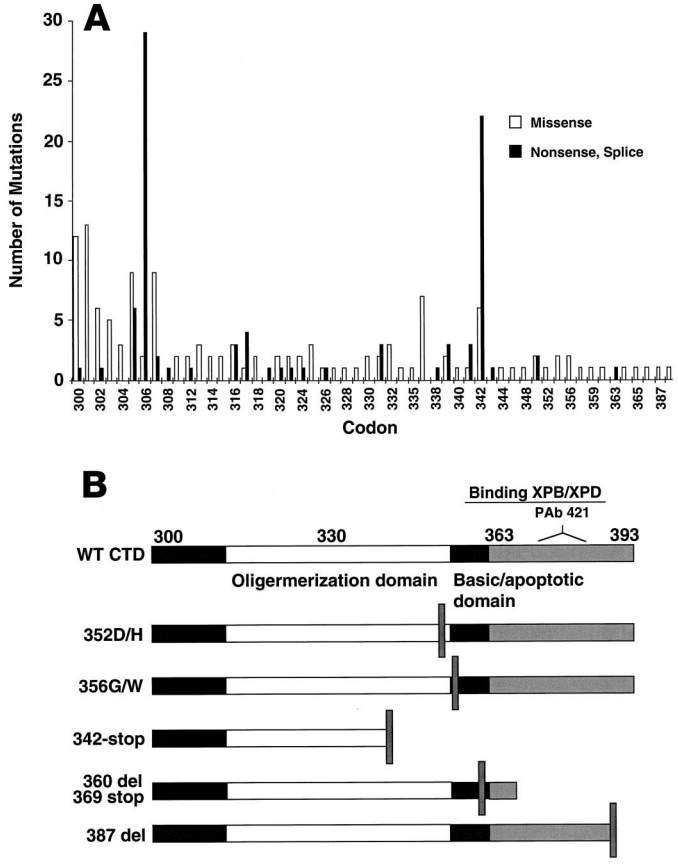

Fig. 1. Mutational spectrum found in p53 CTD (*A*) and p53 CTD and tumor-derived p53 CTD mutants used in this study (*B*). p53 CTD can be subdivided into three regions: flexible linker (residues 300–320), oligomerization domain (320–360), and basic domain (363–393). Mutant *352D/H* was derived from an esophageal squamous cell carcinoma, *356G/W* from an endometrial carcinoma of uterus, and *387-del* from a bladder carcinoma. The *342-stop* mutation occurred in several different cancer types. A 1-bp deletion at 360 was found in hereditary, nonpolyposis colorectal cancer, which created a new stop codon at residue 369, altered seven amino acids, and truncated the last 25 amino acids.

and the CTD mutants were inserted into the pcDNA3 vector under the control of the cytomegalovirus early promoter.

Cell Culture, Microinjection, and Immunocytochemistry Analysis. Primary human fibroblasts and an immortalized p53-null LFS fibroblast cell line (041) derived from the skin biopsies of a patient with LFS were grown in Ham's F10 medium supplemented with 10% fetal bovine serum. Cells were seeded onto coverslips and incubated for 1–2 days prior to microinjection. pcDNA3 vectors containing wild-type p53, the CTD mutants, or *LacZ* as a negative control at a concentration of 200 ng/μl, were microinjected into the nuclei of cells, using a microcapillary glass needle. For each experiment, at least 100 cells were injected, and a typical experiment yields at least 50 positive cells for analysis. After incubation for 24 h, cells were fixed with 4% paraformaldehyde followed by methanol treatment. p53 was visualized by staining the cells with the anti-p53 polyclonal CM-1 antibody (Signet Labs, Dedham, MA), and followed by fluorescein-conjugated antirabbit IgG (Vector Labs, Burlingame, CA) as described previously (17). Nuclei were stained with 4′, 6-diamidino-2-phenylindole. Apoptotic cells that were p53-positive were scored by their smaller size, condensed chromatin, and fragmented nuclei, as compared with control cells.

Cell Culture and Reporter Gene Assay. LFS fibroblasts cultured in 12-well plates at 60% confluence were transfected transiently with 1 μg of pcDNA3 p53 expression vectors, using Lipofectin (Life Technologies, Gaithersburg, MD). The p53 transcriptional transactivities were examined with the Dual-Luciferase Reporter system (Promega, Madison, WI) by cotransfecting 0.025 μg of Renilla Luc vector SV 40 (as an internal control) and 0.5 μg of p53-responsive reporter vectors, WWP-Luc-p21, PGL3-Luc-Bax, or pUHC13–3-Luc-IGF BP3-BOX B (26, 43, 44). Five h after transfection, the medium was changed. After an additional 15 h of incubation, the cells were lysed and the lysates were collected for the activity measurement. The activity of luciferase was quantified with a Luminometer (Analytical Luminescence Lab., Ann Arbor, MI).

To evaluate the expression level of wild-type and CTD-mutated p53 after transfection, the cells were lysed in RIPA buffer [150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0)]. Forty μg of total cellular protein were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a purified, biotinylated mouse monoclonal antibody that recognizes the p53 protein (Oncogene Research Products, Cambridge, MA).

Expression of the p53 Protein Constructs and EMSA. To generate p53 proteins for EMSA, 1 μg of each of the pcDNA3-p53 constructs was used for the *in vitro* transcription-translation assay, according to the procedure described by the Promega manufacturer for the TNT T7 Quick Coupled Transcription/Translation System (Promega). The DNA-binding activities of the wild-type and the CTD-mutated p53 proteins were analyzed by EMSA. The consensus sequence oligonucleotide, TCGAGAGGCATGTCTAGGCATGTCTC (44) was synthesized, prepared in double-stranded form, and end-radiolabeled with ^{32}P-ATP. Four μl of the translated protein products were mixed with 20 fmol of radiolabeled DNA, 1 μl of PAb-421 ascitic fluid (when indicated; +), 2 μg (2 μl) of poly dI-dC as nonspecific DNA, and one-half of the reaction volume of buffer (25 mM Tris-HCl, 100 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol). The reactions were incubated for 15 min on ice and for an additional 15 min at room temperature. The samples were analyzed in 4% polyacrylamide gels electrophoresed at 350 V in 0.5 Tris-borate-EDTA buffer at 4°C.

Colony Formation Assays. Both p53-null LFS fibroblast cells (041) and Hep 3B cells were cultured in EMEM medium supplemented with 10% fetal bovine serum. Five μg of pcDNA3 containing wild-type p53 or the CTD mutants were transfected into the cells at 60% confluence in 60-mm dishes. Forty-eight h after transfection, G418 was added for the selection. The cells were maintained in the G418-containing medium for 10–14 days, and the colonies were counted. The experiments were repeated at least three times. The significant difference (*P*) was tested by the Student's *t* test.

RESULTS

Induction of Apoptosis by p53 CTD Mutants. To test the biological significance of the CTD mutations selected during tumorigenesis, we investigated five tumor-derived p53 CTD mutants (Fig. 1*B*), including a mutation hotspot, 342-stop. These nonsense, missense, deletion, or frameshift mutations occurred in either the oligomerization domain or the apoptotic domain of CTD.

Consistent with our previous data, the wild-type p53 induced apoptosis in ~20% of the p53-positive cells in both primary human

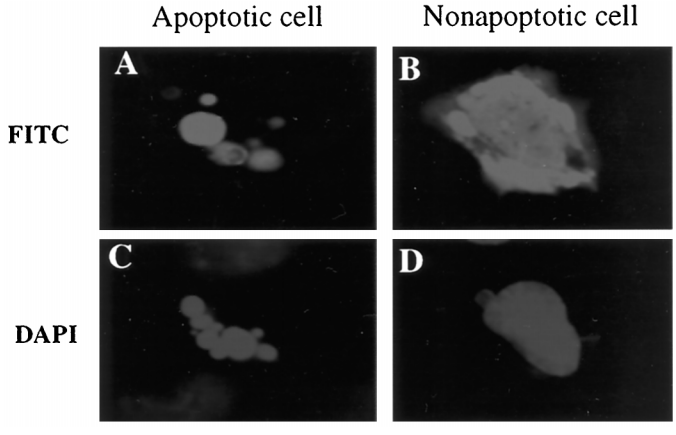

Fig. 2. Induction of apoptosis in LFS fibroblasts (p53 null) by WT-p53. p53 protein was stained with CM-1 antibody (*FITC*), and nuclei were stained with 4′,6-diamidino-2-phenylindole (*DAPI*). Apoptotic cells showed a smaller size, condensed nuclei, and chromatin when compared with the nonapoptotic cells.

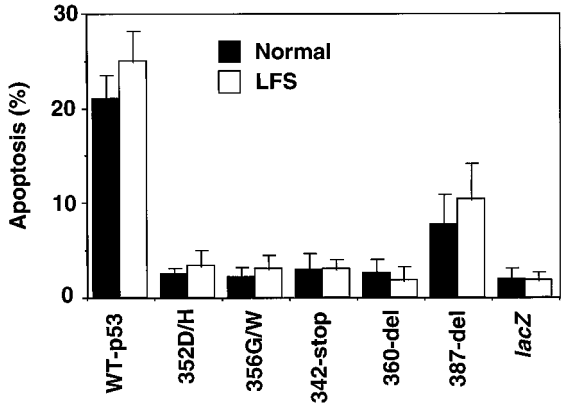

Fig. 3. Induction of apoptosis by wild-type p53 (*WT-p53*) and tumor-derived p53 CTD mutants. Apoptotic cells among p53-positive cells were scored by the morphological characteristics 24 h after microinjection. All data are an average of three independent experiments, and a total of ~200 cells were examined. *Bars*, SD.

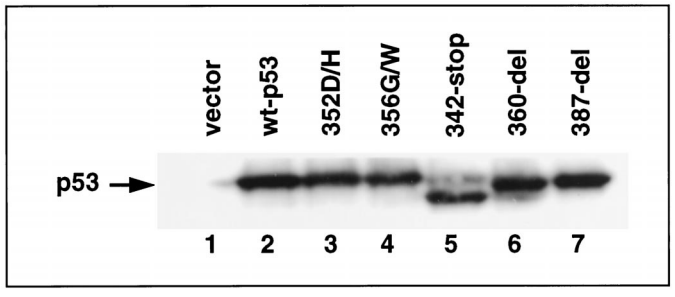

Fig. 4. Western blot of extracts from p53-null LFS fibroblasts transfected with wild-type (*wt-p53*) and CTD-mutated p53. The similar levels of p53 expression are shown. Truncated protein representing the *342-stop* mutant can be seen by smaller size.

fibroblast cells and LFS fibroblast cells (p53 null), 24 h after microinjection (Figs. 2 and 3). The cells that were microinjected successfully with either wild-type p53 or the CTD mutants showed expression of p53 by antibody CM-1 staining (Fig. 2, *A* and *B*). p53 localized mainly in both the nucleus and cytoplasm. No significant alterations of localization between wild-type p53 and CTD mutants were observed. p53 immune-positive and apoptotic cells showed fragmented nuclei and condensed chromatin (Fig. 2*C*). Interestingly, most of the p53 CTD mutants have a significant reduction in apoptotic activity that was similar to the background rate induced after microinjection of a *LacZ* expression vector (Fig. 3). The p53 CTD mutant 387-del displayed a slight reduction in apoptotic rate.

Transcriptional Transactivation Activity of p53 CTD Mutants. p53 is a DNA-binding-dependent transcriptional transactivator, and the p53 CTD is a regulator of sequence-specific DNA binding (12, 15, 22, 23, 26, 27). To test the transcriptional transactivation activity of the tumor-derived p53 CTD mutants, the expression vectors encoding wild-type p53 or the CTD mutants were transfected into p53-null LFS fibroblasts along with reporter genes containing promoter elements from genes transcriptionally regulated by p53. In the transient transfection assay, the protein expression levels of the CTD mutants were similar to that of wild-type p53 (Fig. 4). However, the effects on transcription of the reporter genes was variable. The mutant 342-stop showed an attenuated transcriptional activation of the $p21^{Waf1/Cip1}$ promoter, but the other tumor-derived p53 CTD mutants activated transcription similar to wild-type p53 (Fig. 5*A*). All of the tumor-derived p53 CTD mutants demonstrated a modest attenuated activation of the *Bax* and *IGF-BP3* promoters (Fig. 5, *B* and *C*). The mutant 342-stop showed no detectable transcriptional transactivity of these genes relative to the vector control (Fig. 5, *B* and *C*).

DNA Binding Affinity of p53 CTD Mutants. p53 sequence-specific DNA binding is required for its transcription transactivation function (26, 45). To evaluate the specific DNA-binding activity of the various COOH-terminally modified p53 mutants, the binding of p53 CTD mutant proteins to p53 consensus sites was determined by EMSA. *In vitro* translated p53 proteins are shown in Fig. 6*A*. As expected, the *in vitro* translated wild-type p53 protein does not bind to DNA spontaneously; however, this activity is enhanced by the anti-p53 antibody PAb-421 (Fig. 6*B*). The p53 CTD mutant 342-stop did not bind the p53 responsive element either in the absence or presence of PAb 421 (Fig. 6*B*). In contrast to the 342-stop, the mutant 360-del has spontaneous DNA binding activity. Neither the 352D/H mutant nor 356G/W affected the DNA binding activity of those proteins (Fig. 6*B* and Table 1).

Inhibition of Cell Growth by Wild-Type p53 and the CTD Mutants. The inhibition of cell growth and induction of cell cycle arrest by p53 depend on p53-mediated transcriptional activation (46). Transcriptional activation of $p21^{Waf1/Cip1}$ appears to contribute to cell growth inhibition (5). The 342-stop mutant, which showed no transcriptional activation from the $p21^{Waf1/Cip1}$ promoter (Fig. 5*A*), lacked the growth inhibition function in both LFS fibroblast and Hep 3B cells, whereas the other mutants retained growth suppressor activity similar to wild-type p53 in both cell

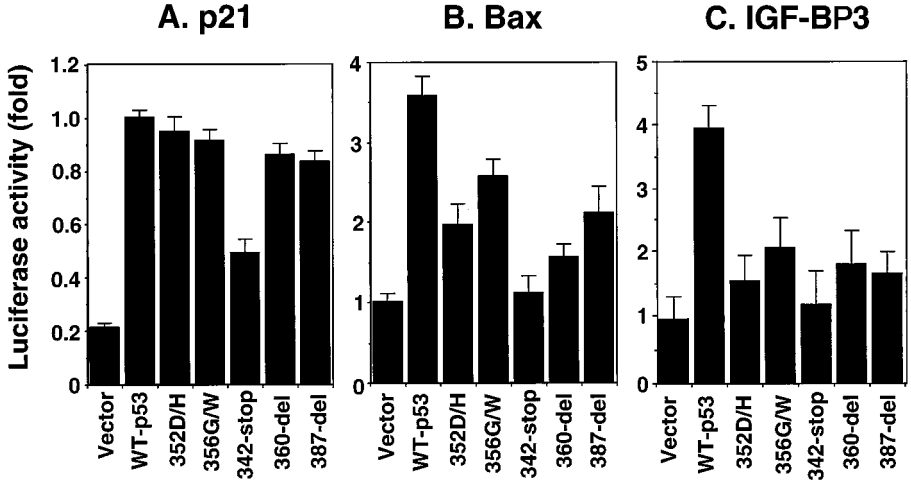

Fig. 5. Sequence-specific transcriptional activation by wild-type p53 (*WT-p53*) and the CTD mutants in LFS fibroblasts. Activities of the *p21* promoter WWP-Luc-p21 (*A*), *Bax* promoter PGL3-Luc-Bax (*B*), and *IGF-BP3* promoter pUHC13–3-Luc-IGF BP3-Box B (*C*) were detected by cotransfection of the p53 plasmid. Results represent the mean of three independent experiments; *bars*, SD.

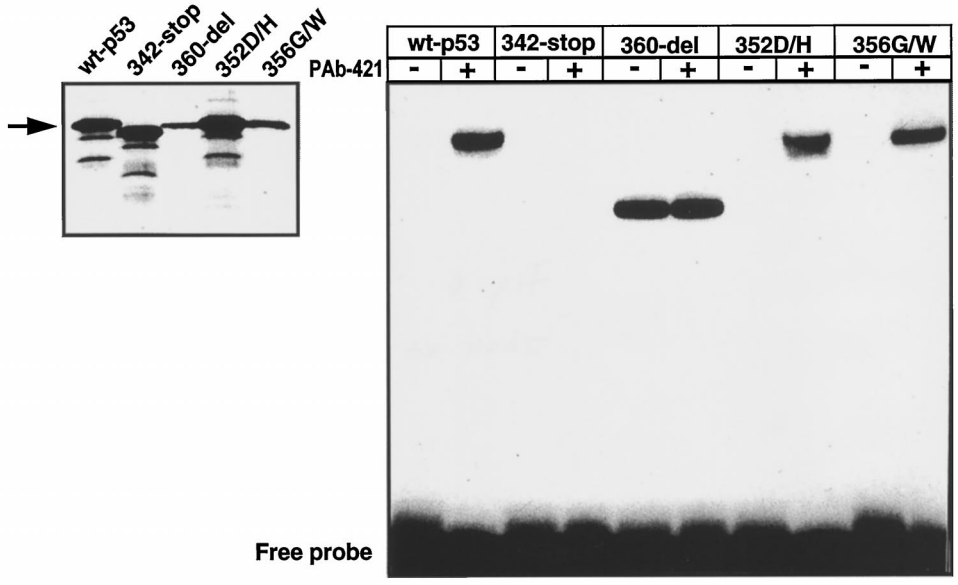

Fig. 6. *A*, p53 proteins translated *in vitro*. The different ^{35}S-methionine-labeled proteins were run in a SDS-PAGE. The *arrow* points to the p53 expected proteins. *B*, sequence-specific DNA binding affinity of *in vitro*-translated wild-type p53 (*wt-p53*) and CTD mutants. DNA-binding activities of *in vitro*-translated p53 proteins are enhanced by the *PAb-421* antibody. Oligonucleotides containing the p53 consensus binding sequence was used. *342-stop* is not able to bind the p53 responsive element, and *360-del* shows spontaneous bingeing activity with or without PAb-421 antibody.

types (Fig. 7). The effects of the tumor-derived p53 CTD mutations on various biological functions measured are summarized in Table 1. All of the tumor-derived p53 CTD mutants were defective in the induction of apoptosis. The p53 CTD mutant 342-stop with no inhibition on the cell growth showed neither transcriptional activation on target genes tested nor sequence-specific DNA binding. Each of the p53 CTD mutants 352D/H, 356G/W, 360-del, and 387-del bound to the p53 consensus sequence and retained most of its transcriptional transactivity. The p53 CTD mutant 360-del activated the sequence-specific binding to the $p21^{Waf1/Cip1}$ (data not shown) and p53 consensus binding site PG13.

DISCUSSION

p53 responds to cellular stress and maintains genomic stability (1, 47–50). However, this multifunctional property of p53 makes it difficult to determine the precise mechanism(s) by which p53 functions as a tumor suppressor. p53 may also induce apoptosis by transactivating proapoptotic genes such as *Bax* and *IGF-BP3* (26, 27) and by transrepressing antiapoptotic genes such as *bcl-2* (51). IGF-BP3 antagonizes the activity of insulin-like growth factor 1, and a reduction in the level of the insulin-like growth factor 1 type II receptor has been shown to result in apoptosis (27). In our study, the tumor-derived p53 CTD mutants have diminished apoptotic activity and reduced transcriptional transactivity of *Bax* and *IGF-BP3*, suggesting the interactive effects of p53 transcription-dependent and transcription-independent apoptotic pathways in normal and LFS fibroblasts. We explored the significance of the p53 CTD by examining several of the biological functions of tumor-derived p53 CTD mutants. A deficiency in apoptosis was a common characteristic of all of the tumor-derived p53 CTD mutants examined. This finding is consistent with the model that apoptosis is an important function for p53-mediated tumor suppression and that loss of this function by mutation at the p53 CTD favors tumorigenicity.

Previously, we reported that the COOH-terminal alternatively spliced p53 product altered the apoptotic function at different time points (52). We showed recently that microinjection of the p53 CTD polypeptide 319–393 into normal primary human fibroblasts, mammary epithelial cells, and p53-null LFS induced apoptosis (17). p53 CTD polypeptides can also induce apoptosis in human cancer cell lines (13). These data indicate that the p53 CTD can be sufficient to induce apoptosis. The CTD of p53 binds to both cellular and oncoviral proteins that can alter p53 functions (15, 53). For example, the p53 CTD can recognize damaged DNA and may influence DNA repair mechanisms through an interaction with replication protein A and TFIIH (16, 54). TFIIH is a multiprotein complex involved in transcription (55), nucleotide excision repair (33, 56), and apoptosis (17). p53 binds to four proteins, XPD, XPB, p36, and p62, in the TFIIH complex (33, 34, 57, 58). The COOH terminus of p53 binds specifically to the DNA helicases XPB and XPD in TFIIH, which are essential for transcription and nucleotide excision repair and contribute to apoptosis (17). p53-mediated apoptosis does not require RNA and protein synthesis (59, 60), and thus supports the hypothesis that p53-dependent apoptosis can occur through a transcription-independent pathway (31, 61–63). Our data are consistent with this hypothesis, indicating that the p53 CTD contains an apoptosis domain and that its induction of apoptosis may be mediated through its binding to TFIIH and other cellular protein mechanisms.

Table 1 *Biologic effects of wild-type p53 or tumor-derived CTD mutants in human fibroblasts*

			Transcriptional activity			DNA-binding affinity
p53	Apoptosis	Growth arrest	*p21*	*Bax*	*IGF-BP3*	p53 consensus binding site
WT-p53[a]	++	++	++	++	++	++
352D/H	−	++	++	+	+	++
356G/W	−	++	++	+	+	++
342-stop	−	−	−	−	−	−
360-del	−	++	++	+	+	++
387-del	−/+	++	++	+	+	++

[a] WT, wild-type; ++, same level as wild-type p53; −, same level as vector only; +, reduction compared with wild-type p53; del, deletion; −/+, significant reduction compared with wild-type p53 ($P < 0.01$).

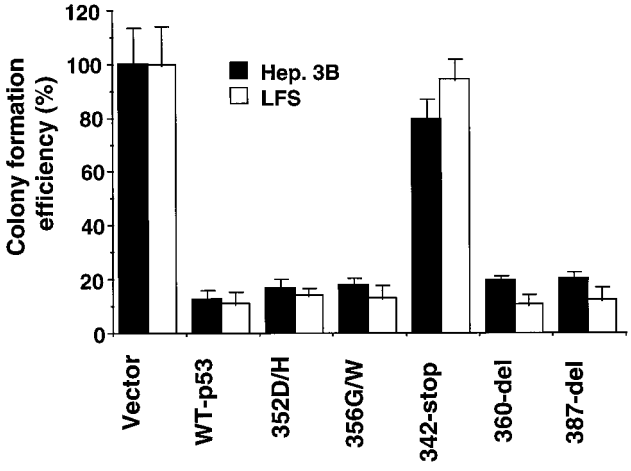

Fig. 7. Inhibition of growth of LFS fibroblasts and Hep 3B cells by wild-type p53 (*WT-p53*) and the CTD mutants. Cells were transfected with expression vectors; *colony formation efficiency* is represented by colony numbers counted after 2 weeks of G418 selection. Data represent the mean of three independent experiments; *bars*, SD.

One of the important functions of p53 is its ability to act as a transcriptional transactivator. The CTD mutant 342-stop abolished both suppression of the cell growth and transactivation of $p21^{Waf1/Cip1}$, *Bax*, and *IGF-BP3* in p53-null LFS fibroblasts. Failure to transactivate was associated with reduced ability of the mutant to bind the p53-responsive DNA sequences present in the target genes. The other tumor-derived p53 CTD mutants activated $p21^{Waf1/Cip1}$ transcription to the same extent as wild-type p53; however, the transcriptional transactivity of *Bax* and *IGF-BP3* was reduced moderately. The heterogeneity of the transcriptional transactivities on p53-responsive genes has been reported previously (45, 46, 64). These reports and the data presented here indicate that there are individual pathways to regulate different p53 downstream target genes, which are necessary for the different biological functions of p53. The lack of transcriptional transactivity by the tumor-derived mutation 342-stop indicates that the last 50 amino acids are critical for the transcriptional transactivation.

The transcriptional transactivity of p53 is dependent on the specific recognition of DNA sequences located in the promoter regions of the target genes, and p53 requires activating factors to confer effective sequence-specific DNA binding activity (22, 44, 54, 65). The CTD of p53 has been shown to be important in regulating the activity of the whole protein by modulating its sequence-specific DNA binding and transcriptional transactivity. The modulating factors include mutation, phosphorylation, acetylation, or PAb-421 binding within CTD of p53 (12, 16, 18, 22). Our data are consistent with these findings. The 360-del mutant has seven altered amino acids and a stop codon at 369. This protein has lost the PAb-421 epitope, but kept intact the oligomerization domain, and behaves as the described p53Δ30 mutant (28) or the alternatively spliced p53-encoded protein found in murine cells (29, 66). The truncated protein-product of the 342-stop mutant lost its DNA-binding activity because of the loss of the oligomerization domain. The PAb-421 antibody also has no effect due to the lack of the specific epitope. Our results support the concept that transcriptional transactivity is dependent on sequence-specific binding and that the binding affinity is regulated and governed by the p53 CTD. All mutants, except for 342-stop, retain the cell arrest function, indicating that p53 CTD is involved in the inhibition of cell growth and that this function is not associated with its induction of apoptosis. Taken together, these results are consistent with the hypothesis that a diminution of the apoptotic function of p53 is important in its tumor suppression activity and that the CTD of p53 contributes to this activity.

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