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Here we describe our analysis of the domain requirements for mutant p53 to transactivate promoters of the human epidermal growth factor receptor (EGFR), human multiple drug resistance 1 (MDR-1) and human proliferating cell nuclear antigen (PCNA) genes. We also report the identification of a structural domain required for the "gain of function" property of mutant p53-281G. "Gain of function" is measured as the tumorigenicity (in nude mice) of 10(3) murine cells expressing mutant p53 constitutively. We have generated internal deletion mutants of p53-281G deleting conserved domains I, II, III, IV, and V, individually. All these mutants have significantly activated the PCNA promoter, suggesting that the mechanism of transactivation of the PCNA promoter is different from that of the EGFR and MDR-1 promoters. When expressed constitutively in 10(3) cells, p53-281G del 393-327 was found to be defective in inducing tumor formation in nude mice although intact p53-281G was very efficient. Thus, our results suggest that structural domains near the C-terminus are needed for "gain of function".

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INTRODUCTION

Mutation in the tumor suppressor p53 gene is a common somatic genetic change found in human cancer (Donehower and Bradley, 1993; Lane, 1994; Levine, 1997; Ozbun and Butel, 1995; Ko and Prives, 1996; Hollstein *et al.*, 1994; Greenblatt *et al.*, 1994). The level of wild-type p53 in normal cells is very low, but when cells are exposed to genotoxic stimuli p53 levels rise rapidly and initiate a program of cell death or stop the cell cycle at the G1/S boundary, presumably to allow time for DNA repair. This "guardian of the genome" response of wild-type p53 (Lane, 1994) is lost in many tumor cells as they have either inactivated their p53 genes by mutation or blocked the activity of p53 through the production of proteins that bind to and neutralize it.

Wild-type p53 acts as a sequence-specific DNA-binding protein and transcriptional activator (reviewed in Donehower and Bradley, 1993; Ko and Prives, 1996; Levine 1997). All p53 genes from evolutionarily distant species contain five highly conserved regions designated domains I-V (Soussi *et al.*, 1990, reviewed in Donehower and Bradley, 1993; Ko and Prives, 1996; Levine, 1997) (Figure 1). p53 has three functionally distinct domains. The transcriptional activation domain is located within residues 1-42 (Fields and Jang, 1990; Raycroft *et al.*, 1990; O'Rourke *et al.*, 1990; Unger *et al.*, 1992), while the oligomerization domain is located within residues 320-360 (Wang *et al.*, 1993; Subler *et al.*,1994a; Sturzbecher *et al.*, 1992; Clore *et al.*, 1994; Lee *et al.*, 1994). The C-terminal region of p53 also codes for a nonsequence-specific nucleic acid-binding domain (reviewed by Ko and Prives 1996). The sequence-specific DNA-binding domain is located on the central part of the molecule covering domains II-V (Wang *et al.*, 1993; Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Halazonetis and Kandil, 1993; Cho *et al.*, 1994). p53 mutations found in tumors usually occur within the central part of the molecule such that the mutant proteins are, in general, defective in sequence-specific DNA binding and transactivation (Hollstein *et al.*, 1991; Hollstein *et al.*, 1994; Greenblatt *et al.*, 1994; reviewed in Donehower and Bradley, 1993; Ko and Prives, 1996).

Broadly, three categories of mutations in the p53 gene can be identified (Donehower and Bradley, 1993; Ozbun and Butel, 1995; Levine *et al.*, 1991): (1) "loss of function" where tumor suppressor activities of p53 are abolished; (2) "dominant negative" where hetero-oligomeric complex formation between wild-type and mutant p53 results in the inactivation of wild-type p53; and (3) "gain of function" where mutant p53 procures a dominant oncogenic role that does not depend on complex formation with wild-type p53.

Although wild-type p53 is relatively unstable, mutant p53 proteins accumulate to high levels in many cancer cells (reviewed in Ko and Prives, 1996; Levine, 1997). There seems to be a selective pressure in human cancer for the expression of mutant p53 proteins rather than no p53 expression at all. This selection suggests that p53 mutations may directly contribute to the gain of some growth-promoting functions ("gain of function" phenotype) rather than represent the mere loss of wild-type p53 function.

We and others showed that mutant p53 can transactivate promoters of cellular growth-related genes *in vivo* (Deb *et al.*, 1992; Chin *et al.*, 1992). p53 mutants activate the promoters of genes expressing human *PCNA*, *EGFR*, MDR-1, vascular endothelial growth factor (*VEGF*), human *IL-6*, basic fibroblast growth factor (*BFGF*) and human *HSP70* (Deb *et al.*, 1992; Chin *et al.*, 1992; Subler *et al.*, 1994b; Tsutsumi-Ishi *et al.*, 1995; Zastawny *et al.*,1993; Kieser *et al.*, 1994; Ueba *et al.*, 1994; Marguiles *et al.*, 1993). These observations give rise to the possibility that in cancer cells expressing high levels of mutant p53, the mutant protein may activate transcription of

growth-promoting genes resulting in more aggressive growth of these cells. The molecular basis of mutant p53-induced transactivation remains to be determined.

Lin *et al.* (1995) showed that amino acids in positions 22 and 23 in the N-terminal transactivation domain of the mutant protein p53-281G are required for "gain of function" (as judged by tumorigenesis assays) as well as for transactivation of the MDR-1 promoter. This suggests that the transactivation ability of mutant p53 is required for "gain of function". However, requirements of any other functional domains in transactivation and/or "gain of function" phenotypes of mutant p53 have not been studied.

In this communication, we define the domain required for mutant p53 to transactivate promoters of EGFR, MDR-1 and PCNA genes. Internal deletion mutants of p53-281G have been generated deleting conserved domains I, II, III, IV and V individually, and one deleting amino acids 100 through 300. These mutants have lower transactivation ability than intact p53-281G for EGFR and MDR-1 promoters. Another mutant p53-281G del 393-327 that deletes the oligomerization/nonsequence-specific nucleic acid-binding domain of p53 also has significantly lower transactivation ability for EGFR and MDR-1 promoters. Significantly, when expressed constitutively in 10(3) cells, p53-281G del 393-327 was found to be defective in inducing tumor formation in nude mice although intact p53-281G was very efficient.

MATERIALS AND METHODS

DNA plasmids. The p53 expression plasmids contain mutant human p53 cDNA (or a deletion mutant) (Figure 1) under the regulation of the human CMV immediate-early promoter in the pCMV-Bam expression vector (Hinds *et al.,* 1990; Subler *et al.,* 1992). The C-terminal deletion derivative p53-281G del 393-327 was generated in the context of the human p53-281G cDNA as described (Ludes-Meyers *et al.,* 1996). All the internal deletion mutants were generated by PCR, and their sequence verified by dideoxy sequencing using a Sequenase kit (United States Biochemicals, Inc.). The mutants with deletion of conserved domains I, II, III, and IV respectively, were all generated in the context of p53-281G. The mutants that deleted domain V and amino acids 100-300 eliminated the mutation at amino acid 281.

The CAT plasmids utilized the *Escherichia coli* CAT gene under the transcriptional control of (a) the complete EGFR promoter (-16 to -1100) (EGFR.CAT, Deb *et al.,* 1994); (b) the EGFR basal promoter (-16 to -105) (EGFR del *Ava I.*CAT, Deb *et al.,* 1994); (c) the complete PCNA promoter (+275 to -1269, Deb *et al.,* 1992); and (d) the MDR-1 promoter sequences (-1073 to +1, Chin *et al.,* 1992; Chen *et al.*, 1990; Dittmer *et al.,* 1993).

Cell culture and transfection. 10(3) cells are murine fibroblasts devoid of endogenous p53 (Harvey and Levine, 1991). These cells are maintained as described (Dittmer *et al.*, 1993; Lin *et al.*, 1995). Saos-2 cells were cultured as described previously (Subler *et al.,* 1992). Cells were transfected by the calcium phosphate-DNA coprecipitation method as described (Subler *et al.,* 1992). In a typical experiment 5 x 10^6 cells were cotransfected with 5 μg of a CAT construct and 10 μg of a p53 (p53-281G or one of the deletion mutants) expression plasmid (or 10 μg of the expression vector without p53 sequences as a negative control). All transient transfection experiments were repeated multiple times (three times or more).

Chloramphenicol acetyltransferase assay. Cells were harvested 36-40 hours post-transfection and lyzed by three successive cycles of freezing and thawing. Extracts were normalized for protein concentration and assayed for CAT enzyme activity (Ludes-Meyers *et al.,* 1996). Because wild-type p53 inhibits and mutant p53 activates different promoters to varying extents (Deb *et al.,* 1992; Ginsberg *et al.,* 1991; Lechner *et al.,* 1992; Santhanam *et al.,* 1991; Subler *et al.,* 1992), it was not possible to use an internal control such as pSVβGal or RSVβGal (Ludes-Meyers *et al.*, 1996). A similar situation has been recognized for SV40 T antigen-mediated regulation of promoters (Gruda *et al.,* 1993). Therefore, multiple independent experiments (three or more) were done to determine the standard deviations. CAT activity was detected by thin-layer chromatographic separation of [^{14}C]-chloramphenicol from its acetylated derivatives followed by autoradiography. Quantitation was done using a Phosphorimager (Molecular Dynamics), and standard deviations were calculated.

Establishment of stable 10(3) cell lines expressing p53-281G and p53-281G del 393-327: tumorigenesis in nude mice. 10(3) cells have been transfected with vector alone (pCMV Bam neo, Hinds *et al.,* 1990; Subler *et al.,* 1992) or expression plasmids for the tumor-derived p53 mutant p53-281G or p53-281G del 393-327 using the calcium phosphate precipitation technique (Subler *et al.,* 1992). Forty-eight hours after transfection, cells were washed, trypsinized, subcultured at 1:4, and plated into selective media supplemented with 200 μg/ml G-418 (active concentration). Cells were maintained in the selective media with twice weekly changes of media. After 14 to 21 days in selective media, cells from individual G-418-resistant colonies were isolated

and expanded into cell lines. Western blot analyses were used to show that mutant p53 was expressed in these cell lines.

Tumorigenicity assay. The tumorigenic potential of 10(3) cells either alone (untransfected) or stably transfected with p53-281G or p53-281G del 393-327 (or expression vector alone) was tested using nude mice. Cells (5 x 10^6 per site) were subcutaneously injected into flanks of athymic nude mice (National Cancer Institute) and tumor development was monitored as described (Dittmer *et al.*, 1993).

Western blot analysis. Western blot analysis was carried out after SDS polyacrylamide gel electrophoresis using monoclonal antibodies against p53 and a kit (Promega) for labeling with an alkaline phosphatase-conjugated secondary antibody. Manufacturer's protocol was followed for this procedure.

RESULTS

p53-281G deletion 393-327 differentially activates different promoters. Earlier we have shown that C-terminal deletion of p53-281G up to amino acid 327 causes a significant decrease in transactivation of the human EGFR promoter (Ludes-Meyers *et al.,* 1996) suggesting that the C-terminal oligomerization/nonsequence-specific nucleic acid-binding domain is necessary for mutant p53-mediated transactivation. Therefore, we tested whether the requirement for the C-terminal amino acids holds true for some other promoters that have also been shown to be transactivated by mutant p53; for that purpose we used the PCNA and MDR-1 promoters (Deb *et al.,* 1992; Chin *et al.,* 1992; Dittmer *et al.,* 1993). We cotransfected Saos-2 human osteosarcoma cells that are devoid of endogenous p53 with different promoter-chloramphenicol acetyl transferase (CAT) constructs and p53-281G or p53-281G del 393-327 (Figure 1) expression plasmids (or expression vector alone) as described in Materials and Methods. Figure 2 shows a representative example of CAT assay results. p53-281G transactivated all three promoters significantly. Whereas p53-281G del 393-327 could not significantly transactivate the EGFR and MDR-1 promoters, it transactivated the PCNA promoter efficiently. It seems that the C-terminally located oligomerization domain or nonsequence-specific nucleic acid-binding domain is not essential for transactivation of the PCNA promoter. This suggests that the mechanism of transactivation of different promoters by mutant p53 may be different.

p53-281G lacking the individual conserved domains transactivates the human EGFR promoter with reduced efficiency. Wild-type p53 has five domains (I-V) (Figure 1) that are phylogenetically conserved (Soussi *et al.,* 1990). Domains II-V are critically important for wild-type p53's functions, as most of the tumor-derived p53 mutants are mutated at the central part of p53 (reviewed in Ko and Prives, 1996; Levine, 1997). Involvement of these domains in mutant p53-mediated transactivation has not been studied. We tested whether these phylogenetically conserved domains are required for mutant p53-mediated transactivation of the EGFR promoter using p53-281G as a model tumor-derived p53 mutant. We generated five internal deletion mutants of p53-281G that eliminate conserved domains I-V individually. It is to be noted that the mutant with domain V deleted does not contain the mutated amino acid at 281. These mutated p53 genes were cloned downstream of the human cytomegalovirus (CMV) immediate-early promoter as described in Materials and Methods. Using cotransfection experiments in Saos-2 cells, we tested whether these deletion mutants could transactivate the human EGFR promoter. Multiple transient transfection assays were done, and the data shown in Figure 3 is an example of a typical assay. It is clear that all the internal deletion mutants dramatically reduced the extent of transactivation by p53-281G (compare lane 6 with lanes 1-5). Western blot analysis indicates that all deletion mutants were expressed appreciably (Figure 3, lower panel). This indicates that the difference in the transactivation ability observed between different deletion mutants and p53-281G is a true representation of transcriptional activation capacity and not due to differences in the level of the expressed proteins.

p53-281G lacking the individual conserved domains transactivate the basal human EGFR promoter with reduced efficiency. Next we analyzed whether the full length and the basal EGFR promoters respond in the same way to the conserved domain deletion mutants of p53-281G. We cotransfected Saos-2 cells with the expression plasmid for p53-281G or one of its conserved domain deletion mutants (or expression vector alone) and EGFR.CAT *Ava I* del. The EGFR *Ava I* del promoter defines the basal EGFR promoter (Deb *et al.,* 1994; Ludes-Meyers *et*

al.,1996). CAT assays were performed as described in Materials and Methods, and Figure 4 shows a representative example of the result. It is clear that all the internal deletion mutants reduced the extent of transactivation by p53-281G as in the case of the complete EGFR promoter (Figure 3). In this case also, the Western blot analysis does not indicate major variation in the expression of different deletion mutants.

Effect of deletion of the conserved domains on p53-281G-mediated transactivation of the human MDR-1 and PCNA promoters. We also tested whether the conserved domain deletion mutants of p53-281G could activate transcription from promoters of two other genes, PCNA and MDR-1. These promoters have also been shown to be transactivated by tumor-derived p53 mutants (Deb *et al.,* 1992; Chin *et al.,* 1992). Therefore, we have cotransfected Saos-2 cells with PCNA.CAT (or MDR-1.CAT) and the expression plasmid for p53-281G or one of its deletion mutants (or expression vector alone). We have performed CAT assays as described in Materials and Methods. Examples of the CAT assays are shown in Figure 5. Deletion of conserved domains II-V had insignificant effect on PCNA promoter activity (Figure 5A, compare lane 6 with lanes 2-5). Among the deletion mutants, however, p53-281G del domain I affected the most in some experiments suggesting that perhaps the transactivation domain of p53 may be required for transactivation of the PCNA promoter. On the other hand, transactivation of the MDR-1 promoter was reduced for all the mutants (Figure 5B, compare lane 6 with lanes 1-5). This suggests that although EGFR and MDR-1 promoter activation is sensitive to deletions of conserved domains II-V in p53-281G, the PCNA promoter activation is not.

Effect of deleting amino acids 100-300 from p53-281G on its transactivation ability. Since deletion of the individual conserved domains decreased the efficiency, but did not eliminate p53-281G-mediated transactivation of the EGFR and MDR-1 promoters (moreover the activity of PCNA promoter was not even reduced), we were interested to determine how elimination of several of the conserved domains of p53-281G would affect its ability to transactivate these promoters. Using PCR we deleted the amino acids of p53 281G between positions 100-300 that includes conserved domains II-V (Figure 1) and covers the entire DNA-binding region of wild-type p53 (reviewed in Ko and Prives, 1996; Levine, 1997). Transactivation was assayed by performing transient transcription analysis using CAT assays as described in Materials and Methods. We have used all three promoter-CAT constructs, EGFR.CAT, PCNA.CAT and MDR-1.CAT. Data shown in Figure 6 show that p53 del 100-300 failed to transactivate the MDR-1 promoter (compare lanes 5 and 6), while it could only partially activate the human EGFR promoter (compare lanes 8 and 9). Surprisingly, the PCNA promoter activity was slightly enhanced by this deletion (compare lanes 2 and 3) suggesting, again, that the mode of transactivation of the PCNA promoter by p53-281G is different from that of the EGFR and MDR-1 promoters.

p53-281G deletion 1-58 fails to activate different promoters. Since the PCNA promoter was transactivated by deletion mutants of p53-281G that failed to significantly transactivate the EGFR and MDR-1 promoters, we tested whether the transactivation domain of p53 is required for activation of the PCNA promoter. We cotransfected Saos-2 cells with different promoter-CAT constructs and p53-281G or p53-281G del 1-58 (or expression vector alone) as described in Materials and Methods. Figure 7 shows a representative example of CAT assay results. In this case p53-281G del 1-58 failed to significantly transactivate all the three promoters. This suggests that the transactivation domain of mutant p53 is required for mutant p53-mediated transactivation.

Removal of the C-terminal oligomerization/nonsequence-specific nucleic acid-binding domain reduces the oncogenic potential of p53 281G. We studied the relationship between the

transactivation property of tumor-derived p53 mutant p53-281G and its "gain of function" phenotype. To assay "gain of function" we have utilized 10(3) murine fibroblast cells that are devoid of endogenous p53. These cells are not tumorigenic in nude mice. Earlier it has been shown that if a mutant p53, such as p53-281G, is expressed in these cells constitutively, the mutant p53 expressing cells become tumorigenic (Dittmer *et al*, 1993; Lin *et al*, 1995).

We have generated a number of cell lines derived from 10(3) cells that express p53-281G and p53-281G del 393-327 (Figure 8) and tested whether they generate tumors in nude mice. Results of these experiments are shown in Table 1. 10(3) cells alone (or stably transfected with vector alone) did not generate tumors when observed for more than six months. However, p53-281G expressing cell clones all generated tumors. Visible tumor formation started within 3-4 weeks after injection. Significantly, none of the mice injected with two independent clones of 10(3) cells expressing p53-281G del 393-327 generated any tumor within six months after injection.

Discussion

We have previously shown that certain tumor-derived p53 mutants can transactivate promoters of growth-related genes such as PCNA and EGFR (Deb *et al.*, 1992; Deb *et al.*, 1994; Ludes-Meyers *et al.*, 1996). A number of other laboratories have made similar observations (Chin *et al.*, 1992; Tsutsumi-Ishi *et al.*, 1995; Zatsawny *et al.*, 1994; Kieser *et al.*, 1994; Ueba *et al.*, 1994; Marguiles *et al.*, 1993). Some tumor-derived p53 mutants have "gain of function" properties suggesting that the mutational change in the p53 molecule has induced oncogenic functions (Dittmer *et al.*, 1993; Wolf *et al.*, 1984; Shaulski *et al.*, 1991; Hsiao *et al.*, 1994). Arnold Levine and his coworkers (Dittmer *et al.*, 1993; Lin *et al.*, 1995) showed that p53-281G can induce increased tumorigenicity in 10(3) cells when constitutively expressed under the control of a CMV immediate-early promoter. Further, they demonstrated that a double substitution at amino acids 22 and 23 of p53-281G destroyed its transactivation capacity (as judged by its ability to transactivate the MDR-1 promoter) as well as its "gain of function" property (as judged by decreased tumorigenicity of 10(3) cells constitutively expressing the mutant). This observation suggested a direct relationship between mutant p53's transactivation ability and its "gain of function" property. To analyze which regions of a tumor-derived p53 mutant may contribute to the "gain of function" phenotype, we carried out a series of experiments using p53-281G mutants and a set of promoter-reporter constructs derived from genes implicated in regulation of cell growth such as EGFR and PCNA.

We have demonstrated that the C-terminally located segment 393-327 in p53-281G is required for transactivation of the human EGFR and MDR-1 promoters (Ludes-Meyers *et al.*, 1996; this report). The C-terminal region of p53 between amino acids 393 and 327 contains its oligomerization (tetramerization) and nonsequence-specific DNA-binding domains (reviewed by Ko and Prives, 1996). Thus, it is reasonable to speculate that proper oligomeric forms of mutant p53 are necessary for transactivation. Perhaps proper oligomeric forms are needed to have efficient protein-protein interaction necessary for mutant p53's function. It is also possible that the nonsequence-specific DNA-binding domain of tumor-derived p53 mutants interacts with promoter DNA, helping nucleation of transcriptional machinery. The C-terminal region also has binding sites for different proteins involved in transcription, e.g., TBP, TFIIH (reviewed by Ko and Prives, 1996). Also, the C-terminal region is required for mutant p53-mediated binding to nuclear matrix/scaffold attachment regions (Mueller *et al.*, 1996). Since the C-terminal region of p53 has multiple subdomains, our data do not rule out the possibility that the requirement for the C-terminal segment up to amino acid 327 can be due to one or more of several reasons such as, loss of oligomerization, loss of nonsequence-specific DNA binding, or loss of crucial protein-protein interactions. Further studies are required to establish the role of each functional subdomain near the C-terminus to decipher the mechanism of transactivation mediated by tumor-derived mutant p53.

p53-281G deletion mutants that eliminate individual conserved domains could not transactivate the EGFR and MDR-1 promoters (Figures 3-6, 8) suggesting a role(s) for these regions in mutant p53-mediated transactivation. The central region of wild-type p53 (amino acids 100-300) is involved in sequence-specific DNA binding. Since tumor-derived mutants of p53 are, in general, defective in sequence-specific DNA binding, it gives rise to the possibility that for mutant p53-mediated transactivation the conserved domains are required for a particular structural conformation or for crucial protein-protein interactions. Alternatively, the conserved domains may

be responsible for DNA binding to an unknown sequence element that is directly or indirectly involved in mutant p53-mediated transactivation.

We have observed that p53-281G del 393-327 could transactivate the PCNA promoter although it could not activate the EGFR and MDR-1 promoters. Therefore, the PCNA promoter transactivation perhaps does not require proper oligomerization of p53-281G. Thus, there is a difference in the mechanism of transactivation by p53-281G of the PCNA and EGFR (or MDR-1) promoters. The PCNA promoter activation by p53-281G, however, requires the transactivation domain (Figure 7). Since the PCNA promoter is transactivated by p53-281G deletion mutants that eliminate the conserved domains II-V of p53 (Figure 5A), it is possible that the PCNA promoter activation takes place through protein-protein interactions mediated via the transactivation domain. The transactivation of the EGFR and MDR-1 promoters by p53-281G, however, requires the C-terminal segment from amino acid 393 to 327 as well as the conserved domains (Figures 3-6, 8). These observations strongly suggest that the domain requirements for mutant p53-mediated transactivation may vary depending on the promoter tested. It is also possible that tumor-derived p53 mutants increases PCNA promoter activity through an indirect mechanism, perhaps through their effect on the cell cycle.

Tumorigenicity data presented here and elsewhere (Dittmer *et al.*, 1993; Wolf *et al.*, 1984; Shaulski *et al.*, 1991; Hsiao *et al.*, 1994) indicate that expression of some tumor-derived p53 mutants in cell(s) without endogenous p53 enhances the oncogenicity of these cells. These findings support the "gain of function" hypothesis. When constitutively expressed in 10(3) cells, p53-281G, a transcriptionally active p53 mutant, increases the tumorigenicity of these cells (Table 1). However, deletion of amino acids 393 to 327 renders p53-281G inactive in transactivation of the EGFR and MDR-1 promoters but not of the PCNA promoter. This deletion mutant failed to induce tumorigenicity when expressed constitutively in 10(3) cells, directly correlating transactivation ability with enhancement of oncogenicity. Since p53-281G del 393-327 can still transactivate the human PCNA promoter (Figure 2), the "gain of function" phenotype cannot be assayed by mutant p53-mediated transactivation of the PCNA promoter. Thus, transactivation of the human EGFR or MDR-1 promoter is a better measure for the "gain of function" phenotype than PCNA promoter transactivation. Our tumorigenicity data (Table 1) also show that the C-terminal region (amino acid 393-327) is necessary for the "gain of function" phenotype. Since the transactivation data presented are from experiments that used Saos-2 and not 10(3) cells, we have evaluated the effect expression of p53-281G and its deletion mutants on the EGFR promoter activity using 10(3) cells also. The results of these analyses (data not shown) indicated that in 10(3) cells the regulation of the EGFR promoter by p53-281G and its derivatives used in this study is similar to that seen in Saos-2 cells.

Taken together our data suggest that the "gain of function" phenotype requires the transactivation function of tumor-derived p53 mutants. It is possible that tumor-derived p53 mutants transactivate a number of growth-promoting genes resulting in a tumorigenic phenotype. In the future it might be possible to identify genes that are specifically transactivated by these mutants unraveling pathways of oncogenesis further.

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Table 1. Tumorigenicity of 10(3) cells expressing p53-281G and its deletion mutants.

Effect of overexpression of mutant p53-281G and its deletion mutants on the tumorigenic potential of p53 null 10(3) murine fibroblast cells		
Cells stably transfected with	Number of mice/ Number of sites injected	Number of tumors generated/ Number of sites injected
None	6/12	0/12
Vector alone, clone 3	3/6	0/6
Vector alone, clone 4	3/6	0/6
p53-281G, clone 4	3/6	6/6
p53-281G, clone 13	3/6	6/6
p53-281G del 393-327, clone 15	3/6	0/6
p53-281G del 393-327, clone 10	3/6	0/6

FIGURES

Figure 1. Schematic representation of p53 and its domain structure.

1. Lanyi et al.

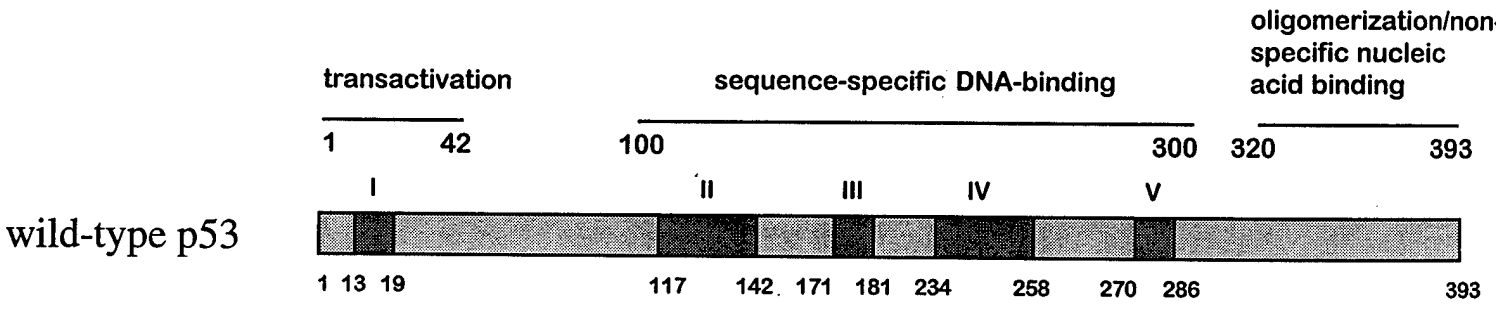

Figure 2. p53-281G deletion 393-327 differentially activates different promoters. Saos-2 cells were transiently transfected with 5 μg of PCNA.CAT, MDR-1.CAT or EGFR.CAT promoter/reporter plasmids and 10 μg of the expression plasmid for intact p53-281G or p53-281G del 393-327 (or expression vector alone). After transfection cells were treated as described in Materials and Methods and CAT assays were carried out. The average percentages of transactivation compared to that induced by p53-281G are as follows: PCNA.CAT: p53-281G -- 100.00, p53-281G del 393-327 -- 166.1±39.2; MDR-1.CAT: p53-281G -- 100.00, p53-281G del 393-327 -- 6.15±2.03; EGFR.CAT: p53-281G -- 100.00, p53-281G del 393-327 -- 24.70±9.30. Compared to vector alone, p53-281G transactivated the PCNA, EGFR and MDR-1 promoters on average 3-, 8.7- and 42-fold, respectively. Top panels show representative autoradiograms of CAT assays. Lower panels show Western blot analyses carried out with equal amounts of protein from each CAT assay extract used for the upper panels. A monoclonal anti-p53 antibody (PAb1801) was used to detect p53. Closed arrowheads indicate the position of p53-281G, while open arrowheads show p53-281G del 393-327.

Fig. 2 Lanyi et al.

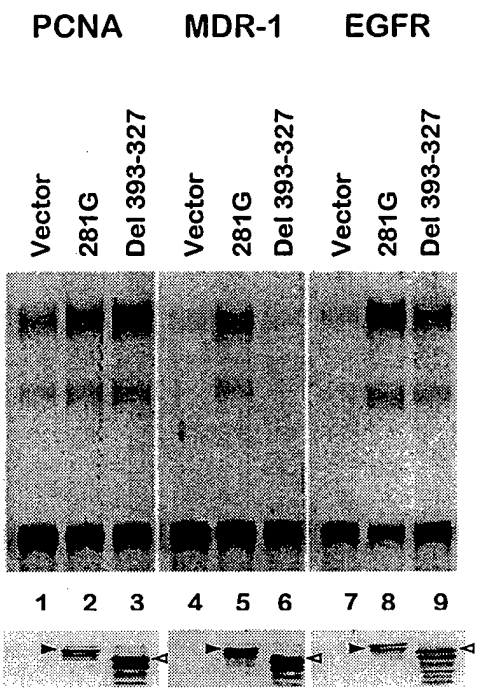

Figure 3. Deletion mutants of p53-281G lacking the individual conserved domains of p53 transactivate the human EGFR promoter with reduced efficiency. Saos-2 cells were transfected with 5 μg of EGFR.CAT promoter/reporter plasmid and 10 μg of the expression plasmid for intact p53-281G, p53-281G del domain I, II, III, or IV, or p53 del domain V (or expression vector alone). After transfection cells were treated as described in Materials and Methods and CAT assays were performed. The average percentages of transactivation compared to that induced by p53-281G are as follows: p53-281G -- 100.00, p53-281G del domain I -- 16.06±5.56, p53-281G del domain II -- 32.40±19.10, p53-281G del domain III -- 54.04±7.64, p53-281G del domain IV -- 52.13±17.02 and p53 del domain V -- 10.37±5.36. On average p53-281G activated the EGFR promoter 42-fold compared to the expression vector alone control. The upper panel shows a representative autoradiogram of CAT assays and the lower panel shows the Western blot analysis carried out with equal amounts of protein from each extract used for CAT assays shown in the figure. Positions of molecular weight markers are shown on left.

Fig. 3 Lanyi et al.

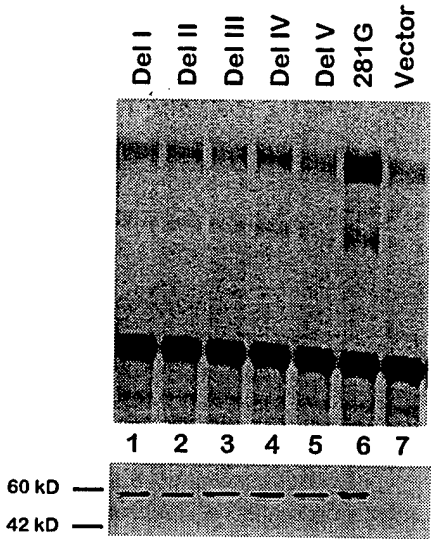

Figure 4. Deletion mutants of p53-281G lacking the individual conserved domains of p53 transactivate the basal human EGFR promoter with reduced efficiency. Saos-2 cells were transfected with 5 μg of EGFR.CAT *Ava I* del and 10 μg of the expression plasmid for intact p53-281G, p53-281G del domain I, II, III, or IV, or p53 del domain V (or expression vector alone). After transfection cells were treated as described in Materials and Methods and CAT assays were carried out. The average percentages of transactivation compared to that induced by p53-281G are as follows: p53-281G -- 100.00, p53-281G del domain I -- 16.45±8.38, p53-281G del domain II -- 16.78±7.67, p53-281G del domain III -- 28.09±9.35, p53-281G del domain IV -- 38.53±18.78 and p53 del domain V -- 21.87±1.21. The average transactivation of the EGFR.CAT *Ava I* del by p53-281G is 38-fold. The upper panel shows a representative autoradiogram of CAT assays, while the lower panel shows the Western blot analysis carried out with equal amounts of protein from each CAT assay extract. Positions of molecular weight markers are shown on left.

Fig. 4 Lanyi et al.

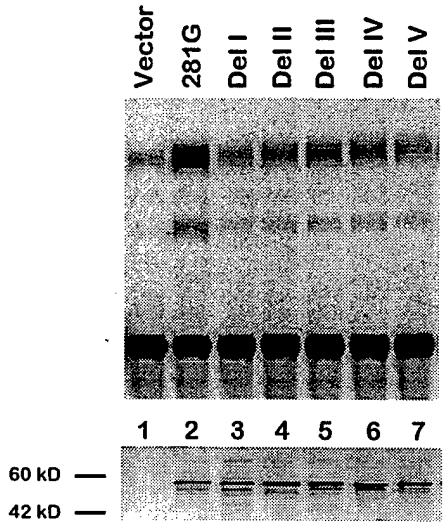

Figure 5. Effect of deletion of the conserved domains on p53-281G-mediated transactivation of the human PCNA and MDR-1 promoters. Saos-2 cells were transfected with 5 μg of PCNA.CAT or MDR-1.CAT and 10 μg of the expression plasmid for intact p53-281G, p53-281G del domain I, II, III, or IV, or p53 del domain V (or expression vector alone). After transfection cells were treated as described in Materials and Methods and CAT assays were carried out. Panels A and B represent assays with the PCNA and MDR-1 promoters, respectively. The average percentages of transactivation compared to that induced by p53-281G are as follows: PCNA.CAT: p53-281G - 100, p53-281G del domain I -- 122.25±84.42, p53-281G del domain II - 95.8±28.74, p53-281G del domain III - 80.1±29.13, p53-281G del domain IV - 121.03±45.07 and p53 del domain V -- 117.25±7.79; MDR-1.CAT: p53-281G -- 100.00, p53-281G del domain I -- 25.85±10.49, p53-281G del domain II -- 18.81±5.79, p53-281G del domain III -- 32.43±9.98, p53-281G del domain IV -- 24.95±4.79 and p53 del domain V -- 12.67±1.63. The average transactivation by p53-281G over vector alone are 3- and 48.57-fold for PCNA and MDR-1 promoters, respectively. Representative autoradiograms of CAT assays are shown at the upper panel. Lower panels show the Western blot analyses carried out with equal amounts of protein from each extract used for CAT assay. Positions of molecular weight markers are shown on left.

Fig. 5 Lanyi et al.

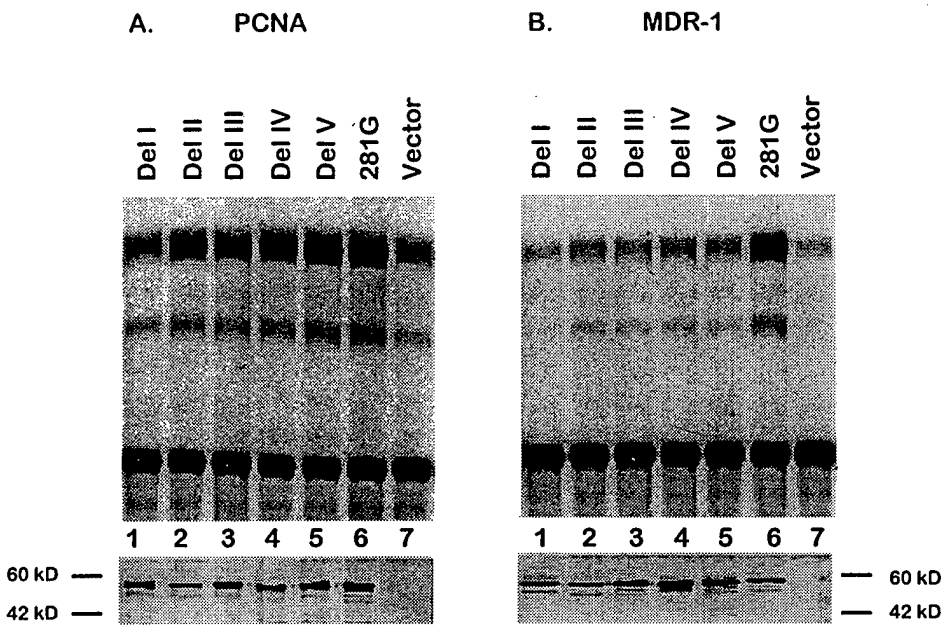

Figure 6. Effect of deleting amino acids 100-300 from p53-281G on its transactivation ability. Saos-2 cells were transfected with 5 μg of PCNA.CAT, MDR-1.CAT or EGFR.CAT and 10 μg of the expression plasmid for intact p53-281G or p53 del 100-300 (or expression vector alone). After transfection cells were treated as described in Materials and Methods and CAT assays were carried out. The average percentages of transactivation compared to that induced by p53-281G are as follows: PCNA.CAT: p53-281G -- 100.00, p53 100-300 -- 196±31.66; MDR-1.CAT: p53-281G -- 100.00, p53 del 100-300 -- 9.20±2.97; EGFR.CAT: p53-281G -- 100.00, p53 del 100-300 -- 33.07±12.07. On average the PCNA promoter was activated 3-fold by p53-281G. Lower panels show Western blot analyses carried out with equal amounts of protein from each CAT assay extract used for panels above. Closed arrowheads indicate the position of p53-281G, while open arrowheads show p53 del 100-300.

Fig. 6 Lanyi et al.

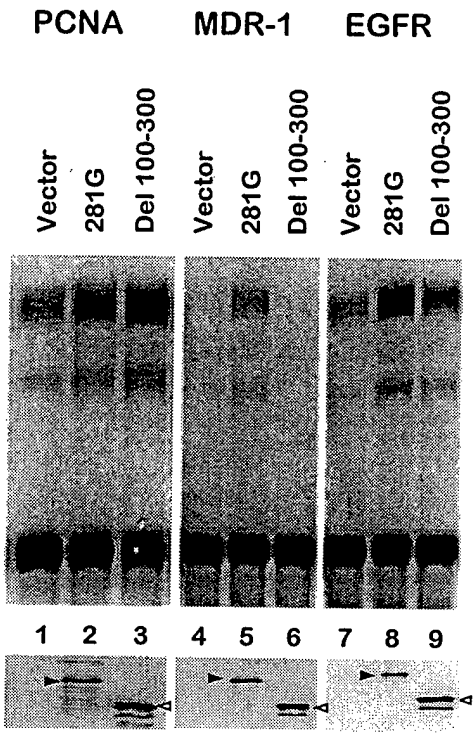

Figure 7. p53-281G deletion 1-58 fails to activate different promoters. Saos-2 cells were transiently transfected with 5 μg of PCNA.CAT, MDR-1.CAT or EGFR.CAT promoter/reporter plasmids and 10 μg of the expression plasmid for intact p53-281G or p53-281G del 1-58 (or expression vector alone). After transfection cells were treated as described in Materials and Methods and CAT assays were carried out. The average percentages of transactivation compared to that induced by p53-281G are as follows: PCNA.CAT: p53-281G -- 100.00, p53-281G del 1-58 – 12.46±11.0; MDR-1.CAT: p53-281G -- 100.00, p53-281G del 1-58 -- 6.80±4.27; EGFR.CAT: p53-281G -- 100.00, p53-281G del 1-58 – 10.86±0.81. Compared to vector alone, p53-281G transactivated the PCNA, MDR-1 and EGFR promoters on average 21.8-, 9.5- and 10.7 -fold, respectively. Top panels show representative autoradiograms of CAT assays. Lower panels show Western blot analyses carried out with equal amounts of protein from each CAT assay extract used for the upper panels. A monoclonal anti-p53 antibody (PAb421) that recognizes the C-terminus was used to detect p53.

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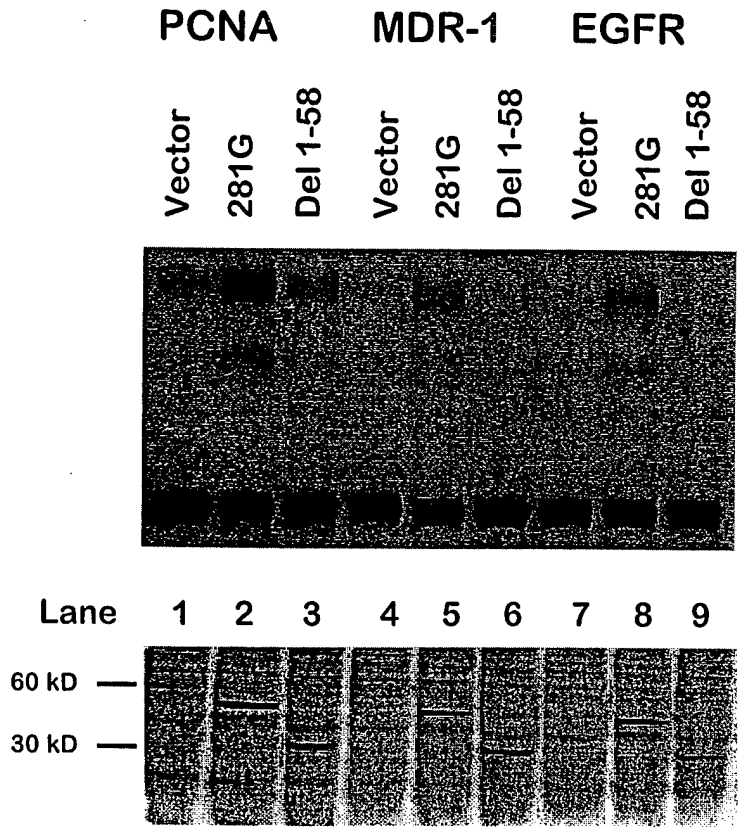
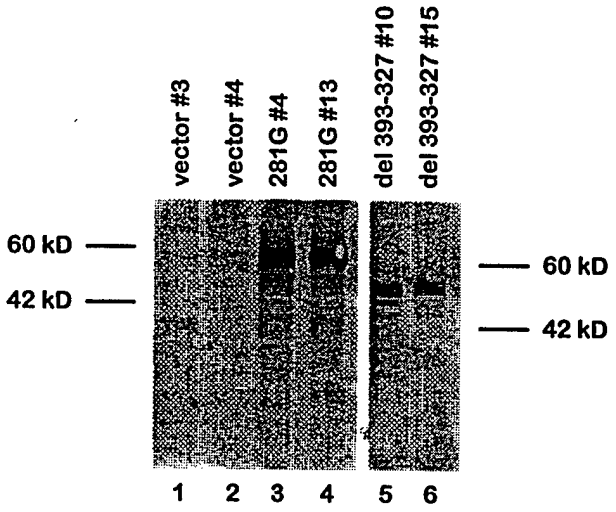


Figure 8. Expression of p53-281G and its deleted form in 10(3) cells stably transfected with p53-281G or with p53-281G del 393-327. 10(3) cell lines stably expressing p53-281G or p53-281G del 327-393 were constructed and maintained as described in Materials and Methods. Protein extracts from cells carrying the expression vector (lanes 1 and 2), p53-281G (lanes 3 and 4) or p53-281G truncated at amino acid 326 (lanes 5 and 6) were obtained by three successive cycles of freezing and thawing. Western blots were prepared by standard procedures (Materials and Methods). Equal amounts of protein were loaded in each well. The blot was developed using the monoclonal antibody PAb 1801. Positions of molecular weight markers are shown.

Fig. 8. Lanyi et al.



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List of personnel receiving pay from the effort

1. Mark A. Subler, Ph.D.
2. John H. Ludes-Meyers, Ph.D.
3. Arpad Lanyi, Ph.D.