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Cooperation of Two Mutant *p53* Alleles Contributes to Fas Resistance of Prostate Carcinoma Cells[1]

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ABSTRACT

Both inactivation of p53 function and loss of sensitivity to Fas contribute to a malignant phenotype and frequently occur during tumor progression. Although in the majority of cases only one of the *p53* alleles is mutated, some tumors acquire mutations in both alleles of the *p53* gene. To determine the biological significance of this phenomenon, we analyzed p53 mutants, $p53^{223Leu}$ and $p53^{274Phe}$, from Fas-resistant prostate carcinoma cell line DU145. Both mutants differed from wild-type p53 in their conformation, transactivation ability, and effect on the growth of p53-deficient cells, with $p53^{223Leu}$ being more similar to wild-type p53 than was $p53^{274Phe}$. Interestingly, the biological effect of coexpression of the DU145-derived mutants was dramatically different from that of each mutant expressed alone. Whereas neither of the two mutants was found to be dominant-negative against wild-type p53, each neutralized the other's growth-suppressive effects and, in combination, were capable of down-regulating Fas expression and converting Fas-sensitive prostate carcinoma cells PC3 into Fas-resistant ones. These results indicate that two different p53 mutants that are separately rather weak can cooperate to generate p53 protein with anti-Fas function that is likely to provide additional selective advantages to the tumor.

INTRODUCTION

Of more than 10,000 different human tumors analyzed to date, 45–50% contained inactivating mutations within the *p53* gene (1) making it the most frequently mutated gene in cancer. Frequencies of mutations vary from one tumor type to another (2) ranging from 60–65% of lung and colon cancers, 40–45% of stomach, esophagus, and bladder cancers, 25–30% of breast, liver, and prostate cancers and of lymphomas, and of 10–15% of leukemias (1). Interestingly, the nature of mutations acquired by p53 in the tumors clearly differentiates this gene from other tumor suppressors that are inactivated predominantly by deletions or transcriptional silencing, resulting in the lack of functional protein. On the contrary, 80–85% of tumor-derived alterations in the *p53* gene are missense point mutations localized within the DNA-binding domain of the protein (1–4). Such mutations result in the accumulation of large amounts of abnormally stable p53 protein that loses specific DNA-binding and transactivation functions. The fact that tumor-derived mutations in *p53* preserve expression of full-length, although altered, proteins suggests that mutant p53 could provide some selective advantages for tumor cells. One such property is the dominant-negative activity of some of the tumor-derived p53 mutants capable of suppressing function of the wild-type allele.

Nevertheless, the wild-type alleles of *p53* are frequently lost during further tumor progression, indicating that the dominant-negative effect of p53 mutants is insufficient for complete inactivation of the wild-type function and that they might have some additional properties contributing to cancer development. In fact, some of the p53 mutants exhibit gain-of-function and can modulate tumor cell phenotype, even in the absence of the wild-type allele, by increasing clonogenic potential, tumorigenicity, and invasiveness (5–8). In addition, some p53 mutants are capable of (*a*) further increasing genetic instability by abrogating the mitotic spindle checkpoint (9); (*b*) changing differentiation status of cells (10); and (*c*) increasing resistance to certain chemotherapeutic drugs (11–13).

The majority among *p53* mutant tumors express a single mutant allele of *p53* (see p53 mutation database).[4] However, *p53* mutation databases contain several cases in which two different *p53* mutations are coexpressed in one tumor, presumably resulting in the formation of mixed proteins built from all possible combinations of mutant p53 monomers. The biological significance of coexpression of two different mutants in one tumor has not been analyzed and became the subject of the present work.

As a model, we chose prostate carcinoma cell line DU145, which carries different mutations in two alleles of *p53* (14) none of which has been characterized before. To understand the biological sense of their coexistence in one tumor, we analyzed the functionality of these mutants. Our approach was to introduce them into p53-deficient cells of prostate and nonprostate origin and to determine phenotypic changes associated with their separate or combined expression. We found that the properties of the p53 protein complex resulting from the coexpression in one cell of two DU145 mutants was different from what could be expected from a simple sum of the properties of two mutants. One of the most remarkable new functions of the new p53 was its ability to confer resistance to Fas-mediated apoptosis, a new gain-of-function activity that presumably reflects the potential mechanism behind the selection of this combination of p53 mutants within a tumor.

MATERIALS AND METHODS

Plasmids. The plasmids used in this study are described in Table 1.

Cell Culture. We used the following cell lines: DU145 and PC3 (both of which are human prostate carcinoma-derived); Saos-2 (human osteosarcoma); AmphoPack-293 (A293-derived packaging cell line; Clontech); 10(1) (BALB/c-derived fibroblast cell line; kindly provided by Arnold Levine); and C8 (E1a+ras-transformed mouse embryo fibroblasts; a gift from Scott Lowe, Cold Spring Harbor Laboratory). Prostate carcinoma cell lines were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 4 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, and 0.1 mM 2-mercaptoethanol. C8 cells were maintained in DMEM (Life Technologies, Inc.) with 10% fetal bovine serum; and Saos-2, 10(1), and AmphoPack-293 cells were cultured in the same medium, but supplemented with 10% of FetaClone (Hyclone Laboratories). All of the media contained 100 units/ml penicillin and 100 μg/ml streptomycin.

Transfection and Retroviral Transduction. Packaging cells plated in 60-mm plates were transfected with 10 μg of retroviral vector DNA using standard calcium phosphate procedure. The medium was changed after 8 h. Virus-containing media supplied with 8 μg/ml of Polybrene (Sigma) were collected at 24 and 48 h posttransfection and were used for infection. Virus-

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[4] Internet address: http://www.iarc.fr/p53/Index.html.

Table 1 *Properties of vectors used in the study*

Construct	Inserts	Selectable marker	Promoter for insert expression	References (base vectors)
pLXSP-p53	Human wt[a] p53	Puromycin	LTR	Modified pLXSN (Clontech) with neo substituted for puro
pLXSP-GSE56	GSE56	Puromycin	LTR	
pPSH-p53	Human wt p53	Hygromycin	LTR	(15)
pPSH-p53-223	p53-223^{Leu}	Hygromycin	LTR	(15)
pPSH-p53-274	p53-274^{Phe}	Hygromycin	LTR	(15)
pLPCwtp53	Human wt p53	Puromycin	CMV	Clontech
pLPC-p53-223	p53-223^{Leu}	Puromycin	CMV	Clontech
pLPC-p53-274	p53-274^{Phe}	Puromycin	CMV	Clontech
pcDNA3	Wild-type p53	G418	CMV	Invitrogene
p21-CAT	CAT	No	Minimal TK + p53-binding site from p21/Waf promoter	(26)
pCMV-β-gal	β-Galactosidase	No	CMV	Stratagene

[a] wt, wild type; TK, thymidine kinase.

transduced cells were selected for the resistance to an appropriate selective agent (G418, hygromycin, or puromycin, depending on the vector) up to a complete death of noninfected cells. Transfections of other cells were carried out with LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer's recommendations.

Isolation and Cloning of Mutant p53 from DU145 Cells. cDNA was synthesized from total RNA of DU145 cells using SuperScript II (Life Technologies, Inc.) reverse transcriptase from oligo(dT) primers. Primers flanking the coding region of the human *p53* gene (left, 5′-acgcttccctggattgg; right, 5′-ggcaggggagggagaga) were used for PCR amplification with synthesized cDNA as a template. The PCR product of 1287 bp was cloned to pCR2.1 vector using TA Cloning kit (Invitrogene). Because it was known that Du145 cells contain different point mutations in each allele of the *p53* gene (223 codon CCT to CTT in one, and codon 274 GTT to TTT in the other; Ref. 14), we screened resulting clones by hybridization with the mutant-specific 17-mer oligonucleotide probes synthesized to contain mutant nucleotides in the middle. Four different 17-mer probes were used for hybridization: two corresponding to the wild-type sequence of codons 223 (5′-TGAGCCG**C**CTGAGGTTG) and 274 (5′-AGGTGCA**G**TTTTGTGCC), and two corresponding to the mutated sequence of the same codons: 223, 5′-TGAGCCGC**T**TGAGGTTG; 274, 5′-AGGTGCA**T**TTTTGTGCC. Hybridization was carried out under conditions calculated to distinguish between perfectly matched hybrids and hybrids with a single mismatch (at 48°C in 4× SSC). Each variant of mutant p53 was recloned in retroviral-expressing vectors with different selective markers; pPSH (15) transgene expression was regulated by LTR[5] and hygromycin resistance gene by SV40 promoter; and pLPCX (Clontech) transgene expression was driven by CMV and puromycin resistance gene by LTR.

Colony, Cell Growth, and Apoptotic Assays. For colony assay, subconfluent cultures in 60-mm plates were transfected or infected with the tested constructs and selected with an appropriate antibiotic (or combination of antibiotics in case of infection or transfection with two different vectors). In parallel, the same transfection solutions or virus-containing media were transferred on Rat1 cells, known to be resistant to transduction with wild-type p53, for estimation of transfection efficiency or virus titer. Cell growth after treatment with apoptosis-inducing agents was estimated by methylene blue staining of methanol-fixed cells attached to the plate with subsequent dissolving of the stain in 0.1 N HCl followed by measurement of absorbance values using ELISA-reader, $\lambda = 600$ nm, and calculated as a percentage of absorbance values of untreated cells.

Apoptosis in cell cultures was monitored by 4′,6-diamidino-2-phenylindole staining of formalin/glutaraldehyde-fixed cultures, calcein, and DNA fragmentation assays as described previously (16). All of the assays were done in 4–6 parallels and repeated at least two times. The activation of caspases 2, 8, and 9 was monitored by Western immunoblotting as described previously (16, 17) using antibodies from Upstate (anti-caspase 2 and 8) and from PharMingen (anti-caspase 9).

FACS Analysis. Staining of DNA with PI for the estimation of DNA content was done as described previously (18). For green fluorescent protein expression analysis, cells were lifted by treatment with 0.5 mM EDTA and washed with PBS, cell pellets were resuspended in PBS with 0.5% of BSA and 1 μg/ml of PI. Results were analyzed using FACSorter (Becton Dickinson) and CellQuest software.

Immunoprecipitation and Western Blotting. For immunoprecipitation, cells in 150-mm plates were lysed in 2 ml of NP40 buffer [50 mM Tris HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 2% NP40) containing 1 mM phenylmethylsulfyl fluoride (Sigma), 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 10 mM sodium orthovanadate (Sigma), and 1 mM NaF (Sigma). Cell lysates were incubated 30 min on ice and centrifuged at 15,000 × *g* for 20 min. Then lysates were precleaned for 1 h at 4°C with the mixture of nonspecific antibodies corresponding to the isotype of the primary antibodies together with protein A/G agarose (Santa Cruz Biotechnology). Ten μg of one of four antibodies (Pab 240 (Santa Cruz), Pab 1620 (Calbiochem), DO1 (Santa Cruz), and nonspecific mouse IgG mixture) were added to 0.5 ml of the lysate and incubated overnight at 4°C, after which 20 μL of protein A/G agarose (Santa Cruz) were added for 1 h. After washings in lyses buffer, agarose pellets were boiled in Laemmli sample buffer, used for Western immunoblotting (see below) and detected with biotinylated anti-p53 antibodies (Boehringer Mannheim) and streptavidin-conjugated horseradish peroxidase. For Western blotting, cells were lysed in radioimmunoprecipitation assay buffer [25 mM Tris HCl (pH 7.2), 125 mM NaCl, 1% NP40, 1% sodium deoxycholate, and 1 mM EDTA] containing 1 mM phenylmethylsulfyl fluoride (Sigma), 10 μg/ml aprotinin (Sigma), and 10 μg/ml of leupeptin (Sigma). Protein concentrations were determined with Bio-Rad Dc protein assay kit. Equal protein amounts were run on gradient 4–20% precast gels (Novex) and blotted onto polyvinylidene difluoride membranes (Amersham). The following antibodies were used: anti-p53–monoclonal mouse DO1 (Santa Cruz) and biotinylated polyclonal rabbit (Boehringer Mannheim); anti-p21–polyclonal rabbit (Santa-Cruz); anti-mdm2–monoclonal mouse (Santa Cruz); anti-CD95–monoclonal mouse (Transduction Laboratories); anti-actin–polyclonal goat (Santa-Cruz). Horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz. Quantitation of the data were performed using Quantity One software from Bio-Rad.

Transactivation Assay. Cells in subconfluent cultures were transfected with a combination of the plasmids carrying wild-type p53 or p53 mutants with reporter construct containing reporter CAT DNA under the minimal thymidine kinase promoter and p53-binding sequence from the promoter of *p21/Waf1* gene (19). A control CMV-LacZ vector expressing β-galactosidase from the CMV promoter was included in all transfections. Parental vector, pBasicCAT, lacking p53 consensus element, was used as a control in the assays. Forty-eight-h posttransfection cell extracts were prepared, normalized according to protein concentrations, and used for the determination of CAT and β-galactosidase (Promega) activities.

RESULTS

Effect of DU145-derived p53 Mutants on Growth of p53-deficient Cells Is Cell-Type-dependent and Different from Wild-Type p53. The accumulation of p53 protein in response to DNA damage or other types of stress may result in the induction of growth

[5] The abbreviations used are: LTR, long terminal repeat; CAT, chloramacetyltransferase; CMV, cytomegalovirus; FACS, fluorescence-activated cell sorter; PI, propidium iodide; mAb, monoclonal antibody.

arrest and/or apoptosis, depending on the cell type and severity of damage. Similar effects can be reached by ectopic expression of p53 in sensitive cells. To compare growth-suppressive properties of DU145-derived mutants with wild-type p53, we used three p53-deficient cell lines, PC3, Saos-2, and 10(1), all known to be p53-sensitive. In Saos-2 and 10(1) cells (human osteosarcoma and a variant of mouse Balb 3T3 cell lines, respectively) both alleles of the *p53* gene are completely lost (5, 20). In PC3 cells (human prostate carcinoma), the only remaining allele of the *p53* gene has a single nucleotide deletion in codon 138, leading to a frameshift that generates a stop codon a few nucleotides downstream of the mutation (14). The resulting aberrant protein is apparently unstable, because it cannot be detected in cell lysates (Fig. 1) even after immunoprecipitation (data not shown). Thus, PC3 cells can also be considered p53-deficient.

PC3, 10(1) and Saos-2 cells were infected with retroviral vectors expressing p53–223^{Leu}, p53–274^{Phe} or wild-type p53. Infection cells were selected for resistance to hygromycin and the numbers of *hygro*-expressing colonies were estimated. Before infection, virus titers were normalized by titration on Rat1 cells known to be resistant to ectopic expression of p53.

As shown in Fig. 1, expression of wild-type p53 was toxic for all of the cell lines tested, as judged by the inhibition of the colony growth of retrovirus-transduced cells. Surprisingly, the effects of the DU145-derived mutants were cell type-dependent. Neither p53–223^{Leu} nor p53–274^{Phe} affected the growth of PC3 cells, whereas the expression of both proteins was confirmed by Western immunoblotting (Fig. 1*a*).

Both mutants caused a growth-suppressive effect on mouse 10(1) cells, although less dramatic than wild-type p53. Consistently, cells, expanded from rare colonies, appeared after the hygromycin selection of virus-transduced 10(1) cells expressed a barely detectable level of mutated p53 protein (Fig. 1*b*).

Saos-2 cells showed the third pattern of sensitivity to DU145-derived mutants. p53–223^{Leu} mutant was as active as wild-type p53 in the inhibition of colony growth of Saos-2, whereas p53–274^{Phe} had no growth-inhibitory effect in these cells (Fig. 1*c*). The inhibition of colony growth in Saos-2 cells by p53–223^{Leu} correlated with the induction of apoptosis by this mutant in a transient-transfection assay. The proapoptotic effect of the p53–223^{Leu} mutant in these experiments was similar to that of wild-type p53, whereas transfection of p53–274^{Phe} did not induce apoptosis (Fig. 1*d*).

Thus, the analysis of biological effects of p53–223^{Leu} and p53–274^{Phe} mutants on p53-deficient cells indicates that both of them clearly differed from wild-type p53. This difference was more pronounced in the case of p53–274^{Phe} mutant, which showed minor growth inhibition in only one cell line and was incapable of inducing apoptosis. Another mutant, p53–223^{Leu}, displays both of these activities in two cell lines. Remarkably, none of the mutants showed any wild-type function in human prostate PC3 cells, suggesting that some unknown tissue-specific factors might modulate p53 activity in different cell contexts.

DU145-derived p53 Mutants Do Not Possess Dominant-Negative Activity against Wild-Type p53. Some tumor-derived p53 mutants, acting as inhibitors of wild-type p53 (Ref. 21, and references therein), display a dominant-negative effect. We used two approaches to estimating the potential dominant-negative activity of DU145-derived mutants: (*a*) testing the toxicity of wild-type p53 transduced into the cells expressing the studied mutants; and (*b*) the suppression of p53-dependent apoptosis by transduction of the mutants.

The first type of assay was done using PC3 cells, stably expressing p53–223^{Leu} or p53–274^{Phe}. They were transduced with a retrovirus carrying human wild-type *p53* cDNA, and the growth of virus-transduced colonies was analyzed. The results of these experi-

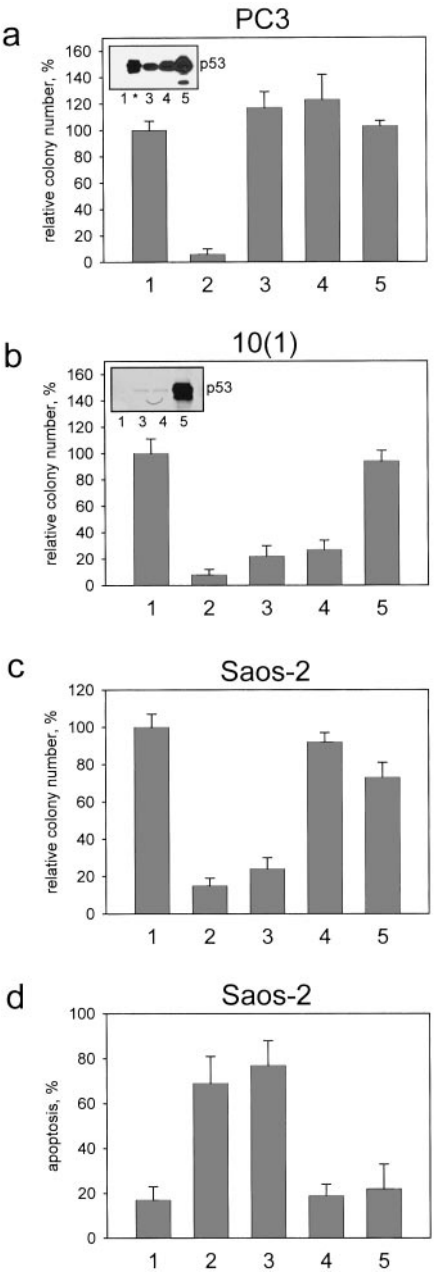

Fig. 1. Influence of Du145-derived p53 mutants on colony growth or viability of PC3 (*a*), 10(1) (*b*), and Saos-2 (*c*) cells. Panels *a–c* show the effects of the transduction of insert free vector (*1*) or vectors, expressing wild-type p53 (*2*), p53–223^{Leu} (*3*), p53–274^{Phe} (*4*), or both p53–223^{Leu} plus p53–274^{Phe} (*5*) on the colony growth. Cells were transduced with combinations of either two insert-free vectors (pPSH and pLPCX), or vector, expressing above-mentioned construct plus insert free vector or two vectors, expressing either p53–223^{Leu} or p53–274^{Phe} mutants. All of the combinations of vectors provide infected cells with simultaneous resistance to hygromycin and puromycin. Colonies were counted after completion of the selection on the combination of hygromycin (120 μg/ml) and puromycin (1 μg/ml) and were presented as a percentage of the colonies formed by the cells transduced with empty vectors. Before infection, virus titers were normalized based on the infection of Rat1 cells, known to be resistant to any form of p53. Experiments were repeated several times using wild-type p53 or p53 mutants, cloned in both vectors, and the data presented are the average of effects, caused by indicated cDNAs, cloned in both vectors. Boxes inside panels *a* and *b* represent Western blots of p53 mutants expression (amount of lysates used were normalized by protein concentration, data not shown). *, lane with the lysate of Du145 cells. *d*, proportion of apoptotic cells among Saos-2 cotransfected by the following constructs (in combination with green fluorescent protein-expressing plasmid): empty vector pPSH (*1*), p53–223^{Leu} (*2*), p53–274^{Phe} (*3*), wild-type p53 (*4*) and p53–223^{Leu} and p53–274^{Phe} together (*5*). Proportion of apoptotic cells among "green" cells was estimated 48 h after transfection by FACS analyses of the level of sub-G_1 cell fraction by using CellQuest software.

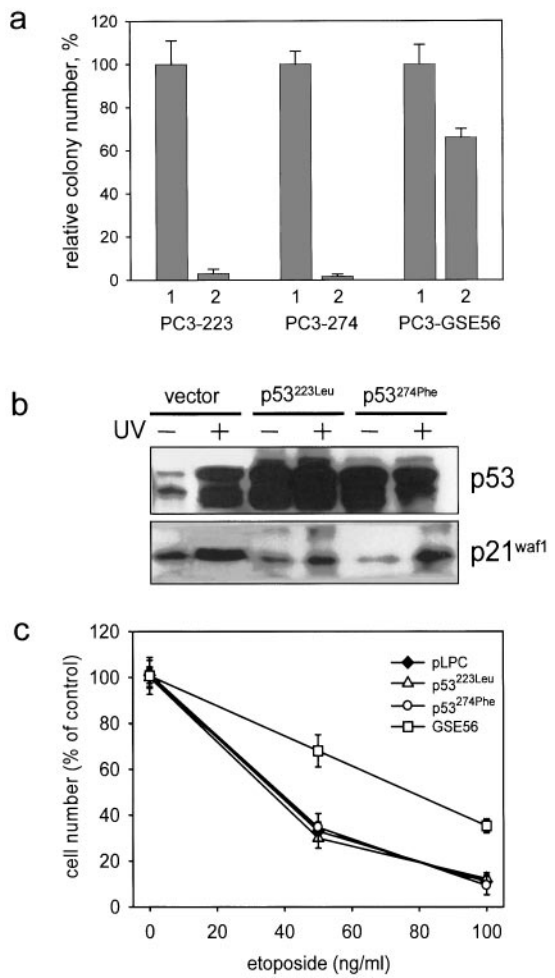

Fig. 2. Lack of dominant-negative activity of p53–223^{Leu} and p53–274^{Phe} mutants against wild-type p53. *a*, growth of colonies of PC3-p53–223^{Leu}, PC3-p53–274^{Phe}, and PC3-GSE56 cells after transduction with wild-type p53. PC3-p53–223^{Leu}, PC3-p53–274^{Phe}, and PC3-GSE56 cells were infected with retroviruses containing empty vector (*1*) and wild-type p53 (*2*). Cells were selected in the medium with appropriate antibiotic, and the number of colonies was counted after 2 weeks and were calculated as a percentage of the number of colonies in the case of empty vector. *b*, C8 cells retain ability to induce p21^{waf1} protein after transduction of the p53–223^{Leu} and p53–274^{Phe} mutants. Western blot analyses of p53 and p21^{waf1} expression in total cell lysates of C8 cells before, or 8 h after, UV treatment. *c*, p53–223^{Leu} and p53–274^{Phe} mutants do not protect C8 cells from etoposide. Cells were plated in 24-well plates in concentration 5×10^4 cells per well. After attachment, medium with etoposide was added, and cell numbers were estimated 72 h later by methylene blue staining and were calculated as a percentage of the cells that were cultivated without etoposide.

ments (Fig. 2*a*) show that neither of the DU145-derived mutants protected PC3 cells from growth inhibition by wild-type p53. Cells expressing previously isolated dominant-negative mutant GSE56 (22) were protected from wild-type p53 activity under these conditions (Fig. 2*a*).

The second type of assay was done in mouse C8 cells (mouse embryo fibroblasts transformed by the combination of *E1A* and *ras* oncogenes), a conventional model for testing p53-dependent apoptosis (23). C8 expressing either p53–223^{Leu} or p53–274^{Phe} were generated by retrovirus transduction. C8 cells could tolerate high expression levels (confirmed by Western blotting; see Fig. 2*c*) of either of the tested mutants without any detectable changes in cell growth or morphology (not shown). Moreover, C8 cells expressing DU145-derived mutants are still capable of inducing p21^{waf1} on DNA damage (UV). However, neither p53–223^{Leu} nor p53–274^{Phe} protected C8 cells from p53-dependent apoptosis caused by serum withdrawal or by DNA-damaging drug treatment (etoposide, doxorubicin), as opposed to control dominant-negative p53 mutants p53–175^{His} or GSE56 (Refs. 24, 25; Fig. 2*b*). Thus, DU145 p53 mutants do not show dominant-negative effect against endogenous wild-type p53 in C8 cells.

p53–274^{Phe} Can Abrogate Growth-suppressive Activity of p53–223^{Leu}. We then tested whether p53–223^{Leu} and p53–274^{Phe} could affect each other's properties if coexpressed within the same p53-deficient cells, thus imitating the situation existing in DU145 cells. p53-deficient cells were infected with the combination of two retroviruses, carrying different selectable markers, followed by the selection of cells that coexpress both constructs. Combinations used included each of the DU145-derived p53 mutants coexpressed with either the insert-free vector or with another mutant.

In PC3 cells, coexpression of both mutants did not have any detectable effect on colony growth (Fig. 1*a*). In 10(1) cells, previously shown to be sensitive to each mutant separately, their combination did not show any toxicity (Fig. 1*b*). In Saos-2 cells, which were sensitive to p53–223^{Leu}, expression of p53–274^{Phe} almost completely neutralized the growth suppressive effect of p53–223^{Leu} (Fig. 1*c*). In transient transfection assays, coexpression of p53–274^{Phe} with p53–223^{Leu} resulted in ~3-fold less proportion of apoptotic cells than in the population transduced with p53–223^{Leu} alone (Fig. 1*d*), indicating that the p53–274^{Phe} mutant can abrogate the residual proapoptotic activity of p53–223^{Leu}.

Thus, we have an interesting example of two tumor-derived p53 mutants that do not possess dominant-negative activity against wild-type p53 but are capable of neutralizing each other's growth inhibitory activity when expressed together. This observation suggests that the DU145-derived mutants could functionally interact with each other, presumably creating p53 protein with new properties.

Conformation of DU145-derived Mutants. Biological activity of p53 largely depends on conformation of DNA-binding domain. Proper folding of DNA-binding domain of p53 can be estimated experimentally by the potency of interaction with the conformation-sensitive antibody capable of distinguishing between wild-type and mutant

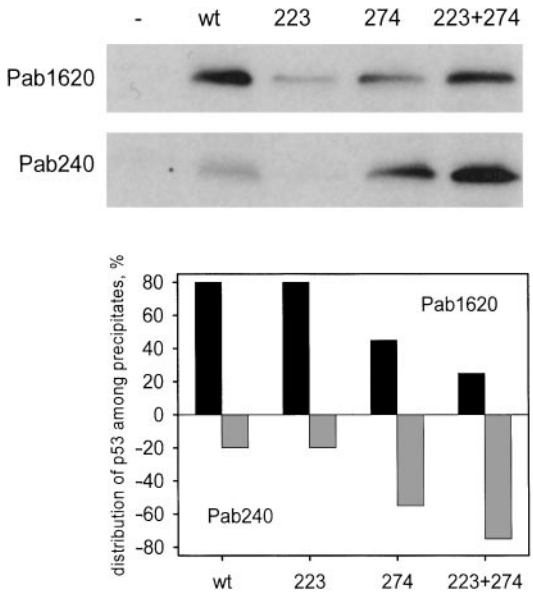

Fig. 3. Conformation of Du145-derived p53 mutants. Immunoprecipitation of the indicated variants of p53 from PC3 cells and wild-type p53 from MCF7 cells by antibodies to folded (Pab1620) or denatured (Pab 240) conformations of p53. *Lanes 1*, PC3 with empty vector. Western blots were probed with biotinylated polyclonal anti-p53 antibodies. For normalization, aliquots from each lysate were precipitated with conformation-insensitive DO1 antibody, and the relative amount of p53 was estimated as the ratios between p53 amounts in the precipitated obtained with Pab1620 or Pab240, and DO1 antibodies. *wt*, wild type.

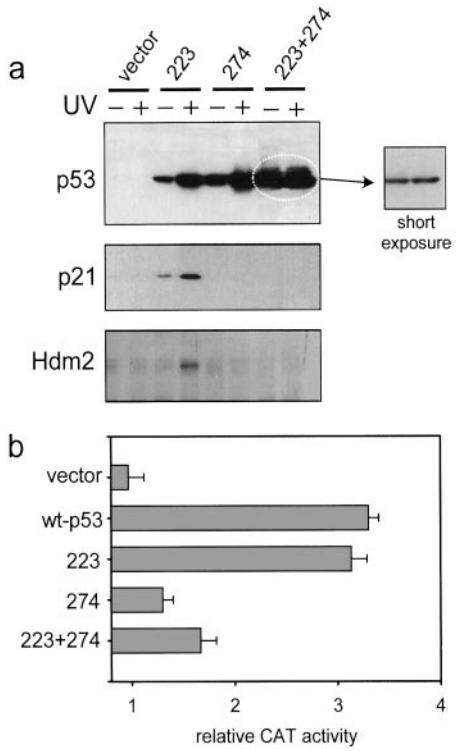

Fig. 4. Transactivation abilities of Du145-derived mutants in comparison with wild-type p53. *a*, induction of p53 protein and p53-responsive proteins in PC3 cells expressing indicated constructs. Western blot of total cell lysates of PC3 cells before, and 8 h after, UV treatment. *b*, CAT activity assay performed 48 h after cotransfection of 10(1) cells with pBasicCAT plasmid containing p53 responsive element and empty vector pLPC (vector), or wild-type p53 (wt-p53), p53–223^{Leu} (223), p53–274^{Phe} (274), combination of p53–223^{Leu} and p53–274^{Phe} (*223+274*). Efficiency of transfection was normalized by the addition of pCMV-β-gal vector to the transfection mixture and estimation of the β-galactosidase activity in cell lysates.

forms of the protein. We analyzed conformation of DU145-derived p53 mutants expressed in PC3 cells, separately and in combination, by immunoprecipitation with conformation-specific antibodies (Pab1620 and Pab240 recognizing preferentially wild-type and mutant p53, respectively) followed by p53 detection by biotinylated polyclonal antibodies on Western blots. As seen in Fig. 3, p53–223^{Leu} mutant retained wild-type conformation as confirmed by its ability to bind with Pab1620 antibodies. However, p53–274^{Phe} was precipitated well by both antibodies. Interestingly, combined expression of both mutants generated p53 protein with predominantly denatured-form DNA-binding domain preferentially binding to Pab 240.

Transactivation of p53-responsive Genes by p53–223^{Leu} and p53–274^{Phe} Mutants. Many p53 functions are mediated by transcriptional regulation of a set of p53-responsive genes. We tested the effect of permanent ectopic expression of each of the studied mutants in PC3 cells, as well as their combination, on the expression of two proteins encoded by major p53-regulated genes *p21/waf1* and *Hdm2* under normal conditions and after genotoxic stress (UV). We could not compare in these experiments the effect of the mutants with that of wild-type p53 because the high toxicity of wild-type p53 for PC3 cells made it impossible to generate a stable p53-expressing cell population. Both of the DU145-derived mutants retain the ability to accumulate in response to UV- (Fig. 4*a*) and doxorubicin-induced (data not shown) DNA damage in PC3 cells (Fig. 4*a*). Hence, although the two mutant proteins studied differed in their conformation, their ability to be accumulated in response to DNA damage makes them similar to wild-type p53. Interestingly, cells expressing a combination of both mutants did not show any changes in the amount of p53 protein after DNA damage (Fig. 4*a*), suggesting that properties of the mixed p53 protein complex formed by a combination of two DU145-derived mutants may be different from the properties of the proteins formed by each of the subunits expressed separately.

Both p21^{waf1} and Hdm2 proteins are expressed at very low levels in PC3 cells, undetectable by Western immunoblotting. p21^{waf1} can be detected in PC3 expressing p53–223^{Leu}; its expression was increased after treatment with UV light. Neither p53–274^{Phe} nor the combination of both mutants induced p21^{waf1} to detectable levels (Fig. 4*a*). Similar results were obtained with Hdm2 expression. Detectable levels of Hdm2 were observed only in the case of PC3 expressing p53–223^{Leu} mutant after treatment with UV (Fig. 4*a*), again indicating that p53–274^{Phe} as well as the combination of mutants were incapable of visible induction of a p53-responsive gene.

Transactivation abilities of DU145-derived mutants were also tested in a direct transactivation assay using transient transfection of different p53-expressing constructs in combination with a CAT reporter construct driven by the promoter with p53-binding site from the *p21^{waf1}* gene (26). In these experiments p53–223^{Leu} was as active as wild-type p53 in transactivation of the reporter construct, whereas p53–274^{Phe} had no detectable activity (Fig. 4*b*). Coexpression of p53–274^{Phe} inhibited transactivation by p53–223^{Leu}, thus correlating with the above-described effects of this mutant on growth-inhibitory

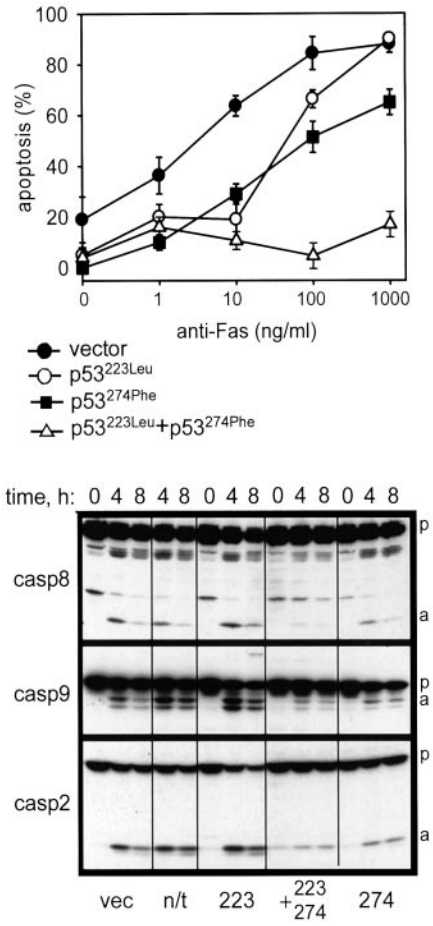

Fig. 5. Sensitivity of PC3 cells, expressing p53 mutants, to treatment with anti-Fas mAb. *Upper panel*, PC3 cells, transduced with indicated constructs, were treated during 48 h with anti-Fas mAb, and then cell death was estimated by calcein assay (see "Materials and Methods"). *Lower panel*, Caspase 8 (*casp8*), -9 (*casp9*), and -2 (*casp2*) activation in PC3 cells, expressing indicated constructs after anti-Fas mAb treatment. Western blot of total cell lysates collected at different indicated time points after addition of anti-Fas mAb. *p*, the position of protein bands corresponding to procaspases; *a*, the position of protein bands corresponding to activated caspases.

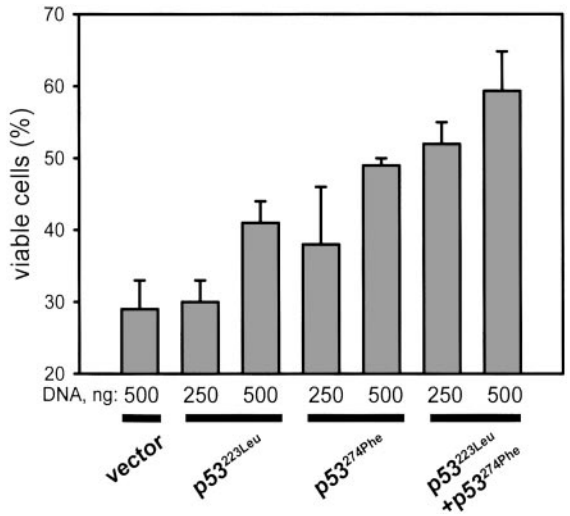

Fig. 6. Effect of combination of p53–223^{Leu} and p53–274^{Phe} mutant expression on cell protection from Fas-induced apoptosis. Cells were treated with 100 ng/ml anti-Fas antibodies 24 h after transfection with different amounts of indicated constructs. Twenty-four h later, nonfixed cells were stained with PI and analyzed by FACS analyses by the use CellQuest software. The proportion of "green" but PI-negative cells among all transfected (green) cells was estimated.

activity of p53–223^{Leu}. The lack of detectable transactivation activity of p53–274^{Phe} suggests that the toxicity of this protein to 10(1) cells is mediated not through p21.

DU145-derived p53 Mutants and Fas Sensitivity of PC3 Cells. PC3 cells and DU145 cells differ dramatically in their sensitivity to Fas-mediated apoptosis (16). Whereas PC3 cells are highly sensitive, DU145 are completely resistant even to a high dose of anti-Fas antibodies. We compared the sensitivity to Fas-mediated apoptosis of parental PC3 cells and PC3 cells expressing p53–223^{Leu}, p53–274^{Phe}, or their combination. Fig. 5 demonstrated the dose dependence of these variants of PC3 cells to the Fas ligand agonist, anti-Fas mAb. Cell death was estimated by calcein and DNA fragmentation assays. Both methods revealed strong protection of PC3 from anti-Fas mAb-induced apoptosis by the combination of two DU145-derived mutants (results of a representative calcein assay are shown in Fig. 5). The results of cellular assays were confirmed by the analysis of caspase activation in p53 mutant-transduced PC3 cell variants (Fig. 5, *bottom panel*). Activated forms of caspases 8, -2, and -9 were detected in cell lysates as early as 4 h after treatment of PC3 cells, transduced with empty vectors or with p53–223^{Leu} mutant. p53–274^{Phe} mutant decreased the level of activated caspases, whereas a combination of both mutants prevented the appearance of activated forms of all of the studied caspases at the 4th and 8th h after adding anti-Fas mAb (Fig. 5).

Because PC3 cells that expressed each of the studied mutants separately demonstrated only partial resistance to Fas, as compared with the cells coexpressing both mutants together, we decided to check whether the effect of combined expression would be stronger than each mutant alone. We, therefore, analyzed the dependence of Fas sensitivity on the dose of mutant p53 in a transient transfection assay combined with the anti-Fas antibody treatment. In these experiments, apoptosis in transfected cells was detected by FACS analysis (proportion of cells in sub-G_1 DNA fraction among transfected cells; Fig. 6). We found that both p53–223^{Leu} and p53–274^{Phe} induced Fas resistance in a dose-dependent manner; however, neither of them alone was as powerful in protection from apoptosis as their combination suggesting that the mutants act in an additive way.

Thus, we were able to transfer Fas-resistant phenotype of DU145 to Fas sensitive PC3 by coexpression of two DU145-derived p53 mutants. Fas resistance of DU145 cells may be overcome by treatment with cycloheximide, suggesting the involvement of a labile inhibitor (16). Interestingly, cycloheximide restored Fas sensitivity in mutant-transduced PC3 cells similarly to what it does to DU145 (Fig. 7*a*). This phenomenon, however, can hardly be attributed to cycloheximide-mediated reduction of the p53-mutant proteins because prolonged (up to 31 h) treatment with cycloheximide showed no dramatic changes in the levels of the mutant p53 (Fig. 7*b*). Thus, if Fas resistance is, in fact, mediated by some unstable protein factor, it is unlikely to be mutant p53 itself but, rather, it could be one of the proteins induced by the p53 mutants.

The most obvious mechanism of Fas resistance of tumor cells could be associated with the loss of membrane Fas. In fact, membranes of PC3 cells that were transduced with p53–274^{Phe} contained only 27% of Fas protein as compared with cells that were transduced with empty vector, as judged by FACS analyses with anti-Fas antibodies (data not shown). To elucidate possible mechanisms of anti-Fas activity of DU145-derived p53 mutants we compared the levels of Fas protein in lysates of PC3 cells transduced with empty vector, or with constructs expressing p53–223^{Leu} and p53–274^{Phe}, either separately or in combination. The results shown in Fig. 8 demonstrate that p53–223^{Leu} expression is associated with a slight decrease in the amount of Fas. The expression of p53–274^{Phe} results in a more remarkable (~2-fold) reduction of Fas expression as compared with that in control vector-transduced PC3. The combination of both mutants has a synergistic effect on the reduction of Fas, exceeding the effect of a "double dose"

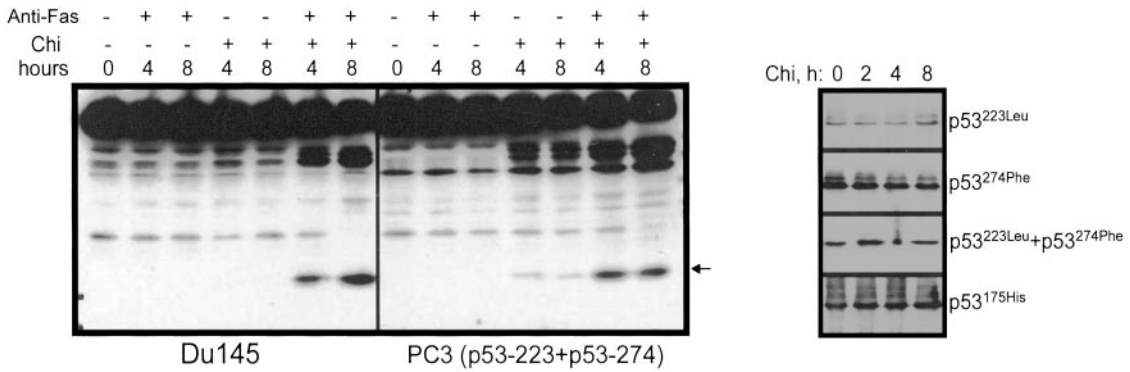

Fig. 7. Cycloheximide (*Chi*) treatment sensitizes Du145 and PC3 cells, expressing combination of the DU145-derived mutants, to Fas-mediated apoptosis. *a*, Western blot detection of caspase 8 activation in Du145 and PC3 cells, expressing combination of DU145-derived p53 mutants, after treatment with anti-Fas mAb (time indicated) in the presence and in the absence of cycloheximide. *b*, stability of p53 protein in PC3 cells in the presence of cycloheximide (3 μg/ml). Western blot analysis (DO1 antibody) of lysates of PC3 cells, expressing indicated constructs, were prepared at different time points after the addition of cycloheximide in cell culture medium.

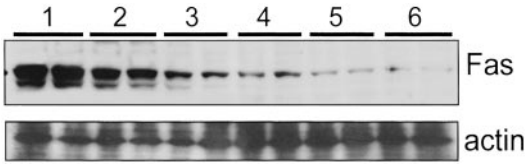

Fig. 8. Determination of Fas protein in PC3 cell lysates by Western immunoblotting (in duplicates). Lysates of PC3 cells transduced with: insert-free vector (*1*), p53–223^{Leu} (*2*), p53–274^{Phe} (*3*), two different p53–274^{Phe}-carrying viruses and expressing double dose of mutant p53 from two different promoters (*4*), and the combination of p53–223^{Leu} and p53–274^{Phe} (*5* and *6*; represent two independently transduced cell cultures).

of p53–274^{Phe} reached by coexpression of two retroviral vectors (pPS-hygro and pLPC) in the same cells.

DISCUSSION

Both alleles of the *p53* gene in prostate carcinoma cells DU145 carry mutations, resulting in the coexpression of two mutant polypeptides with different amino acid substitutions (Pro to Leu in 223, and Val to Phe in 274 codons), both within the DNA-binding domain (14). None of these mutations belong to "hot spots," although codon 274 is located between two frequently mutated codons of p53 (273 and 275). There could be several alternative explanations for the coexistence of two different p53 mutants in the same tumor: (*a*) each mutation results in a simple inactivation of p53 creating a phenotype that is functionally equivalent to p53 deficiency; (*b*) one of the mutants has a gain-of-function activity, whereas another mutation just neutralizes the other allele; and (*c*) there is a certain form of cooperation between the mutants creating additional selective advantages to cancer cells. To distinguish among these possibilities, we characterized the biological properties of each of the DU145-derived mutants in comparison with wild-type p53 after transduction into several p53-deficient cell lines, separately and in combination. We found that both of them are different from wild-type protein in at least some of their properties. Although p53–274^{Phe} has more features of a "real" cancer-derived mutant (maintains the denatured conformation of the DNA-binding domain, lacks transactivation ability) than the p53–223^{Leu} one (wild-type conformation, transactivation ability), they both showed some properties that are unusual for tumor-derived mutants, and they more closely resemble wild-type p53 (can be stabilized by DNA damage, cause growth suppression in some cell types, do not possess dominant-negative activity). However, these relatively "weak" mutants create p53 protein with new structural and functional properties when coexpressed in one cell. p53, formed by the combination of mutants, keeps a predominantly denatured conformation, is accumulated in large amounts in the cell and does not show any increase after genotoxic stress, has no toxicity to any cells tested, and lacks transactivation function. Properties of the protein formed by the combination of mutants are clearly different from what could be expected from a simple superimposition of the individual properties of each of them. Thus, growth-suppressive activity of either of the mutants, detectable in mouse 10(1) (both mutants) and human Saos-2 cells (p53–223^{Leu}), is completely inactivated when they are coexpressed. Synergism of the DU145-derived mutants was revealed in their effect on cell sensitivity to Fas, the most unusual property of these mutants. Whereas each of them alone had some, albeit weak, suppressive effect on the Fas sensitivity of transduced PC3 cells, the combination of p53–274^{Phe} and p53–223^{Leu} caused a strong anti-Fas effect presumably acting by down-regulating Fas protein expression. Transduction of the combination of 274^{Phe} and p53–223^{Leu} mutants into Fas-sensitive PC3 cells makes them resistant to Fas, as is the DU145 cell line from which these mutants are derived, suggesting that they might serve a similar function in DU145. The generation of p53 protein with new properties from a combination of two rather weak mutants and the strong inhibition of Fas-mediated apoptosis by the resulting p53 protein are two major conclusions that came out of the present work.

Inactivation of both p53 and Fas signaling are frequent events in cancer that play an important role in cancer progression. Whereas the suppression of p53 results in genomic instability and rapid progression (27), the blocking of Fas sensitivity contributes to tumor escape from host immune response (28). In addition, both may increase tumor resistance to anticancer treatment through the suppression of apoptosis (23, 29). Thus far, there have been two mechanistic links between these two pathways. In some cells, Fas behaves as a p53-responsive gene (30) and Fas sensitivity could be modulated in a p53-dependent manner after DNA-damaging treatments *in vitro* and *in vivo* (31). p53 protein was also shown to be involved in intracellular transport of Fas (32). In both cases, p53 cooperates with Fas expression. Therefore, p53 deficiency is expected to attenuate Fas-mediated apoptosis. In fact, a correlation between p53 status of the tumor and its sensitivity to Fas has been experimentally confirmed by the analysis of a series of isogenic tumor cell lines differing in their p53 status (31). Presumably, attenuation of sensitivity to Fas is one of the driving forces behind the selection of tumor cell variants with inactivated p53.

Although the inactivation of p53 may cause a reduction in cell sensitivity to Fas-induced apoptosis, it, nevertheless, cannot make the tumor cell completely Fas resistant; Fas sensitivity of p53-deficient PC3 cells (16) is an illustration for this statement. However, transduction with p53 mutants derived from Fas-resistant cells of prostate origin, DU145, converts PC3 into Fas resistance, indicating a new type of Fas-p53 interaction, in which altered p53 protein plays the role of an active inhibitor of Fas-mediated apoptosis, which causes a strong down-regulation of Fas expression. This phenomenon demonstrates a new gain-of-function activity of mutant p53.

Gain-of-function has been reported for many tumor-derived p53 mutants (5, 13, 21). Mutant p53 can acquire alterations in their transactivation capabilities (13, 33) and increase cell resistance to cytotoxic drugs even when transduced on a p53-deficient background. Down-regulation of Fas expression accompanied by resistance to Fas-mediated apoptosis is a new type of gain-of-function by p53 mutants. It is likely that mutants with such unusual properties were picked by natural selection because they provide additional selective advantages to tumor cells *in vivo*. Although each of the DU145-derived mutants possesses this anti-Fas property, their combined expression generates p53 protein with much stronger anti-Fas activity, providing an unusual example of gain-of-function that presumably results from the combination of two mutant p53 subunits in one protein.

Functional interaction of structurally different p53 monomers within one protein has been known for a long time as a phenomenon associated with dominant-negative mutants. It is believed that incorporation of a mutant subunit into a p53 homotetramer may result in its functional inactivation (21). In this case, one allele (inactive) clearly dominates over another one (wild-type). DU145-derived mutants, however, represent another more complicated form of functional interaction of two altered proteins. Although none of them alone showed dominant-negative activity against wild-type p53, in combination they can modify each other's properties, presumably by creating a mixed protein complex consisting of two mutant forms of p53 protein that is not functionally equivalent to the homogeneous complexes formed by any of the subunits.

In principle, another mechanism could be responsible for the observed phenomenon. Instead of the formation of a putative "mixed" protein complex with new properties, Fas resistance may result from combined changes in gene expression caused by each of the mutants.

It this scenario, DU145-derived mutants would cooperate indirectly through their responsive genes. Although we cannot unequivocally choose between the two models, altered biochemical properties of p53 protein, resulting from the coexpression of the studied mutants, argues in favor of the first possibility.

Fas-suppressing p53 mutants can be viewed as therapeutic targets to develop compounds that would cause antitumor effect by sensitization to Fas. However, it remains unclear how common is the anti-Fas activity of tumor-derived p53 mutants; this question is the subject of our ongoing study.

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Cooperation of Two Mutant *p53* Alleles Contributes to Fas Resistance of Prostate Carcinoma Cells

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