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Mammalian cells resistant to tumor suppressor genes

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ABSTRACT **Expression of p53 causes growth arrest or apoptosis in many normal and neoplastic cell types, but the relationship between these two effects has remained obscure. To begin to dissect the underlying mechanisms at a genetic level, we have generated mutant cells resistant to the action of wild-type p53. Rat embryo fibroblasts transformed with *ras* and a temperature-sensitive p53 (tsp53^{135val}) gene were chemically mutagenized and selected for growth at a temperature at which p53 adopts a wild-type conformation (31.5°C). Clones that grew exponentially at 31.5°C were selected. Cell fusion experiments demonstrated that the mutations conferring resistance to p53-mediated growth arrest were dominant. The mutagenized clones were resistant not only to p53-mediated growth arrest, but also to the apoptosis induced by E1A in conjunction with p53, and partially resistant to the retinoblastoma tumor suppressor, pRB. The results suggest that a single downstream pathway can control the induction of growth arrest and apoptosis, and that both p53 and RB function through this pathway.**

Following DNA damage, mammalian cells either undergo growth arrest, or programmed cell death (apoptosis). Both of these responses appear to be mediated by the tumor suppressor gene, p53 (1–4). Cells lacking functional p53 protein fail to arrest in G1 following exposure to ionizing radiation and are resistant to apoptosis induced by DNA damage (5–8). In normal cells, DNA damage stimulates rapid induction of p53, followed by transcriptional transactivation of p53 target genes. p53-mediated control of the cell cycle is associated with transcriptional up-regulation of various target genes, including p21$^{WAF1/CIP1}$ (9). This gene encodes a protein product involved in regulating enzymatic activities necessary for G1 and S phase progression (10, 11).

The variable sensitivities of different cells to DNA damage-induced apoptosis may relate to cell-specific differences in cell cycle regulation. In murine erythroleukemia cells lacking a wild-type (wt) p53, transfection and expression of a temperature-sensitive p53 leads to apoptosis following expression of wt p53 protein (12). In certain cell types, p53-mediated apoptosis is associated with the transcriptional activation of the *bax* gene (13–15). It is proposed that by forming heterodimers with bcl2 and inhibiting bcl2 functions, bax accelerates apoptosis (16). In agreement with these findings, Sabbatini and coworkers have demonstrated the necessity of p53-mediated transcriptional activation in apoptosis (17). However, another study has shown that this activity was not necessary for p53-mediated apoptosis (18). The precise mechanisms that determine if DNA damage causes cell cycle arrest or apoptosis are not well defined but likely involve integration of both intracellular and extracellular signals. For example, Canman and coworkers (4) demonstrated that an interleukin 3-dependent hematopoietic cell line was capable of undergoing either G1 arrest or apoptosis following initiation of DNA damage. The final outcome in this system was determined by the presence or absence of interleukin 3.

To better understand the relationship between p53-mediated growth arrest and apoptosis, we have generated mutant cells resistant to the action of wt p53. A rat embryonal fibroblast cell line, transformed with Ha-*ras* and a temperature-sensitive p53 (tsp53^{135val}) gene (19), was mutagenized and selected for clonal growth of cells at a temperature at which p53 adopts a wt conformation (31.5°C). Clones that grew exponentially at 31.5°C and displayed resistance to the actions of biochemically active p53 protein were identified. The data acquired with these cells imply that a single downstream pathway may control the induction of growth arrest and apoptosis, and that both p53 and RB function through this pathway.

MATERIALS AND METHODS

Cell Culture, Growth, and Transfection Assays. Rat embryo fibroblasts (clone 112) (19) were cultured in DMEM (high glucose) with 10% fetal bovine serum and 1% penicillin/streptomycin. For growth assay experiments, 500 cells were plated and shifted to the indicated temperature following attachment to tissue culture flasks at 38°C for 24 hr.

For transfection experiments, cell lines were transfected with 2.5 μg of indicated plasmid and 18 μg of Lipofectamine (Bethesda Research Laboratories) in 25-cm^2 flasks. Twenty-four hours after transfection, Hygromycin B (Calbiochem) was added to a final concentration of 250 μg/ml. Colonies containing at least 20 cells were counted after 10 days of selection. Individual clones, stably transfected with pCEP4-E1A, were isolated, characterized for E1A protein expression, and subjected to growth analysis as described below.

Expression Vectors. The parental plasmid used in the transfection experiments was pCEP4 (Invitrogen). Construction of the pCEP4 plasmid containing either wt or mutant (mut) p53 cDNAs has been reported (20). pCEP4-E1A was constructed by inserting an *Eco*RI/*Pst*I fragment containing the adenovirus type 5 early region 1A coding region from pC1A (21) into pCEP4. pCEP4-pRB was constructed by subcloning the *Bam*HI fragment containing the pRB coding region isolated from pHRbcSVE (kindly provided by P. Hinds and R. Weinberg, Massachusetts Institute of Technology, Boston, MA) into pCEP4.

Chemical Mutagenesis. Cells (10^9) at 50% confluence were treated with 115 μg/ml methanesulfonic acid ethyl ester (EMS) for 24 hr. Following mutagenesis, the cells were grown in complete medium for 24 hr, after which time the cultures were shifted to the selective temperature (31.5°C). Colonies became apparent 1–3 weeks after temperature shift and were isolated and expanded.

DNA Fragmentation. Total genomic DNA was isolated from the indicated cell lines grown at either 38°C or 31.5°C. The

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Abbreviations: wt, wild-type; mut, mutant; EMS, methanesulfonic acid ethyl ester.

Table 1. Effect of temperature shift on cell growth

Cell line	Colonies formed at the indicated temperature			
	38°C		315°C	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
P3	154, 189	>200, >200	0, 0	0, 0
P7	>200, >200	>200, >200	0, 0	0, 0
B1	183, >200	>200, >200	179, 194	>200, >200
E4	>200, >200	>200, >200	>200, >200	>200, >200

Approximately 500 cells were plated and shifted to the indicated temperature following attachment to tissue culture flasks at 38°C for 24 hr. Two independent determinations are shown for each experiment (Exp.).

integrity of the genomic DNA was evaluated by agarose gel electrophoresis (1.5% agarose) and ethidium bromide staining.

Cell Fusions. Cell lines were transfected with pCEP4 (Invitrogen) and pMSVPAC (22) as described above. Hygromycin B (Calbiochem) or puromycin (Sigma) was added 24 hr later, and colonies were selected after 10 days. Cells (1.3×10^6) of indicated type were mixed, pelleted, and washed in serum-free medium. The cell pellet was resuspended in 0.5 ml of 50% polyethylene glycol 1450 (Sigma), and fusion was terminated 1 min later by addition of culture medium. The hybrid cells were plated (500 cells per flask), and selection was begun 24 hr later.

Immunoblot Analysis. For detection of p53 and E1A proteins, 50 μg of protein was resolved on an SDS/10% polyacrylamide gel, transferred to Immobilon P membrane (Millipore), and incubated with the monoclonal, human-specific anti-p53 antibody PAb1801 and the anti-E1A antibody M73 (Oncogene Research Products, Calbiochem). For detection of p21 protein, 50 μg of total protein per lane was loaded and resolved on an SDS/12% polyacrylamide gel, transferred to Immobilon P membrane, and incubated with a mouse monoclonal anti-p21 antibody, clone 22 (Oncogene Research Products, Calbiochem). For detection of pRB, 50 μg of total protein per lane was loaded and resolved on an SDS/8% polyacrylamide gel, transferred to Immobilon P membrane, and incubated with the monoclonal anti-pRB antibody IF8 (Santa Cruz Biotechnology).

RESULTS

Generation of p53-Resistant Cell Lines. To obtain mutant cells resistant to the action of wt p53, rat embryo fibroblasts transformed with Ha-*ras* and $tsp53^{135val}$ (19) were chemically mutagenized with EMS and selected for growth at 31.5°C, a temperature at which $tsp53^{135val}$ adopts a wt conformation. Of 10^9 cells mutagenized, 19 formed clones that grew exponentially at 31.5°C. Two of the mutagenized clones (B1 and E4) and two unmutagenized clones of parental cells (P3 and P7) were isolated and examined further. Unlike the P3 and P7 cells, mutagenized B1 and E4 cells grew robustly at both 31.5°C (wt p53 conformation) and 38°C (mut p53 conformation) (Table 1).

Confirmation of p53 Biochemical Activity in the Resistant Cell Lines. Several experiments were performed to rule out the possibility that EMS mutagenesis targeted the $tsp53^{135val}$ gene, thus altering the biochemical activity of the p53 protein produced at 31.5°C. Expression plasmids encoding wt or an *in vivo*-derived mutant form of human p53 (Arg-175 → His) (23) were transfected into the cells, and the hygromycin B-resistant colonies were counted 10 days later. P3 and P7 cells transfected with the plasmid expressing wt p53 formed at least 70-fold fewer visible colonies than those transfected with the *in vivo*-derived mutant. In contrast, the B1 and E4 cells transfected with plasmids expressing either wt or mutant p53 formed a similar number of visible colonies (Table 2). Immunohistochemical and Western blot analysis of the cell lines

Table 2. Effect of wt and mut p53 protein expression on cell growth

Cell line	Protein expressed from pCEP4 vector	No. of colonies at 38°C
P3	—	>200, >200
P3	wt p53	3, 2
P3	mut 175	>200, >200
P7	—	>200, >200
P7	wt p53	0, 0
P7	mut 175	>200, >200
B1	—	>200, >200
B1	wt p53	>200, >200
B1	mut 175	>200, >200
E4	—	>200, >200
E4	wt p53	>200, >200
E4	mut 175	>200, >200

The indicated cell lines were transfected with control pCEP4 plasmid (—) or pCEP4 plasmid expressing wt or mut p53 protein. Colonies containing at least 20 cells were counted after 10 days of selection. Two independent determinations are shown.

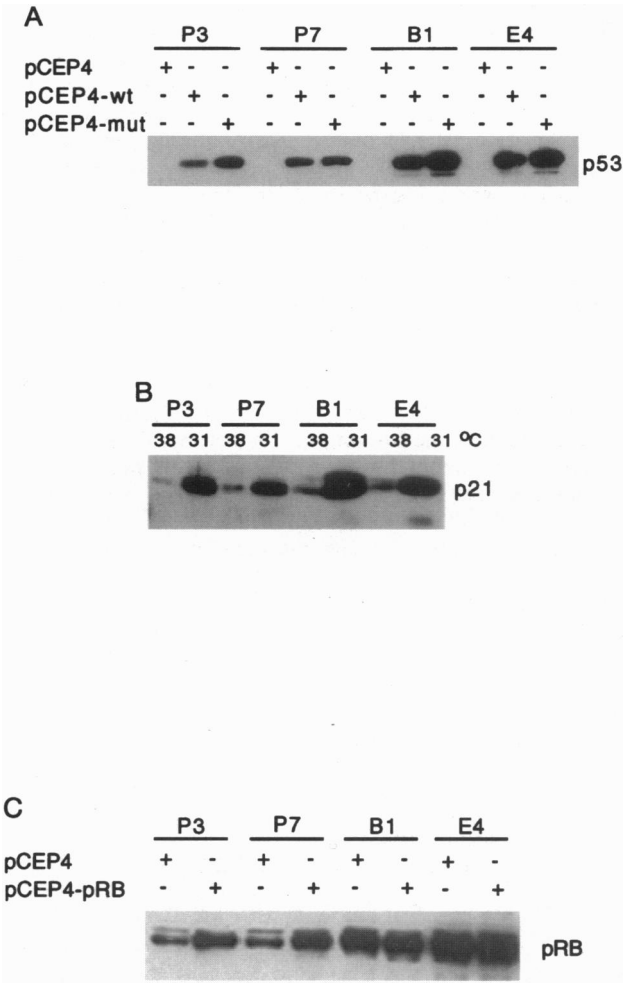

FIG. 1. (*A*) Exogenous p53 protein expression in transfected cells. Immunoblot of p53 protein harvested from cells 24 hr after transfection of indicated expression vectors. (*B*) Effect of temperature shift on p21 protein expression. Expression of p21 protein in P3, P7, B1, and E4 cell lines after growth at the indicated temperatures for 24 hr (P3 and P7) or 7 days (B1 and E4). Expression of p21 in B1 and E4 cells was similar to that in P3 and P7 cells at 24 hr. (*C*) pRB expression in transfected cells lines. Immunoblot of pRB protein harvested from P3, P7, B1, and E4 cells transfected with indicated plasmid.

Table 3. Effect of temperature shift on hybrid cell growth

Hybrid	Cell lines fused to form hybrid: Hygromycin- (pCEP-4)*	Cell lines fused to form hybrid: Puromycin- (pMSCVPAC)*	No. of colonies formed at indicated temperature: 38°C	No. of colonies formed at indicated temperature: 31.5°C
F18-5A	P3	P7	151, 132	1, 3
F18-2A	P3	P7	161, 143	2, 0
F7-1A	P7	P7	218, 232	0, 0
F7-2B	P7	P7	141, 163	0, 0
F1-1A	E4	P7	159, 151	121, 137
F1-2D	E4	P7	187, 175	207, 229
F10-2A	B1	P7	131, 148	115, 135
F10-5B	B1	P7	161, 143	139, 152
F8-1B	B1	E4	175, 191	182, 201
F8-2A	B1	E4	252, 218	241, 221

Two independent determinations are shown.
*Drug resistance conferred to cell line by transfection of indicated vector.

confirmed that p53 protein expressed from the pCEP4 vectors was localized to the nucleus (data not shown) and produced in all cell lines (Fig. 1*A*). The B1 and E4 clones were thus not simply resistant to the effects of the tsp53 gene present during the mutagenesis, but also to exogenously introduced p53.

To ensure that the p53 protein expressed from the $tsp53^{135val}$ gene at 31.5°C retained normal biochemical activity as a transcriptional activator, the level of an endogenous target of p53 transactivation, $p21^{WAF1/CIP1}$ (9), was measured in cells grown at both 31.5°C and 38°C. A similar temperature-dependent increase in p21 protein expression was observed in all four lines, and this expression continued for at least 7 days of exponential growth at 31.5°C in the B1 and E4 cells (Fig. 1*B*). Additionally, the sequence-specific transcriptional activity of the $tsp53^{135val}$ protein was evaluated by transfection of a construct consisting of a p53-DNA binding site upstream of a minimal promoter and chloramphenicol acetyltransferase reporter gene (20). Induction of similar levels of chloramphenicol acetyltransferase activity was observed in all the cell lines at 31.5°C, but no induction was seen at 38°C (data not shown).

p53-Resistance Is Dominant. To determine whether the p53-resistance phenotype in the B1 and E4 cells was genetically dominant or recessive, cell fusion experiments were performed. Each of the four cell lines were transfected with plasmids containing selectable markers for either hygromycin or puromycin. Cell fusions were performed with different combinations of cells containing the selectable markers. The resulting cell-hybrids were analyzed for clonal growth at both 38°C and 31.5°C. Hybrid clones derived from P3 or P7 cells fused to P7 cells were growth-arrested at 31.5°C. However, hybrid clones derived from either B1 or E4 cells fused to P7 cells resulted in hybrids that grew clonally at 31.5°C (Table 3).

Table 4. Effect of E1A protein expression on cell growth

Cell line	Protein expressed from pCEP4 vector	No. of colonies formed at the indicated temperature: 38°C	No. of colonies formed at the indicated temperature: 31.5°C
P3	—	>200, >200	0, 0
P3	E1A	>200, >200	0, 0
P7	—	>200, >200	0, 0
P7	E1A	>200, >200	0, 0
B1	—	>200, >200	>200, >200
B1	E1A	>200, >200	>200, >200
E4	—	>200, >200	>200, >200
E4	E1A	>200, >200	>200, >200

The indicated cell lines were transfected with control pCEP4 plasmid (—) or pCEP4 plasmid expressing E1A protein. Colonies containing at least 20 cells were counted after 10 days of selection. Two independent determinations are shown.

The growth assays with the cell hybrids suggested that the EMS-induced genetic alteration(s) conferring resistance to p53-mediated growth arrest was dominant.

Resistance to p53-mediated Apoptosis. Having demonstrated that the mutagenized cells were resistant to p53-mediated growth arrest, we determined whether the cells were resistant to another p53-mediated activity, apoptosis. It had previously been shown that coexpression of wt p53 with the viral oncoprotein adenovirus E1A (E1A) resulted in apoptosis rather then growth arrest (24–26). The cell lines were transfected with an expression plasmid encoding E1A driven by the cytomegalovirus promoter, and clones expressing E1A were examined for growth and apoptosis at 31.5°C. Pooled clones as well as individual clones of E1A-producing P3 and P7 cells did not form any visible colonies at 31.5°C but formed hundreds of colonies at 38°C (Fig. 2*A*; Tables 4 and 5). When shifted to the permissive temperature, the P3 and P7 subclones expressing E1A displayed a characteristic property of apoptosis, DNA fragmentation (Fig. 2*B*). In contrast, the B1 and E4 subclones expressing E1A were resistant to both p53-mediated growth arrest and apoptosis, as indicated by the presence of viable colonies (Fig. 2*A*, Tables 4 and 5) and by the absence of DNA fragmentation at the permissive temperature (Fig. 2*B*). All clones expressed similar levels of E1A protein (Fig. 2*C*).

Resistance to Another Tumor Suppressor. We next determined the sensitivity of the p53-resistant cells to constitutive synthesis of retinoblastoma protein (pRB). Cells were transfected with an expression plasmid encoding human pRB driven by the cytomegalovirus promoter. Following transfection and 10 days of hygromycin B selection, the P3 and P7 cells did not form any visible colonies, whereas 90–180 colonies formed from the B1 and E4 cells (Table 6). Before transfection, the levels of pRB in the E4 and B1 cells were markedly higher than those in P3 and P7 cells, confirming the resistance of E4 and B1 cells to pRB expression (Fig. 1*C*). After transfection, the

Table 5. Growth of individual clones expressing E1A

Clone	No. of colonies formed at the indicated temperature: 38°C	No. of colonies formed at the indicated temperature: 31.5°C
P3E1A3	203, 229	0, 0
P7E1A2	263, 218	0, 0
B1E1A4	247, 189	137, 150
E4E1A4	140, 167	89, 125

Individual cell lines, stably transfected with pCEP4-E1A, were subjected to growth analysis at the indicated temperatures. Approximately 500 cells were plated in each experiment. Two independent determinations are shown.

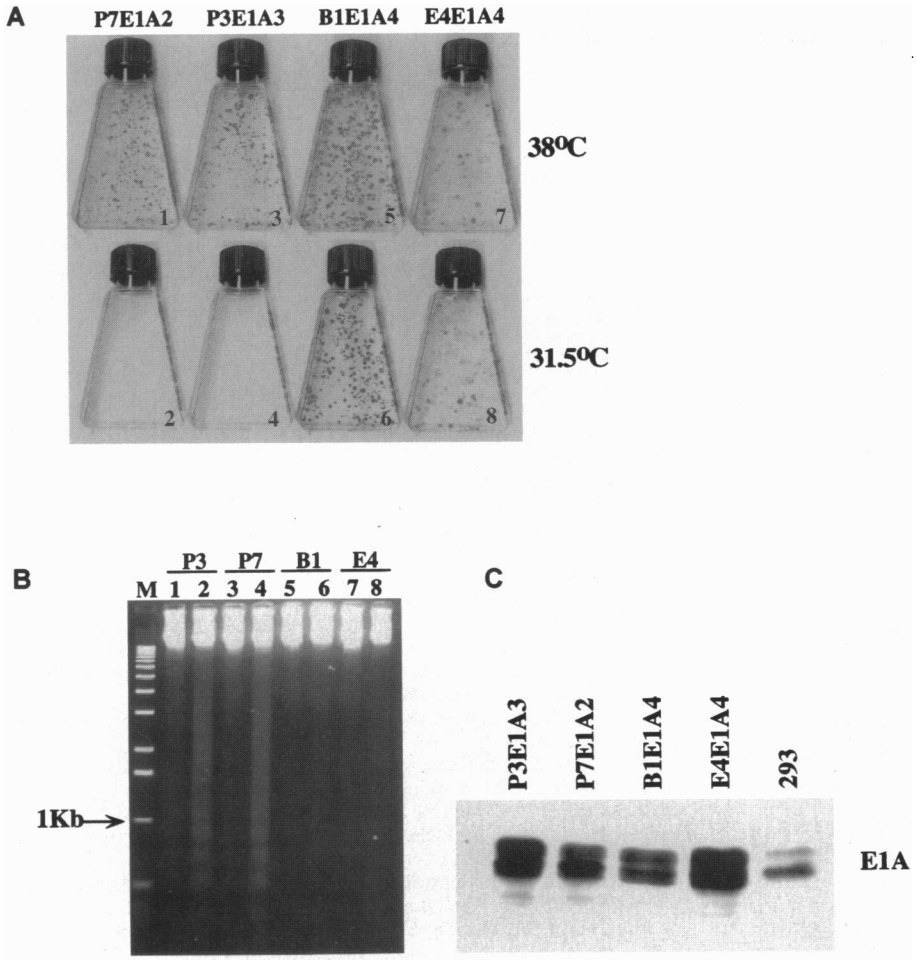

FIG. 2. Effect of E1A on cell growth. (*A*) Representative clones were plated at clonal density and grown at the indicated temperatures until visible colonies formed (10 days). (*B*) DNA fragmentation analysis of clones expressing E1A derived from P3 (lanes 1 and 2), P7 (lanes 3 and 4), B1 (lanes 5 and 6), and E4 (lanes 7 and 8) cells following growth for 18 hr at either 38°C (lanes 1, 3, 5, and 7), or 31.5°C (lanes 2, 4, 6, and 8). M, 1 kb marker (GIBCO/BRL). (*C*) Immunoblot of E1A protein harvested from indicated cells stably expressing E1A. Protein from the 293 cell line was included as a control.

RB protein level increased in P3 and P7 cells, but this increase could not be observed in the context of the high endogenous pRB level in the E4 and B1 cells.

DISCUSSION

The ability of a tumor cell to undergo cell cycle arrest or apoptosis may play an important role in determining the responsiveness of tumors to therapeutic regimes involving induction of DNA damage. Thus, definition of the signal transduction pathways involved in these two distinct processes is necessary for rational therapeutic design. The p53 protein has been demonstrated to control pathways of cell cycle arrest and apoptosis (27). However, results from previous studies have not allowed a clear determination of whether p53-mediated cell cycle arrest and apoptosis represent independent pathways.

Table 6. Effect of pRB protein expression on cell growth

Cell line	Protein expressed from pCEP4 vector	No. of colonies at 38°C
P3	—	>200, >200
P3	pRB	0, 0
P7	—	>200, >200
P7	pRB	0, 0
B1	—	158, 142
B1	pRB	94, 102
E4	—	>200, >200
E4	pRB	161, 181

The indicated cell lines were transfected with pCEP4 plasmid alone (—) or the pCEP4 plasmid expressing pRB protein. Ten days after hygromycin B selection, colonies were counted. Two independent determinations are shown.

Previously, we have demonstrated that the sequence-specific transactivation properties of p53 were necessary for growth suppression (20). Similarly, Sabbatini and coworkers recently described the essential role of p53-mediated transcriptional activity in E1A-mediated apoptosis (17). These observations are consistent with potential shared signal transduction pathways for these two p53-mediated events. However, several other studies have indicated that p53-mediated apoptosis and cell cycle arrest use distinct pathways. For example, p53-mediated cell cycle arrest apparently requires *de novo* gene expression, whereas p53-dependent apoptosis may not (28). Recently, Abrahamson and coworkers have shown that Steel factor can inhibit p53-induced apoptosis and terminal differentiation but not G1/S cell cycle arrest (29).

In this report we demonstrate that cells which are resistant to both p53-mediated growth arrest and E1A-mediated apoptosis can be generated, thus providing evidence that these activities use shared cellular circuitry. If divergent signaling pathways exist for p53-mediated cell cycle arrest and apoptosis, we predict that the target(s) of mutation in the B1 and E4 cell lines, lies upstream of some divergent point. Additionally, the data presented in this study support the theory that both p53 and RB act through a common signaling pathway (30–38). The system described here may provide a valuable tool to study cell cycle regulatory factors as well as candidate downstream components of the p53-mediated signaling pathway.

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