ABSTRACT

The p53 mutant 143Ala is a human temperature-sensitive mutant with two conformational states. To definitively determine whether the Fas signal transduction pathway and the function of the pathway are dependent on p53 status, we have established stable transfectants of p53 mutant 143Ala in two human cancer cell lines: H1299 (lung cancer line) and PC-3 (prostate cancer line), the native state of which contains null p53 status and can grow at 37°C and 32.5°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell cycle analysis showed inhibition of the growth of cells overexpressing p53 mutant 143Ala in the wild-type p53 form at 32.5°C because of induction of G0/G1 arrest. Transfected cells had increased protein expression of p21, Fas, and MDM2 at the wild-type p53 conformation at 32.5°C, but not in the mutant p53 form at 37°C. However, there was no change in protein expression of FADD, FAP-1, Bcl-2, or Bax at 32.5 or 37°C. Assays for apoptosis demonstrated that anti-Fas antibody CH-11 and Fasl-induced apoptosis only in cells that overexpress p53 mutant 143Ala at 32.5°C with the wild-type p53 form. Both caspase-3 and caspase-8 activities were increased by anti-Fas antibody CH-11 only in cells at 32.5°C with wild-type p53. Our results demonstrated that Fas-mediated apoptosis in H1299 and PC-3 cells expressing p53 mutant 143Ala occurred only with the wild-type p53 phenotype. These results support the hypothesis that Fas-mediated apoptosis is dependent, at least partially, on the presence of a functional wild-type p53 state. This model may be a useful tool for dissecting the specific interactions between wild-type p53 and the Fas signal transduction pathway in human cancer cells.

INTRODUCTION

p53 is one of the most commonly mutated genes found in human tumors (1). More than 50% of human malignancies, including breast and lung cancers, are associated with missense mutations or deletions of p53. Most of the missense mutations map to the DNA-binding domain of the protein (2, 3). The function of p53 as a tumor suppressor has been demonstrated by experiments showing that the loss of p53 correlates with the loss of G1-S-phase regulation after DNA damage, increasing genomic instability and gene amplification (4–6). Many signals encountered during tumor development can activate p53, including carcinogen-induced DNA damage, telomere erosion, aberrant proliferative signals, hypoxia, and the loss of adhesion or survival signals. It is, therefore, likely that wild-type p53 plays a role at several stages in the carcinogenic process (7).

Apoptosis, or programmed cell death, is an essential feature of developing and adult organisms. p53 is a sequence-specific transcription factor that binds to DNA as a tetramer and thereby activates or represses transcription from a large, and ever increasing, number of genes. These include p21/WAF1/Cip1, a cyclin-dependent kinase inhibitor known to arrest the cell cycle (8), GADD45 for DNA repair (9), and Bax (10) and Fas/APO-1 (11) to induce apoptosis. Fas-mediated apoptosis begins with the trimerization of Fas; this occurs when Fas is bound either to its ligand (FasL) or to the anti-Fas monoclonal antibody CH-11 (12) or Jo2 (13). Fas signals are transduced via several proteins, including FADD/MORT1 (14, 15) and RIP (16). However, p53-containing mutations in the transcriptional regulation domain of the protein were also shown to be capable of inducing apoptosis (17). MDM2 interacts with the NH2-terminal region of p53 leading to inhibition of the transcriptional activity of p53 and its degradation via ubiquination (18, 19). This property underlies the oncogenic potential of MDM2, which is overexpressed in various human tumors. In addition, MDM2 also has p53-independent activities that are not completely understood (20). Similar to other oncogenes, surveillance pathways may counteract the deleterious effects of deregulated MDM2 expression.

The human “hot-spot” p53 mutant 143Ala (Val-Ala) is temperature sensitive for specific DNA binding and for p53-binding element-mediated transcriptional activation and conformation (2, 21). At 32.5°C, the p53 mutant 143Ala possesses strong DNA-binding ability and functions like wild-type p53; but at 37°C, its ability to bind DNA and activate transcription is severely weakened or lost and acts similar to mutant p53 (22). In addition, the p53 mutant 143Ala, at the permissive temperature (32.5°C), enhanced protein expression of the Fas/APO-1 gene in K562 leukemia cells. However, increased expression of Fas receptor did not induce apoptosis with FasL or CH-11 antibody (11). A study by Muller et al. (23) found that p53 directly activated the promoter of the Fas/APO-1 gene in response to DNA damage by anticancer agents, and the up-regulation of the Fas receptor was observed only in cells with wild-type p53 and not in cells with mutant or null p53.

In this study, we have established stable transfectants of p53 mutant 143Ala in human lung cancer cells H1299 and prostate cancer cells PC-3 to determine whether the expression and function of the Fas signal transduction pathway is causally correlated with p53 status. We found that cells overexpressing p53 mutant 143Ala had increased expression of p21, Fas, and MDM2 at wild-type p53 conformation at 32.5°C. Various apoptosis assays showed that Fas-mediated apoptosis occurred only by Fas antibody CH-11 or Fasl at the wild-type p53 phenotype at 32.5°C. Furthermore, downstream signals of Fas, such as caspase-8 and caspase-3 activities were increased by CH-11 only in the wild-type p53 form.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The null p53 human lung adenocarcinoma cancer cell line H1299 and the null p53 human androgen independent prostate cancer cell line PC-3 were obtained from American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% FCS.

Antibodies. Anti-p53 monoclonal antibody (clone DO-1), anti-p21 polyclonal antibody (clone H-164) and anti-MDM2 monoclonal antibody (clone SMP14) were obtained from Santa Cruz Biotechnologies (San Diego, CA). Anti-Fas polyclonal antibody and anti-Fas monoclonal antibody (clone CH-11) were obtained from MBL International Corporation (MIC, Watertown, MA). Fas ligand was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-α-tubulin monoclonal antibody was obtained from Sigma (St. Louis, MO).
Plasmid and Stable Transfection. Plasmids of pCMV and pCMV/p53-143 were a kind gift of Dr. Albert Deisseroth (Yale University School of Medicine, New Haven, CT). Plasmids were transfected into H1299 and PC-3 cells by using LipofectAMINE (Life Technologies, Inc., Grand Island, NY). The selection of clones was carried out for 2 weeks with 800 μg/ml G418 (Life Technologies, Inc., Grand Island, NY). The insertion of the p53-143 cDNA was determined by Western blot.

MTT Assay. Cells (2000–3000) were seeded per well in 96-well plates and were treated with various concentrations of anti-Fas antibody CH-11 for the time points. The assay was performed with a Cell Proliferation kit I (MTT 3; Roche Diagnostics, IN) according to the manufacturer’s instructions.

Western Blot and Caspase Assays. For Western blot analysis, cell lysates were prepared in 1 ml of lysis buffer [20 mM Tris/HCl (pH 7.6), 1 mM EDTA (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10 μg/ml leupentin, 10 μg/ml pepstatin A, and 1% phenylmethylsulfonyl fluoride] for 15 min on ice and were centrifuged at 10,000 × g for 30 min. Equal amounts of lysates (40 μg) were boiled in SDS sample buffer and loaded onto SDS-PAGE. After transferring, immunoreactive products were detected by ECL system (Amersham Pharmacia, Piscataway, NJ). Anti-α-tubulin monoclonal antibody was used as a control. Caspase-8 and caspase-3 assays were performed with a caspase-8 and CPP32/caspase-3 Fluorometric Protease Assay kit (MBL, Nagoya, Japan) according to the manufacturer’s instructions.

Apoptosis Assays. Cells were preincubated for 16 h at 37°C or 32.5°C and then treated with various concentrations of anti-Fas antibody CH-11 or FasL for different periods of time. After washing with PBS, cells were fixed in 3.7% paraformaldehyde at room temperature for 30 min. For DAPI staining, cells were stained with 50 μg/ml DAPI at 4°C for 1 h. The number of apoptotic cells with nuclear morphology typical of apoptosis was scored in at least 400 cells in each sample by using fluorescence microscopy. The reader was blinded to the actual groups in the fluorescence microscopy. The TdT assay was performed with a MEBSTAIN Apoptosis kit direct (MBL, Nagoya, Japan) according to the manufacturer’s instructions.

RESULTS

Stable Transfectants of p53 Mutant 143Ala in Human Cancer Cells. To determine whether the Fas signal transduction pathway was correlated with p53 status, we established stable transfectants expressing p53 mutant 143Ala in human cancer cells, which were null p53 cell lines H1299 (lung cancer line) and PC-3 (prostate cancer line); both lines grew well at both 37 and 32.5°C. We transfected the mammalian expression construct pCMV/p53-143 to establish the stable transfectants of p53 mutant 143Ala into the two cell lines. After

The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4',6-diamidino-2-phenylindole; TdT, terminal deoxynucleotidyl transferase.

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Fig. 1. Expression of p53 in H1299 and PC-3 stable transfectants. Cell lysates were prepared from H1299 and PC-3 cells and analyzed for p53 by Western blot using a monoclonal anti-p53 antibody (DO-1): A, in H1299, H1299/vector, and H1299/p53-143 nos. 6, 7, and 11 cells; B, in PC-3, PC-3/vector, and PC-3/p53-143 no. 1 cells. Expression of α-tubulin was used as a control.

Fig. 2. Cell growth by MTT assay in H1299, H1299/vector, and H1299/p53-143 nos. 6, 7, and 11 cells. A, cell growth at 37°C and 32.5°C. Cells were grown for 6 days at 37°C and 32.5°C, and cell viability was determined by MTT assay. Results represent mean absorbance ± SD (n = 3). B, time course at 37°C and 32.5°C. Cells were grown for the indicated times at 37°C and 32.5°C, and cell viability was determined by MTT assay. Results represent mean absorbance ± SD (n = 3).

Fig. 3. DNA content analysis by flow cytometry in H1299, H1299/vector, and H1299/p53-143 nos. 6, 7, and 11 cells. Cells were grown for 2 days, stained with propidium iodide, and analyzed by flow cytometry. DNA content is presented as relative fluorescence. Quantitation of the cell cycle distribution is presented in Table 1. wt, wild type.
selection with G418 for 3 weeks, three single clones of H1299 cells (H1299/p53-143 nos. 6, 7, and 11) and one single clone of PC-3 cells (PC-3/p53-143 no. 1) expressing p53 protein were selected by Western blot analysis with anti-p53 monoclonal antibody (DO-1). As shown in Fig. 1, both lines had high expression of p53 protein in the four stable transfectants with p53 mutant 143Ala. Expression of α-tubulin was used as a control.

Cell Growth and Cycle in the Stable Transfectants with p53 Mutant 143Ala. To investigate the cell growth characteristics of stable transfectants with regard to temperature changes, MTT assays were performed at 37°C and 32.5°C after 6 days of growth with the three stable transfectants (H1299/p53-143 nos. 6, 7, and 11). As shown in Fig. 2A, these stable transfectants were grown at 37°C (mutant temperature) along with parental H1299 and H1299/vector alone (control) cells. The control and parental cell lines had similar growth rates. However, the three stable transfectants grew more slowly at 32.5°C (wild-type p53 form). A time course experiment demonstrated that cell growth patterns of H1299/p53-143 no. 6 cells was decreased after 2 days at 32.5°C (Fig. 2B). DNA content analysis showed that p53 mutant 143Ala cells underwent a stable G0/G1 arrest when grown at 32.5°C after 2 days (Fig. 3; Table 1). In addition, the same results as above were also obtained in the PC-3 stable transfectant PC-3/p53-143 no. 1 cells (data not shown). Thus, arrest was not attributable to the lower temperature alone because previous studies of parental H1299 and PC-3, H1299/vector and PC-3/vector cells showed no change in growth rates at 32.5°C.

Induction of p21, Fas, and MDM2. To obtain insight into the molecular mechanism by which p53 mutant 143Ala induced G0/G1 arrest in the wild-type p53 form at 32.5°C and whether p53 status was correlated with other apoptotic signal transduction proteins, we assessed expression of p21, MDM2, Fas, FADD, FAP-1, Bcl-2, and Bax by Western blot analysis. As shown in Fig. 4A, H1299 cells at 32.5°C, overexpressing p53 mutant 143Ala, had increased expression of p21, Fas, and MDM2 at the wild-type p53 conformation after 2 days, but not at the mutant p53 form at 37°C. Fig. 4B shows that expression of MDM2 and p21 were induced as early as 6 h at 32.5°C in H1299/p53-143 no. 6 cells. Similar results were also obtained in PC-3 cells overexpressing p53 mutant 143Ala (PC-3/p53-143 no. 1) at 32.5°C with the wild-type p53 form (Fig. 4C). However, there was no change in expression of FADD, FAP-1, Bcl-2, or Bax at 32.5 or 37°C in either of the stable transfectants lines (data not shown).

Induction of Apoptosis by Anti-Fas Antibody CH-11 and FasL. To further determine whether the Fas signal transduction pathway was functional and correlated with p53 status, we tested selective apoptotic inducers of the Fas pathway, anti-Fas antibody CH-11 and FasL, in H1299, H1299/vector, and H1299/p53-143 clone no. 6. Induction of apoptosis was determined by DAPI staining (Fig. 5, A and B). Treatment with 2 μg/ml anti-Fas antibody CH-11 for 24 h induced apoptosis only in the H1299/p53-143 no. 6 cells with wild-type p53 conformation, but not in the mutant p53 form. Fig. 5B shows the time course in H1299/p53-143 no. 6 cells under the same treatment as shown in Fig. 5A. In addition, DNA fragmentation induced by anti-Fas antibody CH-11 and FasL in H1299/p53-143 cells with the wild-type p53 form at 32°C was determined by TdT assay (Fig. 5, C and D). As shown in Fig. 5C, CH-11 induced ~21% apoptosis with the 0.5 μg/ml concentration and 54% with the 2 μg/ml concentration.
after 24 h of treatment. In addition, FasL induced ~52% apoptosis with 100 ng/ml after 24 h of treatment (Fig. 5D). We also tested the sensitivity of anti-Fas antibody CH-11 in PC-3, PC-3/vector and PC-3/p53-143 no. 1 cells. The results of DAPI staining and TdT assay are shown in Fig. 6, A and B, respectively. In comparison with H1299 stable transfectants, PC-3/p53-143 no. 1 cells were less sensitive to CH-11. The percentage of apoptotic cells by DAPI staining and TdT assay were 15% and 52%, respectively, when the cells were treated with CH-11 at 5 μg/ml for 24 h. These data demonstrated that anti-Fas antibody CH-11 selectively induced apoptosis only in the stably transfected H1299 and PC-3 cells with wild-type p53 conformation at 32.5°C.

Effect of Anti-Fas Antibody CH-11 on Caspase-8 and Caspase-3 Activation. To confirm the mechanism of downstream apoptotic induction by anti-Fas antibody CH-11 for the Fas signal transduction pathway, we tested the effect of CH-11 on caspase-8 and caspase-3 activation in H1299/p53-143 no. 6 cells at 37 and 32.5°C. Cells were treated with 2 μg/ml anti-Fas antibody CH-11 for 24 h. As

Table 1  Cell cycle distribution of H1299 cells

<table>
<thead>
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<th>Cell cycle phase</th>
<th>H1299/vector (37°C)</th>
<th>H1299/p53-143 no. 6 (37°C)</th>
<th>H1299/vector (32.5°C)</th>
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<td>G0-G1</td>
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<td>54 56 60 57 65 80</td>
<td>22 20 21 22 21 20</td>
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<td>S phase</td>
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<td>22 20 21 22 21 20</td>
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<td>24 24 24 20 22 17 8</td>
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<tr>
<td>G2-M</td>
<td>22 21 22 22 21 20</td>
<td>22 20 21 22 21 20</td>
<td>24 24 24 20 22 17 8</td>
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</table>

a Cell cycle distribution of cells incubated for indicated times at 37°C or 32.5°C. Values are from a single experiment from three separate flow cytometry experiments. Representative histograms are shown in Fig. 3. wt p53, wild-type p53; Cont., control.
Results represent mean absorbance from microscopy experiments with DAPI staining performed by a reader working blindly.

DISCUSSION

Mutations in the evolutionarily conserved codons of the p53 tumor suppressor gene are common in diverse types of human cancer. The p53 mutational spectrum differs among cancers of lung, colon, esophagus, breast, pancreas, liver, brain, melanoma, and hematopoietic tissues. Analysis of these mutations can provide clues to the etiology of these diverse tumors and to the function of specific regions of p53 (2).

The conformational status of p53 is vitally important for its biological functions. For example, a mouse temperature-sensitive p53 mutant 135Val exhibits the wild-type conformation at 32.5°C and activates the MDM2 gene (24). In addition, the conformational changes of p53 also appear to be important for cell cycle activity. The mutant form is compatible with cellular proliferation. In contrast, wild-type p53 protein is consistent with its role in inducing cell cycle arrest and apoptosis (24, 25).

The p53 mutant 143Ala was the first human temperature-sensitive mutant that was identified in 1991 (2). The activity differences correlated with the presence of two conformational states of p53, which are differentially recognized by Pab1620, an antibody which is relatively specific for the wild-type form. At 37°C, p53 mutant 143Ala is almost undetectable by Pab1620-mediated immunoprecipitation (22). Furthermore, the p53 mutant 143Ala at the permissive temperature (32.5°C), which was stably transfected into the human myeloid leukemia cells K562, markedly enhanced expression of the Fas/APO-1 gene (11). These cells, although they expressed Fas/APO-1, did not undergo apoptosis when exposed to CH-11 antibody or FasL. The reasons why they failed to induce Fas-mediated apoptosis are not understood but the failure could be attributable to a dissociated Fas pathway downstream of the Fas receptor. Thus, definitive proof that the wild-type p53 form in this model system was causally required for Fas-mediated apoptosis was still lacking.

On the basis of this background, we used the two human cancer cell lines H1299 (lung cancer line) and PC-3 (prostate cancer line), whose native state was null p53, to establish the stable transfectants of p53 mutant 143Ala. MT assay and cell cycle analysis showed that the growth of cells overexpressing p53 mutant 143Ala was inhibited in the wild-type p53 form at 32.5°C because of the induction of G1 arrest, most likely from p21 induction (Figs. 2, A and B, 3, and 4; Table 1). These results correlated with the data published on human myeloid leukemia K562 cells overexpressing p53 mutant, Ala-143, cultured at 32°C, which exhibited G1 arrest with p21 induction (11). p21/WAF1/Cip1 mediates p53-induced growth arrest triggered by DNA damage and arrests cells mainly in G1 phase (25). The MDM2 protein reduces the quantity of p53 available for the induction of cellular proliferation or for the induction of cell death (26). In this report, we have shown that H1299 and PC-3 cells overexpressing p53 mutant 143Ala had increased expression of p21 and MDM2 with wild-type p53 conformation after the incubation at 32.5°C for 2 days (Fig. 4, A and C). In the time course studies, induction of p21 and MDM2 were obtained as early as 6 h (Fig. 4B). However, there was no change in protein expression of FADD, FAP-1, Bcl-2, or Bax at 32.5°C or at 37°C (data not shown). These data suggested that the wild-type p53 conformation is playing a crucial role in expression of some oncoproteins and regulation of p53 targets.

The presence of functional wild-type p53 is closely coupled with the efficient induction of Fas/APO-1-mediated apoptosis in many (27–29) but not all (30, 31) cell types. However, the controls for these experiments were always other cell lines in contrast to the experiments in this report. As shown in Fig. 4, A and C, Fas expression was greatly increased in cells overexpressing p53 mutant 143Ala with wild-type p53 conformation at 32.5°C in comparison with the basal level of Fas. By using these stable transfectant cells as a model, we have demonstrated that anti-Fas antibody CH-11 induced apoptosis solely in cells overexpressing p53 mutant 143Ala at 32.5°C but not at 37°C, by DAPI staining (Figs. 5, A and B, and 6A) and TdT assay (Figs. 5C and 6B). It has been reported that p53 mutant 143Ala, at the
permissive temperature (32.5°C) with wild-type p53 form, enhanced protein expression of the Fas/APO-1 gene in K562 leukemia cells, but its increased expression of Fas receptor did not induce apoptosis with FasL and CH-11 antibody (11). Our results suggested that the induction of apoptosis by CH-11 might be dependent on the cell type. Krammer’s group [Muller et al. (23)] has reported that drug-induced p53 up-regulation is involved in Fas/APO-1 gene induction and apoptosis. Induction of Fas/APO-1 gene transcription by p53 is mediated through a strong p53-responsive element located within the first intron of the gene. This element cooperates with sequences in the Fas/APO-1 promoter to achieve maximal transactivation by wild-type p53. However, up-regulation of FasL, on treatment with anticancer drugs was demonstrated in cell lines containing wild-type, mutant, or null p53 status (23). We also found similar levels of increased FasL expression in PC-3 cells with wild-type, mutant, and null p53 status (data not shown). These data suggested that the regulation of FasL was independent of p53. In addition, the results from the TdT assays showed that anti-Fas antibody CH-11 also induced a small degree of apoptosis (10%) in cells with mutant or null p53 phenotype, suggesting that there may be other minor mechanisms that are not dependent on wild-type p53. There could also be minor amounts of wild-type p53 at 37°C in PC-3 cells causing the Fas expression (Fig. 4C). We also showed that both caspase-8 and caspase-3 activities were increased by anti-Fas antibody CH-11 in H1299 cells overexpressing wild-type p53 at 32.5°C (Fig. 7). These data demonstrated that anti-Fas antibody CH-11 selectively induced Fas expression and downstream Fas-mediated apoptosis in H1299 and PC-3 stable transfectants with wild-type p53 conformation. This report supports the hypothesis that Fas expression and its mediated apoptosis is causally associated with the wild-type p53 conformation by using the temperature-sensitive p53 mutant 143Ala as a model.

The status of the p53 gene has been proposed as one of the major determinants of a tumor’s response to anticancer therapy (32). With regard to the study of anticancer drugs and drug resistance in p53-dependent or -independent pathways, there have been contradictory reports in the literature regarding drug sensitivity and p53 status (33–37). To assess this further and to compare it with the previous results with Fas in the temperature-sensitive p53 model, several anticancer drugs, including paclitaxel, docetaxel, vinorelbine, gemcitabine, cisplatin, and 5-fluorouracil, were studied. Paradoxically, the H1299 transfectant cells exhibited increased resistance to all of the drugs when tested at the wild-type conformation at 32.5°C as compared with the mutant p53 temperature (data not shown). This decrease in sensitivity could be from wild-type p53 induction of p21, which has been shown to increase drug resistance by increasing G0-G1 arrest and time for DNA repair (38). Thus, a possible explanation for the contradictory reports of wild-type p53 tumor cells being more or less sensitive to anticancer drugs may be attributable to whether p21 is induced. Also, at the wild-type p53 conformation, Bax was not induced in the p53 mutant 143Ala stable-transfected H1299 cells. Reasons for this are not known, but without Bax induction, the wild-type p53 cells may have an additional possible mechanism why they are more resistant to the above anticancer drugs. It is interesting to hypothesize that wild-type p53 tumor cells, which on cytotoxic drug exposure induce p21-mediated cell cycle arrest, may be better suited for selective Fas-mediated apoptotic inducers. If the Fas pathway can be activated, then the p21-induced drug resistance could possibly be circumvented.

In summary, our work demonstrated that wild-type p53 status was a necessary prerequisite for Fas expression and a functional downstream Fas-mediated apoptotic pathway in two human cell lines with stably transfected human temperature-sensitive p53 mutant I43Ala. In addition, there may be other, yet undiscovered, mechanism(s) for the induction of Fas-mediated apoptosis that are independent of wild-type status.

Fig. 7. Effect of CH-11 on caspase-8 and caspase-3 activation in H1299/p53-143 no. 6 cells. Cells were treated with 2 μg/ml anti-Fas antibody CH-11 for 24 h at 37°C and 32.5°C, and caspase-8 and caspase-3 activation was detected as described under “Materials and Methods.” A, caspase-8 activation. B, caspase-3 activation. Results represent mean absorbance ± SD (n = 3). wt, wild type.
p53. These model systems could facilitate the investigation of factors and cofactors necessary to further dissect and understand the Fas pathway. This model could also serve to screen novel anticancer drugs for their ability to induce Fas-mediated apoptosis and to determine the cytotoxicity effects of anticancer agents in relation to p53 status.

ACKNOWLEDGMENTS

We thank Dr. Albert Deisseroth for providing the pCMV/p53-143 plasmid and Dr. Paul W. Brandt-Rauf for helpful discussions.

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Fas-Mediated Apoptosis Is Dependent on Wild-Type p53 Status in Human Cancer Cells Expressing a Temperature-Sensitive p53 Mutant Alanine-143

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