Wild-Type Human p53 and a Temperature-Sensitive Mutant Induce Fas/APO-1 Expression

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Received 10 October 1994/Returned for modification 15 November 1994/Accepted 27 February 1995

Fas/APO-1 is a cell surface protein known to trigger apoptosis upon specific antibody engagement. Because wild-type p53 can activate transcription as well as induce apoptosis, we queried whether p53 might upregulate Fas/APO-1. To explore this possibility, we examined human p53-null (H358 non-small-cell lung adenocarcinoma and K562 erythroleukemia) and wild-type p53-containing (H460 non-small-cell lung adenocarcinoma) cell lines. When H358 or H460 cells were transduced with a replication-deficient adenovirus expression construct containing the human wild-type p53 gene but not with vector alone, a marked upregulation (approximately a three- to fourfold increase) of cell surface Fas/APO-1 was observed by flow cytometry. Similarly, K562 cells stably transfected with a plasmid vector containing the temperature-sensitive human p53 mutant Ala-143 demonstrated a four- to sixfold upregulation of Fas/APO-1 by flow-cytometric analysis at the permissive temperature of 32.5°C. Temperature-sensitive upregulation of Fas/APO-1 in K562 Ala-143 cells was verified by immunoprecipitation and demonstrated to result from enhanced mRNA production by nuclear run-on and Northern (RNA) analyses. Stably transfected K562 cells expressing temperature-insensitive, transcriptionally inactive p53 mutants (His-175, Trp-248, His-273, or Gly-281) failed to upregulate Fas/APO-1 at either 32.5° or 37.5°C. The temperature-sensitive transcription of Fas/APO-1 occurred in the presence of cycloheximide, indicating that de novo protein synthesis was not required and suggested a direct involvement of p53. Collectively, these observations argue that Fas/APO-1 is a target gene for transcriptional activation by p53.

The wild-type p53 tumor suppressor can inhibit cellular proliferation, eliminate tumorigenicity (4, 16, 19, 23, 40, 43, 44, 77), and suppress gene amplification and oncogene-mediated cellular transformation. The p53 protein is a transcriptional regulator (22, 57) that can directly bind to DNA sequence-specific sites (9, 17, 20, 26, 34–36, 61, 80, 82, 84) or interact with other transcriptional regulatory factors such as Sp1 (7) and the CCAAT-binding factor (2). Genes known to be positively regulated by wild-type p53 include the *WAF1/Cip1* gene, which encodes an inhibitor of cyclin-dependent kinase (18, 28), the growth arrest and DNA damage-inducible gene *GADD45* (34), the muscle-type creatine kinase gene *MCK* (72), the putative proto-oncogene *mdm-2* (5), the epidermal growth factor receptor gene (15), and the recently identified bax protein-encoding gene (46, 50).

The studies of the effect of wild-type p53 on cellular growth and death have led to the view of p53 as a “guardian of the genome” (38). p53 protein levels increase in response to DNA-damaging agents such as ionizing radiation and result in the delay of cell cycle progression from G₁ into S phase to allow for repair of DNA damage (33, 34). If DNA repair is unsuccessful, programmed cell death (apoptosis) occurs. Although wild-type p53 is known to be involved in the initiation of apoptosis following exposure to etoposide or DNA-damaging ionizing radiation (12, 41), p53-driven programmed cell death is not always dependent upon DNA strand damage. For example, overexpression of exogenous wild-type p53 in cells deficient for p53 expression is also known to lead to apoptosis (32, 54, 56,

60, 62, 70, 79). The mechanism(s) by which p53 orchestrates diverse cellular programs ultimately culminating in apoptotic cell death is the subject of intense investigation. Because p53-dependent apoptosis can occur in the absence of p53 target gene activation, Caelles et al. (8) have proposed that p53 may function to repress cellular survival genes such as *bcl-2* (46) or participate in the enzymatic machinery involved in intranucleosomal cleavage of DNA (38, 76). It is conceivable, however, that p53 can also transcriptionally activate unidentified target genes which can mediate apoptosis.

Fas/APO-1, a member of the tumor necrosis factor receptor superfamily expressed on the cell surface of normal and malignant cells, can transduce a signal for apoptosis upon specific ligand or antibody engagement (31, 49, 64, 68, 78). Previous investigations with mice homozygous for the *lpr* or *gld* mutations have established the biologic relevance of the Fas/Fas ligand system in autoimmune disease pathogenesis. In both cases, mice develop circulating autoantibodies, glomerulonephritis, and lymphadenopathies (13). The autoimmune phenotype arising as a consequence of the *lpr* mutation appears to result from a loss of Fas function (1, 11, 71, 74) and can be reversed in mice reconstituted with functional Fas under the control of a CD2 promoter (75). Reciprocally, autoimmune disease in *gld* mice appears to result from the loss of a functional Fas ligand (67). Programmed cell death induced by Fas/Fas-ligand has been implicated in the killing of tumor targets by immune cytotoxic effector cells (27, 58, 63, 69) and in the elimination of chronically activated lymphocytes at the periphery by a mechanism known as activation-induced apoptosis (3, 48, 59, 75). However, the spectrum of Fas/Fas ligand-induced apoptosis is unlikely to be unique to the immune system, because these proteins are expressed in a variety of

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normal and malignant nonhematopoietic tissues (39, 51, 52, 64, 78).

To test the hypothesis that the p53 can transcriptionally activate Fas/APO-1 expression, we have investigated the effects of exogenous p53 on Fas/APO-1 expression in human tumor cell lines either homozygously deleted for the p53 allele or expressing wild-type p53. We now report that the introduction of either wild-type or a temperature-sensitive mutant of human p53, Ala-143, (83) can (at the permissive temperature) enhance expression of the Fas/APO-1 gene. Induction of Fas/APO-1 mRNA does not require de novo protein synthesis. These observations provide the first evidence that p53 can directly regulate the expression of an extracellular membrane-bound protein potentially involved in the induction of apoptotic cell death.

MATERIALS AND METHODS

Cells. The p53-negative H358 human lung carcinoma cells and the wild-type p53-containing human lung carcinoma cell line H460 (45, 66) were the generous gifts of A. Gazdar, Southwestern Medical Center, Dallas, Tex. Both H358 and H460 cell lines were infected with a replication-deficient adenovirus construct containing the cytomegalovirus enhancer/promoter and wild-type p53 gene (Ad-p53) or the Rous sarcoma virus promoter and luciferase gene (Ad-luc) maintained as described previously (25). Construction of Ad-p53 and Ad-luc has been previously reported (85). p53-negative K562 human erythroleukemia cells (37), obtained from the American Type Culture Collection, Rockville, Md., were stably transfected with p53 expression plasmids containing the cytomegalovirus enhancer/promoter, the neomycin resistance gene (83), and the p53 mutant Ala-143, His-175, Trp-248, His-273, or Gly-281. Human p53 expression vectors were generously provided by B. Vogelstein, Johns Hopkins School of Medicine, Baltimore, Md. Conditions used for temperature shift experiments of the stably transfected K562 cells have been described previously (83). K562 cells were routinely maintained in RPMI 1640 supplemented with 10% fetal calf serum in a 37.5°C incubator containing 5% CO₂.

Antibodies and flow-cytometric analysis. The murine monoclonal antibody anti-Fas was purchased from Kaimaya Biomedical Co., Thousand Oaks, Calif.; phycoerythrin- or fluorescein-conjugated rat anti-mouse κ -specific antibody was purchased from Becton Dickinson. All antibodies were used as recommended by the manufacturer. Briefly, 10⁶ cells were incubated with 1 μ g of anti-Fas (immunoglobulin M, κ) or isotype-matched control (Calbiochem Corp., La Jolla, Calif.) antibody for 30 min at 4°C, washed twice, and incubated with the secondary antibody for an additional 30 min. After being washed twice, the cells were fixed in phosphate-buffered saline containing 1% paraformaldehyde (pH 7.0) prior to analysis. Flow-cytometric analysis was carried out with a FACScan with the window set to exclude dead cells and debris. Ten thousand cells were examined for each determination. Cell cycle analysis was carried out after propidium iodide staining with the MultiCycle DNA analysis program (Phoenix Flow Systems, San Diego, Calif.).

Immunoprecipitation. Cells (5 $\times$ 10⁶) were extracted at 4°C overnight with 1% digitonin buffer in phosphate-buffered saline containing 15 μ M phenylmethylsulfonyl fluoride, 0.5 μ g of leupeptin per ml, and 0.7 μ g of pepstatin per ml. Cellular debris was removed by centrifugation. Cell lysates were precleared with goat immunoglobulin G bound to recombinant protein G-agarose and then immunoprecipitated with normal goat serum or anti-Fas-specific goat serum bound to protein G. Anti-Fas-specific goat serum raised against purified human Fas:Fc protein was the generous gift of Carl F. Ware, University of California, Riverside. Protein G beads were collected by centrifugation, washed four times with digitonin lysis buffer, resuspended in 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and boiled for 5 min. The samples were run on an SDS-PAGE gel (7.5% polyacrylamide) under nonreducing conditions, and the proteins were blotted to nitrocellulose. Fas/APO-1 proteins were detected with anti-Fas specific rabbit serum and horseradish peroxidase-conjugated donkey anti-rabbit antibody. Anti-Fas-specific rabbit serum raised against purified human Fas:Fc protein was the generous gift of Tom Gessner (Chiron Corp., Emeryville, Calif.). Proteins were visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.) as recommended by the manufacturer.

mRNA isolation and Northern (RNA) analysis. Total cellular RNA was extracted from 10⁷ to 10⁸ tumor cells growing in culture by a guanidinium thiocyanate-hot-phenol method as previously described (55). Poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography, fractionated on denaturing 1% formaldehyde-agarose gels, electrophoretically transferred at 0.6 A to GeneScreen nylon membranes (DuPont Co., Boston, Mass.), and UV cross-linked at 120,000 μ J/cm² with a UV Stratalinker 1800 (Stratagene, La Jolla, Calif.). Hybridizations were performed as described previously (53). Filters were washed three times at 60 to 65°C with 30 mM NaCl-3 mM sodium citrate (pH 7.2)-0.1% SDS. cDNA probes used were (i) a 1.8-kb *Bam*HI cDNA fragment corresponding to the

full-length human wild-type p53 gene (4); (ii) a 1.3-kb *Pst*I gene fragment corresponding to rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (24); and (iii) a 1.4-kb *Eco*RI fragment corresponding to nucleotides 336 to 1806 of human Fas/APO-1 (31). Each DNA fragment was purified by agarose gel electrophoresis, recovered with GeneClean (Bio 101, La Jolla, Calif.), and radiolabeled by the random-primer technique with α -³²P-labeled deoxyribonucleoside triphosphates (21).

For temperature shift experiments, cell lines were cultured at the permissive temperature (32.5°C) overnight. Where indicated, cycloheximide (10 μ g/ml) was added 30 min prior to the temperature shift. For the determination of mRNA half-life, cell lines were cultured at 32.5 or 37.5°C overnight, actinomycin D (20 μ g/ml) was added, and mRNA was isolated sequentially at the times indicated. Fas/APO-1 mRNA levels were quantified in the linear range of the X-ray film on a personal densitometer (Molecular Dynamics, Sunnyvale, Calif.) with the Image Quant software program. Each sample measurement was calculated as a ratio of the average area of the Fas/APO-1 transcript and the control 1.3-kb GAPDH transcript.

Nuclear run-on transcription assays. A total of 2.5 $\times$ 10⁷ to 5 $\times$ 10⁷ Ala-143 K562 or K562 parental (par) cells were harvested after an overnight culture at the permissive or nonpermissive temperature. Nucleus preparation and in vitro transcription were carried out as described previously (73) with the following modifications. Following DNase and proteinase K treatments, the nuclear RNA was isolated by phenol-chloroform-isooamyl alcohol extraction and ammonium acetate precipitation. For the reaction shown, [³²P]RNA was normalized to the lowest sample to achieve 10⁷ cpm/ml of hybridization buffer. Hybridization buffer consisted of 50% formamide, 5 $\times$ SSC (pH 7.0) (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$ Denhardt's solution, 1% SDS, 10 mM sodium phosphate (pH 7.0), 1 mM sodium PPi (pH 7.0), and 100 μ g of yeast tRNA per ml. After hybridization for 72 h, the filters were washed in 2 $\times$ SSC at 65°C, then in 2 $\times$ SSC containing 10 μ g of RNase A per ml at 25°C, and finally in 2 $\times$ SSC containing 0.1% SDS at 65°C. For the hybridization, 5 μ g of Fas/APO-1 cDNA (described above), purified pGEM 11zf(+) vector (Promega, Madison, Wis.), and human transferrin receptor cDNA (American Type Culture Collection) were denatured and slot blotted. For the experiment shown in Fig. 7, the dried filters were exposed to X-ray film overnight. The relative transcription rate was determined as a ratio of the average area of the Fas/APO-1 signal to transferrin receptor signal obtained with a Phosphor Imager (Molecular Dynamics) with the Image Quant software program.

Biologic effects of anti-Fas treatment. Cells (10⁴/200 μ l) were incubated in flat-bottomed 96-well plates in triplicate in the presence of either anti-Fas or control antibody (0.5 μ g/liter) overnight. After incubation, metabolic function was assessed from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction as previously reported (51).

RESULTS

Wild-type or temperature-sensitive mutant p53 regulates expression of Fas/APO-1 in human tumor cell lines. To determine whether exogenous p53 can alter Fas/APO-1 expression, the human non-small cell lung adenocarcinomas H358 and H460 were transduced with wild-type p53 in an adenovirus expression construct under the control of a cytomegalovirus promoter. H358 cells are deficient in p53 expression as a result of a deletion in p53-specific gene sequences, whereas H460 cells contain an endogenous wild-type p53 gene (45, 66). Previous studies in our laboratories have demonstrated that adenovirus transfer was highly efficient and that expression of p53 reached maximal levels 48 h after infection (14, 25). As shown in Fig. 1, Fas/APO-1 expression was markedly enhanced in both H358 and H460 cells transduced with wild-type p53 (Ad-p53) compared with parental cells and vector control (Ad-luc) at 48 h. A 2.9- to 3.7-fold increase in the mean fluorescence intensity (MFI) of cell surface Fas/APO-1 expression, representative of antigenic density on a per-cell basis, was observed in both cell lines after p53 introduction (average of three experiments). In the experiment shown, introduction of the Ad-luc vector alone in both H358 and H460 cells resulted in an increased percentage of cells positive for Fas/APO-1 expression compared with the parental cells. It is important to note, however, that Fas/APO-1 density (MFI) was significantly increased only after transduction with the p53-containing construct. To further explore Fas/APO-1 upregulation by p53, we examined the effects of a temperature-sensitive p53 mutant, Ala-143, stably transfected into p53-null K562 cells (37, 83).

FIG. 1. Fas/APO-1 expression in non-small cell lung carcinomas H358 and H460 48 h after transduction with wild-type p53 in an adenovirus vector. Cells were analyzed by flow-cytometric analysis as described in Materials and Methods. In this experiment, H358 parental cells were 4.7% positive for Fas/APO-1 expression (MFI, 3.0), H358 cells transduced with Ad-luc were 14.6% Fas/APO-1 positive (MFI, 2.4), and H358 cells expressing Ad-p53 were 78.8% positive for Fas/APO-1 expression (MFI, 6.7). Similar results were observed with H460 cells: H460 parental cells were 8.1% positive for Fas/APO-1 expression (MFI, 0.4), H460 Ad-luc cells were 45.6% positive (MFI, 1.7), and H460 Ad-p53 cells were 55.3% positive (MFI, 5.5).

K562 parental cells express virtually undetectable Fas/APO-1 levels by flow cytometry; however, a marked induction of Fas/APO-1 was observed in K562 cells transfected with Ala-143 at the permissive temperature of 32.5°C (Fig. 2). In four experiments, K562 par cells demonstrated an average of $10.1\% \pm 7.5\%$ cells positive for Fas/APO-1 at 37.5°C, and Ala-143- and His-273-containing K562 showed $10.4\% \pm 5.7\%$ and $14.5\% \pm 10\%$ positive cells, respectively. Upon culture at 32.5°C, K562 par and His-273 K562 cells were $8.9\% \pm 4.4\%$ and $6.1\% \pm 2.2\%$ positive for Fas/APO-1 expression, respectively, whereas the Ala-143-containing K562 cells were $34.8\% \pm 16.1\%$ positive for Fas/APO-1 (an approximate four- to sixfold increase). This effect is most probably dependent on the transcriptional activity of Ala-143 at 32.5°C. In this regard, we have previously demonstrated that Ala-143 assumes a wild-type p53 conformation at 32.5°C (as determined by antibody immunoprecipitation experiments) and strongly activates p53-binding-element-mediated transcription (83). Other temperature-insensitive, transcriptionally inactive p53 mutants (His-175, Trp-248, His-273 and Gly-281) fail to activate transcription (83) and to upregulate Fas/APO-1 expression at either temperature (Fig. 2). These results were further confirmed by immunoprecipitation with extracts of Ala-143 K562 cells maintained at the permissive or nonpermissive temperature (Fig. 3). As shown, anti-Fas specific antibody precipitated a 48-kDa Fas/APO-1 protein from Ala-143 K562 cells maintained at 32.5°C but not from cells maintained at 37.5°C. K562 par and the transcriptionally inactive His-273-containing K562 cells were also examined. Consistent with the results from the flow-cytometric analyses, no Fas/APO-1 protein was detected in these cells maintained at either temperature. Collectively, these results demonstrate that exogenous p53 can upregulate Fas/APO-1 when introduced into cells with either a wild-type or p53-null background. Because Ala-143 K562 cells can be specifically and reversibly induced to express Fas/APO-1 in the absence of

FIG. 2. Fas/APO-1 upregulation in K562 cells stably transfected with a temperature-sensitive mutant p53. Cells were harvested after overnight culture at the permissive or nonpermissive temperature and analyzed by flow cytometry as detailed in Materials and Methods. In this experiment, K562 par cells cultured at the permissive temperature overnight demonstrated 10.8% positive cells, Ala-143 K562 cells demonstrated 51.6% positive cells, His-175 K562 cells demonstrated 11.9% positive cells.

cell death, they were used for further analysis of the mechanism(s) underlying p53-driven enhancement of Fas/APO-1 expression.

Elevation of Fas/APO-1 levels in Ala-143 K562 is not related to cell cycle. The failure of stably transfected Ala-143 K562 cells to undergo frank growth arrest at the permissive temperature cannot rule out p53-induced alterations in cell cycle. To address this issue directly and to determine whether Fas/APO-1 induction is correlated with the cell cycle, Ala-143 K562 cells were cultured at the nonpermissive and permissive temperatures. The results from one of three consistent experiments are shown in Fig. 4. It is evident that Ala-143 K562 cells cultured at 32.5°C exhibit a cell cycle profile different from that of K562 par. Thus, temperature-shifted Ala-143 K562 cells show an increased G₁ and G₂/M peak with a decreased S phase compared with Ala-143 K562 maintained at the nonpermissive temperature (38.9% versus 21.1% G₁, 22.2% versus 16.1% G₂/M, and 38.8% versus 61.8% S phase, respectively). Simultaneous analysis of cell cycle position and Fas/APO-1 cell sur-

FIG. 3. Immunoprecipitation of Fas/APO-1 protein from K562 cells stably transfected with a temperature-sensitive mutant p53. Immunoprecipitation was performed on K562 par, His-273 K562, or Ala-143 K562 cells cultured overnight at the permissive (32.5°C) or nonpermissive (37.5°C) temperature with a Fas-specific antibody (Fas) or an isotype-matched control antibody (C). The arrowhead indicates a specific band immunoprecipitated by anti-Fas antibody in the Ala-143 K562 cells maintained at the permissive temperature. MW, molecular mass in kilodaltons.

face expression revealed that Fas/APO-1 was not limited to cells in the G₁ phase and was expressed in G₁, S, and G₂/M (Fig. 5). These observations demonstrate that Fas/APO-1 is expressed throughout the cell cycle and that elevations in Fas/APO-1 protein levels are not solely related to an altered cell

cycle profile in Ala-143 K562 cultured at the permissive temperature.

Increased Fas/APO-1 mRNA levels in Ala-143 K562 cells cultured at 32.5°C are independent of de novo protein synthesis. Increased cell surface Fas/APO-1 in Ala-143 K562 cells cultured at 32.5°C is probably the result of elevated mRNA levels in these cells. To validate this possibility, mRNA harvested from Ala-143 K562 cells cultured at either 37.5 or 32.5°C for 16 h was examined by Northern blot analysis. As shown in Fig. 6, a Fas/APO-1-specific probe hybridized to a 2.5- and 1.9-kb transcript in the Ala-143 K562 cell line, consistent with previous observations in other cell types (31). No Fas/APO-1 transcripts were observed in K562 par cultured at either temperature (Fig. 6, lanes A and B). Interestingly, Fas/APO-1 transcripts were present in Ala-143 K562 cells maintained at both 37.5 and 32.5°C despite the absence of detectable Fas/APO-1 protein in cells cultured at 37.5°C (Fig. 2 and 3). Both Fas/APO-1 transcript types, however, were present at higher levels in Ala-143 K562 cells maintained at 32.5°C than in cells cultured at the nonpermissive temperature of 37.5°C (Fig. 6, compare lanes E and F). A similar temperature downshift failed to enhance Fas/APO-1 mRNA in His-273 K562 cells (lanes C and D). These data demonstrate that temperature-sensitive transcriptional activation of Ala-143 is associated with enhanced Fas/APO-1 mRNA accumulation.

FIG. 4. Cell cycle distribution in parental and Ala-143-containing K562 cells cultured at the permissive and nonpermissive temperatures. Samples were withdrawn after an overnight culture at either 32.5 or 37.5°C and were subjected to cell cycle FACS analysis as detailed in Materials and Methods. The percentage of cells in each cell cycle position is indicated.

FIG. 5. Fas/APO-1 expression as a function of cell cycle position in Ala-143 K562 cells cultured at the permissive and nonpermissive temperatures overnight. Cell cycle position and cell surface Fas/APO-1 expression were determined simultaneously by FACS with using propidium iodide and fluorescein-labeled anti-Fas antibody, respectively, as described in Materials and Methods. The total percentage of Fas/APO-1 positive cells in the gated region is shown in the upper right; the relative cell cycle position of the positive cells is also indicated.

To determine whether de novo protein synthesis was required for the temperature-sensitive induction of Fas/APO-1 mRNA in Ala-143 K562 cells, cycloheximide was added 1 h prior to the temperature downshift and the cells were cultured

FIG. 6. Temperature-sensitive p53 controls expression of Fas/APO-1 independent of de novo protein synthesis in K562 cells. K562 par, Ala-143 K562, or His-273 K562 cells were cultured overnight at the permissive (32.5°C) or nonpermissive (37.5°C) temperature in the presence (+ Cyclohex) or absence (- Cyclohex) of 10 μ g of cycloheximide per ml prior to harvesting of mRNA. Relative levels of Fas/APO-1, p53, or GAPDH mRNA were measured by Northern blot analysis of 5 μ g of poly (A)⁺ mRNA per lane as detailed in Materials and Methods.

FIG. 7. Transcriptional enhancement of Fas/APO-1 mRNA in Ala-143 K562 cells. K562 par or Ala-143 K562 cells were cultured overnight at the permissive (32.5°C) or nonpermissive (37.5°C) temperature, nuclei were harvested, and the rate of nuclear mRNA synthesis was measured for Fas/APO-1 and transferrin receptor as described in Materials and Methods.

for an additional 16 h at either the permissive (32.5°C) or nonpermissive (37.5°C) temperature and then subjected to mRNA extraction and Northern analysis. Under the experimental conditions employed, a 30-min pretreatment with cycloheximide was observed to inhibit de novo protein synthesis by >92% (measured by [³H]methionine incorporation) and the half-life of the Ala-143 p53 protein was shown to be in excess of 8 h (81). As expected, no Fas/APO-1 mRNA was detectable in K562 par or His-273 K562 cells maintained at either 37.5 or 32.5°C (Fig. 6, lanes G and H and lanes I and J, respectively). Importantly, Ala-143 K562 cells incubated with cycloheximide prior to the temperature downshift showed an increase in Fas/APO-1 mRNA levels similar to that of identical cultures maintained at 32.5°C in the absence of the drug (compare lanes F and L). A positive effect of cycloheximide on Fas/APO-1 mRNA levels was observed at the permissive temperature, suggesting the potential for a short-lived transcriptional repressor or other regulatory proteins involved in mRNA degradation. Alterations in Fas mRNA half-life alone are unlikely to contribute to the observed enhancement of Fas/APO-1 mRNA in cycloheximide-treated Ala-143 K562 cells cultured at 32.5°C, because elevated transcript levels were not present in similarly treated cells cultured at 37.5°C (compare lanes E and K) and mRNA half-lives were not found to differ in cycloheximide-treated and untreated Ala-143 cells maintained at 32.5 versus 37.5°C (see below). The same blot was rehybridized with probes specific for human p53 and GAPDH to verify expression and loading efficiency, respectively. Collectively, these results suggest a primary requirement for transcriptional activation, a lack of dependence on de novo protein synthesis, and a possible direct involvement of p53 in the upregulation of Fas/APO-1 mRNA.

Transcriptional enhancement of Fas/APO-1 mRNA in Ala-143 K562 cells. To verify that Fas/APO-1 mRNA accumulation resulted from transcriptional activation of the Fas/APO-1 gene in K562 cells, nuclear run-on assays were performed. For these experiments, K562 par or Ala-143 K562 cells were incubated overnight at the permissive or nonpermissive temperature and their nuclei were harvested for in vitro analysis of transcriptional rate. As shown in Fig. 7, transcriptional activation of the Fas/APO-1 gene was not demonstrable in K562 par cells cultured at either temperature; transcription of the transferrin receptor, however, was easily detectable. Induction of Fas/APO-1 transcription was observed in Ala-143 K562 cells maintained at either 32.5 or 37.5°C, corroborating results of our previous Northern blot analyses (Fig. 6). Somewhat surprisingly, however, in three experiments the specific rate of Fas/APO-1 mRNA synthesis (normalized for transferrin expression) increased only 1.4-fold in Ala-143 K562 cultured at 32.5°C compared with 37.5°C. To rule out differences in Fas/

FIG. 8. Fas/APO-1 mRNA stability in Ala-143 K562 cells is not altered by temperature downshift or the presence of cycloheximide (Cyc). Ala-143 K562 cells were cultured overnight at the permissive or nonpermissive temperature prior to the addition of actinomycin D (Act D). Where indicated, cycloheximide was present 30 min prior to the temperature downshift, the cells were cultured overnight, and actinomycin D was added the next day. mRNA was harvested sequentially from Ala-143 cells maintained at either temperature in the presence and absence of cycloheximide. The relative Fas/APO-1 half-life was determined by scanning densitometry as described in Materials and Methods.

APO-1 mRNA stability, mRNA was sequentially harvested from Ala-143 K562 cultured at the permissive and nonpermissive temperatures after actinomycin D addition. As shown in Fig. 8, Fas/APO-1 mRNA stabilities were similar in Ala-143 K562 cultured at either temperature. Although Fas/APO-1 mRNA accumulation was considerably less in Ala-143 K562 cells cultured at 37.5°C, at either temperature the half-life of Fas/APO-1 mRNA was approximately 5 h. Furthermore, Fas/APO-1 mRNA stability was not altered by pretreatment of the cells with cycloheximide (Fig. 8). Taken together, these results demonstrate that in K562 cells the Ala-143 p53 mutant activates Fas/APO-1 transcription, that the rate of Fas/APO-1 transcription is enhanced approximately 1.4-fold after an overnight culture at the permissive temperature, and that Fas/APO-1 mRNA stability is not significantly altered by temperature downshift or by the presence of cycloheximide.

Biologic function of Fas/APO-1 on K562. To determine the sensitivity of the Ala-143 K562 cells to anti-Fas-mediated apoptosis, cells maintained at 32.5 or 37.5°C were cultured overnight in the presence of 0.5 µg of anti-Fas per ml or isotype-matched control antibody before assessment of cellular metabolic function by MTT reduction as described previously (51). As controls, the Fas/APO-1-positive Jurkat E6 lymphoid cell line and the Fas/APO-1-negative K562 par and His-273 K562 cells were also examined. Results from one of three experiments are shown in Fig. 9. Neither the K562 par, the His-273, nor the Ala-143 K562 cells cultured at either the permissive or nonpermissive temperature demonstrated a significant metabolic inhibition after anti-Fas treatment. As expected, Jurkat cells were consistently inhibited by >80%. Further, Ala-143 K562 cells were unaffected by anti-Fas treatment as measured by cellular survival studies or flow-cytometric DNA fragmentation analyses (data not shown). These results demonstrate that despite cell surface Fas/APO-1 expression, Ala-143 K562 cells are refractory to anti-Fas-mediated signaling for cell death. Interestingly, K562 cells are known to be resistant to a variety of apoptotic stimuli as a result of their expression of the Bcr-Abl fusion protein (42).

DISCUSSION

We have presented data showing that expression of the Fas/APO-1 gene product, a cell surface protein known to trigger apoptosis, can be positively regulated by p53 in both p53-deleted and wild-type p53-containing human cell lines. These

FIG. 9. Ala-143-containing K562 expressing cell surface Fas/APO-1 are resistant to the biological effects of anti-Fas antibody treatment. K562 par, His-273 K562, or Ala-143 K562 cells were cultured overnight at the permissive (32.5°C) or nonpermissive (37.5°C) temperature prior to the addition of anti-Fas antibody or isotype-matched control antibody, immunoglobulin M (IgM), at a concentration of 0.5 µg/ml followed by an additional overnight culture at the permissive or nonpermissive temperature. The next day, metabolic inhibition was assessed by MTT reduction as described in Materials and Methods. Jurkat E-6 cells are shown as a positive control for the biological activity of anti-Fas antibody at both temperatures.

observations provide the first demonstration, to our knowledge, that p53 can activate a gene known to be involved in the initiation of apoptosis.

The Fas/APO-1 protein was initially characterized by its ability to trigger programmed cell death in a variety of normal and malignant cell types upon monoclonal antibody binding (31, 49, 51, 52, 68, 78). Although Fas/APO-1 expression can be induced or upregulated in response to cellular activation or various cytokines (52, 68, 78), there is no available information concerning the molecular events underlying this process. In this report, we document that the Fas/APO-1 gene is a transcriptional target of the tumor suppressor gene p53. Using wild-type p53 transduced into H358 or H460 cells or the human temperature-sensitive p53 mutant Ala-143 stably transfected into K562, we demonstrated that Fas/APO-1 can be markedly induced or upregulated. We have previously demonstrated that the Ala-143 p53 mutant can assume a p53 wild-type-like conformation; Ala-143 has a conformation recognized by monoclonal antibody PAb1620 at 32.5 but not 37.5°C. This mutant also potentially activates reporter constructs containing the p53CON and ribosomal gene cluster elements at the endogenous *WAF1/Cip1* gene, while the parental K562 and other non-temperature-sensitive p53 mutants (His-175, Trp-248, His-273, and Gly-281) fail to activate p53-driven transcription (83). Taken together, these studies suggest that induction of Fas/APO-1 is tightly linked to Ala-143 p53-mediated transcriptional activity. We should stress, however, that Fas/APO-1 expression is not absolutely dependent upon the presence or absence of the p53 protein. For example, Fas/APO-1 is not always expressed in wild-type p53-expressing cells (i.e., H460) and, reciprocally, can be detected in cells homozygously deleted for the p53 allele (14). These findings imply that the Fas/APO-1 gene is controlled at multiple levels, some of which involve p53. Although the effects we observed are probably due to the strong expression of p53 under the influence of the cytomegalovirus promoter/enhancer, other physiologic stimuli

known to induce p53, such as irradiation and chemotherapeutic agents, have also been shown to enhance Fas/APO-1 expression and/or alter the sensitivity to anti-Fas mediated killing (14, 47).

The process of Fas/APO-1 induction by Ala-143 p53 was shown to occur in the absence of de novo protein synthesis, suggesting that Fas/APO-1 is a direct transcriptional target for p53. That Ala-143 p53 induced Fas/APO-1 mRNA after cycloheximide pretreatment and culture at the permissive temperature was not completely unexpected, given the stability of the p53 mutant protein and the known enhancement of DNA binding and transcriptional activity of this mutant at 32.5°C (81, 83). In support of our observation that p53 can enhance Fas/APO-1 gene expression, sequence analysis of the human Fas/APO-1 promoter has revealed the presence of a p53CON element (26) approximately 1.3 kb upstream in the 5'-flanking region of this gene (6, 10). Our laboratory is currently undertaking experiments to further define the p53-mediated regulation of Fas/APO-1 and determine p53 binding within the promoter and intronic sequences of human Fas/APO-1.

The measurements of transcriptional activity, cytoplasmic mRNA, and cell surface protein indicate that Ala-143 p53-mediated Fas/APO-1 gene expression in K562 cells is controlled by at least two regulatory steps. Northern blot analyses revealed that Fas/APO-1 mRNA was detectable at low levels in K562 Ala-143 cells maintained at 37.5°C and that expression was markedly upregulated upon culture at 32.5°C. Parental K562 cells, on the other hand, failed to express Fas/APO-1 mRNA when cultured at either temperature. Nuclear run-on assays confirmed these findings; K562 par cells did not demonstrate Fas/APO-1 expression, whereas Ala-143-containing cells showed transcriptional activation of this gene. These studies strongly support the premise that induction of Fas/APO-1 mRNA is transcriptional in nature and dependent on the presence of Ala-143 p53. In light of the levels of mature Fas/APO-1 mRNA accumulation and cell surface protein expression detected at the permissive temperature, it was somewhat surprising that Ala-143 cells shifted from the nonpermissive to the permissive temperature demonstrated only a modest increase (1.4-fold on average in three experiments) in the transcriptional rate of Fas/APO-1 synthesis. Although alterations in mRNA stability could account for this apparent disparity, we were unable to discern changes in Fas/APO-1 mRNA half-lives in Ala-143 K562 cells maintained at 37.5°C compared with those cultured at 32.5°C. Our in vitro transcription studies did not address possible differences in Fas/APO-1 transcriptional elongation between Ala-143 K562 cells cultured at the permissive and nonpermissive temperatures. Thus, it is conceivable that while transcriptional initiation was roughly equivalent at both temperatures, elongation and synthesis of full-length mRNA transcripts differed. Nuclear run-on assays performed with both 5' and 3' Fas/APO-1 cDNA probes will be required to test this hypothesis. Alternately, other posttranscriptional events (other than mRNA stability) may contribute to the Fas/APO-1 mRNA accumulation observed at the permissive temperature. Despite the apparent complexity regulating Fas/APO-1 cell surface expression, it is clear that the initial event involves transcriptional activation of the Fas/APO-1 gene in the presence of Ala-143 p53.

Because wild-type p53 activity can induce growth arrest in some cell types, we explored whether the induction of Fas/APO-1 was a consequence of the ability of p53 to alter the cell cycle rather than an effect attributable to p53 expression. The simultaneous analysis of cell cycle and cell surface Fas/APO-1 expression revealed that despite marked changes in cell cycle profile (increased G₁ and G₂/M with decreased S phase rela-

tive to Ala-143-containing cells maintained at 37.5°C), Ala-143-transfected cells cultured at the permissive temperature showed enhanced Fas/APO-1 expression throughout the cell cycle without preferential display in G₁ or G₂/M. Whether Fas/APO-1 expression is normally expressed at a particular point in the cell cycle cannot be inferred from these studies, because p53 is constitutively expressed under the cytomegalovirus promoter used for these experiments.

Although Fas/APO-1 can be induced in response to cellular activation (or as shown here by wild-type p53 activity), expression of Fas/APO-1 is not predictive of an apoptotic response initiated by specific antibody engagement. The dissociation of Fas/APO-1 function and expression has been observed in normal and malignant lymphocytes (52, 68) as well as in nonhematopoietic tumors (51, 78). For example, Fas/APO-1 expression is observed on both resting and activated human lymphocytes, although sensitivity to anti-Fas-mediated apoptosis is restricted only to cells which have been chronically stimulated (52). It is not particularly surprising, therefore, that Ala-143 K562 cells failed to undergo anti-Fas-triggered apoptosis despite expression of Fas/APO-1. Importantly, ongoing studies in our laboratories demonstrate that p53-driven Fas/APO-1 upregulation can result in a functional Fas/APO-1 protein in several other cell types including H460 and H358 (14). The nature of the K562 resistance is currently unknown; however, previous experimental evidence has documented the role of protective proteins such as manganese superoxide dismutase (29) and bcl-2 (30) in anti-Fas resistance in other cell types. Another potentially interesting, albeit unexplored, candidate mediating K562 resistance to anti-Fas is the Bcr-Abl fusion protein (42).

The role of apoptosis in the pathogenesis of malignant disease has been underscored by recent observations that p53 can induce apoptosis (32, 54, 56, 60, 62, 70, 79) and that p53-dependent apoptosis can be a critical regulator of tumorigenesis (65). Recently, the molecular mechanisms underlying p53-induced apoptosis have been suggested to involve either alterations in cellular genes necessary for survival or the direct participation of p53 in DNA repair or apoptotic DNA cleavage (8). Thus, several investigators have demonstrated that the 26-kDa intracellular integral membrane protein bcl-2 and its recently identified molecular antagonist bax (50) are transcriptional targets for p53; bcl-2 levels are negatively regulated while bax is positively regulated by p53 (46). Additionally, p53 has been postulated to control apoptotic cell death by participating in DNA repair (36) or intranucleosomal cleavage of DNA (76). In support of the latter hypothesis, Caelles et al. have demonstrated that p53-dependent apoptosis can occur in the absence of transcriptional activation of p53 target genes in the somatotrophic progenitor cell line GHFT1 (8). Our data suggest a previously unrecognized possibility—that p53 can transcriptionally activate target genes known to induce apoptosis. It appears that multiple mechanisms are involved in the regulation of apoptosis in various cell types, namely, p53 dependent (independent of or dependent on p53-mediated transcription) and p53 independent. It is conceivable that autocrine or paracrine apoptotic cell death could be initiated in some cell types following p53-driven upregulation of Fas/APO-1 and interaction with the natural Fas/APO-1 ligand (64). Indeed, p53 may play a critical role in the recently documented Fas/Fas-ligand-dependent "activation-induced" apoptosis of lymphoid cells (3, 48, 59, 75).

ACKNOWLEDGMENTS

This work was supported by grants IM-723 (L.B.O.-S.) and PF-3446 (R.R.) from the American Cancer Society and by grant CA-45187

(J.A.R.), training grant CA09611 (J.A.R.), and The University of Texas M. D. Anderson Cancer Center support core grant CA-16672 from the National Cancer Institute.

We thank Karen Ramirez and Rachael Tsan for excellent technical support in flow-cytometric and mRNA analyses, respectively; Nadine Nelson for her expert secretarial skills; and Kenneth Dunner for his assistance in photographic reproductions.

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