Mutant p53 Cooperates with ETS and Selectively Up-regulates Human MDR1 Not MRP1*

The most frequently expressed drug resistance genes, MDR1 and MRP1, occur in human tumors with mutant p53. However, it was unknown if mutant p53 transcriptionally regulated both MDR1 and MRP1. We demonstrated that mutant p53 did not activate either the MDR1 promoter or the endogenous gene. In contrast, mutant p53 strongly up-regulated the MDR1 promoter and expression of the endogenous MDR1 gene. Notably, cells that expressed either a transcriptionally inactive mutant p53 or the empty vector showed no endogenous MDR1 up-regulation. Transcriptional activation of the MDR1 promoter by mutant p53 required an Ets binding site, and mutant p53 and Ets-1 synergistically activated MDR1 transcription. Biochemical analysis revealed that Ets-1 interacted exclusively with mutant p53s in vivo but not with wild-type p53. These findings are the first to demonstrate the induction of endogenous MDR1 by mutant p53 and provide insight into the mechanism.

The emergence of drug resistance poses a major obstacle to the success of cancer chemotherapy. Tumor cells acquire drug resistance via many routes including alterations in transport, drug targets, metabolism, and/or genes regulating cell survival. The most common alterations in drug transport are increased expression of MDR1 (the gene product is P-glycoprotein (1, 2)) and the multidrug resistance-associated protein (MRP1) (3, 4). Both are energy-dependent anticancer drug efflux pumps and play critical roles in the response to chemotherapeutic drugs (e.g. vinca alkaloids, taxanes, and epipodophyllotoxins). Further, both MDR1 and MRP1 are expressed in colon tumors that frequently express mutant (MT) forms of p53 (5, 6) and are intractable to chemotherapy. Notably, we have shown directly that MDR1 in colon tumors is normally repressed by wild-type (wt) p53 (7). In an analogous fashion Wang and Beck (9) as well as Sullivan et al. (8) have shown that wt p53 represses MRP1. Many clinical studies show that MT p53 expression is associated with increased MDR1 and/or MRP1 expression (5, 10, 11). These findings are fully consistent with a loss of p53 repression leading to MDR1 or MRP1 up-regulation. However, it is just as likely that these genes could be up-regulated by the “gain-of-function” activity of MT p53s (12–14).

p53 deletion or mutation is one of the most frequent alterations in human malignancy and is clearly a critical step in the progression of colorectal cancer (15). Close to 90% of the p53 mutations in human tumors results in a disruption of the DNA binding domain. This not only disrupts transrepression and sequence-specific transactivation but also confers a gain-of-function activity that was first demonstrated for many MT p53s as acquiring the ability to induce tumors (13). This property was associated with the capability of these MT p53s to stimulate the expression of an alternate set of endogenous genes (13, 14, 16) that could potentially promote tumor progression and impair therapeutic response. However, although c-myc has unequivocally been demonstrated to be an endogenous target of MT p53 (16), it is unknown if human MDR1 and/or MRP1 are MT p53 targets. If MT p53 did activate endogenous MDR1 and MRP1, then activation through p53 could occur first by the loss of repression (5–7) and second by MT p53 activation. Currently, it is unknown if MT p53s up-regulate MRP1 and MDR1 expression.

Clearly, given that the mutation of p53 occurs in many tumors that co-express both MDR1 and MRP1, it would be important to know if and how MT p53 affects their expression, because if both these genes are activated by MT p53 it would profoundly affect therapeutic response. In the current study we use a p53 null colon carcinoma, Caco-2 (17) to evaluate how MT p53 affects activation of the promoters and endogenous gene for MDR1 and MRP1. Moreover, we elucidate a mechanism for activation of the MDR1 promoter by MT p53 that demonstrates repression by wt p53 is distinct from activation by MT p53 but that MT p53 requires an Ets-binding site and Ets-1 interacts with MT p53.

MATERIALS AND METHODS

Cell Lines, Antibodies, DNA—The culture conditions for the cell lines have been described previously (11, 17). The human osteosarcoma, Saos-2 (18), and human colon carcinoma, Caco-2, are p53 null cell lines (17). The antibodies for p53 and MRP1 were obtained from Oncogene Science and Signet, respectively. The MDR1 and MRP4 antibodies were described previously (7, 18). The Ets-1-specific antisera, N-276, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western blots were developed by using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

The human p53 expression vectors regulated by the CMV promoter have been described previously (7, 13, 16). QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) was performed on the CMV-p53 plasmid with two oligonucleotide primers designed to change amino acid 281 from aspartate to glycine, and plasmid DNA was sequenced to identify the clones with the desired aspartate to glycine mutation. The Ets-1 cDNA (19) was released from the plasmid by EcoRI digestion and

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§ The abbreviations used are: MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; wt, wild type; CMV, cytomegalovirus; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; Inv, inverted; MT, mutant.
ligated into pcDNA3. The orientation of the insert was confirmed by nucleotide sequence analysis. The MDR1 promoter deletion and mutants were generated by polymerase chain reaction using a previously described MDR1 promoter construct (7) and generated reporters with the 5' positions −107, −76, and −58. The MT Ets site was constructed by site-directed mutagenesis described previously (20) on the −107 to +30 MDR1 promoter. All mutations and deletions were confirmed by DNA sequence analysis. The −1202 and inverted (Inv) CAAT MT MDR1 promoters were provided kindly by Dr. Kathy Scotto (Memorial Sloan-Kettering, New York) (21). The MRP1 luciferase promoter (−2008 to +103) was provided by Dr. Bill Beck (University of Illinois at Chicago). The hot-spot MT p53 expression vectors have been described previously (V143A, R175H, R248W, R273H, and D281G) (13) and were provided kindly by Dr. Arnold J. Levine (Rockefeller University, New York).

Western Immunoblot Analysis—Transfected cells were lysed in reporter lysis buffer as described above and previously (23). Equal amounts of protein (50 μg) were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with a polyclonal anti-p53 antibody (Ab7). Western immunoblot analysis of crude membrane preparations was performed as described previously (7, 18).

Flow Cytometry—Cells were cultured on 60-mm dishes as described above. Subsequently, the cells were washed with PBS and then incubated in warm medium containing rhodamine 123 (1 μg/ml). After a 1-h incubation, the cells were washed with ice-cold PBS, trypsinized, and collected for intracellular rhodamine determination by fluorescence-activated cell sorting (22).

For the analysis of DNA fragmentation, cells were plated onto 60-mm dishes followed by treatment with the indicated compounds. After the treatment interval both floating and attached cells were harvested in a propidium iodide solution (50 μg/ml propidium iodide in 0.1% sodium citrate and 0.1% Triton X-100), treated with 5 μg/ml RNase (Calbiochem, San Diego, CA) for 30 min at room temperature, and then analyzed by flow cytometry on a Becton Dickinson FACscan (Becton Dickinson Immunocytometry, San Jose, CA) using laser excitation at 488 nm. The percentage of sub-G1 cells was determined.

Luciferase Assays—The cells plated on 60-mm dishes were transfected overnight by calcium phosphate co-precipitation with 10 μg of the indicated MDR promoter construct and 50 ng of the wt p53 expression vector or CMV-Neo-Bam (empty vector) to keep the amount of other promoter activities greater than those in Caco-2. Nevertheless, the findings between the two cell lines were quantitatively similar. The cells were lysed by a 15-min incubation in 400 μl of the reporter lysis buffer provided in the luciferase kit (Promega, Madison, WI). Cellular debris was removed by centrifugation, and protein in the supernatant was quantified by using a modified Bio-Rad assay. Luciferase activity was determined in 40 μl of supernatant as described (7, 20), and activity (relative light units/μg of protein) was determined after normalizing for protein content. In some assays we co-transfected a plasmid (pRL-TK (Promega, Madison, WI) 1 μg/60-mm dish) expressing a Renilla luciferase that allows concurrent measurement of both luciferase activities. However, because of almost identical results we did not use it in every assay and instead utilized protein normalization in most cases. Moreover, because many promoters are repressed by wt p53 as reported previously (7, 14) we were unable to use Renilla to normalize the wt p53 assays and used protein alone.

RNase Protection Analysis—Total RNA was isolated as described (7) from cells grown at comparable densities. Analysis of apoptotic and antiapoptotic genes (see Fig. 3) was performed on total RNA by RNase protection using the RiboQuant multiprobe ribonuclease protection assay system (PharMingen, San Diego, CA) according to manufacturer instructions. The protected products were analyzed as described previously (23).

In Vitro Translation and “Pull-down” Assay—GST-Ets-1 fusion protein (GST-Ets-2–440 (19)) was eluted from glutathione-agarose (GSH beads, Sigma) after expression in Escherichia coli. MT p53 expression vectors were digested with a restriction enzyme that linearized the plasmid. The linear plasmid was in vitro transcribed and translated using [35S]methionine and the coupled vectors were digested with a restriction enzyme that linearized the 488 nM. The percentage of sub-G1 cells was determined.

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Immunoprecipitation—Ten micrograms of p53–281 and/or Ets-1 or their appropriate empty vectors were transfected as described (7). After the transfection interval, the cells were harvested and lysed in mammalian protein extraction reagent (MPER) (Pierce, Rockford, IL) containing the protease inhibitor mixture (Complete, Roche Molecular Biochemicals, Indianapolis, IN). The lysate was precleared with protein A-Sepharose followed by a centrifugation at 1500 × g for 10 min. The supernatant was transferred to a fresh tube and 1 μg of p53–281 or empty vector. Subsequently, luciferase activity was determined, and the fold activation was determined after normalization with protein. The value in the bars represents a mean of 5–6 independent experiments each performed in duplicate, and the line represents the standard deviation.
MDR1 is selectively overexpressed in Caco-2 cells expressing mutant p53-281. A, Caco-2 cells were transiently transfected with either MRP1 or MDR1-luciferase (10 μg) reporter construct along with the effecter plasmids (1 μg), p53-281, p53-14,19,281, or the empty vector CMV-Neo-Bam using the standard calcium phosphate co-precipitation. The luciferase activity was normalized to protein, and the fold activation was determined by dividing by normalized control activity. The bars reflect an average of 3–4 independent experiments performed in duplicate. The line above the bar represents the standard deviation. B, Caco-2 cells were stably transfected with either a p53–281 expression vector, a transcriptionally inactive MT, p53–14,19,281, or the empty vector (CMV-Neo-Bam). A total protein lysate was prepared from each of these clones and used for Western blot analysis. Equivalent amounts of protein (200 μg) were fractionated on a SDS-PAGE gel followed by immunoblot analysis for MDR1, p53, MRP4, and MRP1.

### TABLE I

| Cell line     | Mean<sup>a</sup> rhodamine fluorescence | VP-16<sup>b</sup> | VP-16<sup>b</sup> | C6-ceramide<sup>b</sup> |
|---------------|-----------------------------------------|-------------------|-------------------|------------------------|
| Neo1          | 813                                     | 1.0 μM            | 10 μM             | 5.0 μM                 |
| Neo2          | –c                                      | 13                | 21                | 18                     |
| 14,19–281     | 781                                     | 12                | 16                | –c                     |
| 281–1         | 440                                     | 4                 | 10                | 14                     |
| 281–2         | 358                                     | 6                 | 8                 | 15                     |

<sup>a</sup> The cells were incubated for 1 h with the MDR1 substrate rhodamine 123. Afterwards the cells were washed with cold 1 × PBS, trypsinized, pelleted, and resuspended in rhodamine-free medium on ice. The mean fluorescence intensity was determined by FACS analysis (20). The mean fluorescence was the average of two determinations from a representative experiment.

<sup>b</sup> The cells were treated with the indicated concentrations of the VP-16 or C6-ceramide for 24 h. Afterward, cells (both floating and attached) were assessed for the proportion (numbers are percentages) of sub-G<sub>1</sub> cells as described under "Materials and Methods." The variation between each independent determination was less than 18%. These are average values from two independent experiments.

<sup>c</sup> These samples were not evaluated.

onto a gel. The gel-fractionated proteins were transferred to a nitrocellulose membrane and blotted with the murine monoclonal antibody, DO-1, specific for human p53, or the sheep polyclonal antibody AB-7 (panspecific for p53).

**RESULTS**

Repression of the MDR1 Promoter by wt p53 Is Separable from MT p53 Activation—It has been proposed that wt p53 generally represses the MDR1 promoter though interaction with basal transcription factors such as TATA-binding protein (24). However, this seems unlikely given that mutants of p53 in the C and N terminus are still capable of interacting with TATA-binding protein but are unable to repress transcription (25, 26). Because it is unknown if p53-mediated repression of the human MDR1 promoter (7, 24) and its activation by MT p53 are mediated by distinct cis-elements, we used a series of MDR1 promoter deletion constructs to assess both repression by wt p53 and activation by the MT p53 containing an aspartate to glycine at amino acid 281 in p53 (p53-281). This MT p53 has been demonstrated previously to activate an MDR1 promoter construct containing greater than 1 kilobase upstream from the transcription initiation site (13). We demonstrate that the MDR1 promoter at −1202 and −137 upstream from the MDR1 transcription initiation site are strongly repressed (>70%) by wt p53. In contrast, the −107 MDR1 promoter is only weakly repressed (<15%) by p53 (Fig. 1, upper).

An analysis of MDR1 promoter activation by p53–281 reveals, as expected, the −1202 MDR1 promoter is activated readily by p53–281 (Fig. 1, lower). Because the −107 was not repressed by MT p53, we next tested it and additional 5′ deletions at positions −74 and −58 of the MDR1 promoter. We found that −107 was strongly activated by MT p53 over 12-fold as was the −74 MDR1 promoter. In contrast, −58 was minimally activated (<1.5-fold). Cumulatively, these studies indicate that activation of the MDR1 promoter by p53–281 requires 5′ sequences between −74 and −58. Moreover, it reveals that p53–mediated promoter repression and MT p53 (p53–281) activation are functionally distinct.

Selective Transcriptional Up-regulation of the MDR1 Promoter and Endogenous Gene by MT p53 in Caco-2 Cells—To address the issue of MT p53 activating either the MRP1 or MDR1 promoter and their endogenous genes, we utilized Caco-2, a p53 null colon cell line (17) that expresses MDR1 and MRP1, to determine whether MRP1 and MDR1 were up-regulated by MT p53 (Fig. 2). A variety of naturally occurring p53 MTSs (so-called hot-spot MTSs found in many tumors, e.g., V143A, R175H, R248W, and R273H) (13, 14, 16) were tested in transient transfection assays using MDR1 and MRP1 promoter reporters. We found that although other MT p53s activated the MDR1 promoter (see Fig. 5), the p53 aspartate to glycine 281 MT (p53–281) was the most potent activator of MDR1 and that transcriptional activation required an intact transactivation domain because p53–281 containing mutations at amino acids 14 and 19 was ineffective in up-regulating the MDR1 promoter (Fig. 2A). In contrast, the p53–281 MT as well as others (e.g., V143A, R175H, 248, and 273) (data not shown) were unable to activate the MRP1 promoter (Fig. 2A). To test if the p53–281 MT could activate endogenous MRP1 or MDR1, Caco-2 cell lines were developed by stable transfection of either a p53–281 expression vector or a p53–281 containing mutations in the transactivation domain (amino acids 14 and 19, p53–14,19,281) that rendered it incapable of activating transcription (7). After G418 selection, two NEO cell lines, three p53–281 cell lines, and one p53–14,19,281 were clonally isolated and expanded for further characterization. Western blot analysis for MDR1, p53, MRP1, and MRP4 protein levels was performed on each of the
these cell lines (Fig. 1B). Notably, MRP1 and MRP4 expressions were unaffected by p53–281. In contrast, MDR1 was strongly up-regulated in all three p53–281 cell lines. In contrast, the transcriptionally inactive p53–14,19,281 did not up-regulate MDR1. MDR1 mRNA overexpression in p53–281 cells was confirmed by cDNA microarray analysis (data not shown).
These microarray studies also demonstrated that MRP1, -3, -4, and -5 were essentially unchanged (≤2-fold change), a finding confirming the Western blot analysis of MRP1 and MRP4 as well as demonstrating the specificity of MDR1 up-regulation (Fig. 2B).

To confirm that the overexpressed MDR1 was functional the following studies were performed. First, the mean steady-state intracellular fluorescence of cells treated with the MDR1 substrate and fluorescent probe, rhodamine 123 (22), was tested. In two of the p53–281 cell lines the average rhodamine fluorescence was approximately half that found in either a Neo or 14,19,281 cell line, indicating increased rhodamine efflux (Table I), which is consistent with MDR1 overexpression (Fig. 2B). The function of MDR1 was evaluated further by determining VP-16 (VP-16 is a topoisomerase inhibitor and MDR1 substrate (2)) sensitivity based on the accumulation of apoptotic cells displaying less than G1 phase content (see “Materials and Methods”). At both doses of VP-16, p53–281-expressing cells had less than half of the sub-G1 cells as the Neo cells, indicating reduced cytotoxicity consistent with the increased MDR1

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**Fig. 5.** MT p53 requires the Ets site for MDR1 transactivation. The nucleotide sequence of the MDR1 promoter from −99 to −48 with the relevant transcription factor binding sites and the mutations introduced are shown. A, mutations at the inverted CCAAT box were introduced into pMDR1202 to generate 1202-CAATT (23). The MT MDR1 promoter plasmids (1202-CAATT and pMDR107-ets) and the wt plasmids (pMDR1202 and pMDR107) were transfected into cells (10 μg), and the relative activation was determined as described above. B, a panel of p53 missense MTs were tested for their ability to up-regulate the MDR1 promoter or the MDR1 promoter containing an Ets site mutation (pMDR107-ets). C, Western blot analysis was performed with an equivalent amount of protein lysate from each transfected sample. The proteins were fractionated on a SDS-PAGE gel followed by incubation with anti-p53 IgG (Ab7). The bar represents the average value of 3–6 independent experiments, each performed in duplicate. The line indicates one standard deviation.
and Ets-1 in lysates from transfectants in A

standard deviation. The line represents the standard deviation. B, Western blot analysis of the amount of p53
and MT p53
was kept constant by the addition of their respective empty expression vectors. The bar indicates an average of four independent values obtained from two independent experiments. The line represents the standard deviation.

activity (Table I). This does not indicate a general increase in drug resistance, because the p53–281 cells were equally sensitive to C6-ceramide, a compound that induces cell death but is not transported by MDR1 (Table I).

MDR1 overexpression decreases caspase activity and caspase-mediated cell death in hematopoietic cells (1), perhaps by affecting the expression of effectors or inhibitors of cell death. To determine whether this pathway was relevant in Caco-2 cells, an RNA analysis by RNase protection was conducted on the Neo, 14,19–281, and p53–281 cells (Fig. 3). Fas, Fas ligand, and the TNF receptor as well as the adapter TRADD were undetectable in the Caco-2 derivatives, which is consistent with studies showing Caco-2 cells are insensitive to Fas-mediated death (27). Further, the expression of TNF-related receptors, their inhibitors (inhibitors of apoptosis or IAPs (29)), and bcl-2 family members were unchanged. Notably, the level of either Bcl-2 short or long form was unchanged. Furthermore, levels of caspasas 1, 3, 4, 6, and 8 were no different in MDR1-overexpressing cells, an unexpected finding in light of the decreased caspase activation found in hematopoietic cells that overexpress MDR1 (1). Thus, although unexpected, it is clear that MDR1 overexpression secondary to expression of MT p53 does not specifically alter expression of many apoptotic mediators.

The MDR1 Promoter Is ETS-responsive, and ETS-1 Cooperates with p53–281—We had shown in a previous study that the MDR1 promoter was Ets responsive (28). To directly test the role of the Ets site, located between −75 and −58, we specifically mutated this site (gga converted to tta) in the context of the −107 MDR1 promoter (Fig. 4A). We then assessed the ability of Ets-1 to activate the wt −107 and the Ets −107 MDR1 promotor (Fig. 4B). The −107 MDR1 promoter was readily activated by Ets-1 (2.5-fold), whereas the MT MDR1 Ets promotor was virtually unchaged by Ets-1. Ets-2 also activated the MDR1 promotor to a similar extent (data not shown).

Mutation of the Ets site decreased MDR1 basal activity (Fig. 5A) to levels comparable with the inverted Inv-CCAAT (see “Materials and Methods”) MT MDR1 promotor (31). However, the Inv-CCAAT MT was readily activated by MT p53 (−15-fold), a finding indicating MDR1 promotor activation by MT p53 does not require interaction with Inv-CCAAT-binding proteins (e.g. NFY) (Fig. 5A). In contrast, the Ets −107 MDR1 promotor was not activated by MT p53, which is in agreement with the MDR1 promotor deletion analysis (Fig. 1, lower). To determine whether an intact Ets site was required for all MT p53a to activate the MDR1 promotor, we evaluated a variety of naturally occurring hot-spot MT p53s for their ability to activate either the −107MDR promotor or the −107MDR-Etsmut promotor (Fig. 5B). Each of the MT p53s activated the −107MDR promotor to varying degrees, whereas none activated the −107MDR-Etsmut promotor. As a control to verify that the lack of MDR1 activation of 107MDR-Etsmut is not caused by decreased MT p53 expression, we performed immunoblot analysis on the transfectants. We found that each transfectant had comparable MT p53 levels, thus ruling out the possibility that decreased activation was caused by no or dramatically altered MT p53 expression (Fig. 5C). These studies demonstrate that the Ets binding site between −75 and −58 is essential for MT p53 activation of the MDR1 promotor. Cumulatively, these data indicate that MT p53 activation of the MDR1 promotor is independent of interactions with either Inv-CCAAT-binding proteins or the transcription factors that reportedly bind the GC box between −58 and −40 (Sp1 and Egr-1 (30)).

One prediction from these studies is that Ets-1 and MT p53 would synergistically activate the MDR1 promotor. The −107MDR promotor was co-transfected with a combination of plasmids expressing either Ets-1 or p53–281 and/or their respective empty vectors (Fig. 6). By themselves, Ets-1 and p53–281 activated the MDR1 promotor 2.5- and 3.3-fold, respectively. Co-transfection of both Ets-1 and p53–281 produced a range from 6- to 12-fold activation of the MDR1 promotor, which indicates a strong functional interaction (Fig. 6A). Notably, the Western blot analysis of the transfectants indicates that Ets-1 does not increase MT p53–281 expression from its expression vector, and likewise the expression of p53–281 does not alter Ets-1 expression from its vector (Fig. 6B).

Ets-1 Interacts with MT p53—The functional requirement for the Ets site and the synergistic activation of the MDR1 promotor by p53–281 and Ets-1 suggests that these two proteins interact. To determine whether MT p53 interacts with Ets-1 in vitro, we performed an in vitro pull-down assay using recombinant glutathione transerfase-Ets-1 and in vitro translated p53–281 protein (Fig. 7A). The recombinant glutathione transferase-tagged Ets-1 was then incubated with the in vitro translated 35S-labeled p53–281. The recombinant Ets-1 readily pulled-down p53–281. Notably, despite loading equivalent amounts of protein, the glutathione coupled to the agarose beads was unable to retain wt p53 (data not shown). Next, to
Fig. 7. Ets associates only with MT p53 in vitro and in vivo. A, MT p53 expression vectors were linearized and transcribed with T7 polymerase in the coupled in vitro transcription and translation system (Promega) and analyzed as described under “Materials and Methods.” B, 10 μg of p53–281 and/or Ets-1 or their appropriate empty vectors were transfected into the human osteosarcoma cell line, Saos-2, as described before. The cells were lysed and proteins were immunoprecipitated (50 μg) as described under “Materials and Methods.” The proteins from the gel were transferred to a nitrocellulose membrane and used for Western blot analysis using the anti-p53 antibody DO-1. C, a variety of p53–281 MTs (p53–281, p53–281,22,23, and p53–281 delta360) and p53–143 were transfected with or without Ets-1, and immunoprecipitation and Western blot were done as described in A except that the protein input for the immunoprecipitation was increased to 100 μg and the antibody was Ab7.

Mutant p53 associates with ets but not wildtype p53 in vivo and in vitro

A

input p53281

p53281

B

p53

Ig

+CTL + + + + + + - - vector
- - + + - - - - ets-1
- - - - - - - - p53281
- - - - - - - - wt p53

test for in vivo interaction between MT or wt p53 and Ets, cells were transfected with either Ets-1 alone or in combination with p53–281 or wt p53 (Fig. 7B). The cells were then lysed, and protein complexes were immunoprecipitated using an antibody that recognizes the N terminus of Ets. The immunoprecipitated complex was washed extensively, and subsequently Western blot analysis using the N-terminal p53 antibody DO-1 demonstrated that MT p53–281 was immunoprecipitated by the Ets antibody, indicating that these two proteins interact in vivo (Fig. 7B). In contrast, wt p53 was not immunoprecipitated by the Ets antibody demonstrating that wt p53 does not interact with Ets-1. Notably, cells transfected with p53–281 alone have a very weak p53 immunoreactive band, a finding suggesting that the endogenous Ets is limiting in promoting an interaction with MT p53. To test the possibility that MT p53 might interact with endogenous Ets and also to map the regions of MT p53 required for interaction with endogenous Ets, cells were transfected with p53–281, p53–281–22,23 containing mutations in the N terminus, p53–281 delta360 (deletion of the last 33 amino acids), and a conformational MT p53–143 (14). The transfected cells were lysed followed by immunoprecipitation with the N-terminal Ets antibody and Western blot using the panspecific p53 antibody, Ab7 (Fig. 7C). This study demonstrates that both MTs p53–281 and p53–143 retain the ability to interact with endogenous Ets. Similarly, deletion of 33 amino acids from the C terminus of p53 (281 delta360) does not affect complex formation with Ets, whereas mutation in the transactivation domain of p53 effectively abrogates complex
Mutant p53 Activates Endogenous MDR1 But Not MRP1

formation with endogenous Ets. Therefore, MT p53s, and not wt p53, physically interacts with Ets and requires an intact N terminus.

**DISCUSSION**

Many different tumors express MT p53 and overexpress both drug resistance genes MDR1 and MRP1 (6, 10, 11); however, these correlative studies, although suggestive, do not provide direct evidence for MT p53-mediating transcriptional regulation of these genes. Because clinical studies leave uncertainty with respect to the mechanism of how the status of p53 impacts MDR1 or MRP1 expression, we evaluated whether MDR1 and MRP1 promoters and endogenous genes were activated by MT p53. Moreover, we used p53 null cells to eliminate potential transdominant effects (14) and found that MT p53 only up-regulated MDR1 and not MRP1. Further, because MT p53s are defective in specific DNA binding, we reasoned that MT p53 might interact with a cellular protein. Our deletion analysis of the MDR1 promoter led us to a region in the minimal promoter of MDR1 that contained an Ets site that was essential for promoter activation by a variety of hot-spot MT p53s. Further studies determined that Ets-1 interacted with only MT p53 in vitro and in vivo and that wt p53 was unable to interact with Ets-1 in vivo. Cumulatively, these studies form the mechanistic basis for activation of the MDR1 gene by MT p53.

The mechanistic basis of MT p53 activation of human MRP1 and MDR1 transporter expression has become unclear on the basis of recent clinical studies (6, 10, 11, 32, 33). With regard to MDR1, studies in breast, colon, and oral cancers indicate an excellent correlation between immunochemical detection of p53 (presumed MT) and MDR1 (6, 10, 11); however, none of these studies have ruled out the possibility that MDR1 up-regulation is a consequence of the loss of repression secondary to mutation in p53. Further, no study to date has demonstrated that the addition of a MT p53 to a cell line lacking p53 activates expression of the endogenous MDR1. Therefore, because tumor-derived MT p53s are, in general, defective in sequence-specific binding, the activation of different promoters by MT p53 interacting with promoter-specific proteins is a formal possibility. Nevertheless, our data indicate that the Inv-CCAAT box (important for NFY-mediated and basal activity of the MDR1 promoter (31)) is very unlikely to play a role in MDR1 up-regulation by MT p53, because mutation of the Inv-CCAAT box did not affect its activation of the MDR1 promoter.

Nonetheless, our studies are the first to demonstrate that MT p53 promotes and specifically up-regulates MDR1 but not MRP1. In light of our previous findings demonstrating MDR1 expression can be increased by relief from p53-mediated repression (7) and our current findings demonstrating MDR1 up-regulation by MT p53, p53 can be viewed as a double-edged sword for controlling MDR1 expression.

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