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mdm-2 Inhibits the G_1 Arrest and Apoptosis Functions of the p53 Tumor Suppressor Protein

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The *mdm-2* gene encodes a 90-kDa polypeptide that binds specifically to the p53 tumor suppressor protein. This physical interaction results in the inhibition of the transcriptional functions of p53 (J. Chen, J. Lin, and A. J. Levine, Mol. Med. 1:142–152, 1995, and J. Momand, G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine, Cell 69:1237–1245, 1992). Experiments are described that demonstrate the ability of mdm-2 to abrogate both the p53-mediated cell cycle arrest and the apoptosis functions. In addition, the results presented here suggest that mdm-2 binding to p53 and the resultant inhibition of p53 transcription functions are critical for reversing p53-mediated cell cycle arrest. The N-terminal half or domain of the mdm-2 protein is sufficient to regulate these biological activities of p53, consistent with the possibility that the highly conserved central acidic region and the C-terminal putative zinc fingers of mdm-2 may encode other functions.

The p53 tumor suppressor protein is a sequence-specific DNA-binding protein and a transcription factor. p53 plays a critical role in the prevention of malignancies in both humans and mice. More than 50% of human tumors contain alterations of the p53 gene (17), most of which are missense mutations in one allele and the loss of the remaining wild-type allele. Members of families with Li-Fraumeni syndrome contain germ line mutations in one p53 allele and are at high risk for developing cancers at young ages (24, 36). Mice with homozygous deletions of both p53 alleles also develop tumors at young ages (9).

The ability of p53 to prevent malignancies is likely due to its ability to regulate the cell cycle and the apoptotic pathway. In animals or in cell culture, DNA damage or abnormal proliferation of cells results in increased p53 levels and enhanced transcriptional activity (18, 22, 34). These increased p53 levels and enhanced transcriptional activity then initiate either cell cycle arrest or apoptosis. The biochemical activity of p53 responsible for the induction of cell cycle arrest may stem from p53's ability to transcriptionally activate the expression of a cyclin-dependent kinase (cdk) inhibitor, p21/WAF1, as well as other possible growth inhibitory proteins such as GADD45 (10, 18). It is still unclear how p53 induces apoptosis. Certain experiments have suggested that the transcription activation function of p53 may not be required for such an activity (1, 40).

The p53 pathway to cell cycle arrest or apoptosis is regulated by multiple mechanisms. The regulation of p53's half-life, intracellular localization, conformation, DNA binding, posttranslational modification, and interaction with regulatory proteins may all play a role in the regulation of the pathways to cell cycle arrest and apoptosis. The *mdm-2* gene encodes a 490-amino-acid protein that is thought to play a role in the regulation of p53 by directly binding to p53. The mdm-2 protein contains an N-terminal 100-amino-acid region that is necessary for binding to the N-terminal transcription activation domain of p53 (4, 32). The mdm-2 protein also contains a highly conserved central acidic region and three putative zinc finger motifs in the C terminus of the protein that are not required for the binding of p53 (32). Overexpression of mdm-2 inhibits both the transcriptional activation and repression activities of p53 (3, 30). This inhibition is thought to be due to mdm-2 binding to a region of p53 that includes p53 amino acid residues 14, 19, 22, and 23, which interact with transcriptional coactivator proteins such as the transcription factor TAF31 (23, 38).

The binding and inhibition of p53 may be an important mechanism of transformation by mdm-2. Overexpression of mdm-2 in a mouse NIH 3T3 cell line confers enhanced tumorigenic potential in a nude mouse assay (11). *mdm-2* can cooperate with an activated *ras* oncogene to transform primary rat embryo fibroblasts (13). Approximately 30% of human sarcomas contain amplification and overexpression of the *mdm-2* gene, suggesting that mdm-2 can contribute to malignant transformation in these tumors (6, 31). Most of the sarcomas with mdm-2 amplification retained the wild-type p53 gene and protein, suggesting that mdm-2 overexpression may well have bypassed the need to mutate p53 (19). It is also possible that mdm-2 has transforming functions independent of its binding to p53, such as the recently proposed results of mdm-2 binding to L5, Rb, E2F, and DP1 proteins (25, 26, 42).

An important functional domain of p53 is its N-terminal 42-amino-acid region, which functions as a transcription activation domain (12, 35, 39). It has been shown previously that mdm-2 binds to this same region and negatively regulates the transcription functions of p53 (3, 30). If these transcriptional activities are important for mediating cell cycle arrest and apoptosis by p53, mdm-2 should also be able to interfere with these functions. In this report, experiments are described that directly address these questions. It is shown that overexpression of mdm-2 can indeed abrogate the G_1 arrest function of p53. In a cell line that undergoes apoptosis in response to wild-type p53, mdm-2 can efficiently inhibit this apoptosis. Furthermore, it is shown that mdm-2 must be able to bind to the p53 protein in order to abrogate p53-mediated G_1 arrest.

MATERIALS AND METHODS

Plasmids and cell lines. The cell lines H1299 (human non-small-cell lung carcinoma cells [28]), U2-OS and Saos-2 (human osteosarcoma cells [8, 27]), 3T3DM (immortalized tumorigenic BALB/c 3T3 cells [2]), and VM10 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Plasmid pCMV-CD20 was provided by Sander van den Heuvel of Massa-

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chusetts General Hospital (16), pCMV-c-myc was provided by Michael Cole of Princeton University, pSFFV-Bc12 was provided by Eileen White of Rutgers University (5), and pRSV-Tg was provided by James Pipas of the University of Pittsburgh. Human mdm-2 expression plasmids pCHDM1A, pCHDMΔ222-437, and pCHDMΔ440-491 were described previously (3). All *mdm-2* clones used in this study are human *mdm-2* cDNAs except the murine *mdm-2* genomic clone used in the rescue experiment. Mutant p53 plasmids pRC-CMV-SN14/19 and pRC-CMV-SN22/23 were described previously (20a).

Immunoprecipitation. Metabolic labeling with [^{35}S]methionine and immunoprecipitations were performed as described previously (33). For immunoprecipitation of mdm-2, 2 μl of a rabbit polyclonal serum against human mdm-2 was used for each reaction. Monoclonal antibody Pab421 was used for the precipitation of p53. The samples were analyzed by 7.5% denaturing polyacrylamide gels and autofluorography.

Cell cycle analysis. To detect cell cycle arrest of p53, the cells were transiently transfected with 4 μg of pCMV-CD20, 2 to 10 μg of p53 expression plasmids, and 10 μg of mdm-2 expression plasmids. Total DNA was adjusted to 30 μg with sonicated salmon sperm DNA. The cells were transfected by the calcium phosphate procedure, and the cells were incubated in the precipitates for 16 h. Forty-eight hours after removal of the precipitates, the cells were harvested and stained with a fluorescein-conjugated anti-CD20 antibody (Becton Dickinson) and propidium iodide as previously described (16) and analyzed on an EPICS fluorescence-activated cytometer (Coulter). To observe the progression of cells through the cell cycle, nocodazole (Sigma) was added to a final concentration of 20 ng/ml at 32 h after removal of the precipitate and the cells were incubated for 16 h before analysis.

Plating efficiency assay. 3T3DM cells were transfected with 15 μg of the pRC/CMV vector (Invitrogen) alone, wild-type or mutant p53 expression plasmids (all in the pRC/CMV vector), and 5 μg of salmon sperm DNA by the calcium phosphate procedure. Three days after transfection, the cells were seeded at densities of 2.5×10^4 and 5×10^4 per plate and incubated in a medium containing 600 μg of G418 per ml. The cells were re-fed every 5 days, and the colonies were stained and counted after 14 days.

Detection of apoptotic cells. VM10 cells were maintained at 39°C and transfected with 4 μg of pCMV-CD20 and 15 μg of mdm-2, Bcl2, or T-antigen expression plasmids. After a 16-h incubation with the precipitates at 39°C, the cells were washed with phosphate-buffered saline (PBS) and incubated at 39°C for 24 h. Cytochalasin B (Sigma) was added to a final concentration of 5 μg/ml, and the cells were incubated at 32°C for 6 h. The adherent cells were harvested by incubating them for 5 min in PBS with 5 mM EDTA, pelleted, and incubated with 20 μl of anti-CD20 antibody (Becton Dickinson) for 30 min on ice. The cells were washed once with PBS with 1% fetal bovine serum and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and with 80% ethanol for 20 min at room temperature. The cells were stained for DNA fragmentation with reagents from an Apoptaq kit (Oncore) according to directions from the manufacturer except that phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin G (Promega) was included with the fluorescein isothiocyanate–anti-digoxigenin antibody in the final incubation. The cells were suspended in PBS with 0.1 μg of DAPI (4′,6-diamidino-2-phenylindole) per ml–1% fetal bovine serum and centrifuged onto a slide. The slides were mounted with a solution of 90% glycerol, 0.15 M $NaPO_4$ (pH 7.5), and 0.1% *p*-phenylenediamine. Fluorescent photography was performed with a Zeiss fluorescent microscope. For quantitation of apoptosis staining among transfected cells, the transfected cells were first identified with a 540- to 560-nm filter that reveals only PE staining of CD20 cells, and these cells were then examined with a 470- to 490-nm filter, with which both PE and fluorescein isothiocyanate (DNA fragmentation) staining were visible. Approximately 300 $CD20^+$ cells were examined, and the percentages of double-stained cells among the $CD20^+$ cells were then calculated.

RESULTS

The role of transcriptional activation in p53-mediated G_1 arrest. To directly assay G_1 arrest mediated by p53 and to determine if mdm-2 can regulate this function, cells were first cotransfected with a p53 expression plasmid and a CD20 gene expression plasmid. The detection of cells expressing CD20, with a labeled antibody to CD20 and a fluorescence-activated cell sorter (FACS), then allowed identification of transfected cells which coexpress p53. These cells were also analyzed for their DNA content (propidium iodide) by FACS analysis. The nontransfected cells ($CD20^-$) from the same sample were employed as the control group (with no p53). While it remains possible that this $CD20^-$ population (which did not take up DNA) is a special subpopulation of cells, they are quite normal with respect to their cell cycle properties. This is a sensitive assay for testing the ability of p53 to block progression of cells through the cell cycle.

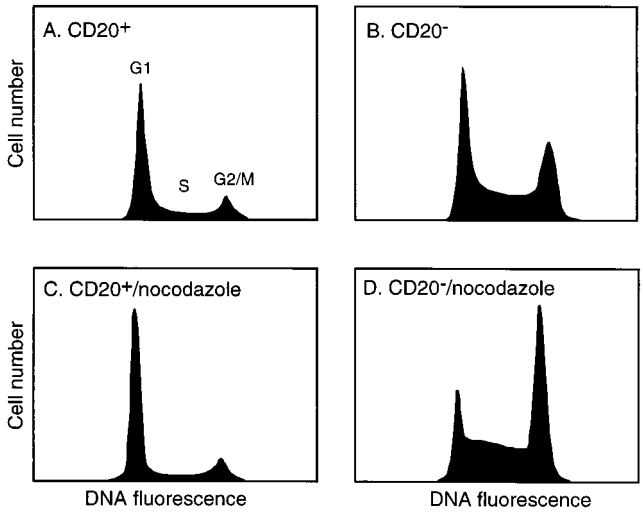

FIG. 1. G_1 arrest of U2-OS cells by human wild-type p53. U2-OS cells were transfected with p53 and CD20 expression plasmids. The cells were harvested 48 h after transfection and stained with anti-CD20 antibody and propidium iodide and analyzed by FACS analysis. (A) Profile of the $CD20^+$ cells (70% in G_1). (B) $CD20^-$ cells (38% in G_1) in the same sample used for panel A. The protocol used for panels C and D was identical to that used for panels A and B except the cells were incubated in medium with nocodazole for 8 h before staining. The $CD20^-$ cells accumulate in the G_2-M phase (25% in G_1); the $CD20^+$ cells remain arrested in G_1 (70% in G_1).

By this assay, the effects of high p53 levels upon cell cycle progression of several cell lines were tested with the human lung carcinoma cell line H1299 and the human osteosarcoma cell line Saos-2, both devoid of endogenous p53, and a human osteosarcoma cell line, U2-OS, which has wild-type p53. Transfection of p53 into all three cell lines results in increased populations of cells in G_1, as shown in Fig. 1, for which U2-OS cells were used as an example. This increase in G_1 cells could be the result of any one of two effects: G_1 arrest or a shortening of the S and G_2-M phases of the cell cycle. To distinguish these possibilities, the cells were treated with the microtubule-disrupting agent nocodazole for 8 h before being stained for FACS analysis to prevent the G_2-M cells from recycling back to G_1. This permits one to observe the rate of cells exiting G_1 during the treatment period. As shown in Fig. 1, p53-expressing cells remain blocked in G_1 in nocodazole whereas the untransfected cells continue to progress into the S and G_2-M phases, confirming that the expression of p53 in these cells specifically caused the G_1 arrest.

Previous studies have constructed and utilized point mutations in the N-terminal transactivation domain of p53 that blocked the transactivation function of p53 and p53's interaction with mdm-2 (20a). These p53 mutants were tested in this cell cycle assay to examine the role of endogenous cellular mdm-2 binding and transcriptional regulation in p53-mediated G_1 arrest. The results of these experiments are shown in Fig. 2. The 14/19 double mutant of p53 (mutations are at amino acid residues 14 and 19) is partially active for the transcription of a test gene, but it is completely defective in mdm-2 binding (20a). This mutant can still cause significant G_1 arrest. The 22/23 mutant of the p53 protein (with mutations at amino acid residues 22 and 23), which is defective for both transcriptional activation and mdm-2 binding, was significantly impaired in G_1 arrest (Fig. 2). As expected, the tumor-derived p53 hot spot mutation at codon 273 commonly observed with cancers has lost most of its p53-mediated G_1 arrest function. These results

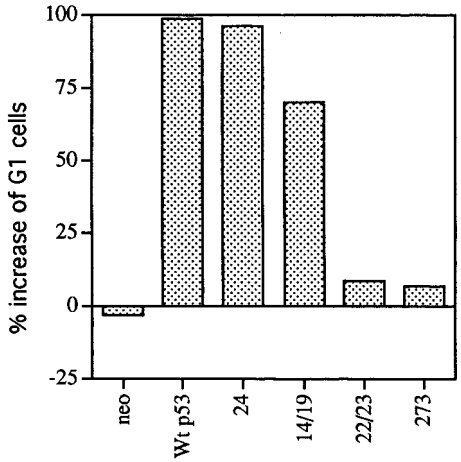

FIG. 2. G_1 arrest by p53 mutants. Saos-2 cells were transiently transfected with p53 and CD20 expression plasmids. The cells were treated with nocodazole and analyzed as described in Materials and Methods. The fractions of G_1 cells in the $CD20^+$ and $CD20^-$ populations were compared, and the difference is shown as percent increase of G_1 cells. Thus, if 80% of the $CD20^+$ cells and 40% of the $CD20^-$ cells are in G_1, the difference will be shown as (80% − 40%)/40% = 100% increase). The p53 mutant at amino acid 24 has a K-to-T change (K24T) and has wild-type (Wt) levels of mdm-2 binding and transactivation; p53 14/19 mutants contain L14Q and F19S mutations and are defective for mdm-2 binding; the p53 22/23 mutant contains L22Q and W23S mutations, and it is defective for both mdm-2 binding and transcription activation; the p53 273 mutant contains an R273H hot spot mutation. neo, pRC/CMV vector alone. Both the p53 22/23 and p53 273 mutants are significantly impaired for G_1 arrest. These results represent averages of two experiments.

suggest that p53 binding to endogenous mdm-2 is not required for G_1 arrest by p53 and that the transcription activation function of p53 is important in mediating this block in the cell cycle.

mdm-2 overcomes G_1 arrest by p53. Next, mdm-2 expression plasmids were cotransfected along with p53 into several different cell lines to determine if mdm-2 could prevent G_1 arrest by p53. Previous experiments indicated that the ratio between the two proteins is critical if one wants to measure the inhibition of p53 transcription activation, probably because of the fact that mdm-2 directly titrates p53 by its binding. Because mdm-2 is poorly expressed in many cells (such as H1299 and Saos-2 cells), high levels of p53 would completely overcome the inhibitory effect of mdm-2. Furthermore, detecting G_1 arrest required significantly higher levels of p53 expression (>1 μg of plasmid added to a 10-cm-diameter culture dish of cells) than detecting transactivation of a chloramphenicol acetyltransferase reporter plasmid (>20 ng of plasmid added to a 10-cm-diameter culture dish of cells) (3). Only the U2-OS cell line, which supported high levels of mdm-2 expression in transient transfections with mdm-2 and p53 plasmids, was found to be useful for examining the ability of mdm-2 to reverse the p53-mediated G_1 arrest.

When a full-length *mdm-2* cDNA was cotransfected with p53 into U2-OS cells, it completely inhibited G_1 arrest by p53 (Fig. 3). To identify the domain of mdm-2 that is important in this inhibition of p53-mediated G_1 arrest, several mdm-2 deletion mutants were cotransfected into these cells. These mutants revealed that only the N-terminal half of mdm-2 is required for this function; mutant mdm-2 proteins with deletions of the acidic region and zinc fingers were still capable of inhibiting p53 G_1 arrest. These data suggest that p53 regulation by mdm-2 is dependent on the binding of the N terminus of mdm-2 to p53. To further test this possibility, the 14/19 double-point mutant of p53 was employed. This mutant is defective for mdm-2 binding, and its transcription function is not inhibited by mdm-2. As expected, the 14/19 mutant was able to cause significant G_1 arrest in the U2-OS cells and coexpression of mdm-2 had no effect on this G_1 arrest (Fig. 3). Therefore, the regulation of p53 G_1 arrest by mdm-2 requires a complex formation between mdm-2 and p53. The expression of various p53 and mdm-2 proteins and their physical interactions in these transfection experiments were confirmed by metabolic labeling of cotransfected cells and immunoprecipitations of p53, mdm-2, and p53–mdm-2 complexes as shown in Fig. 4.

Growth inhibition by the 14/19 mutant is resistant to mdm-2. To further confirm the role of complex formation between mdm-2 and p53 in the regulation of p53 functions, the ability of mdm-2 to overcome p53-mediated inhibition of cell growth in a plating efficiency assay was tested. In this assay, a drug-resistant plasmid is transfected into the cell and stable drug-resistant colonies are selected. When the plasmid also coexpresses wild-type p53, the efficiency of obtaining colonies is significantly reduced, probably because p53 overexpression leads to growth arrest or cell death. To test if high levels of mdm-2 can abrogate this growth inhibition by p53, the 3T3DM cell line was transfected with wild-type p53 and the p53 14/19 mutations were linked in *cis* on a plasmid with a G418 resistance marker. 3T3DM cells contain *mdm-2* gene amplifications on double-minute chromosomes and express high levels of mdm-2 protein (33). Wild-type p53 is not able to reduce the formation of G418-resistant colonies in this cell line, suggesting that the high levels of endogenous mdm-2 in these cells have prevented p53 from exerting its effects (Table 1). In contrast, the p53 14/19 mutant, which does not bind mdm-2, caused an approximately fivefold reduction of colony plating efficiency (Table 1). When these constructs were transfected into the Saos-2 cells which do not overexpress mdm-2, both were able to cause a four- to fivefold reduction of G418-resistant colonies (21). Therefore, growth inhibition of cells in culture by p53 is abrogated by mdm-2 in a mdm-2–p53 complex-dependent fashion.

Construction of a p53-dependent apoptotic cell line. To test the inhibition of p53-mediated apoptosis by mdm-2, a cell line was established that can initiate apoptosis in response to wild-type p53. Previously, a temperature-sensitive p53 mutant, Val135, was introduced into an immortal mouse fibroblast line, 10(1), which had no endogenous p53 (15). The resulting cell line, 10(1)Val5, was growth arrested in G_1 only at 32°C, when p53 was in the wild-type conformation (32a). This cell line was used to introduce a murine c-*myc* oncogene plasmid at 39°C (with the mutant p53 form) to establish a cell line named VM10 that overexpresses c-*myc*. The cell line was maintained at 39°C, at which temperature p53 is in the mutant conformation. After a 2- to 3-h incubation at 32°C, many cells became refractile to light and showed blebbing of their cellular membranes and the formation of apoptotic bodies (Fig. 5D). Agarose gel electrophoresis of the genomic DNA extracted from these cells showed the formation of nucleosome-sized ladders, a hallmark of apoptosis (Fig. 5E). Therefore, overexpression of the c-*myc* oncogene in the presence of high levels of wild-type p53 leads to apoptosis.

Inhibition of p53-dependent apoptosis by mdm-2. To test if mdm-2 overexpression can inhibit the apoptosis function of p53, the VM10 cells were transfected with a cosmid containing the genomic *mdm-2* gene (10). The cells were maintained at 32°C without drug selection for the mdm-2 cosmid, and surviving colonies were isolated. If mdm-2 can rescue the VM10 cells from apoptosis, then transfection should result in surviving colonies that express high levels of mdm-2 without drug selection for the cosmid. When emerging colonies at 32°C were

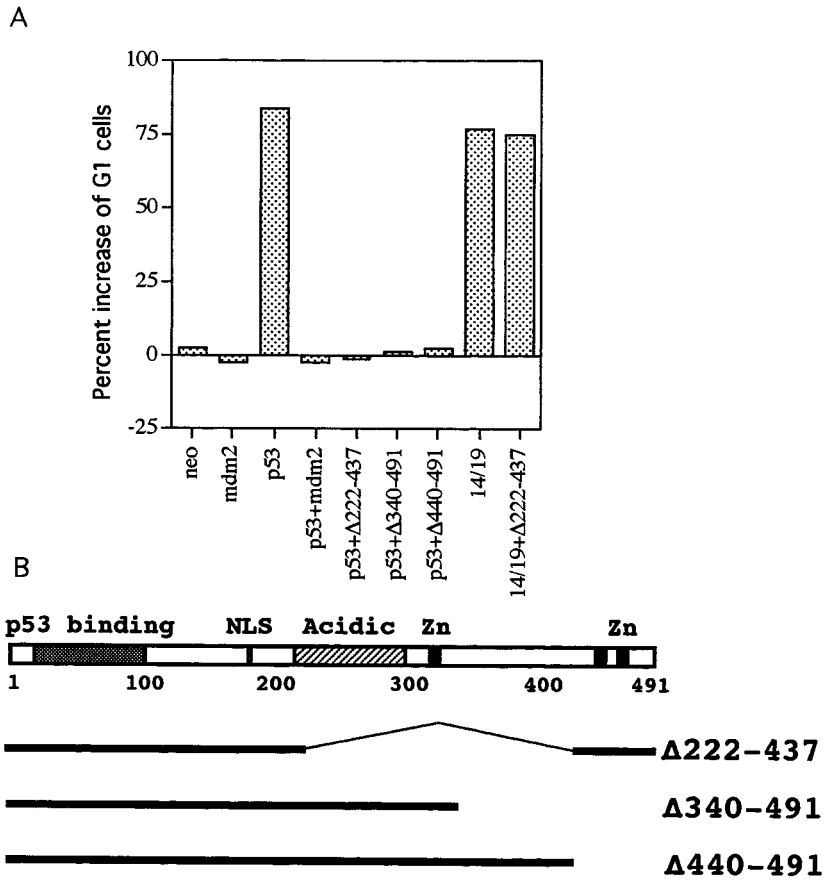

FIG. 3. (A) Inhibition of p53 G_1 arrest by mdm-2. U2-OS cells were transfected with p53, mdm-2, and CD20 and analyzed without nocodazole treatment. The changes in the G_1 population of the transfected cells are shown. Coexpression of full-length human mdm-2 and three mdm-2 mutants prevents G_1 arrest by p53. The p53 14/19 mutant, which does not bind mdm-2, is not inhibited by the mdm-2 Δ222-437 mutant. Full-length mdm-2 also does not inhibit the p53 14/19 mutant (not shown). The data are averages of three experiments. (B) Schematic drawing of the three mdm-2 deletion mutants used in the experiment shown in panel A, showing deletions of the acidic region and the zinc fingers of mdm-2. NLS, nuclear localization signal. neo, pRC/CMV vector alone.

isolated and tested for mdm-2 expression, 3 of 15 (20%) expressed high levels of mdm-2 (data not shown). Therefore, this experiment is consistent with the possibility that overexpression of mdm-2 is among one of the genetic changes that can inhibit the apoptotic function of p53 (at 32°C).

To further confirm and test the ability of mdm-2 to block p53-induced apoptosis and to circumvent the high-level-background problem of this long-term selection assay, a transient transfection assay was established that would permit the detection of transfected cells and quantitation of apoptosis. The VM10 cells were cotransfected with the CD20 and mdm-2 plasmids. Thirty-six hours after transfection, cytochalasin B was added to the medium and the cells were incubated for 6 h at 32°C. The adherent cells were then harvested and stained with antibody against CD20, and the apoptotic cells were identified by TUNEL staining (14). In the TUNEL staining, the DNA ends were labeled with terminal deoxynucleotide transferase and digoxigenin-conjugated dUTP and the labeled cells were then visualized by fluorescein isothiocyanate-conjugated anti-digoxigenin antibody. The double-fluorescence staining allows the quantitation of transfected cells that have undergone DNA fragmentation (Fig. 6). Because the VM10 cells that have initiated apoptosis rapidly disintegrate into multiple apoptotic bodies and detach from the plate, the use of cytochalasin B was necessary to obtain quantitative results. Cytochalasin B disrupts actin filaments and can prevent disintegration of apoptotic cells while having no effect on the other aspects of apoptosis such as condensation and fragmentation of nuclear DNA (7). Cytochalasin B itself does not cause apoptosis of the VM10 cells when they are kept at 39°C.

To demonstrate that the assay can indeed measure transient inhibition of apoptosis, we first tested the phenotypes of the *Bcl2* gene and the simian virus 40 large T antigen, both of which are known inhibitors of p53-mediated apoptosis (5, 37). During the 6-h incubation at 32°C, about 30% of the VM10 cells fragmented their DNA and thus became intensely stained by the TUNEL labeling. Transfection of both *Bcl2* and the simian virus 40 large T antigen resulted in an approximately fivefold reduction of apoptotic cells. Transfection of full-length mdm-2 also consistently resulted in four- to fivefold reduction of TUNEL-positive cells (Fig. 7). Two deletion mutants of mdm-2 with an intact p53 binding domain were also able to provide a threefold inhibition of apoptosis. Perhaps the altered conformation of these mutant proteins reduces their efficiency. Several mdm-2 deletion mutants that fail to bind to p53 expressed only very low levels of mdm-2 in this cell line. This prevented further testing of the correlation between p53–mdm-2 complex formation and inhibition of apoptosis. The inhibitory effects of Bcl2, T antigen, and mdm-2 were specific for these functions, because a cdc2 dominant-negative mutant failed to block apoptosis even though it caused a G_2-M arrest in these cells (not shown). Other genes tested that do not

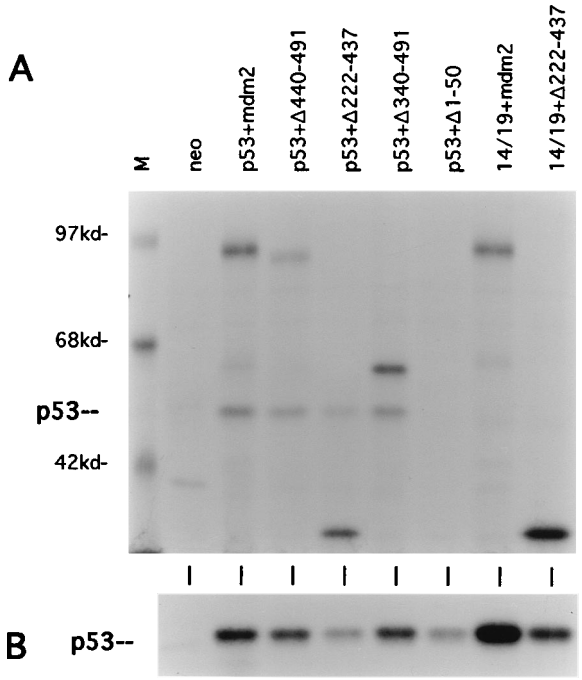

FIG. 4. Expression of mdm-2 and p53 in U2-OS cells. U2-OS cells were transfected as described in the legend to in Fig. 3 and labeled with [^{35}S]methionine 36 h after transfection. (A) Immunoprecipitation of mdm-2 with rabbit anti-mdm-2 serum. The cells were transfected with the combinations of plasmids indicated above the autoradiograph. The p53 proteins that coprecipitated with mdm-2 are indicated. The p53 14/19 mutant did not coprecipitate with mdm-2. The mdm-2 Δ1-50 mutant did not express in this transfection. (B) Immunoprecipitation of p53 with Pab421 of the same lysate used in the experiment shown in panel A, showing the expression of p53 and the p53 14/19 mutant in these transfections. neo, pRC/CMV vector alone.

inhibit the apoptosis of VM10 cells at 32°C include *Rb*, the *ras* oncogene, and the *cdk2* and *cdk3* dominant-negative mutants (16) (data not shown). Therefore, mdm-2 can inhibit the apoptosis formation of p53 and this inhibition does not require the acidic region and zinc finger domains of the mdm-2 protein.

DISCUSSION

Previous experiments have shown that mdm-2 can inhibit the transactivation and repression functions of the p53 protein and that this inhibition requires direct binding between mdm-2 and p53 (3). p53 amino acid residues 14, 19, 22, and 23 are critical for interactions with both mdm-2 and the coactivator TAF31 (20a, 25), suggesting that these interactions are mutually exclusive; i.e., these two proteins compete for the same sites on p53. mdm-2 binds to p53 in vitro with greater strength than TAF31 does (the p53 complex is stable in 500 mM NaCl for mdm-2, compared with 100 mM NaCl for TAF31), and mdm-2 is expected to block the binding of TAF31. If the biological activities of p53, such as G_1 arrest and apoptosis, result from these transcriptional functions, then they will be regulated by mdm-2. The experiments described in this report clearly demonstrate that mdm-2 can negatively regulate these two functional activities, which may be most critical for the tumor-suppressing function of p53.

The ability of p53 to cause G_1 arrest is dependent on the function of its N terminus. Mutations of codons 22 and 23, which inactivate transcription activation, significantly impair G_1 arrest. The binding of mdm-2 to this region of p53 also inhibits G_1 arrest. These results are consistent with the hypothesis that p53 transcriptionally regulates genes such as *p53/WAF1* and *GADD45* that mediate growth arrest. The requirement of p53–mdm-2 binding to block G_1 arrest suggests that this regulation is directly mediated by this protein-protein interaction and is unlikely to be the result of other unknown mdm-2 functions. Consistent with this notion is the observation that the highly conserved zinc finger domain and the acidic domain of mdm-2 are dispensable for this regulatory function.

The ability of the p53 14/19 mutant to cause G_1 arrest suggests that mdm-2 binding is not required for this activity, eliminating the possibility that mdm-2 is a functional partner or positive regulator in this process. The G_1 arrest function of this mutant, similar to its transcription activation function, is not abrogated by overexpression of mdm-2. This may be the reason that this mutant, which is immune to the growth inhibition effects of wild-type p53 because of its overexpression of mdm-2, is able to efficiently reduce the plating of the 3T3DM cells. The phenotypes of this mutant strongly suggest the importance of complex formation in the functional interactions between these two proteins and reinforce the conclusion that mdm-2 regulates p53 by direct binding (30).

The mechanism by which p53 induces programmed cell death is still unclear. Experiments using RNA and protein synthesis inhibitors suggest that active transcription and protein synthesis are not required for this function in some cell types (1, 40). Other studies suggest that p53-induced apoptosis may be mediated by transcription activation of the *Bax* gene and repression of the *Bcl2* gene in certain cell lines (29). This study clearly demonstrates that mdm-2 can inhibit the apoptosis function of p53 in VM10 cells. This would imply that the transcriptional domain of p53 is required for apoptosis in these cells. This could be due either to transcriptional activation or the repression functions of p53 or to the ability of this region to signal to the apoptotic pathway directly. These results also point to the possible involvement of the amino acid sequence in conserved region I of p53 (residues 14 to 23) in mediating this function. Therefore, it would be interesting to see if p53 with mutations in codons 14 and 19 and p53 with mutations in codons 22 and 23 are impaired in the apoptotic function.

Because some of the mdm-2 deletion mutants did not express at significant levels in the VM10 cells, it was not possible to definitively test the role of p53–mdm-2 complex formation in the regulation of p53-mediated apoptosis. However, the ability of two mdm-2 deletion mutants without the acidic region or zinc fingers to block p53-mediated apoptosis suggests, but does not prove, that the mechanism of this regulation requires the binding of mdm-2 to p53 and the masking of p53. Whether mdm-2 has the potential to inhibit other types (in

TABLE 1. Effects of the p53 mutants on the plating efficiency of 3T3DM cells

Transfected DNA	mdm2 binding	No. of colonies/10^5 cells plated[a] in expt						Ratio to vector (avg)
		1	2	3	4	5	Avg	
Vector		404	665	1,072	1,404	560	821	1.0
Wild-type p53	+	407	671	1,088	1,264	544	794	0.97
p53 14,19	−		124	323	202	276	230	0.28
p53 24	+		637	226	1,388	400	663	0.81

[a] 3T3DM cells which overexpress mdm-2 were transfected with human p53 on a neomycin-resistant plasmid, and the G418-resistant colonies were counted. Wild-type p53 did not reduce the plating efficiency of this cell line. The p53 14/19 mutant, which does not bind mdm-2, reduced colony numbers fivefold. The p53 K24T mutant binds mdm-2 with wild-type affinity and is only weakly inhibitory in this assay.

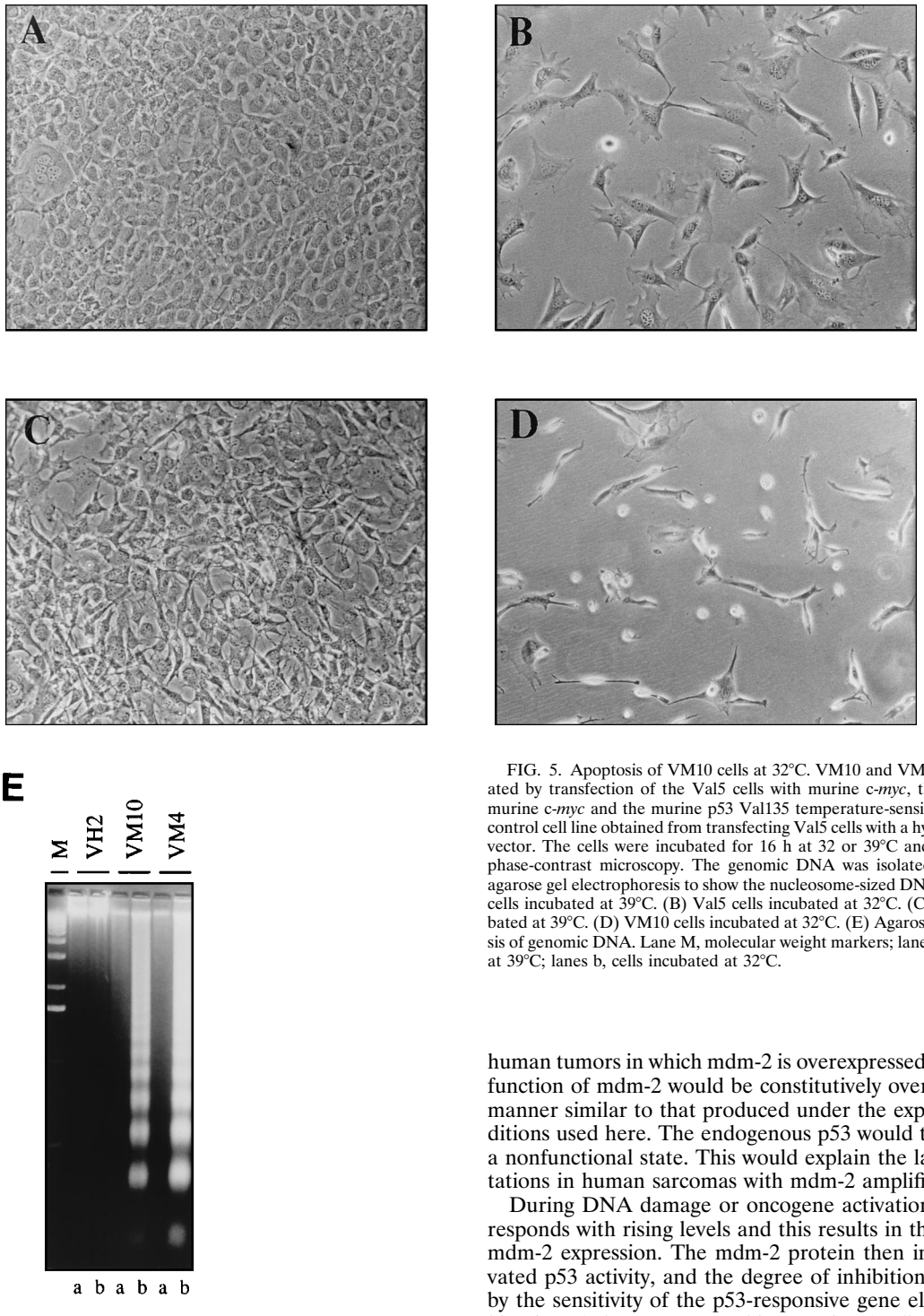

FIG. 5. Apoptosis of VM10 cells at 32°C. VM10 and VM4 cells were generated by transfection of the Val5 cells with murine c-*myc*, thus overexpressing murine c-*myc* and the murine p53 Val135 temperature-sensitive allele. VH is a control cell line obtained from transfecting Val5 cells with a hygromycin-resistant vector. The cells were incubated for 16 h at 32 or 39°C and photographed by phase-contrast microscopy. The genomic DNA was isolated and analyzed by agarose gel electrophoresis to show the nucleosome-sized DNA ladder. (A) Val5 cells incubated at 39°C. (B) Val5 cells incubated at 32°C. (C) VM10 cells incubated at 39°C. (D) VM10 cells incubated at 32°C. (E) Agarose gel electrophoresis of genomic DNA. Lane M, molecular weight markers; lanes a, cells incubated at 39°C; lanes b, cells incubated at 32°C.

other cell types) of p53-dependent and p53-independent apoptosis remains to be tested.

The ability of mdm-2 to negatively regulate the transcription, G_1 arrest, and apoptosis functions of p53 supports the notion that it is a regulator of p53 functions. Although these experiments were carried out in tissue culture with overexpressed levels of mdm-2 proteins, it is likely that this negative regulation reflects physiological conditions. For example, with human tumors in which mdm-2 is overexpressed, the inhibitory function of mdm-2 would be constitutively overexpressed in a manner similar to that produced under the experimental conditions used here. The endogenous p53 would then be kept in a nonfunctional state. This would explain the lack of p53 mutations in human sarcomas with mdm-2 amplification.

During DNA damage or oncogene activation in a cell, p53 responds with rising levels and this results in the activation of mdm-2 expression. The mdm-2 protein then inhibits the elevated p53 activity, and the degree of inhibition is determined by the sensitivity of the p53-responsive gene element and the kinetics of the response. This negative feedback loop alone cannot not completely shut down p53 function, because its very presence is dependent on p53 transcriptional activation of the mdm-2 gene (41). However, in combination with other activities that regulate the mdm-2 basal promoter, p53 and mdm-2 protein half-lives, and posttranslational modifications, mdm-2 may then achieve significant inhibition of p53 function, thus returning the cells to their normal cycles when the DNA damage is repaired.

The p53 binding domain on mdm-2 comprises only 100 amino acids at its N terminus. mdm-2 binding to p53 in cells

FIG. 6. CD20 and TUNEL double staining of VM10 cells. VM10 cells were transiently transfected with the vector or the Bcl2 expression plasmids and the CD20 plasmid. Twenty-four hours after transfection, the cells were incubated at 32°C for 6 h in the presence of cytochalasin B. The cells were harvested and stained for CD20 and DNA fragmentation. (A) Vector-transfected cells. Green fluorescence (with fluorescein isothiocyanate) indicate TUNEL-positive nuclei with fragmented DNA. Orange fluorescence (with PE) is cell surface staining of CD20. Arrows indicate double-positive cells. (B) Bcl2-transfected VM10 cells. The $CD20^+$ cells in this field are all TUNEL negative. (C) DAPI staining of the cells shown in panel A for nuclear morphology. (D) DAPI staining of the cells shown in panel B. The VM10 cells also show weak red autofluorescence in panels A and B.

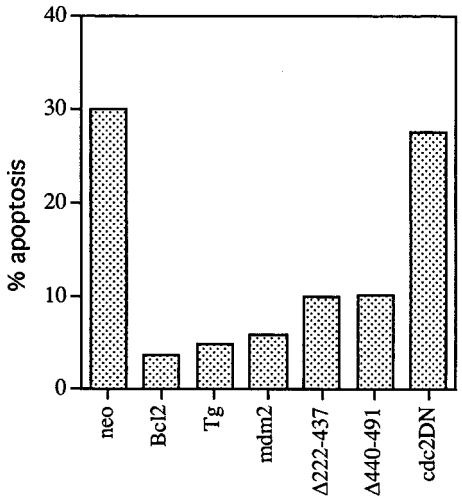

FIG. 7. Inhibition of VM10 apoptosis in transient transfections. VM10 cells were transfected, and apoptosis was quantitated as the percentage of $CD20^+$/$TUNEL^+$ cells among the $CD20^+$ cells. Approximately 30% of vector-transfected cells were TUNEL positive after 6 h of incubation at 32°C. Random counting of $CD20^-$ nuclei yielded similar percentages of TUNEL-positive nuclei (not shown), suggesting that the transfection procedure itself does not affect the apoptosis response. Transfection of Bcl2, simian virus 40 large T antigen (Tg), mdm-2, and two mdm-2 mutants all decreased the percentages of TUNEL-positive nuclei within this period. The cdc2 dominant-negative mutant does not significantly inhibit apoptosis of VM10 cells. The data are averages of three experiments. neo, pRC/CMV vector alone.

and regulation of p53 transcription in vivo also require the mdm-2 nuclear localization signal (3). These are the only elements that are essential for regulation of p53 function by mdm-2 (3, 20). The central acidic domain and three zinc finger motifs near the C terminus are highly conserved through evolution to be similar to those of the p53 binding domain, and this suggests that mdm-2 has other functions besides its regulation of p53. Consistent with this idea are the recent observations that mdm-2 binds to the ribosomal L5 protein (24) and to Rb (41), E2F, and DP1 (25). Clearly, then, mdm-2 has several roles to play at the G_1-S phase transition. This concept suggests that p53, via regulation of mdm-2 gene expression, can play both a positive and negative role at the G_1-S phase border of the cell cycle.

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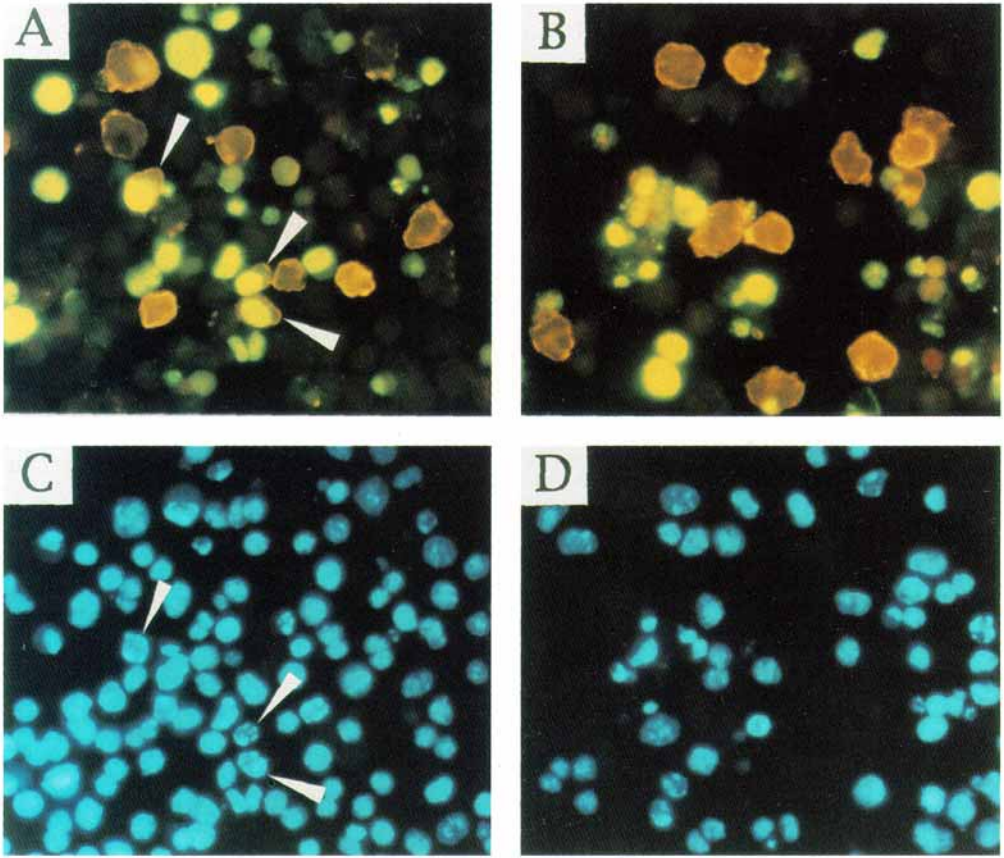
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