The human oncoprotein MDM2 arrests the cell cycle: elimination of its cell-cycle-inhibitory function induces tumorigenesis

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The human oncoprotein MDM2 (hMDM2) overexpresses in various human tumors. If amplified, the mdm2 gene can enhance the tumorigenic potential of murine cells. Here, we present evidence to show that the full-length human or mouse MDM2 expressed from their respective cDNA can inhibit the G0/G1–S phase transition of NIH 3T3 and normal human diploid cells. The protein harbors more than one cell-cycle-inhibitory domain that does not overlap with the p53-interaction domain. Deletion mutants of hMDM2 that lack the cell-cycle-inhibitory domains can be stably expressed in NIH 3T3 cells, enhancing their tumorigenic potential. The tumorigenic domain of hMDM2 overlaps with the p53-interaction domain. Some tumor-derived cells, such as Saos-2, H1299 or U-2OS, are relatively insensitive to the growth-inhibitory effects of MDM2. These observations suggest that hMDM2 overexpression in response to oncogenic stimuli would induce growth arrest in normal cells. Elimination or inactivation of the hMDM2-induced G0/G1 arrest may contribute to one of the steps of tumorigenesis.

Keywords: cell-cycle arrest/human MDM2/oncoprotein/tumorigenesis

Introduction

The mouse double minute-2 (mdm2) gene is evolutionarily conserved among eukaryotes (Fakharzadeh et al., 1991), which suggests that the gene product (MDM2) serves an important function in eukaryotic cells. As is the case for many other oncogenes, the dysfunction of the mdm2 gene was discovered before its normal function. Originally, the gene was identified as an amplified and overexpressed gene in a spontaneously transformed derivative of mouse BALB/c cell line 3T3DM (Fakharzadeh et al., 1991). Since 3T3DM cells overexpress several splice variants of MDM2, it was not clear which form of the protein is capable of inducing tumorigenesis. Amplification of the entire mdm2 gene capable of expressing all the spliced forms was shown to enhance tumorigenic potential of murine cells (Fakharzadeh et al., 1991; Finlay, 1993). The human homolog of the mdm2 gene (hmdm2) is frequently overexpressed in many human cancers (Ladanyi et al., 1993; Leach et al., 1993; Sheikh et al., 1993; Cordon-Cardo et al., 1994; Florenes et al., 1994; Quesnel et al., 1994; Reifenberger et al., 1994; Gudas et al., 1995; Baunoch et al., 1996), suggesting that the genetic alteration may be one of the common causes of oncogenesis. Recently, Lundgren et al. (1997) reported that 16% of transgenic mice that overexpress MDM2 from a mini-gene containing the introns 7 and 8 of mdm2 inserted within its cDNA under a bovine β-lactoglobulin promoter develop breast tumors. These findings suggest that MDM2 has an oncogenic function.

The mdm2 gene product was originally detected in a complex with the tumor suppressor p53 (Momand et al., 1992). Overexpression of hmdm2 gene was found in many cancer cells with wild-type p53 (Oliner et al., 1992; Leach et al., 1993). These findings led to the hypothesis that hMDM2 may induce oncogenesis by inactivating the tumor suppressor p53 (Oliner et al., 1992; Leach et al., 1993). Later studies reveal alteration in the expression of both p53 and hmdm2 genes (Cordon-Cardo et al., 1994), suggesting that abnormal expression of the two genes may confer an additive effect on cell growth. Besides p53, MDM2 also interacts with the retinoblastoma gene product pRb (Xiao et al., 1995), the transcription factors E2F1/DP1 (Martin et al., 1995), ribosomal L5 protein (Marechal et al., 1994), simian virus 40 (SV40) T antigen (Brown et al., 1993) and human TATA-binding protein (Leng et al., 1995a).

Among all the interactions of MDM2 with cellular or viral proteins, the interaction of human or mouse MDM2 with p53 has been investigated the most. Our laboratory, as well as others, have shown that human or mouse MDM2 can interact with the tumor suppressor p53 in cell-free systems (Brown et al., 1993; Chen et al., 1993) or in the whole cell (Brown et al., 1993; Oliner et al., 1993; Haines et al., 1994; Leng et al., 1995b) and inhibit transactivation by wild-type p53. Work from our laboratory showed that the interaction of hMDM2 with p53 is needed for inhibition of p53-mediated transactivation (Brown et al., 1993), and only 127 amino acids (amino acids 28–154) of hMDM2, out of a total of 491, are sufficient to inhibit p53-directed transactivation (Leng et al., 1995b). MDM2 regulates several functions of p53. Human or mouse MDM2 regulates p53-mediated growth suppression and apoptosis (Chen et al., 1994, 1996; Haupt et al., 1996). Also, the p53-regulatory function of MDM2 is required for embryonic development (Jones et al., 1995; Montes de Oca Luna et al., 1995).

Wild-type p53 induces MDM2 expression by recognizing a response element situated downstream of the first exon of the oncogene (Barak et al., 1993, 1994; Juven et al., 1993). Several laboratories (Perry et al., 1993; Chen et al., 1994; Price and Park, 1994; Bae et al., 1995) have shown that ionizing radiation induces MDM2 expression in a p53-dependent manner. Thus, the presence of an autoregulatory feedback loop has been suggested (Barak...
et al., 1993; Otto and Deppert, 1993; Picksley and Lane, 1993; Wu et al., 1993).

At least five to seven MDM2-related polypeptides have been found in cultured mouse or human cells that overexpress MDM2 (Gudas et al., 1993; Olson et al., 1993; Haines et al., 1994; Maxwell, 1994; Sigalas et al., 1996). Two of these five forms (p90, the full length protein and p58, which harbors a deletion at the C-terminus) are capable of binding p53. The presence of alternately spliced forms of MDM2 that cannot bind p53 suggests p53-independent biochemical function(s) of hMDM2. It is not known which form(s) of MDM2 induce tumorigenesis.

As stated above, several laboratories including ours have shown that full-length human or mouse MDM2 expressed from their respective cDNA can interact with p53 and inhibit p53-mediated transactivation (Momand et al., 1992; Brown et al., 1993; Oliner et al., 1993; Haines et al., 1994; Leng et al., 1995b). If hMDM2 induces tumorigenesis by inactivating the tumor suppressor p53, overexpression of full-length hMDM2 from its cDNA should enhance tumorigenic potential of NIH 3T3 cells. Also, the deletion mutants of hMDM2 that interact with p53 to inactivate its transcriptional activation should harbor the same property. To test this hypothesis, we overexpressed full-length hMDM2 or its deletion mutants from their respective cDNA in NIH 3T3 cells and analyzed the growth regulatory properties of the oncoprotein. Our results show that the full-length hMDM2 inhibits the G₀/G₁–S phase transition of the cell cycle and cannot confer growth advantage to NIH 3T3 cells. Some tumor-derived cells are partially insensitive to the hMDM2-mediated cell-cycle inhibition. Deletion of the cell-cycle-inhibitory domains of hMDM2 activates the tumorigenic potential of the oncoprotein. The cell-cycle-inhibitory function of hMDM2 is p53-independent, but the tumorigenic domain overlaps with its p53-interaction domain.

**Results**

**Overexpression of full-length hMDM2 from its cDNA is disadvantageous to the growth of non-tumor cells**

Since full-length hMDM2 expressed from its cDNA can interact with p53 and inhibit p53-mediated transcriptional activation (Brown et al., 1993; Leng et al., 1995b), we wished to determine whether hMDM2 can enhance the tumorigenic potential of NIH 3T3 cells by inactivating p53. As a first step, we attempted to generate stable transfectants of NIH 3T3 cells that overexpress hMDM2. An hMDM2 expression plasmid harboring the hMDM2 cDNA (pCMVneo.hmdm2; Leng et al., 1995b) was transfected into NIH 3T3 cells. Forty-eight hours after transfection, half of the cells were analyzed for transient expression and the other half was selected to generate neomycin-resistant colonies. The results (summarized in Table I) showed that although hMDM2 expressed transiently (Figure 1A) and G418-resistant colonies were generated, hMDM2 expression was not detected in the pooled G418-resistant stable transfectants or in isolated colonies. Similar results were found in several other cell lines that are not derived from tumors (Table I).

Our attempts to generate stable transfectants of some tumor-derived cells overexpressing hMDM2, on the other hand, were successful. Tumor-derived cells, such as Saos-2 (Masuda et al., 1987), H1299 (Mitsudomi et al., 1992) and 21PT (Band et al., 1990), were transfected with the hMDM2 expression plasmid (pCMVneo.hmdm2; Leng et al., 1995b). G418-resistant colonies were selected and expanded. Western blot analysis of the cell extracts showed stable expression of hMDM2 in these cells (Figure 1B). These results suggest that overexpression of hMDM2 is not toxic to all of the cells.

Also, MDM2 has been stably overexpressed in NIH 3T3 and primary rat embryo fibroblast (REF) cells by amplifying a cosmid harboring the entire mdm2 gene (Fakhrazadeh et al., 1991; Finlay, 1993). The MDM2 overexpressing NIH 3T3 cells have been shown to form tumors in nude mice (Fakhrazadeh et al., 1991). These results led us to speculate that MDM2 overexpressed from the cDNA may be functionally different from the oncoprotein overexpressed from the mouse genomic clone. The full-length hMDM2 overexpressed from the cDNA may cause growth disadvantage in cell lines that are not tumor derived.

**hMDM2 blocks the G₀/G₁–S phase transition of non-tumor cells**

To determine how hMDM2 modulates cell growth, we analyzed the effect of transiently overexpressed hMDM2 growth disadvantage in cell lines that are not tumor derived.
Human MDM2 arrests cell cycle

on cell-cycle progression of NIH 3T3 cells. NIH 3T3 cells were transfected with hMDM2 expression plasmid harboring the human cDNA (pCMV.hmdm2; Leng et al., 1995b). Cells were harvested and fixed 48 h after transfection. The hMDM2-overexpressing cells were identified by immunostaining with an anti-hMDM2 monoclonal antibody (2A10; Chen et al., 1993) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The cells were then stained with propidium iodide. The FITC-labeled and unlabeled cells were sorted (30 000 cells in each case) and analyzed for cell-cycle transition by flow cytometry (program PEAKS3.FTN) using a fluorescence-activated cell sorter (FAC Starplus, Becton Dickinson).

Results of several (more than six) independent experiments using three different anti-hMDM2 antibodies showed that transient overexpression of full-length hMDM2 inhibits the G0/G1–S phase progression of the NIH 3T3 cells (Figure 2A). As shown in the figure, >90% of the FITC-labeled cells (shown by a broken line) were arrested at the G0/G1 phase, and only 8% of the cells were cycling (S + G2 + M phases). On the other hand, 38% of unlabeled cells (solid line) were found in the S + G2 + M phases. hMDM2 expression in the FITC-labeled cells was confirmed by Western blot analysis of the sorted FITC-labeled and unlabeled cells. Figure 2B (lane 2) shows that hMDM2 was expressed in the FITC-labeled cells.

To confirm that the hMDM2-mediated inhibition of the G0/G1–S phase transition is not an artifact due to transfection of plasmid DNA or non-specific overexpression of any protein, we transfected NIH 3T3 cells separately with three different plasmids. One of these plasmids (pCMV.CD20) expresses the surface marker CD20 under the control of cytomegalovirus (CMV) promoter, a second plasmid (pCMV.β-gal) expresses the enzyme β-galactosidase under the control of CMV promoter, and the third plasmid (pSV.β-gal) also expresses the enzyme β-galactosidase under the control of SV40 early promoter. Cells overexpressing CD20 or β-galactosidase were identified using their respective antibodies and an FITC-labeled secondary antibody. A monoclonal antibody against CD20 (Becton Dickinson) was used to identify pCMV.CD20-transfected cells, whereas cells overexpressing β-galactosidase were identified using a polyclonal antibody against β-galactosidase (5′ to 3′). Overexpression of the surface marker CD20 (Figure 2C) or the enzyme β-galactosidase
the G0/G1–S phase transition of NIH 3T3 cells. Compared with the overexpression of T antigen from the CMV promoter analyzed as described in the earlier experiment. Transient et al. T antigen (Pab419; Harlow transfected into NIH 3T3 cells. Transfected cells were identified by immunostaining with the respective antibodies and an FITC-conjugated secondary antibody. DNA content of the FITC-labeled (broken line) and unlabeled (solid line) cells were analyzed after propidium iodide staining. The figure shows that overexpression of \( \beta \)-galactosidase from the CMV promoter (A) or from the SV40 early promoter (B) does not block the G0/G1→S phase transition of NIH 3T3 cells (similar percentage of FITC-labeled or unlabeled cells progressed to the S+G2/M phases). Overexpression of T antigen (C) accelerates the G0/G1→S phase transition of NIH 3T3 cells. Compared with the unlabeled cells (32%), higher percentage (72%) of FITC-labeled cells progressed to the S+G2/M phases, whereas mouse MD2 (D) blocks the G0/G1→S phase transition (42.5% unlabeled and 9.9% labeled cells progressed to the S+G2/M phases).

(Figure 3A and B) did not significantly alter the phase distribution of transfected and untransfected cells, suggesting that the hMDM2-mediated inhibition is not due to overexpression of any protein.

To determine whether our flow cytometric assay can recognize acceleration of the G1→S phase transition, we used the viral oncoprotein SV40 T antigen as a positive control. As in the case of hMDM2, SV40 large T antigen is a transforming protein that interacts with the tumor suppressor p53 (Lane and Crawford, 1979; Linzer and Levine, 1979) and inhibits p53-mediated transcriptional activation (reviewed in Levine, 1993; Lane 1994). To determine the effect T antigen overexpression on the G1→S phase transition of NIH 3T3 cells, a T antigen expression plasmid (pCMV.T; Deb et al., 1995) was transfected into NIH 3T3 cells. The T antigen-expressing cells were identified using a monoclonal antibody against T antigen (Pab419; Harlow et al., 1981), sorted and analyzed as described in the earlier experiment. Transient overexpression of T antigen from the CMV promoter enhanced the G0/G1→S phase transition of T antigen expressing cells as expected (Figure 3C).

Although human and mouse MDM2 show high degree of sequence homology (Fakharzadeh et al., 1991; Oliner et al., 1992), overexpression of mouse MDM2 from the genomic clone was shown to enhance the tumorigenic potential of NIH 3T3 cells (Fakharzadeh et al., 1991; Finlay, 1993). Therefore, we tested whether the growth-inhibitory effect is specific for hMDM2. Overexpression of mouse MDM2 from an MDM2 expression plasmid (pCMV.mdm2; Haines et al., 1994) also led to the G0/G1 arrest of NIH 3T3 cells (Figure 3D), suggesting that the hMDM2-mediated growth arrest is not species specific.

To determine whether hMDM2-mediated growth arrest is specific for NIH 3T3 cells, hMDM2 expression plasmid was transfected into two normal human diploid fibroblast cell lines, WI38 and MRC5. The untransfected and hMDM2 overexpressing cells were sorted and analyzed as described above. Transient overexpression of hMDM2 caused G0/G1 arrest in MRC5 and WI38 cells, suggesting that the effect is not specific for NIH 3T3 cells (Table IIA). As in the case of NIH 3T3 cells, overexpression of the surface marker CD20 from the pCMV.CD20 expression plasmid did not induce G0/G1 arrest (Table IIB).

**hMDM2 blocks the G0/G1→S phase transition of NIH 3T3 cells at low or high levels**

Overexpression of MDM2 from the amplified mdm2 gene has been shown to induce tumorigenesis (Fakharzadeh et al., 1991; Finlay, 1993). Although the mdm2 gene was amplified to elevate the normal levels of the protein, in the genomic clone, MD2 was expressed from its own promoter. Therefore, hMDM2-mediated G0/G1 arrest could be a result of high levels of hMDM2 overexpressed from a strong CMV promoter, while the protein may confer growth advantage at low levels. To test this possibility, we analyzed the growth-regulatory effects of hMDM2 at different levels of the protein expressed. We transiently transfected NIH 3T3 cells with 1, 5, 10 and 30 \( \mu \)g of an hMDM2 expression plasmid (pCMV.hmdm2; Leng et al., 1995b). To detect the G0/G1 arrest mediated by low levels of wild-type hMDM2, the cells were blocked at mitosis by treatment with nocodazole after transfection (Giunta and Carlo, 1995; Chen et al., 1996). Nocodazole arrests the cell cycle at mitosis. Therefore, the cells that have completed DNA replication (untransfected cells) will not undergo cell division in the presence of nocodazole, and accumulate at the G2/M phase. However, cells that will not be able to enter the S phase or complete DNA replication will not be able to reach G2/M phase. Thus, hMDM2 expressing cells should not show significant number of cells in the S and G2/M phases, even in the presence of nocodazole.

The transfected cells were identified by immunostaining with a monoclonal antibody against hMDM2 (2A10, Chen et al., 1993) and an FITC-labeled secondary antibody, and then sorted. The intensity of the FITC fluorescence associated with the cells was measured at 530 nm using FACS (fluorescence activated cell sorter) and was plotted against cell numbers. The monoclonal antibody used for this experiment recognizes mouse as well as human MDM2 (Chen et al., 1993). Thus, the FITC fluorescence of the untransfected NIH 3T3 cells should be indicative of endogenous MDM2. The amounts of hMDM2 expressed in the transfected and untransfected cells were estimated from the intensity of fluorescence, and compared. The transfected and untransfected cells were also stained with propidium iodide and their DNA content determined.

The result of this experiment is shown in Table III. When 1 \( \mu \)g hMDM2 expression plasmid was used for transfection, most of the transfected cells showed 1.7- to
1.95-fold higher intensity of green fluorescence than the untransfected cells, while the brightest transfected cells showed 2.5- to 3.7-fold higher fluorescence than the untransfected cells. These results suggest that the levels of hMDM2 expressed in these cells were 1.7- to 3.7-fold higher than the untransfected cells. The DNA content analysis showed that 1.7- to 3.7-fold higher expression of hMDM2 inhibited the G0/G1–S phase progression of cells (Table III).

Similarly, when we used 5 μg hMDM2 expression plasmid, most of the transfected cells expressed 5-fold higher levels of hMDM2, while the brightest cells showed 6.6-fold higher expression 6- to 8.3-fold. Similarly, 10 μg hMDM2 expression plasmid enhanced hMDM2 expression. When we used 30 μg expression plasmid, most of the cells expressed 9.5-fold higher levels of hMDM2, whereas the brightest cells showed 22.6-fold higher levels of hMDM2. Since an increase in the amount of expression plasmid from 5–30 μg showed an increase in the FITC fluorescence, the amount of antibody used was not limiting for cells expressing 5- to 10- fold higher levels of hMDM2. In all the cases, overexpression of hMDM2 resulted in G0/G1 arrest. The efficiency of inhibition increased with 10- to 20-fold increase in the levels of hMDM2, although not proportionally. These results indicate that 2- to 20-fold overexpression of hMDM2 from its full-length cDNA induces G0/G1 arrest in NIH 3T3 cells. Therefore, the hMDM2-mediated G0/G1 arrest could be physiologically relevant when hMDM2 is induced by a factor such as p53. Figure 4 shows the comparative levels of hMDM2 expression in NIH 3T3 cells transfected with 1 or 10 μg hMDM2 expression plasmid, as detected by Western analysis.

**Some tumor-derived cells are partially insensitive to hMDM2-mediated G0/G1 arrest**

Studies in our laboratory and elsewhere suggest that several tumor-derived cells can tolerate stable overexpression of hMDM2 from its cDNA (Figure 1B, lanes 4 and 5; Table I) (Chen et al., 1994). Therefore, we investigated how hMDM2 regulates the G0/G1–S phase transition of these cells. Since it was possible to generate hMDM2-overexpressing stable transfectants of H1299 or Saos-2 cells, these cells were transfected with the hMDM2 expression plasmid pCMV.hmdm2. The hMDM2-expressing cells were identified and analyzed by flow cytometry as described above. The results [Table IV (A)] show that overexpression of hMDM2 reduces the percentage of cycling Saos-2 cells from 54 to 32%, and H1299 cells from 57 to 47%. These results suggest that Saos-2 and H1299 cells are less sensitive to hMDM2-mediated G0/G1 arrest than NIH 3T3, WI38 or MRC5 cells, which show a 3- to 5-fold reduction in the number of cycling cells under similar conditions (Figure 2; Table II). Consistent with this observation, our stable transfection analysis showed that 1.7- to 3.7-fold higher expression of hMDM2 expressed in these cells were 1.7- to 3.7-fold higher than the untransfected cells. The DNA content analysis showed that 2.5- to 3.7-fold higher fluorescence than the untransfected cells, while the brightest transfected cells showed 2.5- to 3.7-fold higher fluorescence than the untransfected cells. These results indicate that 2- to 20-fold overexpression of hMDM2, although not proportionally. These results indicate that 2- to 20-fold overexpression of hMDM2 from its full-length cDNA induces G0/G1 arrest in NIH 3T3 cells. Therefore, the hMDM2-mediated G0/G1 arrest could be physiologically relevant when hMDM2 is induced by a factor such as p53. Figure 4 shows the comparative levels of hMDM2 expression in NIH 3T3 cells transfected with 1 or 10 μg hMDM2 expression plasmid, as detected by Western analysis.

### Table II.

<table>
<thead>
<tr>
<th></th>
<th>WI38</th>
<th>MRC5</th>
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<tr>
<td></td>
<td>Unlabeled Cells</td>
<td>FITC-labeled Cells</td>
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<tr>
<td>%G0/G1</td>
<td>65.57 ± 0.37</td>
<td>89.65 ± 1.03</td>
</tr>
<tr>
<td>%S</td>
<td>17.15 ± 0.19</td>
<td>5.45 ± 0.25</td>
</tr>
<tr>
<td>%G0/G1+M</td>
<td>20.28 ± 0.20</td>
<td>4.9 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>22.81 ± 0.15</td>
<td>8.19 ± 0.28</td>
</tr>
</tbody>
</table>

### Table III. Overexpression of full-length hMDM2 inhibits the G0/G1–S phase transition of NIH 3T3 cells at low or high levels

<table>
<thead>
<tr>
<th>Amount of pCMV.hmdm2 transfected (μg)</th>
<th>Increase in FITC fluorescence in transfected cells</th>
<th>Percent S+G2+M cells</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1.74- 2.54-fold</td>
<td>48.42 ± 0.49</td>
</tr>
<tr>
<td>5</td>
<td>5- 6.6-fold</td>
<td>67.03 ± 0.58</td>
</tr>
<tr>
<td>10</td>
<td>9.55- 22.6-fold</td>
<td>55.48 ± 0.53</td>
</tr>
<tr>
<td>1</td>
<td>1.95- 3.7-fold</td>
<td>62.85 ± 0.57</td>
</tr>
<tr>
<td>5</td>
<td>5.9- 8.3-fold</td>
<td>58.1 ± 0.54</td>
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</tbody>
</table>

### Table IV (A)

<table>
<thead>
<tr>
<th></th>
<th>Unlabeled Cells</th>
<th>FITC-labeled Cells</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1 μg</td>
<td>U F</td>
</tr>
<tr>
<td></td>
<td>10 μg</td>
<td>U F</td>
</tr>
<tr>
<td>B</td>
<td>1 μg</td>
<td>U F</td>
</tr>
</tbody>
</table>
suppression is not dependent on the status of p53 or Rb.

growth suppression. Therefore, hMDM2-mediated growth
three cell lines are partially resistant to hMDM2-mediated
et al.
within amino acid residues 155 to 324
(Mitsudomi
et al.
contains wild-type p53 and Rb (Lee
et al.
, 1990; Diller
et al., 1990; Shew
et al., 1990). Our data suggest that all
three cell lines are partially resistant to hMDM2-mediated
growth suppression. Therefore, hMDM2-mediated growth suppression is not dependent on the status of p53 or Rb.

Growth-inhibitory domains of hMDM2 reside within amino acid residues 155 to 324
To identify the growth-inhibitory domains of hMDM2, a series of C- and N-terminal deletion mutants were individually expressed in NIH 3T3 cells (Figure 5). To detect growth suppression mediated by the mutants of hMDM2 that may have compromised growth-suppression ability, the cells were blocked at mitosis by treatment with nocodazole after transfection. Cells expressing each deletion mutant were identified with appropriate anti-hMDM2 antibodies, sorted and analyzed by flow cytometry. Results of our experiments show that deletion of N-terminal 58 or 190 amino acid residues did not alter hMDM2-mediated G0/G1 arrest significantly (Figure 6C). Deletion up to amino acid residue 276 from the C-terminus (del 491–276) reduced, but did not fully release, the growth-inhibitory activity (Figure 6D). Further C-terminal deletion up to amino acid 221 did not reduce growth inhibition any further (Figure 6E). These results suggest that at least one growth-inhibitory domain resides within the amino acid residues 324–276. We will refer to this domain as inhibitory domain 2 (ID2). Consistent with this observation, results of our stable transfection analysis showed that this C-terminal deletion mutant of hMDM2 (del 491–221) cannot be stably expressed in NIH 3T3 cells (Table I). Further deletion up to amino acid 155 or 131 drastically reduced the growth arrest (Figure 6F and G). Thus, another growth-inhibitory domain resides within the amino acid residues 155–221. We will refer to this domain as inhibitory domain 1 (ID1). As expected, overexpression of full-length hMDM2 induces G0/G1 arrest in this experiment (Figure 6H), whereas overexpression of CD20 did not alter the cell-cycle profile (Figure 6I).

To confirm that hMDM2 harbors two non-overlapping growth suppressor domains, we generated two deletion mutants of hMDM2. The mutant hMDM2 58–220 harbors ID1, whereas the mutant hMDM2 del 1–269 harbors ID2.

### Table IV.

(A) Some tumor-derived cells are partially insensitive to hMDM2-mediated G0/G1 arrest

<table>
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<tr>
<th></th>
<th>Saos2</th>
<th>H1299</th>
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<tr>
<td></td>
<td>Unlabeled cells</td>
<td>FITC-labeled cells</td>
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<tr>
<td>%G0/G1</td>
<td>45.3 ± 0.18</td>
<td>67.7 ± 0.63</td>
</tr>
<tr>
<td>%S</td>
<td>38.2 ± 0.17</td>
<td>23.3 ± 0.37</td>
</tr>
<tr>
<td>%G2 + M</td>
<td>16.5 ± 0.11</td>
<td>9.0 ± 0.23</td>
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(B) U–2OS cells are tolerant to low levels but sensitive to high levels of hMDM2

<table>
<thead>
<tr>
<th></th>
<th>pCMV.hmdm2 (1 μg)</th>
<th>pCMV.hmdm2 (20 μg)</th>
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<tbody>
<tr>
<td></td>
<td>Unlabeled cells</td>
<td>FITC-labeled cells</td>
</tr>
<tr>
<td>%G0/G1</td>
<td>32.27 ± 0.41</td>
<td>29.34 ± 0.50</td>
</tr>
<tr>
<td>%S</td>
<td>45.70 ± 0.48</td>
<td>27.80 ± 0.49</td>
</tr>
<tr>
<td>%G2 + M</td>
<td>22.63 ± 0.34</td>
<td>42.9 ± 0.61</td>
</tr>
</tbody>
</table>

Deletion of 166 amino acids from the C-terminus (del 491–325) did not reduce the efficiency of G0/G1 arrest significantly (Figure 6C). Deletion up to amino acid residue 276 from the C-terminus (del 491–276) reduced, but did not fully release, the growth-inhibitory activity (Figure 6D). Further C-terminal deletion up to amino acid 221 did not reduce growth inhibition any further (Figure 6E). These results suggest that at least one growth-inhibitory domain resides within the amino acid residues 324–276. We will refer to this domain as inhibitory domain 2 (ID2). Consistent with this observation, results of our stable transfection analysis showed that this C-terminal deletion mutant of hMDM2 (del 491–221) cannot be stably expressed in NIH 3T3 cells (Table I). Further deletion up to amino acid 155 or 131 drastically reduced the growth arrest (Figure 6F and G). Thus, another growth-inhibitory domain resides within the amino acid residues 155–221. We will refer to this domain as inhibitory domain 1 (ID1). As expected, overexpression of full-length hMDM2 induces G0/G1 arrest in this experiment (Figure 6H), whereas overexpression of CD20 did not alter the cell-cycle profile (Figure 6I).

To confirm that hMDM2 harbors two non-overlapping growth suppressor domains, we generated two deletion mutants of hMDM2. The mutant hMDM2 58–220 harbors ID1, whereas the mutant hMDM2 del 1–269 harbors ID2.
Human MDM2 arrests cell cycle

Fig. 6. The full-length hMDM2 has more than one growth-inhibitory domain. Flow cytometric analysis of NIH 3T3 cells transiently expressing wild-type and deletion mutants of hMDM2. The deletion mutants used are labelled at the top of the figure. The cell-cycle profile of the FITC-labeled cells are shown by a broken line, and that of the unlabeled cells by a solid line. The 2A10 antibody was used for experiments (A) and (B), SMP14 antibody was used for experiments (C–E) and N-20 was used for (F–H). Expression of the deletion mutants of hMDM2 in the sorted FITC-labeled (lane F) and unlabeled (lane U) cells are shown with each cell-cycle profile. (I) CD20 overexpression did not alter the cell-cycle profile of NIH 3T3 cells in the presence of nocodazole. FITC-labeled or CD20 expressing cells (broken line) showed the same pattern as the unlabeled (solid line) cells. A monoclonal antibody against CD20 (Becton Dickinson) was used to identify the transfected cells.

Fig. 7. hMDM2 harbors two independent growth-inhibitory domains. Flow cytometric analysis of NIH 3T3 cells transiently expressing deletion mutants of hMDM2 harboring (A) growth-inhibitory domain 1 (ID1) or (B) growth-inhibitory domain 2 (ID2). The deletion mutants used are labelled at the top of the figure. The cell-cycle profile of the FITC-labeled cells are shown by a broken line and that of the unlabeled cells by a solid line. Cells expressing hMDM2 59–220 were recognized by the antibody SMP14 (A) and the cells expressing hMDM2 del 1–269 were recognized by the antibody 2A10 (B). Expression of the deletion mutants in the sorted cells are shown by Western analysis. U: unlabeled and F: FITC-labeled cells. (C) pCMV .CD20 transfected cells did not show any significant alteration in the cell-cycle profile.

(Figure 7). Our data suggest that either of the mutants efficiently arrests the G0/G1–S phase transition of NIH 3T3 cells (Figures 7A and B), whereas overexpression of CD20 does not have any significant effect on the cell cycle (Figure 7C). As shown in Figure 6E, the efficiency of growth arrest induced by the hMDM2 deletion mutant del 491–221 that harbors ID1 was less than that induced by hMDM2 58–220. This difference was reproducible in several experiments suggesting that the N-terminal 58 amino acid residues may interfere with the growth arrest function of ID1. The extent of growth arrest induced by different deletion mutants of hMDM2 is summarized in Table V.

The deletion mutants of hMDM2 lacking the putative nuclear localization signals can localize into the nucleus

As shown in Figure 8E, hMDM2 has two putative nuclear localization signals, one (RKRHK) is located in between amino acid residues 181–185, and a second signal,
Table V. Growth arrest induced by expression of different mutants of hMDM2

<table>
<thead>
<tr>
<th>hMDM2 protein expressed</th>
<th>Percent S + G2 + M cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unlabeled cells</td>
</tr>
<tr>
<td>Wild-type</td>
<td>58.1 ± 0.54</td>
</tr>
<tr>
<td>Del 1–58</td>
<td>71.47 ± 0.61</td>
</tr>
<tr>
<td>Del 1–189</td>
<td>78.89 ± 0.64</td>
</tr>
<tr>
<td>Del 1–269</td>
<td>70.04 ± 0.60</td>
</tr>
<tr>
<td>Del 491–325</td>
<td>65.95 ± 0.63</td>
</tr>
<tr>
<td>Del 491–276</td>
<td>71.12 ± 0.61</td>
</tr>
<tr>
<td>Del 491–221</td>
<td>74.04 ± 0.65</td>
</tr>
<tr>
<td>59–220</td>
<td>84.87 ± 0.66</td>
</tr>
<tr>
<td>Del 491–155</td>
<td>73.79 ± 0.73</td>
</tr>
<tr>
<td>Del 491–131</td>
<td>74.20 ± 0.71</td>
</tr>
</tbody>
</table>

KKLKKRNK is located in between amino acid residues 466–473 (Roberts, 1989; Fakharzadeh et al., 1991; Oliner et al., 1992; Sigalas et al., 1996). Thus, all of the deletion mutants (Table V), except hMDM2 del 491–154 and del 491–130, should contain at least one nuclear localization signal. Since only the mutants hMDM2 del 491–154 and del 491–130 do not induce growth arrest of NIH 3T3 cells, it is important to know whether the mutants lacking the nuclear localization signals are localized to the nucleus. To determine the localization of transiently expressed hMDM2, NIH 3T3 cells were transfected with 1 μg of wild-type or del 491–155 expression plasmids. Cells were fixed 48 h after transfection and immunostained using a monoclonal antibody against hMDM2 (Ab1, Oncogene Science), a biotin-conjugated secondary antibody and avidin–biotin alkaline phosphatase complex (Vector Laboratories). The OSACL cells overexpress hMDM2 (Khatib et al., 1993) and were used as positive controls.

**Fig. 8.** Nuclear localization of the wild-type hMDM2 and del 491–155. Wild-type or the deletion mutant of hMDM2 was immunostained to develop red color. (A) OSACL cells immunostained in the absence of primary antibody, (B) OSACL cells and (C) NIH 3T3 cells transfected with 1 μg pCMV.hmdm2, or (D) NIH 3T3 cells transfected with 1 μg pCMV.hmdm2 del 491–155 were immunostained in the presence of Ab1 (Oncogene Science). Presence of hMDM2 was detected by development of red color. Cells that were stained comparatively darker are shown by arrows. Magnification of the photograph is 200×. (E) The putative nuclear localization signals of hMDM2.
Fig. 9. Stable expression of hMDM2 deletion mutants that lack the growth inhibitory domains in NIH 3T3 cells. Lysates prepared from transiently or stably transfected NIH 3T3 cells were subjected to Western blot analysis using an N-terminal anti-hMDM2 antibody (N-20, Santa Cruz). Plasmids used for transfection are shown at the top. Migration of the wild-type or deletion mutants of hMDM2 are indicated by arrowheads. Positions of the mol. wt markers in kDa are shown at the side.

To determine the background staining, OSACL cells were stained in the absence of the primary antibody. As expected, the data show an insignificant amount of background staining (Figure 8A). When the cells were immunostained in the presence of a monoclonal antibody against hMDM2, presence of hMDM2 was detected as red color in the nucleus (Figure 8B). Immunostaining of the NIH 3T3 cells transfected with the wild-type or mutant (del 491–155) hMDM2 expression plasmids are shown in Figure 8C and D, respectively. In both the cases, the red stain was detected in the nuclei indicating nuclear localization of the wild-type and del 491–155.

**Deletion mutants of hMDM2 lacking the growth-inhibitory domains can be expressed stably in NIH 3T3 cells**

Since deletion up to the amino acid residue 155 or 131 released the growth-inhibitory property of hMDM2 (Figure 6F and G), we attempted to express these mutant proteins (del 491–155 or del 491–131) stably in NIH 3T3 cells, as described in Materials and methods. Pooled G418-resistant colonies generated from the transfected cells were analyzed for stable expression of hMDM2 or its deletion mutants. Pooled G418-resistant colonies generated from vector-transfected cells were used as control. Transient expression of the hMDM2 deletion mutants was also tested to confirm that the proteins were expressed transiently. The data obtained is shown in Figure 9. hMDM2 and its deletion mutants were expressed in transiently transfected cells (lanes 2–4). However, only the deletion mutants del 491–155 and del 491–131 were stably overexpressed in the G418-resistant pooled colonies (lanes 7 and 8). Cell extracts prepared from G418-resistant pooled colonies of vector-transfected (lanes 1 and 4) or pCMV.hmdm2-transfected (lane 6) cells did not show expression of wild-type hMDM2 or the mutants. This observation confirms that deletion of both the growth-inhibitory domains releases hMDM2-mediated growth arrest.

**Table VI. Overexpression of hMDM2 deletion mutants lacking the growth inhibitory domains enhances the tumorigenic potential of NIH 3T3 cells**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Protein ectopically expressed</th>
<th>No. mice/No. sites injected</th>
<th>No. tumors generated/No. sites injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nonea</td>
<td>3/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Del 491–155</td>
<td>3/6</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Del 491–131</td>
<td>3/6</td>
<td>6/6</td>
</tr>
<tr>
<td>2</td>
<td>Nonea</td>
<td>3/6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Del 491–155</td>
<td>3/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

*a*Vector transfected cells.

**Deletion mutants of hMDM2 lacking the growth-inhibitory domains enhance the tumorigenic potential of NIH 3T3 cells**

To determine whether deletion of the growth-inhibitory domains of hMDM2 unmasks its tumorigenic property, we analyzed the tumorigenic potential of the NIH 3T3 stable transfectants overexpressing hMDM2 deletion mutant del 491–155 or del 491–131. NIH 3T3 cells stably transfected with the vector were used as control. Cells (1×10⁷) obtained from the G418-resistant pooled colonies were injected subcutaneously into flanks of athymic nude mice, as described in Materials and methods. All the mice injected with cells overexpressing hMDM2 deletion mutant del 491–155 or del 491–131 developed palpable tumors at all the injection sites in three weeks. As expected, vector transfected NIH 3T3 cells did not generate any tumor (Table VI).

These results suggest that elimination of the growth-inhibitory domains of hMDM2 activates its tumorigenic property. Also, the tumorigenic domain of hMDM2 resides within the first 130 amino acids of hMDM2. It is important to note that this domain of hMDM2 is sufficient to inactivate p53-mediated transactivation (Leng *et al.*, 1995b) suggesting that hMDM2 may induce tumorigenesis by inactivating p53. Although located within the first 130 amino acids, the tumorigenic and p53-interaction domains of hMDM2 may, however, function independent of each other. Further analyses are needed to answer this question.

**Discussion**

Results of our experiments show that the human oncoprotein hMDM2 expressed from its cDNA can arrest the cell-cycle progression of untransformed cells at the G0/G1 phase. Elimination of this growth-inhibitory function converts the protein to a tumorigenic form. These properties of hMDM2 are consistent with the general definition of oncoproteins. Proto-oncoproteins, the normal cellular counterparts of oncoproteins, are needed for normal cell-growth regulation, while the oncoproteins induce tumorigenesis if abnormally expressed (reviewed in Bishop, 1991; Hunter, 1991).

A recent report (Serrano *et al.*, 1997) has demonstrated that overexpression of oncogenic ras can induce G1 arrest of primary human and rodent cells. Loss of the growth-arrest function activates its oncogenic property. These properties of oncogenic ras are remarkably similar to that of hMDM2. The growth arrest induced by ras, however,
seems to follow a different pathway from hMDM2-mediated growth arrest. The ras-mediated G1-arrest requires induction of functional p53 and p16. hMDM2 can induce G0/G1-arrest in NIH 3T3 cells which have deleted p16 gene (Linaridopoulos et al., 1995; Quelle et al., 1995) and in p53-/- cells (Table I; Figure 2).

The two apparently opposite functions of hMDM2, tumorigenesis and growth arrest, are intriguing and suggest a novel cell-growth-regulatory mechanism. The cell-cycle-inhibitory function could be a protective mechanism of normal cells against growth proliferation in response to abnormal tumorigenic signals. It is possible that both the functions are necessary at different stages of normal cell growth, and a balance of the two functions is maintained in normal cells. It is unlikely that the tumorigenic forms are derived in normal cells by structural modification of the full-length protein, because in that case we should be able to generate stable transfectants of untransformed cells expressing the full-length protein.

Since the mouse and the human mdm2 gene expresses several splice variants (Gudas et al., 1993; Olson et al., 1993; Haines et al., 1994; Maxwell, 1994; Sigalas et al., 1996), it is possible that some splice variants code for the tumorigenic MDM2, which lacks functional growth-inhibitory domains. This hypothesis agrees with the fact that amplification of the mdm2 gene (Fakhzarzadeh et al., 1991; Finlay 1993) or insertion of introns within its cDNA (Lundgren et al., 1997) is necessary to overexpress the protein in untransformed cells and to induce tumorigenesis (Fakhzarzadeh et al., 1991; Finlay, 1993). Presence of the introns may be necessary to generate the tumorigenic MDM2. This hypothesis is also consistent with our observation that overexpression of deletion mutants of hMDM2 that lack the growth inhibitory domains induces tumorigenicity in NIH 3T3 cells (Table VI).

NIH 3T3 cells should express normal levels of endogenous MDM2. Also, amplification of the mdm2 gene should generate the full-length protein with the growth inhibitory property as well as the tumorigenic form(s). Since the mdm2 gene amplification or overexpression of the hMDM2 deletion mutants induces tumorigenesis in NIH 3T3 cells (Fakhzarzadeh et al., 1991; Table VI), the oncogenic form of MDM2 or hMDM2 should have a dominant effect over the growth-inhibitory form. It is possible that MDM2 expression from the normal gene in normal cells is regulated differently than expression from the amplified gene, and overexpression of the tumorigenic form may be favored when the gene is amplified. Different regulatory factors may induce expression of different splice variants of hMDM2. It is known that the tumor suppressor p53 can induce MDM2 expression (Barak et al., 1993, 1994; Juven et al., 1993; Wu et al., 1993). If the mdm2 gene is amplified, it is likely that the amount of p53 available to induce MDM2 expression from all the templates will be limiting. The tumorigenic form of MDM2 expressed under this condition may be different from the p53-induced form. hMDM2 overexpression in breast cancer cells has been associated with the presence of estrogen receptor (Sheikh et al., 1993; Gudas et al., 1995; Baunoch et al., 1996). Thus, the estrogen receptor may induce the overexpression of the tumorigenic form of hMDM2.

As indicated by our results, some tumor cells are partially insensitive to the growth-inhibitory effect of hMDM2, and hMDM2 can be stably overexpressed in these tumor cells (Tables I and IV and Figure 1B). This observation suggests that some tumor-derived cells could be defective in the ability to respond to hMDM2-mediated growth inhibition. This property of hMDM2 is very similar to the retinoblastoma (Rb) and Rb family of proteins, which do not suppress growth of some tumor-derived cells (Zhu et al., 1993). Tumor-derived cells are often polyclonal in nature. It is also possible that some of the cell populations are fully resistant to hMDM2-mediated growth arrest, while the others are susceptible. The hMDM2-resistant cell population can stably overexpress the oncoprotein (Table I and Figure 1B).

Consistent with this observation, it has been shown that hMDM2 can inhibit p53-mediated growth suppression in tumor-derived cells (Chen et al., 1994, 1996; Haupt et al., 1996). As the growth-inhibitory domains of hMDM2 are non-functional in such cells, the p53-interaction domain would inhibit p53-mediated growth suppression. Thus, the hMDM2 overexpression observed in cancer cells could be indicative of a genetic defect that renders the cells partially tolerant to hMDM2 overexpression, or a result of overexpression of hMDM2 that harbors inactive growth-inhibitory domains. Analysis of the splice variants of hMDM2 overexpressing in human cancer cells may reveal whether one, or both, of the mechanisms are involved in hMDM2-mediated tumorigenesis.

Our deletion analysis suggests that hMDM2 harbors more than one growth-inhibitory domain. These growth-inhibitory domains (ID1 and ID2) do not overlap with the p53-interaction domain of hMDM2 (Leng et al., 1995b) (Figures 6, 7 and 10). Recently, Schlott et al. (1997) have looked for mutations in the hmdm2 gene from several cancer samples that produced elevated levels of the hmdm2 transcript in the absence of gene amplification. Using RT–PCR and nucleotide sequencing analysis, they have identified several mutations between amino acids 302 and 310 that may interfere with ID2 defined by our analyses. Mutations in the growth-inhibitory domains of hMDM2 may be an alternate pathway that allows for overexpression of the protein in cancer cells, and needs to be thoroughly explored.

The tumorigenic domain of hMDM2 overlaps with the domain that inhibits p53-mediated transactivation (Table VI and Figure 10). This finding strengthens the hypothesis that inactivation of p53 contributes to the hMDM2-induced tumorigenesis. However, our data do not exclude the possibility that both the tumorigenic domain and the p53-inactivation domain of hMDM2 reside within the

![Fig. 10. Functional domains of hMDM2. The acidic, basic, Zn-finger and ATP-binding domains have been predicted by computer analysis. The two growth-inhibitory domains are labelled ID1 and ID2. As described in the text, the tumorigenic domain overlaps with the p53-interaction domain.](image-url)
N-terminal 130 amino acid residues and have different sequence requirements or functional domains. hMDM2 harbors two putative Zn-finger domains and an acidic activation domain (Figure 10) (Brown et al., 1993; Fakharzadeh et al., 1991). We have shown that the putative acidic activation domain of hMDM2 interacts with the human TATA-binding protein in vivo (Leng et al., 1995a). Therefore, hMDM2 shows the structural characteristics of a sequence-specific transcriptional activator. We analyzed whether the hMDM2-mediated G0/G1 arrest is associated with induction of one of the growth suppressors such as p53, p21 or p27. The induction of p53, p21 and p27 in sorted hMDM2-expressing and untransfected cells was tested by Western blot analysis using the respective monoclonal or polyclonal antibodies. The results (data not shown) suggest that these proteins are not induced as a consequence of hMDM2 overexpression. Consistent with this observation, the acidic activation domain of hMDM2, involved in binding the TATA-binding protein (Leng et al., 1995a), is not required for the G0/G1 arrest.

It has been reported that p53 regulatory functions of MDM2 are essential for embryonic development (Jones et al., 1995; Montes de Oca Luna et al., 1995). Our data show that MDM2 harbors tumorigenic and growth-inhibitory functions. Both the tumorigenic and the growth-inhibitory forms of MDM2 are capable of interacting with p53. These findings raise the question of which form of MDM2 is important for embryonic development. It is possible that only the tumorigenic form of MDM2 is necessary during embryonic development. However, the full-length MDM2 should arrest growth of normal embryonic cells when induced by another factor such as p53. In normal mouse embryonic cells, the growth-inhibitory function of MDM2 may be necessary to block cell growth in response to DNA damage or similar oncogenic stimuli.

Materials and methods

Plasmids and hMDM2 deletion mutants

The hmdm2 cDNA, mouse mdm2 cDNA and the plasmid pCMV.CD20 were generous gifts from Bert Vogelstein (Oliner et al., 1992), Donna George (Fakharzadeh et al., 1991) and Ed Harlow (Zhu et al., 1993) respectively. The plasmid pCMVβgal is from Clontech and pSVβgal is from Promega. Construction of pCMV.T and the deletion mutants of hmdm2 cDNA has been described earlier in detail (Brown et al., 1993; Deb et al., 1995; Leng et al., 1995a,b). Deletion mutants were generated by polymerase chain reaction (PCR) using primers flanking initiation and termination codons with appropriate start and stop sites. The wild-type or the deletion mutants were cloned in pGEM3zf (–) vector (Promega) under the control of T7 promoter for in vitro expression, and in pUCMV Bam vector (Hinds et al., 1990) under the control of CMV promoter for in vivo expression. The pUCMV Bam vector has been designated as pCMVneo. The pCMV vector has been derived from pHCMV Bam vector and does not harbor the neomycin resistance gene (Subler et al., 1992).

Cells and transfections

NIH 3T3, MRC-5, WI38, SAos-2, H1299 and U-2OS cells were purchased from American Type Culture Collection and were maintained in media as suggested by the suppliers. Normal human keratinocyte cells were purchased from Clonetics and were grown in complete keratinocyte growth medium (Clonetics). Normal and p53-null mouse embryo fibroblasts (MEF, gifts from Lawrence Donehower; Harvey et al., 1993) and 10(S) (gift of Arnold Levine; Dittmer et al., 1995) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supple-

mented with 10% fetal bovine serum. 21PT (gift of Vimla Band; Band et al., 1990) cells were maintained in alpha H and E medium (Band and Sager, 1989). Cells were seeded 18–24 h before transfection at 0.4–1 × 10⁶ cells per 10 cm dish and transfected by the calcium phosphate method (Gorman et al., 1982). The cells were transfected with plasmids that express wild-type or deletion mutants of hMDM2 and confers G418 resistance. With the wild-type or p53-null MEF cells, a plasmid conferring hygromycin resistance (Sugden et al., 1985) was cotransfected. Half of the cells per 10 cm dish were collected after transient expression of hMDM2, and the other half was replated at a ratio of 1:1 or 1:5 to select for G418 (500 μg per ml) or hygromycin (100 μg per ml) resistant colonies. Colonies were isolated and expanded in the presence of appropriate antibiotic. Expression of wild-type or deletion mutants of hMDM2 in pooled and isolated colonies were detected by Western blot analysis of the cell extracts. Western blot analysis was carried out essentially as described previously (Leng et al., 1995b; Shivakumar et al., 1995). Four anti-hMDM2 antibodies, N-20, SMP14 (Santa Cruz), Ab-1 (Oncogene Science) and 2A10 (Chen et al., 1993), recognizing three different domains of hMDM2 (Chen et al., 1993), were used for this purpose.

Flow cytometry

For flow cytometric analysis, 10 μg expression plasmid were used in most of the cases unless stated otherwise. Cells were harvested 48 h after transfection and fixed with 70% methanol for at least 2 h at –20°C. For experiments where the transiently transfected cells were blocked at mitosis, 40 h after transfection, cells were treated with 20 ng per ml of nocodazole (Oncogene Science) for 8 h. The fixed cells were rehydrated with phosphate-buffered saline (PBS) for 30 min at 4°C, blocked with horse serum to prevent non-specific staining and incubated with appropriate antibody for 12–20 h at 4°C. Cells were then washed three times in PBS and incubated with FITC-conjugated goat anti-mouse or anti-rabbit secondary antibody (Oncogene Science) for 60 min at 37°C. Following incubation, the cells were washed thrice in PBS. For cells transfected with pCMV.CD20, antibody incubations were carried out prior to methanol fixation (Zhu et al., 1993). To stain DNA, the cells were incubated with 0.1 mg/ml RNase A, 50 μg/ml propidium iodide and 0.1% NP40 in a trisodium citrate buffer for 30 min. The samples were analyzed by FACScan (Becton Dickinson). Relative levels of hMDM2 per cell were determined by FITC-fluorescence intensity in the green channel (530 nm). Vector-transfected cells were used to determine the background fluorescence. Cells showing higher FITC-fluorescence intensity than vector-transfected cells were sorted and collected. Expression of hMDM2 (or the deletion mutants) in the sorted cells was reconfirmed by analyzing expression of hMDM2 and its deletion mutants by Western blot analysis. The intensity of propidium iodide staining was recorded in the red channel. PEAKS,FTN or Modfit program was used to determine the percentage of G0/G1, S and G2/M phase cells. At least 10 000 FITC-labeled, and an equal number of unlabeled, cells were analyzed in each experiment. Experiments were repeated several times.

The levels of hMDM2 expression in the transfected cells (Table III) was quantitated and compared using a method described earlier (Mittnacht and Weinberg, 1991; Pollice et al., 1992; Sharro et al., 1996). The FITC-fluorescence associated with the transfected cells was compared by using a protocol provided by the instrument’s manufacturer (Becton Dickinson). In our flow cytometer, the difference in fluorescence intensities from 10 to 10⁶ is divided in 512 channels (linear digitized units). We calculated the difference by plotting the data in a semi-logarithmic plot with the channel number in the x-axis and fluorescence intensities (10 to 10⁵) in the y-axis.

Immunostaining

To determine localization of transiently expressed hMDM2, NIH 3T3 cells were transfected with 1 μg of wild-type or del 491–155 expression plasmids. Forty-eight hours after transfection, cells were fixed, heat treated and incubated using a monoclonal antibody against hMDM2 (Ab-1, Oncogene Science), using a protocol provided by Oncogene Science. The cells were then incubated with a biotin-conjugated secondary antibody and avidin–biotin alkaline phosphatase complex (Vector Laboratories) following the supplier’s protocol.

Tumorigenicity assay

For tumorigenicity assay, 1 × 10⁶ cells suspended in 0.2 ml serum free DMEM were injected subcutaneously into the flanks of 4–6-week-old
female athymic mice (National Cancer Institute). Animals were monitored regularly for tumor occurrence and size.

Acknowledgements

We thank Dr Donna George for providing the plasmid expressing mouse MDM2, Dr Arnold Levine for the monoclonal antibody 2A10 and the 10(3) cell line, Dr Lawrence Donohew for normal and p53–/– mouse embryo fibroblasts and Dr Vinna Band for 21PT cell line. This study was supported by funds from Wendy Will Cancer Research Foundation and NIH CA70712. D.B. is supported by a postdoctoral fellowship from Howard Hughes Medical Institute.

References


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\textbf{Human MDM2 arrests cell cycle}

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