The RING Finger Domain of MDM2 Is Essential for MDM2-mediated TGF- β Resistance

Christian Kannemeier, Rong Liao, and Peiqing Sun

Department of Molecular Biology, MB-41, The Scripps Research Institute, La Jolla, CA 92037

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In this study, we attempt to gain insights into the molecular mechanism underlying MDM2-mediated TGF- β resistance. MDM2 renders cells refractory to TGF- β by overcoming a TGF- β -induced G1 cell cycle arrest. Because the TGF- β resistant phenotype is reversible upon removal of MDM2, MDM2 likely confers TGF- β resistance by directly targeting the cellular machinery involved in the growth inhibition by TGF- β . Investigation of the structure-function relationship of MDM2 reveals three elements essential for MDM2 to confer TGF- β resistance in both mink lung epithelial cells and human mammary epithelial cells. One of these elements is the C-terminal half of the p53-binding domain, which at least partially retained p53-binding and inhibitory activity. Second, the ability of MDM2 to mediate TGF- β resistance is disrupted by mutation of the nuclear localization signal, but is restored upon coexpression of MDMX. Finally, mutations of the zinc coordination residues of the RING finger domain abrogates TGF- β resistance, but not the ability of MDM2 to inhibit p53 activity or to bind MDMX. These data suggest that RING finger-mediated p53 inhibition and MDMX interaction are not sufficient to cause TGF- β resistance and imply a crucial role of the E3 ubiquitin ligase activity of this domain in MDM2-mediated TGF- β resistance.

INTRODUCTION

Transforming growth factor β (TGF- β) is a multifunctional extracellular cytokine that regulates important cellular processes such as cell proliferation and differentiation, development, wound healing, and angiogenesis. The signaling pathway through which TGF- β regulates these cellular processes has been elucidated extensively (Siegel and Massague, 2003). There are two cell surface receptors for TGF- β , type I and II, which contain serine/threonine protein kinase activities in their intracellular domains. TGF- β primarily binds to and activates the type II receptor. The activated type II receptor then recruits the type I receptor into the complex, transphosphorylates it, and thereby stimulates its protein kinase activity. Once activated, the type I receptor phosphorylates Smad2 or Smad3, which are members of the Smad transcription factor family. Phosphorylated Smad2 or Smad3 binds to another Smad protein, Smad4. The resulting Smad heterodimer then translocates into the nucleus, where it regulates the transcription of TGF- β responsive genes in a cell type-specific manner.

TGF- β is a potent inhibitor of proliferation in most normal and early-stage tumor cells of epithelial, endothelial, and hematopoietic origins. On the other hand, TGF- β can also facilitate the growth and invasiveness of tumor cells by promoting angiogenesis, suppressing the host immune system, enhancing the tumor cell migration and adhesion, and stimulating the expression of metastasis-promoting proteases. Thus, late-stage metastatic tumors often produce increased amounts of TGF- β and, at the same time, become refractory to TGF- β -induced growth inhibition. The devel-

opment of resistance to TGF- β -induced growth inhibition allows these tumors to escape the negative growth impact of TGF- β while benefiting from its tumor-promoting effects (Siegel and Massague, 2003). Previous studies have shown that tumor cells can develop TGF- β resistance after inactivation of components of the TGF- β signaling pathway (Siegel and Massague, 2003), overexpression of oncogenes such as *c-myc* (Feng *et al.*, 2002) or *cdc25A* (Iavarone and Massague, 1997), or deletion of the *p15^{INK4B}* locus (Hannon and Beach, 1994). In addition, we have demonstrated that overexpression of an oncogene, MDM2, leads to TGF- β resistance in both mink lung epithelial cells (Mv1Lu) and normal human mammary epithelial cells (Sun *et al.*, 1998). In human breast tumor cells, increased MDM2 expression correlates with loss of TGF- β sensitivity, suggesting that MDM2 overexpression is a potential mechanism for TGF- β resistance in human tumors.

MDM2 is a multifunctional oncoprotein, and its ability to inactivate the p53 tumor suppressor protein has been well characterized. MDM2 inhibits p53 functions through multiple mechanisms. The N-terminal p53-binding domain of MDM2 binds the transactivation domain of p53 and inhibits its transcriptional activity (Momand *et al.*, 1992). In addition, the MDM2/p53 complex shuttles from the nucleus to the cytoplasm (Tao and Levine, 1999a), and the E3 ubiquitin ligase activity located in the C-terminal RING finger domain of MDM2 marks p53 and itself for degradation by ubiquitination (Honda *et al.*, 1997; Fang *et al.*, 2000). The MDM2/p53 interaction is regulated by p19/p14^{ARF}, which sequesters MDM2 from p53 and blocks the ability of MDM2 to inhibit p53, leading to increased p53 activity (Honda and Yasuda, 1999; Tao and Levine, 1999b).

p53-independent functions of MDM2 have also been described. MDM2 binds to and ubiquitinates the Retinoblastoma protein (Rb; Xiao *et al.*, 1995; Uchida *et al.*, 2005). This results in Rb degradation and release of the E2F1 transcription factor and cell cycle progression. MDM2 has also been reported to bind E2F1 directly and enhance E2F1 expression

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Address correspondence to: Peiqing Sun (pqsun@scripps.edu).

(Martin *et al.*, 1995). The p53-independent functions of MDM2 are supported by transgenic mouse models, in which overexpression of MDM2 in a p53 null background leads to multiple rounds of S-phase without cell division in mammary epithelial cells (Lundgren *et al.*, 1997) and hyperproliferation of the skin (Alkhalaf *et al.*, 1999). It has also been described that overexpression of MDM2 in mice leads to spontaneous tumor formation in the absence of p53 (Carstens *et al.*, 2004). Moreover, splice variants of the human counterpart of MDM2, HDM2, which contain partial or complete deletions of the p53-binding domain and do not bind p53 *in vitro*, retain significant tumorigenic activity in NIH3T3 cells (Sigalas *et al.*, 1996; Harris, 2005).

The RING finger domain of MDM2 has received special attention because of its multiple functions. It contains an E3 ubiquitin protein ligase activity that is indispensable for the ubiquitination and degradation of p53. In particular, the zinc coordination residues C436, H455, C459, and C473 dictate the formation of the RING fingers and are essential for the E3 activity (Honda *et al.*, 1997; Fang *et al.*, 2000). Interestingly, the ubiquitination and degradation of p53 requires not only the RING finger, but also a central acidic domain of MDM2 (Argentini *et al.*, 2001; Kawai *et al.*, 2003). It is unclear whether the acidic domain is also required for the ubiquitination of other MDM2 substrates. The RING finger has also been described to interact with different proteins, such as MDMX and the transcription factor TAFII250 (Leveillard and Wasylyk, 1997; Tanimura *et al.*, 1999), and a specific 77-nucleotide RNA sequence that may be involved in gene translation (Elenbaas *et al.*, 1996). Furthermore, the RING finger can bind to nucleotides, which facilitates the nucleolar localization of MDM2 (Poyurovsky *et al.*, 2003). In an attempt to gain insights into the mechanism by which MDM2 leads to resistance to TGF- β -induced growth arrest, we performed a structure-functional analysis of MDM2 in the current report. We have confirmed the ability of MDM2 to directly confer TGF- β resistance and further showed that the resistance is due to bypass of the TGF- β -induced G1 cell cycle arrest. Moreover, we have defined the structural requirements for MDM2-conferred TGF- β resistance. The results have indicated the essential roles of the C-terminal half of the p53-binding domain, the nuclear localization motif of MDM2 and the zinc coordination residues in the RING finger domain in TGF- β resistance in epithelial cells of both mink and human origins.

MATERIALS AND METHODS

Antibodies and Western Blotting Analysis

Cell lysates were collected, separated by SDS-PAGE, and analyzed by Western blotting as described previously (Wang *et al.*, 2002). To detect MDM2, an anti-MDM2 mAb (2A10, a kind gift of Dr. Arnold Levine; Xiao *et al.*, 1995) was used in a dilution of 1:100. The hemagglutinin (HA)-tagged MDM2 was detected with an anti-HA antibody (HA11, Covance, Berkeley, CA). Detection of mink p53 was performed with an anti-p53 antibody CM5 (Novo Castra, Newcastle upon Tyne, United Kingdom). Actin was detected using an anti-actin antibody (Sigma, St. Louis, MO). MDMX-FLAG was detected using the anti-FLAG M5 antibody (Sigma). P53 and FLAG-MDMX were immunoprecipitated by an anti-p53 antibody (FL393, Santa Cruz Biotechnology, Santa Cruz, CA) and an anti-FLAG M2 antibody (Sigma), respectively. A goat anti-mouse or a goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) was used as secondary antibodies.

Cell Culture

Mink lung epithelial cells (Mv1Lu), HEK293T and the amphotropic packaging cell line LinXA were grown in Dulbecco's modified essential medium containing L-glutamine, penicillin/streptomycin, sodium-pyruvate, and 10% fetal calf serum (Gemini, Calabasas, CA) at 37°C in a humidified atmosphere containing 5% CO₂. Human mammary epithelial cells (HMECs) were purchased from Cambrex (Walkersville, MD) and cultivated according to the manufacturer's instructions.

Retroviral Vectors and Retrovirus-mediated Gene Transduction

mdm2 was cloned as described previously (Sun *et al.*, 1998). For the deletions of the N- and C-terminal regions of MDM2, cDNA encoding the corresponding mutants were amplified by PCR with the appropriate primers (sequence available upon request) and subcloned into the pWZL-hygro retroviral vector. Point mutations of the RING finger domain of MDM2 were generated using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA). For the internal deletions of MDM2, the 5' region and the 3' region of each deletion were amplified separately by PCR and subcloned into pWZL-hygro containing an N-terminal epitope tag from the HA protein (sequence available upon request) via triple ligation. The deleted residues were replaced by three Ala encoded by nine nucleotides that contained an *NotI* restriction site. All deletions were verified by sequencing analysis. An *mdmx* cDNA was kindly provided by Dr. Jochemsen (University of Ghent, Belgium) and subcloned into a FLAG-containing pBABEPuro vector. Retroviral infection was carried out as described previously (Deng *et al.*, 2004), employing the packaging cell line LinXA. The infection efficiency was determined to be 30–50%. The infected cells were selected with hygromycin (Calbiochem, La Jolla, CA 300 μ g/ml for Mv1Lu or 12 μ g/ml for HMEC) or puromycin (2 ng/ml, Fluka, Buchs, Switzerland). HMEC cells were infected at passage 9–11. For double-infections with the MDM2 deletion mutants and MDMX, cell lines already infected with MDM2 deletion mutants were infected with a puromycin-resistant retrovirus containing a FLAG-tagged cDNA of *mdmx* and then selected with puromycin in the presence of hygromycin.

For the reversion experiment, MDM2 was subcloned into a HygroMarxII vector (Hannon *et al.*, 1999) containing a loxP site in the 3'-LTR (long terminal repeat; see Figure 2A). Cells transduced with this construct were infected with a second retrovirus encoding the Cre recombinase with a puromycin resistance marker. After selection was complete, cells were cultivated for a period of 10–14 d before being subjected to the indicated experiments.

Colony Formation Assay

The colony formation assay was performed as described previously (Sun *et al.*, 1998). Briefly, $2-6 \times 10^3$ of Mv1Lu cells or $2-8 \times 10^3$ of HMEC cells were seeded into six-well plates and treated with 5 ng/ml TGF- β or left untreated for 8–12 d with a replenishment of TGF- β every 4 d. Cells were then washed, fixed, and stained with crystal violet. Each experiment was repeated at least three times.

Analysis of Cell Cycle Profiles Using the Bromodeoxyuridine Incorporation Assay Followed by Fluorescence-activated Cell Sorting Analysis

To determine the cell cycle profiles, 1×10^5 cells were seeded into a 10-cm dish and treated with 5 ng/ml TGF- β for 48 h. For the last 45 min, 20 μ M bromodeoxyuridine (BrdU) was added to the cells. Cells were harvested by trypsinization, washed once in phosphate-buffered saline (PBS), and fixed in 70% ethanol for at least 2 h. Subsequently cells were incubated with 2 M HCl and 0.1% Triton X-100 for 30 min at 20°C, washed first with 0.1 M Na₂B₄O₇, pH 8.5 and then with PBS containing 1% BSA and 0.5% Triton X-100, and incubated with a fluorescein isothiocyanate (FITC)-coupled mouse anti-BrdU antibody (PharMingen, San Diego, CA) for 30 min. After washing with PBS, cells were incubated with 10 μ g/ml 7AAD (Sigma) in PBS containing 1% BSA and 0.1% Triton X-100 for 10–20 min at 20°C, followed by another wash with PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 before being subjected to fluorescence-activated cell sorting (FACS) analysis. Fluorescence of the cells was detected by exciting the FITC and 7AAD spectra, respectively, and measuring at the appropriate emission wavelength. The data were evaluated using the FCSpress software (Ray Hicks, www.fcspress.com). Each experiment was repeated at least three times.

Analysis of TGF- β Sensitivity in Synchronized Cells

Mv1Lu cells transduced with either a control vector or *mdm2* were maintained under confluence for 6 d, with replenishment of medium every 2 d. Cells were then released from contact inhibition by splitting, and seeded at 1.5×10^6 cells/10-cm plate in the presence or absence of TGF- β to allow re-entry into the cell cycle. The cell cycle profiles were determined in cell populations grown under confluence or those that had been released from contact inhibition for 17 or 28 h, using a BrdU incorporation assay followed by FACS analysis as described above.

Analysis of p53 Transcriptional Activity

To determine the transcriptional activity of p53, luciferase reporter assays were performed using the p53 reporter construct PG14 (a kind gift from Dr. Bert Vogelstein; el-Deiry *et al.*, 1992) and the Dual Luciferase Assay Kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, Mv1Lu cells stably expressing the wild-type or relevant mutant MDM2 proteins were transfected with 1.9 μ g of PG14 and 0.1 μ g of a Renilla luciferase reporter driven by an actin promoter. The Firefly luciferase activity

Figure 1. MDM2 confers TGF- β resistance by overcoming TGF- β -induced G1 arrest. (A) Control cells ($n = 4 \times 10^3$; con) or MDM2-expressing cells ($n = 2 \times 10^3$; MDM2) were seeded into six-well plates, grown for 8 d in the absence or presence of 5 ng/ml TGF- β , and stained with crystal violet. (B) Control cells (con) and MDM2-expressing cells (MDM2) were grown for 2 d in the absence or presence of 5 ng/ml TGF- β and labeled with BrdU for 45 min. After staining total DNA with 7AAD and BrdU-incorporated cells with a FITC-conjugated anti-BrdU antibody, the percentage of cells in each cell cycle phase was determined by FACS analysis. (C) To demonstrate the inhibitory effect of TGF- β on G1/S progression in the control cells (con) and MDM2-expressing cells (MDM2), fold reduction in S phase cells by TGF- β was calculated by dividing the percentage of S phase cells (as determined in Figure 1B) in the untreated population by that in the TGF- β -treated population. (D) The cell cycle profiles of the control (con) or MDM2-expressing cells (MDM2) grown under confluence (0h) or 17 h (17h), or 28 h (28h) after being released from contact inhibition in the presence or absence of TGF- β . Each cell population was labeled with BrdU for 45 min before collection, stained with a FITC-conjugated anti-BrdU antibody and 7-AAD, and subjected to FACS analysis. (E) Twenty-five micrograms of a lysate from control cells (con) or MDM2-expressing cells (MDM2) with and without MDMX were subjected to SDS-PAGE followed by Western blot analysis, and the indicated proteins were detected with the appropriate antibodies.

was determined 2 d after transfection and normalized to the Renilla luciferase activity.

Immunofluorescence

Mv1Lu cells expressing the wild type or relevant MDM2 mutant proteins were seeded at a density of 1×10^4 cells/well in an eight-well chamber slide (Nunc, Naperville, IL) and cultivated overnight at 37°C. The cells were fixed with 4% paraformaldehyde in PBS at 4°C, followed by blocking with PBS containing 3% BSA and 0.1% Tween 20 for at least 1 h at 37°C. Subsequently, the cells were incubated for 1–2 h at 20°C with a 1:100 dilution of the mouse anti-HA antibody (HA11) followed by three washes with PBS containing 1% BSA, and 0.1% Tween 20. After that, a FITC-coupled goat anti-mouse antibody (Santa Cruz) was diluted 1:200 and incubated with the cells for 2 h at 20°C followed by extensive washing. The well chambers were dismantled from the slide, and the slide was incubated with anti-fading solution containing DAPI (Vector Laboratories, Burlingame, CA) and analyzed with a fluorescence microscope.

Immunoprecipitation

Cell lysates were prepared in a lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.25% NP40, and Complete protease inhibitors (Roche, Indianapolis, IN). The total protein concentration was determined by a Bradford protein assay (Bio-Rad, Richmond, CA) and 0.2–1 mg of protein was used for immunoprecipitation. The remaining lysate was used as a whole cell lysate control during Western blotting. Antibody, 2–10 μ g, was added to the lysate and incubated at 4°C for 2 h. Protein G-Sepharose, 10–20 μ l of a 50% slurry (GE Healthcare, Waukesha, WI) was added, and the reactions were incubated for an additional hour at 4°C. The beads were pelleted by centrifugation at $200 \times g$, washed four times with lysis buffer, mixed with 50 μ l of SDS sample buffer (Invitrogen, Carlsbad, CA), and incubated at 95°C for 5 min. After cooling, the beads were subjected to SDS-PAGE followed by Western blotting.

RESULTS

MDM2 Mediates TGF- β Resistance by Overcoming TGF- β -induced G1 Arrest

mdm2 has been identified in a functional cDNA library screen designed to search for genes that could bypass TGF- β -induced growth arrest in a mink lung epithelial cell line Mv1Lu (Sun *et al.*, 1998). To confirm the ability of MDM2 to confer TGF- β resistance, Mv1Lu cells were stably transduced with *mdm2* using a retroviral vector and, immediately after selection of infected cells (7–10 d post infection), were tested for TGF- β resistance. After 8 d of TGF- β treatment of sparsely seeded cells, a significantly higher number of TGF-

β -resistant colonies were observed in MDM2-expressing cells than in control cells (Figure 1A), confirming our previous observation that MDM2 confers TGF- β resistance (Sun *et al.*, 1998). We estimated that at least 10% of the cells in the MDM2-transduced population, whereas only <0.1% of the control cells, formed colonies in the presence of TGF- β . The high percentage of TGF- β -resistant cells shortly after the transduction of *mdm2* suggested that MDM2 did not confer TGF- β resistance by promoting the accumulation of secondary mutations, but rather through a direct effect.

It has been demonstrated that TGF- β arrests cell growth in G1 phase (Laiho *et al.*, 1990). To determine whether MDM2 confers TGF- β resistance by abrogating TGF- β induced G1 arrest, we measured the percentage of MDM2-expressing or control cells that had progressed from G1 into S phase in the presence of TGF- β using a BrdU incorporation assay followed by FACS analysis. When treated with TGF- β , the percentage of cells incorporating BrdU (representing those in S phase) in the MDM2 expressing population was 11.8%, compared with 3.7% in the control population, indicating that a significant portion of MDM2-expressing cells were able to exit G1 and progress into S phase in the presence of TGF- β (Figure 1B). Although TGF- β reduced the percentage of control cells in S phase by fivefold, only a twofold reduction in S-phase cells was observed by TGF- β in the MDM2-transduced population (Figure 1C). These results indicated that MDM2 abolished TGF- β -induced G1 arrest, thereby allowing cells to move forward into S phase and proliferate in the presence of TGF- β . Consistent with this notion, the increase in the percentage of S phase cells in the TGF- β -treated MDM2 population was accompanied by a reduction in TGF- β -induced accumulation of G1 cells, compared with that in the control population, although the relative level of this reduction (from 27% in control to 25% in MDM2 cells) was low due to the presence of a high percentage (>60%) of G1 cells in an asynchronous culture.

To further substantiate the ability of MDM2 to overcome TGF- β -induced G1 arrest, we analyzed cells that had been synchronized in G1 by contact inhibition and then released from G1 in the presence or absence of TGF- β . Both MDM2-

ated for 2 wk (MDM2+Cre) were grown for 8 d in the absence or presence of 5 ng/ml TGF-β before staining with crystal violet.

expressing and control cells arrested in G1 phase when grown to confluence, suggesting that MDM2 could not overcome the G1 arrest induced by contact inhibition (Figure 1D). When released from contact inhibition without TGF-β, MDM2-expressing cells entered S phase faster than the control cells. Twenty-eight hours after the release, 32.4% of MDM2-expressing cells had already proceeded from G1 into S phase, whereas there were only 11.2% of S phase cells in the control population. This finding is consistent with a previous report that MDM2 can promote the transition of cell cycle from G1 to S phase (Argentini *et al.*, 2000) and overcome G1 arrest induced by multiple mechanisms (Dubs-Poterszman *et al.*, 1995; Loughran and La Thangue, 2000). More importantly, in the presence of TGF-β, the MDM2 cells progressed into cell cycle upon release, but the reentry of the control cells was completely blocked. Therefore, our results suggest that MDM2 has an intrinsic ability to enhance G1/S transition and that this G1/S promoting activity of MDM2 allows cells to escape the negative regulation of the G1 machinery imposed by TGF-β and proliferate in the presence of TGF-β. Notably, MDM2 did not overcome the G1 arrest caused by contact inhibition, implying that TGF-β and contact inhibition induce G1 arrest through different mechanisms.

Western blot analysis showed distinct MDM2 expression in *mdm2*-transduced cells, but not in control cells (Figure 1E). However, there is no significant decrease in the p53 protein level in MDM2-expressing cells. This coincides with a previous finding that MDM2 overexpression alone is not always sufficient to down-regulate p53 protein levels, but that MDM2 requires MDMX as a cofactor for degradation of the p53 protein (Badciong and Haas, 2002). Indeed, coexpression of MDMX and MDM2 led to a marked decrease in the p53 protein level. This suggests that MDMX and MDM2 work in conjunction to inhibit and degrade p53.

Removal of MDM2 Expression Reverses TGF-β Resistance in Mv1Lu Cells

MDM2 inhibits the functions of p53, which is important for the maintenance of genome stability. Indeed, MDM2 overexpression has been shown to lead to centrosome hyperam-

Figure 2. MDM2-mediated TGF-β resistance is reversible upon removal of MDM2. (A) A cartoon for Cre/loxP-mediated recombination. The *mdm2* cDNA is introduced into the genome between the two LoxP sites located in the LTRs. On transduction of the Cre recombinase, the loxP sites are recombined, and a circular plasmid containing *mdm2* cDNA is excised from the genome. The circular plasmid is lost during cell divisions. (B) Experimental procedures for testing the consequence of loss of MDM2 expression. A retrovirus containing the *mdm2* cDNA between two loxP sites is transduced into Mv1Lu cells. Subsequently this cell line is transduced with a second retrovirus encoding Cre and cultivated for 2 wk to allow the loss of the excised *mdm2* plasmid. TGF-β responsiveness is examined before and after the excision of *mdm2*. (C) Twenty-five micrograms of a lysate from control cells (con), MDM2-expressing cells (MDM2), or MDM2-expressing cells transduced with *cre* (MDM2+Cre) or a vector control (MDM2+Vector) and cultivated for 2 wk were subjected to SDS-PAGE followed by Western blot analysis to detect MDM2. The asterisk indicates an unspecific band. (D) Control cells ($n = 4 \times 10^3$; con), 2×10^3 MDM2-expressing cells transduced with a vector control and cultivated for 2 wk (MDM2+Vector), and 4×10^3 MDM2-expressing cells transduced with *cre* and cultivated for 2 wk (MDM2+Cre) were grown for 8 d in the absence or presence of 5 ng/ml TGF-β before staining with crystal violet.

plification and genome instability in certain tumors (Carroll *et al.*, 1999). Thus, it is conceivable that increased expression of MDM2 may promote genomic instability, resulting in mutations that in turn lead to TGF-β resistance. In an attempt to determine whether MDM2 mediates TGF-β resistance directly or through secondary genomic mutations, a retrovirus was constructed that contained loxP sites flanking the *mdm2* cDNA. The Cre recombinase can recombine the two loxP sites and thus excises the DNA in between (Figure 2A; Hannon *et al.*, 1999). On transduction of loxP-*mdm2*-loxP-infected cells with a second retrovirus encoding Cre, the *mdm2* cDNA will be excised from the genome, and its expression will be lost after cell division (Figure 2, A and B). This allows a direct assessment of the effect of loss of MDM2 expression on TGF-β sensitivity.

Infection of Mv1Lu cells with the loxP-*mdm2*-loxP retrovirus led to MDM2 overexpression (Figure 2C) and TGF-β resistance as detected in a colony formation assay. Subsequently those MDM2-expressing cells were infected with a second retrovirus encoding Cre. After 2 wk of cultivation, MDM2 was no longer detected by Western blotting, whereas those cells infected with an empty vector retained MDM2 expression (Figure 2C). The cells that had lost MDM2 expression upon Cre expression did not confer TGF-β resistance as detected in a colony formation assay (Figure 2D). In contrast, MDM2-expressing cells that were infected with a control vector clearly showed TGF-β resistance. This indicates that the TGF-β-resistant phenotype is reversible upon removal of the exogenously expressed *mdm2* gene. Therefore, our results demonstrated that MDM2 confers TGF-β resistance by directly interfering with the cellular machinery that mediates the growth inhibition in response to TGF-β, rather than by promoting secondary genomic mutations, as has been suggested elsewhere (Blain and Massague, 2000). The high percentage (10%) of TGF-β-resistant cells in an MDM2-expressing population is also inconsistent with the statistical rate of the occurrence of random mutations. The differences between our observations and those obtained by Blain *et al.* may be resulted from the differential experimental conditions used in these studies.

The C-terminal Half of the p53-Binding Domain of MDM2 Is Essential for MDM2-mediated TGF- β Resistance

To identify the functional domains of MDM2 that contribute to TGF- β resistance, we performed a series of deletion analysis of MDM2 (see Figure 6). The first set of deletion mutants targeted the N-terminus of MDM2. The deletion MDM2 Δ N1 represented a known splice variant of HDM2 in the mouse protein, which had lost its p53-binding ability *in vitro*, but retained tumorigenic activity (Sigalas *et al.*, 1996; Harris, 2005). MDM2 Δ N2 targeted the complete p53-binding domain of MDM2. Consistent with our previous report (Sun *et al.*, 1998), MDM2 Δ N1 conferred TGF- β resistance to the same extent as wild-type MDM2 as detected by a BrdU incorporation assay followed by FACS analysis. Both wild-type MDM2 and MDM2 Δ N1 greatly prevented TGF- β -induced reduction in the percentage of cells progressing from G1 into S phase (Figure 3A, top panel). By contrast, TGF- β led to a fivefold reduction in the percentage of cells progressing into S phase in the MDM2 Δ N2-expressing population, the same as in the control cells, indicating that MDM2 Δ N2 failed to confer TGF- β resistance. The differential effects of Δ N1 and Δ N2 deletions on TGF- β responsiveness were also confirmed in colony formation assays in Mv1Lu cells (Figure 3A, bottom panel) and in primary HMECs (Figure 3F). These results indicate that the C-terminal part of the p53-binding domain of MDM2, which was retained in Δ N1 but lost in Δ N2, is required for MDM2-mediated TGF- β resistance. The levels of p53 protein in control cells or the cell lines expressing either wild-type or mutant MDM2 did not differ significantly (Figure 3B), indicating that neither wild type nor the N-terminal deletion mutants of MDM2 were able to down-regulate the p53 protein level in a detectable manner under the current experimental settings in Mv1Lu cells.

To measure the effect of MDM2 on the transcriptional activity of p53, a luciferase reporter assay was conducted using PG14, a Firefly luciferase reporter for p53 containing 14 repeats of a synthetic p53-binding site (el-Deiry *et al.*, 1992). The PG14 reporter was transiently transfected into Mv1Lu cells stably expressing either wild-type MDM2 or one of the deletion mutants of MDM2 to detect endogenous p53 activity. A Renilla luciferase construct driven by a β -actin promoter was cotransfected together with PG14 to allow normalization of the luciferase readout. MDM2 and MDM2 Δ N1 led to comparable levels of down-regulation of transcription from the PG14 reporter, whereas MDM2 Δ N2 had lost its ability to inhibit the p53-dependent transcription (Figure 3C). This observation raises the possibility that, even though MDM2 Δ N1 has been described to have lost its binding affinity to p53 *in vitro* (Sun *et al.*, 1998; Bartel *et al.*, 2004), it may still bind to p53 *in vivo*, thus leading to the inhibition of p53 transcriptional activity.

We tested this hypothesis in a coimmunoprecipitation experiment with endogenous p53. Because of the lack of a suitable antibody for immunoprecipitating p53, we transduced HEK293T or HMEC with a retrovirus encoding MDM2, MDM2 Δ N1, or MDM2 Δ N2. Both MDM2 and MDM2 Δ N1 coprecipitated with p53 in HMEC and 293T cells, although MDM2 Δ N1 coprecipitated with lower affinity compared with MDM2. MDM2 Δ N2 was absent from the p53 complex in 293T cells (Figure 3, D and E). In addition, the protein level of p21, a transcriptional target of p53, was significantly reduced in HMEC expressing MDM2 or MDM2 Δ N1 compared with the control (Figure 3E). This confirms our results from the luciferase assay that MDM2 Δ N1 can still inhibit p53 activity. These observations

Figure 3. The C-terminal half of the p53-binding domain and the RING finger of MDM2 are required for TGF- β resistance. (A) Top, Mv1Lu cells transduced with a vector control (con) or wild-type or mutant *mdm2* (as indicated) were grown for 2 d in the absence or presence of 5 ng/ml TGF- β before being labeled with BrdU for 45 min. Cells were then stained with a FITC-conjugated anti-BrdU antibody and 7-AAD and subjected to FACS analysis. Fold reduction in S phase cells by TGF- β was calculated by dividing the percentage of S phase cells in the untreated population by that in the TGF- β -treated population. Bottom, $2\text{--}4 \times 10^3$ of Mv1Lu cells transduced with vector control (con), wild-type MDM2 (MDM2), or indicated MDM2 mutants were seeded into six-well plates, grown for 8 d in the absence or presence of 5 ng/ml TGF- β , and stained with crystal violet. (B) Twenty-five micrograms of a lysate from control cells (con) or cells transduced with wild-type or indicated deletion mutants of *mdm2* was subjected to SDS-PAGE followed by Western blot analysis. The indicated proteins were detected with appropriate antibodies. (C) The PG14 Firefly luciferase reporter plasmid containing a p53 responsive promoter and a Renilla-luciferase reporter driven by a β -actin promoter were cotransfected into Mv1Lu cells transduced with a vector control or the wild-type or indicated mutant *mdm2*. The Firefly luciferase activity was determined and presented as the p53 activity after normalization to the Renilla luciferase activity. Values are means $\pm$ SD for triplicates. (D) HEK293T cells transduced with *mdm2* or its p53-binding domain mutants were lysed, and p53 was immunoprecipitated. Subsequently, whole cell lysates (Lysate) and p53 immunoprecipitates (IP) were subjected to SDS-PAGE followed by Western blot analysis of the indicated proteins. The asterisk indicates an unspecific band. (E) HMEC transduced with *mdm2* or its p53-binding domain mutants were lysed and p53 was immunoprecipitated. Subsequently, whole cell lysates (Lysate) and p53 immunoprecipitates (IP) were subjected to SDS-PAGE followed by Western blot analysis of the indicated proteins. The asterisk indicates an unspecific band. (F) HMEC cells transduced with vector control ($n = 4 \times 10^3$; con), wild-type MDM2 (MDM2) or indicated MDM2 mutants were seeded into six-well plates, grown for 12 d in the absence or presence of 5 ng/ml TGF- β , and stained with crystal violet.

strongly suggest that MDM2 Δ N1 still binds to p53 and inhibits p53 activity, whereas MDM2 Δ N2 has lost this activity. The molecular basis for the interaction between MDM2 Δ N1 and p53 is currently under investigation. One possibility is that MDM2 Δ N1 still retains some residual p53-binding, either directly or indirectly, *in vivo*. Alterna-

tively MDM2 Δ N1 may bind to p53 *in vivo* through a bridging protein such as endogenous MDMX or MDM2. The lack of binding of p53 to MDM2 Δ N2 suggests that p53 does not interact with a second p53-binding site in the acidic domain of MDM2 as reported recently (Ma *et al.*, 2006).

The Zinc Coordination Residues of the RING Finger Domain of MDM2 Are Indispensable for MDM2-mediated TGF- β Resistance

The RING finger domain of MDM2 has multiple functions. It has been described to have an E3 ubiquitin protein ligase activity, which is important for the ubiquitination of p53 and MDM2 itself (Honda *et al.*, 1997). In addition, the RING finger domain can bind to nucleotides such as ATP and a specific RNA sequence involved in gene translation (Elenbaas *et al.*, 1996; Poyurovsky *et al.*, 2003). Furthermore, the RING finger domain interacts with other proteins such as MDMX (Elenbaas *et al.*, 1996; Tanimura *et al.*, 1999) and TAFII250 (Leveillard and Wasyluk, 1997). To examine the role of the RING finger, we deleted the entire RING finger domain from MDM2 (MDM2 Δ C1) and tested for the ability of this mutant to confer TGF- β resistance. TGF- β treatment led to a fivefold reduction in the percentage of cells progressing from G1 into S phase in the population that overexpressed MDM2 Δ C1, as in the control population (Figure 3A). In addition, MDM2 Δ C1 failed to promote colony formation in the presence of TGF- β in both Mv1Lu cells (Figure 3A) and HMEC cells (Figure 3F). These results indicate that the RING finger domain is essential for the ability of MDM2 to mediate TGF- β resistance.

Western blot analysis revealed that overexpression of MDM2 Δ C1 led to a marked increase in the p53 protein level compared with that of wild-type MDM2 (Figure 3D). This observation is in accordance with a previous report demonstrating that loss of the E3 activity of MDM2 is accompanied by p53 stabilization and increased p53 levels in cells (Fang *et al.*, 2000).

To determine which RING finger-associated activity of MDM2 was required for TGF- β resistance, five point mutants of MDM2 were created within the RING finger domain. Four of these point mutants targeted the zinc coordination residues essential for the E3 activity (MDM2C436L, MDM2H455S, MDM2C459S, and MDM2C473G; Fang *et al.*, 2000), whereas the other targeted the ATP and RNA binding abilities of MDM2 (MDM2G446S; Elenbaas *et al.*, 1996). After the expression of these mutants was confirmed by Western blot (Figure 4B), they were tested for their ability to confer TGF- β resistance. All mutants that targeted the zinc coordination residues of the RING finger destroyed the ability of MDM2 to confer TGF- β resistance. These MDM2 mutants failed to bypass TGF- β -induced cell cycle arrest (Figure 4A) and did not promote colony formation in TGF- β in Mv1Lu or HMEC cells (Figure 4E). In contrast, the G446S mutation did not interfere with the ability of MDM2 to mediate TGF- β resistance (Figure 4, A and E). These results clearly suggest an essential role of the zinc coordination residues within the RING finger and imply that the E3 ubiquitin ligase activity may be required for MDM2-mediated TGF- β resistance. In contrast, the ATP- and RNA-binding activities of the RING finger are dispensable.

Similar to MDM2 Δ C1, all the MDM2 point mutants that have lost one of the zinc coordination residues and thus the E3 activity led to stabilization of p53, whereas MDM2G446S targeting the RNA binding activity failed to do so (Figure 4B). This is again consistent with the previous finding that MDM2 mutants lacking the E3 activity stabilize p53 and lead to increased p53 protein levels in cells (Fang *et al.*,

2000). To determine the effect of the RING finger point mutations on the transcriptional activity of p53, Mv1Lu cells expressing these mutants were subjected to a luciferase reporter assay using the p53-dependent PG14 reporter. MDM2 expression reduced p53 reporter activity by fourfold (Figure 4C). The MDM2G446S mutant also led to a similar level of inhibition of p53 activity. Surprisingly, the MDM2 mutants with altered zinc coordination residues either partially (MDM2H455S and C473G) or completely (MDM2C436L and C459S) retained the ability to inhibit p53 activity. These results indicate that the zinc coordination residues of the RING finger of MDM2 are not essential for the inhibition of p53 transcriptional activity. Indeed, no significant reduction in the p53 protein level was observed in Mv1Lu, 293T, or HMEC cells transduced with the *mdm2* retrovirus (Figures 1D, 3B, 3D, 3E, 4B, and 5B), suggesting that p53 degradation might not play a major role in the inhibition of p53 activity by the retrovirally expressed MDM2. Because these zinc coordination residue mutants have an intact p53-binding domain, it is possible that binding of MDM2 to p53 is sufficient to inhibit p53 activity. On the basis of the observation that the zinc coordination residue mutants of MDM2 inhibited p53 activity but failed to confer TGF- β resistance, we conclude that inhibition of p53 activity by MDM2 is not sufficient to cause TGF- β resistance.

Because the zinc coordination residues may be important for the tertiary structure of the RING finger of MDM2, mutations of these residues may disrupt other functions such as protein-protein interaction, in addition to the E3 activity. Therefore, we tested whether the RING finger point mutants were still able to bind to MDMX. MDMX was coexpressed in Mv1Lu cells together with wild-type MDM2 or MDM2 mutants carrying point mutations or a complete deletion (MDM2 Δ C1) of the RING finger domain. On pull-down of MDMX, MDM2 and all the point mutants of the RING finger domain could be detected in the MDMX complex, whereas MDM2 Δ C1 could not be detected (Figure 4D). These results indicate that the zinc coordination residue mutations of MDM2 retain the interaction with MDMX, although they can no longer confer TGF- β resistance, suggesting that interaction between MDMX and the RING finger of MDM2 is not sufficient to lead to TGF- β resistance. Because the nucleotide binding activity of the RING finger is also not essential, it is likely that the ability of MDM2 to mediate TGF- β resistance relies on the E3 ubiquitin ligase activity.

Analysis of the Central Domains of MDM2: Requirement of the Nuclear Localization of MDM2 for TGF- β Resistance

The central region of MDM2 harbors the nuclear localization and nuclear export signals (NLS and NES, respectively) as well as domains mediating interaction with key growth regulators such as p21^{WAF1} (Jin *et al.*, 2003), Rb (Xiao *et al.*, 1995), p19/p14^{ARF} (Honda and Yasuda, 1999; Bothner *et al.*, 2001) and ribosomal protein L5 (Marechal *et al.*, 1994; Elenbaas *et al.*, 1996). To investigate whether the central region of MDM2 is involved in TGF- β resistance, a series of internal deletion mutants of MDM2 were constructed. MDM2 Δ NLS/p21 targeted the NLS/NES and the region responsible for enhanced turnover of the p21^{WAF1} protein (amino acids 150–230). MDM2 Δ NLS included a deletion of the NLS/NES only (amino acids 177–192). MDM2 Δ AD lacked the acidic domain (amino acids 233–285) that was essential for the ubiquitination and degradation of p53 (Argentini *et al.*, 2001; Kawai *et al.*, 2003) and for the binding of MDM2 to ribosomal protein L5 (Marechal *et al.*, 1994; Elenbaas *et al.*, 1996;

Figure 4. The zinc coordination residues in the RING finger of MDM2 are essential for TGF- β resistance. (A) Cells transduced with a vector control (con) or wild-type or mutant *mdm2* (as indicated) were grown for 2 d in the absence or presence of 5 ng/ml TGF- β before being labeled with BrdU for 45 min. Cells were then stained with a FITC-conjugated anti-BrdU antibody and 7-AAD and subjected to FACS analysis. Fold reduction in S phase cells by TGF- β was calculated by dividing the percentage of S phase cells in the untreated population by that in the TGF- β -treated population. (B) Twenty-five micrograms of a lysates from control cells (con) or cells transduced with wild-type or indicated deletion mutants of *mdm2* were subjected to SDS-PAGE followed by Western blotting analysis. The indicated proteins were detected with the appropriate antibodies. The asterisk indicates an unspecific band. (C) The PG14 Firefly luciferase reporter plasmid containing a p53-responsive promoter and a Renilla luciferase reporter driven by a β -actin promoter were cotransfected into Mv1Lu cells transduced with a vector control or the wild type or indicated mutant *mdm2*. The Firefly luciferase activity was determined and presented as the p53 activity after normalization to the Renilla luciferase activity. Values are means $\pm$ SD for triplicates. (D) Mv1Lu cells transduced with *mdm2* or its RING finger mutants and with and without MDMX were lysed, and MDMX was immunoprecipitated. Subsequently, whole cell lysates (Lysate) and MDMX immunoprecipitates (IP) were subjected to SDS-PAGE followed by Western blot analysis of the indicated proteins. The asterisk indicates an unspecific band. (E) Mv1Lu cells ($2-4 \times 10^3$) or HMEC cells ($2-8 \times 10^3$) transduced with vector control (con), wild-type MDM2 (MDM2), or indicated MDM2 mutants were seeded into six-well plates, grown for 8 d (Mv1Lu) or 10 d (HMEC) in the absence or presence of 5 ng/ml TGF- β , and stained with crystal violet.

Marechal *et al.*, 1997). MDM2 Δ p21/Rb contained deletion of a region downstream of the acidic domain inheriting the binding sites for p21^{WAF1}, Rb, and p19/p14^{ARF} (amino acids 271–385). These deletion mutants were transduced into Mv1Lu cells, and those cells were tested for TGF- β responsiveness.

The deletion of either the NLS/NES and the p21 degrading activity (MDM2 Δ NLS/p21) or the NLS/NES alone (MDM2 Δ NLS) led to abrogation of the ability of MDM2 to mediate TGF- β resistance in both BrdU incorporation assays (Figure 5A) and colony formation assays in both Mv1Lu and HMEC cells (Figure 5F). To confirm the aberrant localization of MDM2 Δ NLS, immunofluorescence analysis was performed. Deletion of the NLS/NES indeed led to abrogation of nuclear localization of MDM2 in Mv1Lu cells. While the wild-type MDM2 and the MDM2 Δ AD mutant were exclusively localized in the nucleus, MDM2 Δ NLS was mainly cytoplasmic (Figure 5C). These results suggest that MDM2-mediated TGF- β resistance requires the NLS/NES of MDM2 and its nuclear localization. Thus, the ability of MDM2 to confer TGF- β resistance may rely on a nuclear function.

In an attempt to clarify the role of MDMX in MDM2-mediated TGF- β resistance, we expressed MDMX alone or

together with MDM2 or its deletion mutants of the NLS. MDMX alone did not confer TGF- β resistance and did not further enhance TGF- β resistance conferred by MDM2. Interestingly, when MDMX was coexpressed with deletion mutants of the NLS of MDM2, TGF- β resistance was observed (Figure 5D, top). To further assess the function of MDMX, the PG14 reporter assay was used in Mv1Lu cell lines expressing MDM2 or its NLS deletion mutants with or without MDMX. Compared with wild-type MDM2, the ability of MDM2 Δ NLS and MDM2 Δ p21/NLS to inhibit p53 activity was greatly reduced (Figure 5D, bottom), suggesting that nuclear localization of MDM2 is crucial for p53 inhibition. MDMX alone also only modestly inhibited p53 activity. However, MDMX in combination with MDM2 Δ NLS or MDM2 Δ p21/NLS led to p53 inhibition at a level comparable to wild-type MDM2 (Figure 5D, bottom). Taken together with our observation that coexpression of MDMX and MDM2 Δ NLS or MDM2 Δ p21/NLS conferred TGF- β resistance, these findings suggest that a threshold of p53 inhibition must be reached in order to establish TGF- β resistance in cells. Therefore, the inhibition of p53 may be essential for MDM2-mediated TGF- β resistance, although p53 inhibition by MDM2 alone is not sufficient to evade the growth arrest

Figure 5. The nuclear localization of MDM2 is required to confer TGF- β resistance. (A) Cells transduced with a vector control (con) or HA-tagged, wild-type or mutant *mdm2* (as indicated) were grown for 2 d in the absence or presence of 5 ng/ml TGF- β before being labeled with BrdU for 45 min. Cells were then stained with a FITC-conjugated anti-BrdU antibody and 7-AAD and subjected to FACS analysis. Fold reduction in S phase cells by TGF- β was calculated by dividing the percentage of S phase cells in the untreated population by that in the TGF- β -treated population. (B) Twenty-five micrograms of a lysates from control cells (con) or cells transduced with HA-tagged, wild-type or the indicated deletion mutants of *mdm2* were subjected to SDS-PAGE followed by Western blot analysis. Antibodies against HA, p53, and actin were used to detect MDM2, p53, and actin, respectively. (C) Cells expressing HA-tagged, wild-type MDM2, MDM2 Δ NLS, or MDM2 Δ AD were cultivated in an eight-well cover slide, fixed, and stained for the subcellular localization of MDM2 with an anti-HA antibody and a FITC-conjugated secondary antibody. The cells were mounted in DAPI-containing medium and analyzed by fluorescence microscopy. (D) Top, cells transduced with a vector control (con) or HA-tagged, wild-type or mutant *mdm2* (as indicated) with and without MDMX were grown for 2 d in the absence or presence of 5 ng/ml TGF- β before being labeled with BrdU for 45 min. Cells were then stained with a FITC-conjugated anti-BrdU antibody and 7-AAD and subjected to FACS analysis. Fold reduction in S phase cells by TGF- β was calculated by dividing the percentage of S phase cells in the untreated population by that in the TGF- β -treated population. Bottom, the PG14 Firefly luciferase reporter plasmid containing a p53 responsive promoter and a Renilla luciferase reporter driven by a β -actin promoter were cotransfected into Mv1Lu cells transduced with a vector control or HA-tagged wild-type or indicated mutants of *mdm2* with and without MDMX. The Firefly luciferase activity was determined and presented as the p53 activity after normalization to the Renilla luciferase activity. Values are means $\pm$ SD for triplicates. (E) The PG14 Firefly luciferase reporter plasmid containing a p53-responsive promoter and a Renilla luciferase reporter driven by a β -actin promoter were cotransfected into Mv1Lu cells transduced with a vector control or HA-tagged wild-type or indicated mutants of *mdm2*. The Firefly luciferase activity was determined and presented as the p53 activity after normalization to the Renilla luciferase activity. Values are means $\pm$ SD for triplicates. (F) Mv1Lu cells ($2-4 \times 10^3$) or HMEC cells ($2-8 \times 10^3$) transduced with vector control (con), wild-type MDM2 (MDM2), or indicated MDM2 mutants were seeded into six-well plates, grown for 8 d (Mv1Lu) or 10 d (HMEC) in the absence or presence of 5 ng/ml TGF- β , and stained with crystal violet.

by TGF- β (Figure 4, A, C, and E). MDM2, thus, may rely on multiple activities to confer TGF- β resistance.

It has been reported that the acidic domain of MDM2 is important for the ubiquitination and degradation of p53 (Argentini *et al.*, 2001; Kawai *et al.*, 2003). However, the acidic domain did not seem to be important for MDM2 to confer TGF- β resistance. The MDM2 Δ AD mutant behaved in a manner similar to wild-type MDM2 in preventing TGF- β -induced G1 arrest in Mv1Lu cells (Figure 5A) and in promoting colony formation in the presence of TGF- β in both Mv1Lu and HMEC cells (Figure 5F). This result sug-

gests that the ubiquitination and degradation of p53 by MDM2 is not essential for TGF- β resistance. Furthermore, similar to the MDM2 mutants lacking E3 activity (the zinc coordination residue mutants), the MDM2 Δ AD mutant also increased p53 protein level (Figure 5B) but inhibited p53 activity (Figure 5E) in Mv1Lu cells. Because both the E3-defective mutants and the Δ AD mutant fail to degrade p53 but remain bound to p53, it is likely that, at least in Mv1Lu cells, binding of p53 to a MDM2 mutant that is unable to degrade p53 results in stabilization of p53 in its inactive form. Because the acidic domain also harbors the binding

Figure 6. Schematic representation of the MDM2 protein showing its interaction partners and domains essential for MDM2 to confer TGF- β resistance (hatched bars above the MDM2 molecule). The deletions mutants of MDM2 used in this study are indicated with gray bars under the MDM2 molecule.

site for ribosomal protein L5 (Elenbaas *et al.*, 1996), our result also indicates that binding to L5 is not required for TGF- β resistance.

The deletion of a region between residues 271 and 385, which contained binding sites to p21^{WAF1}, Rb and p19/p14^{ARF}, did not significantly interfere with TGF- β resistance conferred by MDM2 either in the cell cycle analysis (MDM2 Δ p21/Rb, Figure 5A) or in the colony formation assay (Figure 5F). Therefore, binding of MDM2 to p21^{WAF1}, Rb, and p19/p14^{ARF} is not required for TGF- β resistance.

The expression of these internal deletion mutants was confirmed by Western blot analysis (Figure 5B). Their expression levels were either comparable to or higher than that of wild-type MDM2, with the exception of MDM2 Δ p21/Rb. However, MDM2 Δ p21/Rb still conferred TGF- β resistance in spite of its lower expression level. All of the deletion mutants that failed to confer TGF- β resistance (Δ NLS/p21 and Δ NLS) showed a robust expression, indicating that their inability to cause TGF- β resistance was not due to insufficient expression levels.

DISCUSSION

Increased expression of MDM2 is a potential mechanism for TGF- β resistance in tumors (Sun *et al.*, 1998). The current study reveals a direct impact of MDM2 on cellular responses to TGF- β and demonstrates that the ability of MDM2 to cause TGF- β resistance relied on at least three elements of the MDM2 protein: the C-terminal half of the p53-binding domain, the nuclear localization, and the zinc coordination residues in the RING finger domain (Figure 6). These results were obtained in both mink lung epithelial cells and primary human mammary epithelial cells and thus may represent general molecular mechanisms underlying the ability of MDM2 to confer TGF- β resistance.

We found that an N-terminal deletion of the p53-binding domain of MDM2 (MDM2 Δ N1), which does not bind p53 in vitro (Sigalas *et al.*, 1996), was able to confer TGF- β resistance. This mutant showed intact, although reduced, binding to p53 in vivo and inhibited p53 transcriptional activity to a level comparable to wild-type MDM2. In contrast, MDM2 Δ N2, which had completely lost p53-binding and inhibition in vivo, fails to confer TGF- β resistance. This finding raises the possibility that inhibition of p53 transcriptional activity may be essential for TGF- β resistance. It is unclear how MDM2 Δ N1 binds to p53 in vivo. In accordance with the notion that only a MDM2 protein that binds and inhibits p53 is capable of mediating TGF- β resistance, we present data suggesting that a threshold of p53 inactivation

has to be surpassed in order for TGF- β resistance to occur. All cell lines showing a strong inhibition of p53 activity were resistant to TGF- β -induced growth arrest (MDM2, MDM2 Δ NLS+MDMX, MDM2 Δ p21/NLS+MDMX). On the contrary, cell lines with a reduced level of p53 inhibition did not show TGF- β resistance (MDMX, MDM2 Δ NLS, MDM2 Δ p21/NLS). This implicates that p53 transcriptional activity has to be inhibited to a sufficiently low level in order for MDM2 to mediate TGF- β resistance. On the other hand, inhibition of p53 transcriptional activity alone does not lead to TGF- β resistance. Although the RING finger point mutations MDM2C436L and MDM2C459S inhibited p53 activity to the same extent as MDM2, they did not confer TGF- β resistance. Therefore, inhibition of p53 transcriptional activity by MDM2 on its own is not sufficient, although it might be essential, for MDM2 to confer TGF- β resistance. Thus, MDM2 may require multiple activities to mediate TGF- β resistance.

The inability of the zinc coordination residue mutants of MDM2 to confer TGF- β resistance suggests an essential role of the E3 ubiquitin ligase activity of MDM2. Supporting this notion, MDMX, which lacks an E3 activity, did not restore the ability of the zinc coordination residue mutants of MDM2 to mediate TGF- β resistance (data not shown), even though it can still bind to those mutants. However, the ability of MDM2 to ubiquitinate and degrade p53 did not seem to be essential for TGF- β resistance, because an MDM2 mutant lacking the acidic domain (MDM2 Δ AD) failed to degrade p53 (Argentini *et al.*, 2001; Kawai *et al.*, 2003), but still led to TGF- β resistance. These observations have clearly demonstrated that TGF- β resistance can be uncoupled with the ability of MDM2 to ubiquitinate and degrade p53. It is attempting to speculate that the E3 activity of MDM2 may target an unidentified protein that mediates TGF- β -induced growth arrest.

Consistent with previous reports that MDMX stimulates MDM2-mediated p53 degradation (Gu *et al.*, 2002; Linares *et al.*, 2003), coexpression of MDM2 and MDMX reduces p53 protein levels (Figure 1E) and causes a stronger inhibition of p53 transcriptional activity compared with MDM2 or MDMX alone (Figure 5D). However, the additive effect of MDM2 and MDMX on p53 does not lead to a more pronounced TGF- β resistance (Figure 5D), suggesting that MDM2 alone already has the highest possible effect on TGF- β resistance in this system. In contrast to previous reports demonstrating that MDM2 alone down-regulates p53 protein levels when transfected into cells, we consistently found that the steady state protein level of p53 was reduced only in cells cotransduced with MDM2- and MDMX-expressing retroviruses, but not in cells transduced with MDM2 or MDMX alone. This observation was made not only in mink Mv1Lu cells (Figure 1E), but also in human HEK293T cells (Figure 3D) and HMEC cells (Figure 3E), and thus is not mink- or cell type-specific. A difference between our and previous studies is the expression system for MDM2. In our study, expression levels of MDM2 from single-copy retroviruses are much lower in cells, in comparison with the conventional transfection methods used by previous reports. It has been reported that at low concentrations of MDM2, p53 is not degraded unless MDMX is present (Linares *et al.*, 2003). Therefore, in these cells, endogenous MDMX may be rate-limiting, in that it is not expressed at high enough levels to enhance the degradation of p53 by ectopically expressed MDM2 from a single-copy retrovirus.

It has been reported that in both murine and human cells, MDMX stabilizes p53 protein, possibly by competing with MDM2 for p53 binding and inhibiting the degradation of

p53 by MDM2 (Jackson and Berberich, 2000; Stad *et al.*, 2000; Gu *et al.*, 2002). On the other hand, efficient degradation of p53 requires both MDM2 and MDMX (Gu *et al.*, 2002; Linares *et al.*, 2003). Our results agree with both of these 2 notions, in that MDMX stabilizes p53, whereas degradation of p53 requires both MDM2 and MDMX. We propose a possible scenario that may reconcile these observations. In this model, the MDM2-MDMX complex is more active than MDM2 alone in ubiquitinating and degrading p53. The endogenous MDMX level is rate-limiting and are all bound to endogenous MDM2, thus maintaining p53 protein at a basal steady state level. Ectopic expression of MDM2 alone does not further decrease the p53 level since there is no extra MDMX available in cells, unless MDM2 is expressed at very high levels (such as those achieved by transfection) that allow the bypass of the requirement for MDMX. On the other hand, ectopically expressed MDMX leads to p53 stabilization by competing with endogenous MDM2 for p53 binding and preventing endogenous MDM2/MDMX-mediated p53 degradation. When both MDM2 and MDMX are ectopically expressed, increased amount of MDM2-MDMX complexes leads to further degradation of p53. This model also explains why mutations of the zinc coordinating residues leads to increased p53 and MDMX protein levels (Figure 4, B and D). These mutations disrupt the E3 activity of MDM2 without affecting its ability to bind p53, thus effectively converting MDM2 into MDMX-like proteins, which stabilize p53 by competing with endogenous MDM2 for p53 binding. MDM2 also mediates the ubiquitination and degradation of MDMX (de Graaf *et al.*, 2003; Pan and Chen, 2003). Because these MDM2 mutants are defective in the E3 activity but retain the ability to bind MDMX (Figure 4D), they may also stabilize MDMX by competing with endogenous wild-type MDM2 for MDMX binding and thus inhibiting MDMX degradation by endogenous MDM2.

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