The Mdm2 gene is amplified in approximately one-third of human sarcomas and overexpressed in a variety of other human cancers. Mdm2 functions as an oncprotein, in part, by acting as a negative regulator of the p53 tumor suppressor protein. Multiple spliced forms of Mdm2 transcripts have been observed in human tumors; however, the contribution of these variant transcripts to tumorigenesis is unknown. In this report, we isolate alternative splice forms of Mdm2 transcripts from sarcomas that spontaneously arise in Mdm2-overexpressing mice, including Mdm2-b, the splice form most commonly observed in human cancers. Transduction of Mdm2-b into a variety of cell types reveals that Mdm2-b promotes p53-independent cell growth, inhibits apoptosis, and up-regulates the RelA subunit of NFkB. Furthermore, expression of Mdm2-b induces tumor formation in transgenic mice. These results identify a p53-independent role for Mdm2 and determine that an alternate spliced form of Mdm2 can contribute to formation of cancer via a p53-independent mechanism. These findings also provide a rationale for the poorer prognosis of those patients presenting with tumors harboring multiple Mdm2 transcripts.

Mdm2 was initially identified in a screen for genes amplified on double minute chromosomes found in spontaneously transformed BALB/c 3T3 cells (1). When overexpressed, the Mdm2 oncprotein has been demonstrated to immortalize rodent primary fibroblasts, to increase the rate of cellular proliferation, and to induce cellular transformation (2). A large body of evidence has established the Mdm2 oncprotein as a potent negative regulator of the p53 tumor suppressor protein. The N terminus of Mdm2 binds the transactivation domain of p53, and Mdm2-p53 complex formation can inhibit p53 modification and p53-mediated transcriptional regulation of heterologous gene expression (3–5). In addition, Mdm2 contains a C-terminal zinc RING domain and functions as an E3-ligase to induce p53 ubiquitination and degradation (6, 7). The ability of p53 to induce the expression of the Mdm2 gene suggests that Mdm2 and p53 form a negative feedback loop to regulate p53 activity in the cell (8, 9). Mdm2-mediated destabilization of p53 is regulated by binding of Mdm2 to the p19 (ARF) tumor suppressor protein and by ATM-induced phosphorylation of Mdm2 and p53 (10,11). The importance of Mdm2 in regulating p53 function has been demonstrated in mice, where the early embryonic-lethal phenotype of Mdm2-null mice is rescued by deletion of p53 (12, 13). The absence of both Mdm2 and p53 under normal development, are viable, and are fertile, suggesting that any functions possessed by Mdm2 aside from its ability to regulate p53 are dispensable for normal cell growth and development.

The human MDM2 gene is amplified to high copy numbers in approximately one-third of all human sarcomas (14), and is overexpressed in a wide range of human cancers (15, 16). As many of these tumors retain a wild-type p53 gene, it is presumed that overexpression of Mdm2 serves to inactivate p53 function in these tumors. However, tumors have been identified that have both Mdm2 amplification and p53 loss; a seemingly redundant set of mutations (17). Interestingly, these rare sarcomas are much more aggressive than those tumors with alterations in only Mdm2 or p53, suggesting that there may exist a p53-independent role for Mdm2 when overexpressed in these tumors.

Several additional lines of evidence suggest that Mdm2 may regulate growth not only by inhibiting p53 function, but through p53-independent mechanisms as well. Human MDM2 has been reported to form a complex with the major (p110) Rb1 tumor suppressor protein and with E2F1 and DP1 transcription factors (18, 19), and can alter transcription of E2F1-induced reporter genes in cell transfection assays. Other cell cycle regulatory proteins that bind with Mdm2 include Numb, MTBP, MAD transcription factors, TIP60, and β-arrestin; a β2-adrenergic receptor regulator (20–24). Genetic evidence for a p53-independent role for Mdm2 in cell growth has been provided through analysis of transgenic mice. Overexpression of MDM2 cDNA in the mammary epithelium of transgenic mice was found to inhibit development of the mammary gland by inducing multiple rounds of S phase without completion of mitosis (25). The uncoupling of S phase from mitosis was found to inhibit development of the mammary gland by inducing multiple rounds of S phase without completion of mitosis (25). The uncoupling of S phase from mitosis was seen in transgenic mice, which were either wild type or deficient for p53, indicating a p53-independent role for Mdm2 in the regulation of DNA synthesis. We have also provided genetic evidence for a p53-independent role for Mdm2 in sarcoma formation. Transgenic mice were generated using mouse genomic DNA encoding the entire Mdm2 gene under control of its native promoter region. These mice displayed a 4-fold increase in the level of Mdm2 expression and were found to have increased...
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Predominantly into spontaneous sarcoma formation regardless of the p53 status of the mice (26). A variety of human tumors that overexpress Mdm2 has revealed the presence of multiple, alternatively spliced forms of Mdm2 message (27). In some cases, the presence of these spliced Mdm2 forms has been correlated with a more aggressive disease state (28, 29). Interestingly, some of these transcripts encode Mdm2 proteins that lack the p53-binding domain and are incapable of complexing with p53, yet can induce foci formation in 3T3 cells in culture, suggesting that these tumor-isolated Mdm2 isoforms may contribute to transformation in a p53-independent manner (30). More recently, several groups have characterized several spliced isoforms of Mdm2 transcripts isolated from mouse or human tumors and have reported that many of these isoforms appear to inhibit cell proliferation, though the precise mechanism of growth inhibition remains unclear (31, 32).

In order to assess the potential role of Mdm2 isoforms in tumorigenesis, we have analyzed sarcomas isolated from our Mdm2-transgenic mice. We have detected numerous spliced isoforms of Mdm2 transcripts in the tumors, including the murine equivalent of the B isoform; the most prevalent isoform observed in human cancers. Mdh2-B has been previously detected in high grade bladder and uterine cancers, lacks the p53-binding region present in full-length Mdm2, and was found to be incapable of complexing with the p53 protein (30). In this report, we examine the functional significance of the Mdm2-b form in cells and in mice. Our results indicate that this Mdm2 isoform encodes a variant Mdm2 protein that lacks the p53-binding domain and contains only the C-terminal RING domain. This Mdm2 protein is found to induce cell proliferation and to interfere with apoptosis in a p53-independent manner in cultured cells, and induce spontaneous tumorigenesis in transgenic mice. Interestingly, expression of either Mdm2 or Mdm2-b increases the level of the RelA (p65) protein in cells, and Mdm2-b can increase NF-kB-dependent transcription in transduced cells and potentiate the response of these cells to TNF-mediated apoptosis. These results identify a p53-independent role for Mdm2 in modulating cell proliferation and apoptosis, demonstrate that a splice isoform of Mdm2 can induce tumor formation in vivo, and further suggest that the presence of this splice isoform of Mdm2 contributes to the neoplasia induced by Mdm2 overexpression in human cancers.

EXPERIMENTAL PROCEDURES

Isolation of Alternatively Spliced Mdm2 Transcripts—Total RNA was isolated from duodenal homogenized, snap frozen transgenic tissue using TRIzol (Invitrogen). RT-PCR was performed using Superscript First-Strand Synthesis System (Invitrogen). RNA was reverse-transcribed using an oligo(dT) primer. The resulting cDNA was used in nested PCR utilizing primer pair Ex2forward (5′-AGAGACTCTGGGAATGCTTGCTGC-3′) and Ex2reverse (5′-GTTGACAAGACAGTATCTTCG-3′) for the first amplification of 25 cycles followed by a second amplification with primer pair Ex2nest (5′-GGCGGCTCTGGAGTACGGCCGC-3′) and Ex212reverse (5′-GTTGAGGTCAGTTCTAGTGCA-3′) for a total of 35 cycles of 94°C for 2 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. PCR products were resolved on 1% agarose gels, excised, purified (GENECLEAN), and cloned into pGEM-T Easy Vector (Promega) for sequencing. PCR identification of specific Mdm2 isoform was done using primer pair MBforward (5′-AAGAGACTCTGGGAATGCTTGCTGC-3′) and TGreverse (5′-ATGGGAGATGGATGGAACAT-3′). DNA sequencing of cDNAs was performed by the University of Massachusetts Nucleic Acid Facility to identify Mdm2-specific isoforms.

Cloning and Expression of the Mdm2-b Isoform—Mdm2-b and Mdm2-B cDNAs were cloned into the EcoRI sites of pBabe-Puro and pcDNA3.1HisC expression plasmids (Invitrogen). Mdm2-b protein product was confirmed using an in vitro transcription and translation system in rabbit reticulocyte lysates (Promega).

Cell Culture, Cell Lines, and Antibodies—NIH3T3 cells were purchased from the ATCC. Mouse embryonic fibroblasts (MEFs) null for pRB or p53 were generated using standard protocols. p19(ARF)-null MEFs were kindly provided by Dr. J. Kowalik laboratory at the University of Massachusetts Medical School. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, (100 units/ml) of penicillin and (100 μg/ml) of streptomycin. Stable cell lines were passaged in media containing (3 μg/ml) puromycin (Sigma). Stable transfection of NIH3T3 cells were performed in 100-mm plates using 10 μg of linearized expression plasmid DNA along with FuGENE 6 reagent (Roche Applied Science), according to the manufacturer’s protocol. Following puromycin drug selection, surviving colonies were pooled for the generation of stable cell lines. For immunolocalization studies, 2 × 105 cells were seeded onto coverslips in the bottom of 6-well plates. Transient transfections were performed using 1 μg of Express-Mdm2-b, Express-AMLM3, or Express-empty vector using LipofectAMINE (Invitrogen). Foci formation was scored in NIH3T3 stable cell lines following methanol fixation and 0.1% crystal violet after 2 weeks of culture.

Boscs293 cells at 80% confluence were transfected with 10 μg of pBabe-Mdm2-b, pBabe-Hdm2-b, or pBabe-control using LipofectAMINE to generate recombinant retroviruses. Forty-eight hours following transfection, retroviral particles were collected and used for the viral transduction of primary MEFs or NIH3T3 cells seeded at 1 × 105 cells per 100-mm plate.

Polyclonal antibodies against p65 (C-20) and against the C terminus of Mdm2 (C-18) were purchased from Santa Cruz Biotechnology, Inc. Primary polyclonal anti-p53 (Oncogene Research Products) was used to detect p53, followed by secondary biotin-conjugated rabbit anti-sheep IgG (Oncogene Research Products) and tertiary horseradish peroxidase-conjugated Streptavidin (Zymed Laboratories Inc. Anti-Xpress-FITC antibody (Invitrogen) was used for immunolocalization studies. An anti-BrdUrd antibody (BD Biosciences) was used to label cells for FACS analysis. Anti-tubulin monoclonal antibody (Sigma) was used for protein loading control.

Analysis of Cell Proliferation—Growth curves were performed with triplicate plating of either NIH3T3 stable cell lines, p53−/−, p19−/−, or pRB−/− early passage MEFs. Cells were seeded at a density of 2 × 105 cells per 60-mm plate and counted every 24 h using a Beckman Coulter Counter. For the determination of apoptosis, cells were plated at 1 × 105 cells per 100-mm plate and pulsed 24 h later with 10 μM BrdUrd for 1 h. FACS analysis was performed on cells stained for BrdUrd and propidium iodide.

Analysis of Cell Death—NIH3T3 cell lines were 50–60% confluent when treated with (500 ng/ml) doxorubicin (Sigma). Triplicate samples of cells were harvested 24–36 h later and analyzed for propidium iodide uptake by FACS analysis.

NF-κB Activity Assays—293T cells were seeded into 6-well plates at a density of 5 × 105 cells in 2 ml of medium and transfected with 50 ng of each of an internal β-galactosidase transfection efficiency control plasmid and either a β-responsive luciferase reporter plasmid containing two canonical kB sites or a control plasmid lacking kB sites together with Mdm2-b or control pcDNA3.1 expression plasmids. Cells were treated with recombinant TNF-α (Roche Applied Science) and 24–36 h following transfection, luciferase assays (Promega) were performed using a luminometer as previously described (33).

Immunolocalization Assays—Forty-eight hours following transient transfection, cells on coverslips were fixed with (5.7%) formaldehyde in PBS, permeabilized with (0.25%) Triton X-100 in PBS, and blocked in 0.5% bovine serum albumin in PBS prior to a 1-h incubation with an anti-Xpress-FITC-conjugated antibody for the recognition of Mdm2-b or AML3. Cell nuclei were stained with DAPI (0.5 μg DAPI in 0.1% Triton X-100-FBSA). Cells were visualized using a Zeiss Confocal Microscope.

Expression of Transgenic Mice—Mdm2-b cDNA was cloned into the EcoRI sites of transgene cassettes pCAGGs and glial fibrillary acidic protein (GFAP). Transgenic mice were generated via pronuclear injection using standard procedures. Identification of GFAP-Mdm2-b founder mice and transmission of the transgene was determined by PCR and Southern analyses. The PCR primers used for genotyping span the p53 binding site of the transgene cDNA to MP-1 (5′-CCAGCTACAGGACTGATTCG-3′) and 12 reverse (5′-GGACGTCAGTTCTAGGC-3′). DNA sequencing of cDNAs was performed by the University of Massachusetts Nucleic Acid Facility to identify Mdm2-specific isoforms.

RESULTS

Isolation and Characterization of Mdm2-b—To determine whether Mdm2 splice variant transcripts are present in our Mdm2 transgenic mouse tumors, RNA was extracted from 14
frozen tumor samples, and RT-PCR was performed using
nested PCR amplification. PCR products were analyzed by
gel electrophoresis (Fig. 1A), and Southern hybridization using
various Mdm2 oligonucleotide probes spanning the Mdm2 cod-
ing sequences. The majority of spliced variants hybridized to
3’-probes corresponding to exon 12 of the Mdm2 gene (data not
shown). In order to isolate individual spliced variants, nested
Mdm2 PCR products were purified and 72 transcripts were
subcloned into plasmid vectors. Subsequent DNA sequencing of
the cDNA clones revealed a wide range of Mdm2 spliced vari-
ants and included both aberrant transcripts resulting from
cryptic splice sites within introns and exons as well as tran-
scripts generated from the donor and acceptor splice sites lo-
cated at the Mdm2 intron-exon boundaries (28). The most
prevalent transcript observed is identical to Hdm2-B, the most
frequently detected Mdm2 spliced variant found in human
tumors (29, 30). This mouse Mdm2-b isoform was detected in
all 14 analyzed tumor samples and was not detected in wild-
type tissue in these experiments (Fig. 1B).

The Mdm2-b transcript encodes for sequences present in
Mdm2 exons 2–3 and exon 12, with RNA splicing between
exons 3 and 12 occurring at the precise exon splice donor-
acceptor motifs. The predicted protein alignment between
Mdm2-b and Hdm2-B is illustrated (Fig. 1C). Amino acid iden-
tity between the two proteins is 82%. The encoded Mdm2-b
protein lacks the p53-binding, p300-binding, pRb-binding, and
p19(ARF) binding domains present on full-length Mdm2, as
well as the Mdm2 nuclear localization and nuclear export sig-
nals. Mdm2-b does contain the complete C-terminal zinc
finger, Ring finger domain, and Mdm2 residues that have been iden-
tified as targets for phosphorylation by ATM (11, 35) and c-Abl
(36).

To confirm that the Mdm2-b spliced transcript encodes for a
protein product, Mdm2-b cDNA was cloned into pcDNA3.1 in
frame with an N-terminal Xpress epitope tag (Invitrogen) and
expressed the protein in an in vitro transcription/translation
expression system (Promega). The Mdm2-b transcript encodes
a protein product of ~47 kDa in size, when the size of the

Fig. 1. Isolation of Mdm2 spliced variants from mouse tumor samples. A, nested PCR products generated from
Mdm2 transgenic mouse tumor numbers 20, 103, 186, 238, 98, and 110. Wild-type tissue (WT) sample and marker (M) are
on right. B, Mdm2-b isoform-specific primers used to PCR tumor cDNAs revealed that Mdm2-b is present in all tu-
mor samples analyzed; Gapdh PCR was used as a control to confirm the presence of intact cDNA. C, Mdm2-b and Hdm2-B
proteins share 82% amino acid identity. D, in vitro transcription and translation of Xpress-Mdm2-b produces a 47 kDa pro-
tein (minus the 3.5-kDa Xpress epitope). E, immunolocalization using DAPI for nuclear staining and FITC-Anti-Xpress
Antibody for detection of Xpress-Mdm2-b reveals Mdm2-b is localized predominantly in the cytoplasm. Xpress-AML3
was used as a positive control for nuclear localization.
3.5-kDa Xpress tag is subtracted (Fig. 1D). To determine the cellular location of the Mdm2-b protein, the pcDNA-XpressMdm2-b vector was transiently transfected into NIH3T3 cells and immunofluorescence microscopy was performed using an α-Xpress-FITC-conjugated antibody against XPRESS-Mdm2-b. Mdm2-b was determined to localize predominantly in the cytoplasm of the transfected NIH3T3 cells (Fig. 1E), in keeping with the absence of a nuclear localization signal on Mdm2-b. An XPRESS-tagged-AML3 expression plasmid that encodes a protein that localizes to the nucleus was used in parallel as a control in this experiment.

Expression of Mdm2-b Increases Cell Proliferation and Transformation—Numerous spliced forms of Mdm2, including the b isoform, have been identified previously in human tumors (30, 32). However, there have been contradictory reports as to the effect of the splice forms upon cell growth (30–32). Therefore, we sought to examine if the presence of the Mdm2-b isoform might contribute to the malignant phenotype of our Mdm2 transgenic mice. Hdm2-B and Mdm2-b cDNAs were cloned separately into the pBabe retroviral expression vector and stably transfused into NIH3T3 cells to examine if Hdm2-B or Mdm2-b is capable of altering cellular growth characteristics. Selection for puromycin-resistant clones indicated a transduction frequency of ~90%. Following drug selection, the stable transfectedants were pooled and the expression of spliced variants was confirmed with RT-PCR and Northern blot analysis (data not shown).

Transduction of Mdm2-b into NIH3T3 cells was found to promote rapid cell proliferation. BrdU staining of asynchronous growing cells transfused with Mdm2-b or with control (pBabe-empty vector) revealed an increase in the numbers of Mdm2-b-transduced cells present in S phase of the cell cycle relative to control transfused cells (Fig. 2A). To confirm the positive effects of the B splice form on cell growth, cell proliferation assays were performed using triplicate plates of NIH3T3 cells transfused with Hdm2-B, Mdm2-b, or empty vector (pBabe) (Fig. 2B). Three repeat experiments confirmed that the presence of either Hdm2-B or Mdm2-b increased the proliferation rate and saturation density of NIH3T3 cells.

To determine whether Mdm2-b could contribute to cellular transformation, Mdm2-b NIH3T3 cells and control pBabe NIH3T3 cells were seeded onto 60-mm dishes and maintained in culture for 2 weeks. Following crystal violet staining, foci formation was scored from six representative plates of each cell line (Fig. 2C). Mdm2-b expression induced larger and more numerous foci in the monolayer (44.6 ± 4.5 foci per plate) than did transduction with pBabe alone (18.2 ± 5.7 foci per plate). Thus, expression of Mdm2-b in NIH3T3 cells accelerates the rate of cell proliferation and interferes with growth suppression induced by contact inhibition.

Mdm2-b Increases Cell Proliferation Independent of p53, p19 (ARF), and Rb—Unlike full-length Mdm2, Mdm2-b lacks the p53 binding domain of Mdm2, and Hdm2-B has been previously demonstrated to be incapable of complexing with p53 (30). However, it remains possible that Mdm2-b still alters p53 functions, possibly by complexing with full-length Mdm2 (32). To determine if Mdm2-b functions through p53 to increase cell proliferation, recombinant retroviruses were used to transduce early passage, primary mouse embryonic fibroblasts (MEFs) derived from p53-null mice. Transient selection of the MEFs in puromycin indicated a 95% transduction frequency. The pooled MEFs were triplicate plated in 60-mm dishes, and growth rates were monitored for each cell type over a period of 5 days in culture. Results of the growth curves clearly demonstrate the ability of both Hdm2-B and Mdm2-b to increase the rate of cellular proliferation when p53 is absent (Fig. 2D).

In addition to the p53-binding region, Mdm2-b lacks both p19ARF and pRb binding domains. In order to determine the proliferative effect this spliced variant has on cells lacking either p19ARF or pRb, we infected early passage p19ARF-null MEFs or Rb-null MEFs with Hdm2-B or pRb-control retrovirus, pooled those cells surviving drug selection, and used resulting cells for proliferation curves. Similar to results obtained with p53-null cells, Hdm2-B accelerates growth in the absence of either p19ARF or pRb (Fig. 2E). These data indicate that the b form does not depend upon the presence of p53, p19, or Rb to increase the rate of cell growth.

Expression of Mdm2-b Interferes with Apoptosis—To determine if Mdm2-b is interfering with p53-mediated apoptosis, we examined the levels of p53 in the transduced 3T3 cells following treatment with the topoisomerase inhibitor, doxorubicin. Doxorubicin is an anthracycline analogue reported to induce apoptosis through p53-dependent and p53-independent mechanisms (37). Although p53 protein levels are elevated in the control-transduced cells, Mdm2-b-transduced cells, and in the Hdm2-B-transduced cells 18 h after doxorubicin treatment, no reduction was observed in p53 protein levels in cells transfused with the b isoforms relative to the control cells, indicating that Mdm2-b or Hdm2-B does not alter p53 levels in these cells (Fig. 3A). Furthermore, the presence of Mdm2-b in 3T3 cells does not inhibit p53-mediated induction of genes such as p21 (Waf1/Cip1) following treatment with 80 Gy ionizing radiation (IR) and Mdm2-b-transduced cells undergo a G1 arrest in response to IR (data not shown). These data suggest that the Mdm2-b splice variant does not alter p53 stability or activity.

Another potent regulator of apoptosis is the NFκB transcription factor, a dimeric complex composed of the transcriptionally inactive p50 subunit and the p65 (RelA) subunit, which contains a potent transactivation domain (38). The activity of NFκB is suppressed by interaction with IκB proteins that sequester NFκB in the cytoplasm (39). NFκB suppresses apoptosis induced by doxorubicin, TNF, and by other apoptotic stimuli by inducing the expression of several anti-apoptotic genes, including Bcl-xL, cIAP1 and 2, TRAF 1 and 2, and A1/Bfl2 (40–42), and NFκB has been implicated in up-regulation of other cell cycle regulatory genes with potential oncogenic functions (43). Furthermore, full-length Mdm2 has been recently reported to bind to SP1 sites present in the promoter region of RelA and to induce transcription of the RelA gene (44). Although the Mdm2-b form lacks the putative acidic activation domain of full-length Mdm2 and is unlikely to induce expression of heterologous genes such as RelA, the anti-apoptotic effects of RelA led us to examine RelA levels in our transduced cells. RelA was strongly elevated in the control-transduced 3T3 cells following doxorubicin treatment of the cells (Fig. 3A).

Interestingly, the presence of Mdm2-b or Hdm2-B correlated with a large increase in the level of RelA in the transfused cells in the absence of any treatment, and a further increase in the amount of RelA in transfused cells treated with doxorubicin (Fig. 3A). Furthermore, both Mdm2-b and full-length Mdm2 up-regulated RelA protein levels in transduced p53-deficient MEFs (data not shown). However, Northern analysis and real-time PCR of p65 message levels in 3T3 cells mock-transduced or transfused with Mdm2-b indicated that up-regulation of p65 by Mdm2-b does not occur at the level of transcription or message stability (data not shown). Interestingly, we observed that the presence of the b form correlates with a decrease in IκBα protein levels (Fig. 3B), suggesting that Mdm2-b might induce NFκB activity by interfering with the negative regulator of p65, which, in turn, leads to increased levels of p65 in the cell.

To confirm that the Mdm2-b form alters NFκB activity, we
performed transient transfection assays to analyze the effects of Mdm2-b on NFκB-mediated gene expression. For these experiments we elected to use 293T cells in order to examine Mdm2-b effects in non-fibroblast cells and because TNF-induction of NFκB activity has been well studied in this system. Furthermore, p53 is functionally inactivated in these cells (45), thus any effects of the b isoform on NFκB activity should be independent of the Mdm2-p53 signaling pathway. Cotransfection of Mdm2-b along with a luciferase gene placed under transcriptional control of a promoter containing canonical NFκB recognition sequences was performed in 293T cells (Fig. 3C). The presence of Mdm2-b resulted in increased expression of the NFκB-induced reporter gene, suggesting that the elevated levels of RelA induced by Mdm2-b leads to activation of the NFκB-responsive promoter. Furthermore, the presence of Mdm2-b increased the response of the NFκB promoter to TNF-α stim-
population (2.4-fold stimulation in pcDNA3.1 control-transduced cells versus 4.8-fold stimulation in Mdm2-b-transduced cells) in this experiment and in three duplicate experiments.

To determine if induction of p65 by the Mdm2-b form alters the apoptotic response of cells, three separate, pooled 3T3 cell lines transduced with either Mdm2-b or control empty vector...
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Fig. 4. Generation of Mdm2-b transgenic mice. A, CMV/β-Actin, Mdm2-b and GFAP-Mdm2-b transgene constructs used in pronuclear injection experiments. B, analysis of transgene expression in multiple tissues of representative GFAP-Mdm2-b transgenic mouse by RT-PCR (top panel) and by Southern analysis with an Mdm2-b-specific oligonucleotide probe (bottom panel) reveals high levels of transgene expression in brain and spleen.

We generated (pBABE) were treated with doxorubicin and the apoptotic response of the cells was compared. Cells were ~50–60% confluent when doxorubicin was added to cell culture media (500 ng/ml final concentration). Thirty-six hours following treatment, propidium iodide staining and FACS analysis of the cells revealed that 37% of control-transduced, 3T3 cells were undergoing apoptosis in comparison to 15% of Mdm2-b-transduced, 3T3 cell lines (Fig. 3D). To confirm that apoptosis was altered by transduction of Mdm2-b and to explore the anti-apoptotic role of the RING finger domain of Mdm2-b, an Mdm2-b point mutant (Mdm2-b C449S) was generated which replaces a cysteine residue at Mdm2-b amino acid position 176 with an arginine residue. This amino acid has been previously mutated in Mdm2 isoforms and has been shown to interfere with ubiquitin ligase activity (46). To avoid putative embryonic lethality, the glial fibrillary acidic protein GFAP promoter was fused to the Mdm2-b to generate transgenic mice that would display tissue-restricted expression of Mdm2-b (Fig. 4A). Seven lines of GFAP-Mdm2-b transgenic mice were identified by tail biopsy, one of which was infertile, and three of which were eliminated from the study because they did not express the transgene in any tissue. Three remaining independent lines of Mdm2-b transgenic mice (B19, B31, and B45) were expanded for analysis and for tumor studies. Densitometry experiments performed on Southern blots of representative mice from each line indicated that lines B19, and B31 integrated 2 and 4 copies of the transgene while line B45 had integrated 25 copies of the transgene (data not shown); however, spatial expression of the transgene was similar in all lines, and the levels of transgene expression was likewise similar across all lines. Northern analysis of RNA isolated from a variety of tissues indicated that all lines of mice displayed highest levels of GFAP-Mdm2-b expression in the brain and, to a lesser extent, the spleen (data not shown). However, lower levels of transgene expression could be readily detected in liver, kidney, ovary, and testes using RT-PCR against total RNA isolated from these tissues (Fig. 4B). The specificity of the resulting PCR products was confirmed by Southern analysis with an internal oligonucleotide. This pattern of transgene expression is typical of the expression pattern seen in other transgenic mouse studies using the GFAP promoter (45, 46).

Approximately 20% of the GFAP-Mdm2-b mice from transgenic line B19 and B31 displayed uncoordinated motor movements, erratic circling behaviors, head-tilting, and weak muscular strength; although these behaviors are not completely penetrant, and not all transgenic offspring from these affected mice displayed this phenotype. Furthermore, a small subset (8%) of GFAP-Mdm2-b mice from these two lines died from hydroencephaly between 4 weeks and 24 weeks of age. Histologic analysis of brain tissue revealed that the hydroencephalic mice display highly dilated ventricles with intraventricular bleeding and macrophage infiltration (data not shown). Other lines of transgenic mice expressing reporter genes from the GFAP promoter do not present similar phenotypes in brain, thus it is likely that robust Mdm2-b expression induced by the GFAP promoter in the brain is the underlying cause of these abnormalities.

We monitored cohorts of all 3 lines of Mdm2-b transgenic mice for spontaneous tumor development (Fig. 5A). Mice typically displayed large abdominal masses between 50w and 104w, with a mean time to tumorigenesis of 80 weeks for the B45 transgenic line, 84 weeks for the B19 transgenic line, and 100 weeks for the B31 transgenic line of mice. Portions of each tumor harvested from moribund mice at time of sacrifice were sent for histological examination as well as snap frozen for RNA and protein isolation. RNA isolated from several tumor samples confirms Gfap-Mdm2-b transgene expression (data not shown). Histologic analysis of tumors in the three lines of mice revealed that 70% of the tumors were myeloid sarcomas, a tumor mass of immature myeloid cells that occurred in an extramedullary site (see Table 1 for representative listing of tumors). These tumors stained negative for B220 surface antigens and positive for chloroacetate esterase. Histopathology and B220 antibody staining confirmed that the remaining 30% of the tumors were B-cell lymphomas (Fig. 5B). To confirm the presence of the Mdm2-b in the tumors, a C-terminal Mdm2 antibody (C-18) was used for detecting Mdm2-b protein. Three representative tumors obtained from Mdm2-b transgenic mice (one from each line) were analyzed for the presence of the Mdm2-b protein. A 47-kd band corresponding to the Mdm2-b form was detected in all three samples and was absent in wild-type, non-transgenic mouse tissue (Fig. 5C).
Mdm2 is overexpressed in a wide variety of human cancers, including osteosarcomas, malignant fibrous histiocytomas, rhabdomyosarcomas, liposarcomas, leiomyosarcomas, glioblastomas, astrocytomas, myeloid leukemias, B-cell lymphomas, and oral squamous cell carcinomas (reviewed in Ref. 29). Mdm2 has been well established as a critical regulator of p53 tumor suppressor function: Mdm2-p53 complex formation has been found to inhibit p53 transcriptional activities (3–5), and to ubiquitinate p53, targeting it for nuclear export and degradation in the proteosome. Inhibition of p53 by Mdm2 requires both the N-terminal, p53-binding domain and, in the case of p53 destabilization, the C-terminal, RING domain of Mdm2 (47). Given that p53 is perhaps the most commonly mutated gene in human cancers, and the absence of p53 mutations in many sarcomas that display Mdm2 amplification (14), it is highly likely that Mdm2-mediated inhibition of p53 is an important mechanistic step in the generation of these tumors.

More recently, there has been an increasing amount of evidence for p53-independent roles for Mdm2 in regulation of cell growth and in tumorigenesis. Numerous reports are present in the literature indicating that Mdm2 can bind to cell cycle regulatory proteins other than p53, including E2F1 and Rb, though the significance of these interactions in normal cell growth is not clear. We have previously generated and studied mice deficient for both Mdm2 and p53 (12). Analysis of the cell cycle characteristics of MEFs derived from these mice suggest that absence of Mdm2 does not induce any additional growth defects beyond what is observed in p53-deficient MEFs, and Mdm2/p53 double-deficient mice phenocopy p53-null mice (48). These finding suggest that Mdm2 functions solely by regulating p53 in normal cell growth and development. However, as absence of p53 dramatically increases cell growth and interferes with apoptosis, more subtle effects of Mdm2 on cell growth or death distinct from its ability to regulate p53 might be difficult to observe in these experiments.

Although p53-independent roles of Mdm2 in regulation of normal cell cycling remains uncertain, genetic evidence provided by analysis of human tumors and from studies involving transgenic mice indicates that Mdm2 can induce tumorigenesis via both p53-dependent and independent mechanisms. Sarcomas have been identified from patients displaying both Mdm2 amplification and p53 loss, a seemingly redundant set of mutations (17). These tumors appear to be more aggressive than tumors with alterations in either Mdm2 or p53, suggesting a p53-independent role for Mdm2. Furthermore, we have previously reported that p53-null mice bearing amplified copy numbers of the Mdm2 gene display a 4-fold increase in spontaneous
formation of sarcomas relative to p53-null mice (26). These findings indicate that there is a p53-independent role for Mdm2 in tumorigenesis when Mdm2 is overexpressed. However, the relative contributions of the p53-dependent and independent mechanistic pathways to tumor formation are unknown.

Analysis of human tumors has revealed the presence of multiple spliced forms of Mdm2 transcripts that were generated using both canonical splice acceptor/donor signals as well as cryptic splice signals present within the Mdm2 gene. These aberrant transcripts have been observed in a variety of human cancers, including ovarian and bladder carcinoma, astrocytoma, glioblastoma, breast carcinomas, lung cancers, and in both bone and soft-tissue sarcomas (27), and the presence of these transcripts has been correlated with a more severe disease state (29). Several groups have isolated alternate Mdm2 transcripts from either mouse or human tumor tissues for further study (30–32). However, analysis of the effect of several different Mdm2 alternate transcripts on cell growth gave apparently conflicting results: one study found that several of the isoforms could induce foci formation in transduced Saos-2 cells suggesting that these isoforms might have oncogenic potential, while two other studies indicated that some isoforms of Mdm2 inhibit cell proliferation in a p53-dependent manner. Thus, the significance of these alternate Mdm2 transcripts and their contribution to cell growth or neoplasia remained unresolved.

In this study, we have isolated and explored the functional significance of the major alternate spliced form observed in a mouse model for Mdm2 amplification. This isoform, Mdm2-b, is the murine equivalent of the human Hdm2-B, the most commonly observed isoform in human cancers that overexpress Mdm2. Mdm2-b is generated by precise splicing between exon 3 and 12 of the Mdm2 gene and maintains the reading frame of the protein (34). This alternate transcript encodes a 47-kDa protein that lacks the nuclear localization signal found on full-length Mdm2 and was determined to localize to the cytoplasmic compartment. In addition, Mdm2-b lacks the N-terminal p53 binding domain and central putative acidic activation domain of Mdm2, and contains only the C-terminal zinc finger and zinc-RING domain of Mdm2. Mdm2-b increases the proliferation of transduced NIH3T3 cells without altering p53 stability in the cells, and transduction of Mdm2-b increases the proliferation of p53-null MEFs, as well as Rb- null MEFs and p19(ARF)-null MEFs. These results indicate that Mdm2-b increases cell proliferation via a p53-independent mechanism that furthermore does not require the presence of pRB or p19(ARF) to function. In addition, Mdm2-b interferes with cell death, and can induce foci formation in cultured cells, indicating that Mdm2-b is oncogenic. This was confirmed by the results of the transgenic study. Expression of Mdm2-b in transgenic mice induced spontaneous tumor formation in myeloid progenitor cells and B-lymphocytes. The tissue specificity of tumor formation is likely due to the choice of promoter used in construction of the transgene. The GFAP promoter induced the highest levels of Mdm2-b expression in the brain, with a lesser amount of expression in the spleen in all three lines of transgenic mice. Although a subset of the Mdm2-b transgenic mice suffered lethality resulting from hydrocephaly or exhibited an aberrant gait, no tumors were detected in brain tissue of Mdm2-b transgenic mice. The relatively long latency of tumor onset in the transgenic mice suggests that additional mutations are required for Mdm2-b induced tumorigenesis to occur.

It is likely that tumor formation in Mdm2-b transgenic mice is a p53-independent event, as Mdm2-b neither binds to p53, alters p53 levels or p53-transactivation, nor requires the presence of functional p53 in order to increase cell proliferation. In addition, Mdm2-b lacks residues involved in complex formation with other p53 family members. The results of our tissue culture experiments indicate that Mdm2-b alters NFκB signaling and inhibits apoptosis. Thus, Mdm2-b may induce tumorigenesis by upregulating NFκB activity in the transgenic mice. Many tumor cell lines, including myeloid and lymphoma cell lines, have been found to contain persistent NFκB activity. Increased NFκB activity was due to mutations leading either to constitutive activation of upstream signaling kinases or to inactivation of inhibitory IκB subunits (46). NFκB has also been observed to be up-regulated in primary tumor samples (50). In our study, expression of Mdm2-b correlates with a reduction in IκBα levels and increases an increase in RelA, the p65 subunit of NFκB, in both transduced cells and in tumor tissues isolated from the transgenic mice. Furthermore, Mdm2-b was capable of increasing NFκB-dependent transcription and potentiating the effects of TNF-α on RelA-mediated transcription in transfected cells.

Mdm2 has been proposed to act as a transcription factor, binding to sp1 sites present in the RelA promoter and inducing RelA expression (40). Given the cytoplasmic location of Mdm2-b and the lack of an acidic activating domain, it is unlikely that Mdm2-b is altering RelA expression directly, and Northern analysis and real-time PCR confirm that Mdm2-b does not induce an increase in p65 transcripts. However, expression of Mdm2-b was found to correlate with lower levels of IκBα, the negative regulatory binding partner of p65. It is presently unclear if negative regulation of IκBα by Mdm2-b leads to an increase in p65 protein levels, or whether the reduction in IκBα levels reflects any negative regulation of IκBα resulting from increased RelA levels. Experiments testing the ability of the Mdm2-b to alter IκBα-mediated inhibition of p65-induced gene expression by altering IκBα stability are ongoing.

It has recently been reported that induction of p53 activates NFκB and correlates with the ability of p53 to induce apoptosis (51). However, using a p53-inducible, Saos-2 cell system, expression of RelA in cells was found to protect cells from TNF-α-induced death without significantly altering p53-induced apoptosis. This suggests that induction of RelA and inhibition of cell death by the Mdm2-b isoform in our experiments is unlikely to affect p53-mediated apoptosis, in agreement with our proposal for a p53-independent mechanism for Mdm2-b-induced tumorigenesis. In addition, NFκB has been implicated in up-regulation of other cell cycle regulatory genes with potential oncogenic functions (43). However, the role of Mdm2-b-induced RelA in cell proliferation in the presence or absence of p53 remains to be tested.

A p53-independent role for Mdm2-b in cell growth and death has significant clinical importance. Not only do these results provide a rational for the poorer prognosis of those patients presenting with tumors harboring multiple Mdm2 transcripts, but the full-length Mdm2 protein also contains all of the sequences present on the Mdm2-b isoform, and transduction of full-length Mdm2 was also found to up-regulate RelA in primary cells. Thus, any p53-independent functions for Mdm2-b may also exist for Mdm2 itself, suggesting that the use of small molecule inhibition to restore functional p53 in tumors with increased Mdm2 expression levels may not be sufficient to ameliorate all of the tumorigenic effects of Mdm2.

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