A Chromatin-associated and Transcriptionally Inactive p53-Mdm2 Complex Occurs in mdm2 SNP309 Homozygous Cells*

Nicoleta C. Arva†‡§, Tamara R. Gopen‡§, Kathryn E. Talbott‡, Latoya E. Campbell¶, Agustin Chicas†‡§, David E. White‡, Gareth L. Bond‡§, Arnold J. Levine‡§, and Jill Bargonetti†‡§

From the †Institute for Biomolecular Structure and Function and Department of Biological Sciences, Hunter College and Graduate School, City University of New York, New York, New York 10021 and the ‡Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, New Brunswick, New Jersey 08903

The p53 tumor suppressor protein plays a central role in the prevention of cancer development by causing growth arrest and/or apoptosis during stress (1). When the p53 pathway is inhibited, tumorigenesis is accelerated. Often p53 is inhibited by mutations within the DNA binding domain of p53 that lead to loss of the p53 tumor suppressor activity (2, 3). Additionally, p53 protein function is inhibited when oncogenes bind to p53. The Mdm2 oncoprotein binds to p53 and inactivates the p53 tumor suppressor activity (4). Interestingly, the Mdm2 protein is part of a negative feedback loop with p53; p53 activates mdm2 transcription, and then the Mdm2 protein inhibits p53 function. The Mdm2 protein functions to inactivate p53 in at least two ways. Mdm2 is an E3 ubiquitin ligase for p53 and targets the tumor suppressor for degradation by the ubiquitin proteolysis pathway (5, 6). The p53 protein is maintained at low levels in normally dividing cells in part through its interaction with Mdm2 (7). This protein-protein interaction also blocks the p53 trans-activation domain and thereby inhibits p53 transcriptional activity (8–10). In addition, under certain situations the overexpression of Mdm2 results in p53-Mdm2 complexes that fail to bind tightly to DNA (11) and thus causes subsequent inhibition of p53 activity.

Many cancer cells have high levels of the oncopgenic Mdm2 protein because of either increased expression (12) or amplification of the mdm2 gene (13). Mdm2 overexpression also results from the P2 promoter when a single nucleotide polymorphism (SNP)1 at position 309 in the first intron of the mdm2 gene causes increased affinity for the ubiquitous transcription factor SP1 (14). In cells homozygous for SNP309 the p53 pathway is compromised.

In an effort to closely examine the compromised signaling from p53 in cells homozygous for mdm2 SNP309, DNA damage was used to activate the checkpoint pathway and p53 functional activities were monitored. In doing so we found that p53 in cell lines homozygous for SNP309 could be significantly stabilized after 6 h of DNA damage treatment with different drugs. This p53 remained associated with the cellular Mdm2 protein in the nucleus and was not sequestered to the nucleolus. Importantly, this p53 bound to chromatin in conjunction with Mdm2 and was unable to activate transcription of downstream target genes. As discussed herein, we addressed the inhibitory actions of Mdm2 by showing that a DNA-bound form of p53 is blocked for trans-activation activity in the presence of high levels of Mdm2.

1 The abbreviations used are: SNP, single nucleotide polymorphism; CTF, camptothecin; ETOP, etoposide; MC, mitomycin C; Ab, antibody; PBS, fetal bovine serum; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; LLaL, N-acetyl-Leu-Leu-Nor-leu-al; RT, reverse transcriptase; AMV, avian myeloblastosis virus; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; BGS, ribosomal gene cluster; ChIP, chromatin immunoprecipitation; FACS, fluorescence-activated cell sorter; PDAR, pre-developed assay reagents; siRNA, small interference RNA; SCS, superconsensus site; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* This work was supported by Grants MCB-0212761 and MCB-9722326 from the National Science Foundation (to J. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Both authors contributed equally to this work.

‡ Supported in part through National Institutes of Health SCORE (1S06 GM60754).

§ Student of the Hunter College Minority Access to Research Careers Program.

** Supported by the Research Centers in Minority Institutions Award RR-03037 from the National Center for Research Resources of the National Institutes of Health, which supports infrastructure and instrumentation in the Biological Sciences Department at Hunter College.

¶ To whom correspondence should be addressed: Dept. of Biological Sciences, Hunter College and Graduate School, City University of New York, 695 Park Ave., New York, NY 10021. Tel.: 212-650-3519; Fax: 212-772-5227; E-mail: bargonetti@genectr.hunter.cuny.edu.
Inhibition of DNA-bound p53 by Mdm2

MATERIALS AND METHODS

Reagents—Camptothecin (CPT), propidium iodide, calpain inhibitor, N-acetyl-Leu-Leu-Norleucinal (LLeuLALLN), etoposide (ETOP), and anti-actin antibody were purchased from Sigma. Bristol-Myers Squibb Co. provided mitomycin C (MC). TRFoz was purchased from Invitrogen. The IgG anti-rabbit polyclonal antibody was purchased from Cell Signaling Technology. The monoclonal PARP antibody was purchased from PharMingen. The p53 antibodies 240, 1801, 421, and the Mdm2 antibody 2A10 were from monoclonal supernatants as described previously (15). The p53 antibody 6 (Ab6) was purchased from Calbiochem, and the p53 polyclonal antibody from Santa Cruz Biotechnology. The Mdm2 antibodies SMP14 and D7 are also from Santa Cruz Biotechnology. The p53 polyclonal antibody from Santa Cruz Biotechnology. The Mdm2 (15). The p53 antibody 6 (Ab6) was purchased from Calbiochem, and the monoclonal PARP antibody was purchased from PharMingen. The p53 antibodies 240, 1801, 421, and the Mdm2 antibody 2A10 were from monoclonal supernatants as described previously (15).

Immunofluorescence—Cells were plated onto glass coverslips using poly-L-lysine (Sigma), fixed with 4% paraformaldehyde for 30 min at room temperature, and then permeabilized with Triton X-100 1% for 30 min at 4 °C. The cells were washed with phosphate-buffered saline and collected into the supernatant. The pellets were resuspended (10 mM Tris-Cl, pH 8.0, 0.25% Triton X-100, 0.5% deoxycholate) in ice and centrifuged at 13,000 rpm at 4 °C. The supernatant was then mounted using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories) and observed using a fluorescence microscope.

Quantitative RT-PCR—For each sample, 5 μg of RNA were obtained using the Qiagen RNeasy kit, per the manufacturer’s protocol. Each sample was then diluted up to 50 μl with water. The samples were then rehydrated with the cDNA Archive Kit (Applied Biosystems). The mixture contained RT buffer, dNTP mix, random primers, and multiscr ipt RT. The 2× RT Master Mix, along with the cDNA, was used to adjust the room temperature for 10 min and then incubated with 20 μl of random primers at 95 °C. cDNA was denatured at 95 °C for 2 min, 60 °C for 1 min, and 72 °C for 2 min, continuing for 30 cycles. PCR products were then run on a 1% agarose gel and excised using a scalpel. Excised products were purified using the Qiagen Quick gel purification protocol per the manufacturer’s instructions. The following primers were used for sequencing of the complete p53 gene: primer set 1: 5′-GGAATTCCACACCCCGCGC-3′, 5′-GGAATTCATGGCCCATGCCA-3′, primer set 2: 5′-GAATTCTGATGCACATCCATCC-3′, 5′-GAATTCTCCATCAAGTGTTTGTC-3′, primer set 3: 5′-GAATTCAC -GAGTCGGAAGTTC-3′, 5′-GAATTCACAGCAGCTGGT-3′, primer set 4: 5′-GAATTCACACACACGACCTG-3′, 5′-GAATTCACGTACCACAAG-3′.

Flow Cytometry—FACS analysis was carried out on a BD Biosciences FACS scan. Cells were spun down at 2000 rpm for 7 min, washed twice with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA), and then in 50% ethanol. Propidium iodide staining and RNase treatment were carried out at 37 °C for 30 min prior to analysis.

Chromatin Immunoprecipitation—Cells were cross-linked with 1% formaldehyde at 37 °C for 30 min then quenched with glycine to 125 mM. The cells were washed with phosphate-buffered saline and collected into 100 mM Tris-Cl, pH 9.4, 10 mM dithiothreitol. The cell pellet was resuspended (10 mM Tris-Cl, pH 8.0, 0.25% Triton X-100, 0.5% Nonidet P-40, 10 mM EDTA, 0.5 mM EGTA, 1 mM PMSF) and incubated on ice for 10 min. Nuclei were collected by centrifugation, washed (10 mM Tris-Cl, pH 8.0, 0.2 mM NaCl, 1 mM EDTA, 0.5 mg EGTA, 1 mM PMSF) and resuspended in the same buffer without NaCl. Samples were sonicated (10×, 10×, each), centrifuged, and 0.10 volume of 10× precipitation buffer (10 mM Tris-X-100, 1% sodium deoxycholate, 10 mM NaCl) was added to the supernatants. One-fifth of each sample was saved and designated as “input.” Immunoprecipitations were carried out overnight with 2 μg of Mdm2-specific antibody (SMP14), 2 μg of p53-specific antibody Ab6, or 1:200 dilution of p5-5erP phosphospecific antibody. The next day protein A plus G-Sepharose beads (Amersham Biosciences) were added 2 h with rocking at 4 °C. The subsequent washes were as described (19). The washed resin was resuspended (100 μl of 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA) and reverse-cross-linked at 65 °C overnight. DNA fragments were purified (QiAQuick spin kit) and PCR-amplified using primers designed to amplify the p53 binding sites in the mdm2 gene (forward primer: 5'-GGAGGTTACAGGTTAAGGTT-3', reverse primer: 5'-ACAAGGTCGTG-GCTTACCGT-3') or p21/raf1 gene (forward primer: 5'-GGTGGTCCCTG-ATTGGCTTCTG-3', reverse primer: 5'-CTGAAAAAGCCAGGCCCAG-3'). [32P]dCTP (PerkinElmer Life Sciences) was added to the PCR.
reaction. Amplified products were analyzed by acrylamide gel electrophoresis. Gels were dried for 1 h at 55 °C, and autoradiography was performed. Quantitative PCR using CHP samples was carried out on a PE9700 PCR machine using a TaqMan Master Mix (Applied Biosystems). The PCR cycles were: 50 °C for 2 min, 95 °C for 10 min, then cycles of 95 °C for 15 s, and 60 °C for 1 min repeated 40 times. The fold change in the specific binding was normalized to GAPDH and Mock IP values. The probe and primer sequences are given below. Primers are in italics, TaqMan probe is in bold, and the p53 binding site is underlined:

\[5^\prime}-\text{H11032}^\prime\]

- **mutant:** Top, \(5^\prime\)-GGGTGGCTTGGCTTGTCAGGGCTTGTCCAGGAGC-3\'; Bottom, \(5^\prime\)-TCCTGGACAAGCCCTGACAAGCCAAGCCAC-3\';

- **fas:** Top, \(5^\prime\)-CCGGGGAGACAAGTCAGGACTTAACTCCTTTTACTGCA-3\'; Bottom, \(5^\prime\)-CCGGGGGAGACAAGTCAGGACTTAACTCCTTTTACTGCA-3\';

**Electrophoretic Mobility Shift Assay (EMSA)**—Custom oligonucleotides for DNA binding analysis were ordered from Operon Technologies and for SCS and RGC mutant as described (20). The consensus site (SCS) contained three adjacent p53 half sites. The sequence of the oligonucleotide was: Top, \(5^\prime\)-TGGCAATGGAGTACTTCGGGC-3\'; and Bottom, \(5^\prime\)-TGGCAATGGAGTACTTCGGGC-3\'; Labeling of the oligo was performed using the large fragment of DNA polymerase and [\(\alpha\)]p21

**Inhibition of DNA-bound p53 by Mdm2**

The p53 Protein Is Compromised for Activating Downstream Target Genes in MANCA or A875 Cells—The p53 protein induces the cyclin-dependent protein kinase inhibitor p21/Waf1 (23), and its increase is part of the cell cycle checkpoint (24). As one indicator of checkpoint activation after DNA damage, we

**RESULTS**

Endogenous Overexpression of Mdm2 via a Naturally Occurring SNP Inhibits Apoptosis following Chemotherapeutic Drug Treatment—We previously documented induced p53 stabilization and subsequent apoptosis in ML-1 cells treated for 6 h with CPT, ETOP, or MC, and no apoptosis induction in the p53-deficient human cell line K562 treated with the same drugs (21). A naturally occurring SNP at position 309 in the promoter 2 region of the mdm2 gene (Fig. 1A) results in overexpression of Mdm2 protein, and inhibition of drug-induced apoptosis occurs in homozygous mdm2 SNP309-containing cell lines MANCA and A875 (14). Both cell lines overexpress Mdm2 at the protein and RNA levels, which results in the attenuation of p53 stabilization in the first 3 h after ETOP treatment (14).

To closely examine the compromised molecular signaling, we compared p53 and Mdm2 protein levels in ML-1, MANCA, and A875 cell lines after 6 h of drug treatment with expression of the proteins in the documented wild-type p53-expressing cell line ML-1 (which is homozygous wild-type for mdm2) (14). Cells were treated with chemotherapeutic DNA-damaging drugs or were left untreated, and extracts were analyzed by Western blot for p53 and Mdm2 proteins (Fig. 1B). The drugs CPT, ETOP, and MC increased the level of p53 in ML-1 cells as well as in MANCA and A875 cells (Fig. 1B, lanes 1–12), whereas no p53 was expressed in the p53-negative control cell line K562 (Fig. 1B, lanes 13–16). We reproducibly noticed that the level of p53 protein in MANCA cells before drug treatment was high relative to other cell lines examined, and we do not have an explanation for this increased basal p53 in MANCA cells at this time. The p53 in MANCA and A875 is wild-type (14), as seen by DNA sequence analysis of p53 exons 1–11 described under “Materials and Methods.”

An examination of apoptosis indicators (PARP cleavage and FACS analysis with propidium iodide staining to score sub-G1 DNA content) was used to compare drug-induced apoptosis. Western blot analysis demonstrated p53 cleavage in ML-1 cells after 6 h of CPT and ETOP treatment and slight cleavage of PARP after 6 h of MC treatment but no PARP cleavage was detected in the other cell lines treated with the drugs (Fig. 2A). FACS analysis demonstrated an increased sub-G1 population (indicated by the M1 gate) up to 55% only in the ML-1 cells treated with drugs and not in any of the other drug-treated cell lines (Fig. 2, B and C).

**p53 Protein Is Phosphorylated at Ser^{15} in Cells with mdm2 SNP309**—The signal transduction pathway toward p53 involves a critical phosphorylation event at Ser^{15}, which helps not only to stabilize the p53 but also to activate the transcriptional activity of the protein (22). To examine kinase signaling to the p53 protein in the cell lines examined, Western blot analysis with antibody specifically recognizing Ser^{15}-phosphorylated p53 was carried out. Phosphorylation of p53 at Ser^{15} in the MANCA and A875 cells was reproducibly detected (Fig. 3A). We examined the ability of the p53 protein to be phosphorylated in MANCA and A875 cells after 6 h of chemotherapeutic treatment because we knew p53 activation in ML-1 cells occurred at this time point (21). Interestingly, we observed significant p53 phosphorylation in cell lines homozygous for the mdm2 SNP309 after CPT, ETOP, and MC treatment (Fig. 3A, lanes 5–12). In response to drug treatments the level of p53 phosphorylation in the ML-1 cell line increased as previously described (21), and MANCA and A875 cells demonstrated p53 phosphorylation greater than ML-1 cells (Fig. 3A, compare lanes 1–4 to lanes 5–12). The p53 stabilization was not greater in MANCA and A875 cells, as shown in Fig. 1.

p53 localized to the nucleus in both wild-type and SNP309 homozygous cells, as seen in immunofluorescence experiments (Fig. 4). Additionally, immunofluorescence studies indicated that the nuclear p53 levels increased following drug treatment of ML-1, MANCA, and A875 cells (Fig. 4).
examined the levels of p21/Waf1 protein. As expected, treatment of the ML-1 cells with CPT, ETOP, and MC resulted in an increase in p21/Waf1 protein, whereas in K562 cells the absence of p53 resulted in no p21/Waf1 increase (Fig. 3, lanes 1–4 and lanes 13–16). p21/Waf1 protein levels in MANCA and A875 cells showed great variation with severely attenuated DNA damage induction by CPT, ETOP, and MC treatment. Densitometric analysis demonstrated a 2.25-fold induction of p21/Waf1 levels in ML-1 cells treated with CPT and MC and a 7.3-fold induction after ETOP treatment. In MANCA and A875 cells, CPT and MC treatments did not produce any increase in p21/Waf1 levels. ETOP treatment gave 1.56-fold induction of the checkpoint protein in MANCA cells and 1.74-fold increase in A875 cells (data not shown).

We examined the ability of p53 target genes to be activated after the DNA damage treatments that resulted in p53 stabilization. Examination of p53 downstream target genes activation by quantitative RT-PCR revealed compromised activation in the MANCA and A875 cells, even though p53 stabilization was achieved (Fig. 5). Whereas the stabilized p53 in ML-1 cells activated p21/waf1, gadd45, fas, and mdm2, we saw compromised activation of the genes in MANCA and A875 cells (Fig. 5). Comparison of activation of these p53 targets in the two homozygous mdm2 SNP309 cell lines was not functioning correctly.

We asked if exogenously introduced p53 was capable of activating transcription from constructs with either mdm2 or p21/waf1 p53 responsive elements in the MANCA and A875 cells to closely examine the ability of wild-type p53 to be inhibited in the SNP309 cell lines. Transient transfection experiments were carried out with p53 protein provided from the plasmid SN3 (a generous gift from Bert Vogelstein). Both reporters were activated by the exogenously introduced p53 in the K562 and H1299 cells, neither of which is homozygous for mdm2 SNP309. These two cell lines do not express endogenous p53. The H1299 adherent cells were compared with A875 whereas the K562 suspension cells were compared with MANCA. We used p53-null cell lines for comparison in the transfection study because our quantitative PCR data demonstrated that p53 target gene activation in SNP309 homozygous cells resembled the activation seen in cells with no p53 expression. Activation from both reporter constructs occurred with p53 protein in the two homozygous mdm2 SNP309 cell lines in the transfection study because our quantitative PCR data demonstrated that p53 target gene activation in SNP309 homozygous cells resembled the activation seen in cells with no p53 expression. Activation from both reporter constructs occurred with p53 protein in the two homozygous mdm2 SNP309 cell lines in the transfection study because our quantitative PCR data demonstrated that p53 target gene activation in SNP309 homozygous cells resembled the activation seen in cells with no p53 expression. Activation from both reporter constructs occurred with p53 protein in the two homozygous mdm2 SNP309 cell lines in the transfection study because our quantitative PCR data demonstrated that p53 target gene activation in SNP309 homozygous cells resembled the activation seen in cells with no p53 expression.
FIG. 3.

The kinase signaling cascade to p53 is intact in cells homozygous for the mdm2 SNP309, but induction of the p21/Waf1 checkpoint protein is severely compromised. Western blot analysis of ML-1, MANCA, A875, and K562 samples. Cells were either left untreated (lanes 1, 5, 9, and 13) or were treated with 0.5 μM CPT (lanes 2, 6, 10, and 14), 8 μM ETOP (lanes 3, 7, 11, and 15), or 5 μM MC (lanes 4, 8, 12, and 16) for 6 h, and nuclear extracts were then prepared from these samples. 50 μg of nuclear protein were subjected to SDS-PAGE (10%) and Western blot analysis. The nitrocellulose membrane was probed with the p53-Ser15 phosphospecific antibody (A), the p21/Waf1-specific monoclonal antibody (Ab-1, B), and anti-actin (C).

C)

A) Ser-15 p53

B) p21/Waf1

C) Actin
cleoplasmm (Fig. 4), and therefore we examined if this p53 was associated with the DNA. To determine if the p53 protein that was compromised for activating transcription was able to bind to p53 responsive elements in chromatin, we compared p53 ChIP in ML-1, MANCA, K562, and A875 cells (Fig. 7A). Increased mdm2 and p21/waf1 chromatin was immunoprecipi-

**Fig. 4.** p53 protein is localized in the nucleoplasm in both mdm2 wild-type and SNP309 homozygous cells. Immunofluorescence was carried out in ML-1 (A), MANCA (B), and A875 (C) cells. Cells were left untreated or treated with 8 μM ETOP (ML-1 and MANCA cells) or 5 μM MC (A875 cells) to induce the p53 protein.
uated with a p53-specific antibody in ML-1 cells treated with ETOP (Fig. 7, lane 4) and some p53 localized on the chromatin of these genes prior to DNA damage (Fig. 7, lane 3). The same experiment was carried out in MANCA, A875, and K562 cells. Comparison of the input chromatin and mock immunoprecipitation for all samples demonstrated that the sets were normalized and gave barely detectable background (Fig. 7A, lanes 1, 2, 5, 6, 9, 10, 13, and 14). In MANCA cells p53 protein was associated with the \textit{mdm2} gene with no evident increase after DNA damage (Fig. 7A, lanes 7 and 8, \textit{mdm2} gene). Barely detectable \textit{p21/waf1} chromatin was immunoprecipitated in MANCA cells using an antibody recognizing the N terminus of p53 and was only evident by phosphorimager analysis (Fig. 7A, lanes 7 and 8, \textit{p21/waf1} gene and data not shown). It appears that this epitope was masked in the MANCA cell chromatin-associated p53, as an antibody to phosphorylated p53 indicated the association of the protein with \textit{p21/waf1} chromatin (Fig. 8B, compare lanes 7 and 8, \textit{p21/waf1} gene). Increased \textit{mdm2} and \textit{p21/waf1} chromatin were immunoprecipitated with the p53-specific antibody in A875 after ETOP treatment (Fig. 7A, compare lanes 15 and 16). In the p53-null cell line K562, p53 antibody did not precipitate any chromatin-containing p53 responsive elements before or after ETOP treatment (Fig. 7A, lanes 11 and 12).

We compared the ability of ML-1, MANCA, and A875 p53 protein in nuclear extracts to bind to the SCS using an EMSA. The p53 DNA binding activity in the presence of the p53-specific antibody 421 is known to be activated in EMSA (25), and such activated binding was assayed using nuclear extracts from CPT-, ETOP-, and MC-treated cells. In the presence of the p53 antibody 421, we observed an induced p53 shift in the ML-1, MANCA, and A875 nuclear extract samples (Fig. 7B, compare odd lanes that contain no 421 antibody to even lanes where the 421 antibody is present and Fig. 7C, compare lanes 1–2 and 3–4). Interestingly, whereas the p53 binding activity was activated in the ML-1 cells after drug treatment, p53 binding activity was present in the MANCA nuclear extract prior to drug treatment, and no further increase was evident after drug treatment (correlating with the Western blot and immunofluorescence data showing high basal levels of p53, as well as with ChIP results). In A875 cells the p53 binding activity was activated after ETOP treatment, and this binding was specific as demonstrated by competition with oligonucleotides containing p53 responsive element sequence but not with
A mutant oligonucleotide sequence (Fig. 7C). This DNA binding specificity for p53 in EMSA was also seen for MANCA cell p53 (data not shown).

Fig. 6. The p53 protein provided in trans is transcriptionally inactive in the mdm2 SNP309 homozygous cells. Transient transfection in K562 and MANCA cell lines (A and B) or H1299 and A875 cells (C and D) was carried out with increasing amounts (50–400 ng) of a plasmid for expression of the wild-type p53 cDNA (SN3) and 2 μg of the plasmid containing the human mdm2 (A and C) or p21//waq1 (B and D) p53 binding site adjacent to a luciferase reporter. The amount of DNA co-transfected in each sample was normalized using a carrier plasmid, pGL2. Fold induction in luciferase activity was measured and normalized to total protein concentration. Results are representative of three independent experiments. Western blot analysis (E and F) was performed to assess the transfection efficiency and p53 expression in H1299 and A875 cells.

Whole cell protein extract was prepared from the co-transfected cells, and 50 μg of extract were subjected to SDS-PAGE (10%) and Western blot analysis. The nitrocellulose membrane was probed with a mixture of the p53-specific monoclonal antibodies (240, 421, 1801; E) or anti-actin (F). Lanes 1 and 5 represent non-transfected samples; lanes 2 and 6 are protein extracts from a 50-ng SN3 transfection, lanes 3 and 7 from a 100-ng SN3 transfection, and lanes 4 and 8 from a 400-ng SN3 transfection.

Increased Mdm2 Protein Binds to Chromatin with p53 Responsive Elements in MANCA and A875 Cells—Although a bimodal mechanism for the inhibition of p53 by Mdm2 has been
described, the capacity of Mdm2 to target p53 for degradation often overshadows the capacity of Mdm2 to inhibit p53 transcription activity. Recently however it has been seen that Mdm2 protein is present at p53 binding sites in chromatin in the Mdm2-overexpressing cell line SJSA-1 (26). This Mdm2-chromatin association was p53-mediated and caused gene silencing because of histone ubiquitylation. Our transient transfection experiments pointed toward a trans-acting inhibitory factor of the DNA-bound p53. We carried out ChIP studies in wild-type or mdm2 SNP309 homozygous cells to examine if Mdm2 was associated with p53 bound to chromatin in MANCA and A875, but not in ML-1 cells. In ML-1 cells we were unable to identify Mdm2 bound to p53 responsive elements of the mdm2 or p21/waf1 genes in both untreated samples or after 3 h of ETOP treatment (Fig. 8A, lanes 3 and 4). In MANCA and A875 cells there was a small amount of Mdm2 bound to p53 responsive elements in both untreated samples and after 3 h of ETOP treatment (Fig. 8A, lanes 3 and 4). Quantitative PCR using the precipitated chromatin showed a 2.5-fold increase above background in untreated samples and went up to a 8-fold increase in ETOP-treated cells (data not shown; fold increase represents the average of three independent experiments). No Mdm2 protein was present at the mdm2 or p21/waf1 responsive elements in the p53-null cell line K562 (Fig. 8A, lanes 11 and 12). We examined if the recruitment of Mdm2 to the p53 responsive elements was mediated by the p53 protein. ChIP with p53-Ser15 phosphospecific antibody showed enhanced binding of p53 protein after DNA damage in ML-1 cells (Fig. 8B, compare lanes 3 and 4) and also in MANCA and A875 cells (Fig. 8B, compare lanes 7 and 8 and Fig. 8C, compare lanes 5 and 6). As expected, p53-Ser15 ChIP did not demonstrate any signal in the K562 cell line (Fig. 8B, lanes 11 and 12). The ChIP data argue that Mdm2 can localize to p53 responsive elements in the MANCA and A875 cells and increases with increased p53 localization.

We examined the soluble p53-Mdm2 complex present in the nucleus of the tested cell lines before and after DNA damage. Co-immunoprecipitation studies were carried out using nuclear extracts derived from the cells treated with the DNA-damaging agents to confirm that Mdm2 was associated with the stabilized p53 that had attenuated function. ML-1, MANCA, and A875 nuclear extracts were compared for p53-Mdm2 complex as complexes with the p53-responsive elements were measured.

Co-immunoprecipitation studies were carried out using nuclear extracts derived from the cells treated with the DNA-damaging agents to confirm that Mdm2 was associated with the stabilized p53 that had attenuated function. ML-1, MANCA, and A875 nuclear extracts were compared for p53-Mdm2 complex as complexes with the p53-responsive elements were measured.

The ChIP data argue that Mdm2 can localize to p53 responsive elements in the MANCA and A875 cells and increases with increased p53 localization.

The ChIP data argue that Mdm2 can localize to p53 responsive elements in the MANCA and A875 cells and increases with increased p53 localization.

We examined the soluble p53-Mdm2 complex present in the nucleus of the tested cell lines before and after DNA damage. Co-immunoprecipitation studies were carried out using nuclear extracts derived from the cells treated with the DNA-damaging agents to confirm that Mdm2 was associated with the stabilized p53 that had attenuated function. ML-1, MANCA, and A875 nuclear extracts were compared for p53-Mdm2 complex as complexes with the p53-responsive elements were measured.
cells were treated with ETOP but not before treatment (Fig. 8D, lanes 4–7). These co-immunoprecipitation results indicate that the increase in chromatin-associated Mdm2 correlates with an increased Mdm2-p53 protein association and strengthens the argument that Mdm2 is recruited to chromatin only in complex with p53. The reciprocal co-immunoprecipitation experiment was done using an antibody specific for the p53 protein, and this analysis demonstrated a greater interaction of p53 with Mdm2 in MANCA and A875 cells than in ML-1 cells (data not shown).

We performed mdm2 siRNA experiments to ensure that the Mdm2-elevated protein levels were required to attenuate the p53-mediated transcriptional activation in cells homozygous for SNP309. Down-regulation of Mdm2 (Fig. 8E, lane 3, Mdm2 Western blot) did not lead to any increase in the p53 protein levels (Fig. 8E, lane 3, p53 Western blot). However, p21/Waf1 protein levels were increased when Mdm2 was down-regulated (Fig. 8E, lane 3, p21/Waf1 Western blot). Mdm2 has been shown to be a p21/Waf1-negative regulator independently of p53, by facilitating the interaction of p21/Waf1 with the pro-
teasomal C8 subunit (27). To make certain that the increase in p21/Waf1 protein was caused by reactivation of the p53 transcriptional activity, we looked at p21/waf1 mRNA levels after mdm2 siRNA treatment. Mdm2 down-regulation led on average to a 3-fold increase in p21/waf1 mRNA levels (Fig. 8F), illustrating reactivation of the p53 transcription factor ability. Re-transfection of cells with two p53-dependent reporter constructs after mdm2 siRNA treatment also showed enhanced p53 function in the absence of Mdm2, as illustrated by the fold change in the luciferase readings (Fig. 8G).

DISCUSSION

The interaction between soluble Mdm2 and p53 has been well described in experimental systems with forced overexpression of both proteins. However the chromatin-bound associations between p53 and Mdm2 proteins have only begun to be addressed (26). It has been shown that Mdm2 regulates p53 by at least two mechanisms. The interaction of Mdm2 with p53 blocks the trans-activation domain of p53 and inhibits the protein transcriptional activity (8). Additionally, Mdm2 is an E3 ubiquitin ligase for p53, helping to target p53 for degradation (27). To make certain that the increase in p21/Waf1 protein was caused by reactivation of the p53 transcriptional activity after mdm2 siRNA treatment. Mdm2 down-regulation led on average to a 3-fold increase in p21/waf1 mRNA levels (Fig. 8F), illustrating reactivation of the p53 transcription factor ability. Re-transfection of cells with two p53-dependent reporter constructs after mdm2 siRNA treatment also showed enhanced p53 function in the absence of Mdm2, as illustrated by the fold change in the luciferase readings (Fig. 8G).

We present a model for how Mdm2 blocks the ability of p53 to activate target genes (Fig. 9). In cells that have wild-type p53 we see stabilization of p53 protein after DNA damage. In the ML-1 cell line, which does not have a SNP at position 309 of the mdm2 gene, we see dissociation of p53-Ser15 from Mdm2. This allows for the activated p53 protein to behave as a viable transcriptional activator, allowing for the activated p53 protein to behave as a viable transcriptional activator.
transcription factor and turn on downstream target genes. In cells overexpressing Mdm2, such as cells homozygous for mdm2 SNP309 (MANCA and A875), there is increased association of the p53-Ser15-Mdm2 complex on chromatin after DNA damage. This p53, in the presence of Mdm2 on chromatin, does not act as a viable transcription factor.

We have begun to look for other proteins that might be part of the chromatin-associated complex together with p53 and Mdm2. In vivo footprinting results have suggested that a large complex protects the p53 responsive element regions (28). Co-immunoprecipitation with the p53 antibody 421 and subsequent Coomassie Blue staining demonstrated that a number of high mobility bands were specifically co-immunoprecipitated in SNP309 cells. Mass spectrometry analysis revealed that the nucleolin protein was one of the interacting proteins in MANCA cells, whereas this same band was not detected in the ML-1 samples (data not shown). Western blot analysis of co-immunoprecipitated samples to compare the interaction between p53, Mdm2, and nucleolin indicated an interaction in the MANCA and A875 cells and none in the ML-1 cells (data not shown). In Fig. 4 we demonstrated no p53 nucleolar sequestration despite its association with the nucleolar protein. Nucleolin has previously been described to interact with p53 and turn on downstream target genes. In vivo inhibition of DNA-bound p53 by Mdm2

Acknowledgments—We thank members of the Bargonnetti laboratory (past and present) for advice, comments, and technical support during the course of this work.

REFERENCES
1. Bargonetti, J., and Manfredi, J. J. (2002) Curr. Opin. Oncol. 14, 86–91
2. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., and Devilee, P. (1989) Nature 342, 705–708
3. Bargonetti, J., Manfredi, J. J., Chen, X., Marshak, D. R., and Prives, C. (1993) Genes Dev. 7, 2565–2574
4. Freedman, D. A., Wu, L., and Levine, A. J. (1999) Cell Mol. Life Sci. 55, 96–107
5. Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000) J. Biol. Chem. 275, 8945–8951
6. Grossman, S. R., Drato, M. E., Brignone, C., Chan, H. M., Kung, A. L., Tagami, H., Nakatani, Y., and Livingston, D. M. (2003) Science 306, 342–344
7. Haux, T., Maya, R., Kazaz, A., and Oren, M. (1997) Nature 387, 296–299
8. Thut, C. J., Godfrich, J. A., and Tjian, R. (1997) Genes Dev. 11, 1974–1986
9. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) Cell 69, 1237–1245
10. Oliner, J. D., Pieterse, J. A., Thiangalingam, S., Gyrus, J., Kizler, K. W., and Vogelstein, B. (1993) Nature 362, 857–860
11. Zauberam, A., Barak, Y., Ragain, N., Levy, N., and Oren, M. (1993) EMBO J. 12, 2799–2808
12. Landers, J. E., Haines, D. S., Strauss, J. F. 3rd, and George, D. L. (1994) Oncogene 9, 2745–2750
13. Momand, J., Jung, D., Wilczynski, S., and Niland, J. (1998) Nucleic Acids Res. 26, 3453–3459
14. Bond, G. L., Hu, W., Bond, E. E., Robins, H., Lutzker, S. G., Arva, N. C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., Onel, K., Yip, L., Hung, S. J., Strong, L. C., Lazano, G., and Levine, A. J. (2004) Cell 119, 591–602
15. Chen, J., Marechal, V., and Levine, A. J. (1995) Mol. Cell. Biol. 15, 4107–4114
16. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuijen, P., Leeserberger, D. H., Barker, D. F., Nakamura, Y., White, R., and Vogelstein, B. (1989) Science 244, 217–221
17. Baker, S. J., Preisinger, A. C., Jessup, J. M., Parasekova, C., Markowitz, S., Willson, J. K., Hamilton, S., and Vogelstein, B. (1990) Cancer Res. 50, 7175–7222
18. Datto, M. B., Yu, Y., and Wang, F. (1995) J. Biol. Chem. 270, 28623–28628
19. Burakov, D., Crofts, A. L., Chang, C. P., and Freedman, L. P. (2002) J. Biol. Chem. 277, 14359–14362
20. Momand, J., Cain, C., and Bargonetti, J. (2003) Methods Mol. Biol. 234, 151–170
21. Abbas, T., Olivier, M., Lopez, J., Houser, S., Xiao, G., Kumar, G. S., Tomas, M., and Bargonetti, J. (2002) J. Biol. Chem. 277, 40518–40519
22. Danz, N., and Meek, D. W. (1999) EMBO J. 18, 7002–7010
23. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kizler, K. W., and Vogelstein, B. (1993) Cell 75, 817–825
24. Waldman, T., Kizler, K. W., and Vogelstein, B. (1995) Cancer Res. 55, 5187–5190
25. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) Cell 71, 875–886
26. Minsky, N., and Oren, M. (2004) Mol. Cell 16, 631–639
27. Zhang, Z., Wang, H., Li, M., Grawal, S. C., Chen, X., and Zhang, R. (2004) J. Biol. Chem. 279, 16900–16906
28. Xiao, G., White, R., and Bargonetti, J. (1998) Oncogene 17, 1171–1181
29. Daniely, Y., Dimitrova, D. D., and Borowicz, J. A. (2002) Mol. Cell. Biol. 22, 6014–6022
30. Tuteja, R., and Tuteja, N. (1998) Crit. Rev. Biochem. Mol. Biol. 33, 407–436
31. Chene, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., and Devilee, P. (1989) Nature 342, 705–708
32. Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammleitl, U., Lukacs, C., Klein, C., Potushi, N., and Liu, E. A. (2004) Science 303, 844–848
