

## Phosphorylation of p53 on Key Serines Is Dispensable for Transcriptional Activation and Apoptosis\* ♦

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**The p53 tumor suppressor is a key mediator of the cellular response to stress. Phosphorylation induced by multiple stress-activated kinases has been proposed to be essential for p53 stabilization, interaction with transcriptional co-activators, and activation of p53 target genes. However, genetic studies suggest that stress-activated phosphorylation may not be essential for p53 activation. We therefore investigated the role of p53 phosphorylation on six key serine residues (Ser<sup>6</sup>, Ser<sup>15</sup>, Ser<sup>20</sup>, Ser<sup>37</sup>, Ser<sup>46</sup>, and Ser<sup>392</sup>) for p53 activation using nutlin-3, a recently developed small molecule MDM2 antagonist. We show here that nutlin does not induce the phosphorylation of p53. Comparison of the activity of unphosphorylated and phosphorylated p53 induced by the genotoxic drugs doxorubicin and etoposide in HCT116 and RKO cells revealed no difference in their sequence-specific DNA binding and ability to transactivate p53 target genes and to induce p53-dependent apoptosis. We conclude that p53 phosphorylation on six major serine sites is not required for activation of p53 target genes or biological responses *in vivo*.**

The tumor suppressor p53 is a transcription factor that coordinates a complex network of cellular proteins evolved to protect cells from malignant transformation (1, 2). In response to diverse stress factors, p53 induces the expression of different subsets of genes leading to cell cycle arrest, apoptosis, DNA repair, or senescence. In cells that are not under stress, p53 activation could suppress their growth or induce apoptosis were it not for the tight regulation elicited by MDM2 (3, 4). The MDM2 gene expression is regulated in part by a p53-responsive promoter. In turn, MDM2 protein binds the p53 N-terminal transactivation domain and negatively regulates tumor suppressor function by compromising transcriptional regulation. Additionally, as MDM2 is an E3 ubiquitin ligase for p53 and itself, MDM2 controls p53 half-life via ubiquitin-dependent degradation. This negative feedback control mechanism assures that both p53 and MDM2 proteins are kept at very low levels in proliferating cells (4).

In response to stress, the p53-MDM2 interaction must be disrupted to enable p53 to associate with factors needed for

activation of its target genes. Stress-induced p53 activation involves post-translational modification of p53 on multiple sites by phosphorylation, acetylation, and sumoylation (2, 5) and modifications to MDM2 that can enhance MDM2 auto-ubiquitination and degradation (6). With regard to p53 modifications, phosphorylation has been studied most intensively and has been proposed to play a critical role in the stabilization and activation of the tumor suppressor. These studies have been greatly facilitated by the availability of antibodies that recognize p53 modified on specific phosphoserine or phosphothreonine residues (for review, see Ref. 7). Multiple serine (6, 9, 15, 20, 33, 37, 46, 315, 371, 376, 378, and 392) and three threonine residues (18, 55, and 81) have been reported to undergo phosphorylation in response to diverse stresses. Multiple serine/threonine kinases have been implicated in the upstream signaling leading to p53 phosphorylation (ATM, ATR, DNA-PK, Chk1, Chk2, CK1, p38, CDK2, PKC, JNK), but the precise mechanism of this signaling and its regulation are not well understood (8, 9). Residues from the N-terminal MDM2 binding domain of p53 (Ser<sup>20</sup> and Thr<sup>18</sup>) have been shown to play a critical role in the interaction between the two proteins and their stress-induced phosphorylation decreases substantially the affinity between p53 and MDM2 when analyzed *in vitro* using peptide substrates (10–12). These and other studies (7, 13) have led to the conclusion that phosphorylation of p53 is a key mechanism responsible for activation of the tumor suppressor in response to cellular stress.

In addition to its proposed role in abrogation of p53-MDM2 binding and stabilization of the protein, p53 phosphorylation has also been implicated in regulation of its activity. However, this aspect of p53 phosphorylation is still poorly understood and controversial. Transcriptional activity of p53 is of principal importance for its function as a tumor suppressor. It has been suggested that phosphorylation at specific residues can affect the transcriptional activity of p53 and/or its selectivity toward different subset of genes thus determining the specific type of cellular response to stress (5, 8). Activity of p53 as a transcription factor may be influenced by several factors: (a) ability to form active tetramers, (b) sequence-specific DNA binding, and (c) interaction with other components of the transcriptional machinery. It has been reported that Ser<sup>315</sup> and Ser<sup>392</sup> phosphorylation may regulate the oligomerization of p53 and thus its sequence-specific DNA binding (14–16). Ser<sup>15</sup> phosphorylation has been shown to enhance the interaction of p53 with transcriptional co-activators CBP and PCAF (10, 17–19). Stress-induced phosphorylation of Ser<sup>46</sup> has been implicated in the activation of p53-dependent apoptotic response (20, 21). Recently, prolyl isomerase Pin1 has been reported to bind p53 and enhance its DNA binding and transcriptional activity. This binding is dependent on DNA damage-induced phosphoryla-

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tion of p53 (22, 23). Taken together, these observations suggest that p53 phosphorylation may play an important role not only in stabilization of p53 but also in modulation of its transcriptional activity. On the other hand, experiments in which almost all phosphorylation sites in p53 have been mutated demonstrated that transiently expressed phosphorylated and unphosphorylated p53 do not differ significantly in their stability or ability to transactivate reporter genes in p53-null cells (24). Furthermore, studies using mouse mutants with substitutions of Ser<sup>15</sup> or Ser<sup>20</sup> suggest that these residues are not essential for p53 activation (25–27).

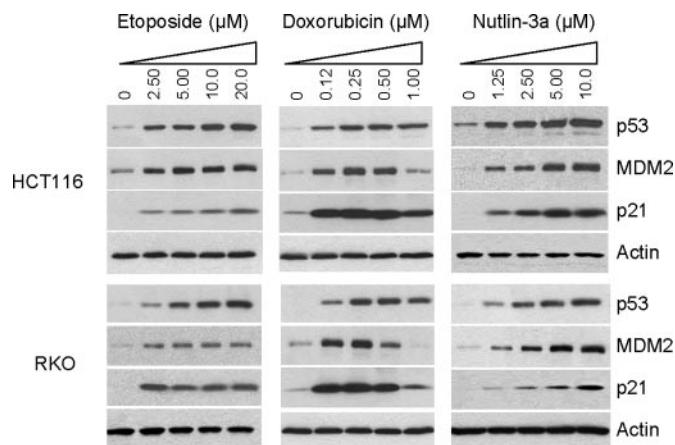
Recently, we reported the identification of the first potent and selective small molecule inhibitors of p53-MDM2 interaction, the nutlins (28). These compounds bind MDM2 in the p53 binding pocket with high selectivity and can release p53 from negative control leading to effective stabilization of p53 and activation of the p53 pathway *in vitro* and *in vivo*. Nutlins are non-genotoxic and activate p53 by preventing it from binding to MDM2 (28). They do not bind to p53 protein and do not interfere with its activities. Treatment of cultured cells with MDM2 antagonists cause accumulation of p53 protein that is free of phosphorylation on Ser<sup>15</sup> (28). Therefore, nutlins may represent valuable molecular tools for studying the role of p53 phosphorylation in its natural cellular context. Here, we show that p53 induced by the MDM2 antagonist, nutlin-3, is not phosphorylated on six key serine residues. Despite the lack of detectable phosphorylation, nutlin-induced p53 showed equal or better sequence-specific DNA binding, ability to transactivate p53 target genes, and p53-dependent apoptotic activity than phosphorylated p53 induced by the genotoxic drugs doxorubicin and etoposide. Our results provide further support to the notion that separating MDM2 from p53 is an important step in p53 activation, but phosphorylation is not required for execution of p53 biological functions.

#### EXPERIMENTAL PROCEDURES

**Cells and Drug Treatment**—HCT116 cells were purchased from ATCC (Manassas, VA), and RKO cells were a gift from Dr. B. Vogelstein (Johns Hopkins Oncology Center). Both cell lines have been derived from human colon cancer and possess wild-type p53. Cells were grown in the recommended media supplemented with 10% heat-inactivated fetal bovine serum. Media and serum were purchased from Invitrogen. For drug treatment,  $1.5 \times 10^6$  cells were seeded in 75-cm<sup>2</sup> tissue culture flasks in 10 ml of growth medium 24 h prior to treatment. They were incubated with doxorubicin or etoposide (Sigma, 10 mM stock solution in Me<sub>2</sub>SO) at various concentrations for 24 h. Control cells were treated with an equivalent amount of Me<sub>2</sub>SO. RKO-R cells were generated by continuous passage of RKO cells in media containing increasing concentrations of nutlin-3 (0.5–10  $\mu$ M) over a 90-day period. The resistant cell population was maintained in the presence of 10  $\mu$ M nutlin-3. p53 gene status was determined by the GeneChip p53 assay (Affymetrix, Santa Clara, CA) as described previously (29).

**Western Blot Analysis**—Cells were harvested by centrifugation and resuspended in lysis buffer containing 20 mM HEPES, 350 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 1% Nonidet P-40, phosphatase inhibitor mixture, and protease inhibitor mixture. Cell pellets were sonicated briefly and cell debris sedimented by brief centrifugation (15,000 rpm) at 4 °C. Supernatants were transferred to fresh tubes, and protein content was determined by the Bradford assay (Bio-Rad). For Western analysis, 10  $\mu$ g of total protein was loaded onto 4–12% Tris-glycine polyacrylamide gels and subjected to electrophoresis. Proteins were visualized by ECL chemiluminescence reagents (Amersham Biosciences) using primary antibodies specific for human p53 (SC-263, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-p53 (Ser<sup>6</sup>, Ser<sup>15</sup>, Ser<sup>20</sup>, Ser<sup>37</sup>, Ser<sup>46</sup>, Ser<sup>392</sup>, catalog number 9919, Cell Signaling, Beverly MA), p21 (OP64, Oncogene Research Products, Boston, MA), MDM2 (SC-965, Santa Cruz Biotechnology), and  $\beta$ -actin (Sigma). Secondary antibodies used were anti-mouse IgG horseradish peroxidase-linked whole antibody from sheep (NA931V; Amersham Biosciences) and anti-rabbit Ig horseradish peroxidase-linked donkey F(ab')<sub>2</sub> fragment (NA9340V; Amersham Biosciences).

**p53-DNA Binding Enzyme-linked Immunosorbent Assay**—TransAM™



**FIG. 1. Stabilization of p53 and activation of p53 target genes in human colon cancer cells treated with genotoxic drugs and MDM2 antagonist.** Exponentially growing HCT116 and RKO cells (wild-type p53) were incubated with the indicated concentrations of etoposide, doxorubicin, or the active enantiomer of MDM2 antagonist nutlin-3a for 24 h and the levels of p53, p21, MDM2, and  $\beta$ -actin were analyzed in cell lysates normalized for total protein by Western blotting.

p53 transcription factor assay kit (Active Motif, Carlsbad, CA) was used following manufacturer's protocol. Cell lysates from treated cells were diluted to 2  $\mu$ g/ml total protein with lysis buffer and applied to plates containing immobilized oligonucleotide containing the p53 consensus binding site (5'-GGACATGCCCGGGCATGTCC-3'). After 1-h incubation at room temperature, plates were washed and incubated with diluted p53 antibody (1:1000) for another hour. Diluted anti-rabbit horseradish peroxidase-conjugated antibody (1:1000) was then added to previously washed plates and developing solution was added and incubated for 8 min to allow color development. The reaction was stopped and absorbance read at 450 nm with a reference wavelength of 650 nm.

**Quantitative PCR**—Cells were seeded in 96-well plates ( $10^4$  cells/well) 24 h prior to treatment. They were lysed and total RNA extracted using the ABI 6700 robotic work station (Applied Biosystems, Foster City, CA). Aliquots containing 5  $\mu$ g of total RNA were converted to cDNA using the TaqMan reverse transcription reagents kit (Applied Biosystems). The relative quantity of the p53, p21, and MDM2 transcripts was determined by TaqMan using gene-specific primer/probe sets and 18 S RNA as a normalization control. The sequence of the primers and probes was as follows: p53 (forward, CTG-GGA-CGG-AAC-AGC-TTT-GA; reverse, CCT-TTC-TTG-CGG-AGA-TTC-TCT-TC; probe, CTG-TGC-GCC-GGT-CTC-TCC-CAG-TA), P21 (forward, CTG-AGA-CTC-TCA-GGG-TCG-AA; reverse, CGG-CGT-TTG-GAG-TGG-T-AG-AA; probe, TTG-GCT-CAC-TGC-AAG-CTC-GCC-CTT), MDM2 (forward, GCT-TGC-GTC-CAG-TGG-GTG-AT; reverse, GAT-GAC-TGT-AGG-CCA-AGC-TAA-TTG; probe, TGG-CTC-ACT-GCA-AGC-TCT-GCC-CT), MIC-1<sup>1</sup> (macrophage inhibitory cytokine-1) (forward, CCA-TGG-TGC-TCA-TTC-AAA-AGA-C; reverse, GGA-AGG-ACC-AGG-AC-T-GCT-CAT; probe, TGA-C TT-GTT-AGC-CAA-AGACTG-CCA-CTG-CA).

**Apoptosis Assays**—Cells were seeded in 24-well tissue culture plates ( $5 \times 10^4$  cells/well) 24 h prior to drug treatment and incubated with the drug for additional 48 h. No treatment controls were established in parallel for each cell line. Culture medium that may contain detached cells was collected and attached cells were trypsinized. Cells were combined with corresponding medium and collected by centrifugation at 1500 rpm for 10 min at 4 °C. Annexin V-positive cells were quantified using Guava Nexin™ kit and the Guava personal cell Analyzer (Guava Technologies, Hayward, CA.) as recommended by the manufacturer.

#### RESULTS

**MDM2 Antagonists Stabilize p53 and Activate p53 Target Genes**—Recently, we developed a class of potent and selective inhibitors of p53-MDM2 interaction (28). These compounds, called nutlins, bind MDM2 at the p53 pocket with high specificity and can displace p53 from its complex with its negative

<sup>1</sup> The abbreviation used is: MIC-1, macrophage inhibitory cytokine-1.

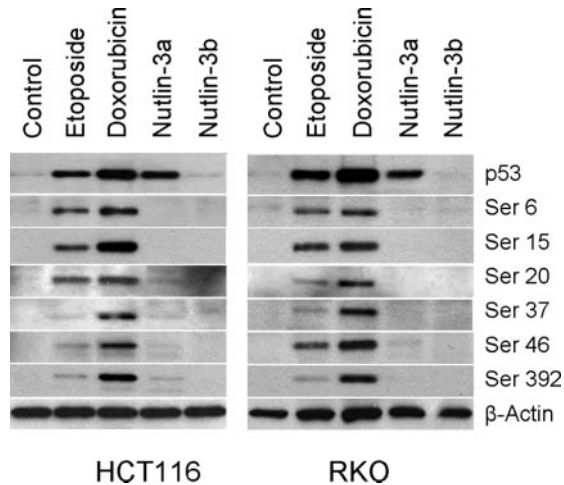
regulator. Treatment of cells encoding wild-type p53 with nutlins leads to p53 stabilization, accumulation, and activation of the p53 pathway. As nutlins activate p53 by preventing its physical interaction with MDM2, they should not alter the post-translational modification status of p53. Consequently, they represent valuable molecular probes to study the contributions of post-translational modifications to p53 function.

Using the MDM2 antagonist nutlin-3, we aimed at studying the functional activity of unmodified p53 compared with the activity of p53 modified in response to treating cells with the

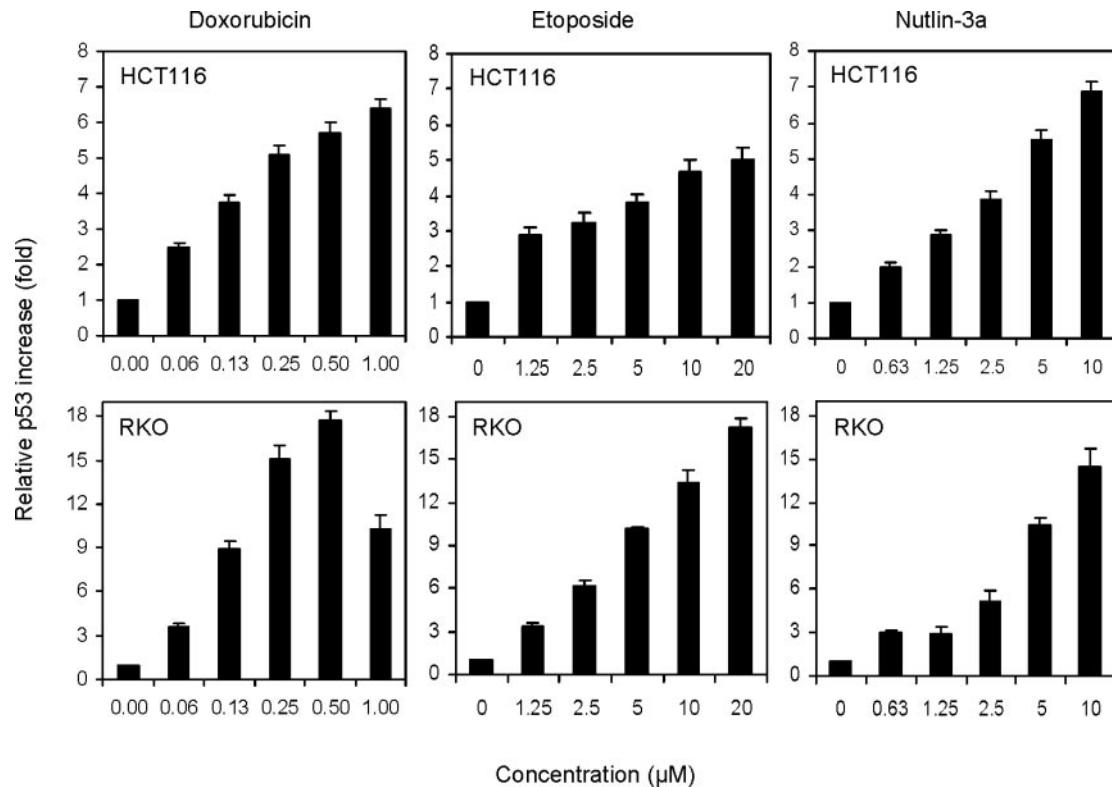
genotoxic drugs etoposide and doxorubicin. We chose the colon cancer cell lines HCT116 and RKO as they possess wild-type p53 and respond to genotoxic stress by p53 stabilization and activation of the p53 pathway (28). To find the optimal treatment condition, we incubated exponentially growing cells with a range of concentrations of etoposide, doxorubicin, and the active enantiomer of nutlin-3 (nutlin-3a) for 24 h. These ranges included the IC<sub>50</sub> and IC<sub>90</sub> values previously determined by a proliferation/viability assay (28). Western analysis of the cell lysates from both cell lines revealed a dose-dependent accumulation of p53 and its target gene products MDM2 and p21<sup>Waf1/CIP1</sup> (Fig. 1). The observed decrease in the MDM2 and p21 level at the high doxorubicin concentrations is most likely due to protein degradation in cells undergoing apoptosis. This experiment showed that nutlin-3a treatment of HCT116 and RKO cells activates p53 comparably with the genotoxic drugs etoposide and doxorubicin.

*p53 Induced by MDM2 Antagonists Is Not Modified on Key Phosphorylation Sites*—We previously showed that nutlin-1 does not cause p53 phosphorylation at Ser<sup>15</sup>, a site typically modified in response to genotoxic stress (28). This observation is consistent with the notion that MDM2 antagonists are non-genotoxic and should not activate the damage-responsive kinases that trigger p53 phosphorylation. However, it is conceivable that other nutlins could possess off-target activities that generate DNA damage or activate stress-related kinases to induce p53 modification. We therefore determined whether nutlin-3a induced p53 phosphorylation on six key serine residues (Ser<sup>6</sup>, Ser<sup>15</sup>, Ser<sup>20</sup>, Ser<sup>37</sup>, Ser<sup>46</sup>, and Ser<sup>392</sup>) using phosphoserine-specific antibodies. The inactive enantiomer nutlin-3b, which has a 150-fold lower affinity to MDM2 *in vitro*, was used as a negative control (28).

Western analysis showed a comparable accumulation of p53 in both HCT116 and RKO cells treated with etoposide, doxo-



**FIG. 2. Stabilization of p53 by MDM2 antagonists does not involve phosphorylation of key serine residues.** Exponentially growing HCT116 and RKO cells were incubated with etoposide (10  $\mu$ M), doxorubicin (1  $\mu$ M), nutlin-3a (10  $\mu$ M), or nutlin-3b (10  $\mu$ M) for 24 h, and the levels of total p53 and p53 phosphorylated on specific serine residues were analyzed by Western blotting. Actin was used as a normalization control.



**FIG. 3. Binding of p53 to its consensus DNA sequence is not affected by its phosphorylation status *in vivo*.** HCT116 and RKO cells were incubated with doxorubicin, etoposide, and nutlins-3a for 24 h, and the level of p53 protein present in the cell lysates that can bind to its consensus recognition sequence was determined by the TransAM<sup>TM</sup> p53 enzyme-linked immunosorbent assay and calculated as fold increase relative to the control samples.

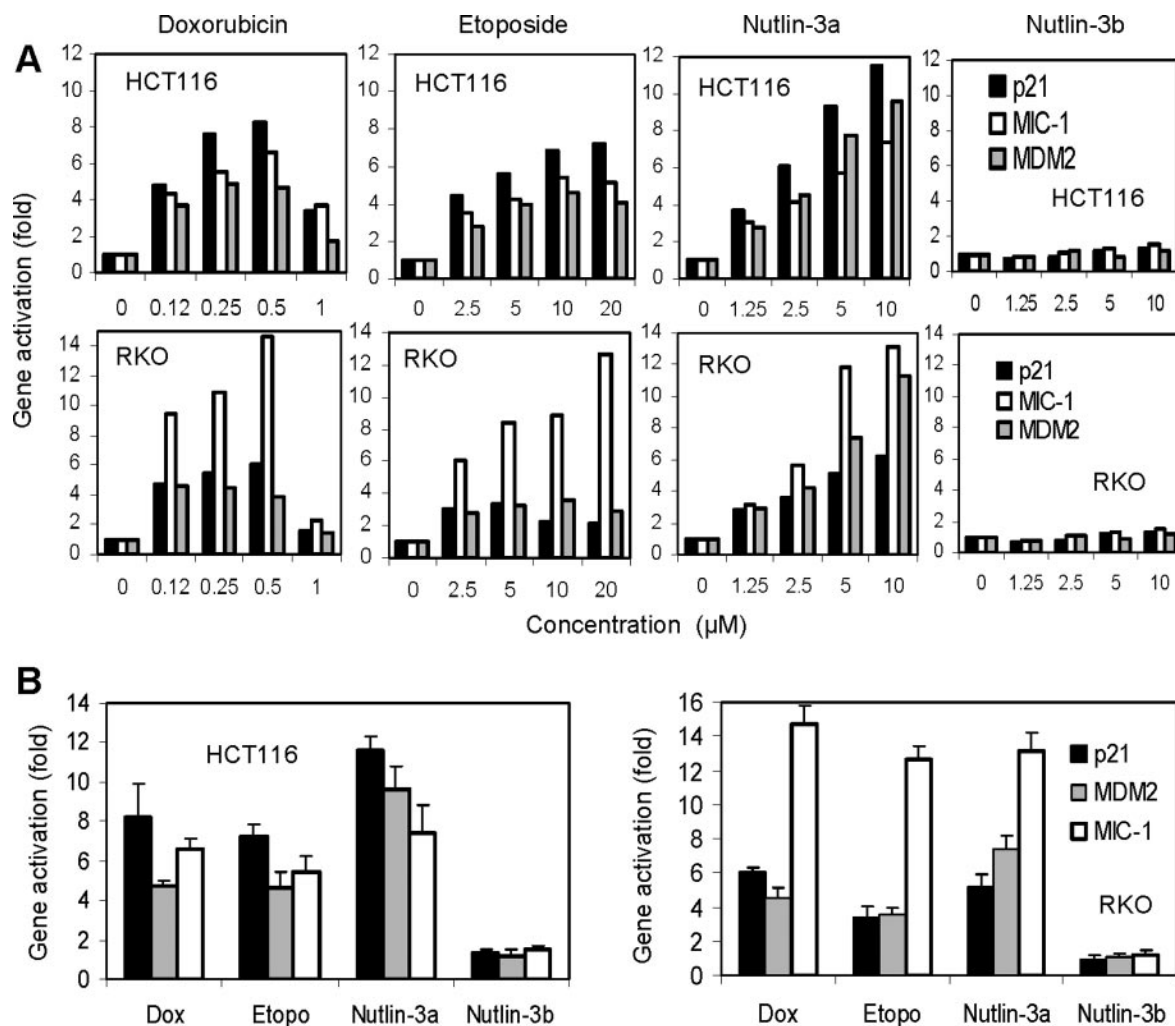


FIG. 4. Activation of p53-regulated genes in cancer cells does not depend on the phosphorylation status of p53. Exponentially growing HCT116 and RKO cells were incubated with the indicated concentration of doxorubicin (*Dox*), etoposide (*Etopo*), nutlin-3a, and nutlin-3b for 24 h, and the relative expression of three p53-regulated genes (*p21*, *mdm2*, and *mic-1*) was determined by quantitative PCR. They were plotted as a relative increase in gene activity.

rubicin, and nutlin-3a, but no accumulation was observed with nutlin-3b. Doxorubicin treatment induced the phosphorylation of all examined serine residues of p53 (Fig. 2). Etoposide showed a strong phosphorylation signal on Ser<sup>6</sup>, Ser<sup>15</sup>, and Ser<sup>20</sup> in both cell lines and Ser<sup>46</sup> in RKO cells and weaker but detectable phosphorylation on Ser<sup>37</sup> and Ser<sup>392</sup>. In contrast, phosphorylation of all p53 serines was undetectable in the lysates from both cell lines incubated with nutlin-3a and nutlin-3b. This result confirmed and extended the previous observation made with nutlin-1 that MDM2 antagonists do not induce stress-related modifications previously correlated with p53 activation. Therefore, nutlin-activated p53 provides an opportunity for studying the functional contributions of phosphorylation to p53 function in living cells.

**DNA Binding Activity of p53 Does Not Depend on Its Phosphorylation Status**—p53 transcriptional activity is of paramount importance for its function as a tumor suppressor. We therefore evaluated the ability of p53 to bind its DNA recognition sequences as an indication of the transcriptional activation potential of the transcription factor. We used the TransAM™ p53 enzyme-linked immunosorbent assay that measures the relative amount of p53 in cell lysates that can bind to a 20-mer oligonucleotide containing a p53 consensus binding site. HCT116 and RKO cells were incubated with a range of concentrations of etoposide, doxorubicin, and nutlin-3a for 24 h, and

DNA-bound p53 was assayed in the cell lysates (Fig. 3). In agreement with the Western analyses (Fig. 1), the levels of DNA-bound p53 increased in a dose-dependent manner in both cell lines treated with all three drugs. The 14–17-fold elevation of p53 in RKO cells was more dramatic, while HCT116 cells showed a more moderate (5–6-fold) increase that reflects the higher basal level of p53 in these cells. The level of p53 induced by nutlin-3a was higher than the level induced by either etoposide or doxorubicin in HCT116 cells and comparable with that induced by these drugs (14-fold versus 16–17-fold) in RKO cells. These data suggest that the lack of detectable phosphorylation on six major phosphorylation sites does not affect the ability of p53 to bind effectively its DNA response elements.

**Transcriptional Activity of p53 Is Not Affected by Its Phosphorylation Status**—We next compared the transcriptional activities of phosphorylated and unphosphorylated p53 after treatment of HCT116 and RKO cells with increasing concentrations of etoposide, doxorubicin, nutlin-3a, and nutlin-3b for 24 h. We measured the expression of three p53 target genes (*p21<sup>Waf1</sup>*, *mdm2*, and *mic-1*) by quantitative real-time PCR. These genes contain p53 recognition sequences in their promoter regions, strongly depend on p53 for transcriptional regulation, and represent diverse functions of the p53 pathway: *p21<sup>Waf1/CIP1</sup>* encodes a potent cyclin-dependent kinase inhibitor that plays a key role in the p53-mediated cell cycle arrest

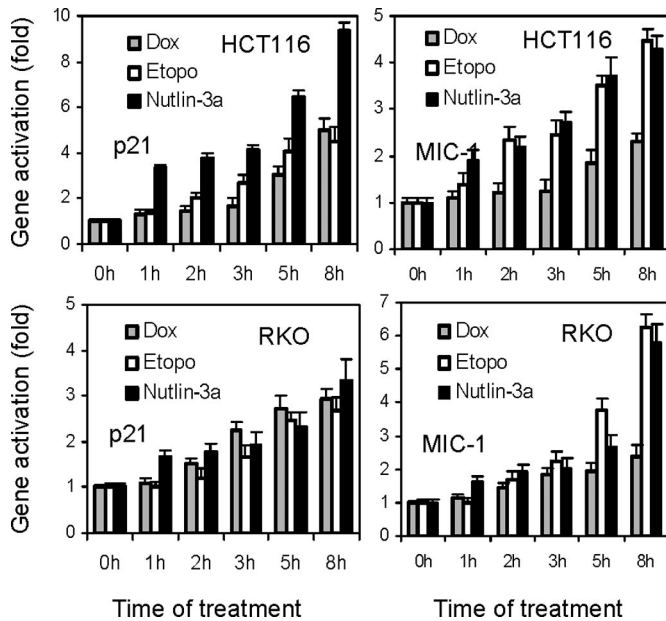


FIG. 5. Kinetics of induction of p53 target genes does not depend on p53 phosphorylation. HCT116 and RKO cells were exposed to doxorubicin (*Dox*) (1  $\mu$ M), etoposide (*Etopo*) (20  $\mu$ M), and nutlin-3a (10  $\mu$ M) for the indicated time periods, and the expression of p21 and MIC-1 was determined by quantitative PCR and expressed as a gene activation relative to untreated controls.

(30); MDM2 is a p53 negative regulator (31); and the recently discovered transforming growth factor- $\beta$  superfamily member, MIC-1, is a secreted protein with poorly understood function (32, 33).

Dose-dependent accumulation of p53 in drug-treated cells elicited a dose-dependent activation of all three genes in both cell lines (Fig. 4A). The relative increase in gene expression was different in each cell line, probably reflecting differences in the basal level of expression. p21 was most highly elevated in HCT116, while MIC-1 showed the strongest induction in RKO cells. To compare the transcriptional activity of p53 between drug-treated cells the highest level of induction within the concentration range was plotted for each drug and cell line (Fig. 4B). The level of gene induction by nutlin-3a (10–12-fold) was the highest for all three genes in HCT116 cells and for p21 and MDM2 in RKO cells. Only the expression of the MIC-1 gene was slightly higher in doxorubicin-treated RKO cells (Fig. 4B). The inactive enantiomer (nutlin-3b) did not show significant transcriptional activation of any of the genes in either cell line, confirming that the activation of p53 target genes by nutlin-3a is due to inhibition of MDM2-p53 interaction (28).

Expression of p53 target genes was measured after 24 h of drug treatment to reach a steady-state level of p53 and to avoid possible differences in the timing of p53 induction. However, one could argue that p53 phosphorylation accelerates p53 activation by both antagonizing MDM2 interaction and enabling recruitment of co-activators. On the other hand, if the critical step in p53 activation involves preventing MDM2 binding, then nutlin-3a may provide a more direct and rapid route to activation, since kinase activation, p53 modification, and damage-induced degradation of MDM2 (6) would not be required as intermediate steps. We investigated these possibilities by determining the kinetics of transcriptional activation of the *p21* and *mic-1* genes (up to 8 h) after addition of doxorubicin (1  $\mu$ M), etoposide (20  $\mu$ M), and nutlin-3a (10  $\mu$ M). Previous studies have demonstrated that 1  $\mu$ M doxorubicin is the optimal dose for p53 induction within an 8-h time frame (33). The results from this experiment showed that the levels of p21 and MDM2 expres-

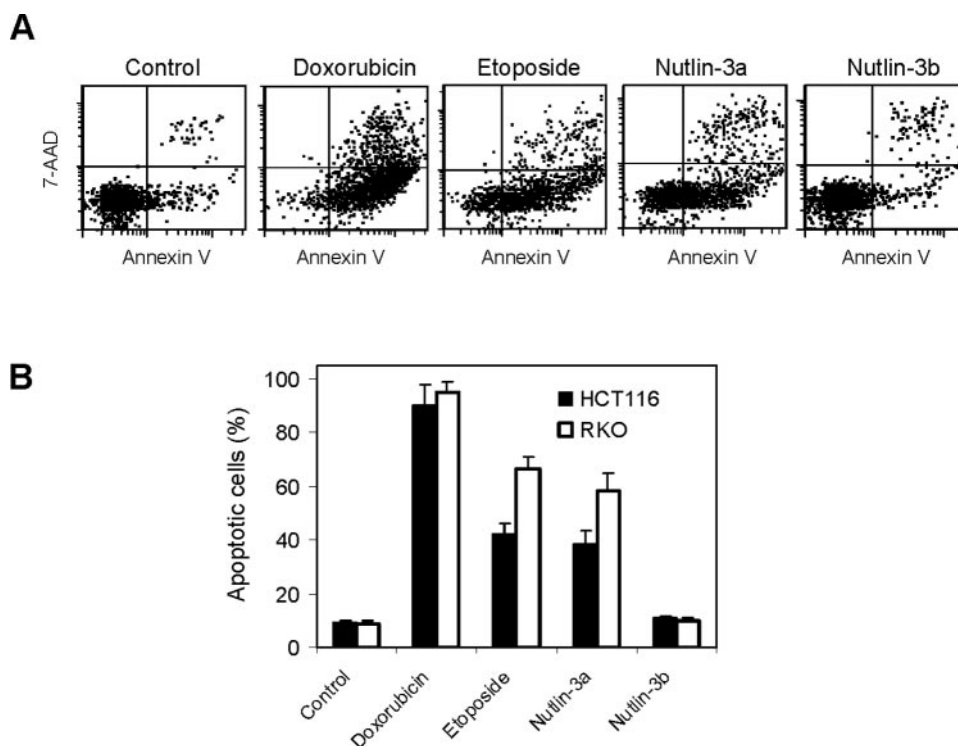
sion were higher or equivalent in nutlin-3a-treated cells than in cells treated with doxorubicin or etoposide (Fig. 5). Importantly, nutlin-3a induced these p53 target genes slightly faster than the genotoxic drugs. This is consistent with its mechanism of action involving direct interference with p53-MDM2 binding. Since nutlin-induced p53 was not detectably phosphorylated on the six residues analyzed (Fig. 2), while doxorubicin-induced p53 was phosphorylated on all six, and etoposide-induced p53 was phosphorylated strongly on at least three residues, we conclude that p53 phosphorylation on key reported phosphorylation sites is dispensable for timely and robust activation of its major downstream transcriptional targets *in vivo*.

**Apoptotic Activity of Phosphorylated and Unphosphorylated p53**—Next, we examined the effect of p53 phosphorylation on its ability to induce apoptosis, since this function is critical for suppression of tumor formation in several mouse models (34, 35). Although p53 phosphorylation on key serine residues appears to be dispensable for transcriptional activation, it may play a role in functions of the tumor suppressor in specific tissues or perhaps in proposed transcription-independent mechanisms of apoptosis (36, 37). As the mechanisms of apoptosis induction by p53 may involve activation of multiple pathways, we decided not to measure a particular pathway but rather to use annexin V staining as a well accepted marker for apoptosis induction (38). We treated exponentially growing HCT116 and RKO cells with the concentration range of etoposide, doxorubicin, nutlin-3a, and nutlin-3b used in the previous tests (see Fig. 4) for 48 h and determined the percentage of annexin V-positive cells. Previous studies (28) and unpublished data<sup>2</sup> have suggested that 48 h is the optimal timeframe for detection of p53-dependent apoptosis. In agreement with the previous results, both HCT116 and RKO cells showed an increase in the population of apoptotic cells upon treatment with etoposide, doxorubicin, and nutlin-3a but not nutlin-3b (data not shown). The samples with the highest level of apoptosis in each dose group were chosen for comparison of the apoptotic activity of p53 (Fig. 6). Doxorubicin induced massive apoptosis with ~90% of both HCT116 and RKO cells testing positive for annexin V. Etoposide and nutlin-3a were comparable in apoptotic activity, and nutlin-3b did not show significant increase in the apoptotic fraction.

At face value, these data suggested that apoptotic activity correlates with the level of phosphorylation, since doxorubicin, which produces the highest level of serine phosphorylation, had the best apoptotic response. However, doxorubicin and etoposide are drugs with known p53-independent cytotoxicity. It has been well documented that DNA damaging drugs can induce apoptosis in cells in which p53 is mutant or deleted (39). Therefore, it is difficult to separate p53-dependent and -independent apoptotic activity of genotoxic agents. We tested the apoptotic activity of doxorubicin and etoposide in the colon cancer cell line SW480 in which the p53 pathway is disabled due to a mutation in the DNA binding domain of p53. Over 80% of doxorubicin-treated and 65% of etoposide-treated SW480 cells tested positive for apoptosis confirming the ability of doxorubicin and etoposide to induce p53-independent apoptosis (data not shown). This result indicates that the population of apoptotic cells in the doxorubicin-treated HCT116 and RKO cells consists of cells undergoing both p53-dependent and independent apoptosis.

In an attempt to determine the p53-dependent apoptotic activity of doxorubicin and etoposide, we used a variant of the RKO cell line (RKO-R) in which p53 has been disabled by

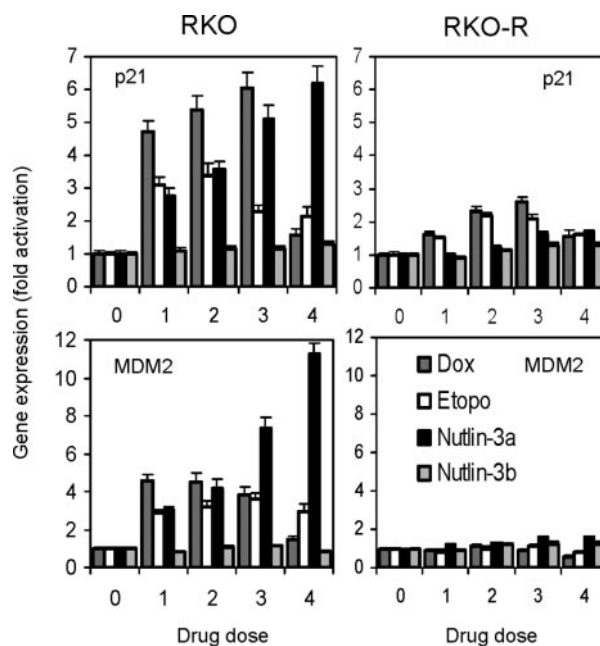
<sup>2</sup> T. Thompson, C. Tovar, H. Yang, and L. T. Vassilev, unpublished data.



**FIG. 6. Unphosphorylated p53 induces apoptosis in cancer cells.** A, detection of apoptotic activity by annexin V staining. Proliferating RKO cells were incubated with doxorubicin (1  $\mu\text{M}$ ), etoposide (20  $\mu\text{M}$ ), nutlin-3a (10  $\mu\text{M}$ ), and nutlin-3b (10  $\mu\text{M}$ ) for 48 h, and the annexin V positive cells were determined using the GuavaNexin kit and Guava personal cell analyzer. Annexin V reactivity indicates that the cells are in the early stages of apoptosis. DNA binding dye 7-aminoactinomycin D can penetrate only cells with compromised integrity and is used to distinguish between live and dead cells. Lower left quadrant, normal cells; lower right, live apoptotic cells; upper right, dead apoptotic cells. B, apoptosis induced by doxorubicin (0.5  $\mu\text{M}$ ), etoposide (20  $\mu\text{M}$ ), nutlin-3a (10  $\mu\text{M}$ ), and nutlin-3b (10  $\mu\text{M}$ ) in HCT116 and RKO cells after 48 h of treatment. The apoptotic fraction was calculated as the sum of annexin-positive live and dead cell (upper and lower right quadrants) and expressed as a percentage of the total cell population.

spontaneous mutations in the p53 gene occurring during a prolonged incubation of RKO cells in the presence of nutlin-3. These include an insertion of a stop codon at position Gln<sup>144</sup> and a mutation in the DNA binding domain of p53 (S240G). As a result, RKO-R cells are resistant to nutlin-3a, which only works through inhibition of MDM2 in cells with wild-type p53 (28). At the same time, RKO-R cells are only partially resistant to doxorubicin and etoposide due to their p53-independent activities. Treatment of RKO-R cells with doxorubicin, etoposide, and nutlin-3 for 24 h showed a strongly attenuated response to all three drugs. Compared with RKO cells, p53-regulated gene p21 was only partially activated in doxorubicin- and etoposide-treated RKO-R cells but not in the cells exposed to nutlin-3a (Fig. 7). The expression level of MDM2 in RKO-R cells was not affected by the drug treatment. This indicates that p53 response is disabled in RKO-R cells. Therefore, RKO-R cells, which are genetically very closely related to RKO cells, offer a good control for p53-independent cellular activity.

To assess the apoptotic activity of doxorubicin, etoposide, and nutlin-3a, we incubated RKO and RKO-R cells with the previously established dose range (Fig. 3) for 48 h and quantified the annexin V-positive cell fraction (Fig. 8). Doxorubicin treatment of RKO-R cells induced apoptosis at half the rate in the parental cell line at 0.12–0.25  $\mu\text{M}$  concentration but at an equally high rate at the 0.5–1.0  $\mu\text{M}$  range. A substantial fraction of the apoptotic population was also found in etoposide-treated cells. Nutlin-3a did not induce detectable apoptosis compared with untreated control. These data confirmed that a substantial fraction of the apoptosis in the RKO cells treated with high doses of doxorubicin and etoposide is induced by p53-independent pathways. Therefore, if p53-independent apoptotic activity of the two genotoxic drugs (*e.g.* in RKO-R



**FIG. 7. p53 pathway is disabled in RKO-R cells.** Parental RKO and the resistant RKO-R cells were incubated with doxorubicin (Dox) (1, 0.13; 2, 0.25; 3, 0.5; and 4, 1.0  $\mu\text{M}$ ), etoposide (Etopo) (1, 2.5; 2, 5.0; 3, 10; and 4, 20  $\mu\text{M}$ ), or nutlin-3a and nutlin-3b (1, 1.25; 2, 2.5; 3, 5.0; and 4, 10  $\mu\text{M}$ ) for 24 h, and the induction of p21 and MDM2 expression was determined by quantitative PCR.

cells) is deducted from the total apoptotic activity in RKO cells, the residual value should represent p53-dependent apoptosis. This value is equal to or lower than that of nutlin-3a. These

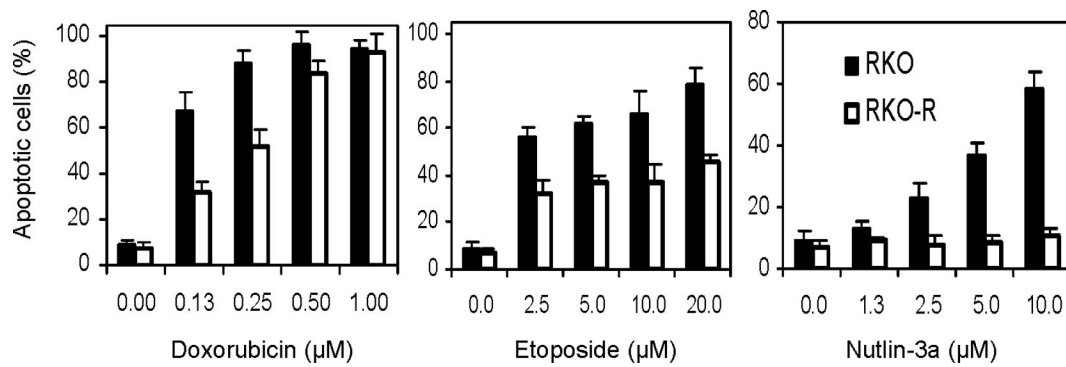


FIG. 8. **Apoptotic activity of p53 is not affected by its phosphorylation status.** Exponentially growing RKO and RKO-R cells were treated with increasing concentration of doxorubicin, etoposide, and nutlin-3a for 48 h, and the annexin-positive apoptotic cell fraction was calculated as described in the legend to Fig. 6.

results support the conclusion that nutlin-activated p53 that is lacking detectable phosphorylation on six key serine residues is competent to induce p53-dependent apoptosis in cancer cells with wild-type p53 at levels comparable with that induced by phosphorylated p53.

#### DISCUSSION

The role of phosphorylation in p53 activation and function has been a subject of intense studies in the last decade. However, despite the abundant data from numerous studies addressing specific phosphorylation events in response to diverse stress factors, the picture is still incomplete. Although several lines of evidence point to N-terminal phosphorylation as a major mechanism for abrogation of p53-MDM2 binding, the role of phosphorylation in modulation of p53 functions is still controversial. In this study, we have used recently discovered MDM2 antagonists as tools for accumulation of unphosphorylated p53 in two cancer cell lines and have compared the functional activity of unphosphorylated p53 with that of stress-induced phosphorylated p53 in the same cellular context. Our data demonstrate that unphosphorylated p53 is fully capable of activating downstream signaling in the p53 pathway equivalent to phosphorylated p53. We propose, therefore, that phosphorylation at the residues tested is not required for p53 activation. This conclusion is compatible with recent studies showing that drugs such as leptomycin B also activate p53 in the absence of serine 15 phosphorylation (6, 40).

Nutlin-treated HCT116 and RKO cells accumulated p53 protein at levels comparable with those levels induced by two genotoxic drugs, doxorubicin and etoposide (Fig. 1). However, as predicted by the mechanism of action, p53 induced by interference with p53-MDM2 binding did not show detectable phosphorylation on six serine residues previously reported as major phosphorylation sites (Ser<sup>6</sup>, Ser<sup>15</sup>, Ser<sup>20</sup>, Ser<sup>37</sup>, Ser<sup>46</sup>, and Ser<sup>392</sup>). At the same time, p53 was phosphorylated on all six serine residues in doxorubicin-treated cells (Fig. 2). Etoposide-induced p53 showed a comparable level of phosphorylation on some serine residues but much weaker to undetectable on others, reflecting differences in the upstream signaling by these drugs (41). The fact that nutlin-induced p53 is not phosphorylated on six highly conserved phosphorylation sites makes it unlikely that other potential sites could be phosphorylated by MDM2 antagonists. Therefore, it is reasonable to speculate that p53 stabilized by nutlin-3 is free of phosphorylation and perhaps from other post-translational modifications.

Nutlin-induced p53 in HCT116 and RKO cells showed comparable binding activity to its consensus DNA sequence indicating an equivalent transactivation potential (Fig. 3). However, transactivation properties depend on multiple factors and are best measured on native p53-regulated genes *in vivo*. To

this end, we examined the activation of three different p53 target genes by quantitative PCR. All three have been reported as immediate downstream genes directly regulated by p53. This test revealed that unphosphorylated nutlin-induced p53 had equal or superior activity (Fig. 4). Together with the previous result, this experiment confirmed that the phosphorylation status does not confer to p53 higher transactivation potency. On the contrary, it appears that unphosphorylated p53 is an equivalent or possibly better activator of all three genes in both cell lines. Only MIC-1 is activated slightly higher by doxorubicin in RKO cells.

Although we did not see effects of p53 phosphorylation on the transcriptional activity of p53 on three genes in two cell lines, we cannot exclude the possibility that differences might be detected in the large constellation of p53 regulated genes or under specific conditions in specific tissues or cell lines. However, the results from a recent study published during the preparation of our manuscript render this possibility unlikely. Jackson *et al.* (42) have used isopropyl  $\beta$ -D-thiogalactopyranoside-induced p14<sup>ARF</sup> as a means to sequester MDM2 and stabilize p53 in the osteosarcoma cell line U2OS. ARF-induced p53 has been found phosphorylation-free on five N-terminal serine residues (Ser<sup>6</sup>, Ser<sup>9</sup>, Ser<sup>15</sup>, Ser<sup>20</sup>, and Ser<sup>37</sup>) but still phosphorylated on the C-terminal Ser<sup>392</sup>. Comparison of the global gene expression activated by this partially phosphorylated p53 with fully phosphorylated doxorubicin-induced p53 by DNA array analysis revealed that most genes are up-regulated similarly. These authors concluded that N-terminal phosphorylation is not required for transcriptional activation of most p53 target genes. The data from this and our study derived by different tools in different cell systems support the conclusion that p53 phosphorylation is dispensable for transcriptional activation of p53-regulated genes *in vivo*.

It has been established that p53 functions primarily as a transcription factor that regulates multiple genes involved in regulation of cell growth and homeostasis. However, transcription-independent p53 functions have also been reported to mediate apoptosis under some experimental conditions (36, 37). p53 can bind directly to mitochondrial proteins and induce apoptosis in a transcription-independent manner (43). Apoptotic activity of p53 is the most complex and least understood of its functions. There are multiple and frequently overlapping apoptotic pathways downstream of p53, and studying one or several of them may not be of significant value. Fortunately, all apoptotic pathways converge at the point of irreversible commitment to apoptosis. This can be quantified by measuring an established apoptotic marker such as annexin V reactivity. Treatment of HCT116 and RKO cells with the active enantiomer of nutlin-3 for 48 h induced apoptosis in a substantial

fraction of the cell population that is p53-dependent, since the inactive enantiomer did not contribute to apoptosis (Fig. 6). The apoptotic fraction in etoposide-treated cells was slightly higher, while doxorubicin induced massive apoptosis (90%) in both cell lines. We found that a significant fraction of the apoptosis induced at high doses was p53-independent by comparing the apoptotic fractions in isogenic variants containing (RKO) and lacking (RKO-R) a functional p53 pathway (Fig. 8). If the apoptotic activity of doxorubicin and etoposide in RKO-R cells is subtracted from their activity in the parental RKO cell line, the residual activity should represent the p53-dependent component of the apoptotic activity of both drugs. The maximal residual (p53-dependent) activity for both doxorubicin and etoposide is equal or lower than the activity of nutlin-3a. This result suggests that the p53-dependent apoptotic activity of nutlin-induced unphosphorylated p53 is equal or better than the activity of phosphorylated p53 induced by doxorubicin or etoposide. However, one needs to acknowledge that such normalization for p53-independent activity could only be used as an approximation, since both p53-dependent and -independent mechanisms may utilize the same apoptotic pathways. Despite the possible inaccuracy, to our knowledge, this is the first attempt to reveal differences in the apoptotic activity of unphosphorylated and phosphorylated p53. Further studies, however, are needed to assess the possible effect of phosphorylation on transcription-independent apoptotic activity of p53.

Comparison of the activities of p53 phosphorylated on six key serine residues in response to genotoxic insult with unphosphorylated p53 induced by MDM2 antagonists revealed that phosphorylation status of p53 does not affect the main functions of the tumor suppressor. Unphosphorylated p53 is indistinguishable from phosphorylated p53 by its ability to bind DNA in a sequence-specific manner and activate transcription of target genes and apoptosis *in vivo*. This study confirms and extends previous observations (24, 42) using an experimental system that allows assessing p53 function in its natural cellular context and reinforces the notion that phosphorylation is dispensable for activation of p53 as a transcription factor. The data also demonstrate the importance of separating MDM2 from p53 as a key requirement for p53 activation. They raise the question of how this is achieved *in vivo* in light of studies showing that p53 mutants that cannot be phosphorylated can nonetheless be activated equivalently to wild-type p53 (24). Recent studies imply that a key event may actually be the damage-induced phosphorylation of MDM2 itself, which triggers the preferential degradation of MDM2. This provides an effective and rapid means for removing MDM2 from p53 *in vivo* and for enabling p53 to engage the transcriptional apparatus for target gene induction (6).

The possibility still remains that phosphorylation of p53 may affect p53 activity independent of transcription (e.g. in p53-dependent apoptotic pathways). However, previously published data from our laboratory does not support this possibility. Treatment of the SJSA-1 osteosarcoma cell line with 10  $\mu$ M nutlin-3a leads to massive apoptotic death (28) comparable with that in doxorubicin-treated RKO cells (Fig. 5). This higher responsiveness to MDM2 antagonists is most likely due to the fact that *mdm2* gene amplification in SJSA-1 cells is the only defect in the p53 pathway, while cell lines with normal MDM2 level (e.g. HCT116 and RKO) are likely to have defects in the p53-dependent apoptotic pathways.

The conclusion that p53 phosphorylation is not essential for execution of its major functions in the p53 pathway has one important implication for cancer therapy. We have shown that MDM2 antagonists can stabilize p53 and inhibit tumor growth in mouse xenografts models of human cancer (28). However,

these drugs are non-genotoxic and do not cause phosphorylation of p53. If p53 phosphorylation is important for modulation of its transcriptional activity then the effectiveness of MDM2 antagonists may be limited. Our data support the utility of MDM2 antagonists as single therapeutic agents in treating tumors with wild-type p53 that have retained intact signaling downstream of p53.

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