The wild type p53 tumor suppressor protein is rapidly degraded in normal cells by MDM2, the ubiquitin ligase that serves as the key regulator of p53 function by modulating protein stability. Cellular exposure to genotoxic stress triggers the stabilization of p53 by multiple pathways that converge upon interference with MDM2 function. In this study, we first investigated the ability of HDM2 (MDM2 human homologue) to degrade endogenous p53 in neuroblastoma (NB). Although the p53 protein in NB has been reported to be constitutively stabilized, we find that HDM2 in NB is functional and facilitates the rapid turnover of p53 in nonstressed cells via the proteasome pathway. Second, we examined the relationship between p53 and HDM2 in the adriamycin-mediated stabilization of p53 in NB. We demonstrate that while p53 stabilization depends neither upon the phosphorylation of specific N-terminal sites nor upon dissociation from HDM2, it requires inactivation of functional HDM2. In support of this notion, p53 stabilization following adriamycin resulted in an inhibition of both p53 ubiquitination and HDM2 ligase activity. Taken together, these data implicate a requirement for enzymatic inactivation of HDM2 as a novel mechanism for p53 stabilization in the DNA damage response pathway.

The p53 tumor suppressor protein is normally expressed at low levels due to rapid degradation by the ubiquitin-proteasome pathway, but p53 is stabilized in response to various cellular stress stimuli (1–4), resulting in nuclear translocation and transactivation of numerous target genes. One of the p53-induced genes is the ubiquitin ligase HDM2 (5), which acts as a key regulator of p53 activity through its ability to target the p53 protein for proteasomal degradation (6). Thus, p53-mediated up-regulation of HDM2 gene expression creates a negative regulatory loop, whereby HDM2 inactivates p53 and promotes its destruction, presumably after p53 has exerted its cellular functions.

In addition to its role as a ubiquitin protein ligase for p53, HDM2 has also been implicated in facilitating p53 nuclear export, an activity that appears necessary for its ability to degrade the protein (7, 8). DNA damage-induced nuclear translocation of p53 protects the protein from the destabilizing cytotoxic effects of HDM2. Stabilization of p53 can also occur by inhibition of p53-HDM2 complex formation, which is proposed to be negatively regulated by p53 phosphorylation at N-terminal sites (9–11). However, at least two reports demonstrate normal stabilization of p53 mutated at multiple phosphorylation sites (12, 13), suggesting that additional mechanisms may also be utilized. Indeed, HDM2 nucleolar sequestration mediated by p14arf and stress-induced down-regulation of HDM2 are alternative mechanisms resulting in p53 stabilization (14–17).

There is much interest in studying the pathogenesis of neuroblastoma (NB), since this disease is among the most common of pediatric malignancies. Unlike the majority of other tumors, NB is unusual in that the p53 protein is wild type (WT) in the overwhelming majority of cases (18); however, the protein is reported to be abnormally stabilized and overexpressed (19). p53 has also been reported to be primarily cytosolic in NB (20), presumably due to hyperactive nuclear export (21). Neuroblastoma is additionally complex, in that tumors are composed of a mixture of neuroblastic (N-type) and Schwannian (S-type) cell morphologies that differ in tumorigenicity (22) and in p53 subcellular localization (23). Taken together, these observations suggest that subversion of p53 function by mechanisms independent of mutation potentially play a role in the progression of NB.

In this study, we utilized a panel of NB cell lines to examine the relationship between p53 stability and HDM2 function, and we further investigated whether the p53 protein present in N- and S-type cells is equally susceptible to HDM2-mediated degradation. In addition, we examined the dependence for, and regulation of, HDM2 in the stabilization of p53 following treatment with the DNA-damaging agent adriamycin (adr). We conclude that the p53 protein from both NB cell types is inherently unstable, is sensitive to HDM2-dependent degradation, and can be further stabilized by the agents adr, ALLnL, and leptomycin B (LMB). We also provide evidence that N-terminal phosphorylation of p53 and its dissociation from HDM2 may be dispensable for p53 stabilization in NB in response to DNA damage. Instead, we suggest that direct inactivation of HDM2 ubiquitin ligase function may contribute significantly to the stabilization of p53.

EXPERIMENTAL PROCEDURES

Cell Culture—NB cell lines were cultured in Dulbecco’s modified Eagle’s medium/F-12 (1:1) (Biofluids) supplemented with 10% fetal bovine serum plus 1× nonessential amino acids and penicillin/streptomycin and incubated in a humidified chamber at 37 °C with 5% CO2. The cell lines SHEP1, KCN69N, and KCNB1 have been described

1 The abbreviations used are: NB, neuroblastoma; ALLnL, N-acetyl-Leu-Leu-norleucinal; adr, adriamycin; LMB, leptomycin B; CHX, cycloheximide; WT, wild type; IR, ionizing radiation; GFP, green fluorescent protein; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

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p53 Turnover and HDM2 Activity in NB

FIG. 1. p53 turnover is regulated by the proteasome in NB. Cells were treated with proteasome inhibitors for the indicated times and subjected to Western analysis as described. A, immunoblot analysis of p53 from the matched pairs (S-type (S) and N-type (N)), SHEP1 (S), SHSY5Y (N), KCNU (S), and KCN6N9 (N), following treatment with 50 μM ALLnL. Immunofluorescence of p53 was performed with DO-1. B, immunoblot of p53 from the above lines, in addition to LAN5 (N), following treatment with 10 μM lactacystin.

previously (23) and were kindly provided by Dr. R. A. Ross and B. A. Spengler (Fordham University, Bronx, NY). For these experiments, WT p53-containing SHSY5Y was obtained from ATCC. LAN5 cells were originally obtained from Dr. R. C. Seeger (Children’s Hospital, Los Angeles, CA), and p53 null Saos-2 cells were obtained from the ATCC (Manassas, VA). MDM2-deficient MEF cells were kindly provided by Dr. Allan Bradley (HHMI, Baylor College, Houston, TX).

Cell Transfections and Treatments—Cells were treated with either 50 μM ALLnL (Sigma), 10 μM lactacystin (Biomol), 0.2 μg/ml adriamycin (Sigma), or 1 μg/ml LMB (generously provided by M. Yoshida, University of Tokyo, Japan) for the indicated times. For GFP transfections, cells were seeded in 75-cm² flasks at 60% confluency 24 h prior to transfection with Fugene (Roche), according to the manufacturer’s specifications, and cells were lysed as described 24 h following transfection. Where indicated, 0.5 μg of GFP vector (CLONTECH) was included to normalize for transfection efficiencies. Expression constructs expressing either WT p53 (24) or the p5322,23 mutant (25) were subcloned into the GFP expression vector to create GFP-p53 fusion proteins with the GFP tag at the N terminus of p53.

p53 Half-life Determination—To measure p53 stability, cells were plated into six-well plates and treated with 40 μg/ml cycloheximide for the indicated times. For half-life determination following adriamycin treatment, cells were treated for 6 h with 0.2 μg/ml adriamycin, at which time cycloheximide was added for the indicated times. Densitometric analysis of the p53 signal was determined, and protein half-life was calculated using NIH Image software. The quantitated values were graphed on a semilog scale to derive the half-life for p53.

Metabolic Labeling—Cells were washed with methionine-free medium containing diazoylated fetal bovine serum and then incubated in this serum for 1 h, whereupon 150 μCi/ml [35S]methionine/cysteine (Tran35S-label; ICN) was added for 2 h. After the labeling period, CHX (40 μg/ml) was added, as indicated, and the cells were lysed as described after the designated times.

Immunoprecipitation and Immunoblotting—Cells were solubilized in 1× lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were sonicated, clarified by centrifugation, and quantitated for protein, and 30–40 μg of protein was subjected to immunoblot analysis. Blots were incubated with the appropriate primary antibody and developed with a 1:3000 dilution of horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) followed by ECL detection (Pierce). For endogenous p53 detection, a 1:1000 dilution of anti-p53 monoclonal antibody DO-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. For p53 immunoprecipitations, lysates were incubated overnight with a 1:1 mixture of agarose-conjugated 1801 and DO-1 antibodies (Santa Cruz Biotechnology). For HDM2 immunoprecipitation and detection, 1 mg of lysate was incubated with agarose-conjugated SMP14 (Santa Cruz Biotechnology) overnight. The resultant blots were developed with either a 1:200 dilution of 4811 hybridoma supernatant, kindly provided by Arnold Levine (The Rockefeller University, NY) or a 1:500 dilution of Ab-1 (Oncogene Science). For p53 detection following p53 or HDM2 immunoprecipitation, reducing agent was omitted to prevent interference of the IgG antibody heavy chain. GFP antibody (CLONTECH) was utilized at a 1:1000 dilution.

Phosphorylation-specific p53 Antibodies and Phospho-p53 Detection—Rabbit polyclonal antibodies specific for phospho(p53 phosphorylated at Ser6, Ser7, Ser15, Thr18, Thr20, Ser20, Ser22, Ser27, Ser32, Ser33, Ser392) have been described previously (26–28). Cells were either untreated (control) or continuously treated for 8 h with 0.2 μg/ml adriamycin and lysed in ice-cold buffer, and 1.2 mg lysate was immunoprecipitated with p53 antibodies as described (26). The resulting samples were split into four aliquots, each subject to separate immunoblot analysis.

Cell Fractionation—Cells were treated as described, washed with cold PBS, overlaid with low salt lysis buffer (20 mM HEPES, 5 mM NaCl, 5 mM MgCl2, 0.1% Nonidet P-40, 1 mM dithiothreitol, protease inhibitors, and incubated on ice for 5 min. The cells were scraped into microcentrifuge tubes and spun at 3000 rpm 4 min. The cytoplasmic supernatant was removed, and the nuclear pellet was washed with low salt lysis buffer. The nuclei were lysed in hypertonic lysis buffer (1× lysis buffer containing 400 mM NaCl and sonicated, and both fractions were clarified by centrifugation. To verify the integrity of the fractionation, the blots were probed with a 1:500 dilution of topoisomerase II antibody (Oncogene) and a 1:2000 dilution of tubulin antibody (Sigma), representative of nuclear and cytosolic proteins, respectively.

Immunofluorescence—Cells were grown on chamber slides and fixed with cold acetone as described (23). For p53 immunofluorescence, antibodies were used at a concentration of 1 μg/ml and incubated with Cy3-conjugated secondary antibody (Sigma) diluted 1:750. For visualization of nuclear fluorescence, slides were treated for 30 min with 100 μg/ml RNase, incubated for 2 min with 20 μg TOPRO-3 (Molecular Probes, Inc., Eugene, OR), washed with phosphate-buffered saline, and mounted. Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a ×60 lens. Images were processed using LaserSharp software (Bio-Rad). The total magnification is ×80 (objective) times 3 times the zoom factor (1 or 1.5, as specified); and scale bars are shown.

In Vitro Ubiquitin Ligase Assay—Cells were treated for 6 h with either ALLnL or add, and 2.5 μg of protein was immunoprecipitated with SMP14, as described. As a negative control, MDM2 immunoprecipitations were performed from MDM2 null MEF cells. To examine intrinsic HDM2 activity, reactions were carried out in the presence of E1 enzyme (Calbiochem), bacterially expressed wheat UbcH5b (E2), ubiquitination buffer, and either unlabeled or 32P-labeled ubiquitin, as described (29). The samples were subjected to SDS-PAGE and visualized by either autoradiography or immunoblot analysis. For detection of high molecular weight ubiquitinated HDM2 species, Ab-1 (Calbiochem) was used at a concentration of 1 μg/ml.

RESULTS

Proteasome Inhibitors Stabilize WT p53 in NB—In NB, the p53 protein is reported to be stabilized (19) and overexpressed (20) in the absence of mutation, suggesting a dysregulation in its degradation pathway. Furthermore, our prior work suggested that p53 in neuroblastic N-type cells might be more stable and highly expressed than p53 from Schwannian S-type cells (23). We have since sequenced genomic p53 from the stabilized N-type lines SHSY5Y and BE1N, and we unexpectedly discovered point mutations. The details of this study will be presented elsewhere. For the current report, we employed only WT p53-containing cell lines, and we further confirmed that the p53 in LAN5 is WT by sequencing exons 2–11. To further determine whether the p53 protein in N- and S-type lines is equally capable of being efficiently degraded by the proteasome, we treated two matched S/N pairs with the general proteasome inhibitor ALLnL. As shown in Fig. 1A, the p53 protein from both cell types was maximally induced by a 4-h exposure to ALLnL. To confirm that this stabilization was mediated by the proteasome, we treated the same cell lines plus the neuroblastic LAN5 line with the highly specific proteasome inhibitor lactacystin (30). As shown in Fig. 1B, the p53 protein from all of the cell lines was readily stabilized by this.
treatment, thereby demonstrating that the p53 protein in NB is indeed a proteasomal substrate, in accordance with a recent report (31).

Multiple p53-stabilizing Agents Promote Nuclear Accumulation of p53—The p53 protein in NB has been reported to be primarily cytosolic (32), presumably due to hyperactive nuclear export (21). The prevailing notion is that nuclear translocation of p53 following cellular stress allows the protein to escape from MDM2-mediated degradation, which requires the cytosolic localization of both proteins (7, 8). Therefore, it was of interest to determine whether p53 stabilization in NB occurs concomitantly with nuclear translocation. Cells were treated with ALLnL or LMB, an antibiotic that inhibits CRM1 receptor-mediated nuclear export of a large number of proteins, including p53 (33, 34). By examining p53 levels in purified nuclear and cytoplasmic subcellular fractions, we observed that both ALLnL and LMB significantly increased the nuclear accumulation of p53 in all cell lines relative to untreated controls (Fig. 2A), in accordance with previous reports (21, 35). We did not anticipate the nuclear translocation of p53 following ALLnL, since this agent is not thought to mediate a DNA damage response, although similar data have very recently been reported in NB (36) and other cell lines (37). We also examined the effect of the DNA-damaging agent adr on nuclear p53 accumulation and obtained results similar to that with LMB (Fig. 2B and data not shown). These results demonstrate that the p53 stabilizing agents ALLnL, adr, and LMB all promote the nuclear translocation and accumulation of p53 in both cell types.

To confirm these biochemical observations, the subcellular localization of p53 was examined by fluorescence microscopy following treatment with either adr or LMB (Fig. 2B). We and others have previously demonstrated the apparent cytosolic localization of p53 in neuroblastic lines when utilizing the monoclonal antibody PAb1801 (21, 23, 32). Although this antibody was able to detect p53 nuclear translocation in S-type cells, it did not efficiently detect nuclear p53 in N-type cells (data not shown), in contrast to our immunoblot data (Fig. 2A). This result suggests that certain p53 epitopes may be inaccessible or masked in certain cell lines, as has been reported for p53 in NB (38). In support of this notion, at least two reports demonstrate nuclear p53 in NB (39, 40). In support of this notion, at least two reports demonstrate nuclear p53 in NB (39, 40). Therefore, we repeated this experiment using the monoclonal antibody DO-1, which recognizes an alternative N-terminal epitope of p53. As shown in Fig. 2B, DO-1 clearly detects the up-regulation and nuclear accumulation of WT p53 in both N and S-type cells. Specificity is demonstrated by the lack of staining in the p53 null line Saos-2 (Fig. 2B, a) and the strong signal detected in mutant p53-containing SKBR3 cells (Fig. 2B, b). These data demonstrate that various p53-stabilizing agents facilitate the nuclear accumulation of WT p53, which can be readily detected with the
DO-1 antibody. In addition, we demonstrate that antibody choice can dramatically influence the apparent subcellular localization of the protein in NB.

The Rapid Degradation of p53 in NB Is Inhibited by Adriamycin—At least one previous report suggests that the p53 protein in NB is inherently stabilized, with a half-life on the order of several hours (19). However, our results in Fig. 1B suggest that a 2-h treatment with lactacystin is sufficient to significantly elevate p53 levels. In general, the ability of several agents to stabilize p53 suggests that the protein is normally labile. To further examine this issue, cells were treated with the protein synthesis inhibitor CHX to determine the half-life of p53. As shown (Fig. 3B), the p53 protein in all NB lines decreased rapidly after even a short incubation (10 min) with CHX. The half-life of p53 in these lines fell within the range of 10–25 min, and by 1 h, the majority of the p53 protein was degraded.

We have previously reported the half-life of p53 in SHEP1 to be almost 2 h (23), which is in marked contrast to the value of...
19 min as determined with CHX treatment. However, the former calculation was determined following prolonged exposure to radioisotope. We therefore considered whether the addition of radioisotopic label to cells was sufficient to induce a DNA damage response, resulting in the stabilization of p53. We chose to analyze LAN5 cells, since the p53 from this cell type was the most unstable of all of the examined NB lines (Fig. 3A), yet its half-life as determined by metabolic labeling has been reported to be well over 6 h (19). Therefore, LAN5 cells were treated for 2 h with 150 μCi/ml [35S]methionine, which corresponds to the incubation period utilized for the half-life determination both for our prior work (23) and for the previous report (19). Following this incubation period, cells were treated with CHX as in Fig. 3B. Immunoblot analysis of p53 reveals that the presence of radioisotope significantly prolonged the half-life of LAN5 to over 4 h. From these data, we conclude that the p53 protein in NB is subject to rapid turnover and that DNA-damaging agents or metabolic labeling stabilize the protein by extending its half-life severalfold.

Adriamycin Inhibits the Ubiquitination of p53 in NB—The stabilization of p53 in response to adr indicates that mechanisms exist to confer resistance to HDM2-mediated degradation in NB. To investigate this issue, we examined whether the p53 in NB was capable of being ubiquitinated and targeted for degradation by HDM2. Cells were treated with the proteasome inhibitor ALLnL, and p53 immunoprecipitates were immunoblotted with p53 (Fig. 4A). We show that while ubiquitinated p53 ladders are barely detectable from p53 immunoprecipitates in control cells, high molecular weight p53 ladders become prominent following ALLnL treatment. The ability of p53 in NB to become ubiquitinated in the presence of ALLnL is in agreement with a previous report (31) and further supports the notion that HDM2 is responsible for p53 degradation. We confirmed that these bands represented ubiquitinated species by probing the immunoprecipitated p53 with an anti-ubiquitin antibody (data not shown).

The stabilization of p53 following genotoxic stress often correlates with decreased ubiquitination of the protein, as has been observed following UV radiation (44). Therefore, we first sought to investigate whether the adr-mediated stabilization of p53 correlated with its decreased ubiquitination. Cells were treated with ALLnL, adr, or LMB, and the levels of ubiquitinated p53 protein associated with HDM2 were measured. Since this approach detects ubiquitinated species associated with HDM2, it serves to more directly assess the ability of HDM2 to ubiquitinate p53 in response to various treatments. As depicted in Fig. 4B, the p53 protein co-precipitated with HDM2 in SHEP1 and SHSY5Y is visibly ubiquitinated following treatment with ALLnL (lane 2) relative to untreated controls (lane 1), and this is comparable to the levels of ubiquitinated p53 from p53 immunoprecipitates (Fig. 4A). The amount of ubiquitinated WT p53 observed following LMB treatment is also increased relative to untreated cells (Fig. 4B, lane 4), although the levels are less than that observed with ALLnL. This suggests either that some of the residual cytosolic p53 is susceptible to proteasome-mediated degradation in the presence of LMB, a contention supported by the inability of LMB to completely inhibit p53 degradation in the presence of HPV E6 (7), or that a portion of the p53 is ubiquitinated in the nucleus, as several reports have suggested (45, 46). However, in marked contrast to ALLnL and LMB, treatment of cells with adr almost completely inhibited p53 ubiquitination (Fig. 4B, lane 3). To verify this result and to ensure that the adr-mediated decrease in ubiquitinated p53 was not due to p53-HDM2 complex dissociation, this experiment was repeated with p53 immunoprecipitates. As shown in Fig. 4C, while treatment with ALLnL resulted in ubiquitinated p53 species (lane 1), treatment with either adr alone (lane 2) or adr in combination with ALLnL (lane 3) significantly diminished the levels of ubiquitinated protein. These data therefore support the hypothesis that adr stabilizes p53 by preventing its ubiquitination.

Adriamycin-mediated p53 Stabilization Is Not Due to HDM2 Down-regulation and Is Not Dependent upon Dissociation of p53-HDM2 Complexes—We next explored the mechanism for the adr-mediated stabilization of p53 in NB and investigated the potential role of HDM2 in this pathway. Inhibition of p53 ubiquitination following adr could be explained by a down-regulation of HDM2 protein levels, which has been demonstrated following UV treatment (16, 47), hypoxia (48), or exposure to topoisomerase inhibitors (15). We therefore examined HDM2 expression levels in N- and S-type NB cells in response to the stabilizing agents used in Fig. 4B. As shown in Fig. 5A (top panel), levels of HDM2 are very low in untreated NB cells but generally increase in response to ALLnL (lane 2), adr (lane 3), and LMB (lane 4). The one exception occurs in KCNBU following adr, which was unexpected, since we previously demonstrated up-regulation p53 activity following IR in these cells (23). However, in four of five cell lines tested, the stabilization of p53 paralleled an increase in HDM2 levels.

Dissociation of p53-HDM2 complexes following DNA damage has also been recognized as a mechanism resulting in p53 stabilization (9–11, 49). However, the data in Fig. 4B suggest that the adr-mediated p53 stabilization observed in SHEP1 and SHSY5Y occurs without significant p53-HDM2 complex dissociation. To further explore this relationship, we determined the relative amount of p53 associated with HDM2 for each of these treatments. As shown in Fig. 5A (middle panel), essentially no p53 is detected from HDM2 precipitates in un-
treated cells (lane 1), due to rapid HDM2-mediated p53 degradation. However, there is a marked increase in the p53 associated with HDM2 following ALLnL treatment (lane 2). In fact, ALLnL treatment produced the maximum amount of p53 complexed with HDM2 in all lines tested. LMB treatment also increased the amount of p53 bound to HDM2 (lane 4) relative to control samples. The situation is more complex for the adr-treated samples (lane 3). While significant levels of p53 from SHEP1, SHSY5Y, and KCNBQ remained associated with HDM2, there was a significant dissociation of p53 from HDM2 in both KCNBQ and LANT5 following adr. For comparison, the amount of p53 immunoprecipitated from whole lysates is shown in the lower panel. These results suggest that adr-induced p53 stabilization may be accompanied by, but does not require, HDM2 dissociation.

The amount of p53 co-precipitated with HDM2 following treatment with adr (Fig. 5A, middle panel, lane 3) was less than the amount co-precipitated following treatment with ALLnL (lane 2); however, these treatments stabilized and elevated p53 levels to approximately the same extent (Fig. 5A, lower panel, lanes 2 and 3). Another possibility to explain this difference, independent of p53-HDM2 dissociation, would be differential stabilization of HDM2. To explore this possibility, SHSY5Y cells were treated with either ALLnL or adr for 6 h, followed by treatment with CHX (Fig. 5B). As shown, while adr treatment (lower panel) elevated HDM2 to levels comparable to those following treatment with ALLnL (upper panel), the stability of HDM2 in the adr-treated cells was considerably shorter than from the ALLnL-treated cells. These results suggest that less p53 is associated with HDM2 from adr-treated cells relative to ALLnL-treated cells due to the increased liability of HDM2 in the former.

The data thus far support the notion that p53 turnover in NB is quite rapid and that this liability depends upon HDM2. However, a recent report suggests that the p53 in NB is resistant to HDM2-mediated degradation (31), despite the formation of p53-ubiquitination conjugates following proteasome inhibition. This interpretation was based on data derived from cotransfection of WT p53 and either mutant or WT HDM2. We therefore sought to establish that normal HDM2 function is a requirement for p53 degradation in NB. To circumvent potential difficulties arising from suboptimal ratios of DNA introduced during cotransfection, SHHSY cells were transfected with either GFPwt/p53 or GFPmt/p5322,23 expression constructs, the latter of which is unable to associate with HDM2, resulting in a stabilized protein (49). As shown in Fig. 5C, the transfected WT protein is barely detectable in control cells (upper panel, lane 1), while the mutant protein is expressed at much higher levels. In addition, while the transfected WT protein is stabilized following adr or ALLnL treatment, the mutant protein is not further stabilized by these treatments, resulting in high constitutive expression. Taken together, these data demonstrate the functionality of endogenous HDM2 in these cells, that HDM2 activity is required for the rapid turnover of p53 in nonstressed NB cells, and that inhibition of this activity leads to protein stabilization.

Our results demonstrating HDM2-dependent activity in the degradation of p53 are in disagreement with results demonstrating that introduction of excess HDM2 does not reduce the levels of cotransfected p53 (31). While this discrepancy may appear difficult to reconcile, one possibility involves the relative amount of p53 associated with HDM2 at any given time. For instance, if only a small percentage of p53 is complexed with endogenous HDM2 at steady state, then introduction of excess exogenous HDM2 should have no further effect upon p53 levels, since HDM2 levels would not be the rate-limiting step for p53 degradation. Indeed, when we compared the percentage of p53 associated with HDM2 following ALLnL treatment relative to the total amount of p53 in the cell, we found that ALLnL significantly increased the overall levels of total immunoprecipitated p53 from cells (Fig. 5D). However, HDM2 immunoprecipitation of equivalent amounts of protein revealed that only a small percentage (less than 25%) of total cellular p53 is actually associated with HDM2 following this treatment.

N-terminal p53 Phosphorylation following Adriamycin Treatment Does Not Correlate with Dissociation from HDM2—The results thus far suggest that HDM2 is functional and responsible for the degradation of endogenous p53 in NB. However, arguments have been made suggesting that the p53 protein in NB is subject to posttranslational modification, resulting in a more acidic protein (31). Such modification of p53 has been suggested to be responsible for p53’s inability to be further
and p53 was immunoprecipitated as in A, cells were treated for 8 h with 0.2 μg/ml adr, p53 was immunoprecipitated with a mixture of PAb1801 and DO-1, and immunoblot analysis was performed with phosphospecific p53 antibodies as described. B, cells were either left untreated (lane 1) or treated with ALLnL for 8 h (lane 2) or with adr for 8 h (lane 3), and p53 was immunoprecipitated as in A.

Fig. 6. Wild type p53 in NB is multiply phosphorylated in response to adriamycin. A, cells were treated for 8 h with 0.2 μg/ml adr, p53 was immunoprecipitated as in A, and p53 was extremely responsive to adr and showed a large phosphorylation in each cell line. While the p53 from SHEP1 and SHSY5Y were either left untreated or treated with ALLnL or adr, and p53 was detected as in Fig. 6A. It is clear from the signal obtained with DO-1 that the levels of cellular p53 induced by either ALLnL or adr are roughly comparable. However, despite the elevation of p53 levels produced by ALLnL treatment, essentially no phosphorylation of p53 at any sites is observed. This confirms the notion that the absence of signal at multiple sites in control cells indicates the hypophosphorylated status of the endogenous protein and further demonstrates that a DNA damage signal is necessary for the induction of p53 phosphorylation in NB.

p53 Stabilization following Adriamycin Correlates with HDM2 Inactivation—The data thus far suggest that there are no unusual phosphorylation events that might render the p53 protein inherently resistant to HDM2-mediated degradation in NB. We therefore turned our attention to the activity of HDM2 in these cells. While our data strongly suggest that HDM2 is responsible for the degradation of p53 in NB, it remains unclear how the p53 protein escapes HDM2-mediated ubiquitination following adr. We speculated that adr itself may inhibit the function of HDM2, resulting in decreased ubiquitinated p53 species (Fig. 4, B and C). There is precedent for this, given a recent report demonstrating decreased HDM2 ligase activity correlating with increased p53 stability following UV treatment (16). In addition, another recent report has documented the rapid phosphorylation of MDM2 following IR (53), which may modulate its function prior to the onset of p53 stabilization.

Therefore, we directly examined HDM2 ubiquitin ligase activity in ALLnL- or adr-treated cells. First, endogenous HDM2 was immunopurified from control or treated SHSY5Y cells. To ensure that equal amounts of HDM2 were immunoprecipitated, the levels of HDM2 were quantitated by immunoblot analysis. As shown in Fig. 7A (bottom panel), comparable amounts of HDM2 were immunoprecipitated from treated cells.
A longer exposure of this same blot (upper panel) reveals high molecular weight HDM2 species in ALLnL-treated cells that are virtually absent in the adr-treated sample. These high molecular weight ladders are suggestive of HDM2 ubiquitin conjugates and are consistent with HDM2’s ability to ubiquitinate itself (29). To further demonstrate that these bands represent autoubiquitinated HDM2, identical aliquots of immunopurified HDM2 from SHSY5Y served as the E3 enzyme in an in vitro ubiquitination assay to which purified E1, E2 (UbcH5b), and unlabeled ubiquitin protein were added. As shown in Fig. 7B, the addition of ubiquitin significantly increased the high molecular weight HDM2 species, indicative of autoubiquitination, when HDM2 was immunopurified from ALLnL-treated SHSY5Y cells, while there was no signal from MDM2-deficient MEF cells. Strikingly, the addition of ubiquitin to HDM2 immunopurified from adr-treated cells was not able to promote the appearance of high molecular weight HDM2 ladders, providing compelling evidence that adr treatment inhibits the ubiquitin ligase activity of HDM2. This experiment was repeated in LAN5 cells, since, unlike in SHSY5Y, adr facilitated p53-NDM2 complex dissociation in this line. Similar to SHSY5Y, the presence of adr markedly inhibited HDM2 autoubiquitination in LAN5.

To confirm that these high molecular weight HDM2-immunoreactive bands were reflective of intrinsic HDM2 ubiquitin ligase activity, we repeated the in vitro ubiquitination assay using 32P-labeled ubiquitin in place of unlabeled ubiquitin, and the blot was probed with HDM2 Ab-1. The lower panel represents a shorter exposure of the same blot, demonstrating equivalent HDM2 immunoprecipitation. B, SHSY5Y or LAN5 cells were treated with either ALLnL or adr, and HDM2 was immunodetected as in A. These immunoprecipitates were utilized in an in vitro ubiquitin ligase assay, as described, in the presence or absence of unlabeled ubiquitin and purified E1 and E2 proteins. Hdm2 MEFs were utilized as a negative control. C, the experiment in B was repeated using [32P]ubiquitin, and ubiquitinated species were visualized by autoradiography.

A discussion of the main purposes of this study was to elucidate the involvement of HDM2 in p53 turnover in NB and to characterize the mechanism(s) promoting p53 stabilization following DNA damage. While exploring these fundamental questions, we were also interested in determining whether these stabilization mechanisms were similar in both N- and S-type cell lines. Our data demonstrate that the p53 protein from both N- and S-type NB cells is inherently unstable due to efficient proteasome-mediated degradation that is dependent upon normal HDM2 function. First, we detect essentially no WT p53 complexed with HDM2 in untreated cells, supporting our contention that p53 is rapidly degraded by HDM2 in these cells. Second, we find maximal levels of ubiquitinated cellular p53 as well as ubiquitinated p53 complexed with HDM2 in the presence of ALLnL, thereby demonstrating that these unstable ubiquitinated species are normally targeted for rapid degradation and can be detected only upon inhibition of the proteasome. Third, transfected mutant p53 unable to bind HDM2 is intrinsically stabilized, while transfected WT protein is further stabilized by either ALLnL or adr, thereby demonstrating a requirement for HDM2 function in p53 turnover in NB. Finally, treatment of cells with adr concomitantly inhibits both p53 ubiquitination and HDM2 ligase activity, thereby further demonstrating a requirement for HDM2 activity in the turnover of p53 in NB.

We recognize that our present results are in contrast to some of our prior work (23) as well as to two other reports (19, 31), and we offer several explanations to account for these differences. One resolved issue concerns the p53 genetic status of the SHSY5Y cells used in our prior work (23). As previously mentioned, the p53 gene from cells used in those experiments contained a previously unrecognized point mutation that rendered the protein stable. Another issue involves the discrepant values for p53 half-life in NB. In the work reported herein, the half-life of p53 in all cell lines examined was less than 25 min; however, we previously reported the half-life of SHEP1 to be on the order of 120 min (23). In the present study, p53 half-life was determined using CHX to inhibit protein synthesis, whereas in the former work (19, 23), p53 half-life was determined by radioisotopic labeling. We demonstrate here that the use of radioisotope in NB leads to a DNA damage response, as has been well documented for other cell types (41, 42). Therefore, previous studies of p53 half-life in NB have been influenced by the effects of radioisotope. However, our results do not preclude the possibility that the endogenous levels of p53 are higher compared with other cell lines due to stabilized and/or elevated levels of mRNA, as suggested by the fact that NB cells contain abundant p53 mRNA (19), or to better translation efficiencies. Our results also support the notion that the rapid degradation of p53 in NB is absolutely dependent upon normal HDM2 function. These results differ from those of another report (31). Zaika et al. (31) propose that p53 in NB is resistant to HDM2-
mediated degradation based on the result of the failure of exogenously added MDM2 to further degrade p53. However, in that work, only the steady state levels of p53 were examined, whereas calculation of half-life would provide a more quantitative measure of the effect of excess HDM2 upon p53 stability. In this work, we determined that the p53 in NB is not phosphorylated at any of the known sites, thereby eliminating this as a possible mechanism for any potential resistance of p53 to HDM2. Second, in agreement with Zaika et al. (31), we detected efficient HDM2-mediated ubiquitination of p53, suggesting that HDM2 can target p53 for degradation. While it is possible that a defect in the degradation of p53 could exist in the step following HDM2-mediated ubiquitination, prior to recognition by the proteasome, this possibility seems unlikely based on the rapid degradation of p53 in the NB lines and on the rapid response of p53 to proteasome inhibition. We instead propose that HDM2 is continuously degrading the p53 protein in NB; however, at any given time, only a small percentage of the total cellular pool of p53 protein is associated with HDM2. Therefore, if the concentration of endogenous HDM2 is not rate-limiting, introduction of excess exogenous HDM2 would not be expected to further diminish p53 levels.

In addition to examining the stability of p53 in nonstressed NB cells, we explored the mechanisms involved in the DNA damage-mediated stabilization of p53 in response to adr. Our previous data (23) agree with results from several groups (32, 40, 54) in demonstrating elevated p53 levels following IR, suggesting that the p53 in NB is competent to respond to DNA damage and that the protein can be further stabilized. We now demonstrate that adr treatment effectively elevates p53 levels in all NB cell lines tested and that this correlates with a severalfold increase in p53 half-life. Interestingly, adr-mediated stabilization of p53 is associated with inhibition of p53 ubiquitination in all lines. Two separate mechanisms that may account for this result are 1) inhibition of HDM2 association via p53 phosphorylation, or 2) direct disruption of HDM2 function by posttranslational modification or interaction with cellular proteins. Our data do not support the first possibility, since the WT p53 protein from five NB lines is uniformly stabilized following adr treatment, although significant dissociation from HDM2 is only observed in two of these five lines. These data strongly suggest that dissociation of HDM2-p53 complexes is not the only mechanism that mediates p53 stabilization following DNA damage. Even more surprising was the observation that in the NB lines undergoing adr-mediated p53-HDM2 complex dissociation, p53 is not hyperphosphorylated at any N-terminal sites proposed to play a role in this process, further limiting the importance of N-terminal p53 phosphorylation as a regulator of HDM2 association. At least two other reports have demonstrated p53 stabilization in response to DNA damage without a necessity for phosphorylation at multiple N-terminal sites (13, 15), further emphasizing that these sites are not necessary for regulation of p53-HDM2 interactions in all cell lines.

Our data demonstrating decreased p53 ubiquitination following adr support the hypothesis that direct inhibition of HDM2 activity serves as an additional mechanism to mediate p53 stabilization and further emphasize the necessity for HDM2 activity in the turnover of p53 in NB. Hdm2 function can be inhibited by interaction with a growing list of cellular proteins such as p14arf (55–57), c-Abl (58), Rb (59), MdmX (60), and the estrogen receptor (61). Alternatively, HDM2 activity can be inhibited by its nuclear localization (14, 17) or by down-regulation of HDM2 protein levels in response to cellular stimuli such as UV or IR (16, 47), hypoxia (48), and topoisomerase inhibitors (15). Our data utilizing endogenous HDM2 in an

\textit{in vitro} ubiquitin ligase assay clearly show that HDM2 isolated from ALLnL-treated NB cells is enzymatically active, while HDM2 isolated from adr-treated cells exhibits significantly less activity. Interestingly, adr inhibited HDM2 ligase function in LAN5 cells, in which dissociation of p53-HDM2 complexes was evident, as well as in SHSY5Y cells, which retain p53-HDM2 complexes. This suggests that inhibition of HDM2 may be as important or more important a determinant of p53 stabilization than its dissociation from p53. This finding thus uncovers a previously unrecognized mechanism by which DNA damage can regulate p53 stabilization.

To our knowledge, only one previous report has implicated direct effects of DNA damage upon HDM2 ubiquitin ligase activity (16). This study demonstrates a correlation between UV irradiation and decreased SUMO-1 conjugation to HDM2, resulting in a shift from p90 to p75 HDM2 species (16). Decreased sumoylation of HDM2 is also presumed to enhance the degradation of HDM2 by rendering the protein more susceptible to ubiquitination. However, our results suggest that a mechanism other than sumoylation is involved in regulating the adr-mediated inhibition of HDM2. We show that p53 stabilization following adr is accompanied by both p90 HDM2 down-regulation and increased HDM2 self-ubiquitination and degradation. In fact, our results demonstrate that elevated levels of p90 (presumably sumoylated) HDM2 may coexist with stabilized p53, implicating a direct inhibition of HDM2 activity by an alternative pathway. It is not yet clear whether the adr-induced inhibition of HDM2 ubiquitin ligase activity is due to a posttranslational modification of HDM2 or to stimulation of its interaction with another cellular protein. Regardless of the precise mechanism, inhibition of HDM2 ligase activity following adr offers a unifying explanation for our observations that p53 can be stabilized without a strict dependence upon its phosphorylation state or its association with HDM2.

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