MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins

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p300 acetylates and activates the tumor suppressor p53 after DNA damage. Here, we show that MDM2, a negative-feedback regulator of p53, inhibited p300-mediated p53 acetylation by complexing with these two proteins. First, we purified a p300–MDM2–p53 protein complex from HeLa nuclear extracts, which was inactive in p53 acetylation, but active in histone acetylation. Also, wild-type, but not N-terminally deleted, MDM2 inhibited p53 acetylation by p300 *in vitro* and *in vivo*. This inhibition was specific for p53, because MDM2 did not affect acetylation of histones or the C terminus of p73 by p300. Consequently, wild-type, but not the mutant, MDM2 repressed the p300-stimulated sequence-specific DNA-binding and transcriptional activities of p53. These results demonstrate that an additional mechanism of p53 inactivation by MDM2 is to inhibit p53 acetylation by p300.

In response to genotoxic agents, the tumor suppressor p53 is stabilized and activated as a transcriptional regulator, mainly inducing expression of genes critical for cell growth arrest and apoptosis (1, 2). Posttranslational modifications of p53 play an important role in regulating p53 stability and activity after DNA damage (3). Phosphorylation of p53, stimulated by UV and γ irradiation, prevents MDM2 binding and activates p53 activity (4–14). In addition to phosphorylation, acetylation plays a key role in modulating p53 function. It has been shown that p53 acetylation at several C-terminal Lys residues by p300 and possibly by P/CAF occurs after DNA damage (15), leading to activation of sequence-specific DNA binding of p53 (16) and transcriptional activities (17–19). Interestingly, it was also shown that p300 can form a complex with MDM2 *in vitro* and *in vivo* (20, 21), a negative regulator of p53 (22), and this complex was shown to facilitate MDM2-mediated p53 degradation (20). However, p300 was also shown to stabilize p53 after DNA damage (23). How p300 exerts two opposite effects on p53 in cells remains puzzling.

Two roles have been suggested for MDM2 in regulating p53 stability. First, MDM2 shuttles p53 from the nucleus to the cytosol (24). Blocking this shuttling by the tumor suppressor protein $p14^{arf}$ ($p19^{arf}$ for the mouse version) results in p53 accumulation (25–28). Second, MDM2 possesses an E3-like ubiquitin-transferase activity (29, 30), marking p53 as a target of cytoplasmic proteosomes for degradation (31, 32). Whether p300 is involved in these processes or not is unclear.

To elucidate the functional relationship between MDM2 and p300 in regulating p53 function, we characterized a nuclear MDM2-associated protein complex. First, we purified a ternary complex composed of p300, MDM2, and p53 from HeLa nuclear, but not cytoplasmic, extracts. Surprisingly, we found that this complex was unable to acetylate p53 but was still active in histone acetylation. Consistent with this observation, MDM2 inhibited p53 acetylation by p300 both *in vitro* and *in vivo*. Functionally, MDM2 blocked the ability of p300 to stimulate the sequence-specific DNA-binding and transcriptional activities of p53. Interestingly, UV and γ irradiation differentially regulate the MDM2 level, which is reciprocal to the level of p53 acetylation. These results demonstrate that formation of the p300–MDM2–p53 ternary complex leads to suppressing p53 acetylation and activation.

Materials and Methods

Purification of the MDM2-Associated Complex from HeLa Nuclear Extracts. HeLa cells (24 liters; $\approx 0.6 \times 10^{10}$) were harvested for preparation of nuclear extracts. Nuclear extracts were then fractionated through phosphocellulose (P11) and DEAE-Sepharose columns as described (33). MDM2 was monitored by using Western blot (WB) with polyclonal anti-MDM2 antibodies throughout the entire procedure. We detected MDM2 in the 0.5 M wash of a P11 column and 0.35 M fraction of the DEAE-Sepharose column. The immunoreacting fractions were pooled and dialyzed against buffer C [40 mM Tris·HCl, pH 8.0/20% glycerol (vol/vol)/1 mM DTT/0.1 mM EDTA/0.2 mM PMSF/0.5 μg/ml pepstatin A/2 μg/ml leupeptin/100 mM KCl]. Dialyzed proteins were loaded onto a HPLC HS-20 column (BioCad Sprint system). Proteins were eluted with a linear gradient from 100 to 700 mM salt in buffer C as described above. The MDM2 antibody-positive fractions were pooled and fractionated on an anionic exchange Mono Q column. Proteins were eluted with a linear gradient from 100 to 600 mM salt in buffer C. Fractions immunoreactive with anti-MDM2 antibodies were pooled and loaded on a sizing column Superdex 200 (SMART HPLC system; Amersham Pharmacia). Proteins in the fractions were analyzed by using acetylation assays as described below. MDM2 antibody-positive fractions were subjected to SDS/PAGE followed by silver staining.

Purification of Recombinant p300, MDM2, and p53. MDM2 and p300 were purified from baculovirus-infected SF9 insect cells by using immunoaffinity columns as described (21). His-p53 was purified from bacteria by using a Ni-NTA column as described (34).

p53 Acetylation Reaction. p53 acetylation assays were carried out according to the published method (16). Reaction mixture (20 μl) contained 50 mM Tris·HCl, pH 8.0/10% glycerol (vol/vol)/0.1 mM EDTA/1 mM DTT/10 mM Na butyrate/[1-^{14}C]acetyl CoA or 500 nM acetyl CoA (Sigma)/50 ng of p53, p300, and MDM2 (see figure legends for the amount of p300 or MDM2 used in each reaction). p300, p53, wild-type, and amino acids 58–89-deleted mutant MDM2 were purified as described (21). MDM2 was preincubated with p300 in ice for 30 min before being added into the reaction mixture containing acetyl CoA.

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Abbreviations: WB, Western blot; EMSA, electrophoretic mobility-shift assay.

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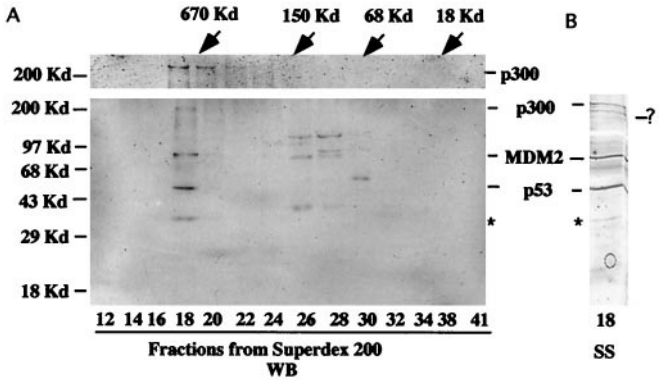

Fig. 1. Association of MDM2 with p300 and p53 in the nucleus. (*A*) Purification of the MDM2-associated complex from HeLa nuclear extracts. The MDM2-associated protein complex was purified from human HeLa nuclear extracts through chromatography as described in *Materials and Methods*. Fractions (25 μl) from the last Superdex 200 column were analyzed on SDS/PAGE, followed by WB by using antibodies against p300 (*Upper*), MDM2, and p53, respectively, in this order. Molecular weight markers at the top of *A* indicate the fractions where corresponding size markers coeluted. Fraction 18 was analyzed on SDS/PAGE and by silver staining, as shown in *B*.

The mixture was then incubated at 30°C for 60 min and analyzed on SDS/PAGE afterward. Acetylated p53 was detected by either autoradiography or WB by using the polyclonal antiacetylated Lys antibody purchased from Upstate Biotechnology (Lake Placid, NY), monoclonal anti-p53 antibody 421, polyclonal anti-MDM2 antibody, and polyclonal anti-p300 antibody from Upstate Biotechnology.

Electrophoretic Mobility-Shift Assay (EMSA). This assay was conducted as described (33). Proteins as indicated in figure legends were preincubated in the presence or absence of acetyl CoA at 30°C for 30 min as described above before being mixed with a DNA-binding mixture containing 10 mM Hepes, pH 7.5/4 mM $MgCl_2$/60 mM NaCl/0.1 μg of poly(dIdC)/0.1% Nonidet P-40/0.1 mM EDTA/5′/3′ ^{32}P-end-labeled DNA fragments harboring two copies of the p53RE sequence derived from the MDM2 promoter (35) (5,000 cpm; 1.0 ng DNA per assay). The reaction was incubated at room temperature for 30 min and directly loaded onto a 4% nondenatured gel.

Irradiation or Treatment of Cells. Human testicular carcinoma Tera-2 or murine embryonic testicular carcinoma F9 cells were irradiated with 10 and 20 J/m^2 UV, or 7 or 14 gray of γ ray. Irradiated cells were harvested 6 h postirradiation for immunoprecipitation-WB and EMSA analyses.

Immunoprecipitation-WB Analysis. Transfected or irradiated cells were harvested for preparation of nuclear extracts. Immunoprecipitation-WB analysis was carried out as described (21).

Results

Identification and Purification of the MDM2–p300–p53 Ternary Complex from HeLa Nuclear Extracts. In our previous studies, we observed that MDM2 could form a complex with p300 and p53 *in vitro* (21). The p300–MDM2 association was direct, because MDM2 specifically interacted with the CH1 domain of p300 (data not shown) (20). To understand the functional significance of this ternary complex, we purified the MDM2-associated complex from HeLa nuclear extracts via biochemical fractionation. After five conventional and HPLC columns, monitored by WB with anti-MDM2 antibodies, MDM2 coeluted with a molecular marker of 670 kDa on the Superdex 200 column (Fig. 1*A*), indicating that MDM2 was associated with other proteins in a large complex. Indeed, silver-staining revealed five polypeptides concentrated in fraction 18 (Fig. 1*B*). WB analysis with anti-p300, anti-MDM2, or anti-p53 antibodies showed that two of these bands represent p300 and p53 (fraction 18). The band with a question mark below p300 could be a shortened fragment of p300 lacking the N terminus, which is the epitope for the anti-p300 antibody (Fig. 1). Alternatively, it might be a nonspecific band. The band below the 43-kDa marker (*) was a degraded form of MDM2, because it immunoreacted with the anti-MDM2 antibody (lane 18). p14^{arf} was not detected in this fraction by anti-p14^{arf} antibodies (data not shown). Similar fractionation was also performed with HeLa cytoplasmic extracts. On the sizing column Superdex 200, we also observed a large MDM2-associated complex with a molecular mass of ≈800 kDa, but this complex did not contain p300 or p53 (unpublished observations).

Because p53 exists in cells as a homotetramer with a native mass of ≈212 kDa, MDM2 displays a molecular mass of ≈90 kDa on a SDS gel, and p300 is a ≈400-kDa polypeptide, we conclude that the purified complex consists of p300, MDM2, and p53 with a native molecular mass of ≈700 kDa (Fig. 1*A*). Approximately 10% of MDM2 proteins were detected in the ternary complex (unpublished data). This estimation may vary, because free MDM2 is very unstable during fractionation. These results indicate that MDM2 can form a ternary complex with p300 and p53 both *in vitro* and in the nucleus, but not in the cytosol of HeLa cells.

MDM2 Inhibits p300-Mediated p53 Acetylation *in Vitro*. We next tested whether the purified protein complex could acetylate p53 or MDM2. We first set up an acetylation assay based on published methods (16), by using recombinant p300 and p53 purified from the baculovirus system and bacteria, respectively (21). As shown in Fig. 2 *A* and *B*, the purified p300 was able to efficiently acetylate p53-dependent on acetyl CoA in a dose-dependent fashion. However, the purified p300–MDM2–p53 complex was unable to acetylate recombinant p53 or the associated p53 and MDM2 (Fig. 2*B*). Surprisingly, this complex was still able to acetylate histones (Fig. 2*B*, lane 5). Thus, p300, once complexed to MDM2, may not be able to target the p53 Lys residues.

We next tested whether MDM2 could affect p53 acetylation by p300 in a reconstituted reaction, by using recombinant MDM2 purified from a baculovirus system as described (21). The purified MDM2 was preincubated with the purified p300 on ice for 30 min, before addition to the reaction mixture containing acetyl CoA. As shown in Fig. 2*C*, MDM2 inhibited p53 acetylation by p300 in a dose-dependent fashion (compare lane 2 with lanes 3–5 of the second bottom panel). The loss of p53 acetylation was not caused by operational errors or degradation, because equal amounts of p53 (Fig. 2*C Bottom*) or p300 (Fig. 2*C Top*) remained at the end of the reaction. Despite the interaction between p300 and MDM2, p300 did not use MDM2 as a substrate (Fig. 2*C Middle*). This result indicates that MDM2 can inhibit p53 acetylation by p300 probably through direct association.

Association of MDM2 with p53 Is Required for Its Inhibitory Effect on p53 Acetylation. To test whether this inhibition requires the association of MDM2 with p53, we compared wild-type MDM2 with its N-terminally deleted form lacking amino acids 58–89 in p53 acetylation assays. As shown in Fig. 2*D*, again, wild-type MDM2 inhibited p53 acetylation by p300. In contrast, the MDM2 deletion mutant was without effect on this acetylation (compare lanes 2–4 with lanes 6–8 of Fig. 2*D*). This suggests that for MDM2 to inhibit p300-mediated p53 acetylation, it must form a ternary complex with p53 and p300. To verify this notion,

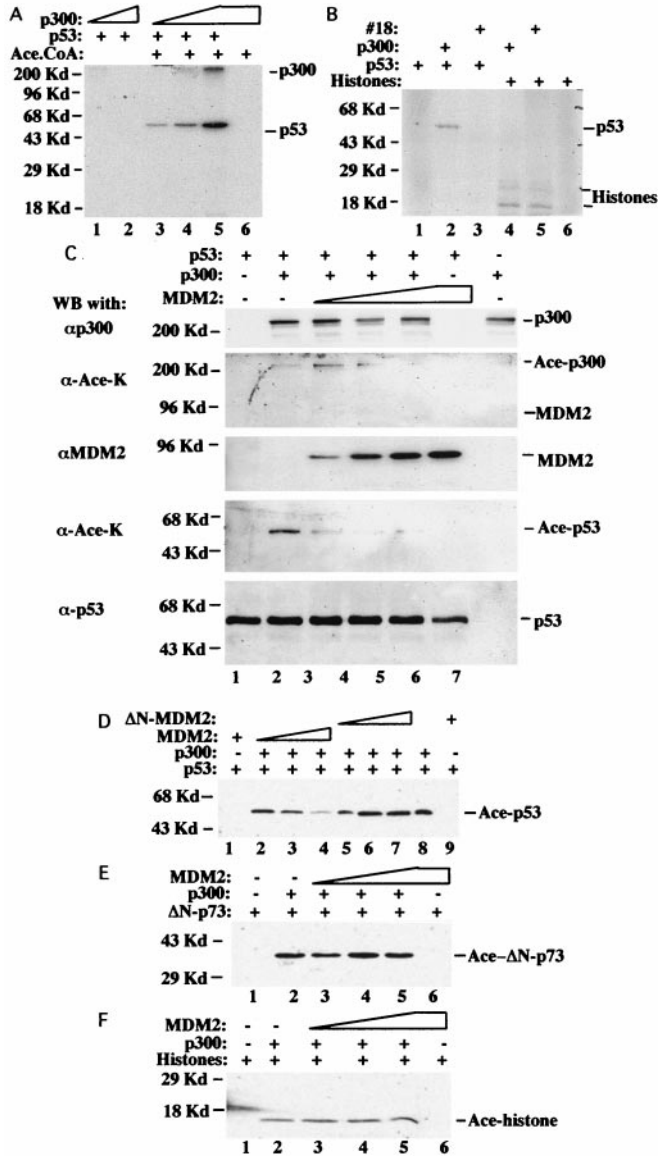

Fig. 2. Inhibition of p300-mediated p53 acetylation by MDM2 *in vitro*. (*A*) Establishment of p53 acetylation by p300 *in vitro*. Recombinant human p53 (50 ng) purified from bacteria and 250 nM acetyl CoA were used in the reaction as indicated; 100 ng (lanes 1 and 3), 200 ng (lanes 2 and 4), and 300 ng (lanes 5 and 6) of recombinant p300 purified from baculovirus-infected insect cells were used. Acetylated p53 was detected by WB by using antiacetylated Lys antibodies (Upstate Biotechnology). (*B*) Histone and p53 acetylation by recombinant p300 or the native p300 complex purified from HeLa nuclear extracts (fraction 18 of Fig. 1 *A* and *B*); 1 μl of ^{14}C-labeled acetyl CoA instead of nonlabeled acetyl CoA was used in each reaction. Histones (100 ng; Sigma), 50 ng of p53, 100 ng of p300, or 200 ng of the p300-containing fraction 18 from Superdex 200 were used in this experiment as indicated. Protein acetylation was detected by autoradiography. (*C*) MDM2 inhibits p53 acetylation by p300. In the acetylation reaction, 75 ng of p53, 200 ng of p300, and 250 nM acetyl CoA were used in this reaction as indicated; 200, 400, and 600 ng of recombinant MDM2 purified from baculovirus were used in lanes 3–5, respectively, and 600 ng of MDM2 for lane 6. Acetylated p53 was detected by antiacetylated Lys antibodies. (*D*) N-terminally deleted mutant MDM2 does not inhibit p53 acetylation by p300. The same acetylation reaction was performed as that in *C*; 200, 400, and 600 ng of either MDM2 or its N-terminal truncated mutant (ΔN-MDM2) were used as indicated. (*E*) MDM2 does not affect acetylation of the p73 C-terminal domain by p300; 75 ng of the p73 C-terminal fragment purified from bacteria (X.Z. and H.L., unpublished data) was used as a substrate. The same amounts of p300 and MDM2 as those in *C* were used in this experiment. (*F*) MDM2 does not affect acetylation of histones by p300. The same assay was conducted as that in *E* except that 40 ng of histones (Sigma) was used in this assay.

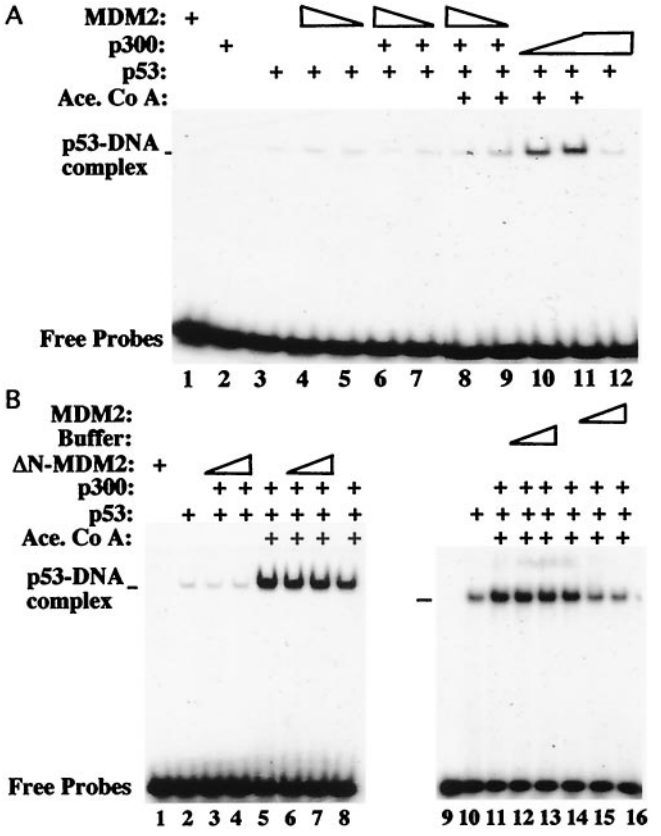

Fig. 3. Wild-type but not the N-terminally deleted mutant MDM2 reduces enhancement of the sequence-specific DNA-binding activity of p53 by p300 *in vitro*. (*A*) Effect of MDM2 on sequence-specific DNA-binding activity of p53. In the EMSA experiment, 50 ng of p53, 250 nM acetyl-CoA, 100 ng (lane 10) or 200 ng (other lanes with +) of p300, and 200 ng (lanes 5, 7, and 9) or 400 ng (other lanes with +) of MDM2 were used as indicated on top. (*B*) Wild-type but not the N-terminally deleted mutant MDM2 inhibits the enhancement of DNA-binding activity by p300 of p53. The same EMSA reaction was carried out as that in *A*, except two controls were included here, the N-terminally truncated MDM2 mutant (200 or 400 ng as indicated on top) and the buffer (the same volumes as those for MDM2 proteins) used for preparation of MDM2 proteins.

we tested whether MDM2 could influence the p300 acetylase activity on different substrates that do not interact with MDM2. Two such substrates used in this test were histones, known p300 acetylase targets (36), and the p73 C-terminal domain, which we recently found to be acetylated by p300 *in vitro* (X.Z. and H.L., unpublished work). MDM2 did not affect the acetylation of either histone or the p73 C-terminal domain by p300 (Fig. 2 *E* and *F*). Because neither of the two substrates interacts with MDM2 (data not shown) (21), this result indicates that forming a ternary complex with p300 and p53 enables MDM2 to specifically inhibit p53 acetylation (Fig. 1). This also suggests that MDM2 may not generally affect the ability of p300 to mediate acetylation of other transcriptional factors.

MDM2 Eliminates the Ability of p300 to Stimulate Sequence-Specific DNA-Binding Activity of p53 *in Vitro*. To test the functional consequence of the inhibition of p300-mediated p53 acetylation by MDM2, we examined the effect of MDM2 on p300-stimulated p53 sequence-specific DNA-binding activity. As shown in Fig. 3*A*, MDM2 did not affect formation of the p53–DNA complex in the absence of acetyl CoA regardless of whether p300 was added or not (lanes 2–7). In the presence of acetyl CoA, p300 markedly stimulated the formation of p53–DNA complexes (lanes 10 and 11), whereas MDM2 blocked this stimulation in a

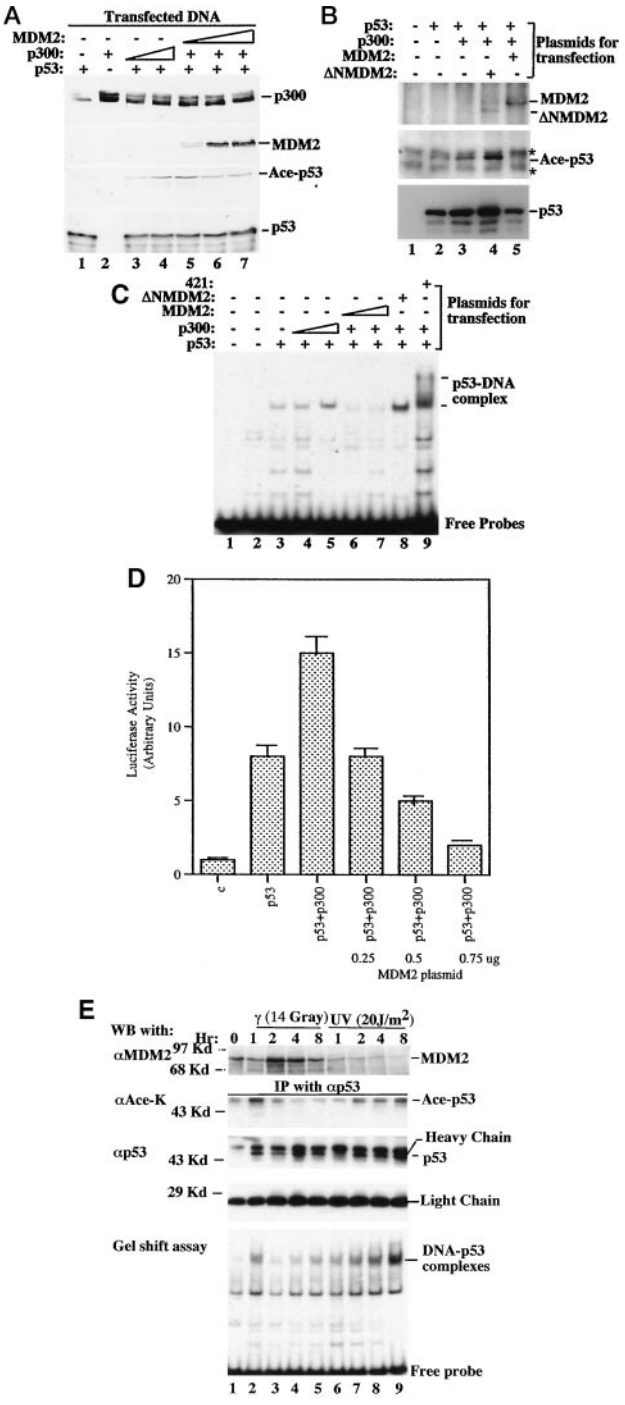

Fig. 4. MDM2 inhibits p53 acetylation and activation by p300 *in vivo*. (*A*) MDM2 reduces p300-mediated p53 acetylation in cells. H1299 cells were transfected with plasmids encoding p53 (0.3 μg), p300 (0.5 μg), and/or MDM2 (0.5, 1, or 2 μg) as indicated on top. MG132 (5 μM) was added into media 12 h before harvesting. Cell lysates were prepared 36 h posttransfection for WB analysis; 400 μg of proteins were loaded onto a 10% SDS gel. (*B*) Wild-type but not N-terminally deleted MDM2 reduces p53 acetylation and level. The same transfection as that in *A* was conducted except the N-terminally deleted MDM2 (ΔNMDM2; 2 μg) was used without MG132 in this experiment. Asterisks indicate nonspecific signals. (*C*) MDM2 reduces the sequence-specific DNA-binding activity of p53 in cells. The exact same transfection as that in *A* was carried out. Nuclear extracts were prepared for EMSA analysis. Proteins (15 μg) were used for each reaction except lane 1; 1 μg of 421 was used in lane 9. (*D*) MDM2 reverses the enhancement of p53-dependent transcription by p300 in cells. As indicated, plasmids encoding no protein as a control (1 μg; *C*), p53 (50 ng) alone, or with p300 (0.15 μg) or with MDM2, and with a luciferase reporter gene (0.2 μg) driven by the p53RE motif derived from the MDM2 promoter (35), as well as a β-galactosidase reporter plasmid (0.1 μg) as an internal control, were introduced into H1299 cells (5×10^4 cells per 35 mm dish) by using Lipofectamine (GIBCO/BRL). Posttransfection (48 h) and 12 h after MG132 treatment (5 μM), cells were harvested for luciferase assays. Each column represents the mean data of three experiments. The bars denote that deviation of errors. (*E*) UV and γ irradiation differentially regulate p53 acetylation and DNA-binding activity, which are reciprocal to the MDM2 level. Tera-2 cells were irradiated with UV or γ ray, as indicated on top and harvested at different time points postirradiation for immunoprecipitation-WB. Proteins (300 μg) of the cell lysates from each time point were used for immunoprecipitation-WB by using antibodies as indicated (two middle panels). Nuclear extracts with 150 μg of proteins were directly loaded onto a 10% SDS gel for WB by using an anti-MDM2 antibody (*Upper*). EMSA (*Lower*) was carried out by using 15 μg of proteins in nuclear extracts and the ^{32}P-labeled p53RE-containing DNA probes as described in *Materials and Methods*. αAce-K denotes the antibody specifically against the acetylated Lys. Similar results to that of *E* were also obtained by using F9 cells. All of these experiments were reproducible.

dose-dependent manner. As a result, the amount of p53–DNA complexes was reduced to the basal level (lanes 8 and 9 of Fig. 3*A* and lanes 14–16 of Fig. 3*B*). In contrast, neither the N-terminally deleted MDM2 mutant nor the buffer used for MDM2 preparation affected formation of the p53–DNA complex (Fig. 3*B*). These results indicate that although MDM2 did not directly affect the sequence-specific DNA-binding activity of p53, it abrogates the ability of p300 to stimulate this activity by inhibiting p300-catalyzed p53 acetylation.

MDM2 Inhibits p53 Acetylation and Activation Mediated by p300 *in Vivo*. To examine whether MDM2 inhibits p53 acetylation by p300 in cells, we transiently transfected human p53 null small cell lung carcinoma H1299 cells (37) with different combinations of plasmids encoding p53, MDM2, or p300. As shown in Fig. 4*A*, exogenously expressed p300 stimulated p53 acetylation, whereas overexpression of transfected MDM2 reduced p53 acetylation (Fig. 4*A Middle*). This reduction was not caused by the decreased level of p53, because the total amount of the exogenous p53 was approximately equal in all of the lanes (Fig. 4*A Bottom*). p53 was not degraded, because a proteosome inhibitor MG132 was added into cell culture media 12 h before harvesting, and also, p53 was overexpressed in this specific experiment. When MG132 was not present in the culture, MDM2 led to the decrease in p53 level (Fig. 4*B*), consistent with previous studies (31, 32), whereas p300 stimulated p53 acetylation as well as its protein level (compare lanes 2 and 3 of Fig. 4*B*). In contrast, the N-terminally deleted MDM2 did not inhibit p53 acetylation, as in the presence of this MDM2 mutant, p53 acetylation as well as its protein level was virtually elevated (Fig. 4*B*).

To test whether inhibition of p53 acetylation by MDM2 affects sequence-specific DNA-binding activity of p53 *in vivo*, nuclear extracts were prepared from the transfected cells under the exact same condition as that in Fig. 4*A* for EMSA analysis. As shown in Fig. 4*C*, p300 enhanced the p53–DNA complex in a dose-dependent fashion (lanes 3 and 4), whereas wild-type but not the N-terminally deleted MDM2 reduced the level of this complex (compare lanes 6 and 7 with lane 8). This reduction was not caused by the low level of p53, because the p53 protein was approximately equal (Fig. 4*A*). This complex was formed with p53, because the anti-p53 antibody Pab421 specifically supershifted this complex (lane 9) and such a complex was not detected in nuclear extracts prepared from the mock-transfected H1299 cells (lane 2). These results indicate that MDM2 also inhibits sequence-specific DNA-binding activity of p53 in cells. Because MDM2 did not directly influence this activity (Fig. 3), this inhibition must be caused by the negative effect of MDM2 on p53 acetylation (Figs. 2 and 4). This is correlated with the abrogation of p300-stimulated p53-dependent transcription by

MDM2 (Fig. 4*D*) (38). Because the p53 level did not change in the presence of MG132 (Fig. 4*A*), this abrogation was unlikely caused by MDM2-mediated p53 degradation. Hence, these results indicate that MDM2 inhibits p300-mediated p53 acetylation and activation *in vivo*.

UV and γ Irradiation Differentially Regulate MDM2 Expression and p53 Acetylation. Recently, we observed that UV and γ irradiation differentially regulated MDM2 expression, leading to different phenotypes (39). To further test how p53 acetylation is correlated with the MDM2 level under these stress conditions, we examined p53 acetylation and protein levels, as well as MDM2 expression in human testicular carcinoma Tera-2 cells, which harbor wild-type p53 (40), postirradiation. Consistent with our previous study (39), MDM2 expression was repressed by UV but not by γ irradiation (Fig. 4*E Top*); p53 levels were induced gradually by UV, but with a typical dual peak pattern by γ irradiation (Fig. 4*E Middle*). Correspondingly, p53 acetylation was stimulated by UV (lanes 6–9 of Fig. 4*E* second *Top*), but increased only within the first hour (lane 2) and declined significantly later on post γ irradiation (Fig. 4*E* second *Top*, lanes 3–5). The decrease was not caused by reduction of p53 protein because p53 protein was detected (Fig. 4*E Middle* lanes 4–5), instead, it was perhaps caused by the higher level of MDM2 (Fig. 4*E Upper*). Consistent with these results, the sequence-specific DNA-binding activity of p53 was also differentially affected by UV and γ irradiation (Fig. 4*E Bottom*). More p53–DNA complexes were formed 1 h post γ-irradiation and since then, the level of this complex decreased significantly (compare lane 2 with lanes 3–5). Also, the MDM2 level gradually decreased when p53 became less acetylated and less active at the later time postirradiation (compare lane 3 with lanes 4 and 5 of Fig. 4*E*). In striking contrast, the p53–DNA complexes gradually increased, well correlated with the p53 level and its acetylation post UV irradiation (Fig. 4*E*, lanes 6–9). These results, together with those in Fig. 4 *A* and *B* and also repeated with murine testicular carcinoma F9 cells, demonstrate that MDM2 inhibits p53 acetylation *in vivo*.

Discussion

The MDM2–p53 feedback loop represents a cellular autoregulatory paradigm, finely tuning p53 function (41). Here, we document a mechanism for the MDM2–p53 loop, i.e., MDM2 inhibits p53 acetylation and transcriptional activation by p300. First, we identified and purified a ternary protein complex composed of p300, MDM2, and p53 from HeLa nuclear, but not cytoplasmic extracts, and found this complex was unable to acetylate p53 but was still active in histone acetylation. Also, MDM2 inhibited p300-dependent acetylation of p53 but not of histone or the C terminus of p73. Consistently, MDM2 inhibited the ability of p300 to stimulate sequence-specific DNA-binding activity of p53 *in vitro*. Moreover, overexpression of MDM2 in H1299 cells reduced p300-mediated p53 acetylation. Interestingly, this inhibition was correlated with the induction of MDM2 by γ irradiation, whereas the repression of MDM2 by UV irradiation was coincident with hyperacetylation of p53. This correlation was also seen during the cell cycle (42). p53 acetylation occurred primarily in the G_0 and G_1 phases, whereas the MDM2–p300–p53 complex was detected in the G_2/M phase (42). Hence, these results demonstrate that MDM2 inhibits p53 acetylation by p300 *in vitro* and *in vivo*. This may interpret that the *in vivo* complexes that contain p53 and MDM2 do not bind to DNA in a sequence-specific manner (43). Because MDM2 also may interfere with the interaction between p53 and TAFII31/TAFII70 (44, 45), how much this interference and the inhibitory effect of MDM2 on p53 acetylation contribute to the repression of p53-dependent transcription still needs to be studied.

However, MDM2 does not appear to generally affect the acetylase activity of p300 on other substrates. First, we found that MDM2 specifically inhibited p300-mediated acetylation of p53, but not of histones or the C terminus of p73, neither of which directly interacts with MDM2. Also, the N-terminally deleted MDM2 mutant, which does not bind to p53 (46), had no effect on p53 acetylation by p300. Moreover, a ternary protein complex composed of p300, MDM2, and p53, inactive in p53 acetylation but active in histone acetylation, was identified and purified from HeLa nuclear but not cytoplasmic extracts (Fig. 1; data not shown). Thus, without a direct interaction, MDM2 does not affect acetylation of other transcriptional activators by p300 (47).

It is perplexing how p300, on one hand, acetylates p53 and activates its function (16, 23), but on the other hand, binds to MDM2 and assists it in degrading p53 and down-regulating its function (20). Our study may reconcile those seemingly contradictory data. Because both ubiquitination and acetylation of p53 occur at the Lys residues in its C terminus (16, 29), acetylation may prevent p53 ubiquitination at these residues. By this rationale, inhibition of p300-mediated p53 acetylation by MDM2 would result in more efficient ubiquitination, consequently leading to the degradation of p53. Although it is still under current investigation whether acetylation of p53 inhibits its ubiquitination, the facts that UV irradiation resulted in a significant induction of p53 acetylation (Fig. 4) (15) and reduction of its ubiquitination (48) strongly support the idea that acetylation and ubiquitination are reciprocal events. Because MDM2 inhibited p53 acetylation in an *in vitro* reconstituted assay in which ubiquitination would not occur (Fig. 2), this protein does not require its ubiquitination activity to negatively modulate p53 acetylation.

Phosphorylation is known to play a role in regulating p53 function after DNA damage (3). It is intriguing how acetylation is related with this pathway. It has been shown that in response to DNA damage, p53 phosphorylation at its N-terminal Ser-33 and Ser-37 was stimulated and followed by acetylation at Lys-382 by p300 or Lys-320 by P/CAF (15). Although how phosphorylation at the N terminus affects acetylation at the C terminus of p53 is unclear, phosphorylation of p53 at Ser-15 was shown to gain a higher affinity to p300/CBP (CREB-binding protein; ref. 49). Also, phosphorylation of p53 at Ser-20 prevents p53 from binding to MDM2 (13, 50, 51), suggesting that MDM2 may preferentially recognize the nonphosphorylated form of p53. This also implicates that MDM2, once binding to p53, may inhibit p53 phosphorylation. These studies suggest that phosphorylation at the N terminus of p53 excludes MDM2 and recruits p300 that acetylates p53. By the same token, hypophosphorylated p53 molecules, with a higher affinity to MDM2, tend to be less acetylated because of the inhibitory effects of MDM2, and thus, are prone to be degraded. Nevertheless, it remains to be addressed whether MDM2 inhibits p53 acetylation by P/CAF and whether C-terminal phosphorylation affects p53 acetylation.

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