Inhibition of p53 degradation by Mdm2 acetylation

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Abstract Mdm2 is a RING finger E3 ubiquitin ligase, which promotes ubiquitination and proteasomal degradation of the p53 tumor suppressor protein. Acetylation of p53 regulates p53's transcriptional activity and inhibits Mdm2-mediated p53 ubiquitination and degradation. We now report that Mdm2 is also a target for acetylation. Mdm2 is acetylated in vitro by CREB-binding protein (CBP) and to a lesser extent by p300, but not by p300/CPB-associated factor. Acetylation occurs primarily within the RING finger domain of Mdm2. In vivo acetylation of Mdm2 was detected easily with CBP but not p300. Efficient in vivo acetylation required the preservation of the RING finger. An Mdm2 mutant (K466/467Q) mimicking acetylation is impaired in its ability to promote p53 ubiquitination, as well as Mdm2 autoubiquitination. Moreover, K466/467Q is defective in promoting p53 degradation in living cells. We thus suggest that acetyltransferases may modulate cellular p53 activity not only by modifying p53, but also by inactivating Mdm2.

Key words: p53; Mdm2; CREB-binding protein; p300; Acetylation

1. Introduction

Histone acetyltransferases (HATs) such as p300, CREB-binding protein (CBP) or p300/CPB-associated factor (P/CAF) act as coactivators for sequence-specific transcription factors. HAT-mediated facilitation of gene expression usually involves acetylation by HATs of core histones at the target promoters, generating a more accessible chromatin structure for transcription factors [1,2]. Acetylation can also occur on the transcription factors themselves [3]. The tumor suppressor p53 was the first transcription factor shown to be acetylated [4]. This stimulated a search for additional non-histone targets of HATs, as a result of which many novel HAT target proteins involved in different aspects of cellular regulation have been identified [3].

p53 occupies a central position in an intricate stress signaling network [5–9]. Various types of oncogenic stress provoke a cellular p53 response manifested, in most cases, by stabilization of this short-lived protein. Once activated, p53 transactivates or transrepresses different sets of target genes, leading to a series of cellular consequences including growth arrest at G1 and G2, apoptosis, senescence, differentiation and DNA repair. Because p53 is a potent inhibitor of cell growth as well as a potent inducer of cell death, its cellular activity must be held in tight control. A major role in the control of p53 activity has been assigned to the Mdm2 oncoprotein. Mdm2 participates in a negative autoregulatory feedback loop with p53, which works as a sensitive switch to turn p53 on or off [5,10,11]. To a large extent, Mdm2 exerts its inhibitory effect on p53 through controlling p53 protein levels [12,13], acting as an E3 ubiquitin ligase to promote p53 ubiquitination and proteasomal degradation [14,15]. The E3 function of Mdm2 requires its RING domain, located at the C′-terminal part of the protein. In addition, Mdm2 can inhibit p53's transcriptional activity by a direct interaction that occludes the p53 transactivation domain [11,16].

Recent studies suggest the existence of a complex interplay between Mdm2 and acetyltransferases. Mdm2 can associate with the C/H1 region of p300 and the N-terminal region of CBP [17,18]. On the one hand, this interaction can enhance the ability of Mdm2 to promote p53 polyubiquitination and degradation [17,19]. On the other hand, CBP binding to Mdm2 was proposed to reduce the availability of CBP for p53 interaction, resulting in reduced transcriptional activity of p53 [18]. Furthermore, Mdm2 has been shown to reduce HAT-mediated p53 acetylation, either by directly interfering with the HAT activity of these proteins or through its recruitment of the histone deacetylase HDAC1 [20–22]. In an effort to gain more insight into the functional interaction between p300/CBP and Mdm2, we explored the idea that Mdm2 may not only modulate p53 acetylation, but also be itself a direct substrate for regulatory acetylation by HATs. We report that Mdm2 can be acetylated in vitro by CBP and, to a lesser extent, by p300; furthermore, Mdm2 is acetylated within living cells, and this is enhanced by CBP. An Mdm2 mutant, mimicking the acetylated state, exhibits impaired E3 ligase activity and is deficient in promoting p53 degradation. Thus, HAT-mediated acetylation may impinge on the p53 pathway not only through modification of p53, but also through inactivation of Mdm2.

2. Materials and methods

2.1. Cells and transfections

H1299 cells (ATCC) were maintained at 37°C in RPMI medium (Sigma) supplemented with 10% fetal calf serum (FCS, Sigma). p53/mdm2 double null mouse embryonic fibroblasts (MEFs) (clone 174-2, kindly provided by G. Lozano) and HEK-293 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma) and antibiotics. MEFs were maintained in DMEM supplemented with

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10% heat-inactivated FBS, non-essential amino acids and β-mercaptoethanol (at 0.4 μl/100 ml). H1299 cells and HEK-293 cells were transfected with the aid of either calcium phosphate or the JetPEI reagent (PolyPlus Transfection).

2.2. Immunoblotting analysis

Steady-state protein levels were monitored as described [12]. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer. Following three rounds of vigorous vortexing at 5 min intervals, lysates were cleared by centrifugation at 4°C for 10 min. Aliquots containing 20 μg total protein (Bio-Rad protein assay) were resolved on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to nitrocellulose membranes (BA83, Schleicher& Schuell). Samples for p53 ubiquitination assays were run on 8% SDS–polyacrylamide gel electrophoresis (PAGE) gels. Membranes were probed with the indicated antibodies and developed with the enhanced chemiluminescence (ECL) kit (Amersham).

2.3. PreScission protease and protein purification

Glutathione-S-transferase (GST)-Mdm2 or GST-Mdm2/4KR solutions, eluted from glutathione beads, were dialyzed against 20 mM Tris–Cl pH 7.5, 1 mM dithiothreitol (DTT), 10% glycerol overnight at 4°C. 1.5 mg of GST fusion protein was cleaved in 1 ml of buffer containing 150 mM, 0.01% Triton X-100, 1 mM DTT, and 40 units PreScission protease (Amersham), incubated at 4°C for 5 h. To remove the released GST from the solution, 1 ml glutathione agarose beads were washed twice with 10 ml of cell lysis buffer (20 mM Tris pH 8.0, 120 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5% NP40 10 μM trichostatin A (TSA) and Sigma protease inhibitor cocktail diluted 1:200), coated for 2 h at 4°C by gentle agitation with 10 ml of 3% bovine serum albumin (BSA) (FV) in cell lysis buffer containing 10% glycerol, 1 mM DTT, and finally washed again three times with lysis buffer. Coated beads were incubated with the cleavage products at 4°C for 2 h, followed by centrifugation and collection of GST-free protein in the supernatant. Aliquots were stored at −80°C.

2.4. In vitro acetylation

In vitro acetylation was performed as described [4]. Recombinant CBP/cf was generated by fusing a CBP catalytic fragment (residues 1196–1718) to GST [23]. A catalytically inactive derivative was made by introducing Leu1690Lys and Cys1691Leu mutations into CBP/cf. For in vitro acetylation assays using anti-p300 or anti-CBP immunoprecipitates, cells from three 15-cm dishes of the p53/mdm2 double null MEFs at 85% confluence, or 10 10-cm dishes of HEK-293 cells transfected with 15 μg/dish of p300-HA or CBP-HA expression plasmid DNA, were lysed by incubation on ice for 20 min in 1.5 ml of lysis buffer, followed by three rounds of freeze-thawing. The cleared supernatant was taken for immunoprecipitation with protein A Sepharose 4B fast flow beads (Sigma) at 4°C for 1 h, using anti-CBP (Santa Cruz, monoclonal C1) or a monoclonal anti-HA (HA.11, Babco) antibody for MEFs or transfected HEK-293 cells, respectively. The beads were washed three times with lysis buffer+1 mM DTT, then divided into aliquots and stored at −80°C. Loaded beads were employed in in vitro acetylation assays, in combination with 2 μg of purified bacterially expressed GST-Mdm2, from which the GST portion had been cleaved off with PreScission protease (Amersham), in a total reaction volume of 30 μl. Reaction products were resolved by SDS–PAGE. Gels were stained with Coomassie brilliant blue, scanned, and then treated with Amplify (NAMP100, Amersham) at room temperature for 30 min, dried and exposed to Kodak BioMAX-MS film.

2.5. In vivo acetylation

In vivo acetylation assays were performed as described [4]. Transfected HEK-293 cells (2×10^6/10 cm dish, a total of 10 dishes) were treated with MG132 (25 μM) for 4 h, and resuspended in 2-ml microcentrifuge tubes in 1.5 ml of labeling medium containing 2 mCi/ml [^{3}H]acetate, 25 μM MG132, 5 μM TSA, 5 mM nicotinamide and 50 μg/ml cycloheximide, in fully complemented DMEM. The tubes were rotated at 37°C for 45 min (without cycloheximide) or 90 min (with cycloheximide). Following washing with 1 ml cold PBS, cells were lysed in 400 μl of 1% SDS–triethanolamine-buffered saline (TBS) by boiling twice for 5 min after vigorous vortexing. 800 μl of TBS containing 1.5% Triton X-100, 5 μM TSA and 5 mM nicotinamide was added to each tube, and the supernatant was collected after centrifugation at 15 000 rpm for 15 min at room temperature. Immunoprecipitation of HA-Mdm2 from HEK-293 cell extracts was performed with a monoclonal anti-HA antibody (HA.11, Babco). Mdm2 was brought down with a mixture of monoclonal antibodies 4B2, SMP14, 2A10, 2A9 and 4B11 [24]. One 10th of each immunoprecipitate was analyzed by Western blotting, whereas the rest of the material was subjected to SDS–PAGE and autoradiography: gels were fixed at room temperature for 30 min in a solution containing 25% MeOH–7% acetic acid, treated with Amplify (Amersham Pharmacia, NAMP100) at room temperature for 30 min, dried at 75°C for 2 h, and then exposed to Kodak BioMAX-MS film.

2.6. In vitro ubiquitination

Reactions were carried out in a total volume of 15 μl, containing bacterially expressed human E1, UbcH5, GST-Mdm2 (400 ng) and 10 μg ubiquitin (Sigma), and 1 μg/ml adenosine triphosphate (ATP)γS (Boehringer), in ubiquitination buffer containing 40 mM Tris pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, and 25 ng/ml ubiquitin aldehyde (Ubal; Affinity). p53 protein (1 μl) produced in a wheat germ transcription-coupled in vitro translation system (Promega) was added to the reaction mixture. The reaction was allowed to proceed for 60 min at 30°C, then stopped by boiling for 5 min after addition of 15 μl of 1.5× protein sample buffer. p53 ubiquitination was analyzed by 8% SDS–PAGE and Western blotting with a mixture of anti-p53 monoclonal antibodies DO-1 and PAb1801. To monitor Mdm2 autoubiquitination, the membrane was reprobed with a rabbit polyclonal anti-Mdm2 antibody generated against a fusion protein between GST and residues 277–351 of human Mdm2.

2.7. In vivo ubiquitination

p53/mdm2 double null MEFs were plated at 0.75×10^6/10 cm dish, and transfected 12 h later with various plasmid combinations, employing JetPEI. The total amount of DNA in each transfection was adjusted to 6 μg/10 cm dish by addition of pCDNA3 empty vector DNA. 20 h later, cells were treated with MG132 (25 μM) for 4 h, harvested and lysed in 200 μl of 1% SDS–TBS by boiling twice for 5 min after vigorous vortexing. Next, 400 μl of TBS containing 1.5% Triton X-100 was added to the lysate. After centrifugation for 10 min at 15 000 rpm at room temperature, the supernatant was collected for immunoprecipitation with a mixture of anti-p53 monoclonal antibodies DO-1 and PAb1801, followed by Western blot analysis with anti-HA antibody (Babco).

3. Results

3.1. Modulation of acetylation enhances the p53 response to DNA damage and rescues p53 from Mdm2-mediated degradation

To evaluate the overall contribution of acetylation to stabilization of p53 after DNA damage, MEFs were pretreated for 4 h with the deacetylase inhibitor TSA prior to exposure to ionizing radiation. As seen in Fig. 1A, pretreatment with TSA caused a more robust and longer-lasting induction of p53. Next, steady-state levels of p53 were examined in p53 null H1299 cells, following transfection of p53 together with Mdm2 or Mdm2 plus CBP. As expected, Mdm2 reduced markedly the steady-state levels of p53 (Fig. 1B, lane 3). Importantly, inclusion of CBP significantly attenuated this reduction (Fig. 1B, lane 4). p53 acetylation on C′-terminal lysines can prevent p53 ubiquitination and degradation [25]. To find out whether this is the only mechanism whereby CBP may protect p53 from Mdm2-mediated degradation, we took advantage of the 4KR p53 mutant, in which lysines 372, 373, 381 and 382, the major sites for acetylation by p300/CBP [4], were mutated to arginine. As shown in Fig. 1C (left panel), p53/4KR did not exhibit detectable C-terminal acetylation by cotransfected CBP (lane 3), unlike wild-type p53 whose acetylation was significantly enhanced by CBP (lanes 1, 2). p53 remained susceptible to Mdm2-mediated deg-

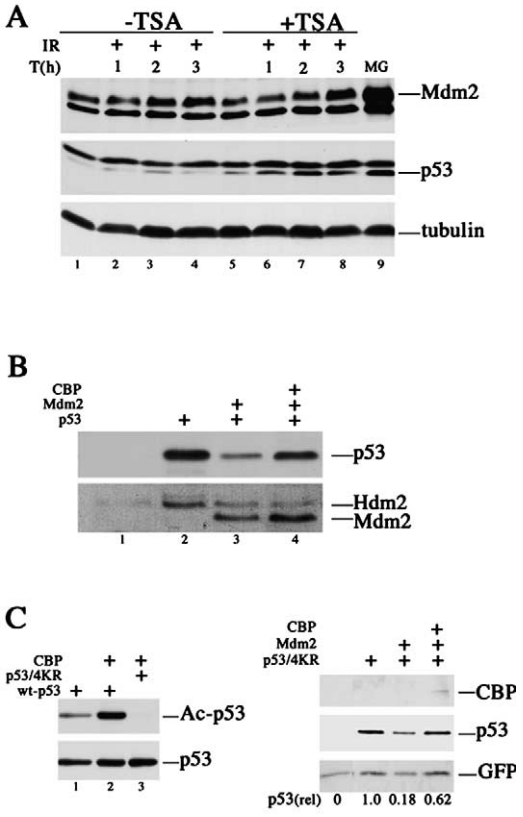

Fig. 1. Regulation of p53 by HAT activity. A: MEFs were pretreated with 100 nM TSA for 4 h or left untreated, and then exposed to 1 Gy of γ-irradiation. Cell lysates were prepared at the indicated time points after irradiation. MEFs treated with the proteasome inhibitor MG132 (MG, 25 μM for 4 h) served as a positive control for p53 and Mdm2 stabilization. A mixture of the monoclonal antibodies PAb421 and PAb248 was used for immunoblotting of mouse p53, and the 2A10 monoclonal antibody was used for immunoblotting of Mdm2. Tubulin served as a loading control. B: H1299 cells were transfected by the calcium phosphate method with 0.5 μg/6 cm dish of human p53 expression plasmid, either alone or with 0.5 μg of a plasmid encoding mouse Mdm2, or with 0.5 μg of mouse Mdm2 expression plasmid together with 5 μg of CBP expression plasmid. Western blot analysis for p53 was done with a mixture of monoclonal antibodies PAb1801 and DO-1, and for Mdm2 with monoclonal antibody 2A10. Transfected mouse Mdm2 (Mdm2) and endogenous human Mdm2 (Hdm2) are indicated. C: H1299 cells were transfected by the JetPEI procedure with 100 ng/6 cm dish (left panel) or 20 ng/6 cm dish (right panel) of the indicated human p53 expression plasmid, either alone or with 40 ng/6 cm dish of human Mdm2 expression plasmid (right panel), together with 5 μg/6 cm dish (left panel) or 2 μg/6 cm dish (right panel) of CBP-HA expression plasmid. The p53/4KR protein contains lysine to arginine substitutions at positions 372, 373, 381 and 382 of p53. In the right panel, green fluorescent protein (GFP) expression plasmid was included as an internal transfection control. Western blot analysis for acetylated p53 was done with a polyclonal anti-pan-acetylated p53 [25] (left panel, Ac-p53). Total amount of p53 was monitored by reprobing the membrane with the polyclonal anti-human p53 antibody CM1 (left panel, p53). In the right panel, CBP-HA and p53 were detected with a monoclonal anti-HA antibody (CBP) or a mixture of PAb1801 and DO-1 (p53), respectively. Numbers at the bottom represent relative p53 levels, after normalization for GFP.

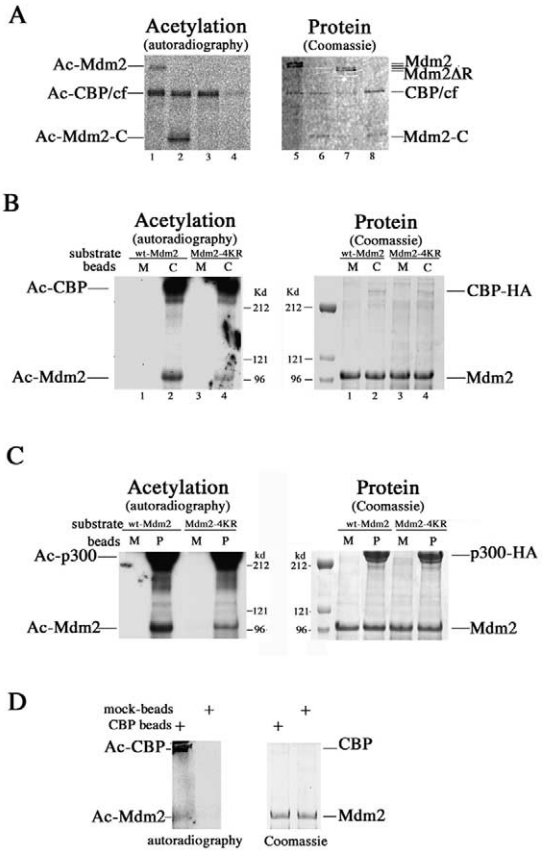

Fig. 2. Mdm2 acetylation in vitro. A: Recombinant fusion proteins between GST and either full-length Mdm2 (Mdm2) or residues 2–442 of Mdm2, lacking the RING finger domain (Mdm2ΔR), were incubated with recombinant catalytic fragment of CBP (CBP/cf, lanes 1–3 and 5–7) or a catalytically inactive mutant version thereof (lanes 4, 8), in the presence of [^{14}C]acetyl-coenzyme A (CoA), as described in Section 2. Reaction products were resolved by 10% SDS–PAGE. Coomassie blue staining (right) of the gel indicates the input protein bands. The autoradiogram shows the acetylation signals (left panel), due to incorporation of radioactive acetyl groups. Mdm2-C is a C-terminal fragment of Mdm2, comprising residues 356–482 fused to GST. Catalytically inactive CBP fragment was produced by mutating leucine 1690 and cysteine 1691 into lysine and leucine, respectively. Ac-CBP/cf indicates the position of autoacetylated CBP/cf. B: In vitro Mdm2 acetylation with cell-derived full-length CBP was carried out following immunoprecipitation of CBP-HA from transfected HEK-293 cells. 10 10-cm dishes of HEK-293 cells, each transfected with 15 μg of HA-tagged CBP expression plasmid DNA, were harvested 48 h post-transfection. Following immunoprecipitation with anti-HA antibodies attached to protein A beads, the beads were split in two equal parts and used for an in vitro acetylation assay of GST-free recombinant wild-type Mdm2 or Mdm2-4KR, as indicated, in the presence of [^{14}C]acetyl-CoA. M and C refer to mock protein A beads and beads carrying immunoprecipitated CBP, respectively. GST-free Mdm2 was prepared by cleavage with PreScission protease (Section 2). Mdm2-4KR has lysines 466, 467, 469 and 470 replaced by arginines. Ac-Mdm2 and Ac-CBP indicate the acetylated Mdm2 and autoacetylated CBP, respectively. C: An experiment similar to the one in B was performed, except that p300-HA was used instead of CBP-HA. M and p refer to mock protein A beads and beads carrying immunoprecipitated p300, respectively. D: Extracts of p53/mdm2 double null MEFs were subjected to immunoprecipitation of endogenous CBP with monoclonal antibody C-1 (Santa Cruz). In vitro acetylation of GST-free recombinant Mdm2 and subsequent analysis were carried out as in B.

radation, even though the effect of Mdm2 was apparently attenuated, presumably owing to ablation of several lysine residues critical for optimal p53 ubiquitination (Fig. 1C, right panel, lanes 2, 3). Importantly, despite the absence of detectable acetylation on p53/4KR, the mutant protein was nicely protected by CBP against Mdm2-mediated downregulation (compare lanes 3, 4). This suggests that, besides direct acetylation of p53, CBP can also contribute to the p53 stabilization by additional mechanism(s).

3.2. *Mdm2 is acetylated in vitro in its RING finger domain*

To find out whether Mdm2 may be a direct target for inhibitory acetylation by CBP, we tested the acetyltransferase activity of CBP towards Mdm2 in vitro. A bacterially expressed catalytic fragment of CBP acetylated recombinant full-length human Mdm2 (Fig. 2A, lane 1), as well as a short fragment of Mdm2 carrying the RING finger domain (lane 2), but not an Mdm2 mutant lacking the RING finger (Mdm2-C; lane 3). No acetylation of Mdm2-CT was obtained with a catalytically inactive CBP fragment (lane 4). Thus, the RING domain is the prime target for Mdm2 acetylation by the catalytic fragment of CBP, consistent with a report that the C′-terminal region of Mdm2 (residues 370–491), can be acetylated by recombinant p300 HAT domain [26]. Indeed, we could detect Mdm2 acetylation by a catalytically active fragment of p300, albeit less efficient than by CBP, whereas a corresponding fragment P/CAF failed to acetylate Mdm2 while being very active towards p53 (data not shown). The Mdm2 RING finger encompasses a cluster of lysine residues including lysines 466/467 and 469/470, which might be potential targets for acetylation. To address this possibility, GST fusions with short Mdm2 RING finger-derived peptides were used as substrates for in vitro acetylation by CBP catalytic fragment. Mutation of single individual lysine residues to arginine did not affect acetylation; however, double mutations at either K466/467 or 469/470 showed a significant reduction in acetylation (data not shown).

The acetylation assay in Fig. 2A was performed with a recombinant catalytic fragment of CBP. To find out whether the full-length enzyme can also acetylate Mdm2, HA-tagged CBP was immunoprecipitated from transfected HEK-293 cells and assayed for acetylation of recombinant Mdm2 in vitro. As shown in Fig. 2B, acetylation of wild-type Mdm2 by immunoprecipitated CBP was clearly detectable (lane 2, Ac-Mdm2). Acetylation was markedly lower when Mdm2 carrying lysine to arginine substitutions at positions 466, 467, 469 and 470 (Mdm2-4KR) was used as substrate (lane 4). Hence, Mdm2 is targeted by an HAT activity associated with intact cellular CBP, and efficient acetylation in vitro requires lysine residues located within the RING finger cluster. HA-tagged p300, immunoprecipitated from transfected HEK-293 cells, also acetylated Mdm2 (Fig. 2C). However, while the amount of immunoprecipitated p300 was much higher than that of CBP (Fig. 2C, right panels, compare CBP-HA to p300-HA), as was also the extent of its autoacetylation (left panels, compare Ac-CBP to Ac-p300), Mdm2 acetylation was comparable in both cases. Hence, p300 appears to be substantially less potent than CBP as an Mdm2 HAT.

Endogenous CBP, immunoprecipitated from p53/Mdm2 double knockout MEFs, also promoted Mdm2 acetylation, albeit less than the more abundant transfected CBP (Fig. 2D). Together, all these observations imply that CBP and to

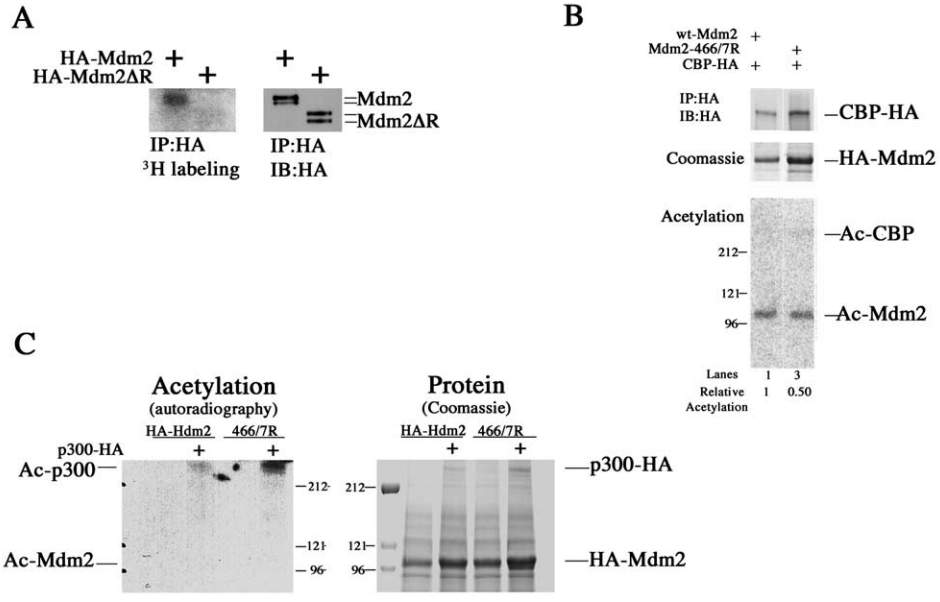

Fig. 3. Mdm2 acetylation in vivo. A: HEK-293 cells were transfected with HA-Mdm2 or HA-Mdm2ΔRING (Mdm2ΔR), containing residues 1–436 of human Mdm2. 48 h later, cells were treated with 25 μM MG132 for 4 h, and labeled with [^{3}H]acetate for 45 min at 37°C. Cell lysates were immunoprecipitated with a monoclonal anti-HA antibody (Babco). Right: Mdm2 immunoblot; left: autoradiography. Exposure: 60 days. B: HEK-293 cells were transfected with CBP-HA together with HA-Mdm2 or HA-Mdm2-466/467R. 48 h later, cells were treated with 25 μM MG132 for 4 h, and then with cycloheximide (50 μg/ml) for 30 min at 37°C and labeled with [^{3}H]acetate for 90 min at 37°C. Cells were lysed in denaturing buffer (Section 2). Lysates were immunoprecipitated with a monoclonal anti-HA antibody (Babco). Amounts of CBP and Mdm2 in the immunoprecipitates, visualized by Western blot for HA and Coomassie staining, respectively, are shown above the gel autoradiography. To calculate relative acetylation, the intensity of the scanned acetylated signal was divided by the intensity of the stained corresponding Mdm2 band. Exposure: 14 days. C: HEK-293 cells were transfected with HA-Mdm2 or HA-Mdm2-466/467R, with or without p300-HA. Analysis was as in B. Ac-p300, autoacetylated p300.

a lesser extent p300, but not P/CAF, acetylate Mdm2 preferentially within the lysine cluster of the RING domain.

3.3. Mdm2 is acetylated in vivo

To assess Mdm2 acetylation in vivo, HEK-293 cells were transfected with HA-tagged human Mdm2, either wild-type (HA-Mdm2) or a deletion mutant lacking the RING domain (HA-Mdm2ΔRING). Following treatment with the proteasome inhibitor MG132 to block Mdm2 degradation, cells were labeled briefly with [^{3}H]acetate. Mdm2 was precipitated with anti-HA antibodies, followed by gel electrophoresis and autoradiography. As seen in Fig. 3A, full-length Mdm2 was appreciably acetylated under those conditions, whereas a mutant lacking the RING finger underwent only very minimal labeling.

We next compared CBP-directed in vivo acetylation of wild-type Mdm2 and an Mdm2 mutant carrying lysine to arginine substitutions in two of the lysines located within the RING finger cluster. Cells were transfected with CBP-HA together with either HA wild-type Mdm2 or HA-Mdm2-466/467R. Incorporation of radioactive acetyl groups was monitored essentially as in Fig. 3A. The relative extent of acetylation was calculated as detailed in the legend to Fig. 3B; it is of note that Mdm2 acetylation was practically undetectable under these conditions when a CBP expression plasmid was not included in the transfection (data not shown). Mutation of lysines 466 and 467 reduced by about two-fold the relative extent of acetylation of Mdm2 (Fig. 3B, compare lanes 3 and 1). Thus, these residues appear to be major sites for CBP-directed acetylation of Mdm2 also in living cells.

In contrast to CBP, cotransfection with p300-HA did not enhance acetylation of Mdm2 in vivo (Fig. 3C), although the transfected p300 underwent substantial autoacetylation (Fig. 3C, Ac-p300). Thus, CBP is remarkably more effective than p300 in promoting Mdm2 acetylation in vivo.

3.4. Mutations mimicking Mdm2 acetylation impair p53 ubiquitination and degradation

The RING finger of Mdm2 is crucial for its E3 ubiquitin ligase activity. Given that Mdm2 acetylation occurs primarily within the RING finger domain, it was conceivable that Mdm2 acetylation might hamper its E3 ligase activity. To assess the impact of acetylation on Mdm2 activity, lysines 466 and 467 of Mdm2 were mutated to glutamine to mimic constitutive acetylation. In a cell-free assay for E3 ligase activity, wild-type Mdm2 promoted the polyubiquitination of p53 in a dose-dependent manner (Fig. 4A, upper panel, lanes 3–5, polyub-p53). Under the same conditions, the 466/467Q acetylation mimic mutant displayed a reduced ability to drive p53 polyubiquitination, particularly at relatively high Mdm2 concentrations (compare lanes 7, 8 to lanes 4, 5); in contrast, p53 monoubiquitination was not affected by the mutations. Interestingly, Mdm2-466/467Q also exhibited a partial defect in autoubiquitination (Fig. 4A, bottom panel; compare lanes

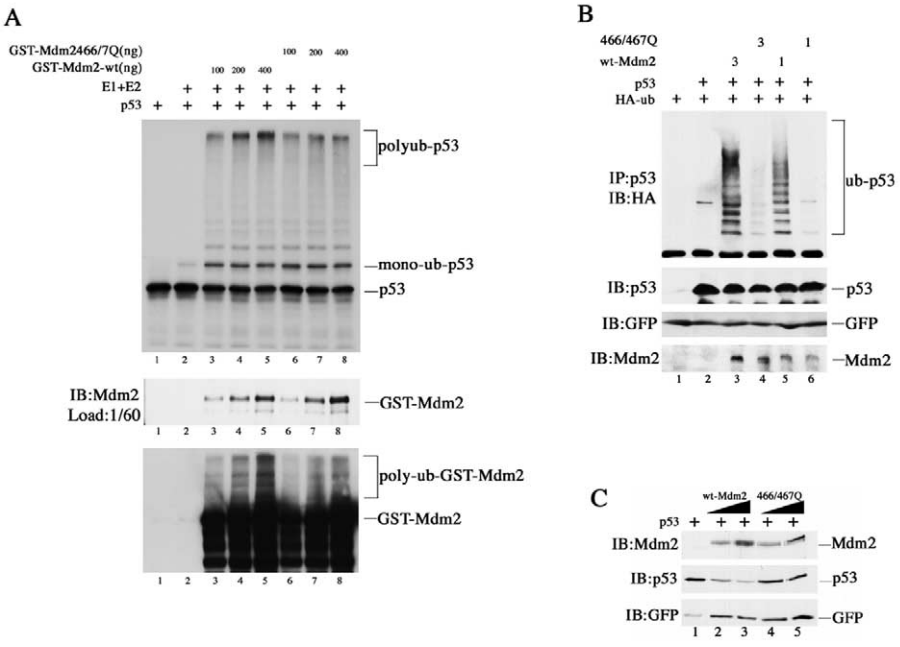

Fig. 4. Mutants mimicking Mdm2 acetylation possess reduced E3 activity and are deficient in p53 degradation. A: In vitro p53 ubiquitination was carried out as described in Section 2, in the presence of increasing amounts of GST-Mdm2 or GST-Mdm2-466/467Q. p53 was visualized by immunoblotting with a mixture of the p53-specific monoclonal antibodies DO-1 and PAb1801 (upper panel). polyub-p53, polyubiquitinated p53. Mdm2 autoubiquitination was examined by reprobing the same membrane with a polyclonal anti-Mdm2 antibody (bottom panel). Amounts of GST-Mdm2 and GST-Mdm2-466/467Q were monitored by Western blot analysis with a mixture of the Mdm2-specific monoclonal antibodies 4B11 and 2A9, following the loading of 1/60 of the reaction contents on an SDS–PAGE gel (middle panel). B: p53/mdm2 double null MEFs were transiently transfected with expression plasmids for human p53 and HA-ubiquitin, together with wild-type Mdm2 or Mdm2-K466/467Q (466/467Q). A GFP expression plasmid was also included. 24 h later, p53 immunoprecipitation was performed with a mixture of DO-1 and PAb1801. Covalent conjugates of p53 with HA-tagged ubiquitin (ub-p53) were visualized with an anti-HA antibody (upper panel). Transfection efficiency was monitored by analysis of cotransfected GFP. p53 and Mdm2 were monitored with a mixture of DO-1 and PAb1801 and a mixture of 2A9 and 4B11, respectively. C: p53/mdm2 double null MEFs were transiently transfected with a fixed amount of p53 expression plasmid (50 ng/6 cm dish) and increasing amounts (0.5 and 1 μg, respectively) of HA-Mdm2 or HA-Mdm2K466/467Q expression plasmids, together with a GFP expression plasmid. p53 and Mdm2 were monitored as in B.

6–8 to lanes 3–5). Hence, mutations that mimic the acetylated state of Mdm2 partially compromise its E3 activity.

To investigate whether Mdm2 acetylation affects its ability to ubiquitinate p53 in vivo, p53/mdm2 double null MEFs were transfected with p53 in combination with either wild-type or mutant Mdm2. While wild-type Mdm2 promoted efficient p53 ubiquitination (Fig. 4B, upper panel, lanes 3, 5), the 466/467Q mutant was severely compromised (lanes 4, 6). In fact, the disabling effect of the mutations on p53 ubiquitination was much more pronounced in vivo than in vitro, suggesting that Mdm2 acetylation may affect the interaction of Mdm2 with additional proteins, absent from the cell-free system, which modulate p53 polyubiquitination within cells.

To determine whether the reduced ubiquitination of p53 compromises its degradation, p53 levels were monitored in p53/mdm2 double null MEFs transfected with a fixed amount of p53 expression plasmid, together with increasing amounts of a plasmid encoding either wild-type Mdm2 (Fig. 4C, lanes 2, 3) or the 466/467Q mutant (lanes 4, 5). Whereas wild-type Mdm2 decreased markedly the steady-state levels of p53 (Fig. 4C, compare lanes 2, 3 to lane 1), comparable levels of Mdm2-466/467Q were relatively inefficient in downregulating p53 (lanes 4, 5). Thus, Mdm2 mutations that mimic acetylation within the RING finger domain severely reduce the E3 activity of Mdm2, as well as its ability to promote p53 degradation.

4. Discussion

We report that Mdm2 can be acetylated in vitro and in vivo. Several HATs, including p300, CBP and P/CAF, can efficiently acetylate p53 [21,27,28]. Of those, we show that CBP is the most efficient mediator of Mdm2 acetylation and p300 is substantially less efficient, whereas P/CAF does not target Mdm2 at all, although it binds Mdm2 [29]. Mdm2 binds both CBP and p300. Furthermore, in vitro acetylation of Mdm2 by recombinant HATs or HAT fragments has also been reported [21,26], although others have challenged the relevance of those findings [30]. We find that acetylation occurs mainly within the RING finger domain of Mdm2. Yuan and coworkers reported that, in addition to a C-terminal fragment (370–491), p300-dependent in vitro acetylation can also occur within a central fragment (amino acids 124–246) of Mdm2 [26]; this may account for the residual CBP and p300-dependent acetylation signal observed with the Mdm2/4KR mutant. Of note, a recent study did not observe acetylation of Mdm2 by p300, neither in vitro nor in vivo [30]. Since p300 is a less potent HAT for Mdm2 than CBP, the extent of Mdm2 acetylation by p300 may have been below detection in the study of Zeng et al.

Mdm2 acetylation was mapped in part to lysines 466/467, which reside within the proposed nucleolar localization signal (NoLS) of the protein [31]. It was thus tempting to speculate that Mdm2 acetylation, through changing the charge of this basic NoLS, might serve to modulate the intracellular localization of the protein. However, Mdm2-466/467Q was translocated into the nucleolus as efficiently as wild-type Mdm2, when assayed in the presence of $p14^{ARF}$ (data not shown). On the other hand, Mdm2-466/467Q displayed a pronounced defect in p53 ubiquitination and degradation, as well as in autoubiquitination in vitro. This suggests that Mdm2 acetylation, when occurring in vivo, is likely to serve as an additional mechanism for inactivation of Mdm2 and thus more efficient induction of a p53 response.

p53 acetylation is generally regarded as an important component in p53 activation by various types of stress. Our data raise the possibility that Mdm2 acetylation may potentially play a similar role. Thus, regulated protein acetylation may contribute to the p53 response through a dual mechanism, involving simultaneous activation of p53 and inactivation of its negative regulator Mdm2.

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