**MDM2 Inhibits PCAF (p300/CREB-binding Protein-associated Factor)-mediated p53 Acetylation**

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Our previous study shows that MDM2, a negative feedback regulator of the tumor suppressor p53, inhibits p300-mediated p53 acetylation. Because PCAF (p300/CREB-binding protein-associated factor) also acetylates and activates p53 after DNA damage, in this study we have examined the effect of MDM2 on PCAF-mediated p53 acetylation. We have found that MDM2 inhibited p53 acetylation by PCAF in vitro. In addition, when overexpressed, MDM2 inhibited PCAF-mediated p53 acetylation in cells. MDM2 interacted with PCAF both in vitro and in cells, as assessed using GST fusion protein interaction and immunoprecipitation assays, respectively. Consistent with the above results, MDM2 significantly repressed the activation of p53 transcriptional activity by PCAF without apparently affecting the level of p53. In addition, MDM2 co-resided with p53 at the p53-responsive mdm2 and p21waf1/cip1 promoters, inhibiting expression of the endogenous p21waf1/cip1. These results demonstrate that MDM2 can inhibit PCAF-mediated p53 acetylation and activation.

The tumor suppressor p53 protein is a transcriptional activator that induces expression of many target genes whose protein products mediate p53-dependent cell growth arrest and apoptosis, thus suppressing cell transformation and tumorigenesis (1, 2). This protein is tightly regulated under physiological and pathological conditions. Post-translational modifications of p53 play crucial but different roles in regulating p53 stability and activity (3). For example, phosphorylation of p53, stimulated by UV and γ irradiation, stabilizes p53 and subsequently activates its activity (4–11). In addition, p53 is acetylated at several C-terminal lysines in response to genotoxic agents (12–14). One group of acetylases, p300 and CBP, has been shown to acetylate p53 in vitro and in cells (12, 14–16). Thus, by acetylating p53, p300/CBP may stabilize and activate p53 (17–20). Although less studied, the p300/CBP-associated factor (PCAF) has also been shown to specifically acetylate the lysine 320 of p53, leading to the enhancement of the sequence-specific DNA binding activity of p53 in vitro (13). The acetylation at lysine 320 is responsive to DNA damage as well (12, 13). Hence, these types of modifications generally result in up-regulation of p53 function. Consistent with this, deacetylation of p53 by HDAC1 and Sir2a deacetylases has been recently shown to inactivate p53 function (21–24). Additionally, adenovirus E1A and E1B 55-kDa oncoproteins inhibit p53 acetylation catalyzed by PCAF (25, 26).

However, p53 is also negatively regulated by post-translational ubiquitination. This modification is mediated by the p53 suppressor, MDM2, which possesses a Ring finger E3-like ubiquitin ligase activity (27–29). It is believed that under physiological or normal conditions, the turnover rate of p53 is controlled by MDM2 through the ubiquitin-mediated proteasome system (30–32). Upon cellular insults, p53 is phosphorylated and/or acetylated (3). These modifications prevent p53 from attack by MDM2. Thus, p53 becomes stabilized and activated, transcriptionally activating the expression of its target genes. Because mdm2 is a p53 target (33, 34), more MDM2 proteins are produced after DNA damage to repress p53 function. Hence, under pathological or abnormal conditions, MDM2 must antagonize multiple enzymes, including kinases and acetylases, in order to monitor p53 function. How these proteins interplay to finely tune p53 activity becomes an important question.

In an attempt to address this issue, we (16) and others (14) have recently shown that MDM2 inhibits 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 (16). Because interaction between p53 and MDM2 or MDM2 and p300 is required for inhibiting p300-mediated p53 acetylation by MDM2, MDM2 inhibits p53 acetylation by forming a ternary complex with p53 and p300 (16). This finding prompted us to determine whether MDM2 is able to inhibit PCAF-mediated p53 acetylation and activation. In the study described here, we found that MDM2 inhibited p53 acetylation by PCAF in vitro. Furthermore, when overexpressed in cells, MDM2 inhibited PCAF-mediated p53 acetylation in vivo. MDM2 interacted with PCAF both in vitro and in cells, as assessed using GST fusion protein interaction and immunoprecipitation assays. In addition, MDM2 significantly repressed the activation of p53 transcriptional activity by PCAF. These results demonstrate that MDM2 can also inhibit PCAF-mediated p53 acetylation and activation. These studies support the idea that after DNA damage, MDM2, once induced by p53, needs to prevent p53 acetylation in order to efficiently ubiquitinate and degrade this protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Human lung small cell carcinoma H1299 cells and human embryonic kidney epithelial 293 cells were cultured as previously described (35, 36).
Buffers—Lysis buffer consisted of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. SNNTe buffer contained 50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1% Nonidet P-40, 500 mM NaCl, and 5% sucrose. RIPA was comprised of 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate buffered to pH 7.4. Buffers were stored at 4°C. Cross-linking was stopped by incubating the cells on the 0.125 M glycine for 5 min. The cells were then harvested in 1 ml of lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 5 mM EDTA) containing the protease inhibitors peptatin A (10 μg/ml), leupeptin (10 μg/ml), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mM), and 10 m M dithiothreitol. Kidney epithelial 293 cells were transfected with pCDNA3-MDM2, pCMV-p53, pCDNA3-ΔN-MDM2, and pCDNA3-Δ150–230 (MDM2) as described (40). pCX-FLAG-PCAF was described previously (39).

Construction of pRSV-PCAF, pRSV-PCAF/-529, and pRSV-PCAF/-666–466 Plasmids—To express PCAF under the control of the RSV and T7 promoters, Neol and KpnI sites were first introduced at the translation initiation codon and after the stop codon of the PCAF coding sequence. The full-length coding sequence was then cloned between the Neol and KpnI sites of the expression vector pExpress-O (41). pRSV-1–529 was constructed similarly by creating Neol and KpnI sites at the translation initiation codon and after the codon for residue 529, respectively. A stop codon was also inserted between codon 529 and the KpnI site. The resulting 1.6-kb Neof/KpnI fragment was subsequently cloned between the Neol and KpnI sites of pExpress-O (41). pRSV-366–466 was constructed by digesting pRSV-PCAF with PstI followed by subsequent religation. These vectors were used for in vitro translation of PCAF and PCAF deletion mutants with the TNT transcription/translation system (42). Purification of Recombinant p300, PCAF, MDM2, and p53—PCAF and p300 were purified from baculovirus-infected SF9 insect cells using immunoaffinity columns as described (37, 39). His-p53 was purified from bacteria using a nickel-nitrilotriacetic acid column as described (37, 38). MDM2 and p53 were purified using an immunoaffinity column as described (37).

Establishment of HA-DMMD Expression Cell Lines—Human embryonic kidney epithelial 293 cells were transfected with pCDNA3-HA-DMMD or pCDNA3 vector. Transfected cells expressing HA-DMMD were selected in MDM2 expression 293 cells were harvested for preparation of nuclear extracts. Nuclear extracts containing 150 μg of proteins were directly loaded onto an SDS gel; proteins were detected by ECL reagents (Bio-Rad) after Western blotting using antibodies as indicated in the figure legends.

Transient Transfection and Luciferase Assay—H1299 cells (60% confluence in a 12-well plate) were transfected with a pCMV-β-galactosidase reporter plasmid (0.2 ug) and a luciferase reporter plasmid (0.1 ug) driven by two copies of the p53RE motif derived from the mdm2 promoter (43). An MD2 promoter (0.2 μg) together with a combination of different plasmids DNA, 1.0 μg of pCDNA3 vector, and 10 μg of pCDNA3-MDM2 were transfected into the cells. Cells were harvested for luciferase assays as described previously (38). Luciferase activity was normalized by a factor of β-galactosidase activity tested in the same assay.

Immunoprecipitation-Western Blot (IP-WB) Analysis—Transfected H1299, 293, or MDM2 expression 293 cells were harvested for preparation of nuclear extracts. Nuclear extracts containing 250 μg of proteins were used for IP, followed by WB as previously described (37).

Chromatin Immunoprecipitation (ChIP)-PCR—H1299 cells were transfected with pDNA3-p53 or pCMV-MDM2 vector, alone or together. ChIP analysis was performed as described (42, 43) with minor modifications. Briefly, cells were cross-linked with a 1% formaldehyde solution in phosphate-buffered saline for 10 min at room temperature. The cross-linking was stopped by incubating the cells on the 0.125 m glycine for 5 min. The cells were then harvested in 1 ml of lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 5 mM EDTA) containing the protease inhibitors peptatin A (10 μg/ml), leupeptin (10 μg/ml), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mM), and 10 m M dithiothreitol. Kidney epithelial 293 cells were transfected with pCDNA3-MDM2, pCMV-p53, pCDNA3-ΔN-MDM2, and pCDNA3-Δ150–230 (MDM2) as described (40). pCX-FLAG-PCAF was described previously (39).

RESULTS AND DISCUSSION

MDM2 Inhibits PCAF-mediated p53 Acetylation in vitro—It has been shown that MDM2 inhibits p300/CBP-mediated p53 acetylation (14, 16). PCAF-mediated p53 acetylation can also be inhibited by the adenovirus-encoded E1B 55-kDa oncoprotein (26), which like MDM2 represses p53 activity by binding to its N terminus (45) and leads to p53 degradation (46). To determine whether MDM2 is also able to affect PCAF-mediated p53 acetylation, we performed an in vitro acetylation assay using purified proteins. As shown in Fig. 1A, in the case of p53Ac66 (lane 6), MDM2 repressed PCAF-dependent p53 acetylation in a dose-dependent fashion in vitro (lanes 5 and 6). With 2-fold more MDM2 than PCAF in molar ratio, less than 10% of p53 molecules were acetylated based upon densitometry (lane 6). This result was reproducible. By contrast, the p53-binding defective mutant MDM2 in the same molar ratio was unable to inhibit p53 acetylation by PCAF (Fig. 1B). PCAF did not
not appear to acetylate MDM2 in vitro (Fig. 1C). Therefore, these results suggest that MDM2 can also inhibit p53 acetylation by PCAF, which specifically targets lysine 320 (12, 13). This inhibition requires the interaction between p53 and MDM2.

**MDM2 Inhibits PCAF-mediated p53 Acetylation in Cells**—To further determine whether MDM2 inhibits PCAF-mediated p53 acetylation in cells, human p53-deficient small cell carcinoma H1299 cells were transfected with plasmids encoding p53 and/or FLAG-PCAF alone or together. Cells were harvested to assess the levels of these proteins and the acetylation status of p53 lysine 320 using antibodies specifically against acetylated lysine 320. As expected (12, 13), FLAG-PCAF, when overexpressed, acetylated p53 at lysine 320 without significantly affecting the level of p53 (Fig. 2A, compare lanes 2 and 4). The same transfection was carried out in the presence or absence of the MDM2-encoding plasmid. In this experiment, the proteasome inhibitor, MG132, was added to medium 12 h prior to harvesting cells to prevent p53 degradation mediated by MDM2. As shown in Fig. 2B, PCAF again acetylated the lysine 320 of p53 (lanes 3 and 4). However, overexpression of MDM2 significantly reduced the level of this acetylation (lane 5 with lane 6). This reduction was not due to the low level of p53, because the level of p53 did not change in the presence or absence of MDM2. Furthermore, this inhibition was not due to the nonspecific effect of the plasmid, because it was not observed when the MDM2 deletion lacking the amino acid 130–250 region was used (Fig. 2C). This deletion mutant was unable to affect PCAF-mediated p53 acetylation largely because it lacks the nuclear localization sequence and is excluded from the nucleus (data not shown) (47). These results thus indicate that MDM2 inhibits PCAF-mediated p53 acetylation in cells.

**MDM2 Directly Binds to PCAF in Vitro**—The finding that MDM2 inhibits PCAF-mediated p53 acetylation suggests a possible interaction between MDM2 and PCAF. To test this and also to define their potential binding domains, we performed a series of in vitro GST fusion protein-protein association assays. In the first experiment, purified FLAG-PCAF proteins were incubated with GST-MDM2 or GST-MDM2 deletion mutants as described under “Experimental Procedures.” After rigorous washing, bound proteins were analyzed by SDS electrophoresis and Western blot, using anti-FLAG antibodies. As shown in Fig. 3A, PCAF bound to the full-length as well as the N-terminal region from amino acid 1 to 295. These bindings appeared to be specific because PCAF rarely bound to GST alone or GST fusion proteins containing the C terminus (amino acids 384–491) of MDM2. Because PCAF also interacted with the N terminus-deleted MDM2 (data not shown), which lacks amino acids 1–50, these results suggest that PCAF can bind to the amino acid 50–384 region of MDM2.

To map the MDM2-binding domain of PCAF, we carried out a similar GST-MDM2 pull-down experiment using in vitro translated and 35S-labeled PCAF and deletion mutants. As shown in Fig. 3B, PCAF as well as the N terminus (amino acids 1–529), but not the N terminus-deleted, fragment of PCAF bound to the GST-MDM2 protein (compare lanes 1 and 2 with lane 3). This binding was specific because none of these PCAF proteins interacted with the GST-MDM2 C-terminal domain (lanes 4–6). Taken together, these results demonstrate that MDM2 directly interacts with the N terminus of PCAF in vitro, whereas PCAF appears to bind to the same region of MDM2 to which p300/CBP binds (48).

**MDM2 Associates with PCAF in Cells**—To determine the interaction between MDM2 and PCAF in cells, human embryonic kidney 293 cells, which contain endogenous MDM2, were transfected with pCX-FLAG-PCAF or pCX alone. Cell lysates were prepared 48 h after transfection for immunoprecipitation using antibodies against FLAG, MDM2, and Jnk1, followed by Western blot with antibodies against MDM2 and FLAG. As shown in Fig. 4A, endogenous MDM2 proteins were co-immunoprecipitated with FLAG-PCAF by the anti-FLAG antibody in FLAG-PCAF expression cells but not in mock-transfected cells (lanes 1 and 2). Consistently, FLAG-PCAF was also co-immunoprecipitated with the endogenous MDM2 by the monoclonal MDM2 antibody in the FLAG-PCAF expression cells but not in mock-transfected cells (lanes 3 and 4). These results indicate that exogenous FLAG-PCAF associates with MDM2 in cells. This association was specific because none of these proteins was pulled down by the antibody against Jnk1 (lanes 5 and 6).

To make sure that endogenous PCAF interacts with MDM2, we conducted an additional immunoprecipitation-Western blot experiment using 293 cells and an MDM2-expressing 293 stable cell line. The MDM2 cell line was used because the level of
endogenous MDM2 in 293 cells is low (Fig. 4A). As shown in Fig. 4B, both endogenous (lane 2) and exogenous (lane 1) MDM2 proteins were co-immunoprecipitated by the anti-PCAF antibody but not by the anti-Jnk1 antibody (lanes 3 and 4). Thus, this result confirms that MDM2 and PCAF interact with each other in cells.

MDM2 Inhibits PCAF-activated p53 Transcriptional Activity in Cells—To determine the functional consequence of the inhibition of PCAF-mediated p53 acetylation by MDM2, we carried out transient transfection/luciferase assays. As shown in Fig. 5A, expression of the exogenous PCAF enhanced p53-dependent transcription as measured by the luciferase activity that was driven by the p21 promoter. p53 is encoded by a p53RE motif. However, further expression of the exogenous MDM2 reversed the p50 activation by PCAF and brought p53-dependent luciferase activity down almost to the basal level. This decrease was not due to the loss of p53 proteins, because the level of p53 did not change dramatically in the presence of the proteasome inhibitor, MG132. This result, which was reproducible, demonstrates that MDM2 can also inhibit PCAF-enhanced p53 transcriptional activity without apparently affecting its level. Together with our previous report (16), this suggests that MDM2 negatively regulates p53 activity at least in part by inhibiting its acetylation, which is catalyzed by p300/CBP and by PCAF.

MDM2 Co-resides with p53 at the Endogenous p53RE Motifs of the mdm2 and p21 Promoters—Because MDM2 can interact with p53RE-bound p53 in vitro (4B), it is possible that MDM2 may repress p53 activity by directly associating with the promoter-bound p53 in cells. To test this idea, we performed a set of chromatin-immunoprecipitation assays, following transient transfection. As described under “Experimental Procedures,” H1299 cells were transfected with plasmids encoding p53 or MDM2 alone or together. Immunoprecipitations were conducted with polyclonal anti-p53 and monoclonal anti-MDM2 antibodies. PCR reactions were carried out using primers that encompass the p53RE-containing sequence of either the mdm2 or the p21 promoter. The results are shown in Fig. 5B. As expected, p53 resided at both the mdm2 and p21 promoters. Interestingly, MDM2 also localized at these promoters. This must be due to the association of MDM2 with p53, because MDM2 does not bind to DNA. In fact, without p53, overexpression of MDM2 (Fig. 5C) alone did not display detectable PCR products from the promoters (Fig. 5B, lane 5). Furthermore, when nonspecific primers upstream from the mdm2 promoter were used, no PCR product was detected from the immunoprecipitates with either anti-p53 or anti-HA-MDM2 antibodies. Consistently, MDM2 significantly repressed p53-mediated expression of the endogenous p21 promoter (Fig. 5C). Hence, these
results demonstrate that MDM2 indeed associates with the promoter-bound p53, and this association might also play a role in negatively modulating p53 transcriptional activity.

We previously showed (16) that MDM2 inhibited p300-catalyzed p53 acetylation by directly associating with these two proteins. Here, we have extended this work by demonstrating that MDM2 is also able to negate PCAF-mediated p53 acetylation at lysine 320. MDM2 inhibited PCAF-mediated p53 acetylation both in vitro and in cells (Figs. 1 and 2). As in the case of p300 (16), the N-terminal domain of MDM2 is required for this inhibition, suggesting that interaction with p53 is essential for the inhibitory effect of MDM2 on p53 acetylation by the acetylases that have been tested. Interestingly, MDM2 also directly interacted with PCAF in vitro and in cells (Figs. 3 and 4). Similar to p300 (14, 48), PCAF bound to the MDM2 region from amino acids 50 to 350 (Fig. 3A and data not shown). Functionally, MDM2 also repressed PCAF-enhanced p53 transcriptional activity. Because MDM2 co-resided with p53 on the endogenous p53-responsive promoters and repressed p53-dependent expression of the endogenous p21 and Mdm2 (Fig. 5, B and C), MDM2 might block p53 function by interfering with the communication between p53 and the transcriptional machin-
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This study consistent with previous reports (14, 16) suggests that MDM2 may target multiple C-terminal lysines for ubiquitination. If this is true, it is likely that MDM2 might target the same lysine residues of p53 (52), thus leading to p53 degradation through the ubiquitin-proteasome machinery (27, 30, 31), although evidence linking p53 ubiquitination to its degradation has not yet been obtained. One study (53) suggests that MDM2 might target multiple C-terminal lysines for mono-ubiquitination. It is not clear whether MDM2 might target lysine 320 for ubiquitination as well. On the other hand, MDM2 also blocks p53 transcriptional activity by directly associating with this activator on the promoters and by inhibiting its acetylation at lysines 303, 322, and 459 (21). Whether MDM2 promotes this deacetylation is not yet clear. Inhibition of p53 acetylation or deacetylation is not yet clear. Inhibition of p53 acetylation or deacetylation is not yet clear. Inhibition of p53 acetylation or deacetylation is not yet clear. Inhibition of p53 acetylation or deacetylation is not yet clear. Inhibition of p53 acetylation or deacetylation is not yet clear.

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