Murine Double Minute (MDM2) Blocks p53-coactivator Interaction, a New Mechanism for Inhibition of p53-dependent Gene Expression*

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The ability of the p53 tumor suppressor to induce cell cycle arrest and cell death is closely regulated under normal conditions. The transcriptional activity of p53 is negatively controlled by murine double minute (MDM2). p53 requires the coactivator CREB-binding protein (CBP), or its structural homolog, p300, to stimulate transcription of responsive genes. Here we find that the transactivation domain of p53 selectively interacts with the N- and C-terminal regions of CBP/p300. A mutant CBP lacking the N terminus failed to stimulate p53-dependent transactivation. In both p53 null Saos2 cells, and in UV-irradiated MCF7 cells, we observed that MDM2 associates with the N-terminal region of CBP/p300. Because p53 interacts with both MDM2 and CBP/p300 through its trans-activation domain, we examined the role of MDM2 in p53-coactivator interactions. MDM2 blocked CBP/p300 recruitment in vitro and inhibited the interaction of the transactivating region of p53 with both the N- or C-terminal regions of CBP/p300 in a mammalian two-hybrid assay. These observations suggest that MDM2 may be inhibiting p53 trans-activation by shielding its activation domain from the coactivators, a new mechanism for the inhibition of p53-dependent gene expression.

The tumor suppressor p53 plays an important role in cell cycle progression and apoptosis by binding to p53 response elements and functioning as a transcriptional activator (1, 2). Genes with p53 responsive elements include p21 (3), growth arrest and DNA damage (GADD45) (4), Bax1 (5), cyclin G, (6) and murine double minute (mdm2) genes (7, 8). Transactivation of the p21 gene by p53 has been correlated with cell cycle control by inducing G1 arrest or apoptosis (3, 9). In addition to its transactivation function, when overexpressed, p53 can indirectly act as a transcriptional repressor through its interaction with the TATA-binding protein (10), or by squelching coactivators (11).

Transcriptional coactivators p300 and CREB-binding protein (CBP/p300) interact with wild-type as well as mutant forms of p53 (12–14). CBP/p300 function as transcriptional coactivators by linking a number of cellular activators to components of the basal transcription machinery (15, 16). CBP and p300 are 63% identical overall at the amino acid level. Greater similarity is observed in specific regions, including the N-terminal C/H1 region encompassing the CREB binding site (93%), and the C-terminal C/H3 region, which includes the E1A binding site (15, 16). Accumulating evidence suggests that regions within these coactivators may have similar functions. The C-terminal region of CBP/p300 binds to the activation domain of p53, a region where several other basal transcription factors interact with p53 (12–14, 17). These interactions are necessary for p53 to function as a transcription factor (12–14). The CBP associated factor (p/CAF), a CBP-binding protein, is also a coactivator for p53-dependent trans-activation (18). Coactivators like CBP and p/CAF have histone acetyltransferase activity, suggesting a role for histone acetylation in transcriptional regulation (19, 20). p300 acetylates the C-terminal region of p53, resulting in increased DNA binding and transactivation function of p53 (21).

Overexpression of p53 induces the expression of the mdm2 proto-oncogene, the product of which binds to the trans-activation region of p53 and inhibits its ability to stimulate transcription (22–24). Additionally, recent studies have shown that MDM2 is involved in degradation of p53 via the ubiquitin-proteasome pathway (25, 26). This negative feedback loop is necessary to control p53 activity because mdm2 knockout mice die during development, but mice with mutations in both the mdm2 and p53 genes are normal (27). These observations demonstrate a key role for MDM2 in controlling p53 activity, and illustrate the importance of understanding how p53 is regulated by MDM2. Repression of p53-mediated transcription by MDM2 is thought to involve two mechanisms. First, MDM2 may act by inhibiting interactions between the transactivating region of p53 and components of the basal transcriptional machinery (24). Second, MDM2 possesses an additional intrinsic inhibitory function that directly represses basal transcription in the absence of p53 (28). The relative importance of these mechanisms in the repression of p53 activity by MDM2 is not well understood.

Here we focused on the interaction of p53 with a coactivator necessary for its transactivation ability. We found that the transactivation domain of p53 selectively interacts with the N- as well as the C-terminal sections of CBP/p300. The functional significance of the N-terminal region of CBP/p300 in p53 transactivation was suggested by findings from transient transfections with dominant negative forms of the coactivator. A mutant of CBP/p300 lacking just the N terminus was unable to stimulate p53 transactivation. Similarly, overexpression of the N-terminal region of CBP/p300 was able to abrogate p53-dependent transactivation of a p21 promoter-reporter construct. Remarkably, MDM2 also interacts with the N-terminal region of CBP/p300. In both p53 null Saos2 cells, and in UV-irradiated MCF7 cells, we observed that MDM2 can associate with the
N-terminal region of CBP/p300. The functional significance of these interactions was demonstrated in vivo in mammalian cells. MDM2 inhibited the interaction of the transactivating region of p53 with either the N- or C-terminal regions of CBP/p300 in a mammalian two-hybrid assay. These observations suggest that MDM2 may be inhibiting p53 trans-activation by concealing its activation domain from the coactivators, a new mechanism for the inhibition of p53-dependent gene expression.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions—**For in vitro translation, cDNAs for wild-type p53 and p53 mutants were cloned into the BamHI site of the expression vector pSP64A+ (Promega Corp.) GST-p53 was constructed by subcloning wild-type p53 into the EcoRI site of pGEX-1T-1 (Amersham Pharmacia Biotech). Wild-type p53, p53*13–53*21-luciferase and the p21 promoter-luciferase reporter vectors were provided by Dr. Bert Vogelstein (The Johns Hopkins University). The full-length human MDM2 expression vector was provided by Dr. Arnold Levine (Princeton University). A full-length RSV-based CBP expression vector was provided by Dr. Richard Goodman (Oregon Health Science University, Portland). Regions of CBP were assembled into the GAL4 and VP16 vectors provided in the mammalian Matchmaker two-hybrid assay kit (CLONTECH), as described (29).

**Reticulocyte Translation—**Wild-type and deletion mutants of p53 were in vitro translated using [35S]methionine in TNT SP6/T7-coupled rabbit reticulocyte lysate according to the manufacturer instructions (Promega).

**GST Fusion Protein Expression and Pulldown Experiments—**GST fusion proteins were expressed, and extracts were prepared as recommended by the manufacturer (Amersham Pharmacia Biotech). Bacterial extracts containing GST, GST-p53, and GST-CBP were incubated with 20 μl of glutathione-Sepharose in 200 μl of buffer S (phosphate-buffered saline plus 0.1% Nonidet P-40, containing 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1.5 μg/ml pepstatin) for 3 h at 4 °C. The fusion protein bound beads were washed three times with 200 μl of 0.5× buffer 20 (20 mM HEPES, pH 7.6, 500 mM NaCl, 0.5 mM EDTA, and 0.1% Nonidet P-40). For GST pulldown experiments, fusion proteins bound to the beads were incubated with proteins from total cell extracts as well as reticulocyte translated proteins for 3 h at 4 °C. The beads were washed using buffer B containing 150 mM NaCl and eluted with 25 μl of SDS sample buffer (75 mM Tris-HCl, pH 6.8, 1% SDS, 4% beta-mercaptoethanol, 0.1% bromphenol blue), and boiled for 5 min before separating on an 8% SDS-polyacrylamide gel. Eluted proteins were subjected to Western blot analysis.

**Immunoprecipitation/Western Blot—**Whole cell extracts were prepared from transfected Saos2 cells by mechanical disruption in 500 μl of lysis buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1.5 μg/ml pepstatin, 0.2 mM levanolese, 10 mM β-glycerophosphate, and 0.5 mM benzamidine) per 100-mm2 plate of confluent cells. Particular matter was removed by centrifugation at 10,000 × g for 20 min. Extracts were precleared with rabbit or mouse secondary antibody and IgG/Ga-agarose beads. Supernatants were incubated with 10 μg/ml MDM2 (Calbiochem or Santa Cruz Biotechnology) or CBP (Santa Cruz Biotechnology) antibodies, respectively. The antibodies were bound to 50 μl of IgG/Ga-agarose beads. These beads were washed three times with 1 ml of lysis buffer and resuspended in SDS sample buffer. The eluted proteins were boiled for 3 min and separated by SDS-polyacrylamide gel electrophoresis (6–10%). Separated proteins were transferred to nitrocellulose membranes (Schleicher and Schuell), blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Tween 20), and incubated with either anti-CBP or anti-MDM2 antibodies for 3 h at room temperature. Blots were washed three times in TBST buffer, incubated with a donkey anti-rabbit or -mouse secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech), and washed three times in TBST. Bound proteins were visualized using the ECL chemiluminescence reagent (Amersham Pharmacia Biotech) followed by autoradiography for 30 s to 60 min.

**Tissue Culture, Transfections, and Reporter Assays—**Approximately 10° osteosarcoma (Saos2) cells were seeded in 60-mm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transfections were performed by the calcium phosphate procedure (30), as described in the figure legends. All transfections contained 0.5 μg of the plasmid CMV/p63-gal as an internal control to monitor transfection efficiency. Cells were harvested 24–36 h after transfection, and β-galactosidase activity was determined by a colorimetric assay. Mammalian matchmaker two-hybrid assays were performed according to manufacturer protocol (CLONTECH).

**UV Irradiation and Preparation of Cell Extracts—**After removing the media, 80% confluent control and transfected MCF7 cells were exposed to UV light. The source of UV light was a Stratalinker (Stratagene), and total exposure was 20 J/m2 at 254 nm UV.

**RESULTS**

The N-terminal Region of CBP/p300 Interacts with p53—We studied p53-CBP interaction and its effect on p53-dependent transactivation as a model to determine the role of coactivators in p53-dependent cell survival and apoptosis. To define the interaction domains of CBP with p53 in more detail, CBP was divided into six overlapping regions (1–6) which were linked to GST and expressed as fusions in bacteria (Fig. 1A). An in vitro translated labeled wild-type p53 was incubated with the GST-CBP fusion proteins. Wild-type p53 showed an interaction with three different regions of CBP (Fig. 1B), but not GST alone. We found that the C-terminal region of CBP showed strong (amino acids 1452–1892) and moderate (amino acids 1892–2441) interactions with p53, consistent with previous studies (12–14). In addition to these regions of p53 and CBP interaction, the N-terminal region of CBP (amino acids 1–771) also showed strong binding to p53 (Fig. 1B, lane 3). This is consistent with the recent report of a p53 binding site in the N terminus of p300 (15), and the striking homology of the proteins in this region.

To further define the interaction between p53 and CBP, various N-terminal deletion mutants of the coactivator (Fig. 1A) were fused to GST and used in GST pulldown assays with full-length wild-type p53. As described in Fig. 1C, the extreme N-terminal CBP (amino acids 1–100), which is involved in nuclear hormone receptor binding, did not show an interaction with p53 (Fig. 1C, lanes 4 and 5). The N-terminal domain from amino acids 373–771, which is important for c-JUN and c-MYB binding, interacted poorly with p53 (Fig. 1C, lane 6). These results suggest that the N-terminal region of CBP between amino acids 100–446 is required for complex formation with p53.

Interaction between the N Terminus of CBP/p300 and the Transactivation Domain of p53 in Vivo—To understand the importance of the interaction between the N-terminal region of CBP and p53, we verified the binding by mammalian two-hybrid analysis. Wild-type p53, either containing the transactivation domain (amino acids 1–393), or lacking this region (amino acids 79–393), were fused with the transcriptional activator VP16 (Fig. 2A). The N-terminal region of CBP (amino acids 1–771) and another region (amino acids 500–1000) were fused to the DNA-binding protein GAL4 (Fig. 2A) and were tested for interaction with full-length and truncated p53 in transfected Saos2 cells. In control studies, the ability of CBP-GAL4 and VP16-p53 constructs to activate GAL4-luciferase was determined by a colorimetric assay. Mammalian matched two-hybrid assays were performed according to manufacturer protocol (CLONTECH).
results (Ref. 13, and data not shown). Collectively, these observations suggest that the transactivation domain of p53 interacts with the N-terminal section of CBP.

The N Terminus of CBP/p300 Is Required for p53 Transactivation—To determine whether the N terminus of CBP was functionally important in p53 transactivation, two approaches were taken. In the first, overexpression experiments were performed with intact and specifically altered (Fig. 3A) forms of CBP (32). Overexpression of p53 activated transcription from a p21 promoter-reporter construct approximately 10-fold (Fig. 3B, lanes 2 and 3). Co-transfection of expression vectors for intact CBP and p53 further stimulated transcription from this reporter construct about 4-fold (Fig. 3B, lane 5). This suggests that levels of CBP limit p53-dependent gene expression, consistent with previous reports (12–14). Interestingly, both the CBP deletion mutants (Δ468 and ΔC/H3) had reduced ability to stimulate p53-dependent transcription (Fig. 3B, compare lanes 6 and 7, with lane 2). The N-terminal CBP mutant (Δ468) had no activity, either activation or repression (Fig. 3B, compare lane 6 with lane 2), while the ΔC/H3 region retained some activation capacity, relative to full-length CBP (Fig. 3B, compare lane 7 with lane 2).

In the second approach, to demonstrate that the N terminus of CBP/p300 was functionally relevant to p53 transactivation, we determined the effect of overexpressing the N terminus of CBP on p53 transactivation. A small region of CBP (amino acids 100–446) capable of binding p53, but incapable of interacting with MDM2, was used in overexpression studies (see Fig. 4A). Saos2 cells were transiently transfected with a reporter plasmid containing multiple p53 response elements (PG13) and increasing amounts of wild-type p53. The tumor suppressor activated expression of the p53-luciferase reporter (Fig. 3C, lanes 1–4). In the presence of p53, CBP stimulated the activity of the reporter gene severalfold (Fig. 3C, lanes 7 and 8). When the CBP deletion mutant (amino acids 100–446) was co-transfected with p53 and CBP, a dramatic inhibition of CBP co-activation was noted (Fig. 3C, compare lanes 3 with lanes 7 and 10). Western blot analysis demonstrated that this effect was not because of diminished levels of p53 (Fig. 3C, inset). Additionally, the 100–446 region did not inhibit expression of a co-transfected β-galactosidase vector (Fig. 3D), suggesting that the inhibition of CBP co-activation was not a nonspecific effect. Thus, both approaches suggest that the N-terminal section of CBP is necessary for p53 transactivation.

Association of MDM2 and CBP—The cellular proto-oncogene MDM2 forms an autoregulatory loop with p53 because the MDM2 gene contains a promoter that is responsive to wild-type p53 (22). Synthesis of MDM2 protein increases in a p53-dependent manner in response to cellular stress such as DNA damaging agents like UV light. MDM2 interacts with the activation domain of p53 and blocks the transactivation ability of the tumor suppressor. Because MDM2 inhibits p53 transactivation, we determined whether MDM2 interacts with CBP. MCF7 cells were irradiated with UV to induce p53 expression, and total cell extract was used for GST pulldown experiments with the N-terminal deletion mutants of CBP. As shown in Fig. 4A, we observed strong interaction of wild-type p53 with both the 1–446 and 1–771 portion of CBP. UV irradiation also
MDM2 Blocks p53 Interaction with CBP

To demonstrate the physical association of MDM2 with CBP in a cellular context, we overexpressed MDM2 in p53 null Saos2 cells and used immunoprecipitation-Western blot analysis to detect interactions. Extracts from cells transiently transfected with a MDM2 expression vector were immunoprecipitated using an MDM2-specific antibody. The coactivator was detected in immunoprecipitates with the MDM2 antibody, but not with nonimmune IgG (Fig. 4C, lane 2). This supports the finding that MDM2 can interact with CBP in the absence of p53. The same approach was taken in an endothelial cell line (ECV-304) that expresses p53. Whole cell extracts were prepared from control cells, or cells exposed to ultraviolet light. Subsequent immunoprecipitation-Western blot analysis revealed that in these p53-containing cells, the tumor suppressor is contained in the MDM2 immunoprecipitation products (Fig. 4D).

To further demonstrate this association, the complementary study of exchanging the antibodies was done. Cell lysates from MDM2-programmed Saos2 cells were immunoprecipitated with either CBP or p300 antibodies, and Western blot analysis was performed with an anti-MDM2-specific serum. Antibodies specific for either CBP or p300, but not an irrelevant antibody, contained MDM2 in the immunoprecipitated products (Fig. 4E). These studies demonstrate that in a p53 null background, MDM2 can associate with CBP in intact cells. These findings are consistent with a recent report demonstrating that MDM2 can bind to the N terminus of p300 (15).

MDM2 Blocks p53 Interactions with Both the N- and C-terminal Regions of CBP/p300 in Vivo—To study the effect of MDM2 on p53-CBP interactions in vivo, we first verified that MDM2 inhibited p53-dependent transactivation. The transactivation region of p53 (amino acids 1–100) was fused to the GAL4 DNA binding domain and co-transfected with a GAL4-luciferase reporter (PF4). In this one-hybrid assay in Saos2 cells, the p53 GAL4 construct stimulated expression of the reporter (Fig. 5A, lane 2). Expression of MDM2 inhibited transcriptional activation by GAL4-p53 in a concentration-dependent manner (Fig. 5B, lanes 3–5), although vector alone did not inhibit the GAL4 reporter activity (Fig. 5A, lanes 6–8). This suggests that MDM2 blocked p53 transactivation, consistent with previous studies (17, 22).

MDM2 Blocks p53 Interactions with Both the N- and C-terminal Regions of CBP/p300 in Vivo—To study the effect of MDM2 on p53-CBP interactions in vivo, we first verified that MDM2 inhibited p53-dependent transactivation. The transactivation region of p53 (amino acids 1–100) was fused to the GAL4 DNA binding domain and co-transfected with a GAL4-luciferase reporter (PF4). In this one-hybrid assay in Saos2 cells, the p53 GAL4 construct stimulated expression of the reporter (Fig. 5A, lane 2). Expression of MDM2 inhibited transcriptional activation by GAL4-p53 in a concentration-dependent manner (Fig. 5B, lanes 3–5), although vector alone did not inhibit the GAL4 reporter activity (Fig. 5A, lanes 6–8). This suggests that MDM2 blocked p53 transactivation, consistent with previous studies (17, 22).

The findings outlined above demonstrated that MDM2 interacted with the N-terminal region of CBP/p300. As noted previously, this section of the coactivator has endogenous transcriptional activity. To determine whether MDM2 binding blocks this transcriptional activity, we again utilized a one-hybrid approach. As shown in Fig. 5B, the N terminus of CBP activates transcription of a GAL4 reporter construct in Saos2 cells. Expression of MDM2, but not vector, results in repression of reporter gene activity. Because p53 is not present in these cells, the decrease in reporter gene activity may be a direct effect of MDM2 on CBP 1–771 transactivation. This suggests that binding of MDM2 to this region of CBP blocks the endogenous transcriptional activating capacity of the coactivator.

To examine the effect of MDM2 on interactions between p53 and either the N- or C-terminal regions of CBP, we utilized the mammalian two-hybrid system. An MDM2 expression vector was cotransfected with GAL4-CBP-(1–771) and VP16-p53-(1–100) expression constructs into Saos2 cells. A dose-dependent down-regulation of GAL4 reporter construct activity was noted (Fig. 5C, lanes 5–7). Overexpression of an irrelevant vector did not diminish the reporter gene activity (Fig. 5C, lane 8). In control studies, Western blot analysis revealed that levels of the VP16-p53 activator did not diminish with increasing amounts of MDM2 (data not shown). Similarly, levels of β-galactosidase did not diminish with increasing amounts of MDM2 (data not shown).

A similar approach was used to examine the effect of MDM2 on interactions between p53 and the C-terminal region of CBP. In a mammalian two-hybrid assay in Saos2 cells, the MDM2 expression vector was cotransfected with VP16-CBP-(1452–

Fig. 2. The N-terminal domain of CBP functionally interacts with the activation domain of p53. A, structure of the CBP-GAL4 and VP16-p53 fusion constructs. The indicated regions of CBP were cloned into a vector containing the GAL4 DNA binding domain. Full-length (amino acids 1–393) and truncated (amino acids 79–393) forms of wild-type p53 were fused with VP16 activation domain (AD). The indicated regions of CBP were cotransfected with 2 μg of a GAL4-luciferase reporter gene, expression plasmids for GAL4-CBP-(1–771) (25 ng), or GAL4-CBP-(500–1000) (25 ng), either alone, or with an equivalent amount of full-length or truncated p53 (tp53). Total DNA was kept constant at 5 μg per 60-cm tissue culture dish. Data are representative of three different experiments.
MDM2 decreased the expression of the GAL4-dependent reporter construct (Fig. 5D, lanes 10–12). In control studies, levels of β-galactosidase did not diminish with increasing amounts of MDM2 (data not shown), indicating that the inhibition by MDM2 was not a nonspecific effect.

MDM2 Blocks Recruitment of the N Terminus of CBP/p300 to p53—The previous studies suggest that MDM2 blocks the interaction between p53 and both the N- and C-terminal regions of CBP. This could be because of MDM2 binding to p53 and inhibiting the interaction between the activator and CBP. Alternatively, MDM2 may be binding to CBP and blocking its interaction with p53. Because MDM2 does not repress the expression of other CBP-dependent inducible genes in overexpression studies (33), and CBP must be recruited to p53 to perform its role as a coactivator, we investigated whether MDM2 blocked CBP recruitment to p53. GST-p53 beads were first saturated with unlabeled MDM2 transfected Saos2 cell extracts and then were incubated with a labeled region of the N terminus of CBP (amino acids 100–446). As expected, GST protein alone did not show any binding of the CBP, but GST-p53 alone showed significant binding to this portion of the coactivator (Fig. 5E, lane 3). Binding of CBP(100–446) to GST-p53 was blocked by MDM2 (Fig. 5E, lane 4). This suggests that recruitment of the N-terminal region of CBP to p53 can be inhibited by MDM2.

DISCUSSION

In this study we found the transactivation domain of p53, and its inhibitor MDM2, functionally interacts with the N-terminal region of CBP/p300. MDM2 inhibited the interaction between the transactivating region of p53 and both the N- and C-terminal regions of CBP/p300 in vitro in a mammalian two-hybrid assay, and blocked p53 recruitment of CBP/p300 in vitro in an interaction study. These findings provide new insights into how MDM2 negatively regulates p53-dependent gene expression.

Repression of p53-mediated transcription by MDM2 is thought to involve three mechanisms. First, MDM2 could act by inhibiting interactions between the transactivating region of p53 and components of the basal transcriptional machinery (24). This is consistent with studies reporting that the trans-
The activation domain of p53 interacts with various nuclear proteins from basal transcription machinery, including TATA-binding protein, TAFII 31, and TAFII 70 (10, 34, 35). The interaction of the basal factors with p53 involves residues Leu22 and Trp23. This region of p53 is also involved in MDM2 interaction (36), which shows the complexity and importance of this region in transcriptional activity of wild-type p53. A second mechanism by which MDM2 represses p53 transcription involves an intrinsic inhibitory function that directly represses basal transcription in the absence of p53 (28). When MDM2 is recruited to p53, it may provide a potent negative effect on the function of the basal transcriptional machinery. The third mechanism by which MDM2 can regulate p53 function is by stimulating the degradation of the activator. Recent studies demonstrated that MDM2 acts as a ubiquitin ligase E3 for rapid degradation of p53 via ubiquitin-proteasome pathway (25, 41). In this model, MDM2 binds to p53 and inhibits gene expression by stimulating proteosome-mediated degradation of the transcription factor. Association of MDM2 with CBP/p300 may also have an important modulatory role in p53 stability. Each of these mechanisms contributes to the overall inhibitory effect of MDM2, although the relative importance of these p53 control systems is uncertain.

The new findings outlined here indicate that MDM2 has an additional mechanism of action. When p53 stimulates the transcription of target genes, it is probably positioned in a collection of other transcription factors that generates a precise network of interactions that are unique to a given p53 target gene. This assembly of activators presents a distinct surface that is required for CBP/p300 recruitment, as well as a surface that is displayed to the basal transcriptional machinery (16, 37). This leads to cooperative recruitment of RNA polymerase II and chromatin remodeling factors to DNA, and to synergistic activation of transcription (38, 39). Any change in the ability of the transcription factors to interact with CBP has a dramatic effect on the activation of gene expression (40). The data reported here are consistent with this type of model, in that MDM2 inhibited the interaction between both the transactivating re-
MDM2 inhibits p53 interaction with CBP

**A.** MDM2 inhibits GAL4-p53 transactivation. 2 μg of a GAL4-luciferase reporter (pF4) were cotransfected into Saos2 cells with 20 ng of a GAL4-p53 expression vector containing the transactivating region of p53 fused to a GAL4 DNA binding domain either together (lane 2) or with increasing amounts (250 ng, 500 ng, or 1 μg) of an MDM2 expression vector (lanes 3–5), or with equivalent amounts of an empty pCR3 expression vector (lanes 6–8). MDM2 inhibits p53 transactivation by the GALA-p53 fusion protein.

**B.** MDM2 negatively regulates GAL4-CBP-(1–771) mediated transactivation. A GAL4-CBP-(1–771) expression vector (2.0 μg) was cotransfected into Saos2 cells with the GAL4-luciferase reporter (pF4), either alone (lanes 2 and 3), with increasing amounts (500 ng or 1 μg) of an MDM2 expression vector (lanes 4 and 5, respectively), or with equivalent amounts of an empty pCR3 expression vector (lanes 6 and 7). MDM2 inhibits the endogenous transcriptional activity of the N terminus of CBP.

**C.** MDM2 blocks the interaction between p53 and the N terminus of CBP in a mammalian two-hybrid system. In Saos2 cells, 2 μg of the GAL4-luciferase reporter (pF4) was cotransfected with 25 ng of the GAL4-CBP-(1–771) expression vector and 25 ng of the VP16-p53 expression vector (200 ng), either alone (lane 4), or with increasing amounts (100, 200, or 400 ng) of either MDM2 (lanes 5–7) or 500 ng of a control vector (lane 8). Total DNA concentration in all transfected plates was adjusted to 5 μg of total DNA with empty pcDNA expression plasmid. MDM2 inhibits the interaction of p53 with the N terminus of CBP.

**D.** MDM2 blocks the interaction between p53 and the C-terminal region of CBP in a mammalian two-hybrid system. In Saos2 cells, 2 μg of the GAL4-luciferase reporter (pF4) was cotransfected with 20 ng of a GAL4-p53 expression vector, either alone (lane 2), or with increasing amounts of a VP16-CBP-(1452–1892) expression vector (200 or 500 ng), in the absence (lanes 5 and 6), or presence of 400 ng of MDM2 (lanes 7 and 8). Total DNA concentration in all transfected plates was adjusted to 5 μg of total DNA with empty pcDNA expression plasmid. MDM2 inhibits the interaction of p53 with the C terminus of CBP. In panels A–D, data are representative of three different experiments.

**E.** MDM2 bound to p53 blocks binding of the N-terminal 100–446 region of CBP. Glutathione-Sepharose beads containing GST alone, or GST-p53 were incubated with reticulocyte translated [35S]methionine-labeled CBP-(100–446), and GST pulldown assays were performed. Input (lane 1) represents the amount of CBP-(100–446) used in the assay. In lane 4, MDM2-transfected Saos2 total cell extract was incubated with GST-p53 beads; after washing, the beads were further incubated with [35S]labeled CBP-(100–446). Prebinding of MDM2 to p53 blocks the interaction with CBP.
gion of p53 and the N- and C-terminal regions of CBP/p300 and blocked p53 recruitment of the coactivator. The new observations are also consistent with previous mechanisms of MDM2 repression (28). p53 containing enhancer assemblies could also generate another distinct surface that interacts with the transcriptional apparatus, resulting in basal transcriptional machinery. Thus, binding of MDM2 would block two parallel pathways by which p53-containing enhancers engage the transcriptional apparatus, resulting in stringent control of transcription from p53-responsive promoters.

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