MDM2 and Promyelocytic Leukemia Antagonize Each Other through Their Direct Interaction with p53*

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p53 can be regulated through post-translational modifications and through interactions with positive and negative regulatory factors. MDM2 binding inhibits p53 and promotes its degradation by the proteasome, whereas promyelocytic leukemia (PML) activates p53 by recruiting it to multiprotein complexes termed PML-nuclear bodies. We reported previously an in vivo and in vitro interaction between PML and MDM2 that is independent of p53. In the current study, we investigated whether interaction between MDM2 and PML can directly affect p53 activity. Increasing amounts of MDM2 inhibited p53 activation by PML but could not inhibit PML-mediated activation of a p53 fusion protein that lacked the MDM2-binding domain. Conversely, increasing amounts of PML could overcome p53 inhibition by MDM2 but could not overcome MDM2-mediated inhibition of a p53 fusion protein that lacked the PML-binding domain. These results demonstrate that MDM2 and PML can antagonize each other through their direct interaction with p53 and suggest the combined effects of MDM2 and PML on p53 function are determined by the relative level of each protein. Furthermore, these results imply that interactions between MDM2 and PML by themselves have little or no effect on p53 activity.

MDM2 is an oncogene that is frequently overexpressed in various human cancers, including sarcomas, gliomas, melanomas, and breast cancers (1). The primary function of MDM2 is to inhibit the activity of the p53 tumor suppressor protein. p53 inhibits cell proliferation in response to DNA damage and other stresses by activating the transcription of genes that mediate either cell cycle arrest or apoptosis (reviewed in Ref. 2). MDM2 can bind to the transactivation domain of p53 and inhibit its ability to activate transcription, and at least three different mechanisms have been described by which this can occur. First, MDM2 binding can block the interaction between p53 and the basal transcription machinery (3, 4). Second, MDM2 can promote the ubiquitination of p53 and its subsequent degradation by the proteasome, and there is some evidence that MDM2 functions as a ubiquitin-protein isopeptide ligase that can transfer ubiquitin moieties directly to p53 (5–7). Third, MDM2 binding can promote the export of p53 from the nucleus to the cytoplasm (8, 9). In this case, it has been suggested that MDM2-mediated ubiquitination activates or exposes a nuclear export signal in the p53 C terminus, leading to the export of p53 from the nucleus to the cytoplasm.

In addition to inhibiting p53, there is increasing evidence that MDM2 has p53-independent activities that may contribute to its oncogenic properties. This evidence includes the following. First, rare tumors harboring both p53 mutations and MDM2 gene amplifications have been described and are more aggressive than those with alterations in either gene alone (10). This suggests that MDM2 overexpression may accelerate tumor growth even in the absence of functional p53. Second, MDM2 overexpression has been reported to transform cells in culture in the absence of a functioning p53 (11). Third, targeted MDM2 overexpression in the mammary glands of mice leads to abnormal cell proliferation and mammary hypertrophy to a similar extent in both a p53+/− and p53−/− background (12). Accordingly, considerable effort has been aimed at identifying targets other than p53 with which MDM2 can interact.

The promyelocytic leukemia (PML)1 protein is a tumor suppressor and the major component of multiprotein nuclear complexes that have been variably termed Kremer bodies, ND10, PODs (for PML oncogenic domains), and PML-nuclear bodies (PML-NBs). These PML-NBs appear as foci within the nucleus when visualized with antibodies against PML or other NB-associated factors (13, 14). The PML protein has received considerable attention recently due, at least in part, to its ability to activate p53. PML binds directly with p53 and recruits it to PML-NBs (15). Current models suggest that recruitment to PML-NBs activate p53 by bringing it in close proximity with CBP/p300 (16). Acetylation of p53 by CBP/p300 then increases p53 DNA binding affinity, leading to an activation of p53-responsive genes. This activation of p53 likely contributes to the tumor suppressor function of PML. We observed previously (17) an interaction between PML and MDM2 that is independent of p53. In those studies, MDM2 could be immunoprecipitated with PML in cells transiently overexpressing both proteins, and recombinant MDM2 produced in insect cells formed a strong complex with a bacterially expressed GST-PML fusion protein. Moreover, confocal microscopy revealed a low level association between the endogenous PML and MDM2 proteins in cells. These results demonstrated that MDM2 and PML can interact with each other in a manner independent of p53 and suggested that this may be a direct interaction. The purpose of the current study was to investigate further the interaction between PML and MDM2, and to assess whether this interaction can indirectly influence p53 activity. The interaction between MDM2 and PML is complex and mediated by multiple regions of each protein. p53, MDM2, and PML can colocalize in PML-NBs with one another, and this can occur in the absence

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1 The abbreviations used are: PML, promyelocytic leukemia; PML-NBs, PML-nuclear bodies; GST, glutathione S-transferase; GFP, green fluorescent protein; HA, hemagglutinin.
of p53:MDM2 binding. Finally, MDM2 and PML can antagonize each other through their direct interaction with p53.

**EXPERIMENTAL PROCEDURES**

**Plasmid DNAs**—Expression DNA encoding FLAG-tagged, full-length PML IVa (588 amino acids long) was obtained from Zhi-Min Yuan (Harvard School of Public Health). HA-tagged PML IVa was generated from the FLAG-tagged form by PCR. The 3′ primer for PCR was the SP6 primer (Promega), and the 5′ primer was 5′-CGCAATTCC-ACCATGACACCATGTCGCTTACAGGTGCTGCG-3′. DNA encoding GFP-tagged p53 has been described previously. The 5′-ACGAGGACCCG-3′ was obtained from Tyler Jacks (Massachusetts Institute of Technology). DNA encoding wild-type MDM2 and MDM2 Δp53BD was obtained from Steve Grossmann (Dana Farber Cancer Institute). MDM2 Δp53BD lacks the MDM2-binding domain between residues 52 and 96. FLAG-tagged MDM2 (2–202) was generated from wild-type MDM2 DNA as a template. For MDM2 (300–488) the 5′ primer was 5′-CCGGGATCCCTAAAGAAAGAACAGGAGAAGACATGATGAG-3′, and the 3′ primer was 5′-CCGCTCTAGATGCTGTTATT ACCAATCCACGTCCAGCCAGGC-3′. The resulting PCR products were digested with BamHI and NotI restriction enzymes and cloned into the corresponding sites downstream of GAL4 in pCDNA3. The resulting PCR products were digested with BamHI and XbaI, and the 3′-CCGGGATCCCTCATTTCCAATAGTCAGCTA-3′ primer was 5′-CCGGGATCCCACCAAGAAGAAGACAGGAGAAGACATGATGAG-3′. The resulting PCR products were digested with BamHI and NotI, and the 3′-CCGGGATCCCTCATTTCCAATAGTCAGCTA-3′ primer was 5′-GGCGCGGCCGCCTCACCAGGAGAAC CCCAC-3′. The resulting PCR products were digested with BamHI and EcoRI and cloned downstream and in-frame with the FLAG epitope that had been cloned previously into pCDNA3. MDM2 (300–488) contains the SW40SW12 nuclear localization signal encoded within the 5′ primer. For MDM2 (2–202) the 5′ primer was 5′-CCGGGATCCCTGGCACATACCAATGTTTGTGT CA-3′, and the 3′ primer was 5′-CCGGGATCTCTGAGGAAAAGCGAC CACGAC-3′. The resulting PCR products were digested with BamHI and EcoRI and cloned downstream and in-frame with the FLAG epitope in pCDNA3. p53DN:VP16 TAD was generously provided by Jennifer Pietenpol (Vanderbilt University) and encodes the VP16 transactivation domain fused to p53 amino acids 80–393. GAL4 DBD:p53 (1–45) was generated by PCR using wild-type p53 as a template. The 5′ primer will be 5′-CCGGGATCCCTGGCACATACCAATGTTTGTGTCA-3′, and the 3′ primer will be 5′-CCGGGATCTCTGAGGAAAAGCGACACGAC-3′. The resulting PCR product was digested with BamHI and XhoI and cloned into a corresponding site of pCDNA3. The GAL4-luc reporter was generously provided by Donald Bloch (Massachusetts General Hospital) and contains GAL4 DNA-binding sites upstream of the luciferase gene. The p53-responsive luciferase reporter pG13-luc was a gift from Bert Vogelstein (The Johns Hopkins University).

**Tissue Culture, Immunobots, and Immunoprecipitation**—35-2 cells (p53 and MDM2 double knock-out) were grown in minimum essential medium supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. U2OS cells (p53 wild-type) were similarly grown. Transfections were done using the FuGENE-6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. Sixteen to 20 h after starting the transfection, cells were either fixed for immunofluorescence staining or harvested for collection of whole cell lysates. To harvest whole cell extracts, cells were rinsed with phosphate-buffered saline and scraped into 500 μl of lysis buffer (50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin). The scraped cells were lysed on ice for 30 min with occasional light vortexing, followed by 15 min of centrifugation to remove cellular debris. For immunoprecipitations from transfected cells, 300 μg of transfected cell proteins were immunoprecipitated with 0.6 μg of polyclonal anti-FLAG polyclonal antibody (Sigma catalog number F7425). The immunoprecipitates were resolved by SDS-PAGE and transferred to a PolyScreen polyvinylidene difluoride transfer membrane (PerkinElmer Life Sciences). The membrane was probed with an anti-HA monoclonal antibody (HA.11 from Babco).

**Immunoreactivity Staining**—For immunofluorescence staining, cells were plated on glass coverslips and subsequently transfected. The cells were then rinsed with phosphate-buffered saline plus 0.1 mM CaCl₂ and 1 mM MgCl₂ and fixed with 4% paraformaldehyde for 30 min at 4 °C. Paraformaldehyde was then replaced with 50 mM NH₄Cl for 5 min, and cells were permeabilized with 0.1% Triton X-100 plus 0.2% bovine serum albumin. HA- and PML staining was carried out using a rabbit anti-HA monoclonal antibody HA.11 (Babco) as the primary antibody, and rhodamine-conjugated anti-mouse antibody (The Jackson Laboratory) as the secondary antibody. MDM2 staining was carried out using the anti-MDM2 polyclonal antibody N-20 (Santa Cruz Biotechnology) as the primary antibody, and 7-amine-4-methylcoumarin-3-acetic conjugated anti-rabbit antibody (The Jackson Laboratory) as the secondary antibody. GFP fluorescence was visualized without immunostaining. Specimens were examined under a fluorescence microscope.

**GST Fusion Protein Production**—GST-tagged MDM2 wild-type DNA was generated by PCR using HA-PML (wild-type) as a template. The 3′ primer for PCR was 5′-GGCGCGGCGCCGCTTACCAAGAAGAAGACCATGACACCATGTCGCTGCG-3′, and the 5′ primer was 5′-CCGGGATCCCTGGCACATACCAATGTTTGTGTCA-3′. The resulting PCR products were digested with BamHI and NotI restriction enzymes and cloned into the corresponding sites of pGEX-4T-3. DNAs encoding the GST-tagged proteins were used to transform BL21 bacterial cells and transformed cells grown at 37 °C until reaching log phase. GST protein expression was induced by addition of 0.2 mM isopropyl-1-thio-β-galactopyranoside (IPTG) to the growth medium. The resulting strains were lysed by sonication in lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% Triton X-100, 150 mM NaCl), and the resulting lysate was incubated for 12 h at 4 °C with glutathione-Sepharose beads. The beads were pelleted by centrifugation and washed with lysis buffer. For MDM2 binding, the GST-tagged proteins bound to beads were incubated with 500 μl of whole cell lysate from transfected 35-2 cells for 5–6 h. Unbound MDM2 protein was removed by 5 washes (1 ml each) with lysis buffer. Bound proteins were eluted by boiling for 10 min in 1× loading buffer, resolved by SDS-PAGE, and examined by immunoblot analysis with anti-MDM2 or anti-GST antibodies.

**Luciferase Assays**—Luciferase activity was monitored by using either the p53-responsive luciferase reporter gene pG13-luc or the GAL4-responsive luciferase reporter GAL4-luc. 35-2 cells were transfected with either of these two reporter genes and various combinations of p53, MDM2, and PML, as indicated in the text and figure legends. pRL-TK Renilla luciferase reporter DNA (5 ng) was included in all transfections and used to normalize the transfection efficiency. Cell lysates were prepared 20–24 h post-transfection, and luciferase activity was assayed using the dual luciferase assay reporter kit (Promega), according to the manufacturer’s instructions.

**RESULTS**

The activity most associated with PML is its ability to bind multiple different proteins and recruit them to PML-NBs (18). We reported recently (17) an *in vivo* and *in vitro* interaction between PML and MDM2 that is independent of p53. In that study, recombinant and partially purified human MDM2 protein formed a strong complex with a bacterially expressed GST-PML fusion protein. We wished to characterize the binding between MDM2 and GST-PML further. To this end, 35-2 cells (MDM2 and p53 double-null) were transfected with DNAs encoding either full-length wild-type MDM2 or FLAG-tagged forms of MDM2 that encompassed the N terminus (residues 2–202), central region (residues 100–304), or C terminus (residues 300–488). Transfected cell lysates were then mixed with either GST alone or the GST-PML fusion protein, and MDM2 binding was assessed in GST pull-down assays. As shown in Fig. 1A, wild-type MDM2 was bound by the GST-PML fusion protein but not by GST alone. This is consistent with our previous results and indicates that MDM2 can form a complex with PML. The FLAG-tagged N-terminal, central, and C-terminal regions of MDM2 were also bound by GST-PML but not by GST alone (Fig. 1B). This indicates that multiple regions of MDM2 are capable of interacting with PML, at least in these GST pull-down studies.

Given that the function of PML, at least in part, is to interact with and recruit different proteins, it is perhaps not surprising that various regions of MDM2 may associate with one or more regions of PML. We used coimmunoprecipitation experiments to try to map the PML domains that interact with different regions of MDM2 (Fig. 2). Cells were again transfected with DNAs encoding FLAG-tagged MDM2s that encompassed the N terminus (residues 2–202), central region (residues 100–304), or C terminus (residues 300–488). However, in this case, the cells were cotransfected with DNAs encoding HA-tagged fragments of PML that encompassed portions of the N terminus, central region, and C terminus. Our previous results demonstrated that conversion of the lysine at position 160 in PML to
arginine (K160R), which inhibits sumoylation at this site, allows a much stronger interaction between PML and wild-type MDM2 (17). Therefore, we also included a form of PML that harbored the K160R mutation. Association between the MDM2 and PML fragments was assessed by immunoprecipitation with an anti-FLAG antibody, followed by immunoblotting with an anti-HA antibody. As shown in Fig. 2, the N-terminal portion of MDM2 (residues 2–202) displayed relatively weak binding to PML fragments encoding the N terminus (residues 1–200) and C terminus (residues 200–585 and 300–585) but not with PML fragments encoding the C-terminal region (residues 200–453). However, MDM2(300–488) displayed much stronger binding to PML residues 1–200 that harbored the K160R mutation, similar to MDM2(2–202). This suggests that MDM2(300–488) interacts primarily with residues 1–200 in non-sumoylated PML and to a lesser extent with the C-terminal and central PML regions. The central region of MDM2 (residues 100–304) coimmunoprecipitated with the C-terminal fragments of PML (residues 200–585 and 300–585) but not with PML fragments encoding either the N terminus (residues 1–200) or central (residues 200–453) regions. In these studies, interaction between the PML C terminus and MDM2(100–304) was somewhat stronger than interaction between the PML C terminus and either the MDM2 N-terminal or C-terminal fragments. This suggests that interactions between MDM2 and the PML C terminus may occur mostly through the MDM2 central region. The fact that the MDM2 central region does not interact with PML residues 1–200 indicates that interaction with this region of PML is specific to the MDM2 N and C terminus. In total, these results indicate that the interactions between PML and MDM2 are complex and can occur through multiple regions of each protein.

PML and MDM2 have opposing effects on p53. MDM2 binding inhibits p53 and promotes its degradation by the proteasome (5–7), whereas PML activates p53 by recruiting it into PML-NBs (reviewed in Ref. 16). To determine whether MDM2 can prevent p53 recruitment to PML-NBs, cells were transfected with DNAs encoding GFP-tagged p53, HA-tagged PML, and MDM2, and localization of the transfected proteins was monitored by immunofluorescence. p53 and MDM2 displayed a diffuse nuclear localization when expressed alone (Fig. 3A). In contrast, both p53 and MDM2 were recruited to PML-NBs in cells coexpressing p53, MDM2, and PML (Fig. 3B). This indicated that MDM2 did not prevent the recruitment of p53 to PML-NBs. To determine whether recruitment of MDM2 to PML-NBs required its interaction with p53, a similar experiment was performed with an MDM2 mutant that lacks the N-terminal p53-binding domain (MDM2 Δp53BD). As shown in Fig. 3B, MDM2 Δp53BD could also be recruited to PML-NBs with PML and wild-type p53, perhaps through interaction with one or more regions of PML. Together, these results indicate that p53, MDM2, and PML can associate in cells in the same PML-NBs and that this can occur in the absence of p53:MDM2 binding.

Given these results, we wished to test whether PML and MDM2 can antagonize each other in their regulation of p53. First, cells were transfected with a p53-responsive luciferase gene alone, or with increasing amounts of DNA encoding MDM2 or PML. As shown in Fig. 4A, MDM2 inhibited p53 transcriptional activity in a dose-dependent manner. In these experiments, 40 ng of input MDM2 DNA was the minimal amount that caused a marked inhibition of p53 activity, whereas 80 and 200 ng of input MDM2 DNA caused a more robust inhibition of p53. In contrast, PML activated p53 in a dose-dependent manner, with 1000 ng of input PML DNA causing the most pronounced activation of p53 (Fig. 4B). To examine the combined effect of MDM2 and PML, p53 activity was monitored in cells transfected with increasing amounts of MDM2 DNA (40, 80, or 200 ng) and high amounts of PML (500 and 1000 ng). As shown in Fig. 4C, PML blocked p53 inhibition by MDM2 and activated p53 when 40 ng of MDM2 DNA was used in the transfection. PML also blocked p53 inhibition by MDM2 when 80 ng of MDM2 DNA was used, although under these conditions only slight activation of p53 was observed. In contrast, PML could not block p53 inhibition by MDM2 when
200 ng of MDM2 DNA was used in the transfection. These results indicate that the effects of MDM2 and PML on p53 activity are likely to depend on the relative levels of each protein. Namely, PML can overcome p53 inhibition by relatively low amounts of MDM2 but not high MDM2 amounts. In contrast, high amounts of MDM2 can inhibit the PML-mediated activation of p53.

Finally, we wished to test whether p53-independent interactions between PML and MDM2 may indirectly affect p53 activity. To this end, a fusion protein was generated (designated GAL4 DBD-p53-(1–45)) in which the p53 transactivation domain (residues 1–45) is fused to the GAL4 DNA-binding domain. This fusion protein maintains the MDM2-binding function of p53 but lacks the region of p53 (residues 120–290; see Ref. 19) that binds PML. Cells were transfected with GAL4 DBD-p53-(1–45) and a GAL4-responsive luciferase reporter alone or with MDM2 and PML. As shown in Fig. 5, GAL4 DBD-p53-(1–45) activated the GAL4-responsive reporter, and this activation was inhibited in a dose-dependent manner by MDM2. Importantly, a high amount of PML (500 ng) had little to no effect on the ability of MDM2 to inhibit GAL4 DBD-p53-(1–45) under all conditions tested. These results demonstrate that p53-independent interactions between PML and MDM2 alone do not prevent MDM2 from inhibiting the p53 transactivation domain. Thus, PML must bind p53 directly to efficiently overcome the inhibition of p53 activity by MDM2.

We next tested whether MDM2 interaction with PML contributes to the ability of MDM2 to inhibit PML-mediated p53 activation. First, p53-dependent luciferase activity was monitored in cells transfected with the p53-responsive luciferase gene alone or with various combinations of p53, wild-type MDM2, MDM2Δp53BD, and PML (Fig. 6A). As expected, high amounts of wild-type MDM2 DNA (200 and 500 ng) strongly inhibited p53 activity, whereas MDM2Δp53BD failed to inhibit p53. These results indicate that p53-independent interactions between PML and MDM2 alone do not prevent MDM2 from inhibiting the p53 transactivation domain. Thus, PML must bind p53 directly to efficiently overcome the inhibition of p53 activity by MDM2.
nated p53ΔN-VP16 TAD, is fully capable of activating gene transcription but lacks the MDM2-binding region and is therefore unable to bind MDM2 (20). As shown in Fig. 6B, p53ΔN-VP16 TAD could activate the p53-responsive luciferase gene. Furthermore, coexpression of PML increased the transcriptional activity of p53ΔN-VP16 TAD to an extent similar to that of wild-type p53, indicating that p53ΔN-VP16 TAD maintains all the requirements for activation by PML. Importantly, increasing amounts of wild-type MDM2 had no effect on the transcriptional activity of p53ΔN-VP16 TAD when it was either expressed alone (data not shown) or when coexpressed with PML (Fig. 6B). Taken together, these results indicate that MDM2 must bind p53 directly in order to inhibit the activation of p53 by PML.

![Disruption of the p53-mdm2 interaction by pml.](image)

**Fig. 4.** MDM2 and PML antagonize each other to regulate p53. A and B, 35-2 cells were transfected with a p53-responsive luciferase promoter DNA (pG13-luc, 200 ng), wild type p53 DNA (5 ng), and the indicated amounts of wild-type MDM2 (A) and HA PML (B). Luciferase activity was monitored in the transfected cell lysates 24 h post-transfection as a measure of p53 activity. C, 35-2 cells were transfected with the indicated amounts of MDM2 and HA PML DNA. Luciferase activity was monitored in the transfected cell lysates 24 h post-transfection as a measure of p53 activity. RLU, relative light units.

![PML does not block MDM2-mediated inhibition of a GAL4-p53 fusion protein.](image)

**Fig. 5.** PML does not block MDM2-mediated inhibition of a GAL4-p53 fusion protein. 35-2 cells were transfected with a GAL4-responsive luciferase promoter DNA (GAL4-luc, 100 ng), GAL4 DBD-p53(1–45) (200 ng), and the indicated amounts of wild-type MDM2 and HA PML. Luciferase activity was monitored in the transfected cell lysates 24 h post-transfection.

**DISCUSSION**

Wild-type PML is a tumor suppressor protein and potent activator of p53. The most striking feature of PML is its localization to multiprotein nuclear foci complexes termed PML-NBs. PML is essential for NB formation, as other NB-associated proteins fail to congregate in NBs in the absence of PML (21, 22). At present, the molecular functions of PML and PML-NBs are not fully understood. Most current models propose that PML functions as a scaffold, capable of interacting with and recruiting a wide variety of proteins to NBs (14). Consistent with this role, the PML protein contains a series of motifs that may serve as interaction surfaces, including a RING finger domain, two zinc-binding domains referred to as B boxes, and an α-helical coiled-coil (23). Together, these motifs comprise what is referred to as the RBCC domain. PML also contains a serine/proline-rich domain in its C terminus of unknown function. The PML-NBs themselves are believed to serve as either temporary storage sites for catalytically active proteins or as catalytic surfaces where specific biochemistries can occur (14).

Our recent studies identified MDM2 as a novel PML-interacting protein (17). MDM2 could be immunoprecipitated with PML in transiently transfected cells, and partially purified MDM2 formed a strong complex with a GST-PML fusion protein. Furthermore, confocal microscopy revealed an association between the endogenous PML and MDM2 proteins in a small percentage of cells. These results demonstrated that MDM2 and PML can interact with each other in a manner independent of p53 and suggested that this may be a direct interaction. In the current study, we used GST pull-down assays and coimmunoprecipitation experiments to characterize further the PML-MDM2 interaction. These studies revealed a complex set of interactions between PML and MDM2 in which multiple regions of each protein are capable of binding each other. The MDM2 N terminus (residues 2–202) and C terminus (residues 300–488) could interact with the N terminus (residues 1–200),...
central region (residues 200–458), and C terminus (residues 300–588) of PML to varying extents. In contrast, the central region of MDM2 (residues 100–304) could interact with the C-terminal region of PML but not with the PML N-terminal region. This indicates that interaction with the PML N terminus is a property specific to the N- and C-terminal MDM2 regions. One interesting observation concerns the interaction of MDM2 with the N-terminal 200 amino acids of PML. PML can be modified by sumoylation within this region at lysine 160, and our previous studies demonstrated that converting lysine 160 to arginine (K160R), which inhibits sumoylation at this site, allowed a much stronger interaction between full-length PML and wild-type MDM2 (17). Based on these results, we speculated that sumoylation at Lys-160 may somehow inhibit MDM2 binding. In the current study, the MDM2 N terminus (residues 2–202) and C terminus (residues 300–488) could interact in communoprecipitation studies with residues 1–200 of wild-type PML. However, a much stronger association was observed between these regions of MDM2 and a PML–(1–200) mutant that harbored the K160R mutation. These results are consistent with our previous observations and raise the possibility that sumoylation at lysine 160 may inhibit the interaction between the PML N terminus and either the N- or C-terminal regions of MDM2.

PML and MDM2 have opposing effects on p53 activity. MDM2 inhibits p53 by promoting its ubiquitination and subsequent degradation by the proteasome (5–7), whereas PML activates p53 by recruiting it to PML-NBs (15, 16). The current report demonstrates that PML and MDM2 can antagonize each other in their regulation of p53. PML overcame p53 inhibition by MDM2 and activated p53 when relatively low amounts of MDM2 were present and, conversely, MDM2 blocked p53 activation by PML when relatively high amounts of MDM2 were present. A recent study by Louria-Hayon et al. (24) is consistent with our results and also demonstrated that PML can protect p53 from MDM2-mediated inhibition. Our results predict the overall effect of MDM2 and PML on p53 activity is likely to depend on the relative amounts of MDM2 and PML protein. This may be especially relevant during the cellular response to DNA-damaging agents. Certain agents, such as UV radiation and methyl methanesulphonate, cause stabilization of p53 and a subsequent cell cycle arrest by signaling a severe reduction in MDM2 levels (25, 26). These low levels of MDM2 may allow PML to participate fully in p53 activation. In contrast, MDM2 levels rise when DNA repair is completed. These higher levels of MDM2 inhibit p53 and are expected to block any potential activation of p53 by PML, thus allowing cell division to continue.

Given the interaction between MDM2 and PML, we investigated whether the MDM2–PML interaction can indirectly influence p53 activity. For these studies we made use of p53 fusion proteins that maintain different p53 activities but lack either the MDM2- or PML-binding domains. Whereas PML could block MDM2-mediated inhibition of wild-type p53, PML could not prevent MDM2-mediated inhibition of a fusion protein that contained the MDM2-binding domain but lacked the PML-binding region (GAL4 DBD–p53–(1–45)). Similarly, MDM2 could inhibit PML-mediated activation of wild-type p53 but could not inhibit PML-mediated activation of a transcriptionally active p53 fusion protein that lacked the MDM2-binding domain (p53 ΔN–VP16 TAD). Taken together, these data provide strong and compelling evidence that MDM2 and PML must bind p53 directly in order to inhibit the effect of each other on p53. Moreover, these results imply that interaction between MDM2 and PML by itself has little or no effect on p53 activity. We anticipate, therefore, that the physiologic consequence of MDM2–PML interaction may be limited to p53–independent functions of either protein. It will be interesting in the future to determine whether association with MDM2 modulates any of the putative p53–independent functions of PML and, similarly, whether association with PML modulates any p53–independent functions of MDM2.

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