The Central Acidic Domain of MDM2 Is Critical in Inhibition of Retinoblastoma-mediated Suppression of E2F and Cell Growth*

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The retinoblastoma gene is well documented as one of the most frequent targets for inactivation during the course of human tumorigenesis (1). Retinoblastoma (Rb)1 protein has been implicated in many cellular processes including regulation of cell cycle, DNA replication, DNA-damage repair, protection against apoptosis, and cell differentiation and senescence (1–3). The primary function of Rb relies on its ability of interaction with the E2F family members and is essential for G1-S transition (11, 12, 21, 22). Overproduction of MDM2 suppressors. Inactivation of Rb plays a critical role in the development of human malignancies. MDM2, an oncogene frequently found amplified and overexpressed in a variety of human tumors and cancers, directly interacts and inhibits the p53 tumor suppressor protein. In addition, MDM2 has been shown to stimulate E2F trans-activation activity and promote S-phase entry independent of p53, yet the mechanism of which is still not fully understood. In this study, we demonstrate that MDM2 specifically binds to Rb C-pocket and that the central acidic domain of MDM2 is essential for Rb interaction. In addition, we show that overexpression of MDM2 reduces Rb-E2F complexes in vivo. Moreover, the ectopic expression of the wild-type MDM2, but not mutant MDM2 defective in Rb interaction, stimulates E2F transactivation activity and inhibits Rb growth suppression function. Taken together, these results suggest that MDM2-mediated inhibition of Rb likely contributes to MDM2 oncogenic activity.

Rb contains three distinct protein-binding domains, the A-B domain in the middle of Rb protein and the C-terminal domain. The A-B domain called the Rb small pocket (RbSP) binds a set of proteins that contain the Rb-binding LXCXE motif. The large pocket (RbLP), containing A-B and C domains, is required for in vivo interaction with the E2F family members and is sufficient for Rb growth suppression function (6, 7). Importantly, the Rb-E2F interaction is disrupted by the viral oncoprotein E1A, which is critical for adenovirus-mediated cellular transformation (8). On the other hand, the C-terminal domain, called the C-pocket (RbC), has been shown to be important for the suppression of retinoblastoma formation since the deletion of Rb exons 24 and 25 located in the C-pocket causes low-penetrance retinoblastoma (9). The C-pocket is critical for Rb interaction with E2F and for Rb growth suppression (7). In addition, the Rb C-pocket selectively interacts with E2F-1 and this interaction is diminished upon DNA damage (10). Furthermore, the Rb C-pocket interacts with MDM2 (9, 11, 12) and c-Abl (13).

Overexpression of MDM2 has been observed in a variety of human tumors/cancers including ~30% human sarcomas (14). It has been well documented that MDM2 contains a RING finger domain at the C terminus that functions as ubiquitin E3 ligase for p53 and for itself to promote the protein degradation via ubiquitin-proteasome-mediated pathway (15–19). In addition to the critical regulation of p53, MDM2 has been shown to have p53-independent functions (20). MDM2 interacts with a set of cellular proteins, including Rb and E2F-1, to promote cell cycle G1-S transition (11, 12, 21, 22). Overproduction of MDM2 leads to DNA endoreduplication independent of p53 (23). In addition, MDM2 can rescue transforming growth factor-β-induced growth arrest in a p53-independent manner (24).

In this study, we demonstrate that MDM2 interacts with the Rb C-pocket and modulates the formation of Rb-E2F complexes. We show that overexpression of wild-type MDM2, but not mutant MDM2 defective in Rb interaction, activates E2F and inhibits Rb growth suppression function. Thus, these data indicate that MDM2 is probably an important modulator for Rb.

**Experimental Procedures**

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1 The abbreviations used are: Rb, retinoblastoma; RbLP, Rb large pocket; RbC, Rb C-pocket; E3, ubiquitin-protein isopeptide ligase; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; DHFR, dihydrofolate reductase.

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and autoradiography. After incubation on ice for 30 min, 5 μl of in vitro translated [35S]methionine-labeled Rb or E2F-1 was added to the reaction mixture and incubated for 20 min prior to the addition of [35S]methionine-labeled MDM2 or E2F-1 translated in vitro. A comparable amount of input GST or GST fusion protein was directly loaded on the gels as controls. A comparable amount of input GST or GST fusion protein was shown by staining the protein gel with Coomassie Brilliant Blue R-250.

A schematic presentation of Rb protein structure, deletion mutants with regards to their interaction with MDM2. RbSP, Rb small pocket.

EMSAs were performed essentially as described previously (25, 26). C33A cells were co-transfected with expression plasmids (5 μg of pCMV-E2F-1, 1 μg of pCMV-DP1, 10 μg of pCMV-MDM2) and increasing amounts of pCMV-RbLP or pCMV-Rb expression plasmid (0, 1, 0.25, 0.5, and 1.0 μg, respectively) as indicated. Similarly, C33A cells were co-transfected with 3 μg of pCMV-E2F-1, 1 μg of pCMV-DP1, 3 μg of pCMV-Rb, and an increasing amount (6 or 12 μg) of pCMV-MDM2 or pCMV-MDM2(-254–264). 36 h post transfection, nuclear extracts were prepared. An EMSA reaction mixture contained 10 μg of nuclear extract proteins, 1 μl of [35P]-labeled double-stranded DHFR oligonucleotide (25) or an E2F oligonucleotide (22507, Santa Cruz Biotechnology), 3 μg of poly(dI-dC) or poly(dA-dT), 5 μg of bovine serum albumin, and 4 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 40 mM KCl, 2 mM dithiothreitol, 125 μM MnCl2, and 10% glycerol) in a total volume of 20 μl. The reaction mixture was incubated at room temperature for 30 min and followed by electrophoresis on non-denaturing 6% polyacrylamide gels in Tris-EDTA buffer and autoradiography. In the competition assay, cold wild type or mutant E2F-double-stranded oligonucleotide (50-fold in excess) (Santa Cruz Biotechnology) was added in the reaction mixture and incubated for 20 min prior to the addition of [35P]-labeled probe.

Rb Growth Suppression Assay—The Rb growth suppression assay was essentially performed as described previously (6, 27). Saos-2 cells grown on 100-mm plates to 80% confluency were co-transfected using calcium phosphate precipitation (Clontech Laboratories, Inc.) with a DNA mixture containing 5 μg of pCMV-Rb, 2 μg of pCMV-puromycin encoding the puromycin-resistant gene, and 10 μg of pCMV-E2F-1, pCMV-MDM2, or pCMV-MDM2(-254–264). 48 h after transfection, transfected cells were selected in the growth medium supplemented with puromycin (0.5 μg/ml), which were changed every other day. 12 days after selection, cells with a unique large flat morphology induced by ectopically expressed Rb (6, 27) were scored under a microscope and presented as the mean ± S.D. of three independent experiments performed in triplicate.

*Fig. 1.* The amino acid residues 785–803 in the Rb C-pocket are critical for Rb interaction with MDM2 and E2F-1. A and B, 5 μl of in vitro translated [35S]methionine-labeled MDM2 or E2F-1 was added into 800 μl of binding buffer containing GST or a derivative of GST-Rb fusion proteins immobilized on Sepharose-glutathione beads and subjected to GST pull-down procedure followed by SDS-PAGE and autoradiography. 1 μl (20% input) of [35S]methionine-labeled MDM2 or E2F-1 was directly loaded on the gels as controls. A comparable amount of input GST or GST fusion protein was shown by staining the protein gel with Coomassie Brilliant Blue R-250. C, a schematic presentation of Rb protein structure, deletion mutants with regards to their interaction with MDM2. RbSP, Rb small pocket.
presented as the percentage of flat cells (numbers of flat cells over total cells counted) with mean ± S.D. At least three independent experiments in triplicate were performed with at least 300 cells counted for each transfection sample.

RESULTS

The Amino Acid Residues 785–803 in the Rb C-pocket Are Critical for Rb-MDM2 Interaction—We have previously shown that Rb and MDM2 form a stable complex in vitro and in vivo and that the Rb C-pocket (amino acid residues 792–928) is important for Rb interaction with MDM2 (11). To further identify the domains of Rb involved in MDM2 interaction, we used a set of GST-Rb fusion proteins with an internal deletion in the Rb C-pocket to examine its ability of binding to in vitro translated \([35S]\)Met-labeled MDM2 protein. As shown in Fig. 1A, the RbLP (amino acids 379–928) retained the full capacity in interaction with MDM2, whereas a small deletion in the C-pocket led to a clear decrease in Rb-MDM2 interaction. Notably, a deletion mutant in the Rb C-pocket (RbLP-(Δ803–909)) still retained considerable MDM2 binding activity (Fig. 1A, lane 6). In contrast, a mutant with an additional deletion of 19 amino acid residues (RbLP-(Δ785–909)) had little MDM2 interaction (Fig. 1A, lane 7), suggesting that this 19 amino acid segment is important for Rb interaction with MDM2. Indeed, a deletion mutant lacking this segment, RbLP-(Δ803–909), was unable to interact with MDM2 (Fig. 1B, lane 5), demonstrating that the amino acid residues 785–803 in the Rb C-pocket is a critical module for MDM2 interaction. Of note, it has been shown that the region adjacent to the module in the Rb C-pocket (amino acids 841–870) is important for Rb interaction with E2F-1 (7).

The Amino Acid Residues 254–264 in the Central Acidic Region of MDM2 Are Essential for Rb Interaction—To identify the binding site of MDM2 for Rb, we used Rb C-pocket fused to GST (GST-RbC) for interaction with the in vitro translated \([35S]\)methionine-labeled MDM2 protein or its deletion mutant derivatives. As shown in Fig. 2A, Rb C-pocket interacted well with the full-length human MDM2-(1–491) but not with the MDM2 deletion mutant lacking amino acid residues 234–284 (Fig. 2A, lane 5), suggesting that the Rb interaction domain may reside in this segment. Furthermore, Rb C-pocket bound MDM2-\((Δ264–284)\) (Fig. 2A, lane 8) but not MDM2-\((Δ254–284)\) (Fig. 2A, lane 11), indicating that the amino acid segment 254–264 of MDM2 is critical for Rb interaction. Indeed, the deletion of the segment 254–264 totally abolished MDM2 interaction with Rb (Fig. 2B, lanes 5 and 6), whereas it retained the interaction with E2F-1 (Fig. 2B, lanes 7 and 8). Moreover, a synthetic peptide (MP1), consisting of the corresponding sequence of 11

![Figure 2](https://example.com/figure2.png)
MDM2 Inhibits Rb Suppression of E2F

MDM2 Reduces Rb-E2F Complex Formation in Vivo—Because E2F-1 has been shown to preferentially interact with hypophosphorylated Rb (25, 28), we examined whether MDM2 has a preference for interaction with certain species of Rb. As shown in Fig. 3A, MDM2 also preferentially interacted with hypophosphorylated Rb protein species, suggesting that MDM2 selectively targets hypophosphorylated Rb. Given that both MDM2 and E2F-1 bind to the Rb C-pocket in the same region and both interact with hypophosphorylated Rb, we investigated whether MDM2 interferes with Rb association with E2F-1. We first examined the effect of MP1 peptide on Rb interaction with E2F-1 in vitro. As shown in Fig. 3C, the addition of an increasing amount of the MP1 peptide in the reaction mixture led to a marked decrease of Rb association with E2F-1, suggesting that this MDM2 peptide can block Rb interaction with E2F-1 in vitro. We then investigated the effects of MDM2 on Rb-E2F-1 complexes in vivo. We chose the human cervical carcinoma cell line C33A, because it bears a mutant Rb protein incapable of interaction with E2F (29). As expected, the ectopic expression of either RbLP (Fig. 4A, lanes 2–4) or full-length Rb (Fig. 4B, lanes 3–5) in C33A cells led to specific Rb-E2F-1 complex formation in a dose-dependent manner. Co-expression of MDM2 significantly inhibited the Rb-E2F-1-DNA complex formation as shown by EMSA (Fig. 4A, lanes 5–7, and B, lanes 7–9). Furthermore, this inhibitory effect of MDM2 was dependent on its physical interaction with Rb since co-expression of wild type MDM2, but not mutant MDM2 (Δ254–264) defective in Rb binding activity, led to a clear reduction of Rb-E2F-1 complex (Fig. 4C). However, no significant changes in free E2F-1 complexes were observed in MDM2-overexpressing cells. Taken together, these data suggest that overexpression of MDM2 can reduce Rb-E2F-DNA complex formation.

MDM2 Blocks Rb-mediated Suppression of E2F Activity and Inhibits Rb Growth Suppression Function—We next investigated the effects of MDM2 on Rb function. Although the expression of Rb significantly inhibited E2F-1-mediated transactivation activity in U2-OS cells, co-expression of wild type MDM2, but not mutant MDM2 (Δ254–264), effectively blocked the Rb-mediated inhibition on E2F activity (Fig. 5A). It has been well established that the ectopic expression of Rb in Saos-2 cells (Rb null and p53 null) leads to growth arrest at G1, manifested by flat and enlarged cell morphology (6, 27). As shown in Fig. 5B, the expression of Rb in Saos-2 cells resulted in predominantly large flat cells (80 ± 5%). The co-expression of E2F-1, as expected, effectively rescued Rb-induced large flat cell phenotype. Co-expression of wild type MDM2 led to a significant reduction of Rb-induced large flat cells, whereas MDM2 (Δ254–264) exhibited only marginal effects. Taken together, these data indicate that the ability of MDM2 to directly interact with Rb is critical for MDM2 inhibition of Rb growth suppression function independent of p53.

**DISCUSSION**

It is well established that the disruption of the tumor suppressor pathways (Rb and p53) is critical for the development of a majority of human tumors. MDM2 has been documented as a key regulator for p53. In this study, we demonstrate that the binding of MDM2 to Rb reduces the formation of Rb-E2F-1 DNA complex, resulting in the inhibition of Rb growth suppression function, suggesting that MDM2 is also a critical regulator for Rb.

![Fig. 3. MDM2 preferably interacts with hypophosphorylated Rb and affects Rb-E2F interaction in vitro](image)

We have previously shown that Rb-C-pocket is important for MDM2 interaction (11), which were confirmed by several independent studies (9, 12). In this study, we demonstrated that MDM2 activates E2F through its physical interaction with Rb. First, the amino acid segment 785–803 of Rb is important for the interaction of both E2F and MDM2. Second, wild type MDM2, but not Rb-binding mutant MDM2, reduces the Rb-E2F complex. Third, a MDM2 peptide consisting of the Rb-binding module can block Rb interaction with E2F-1 in vitro. Fourth and most importantly, the wild type MDM2 inhibits Rb-mediated suppression of E2F-1. In contrast, mutant MDM2 defective in Rb interaction while retaining the ability of interaction with E2F-1 fails to do so. These data indicate that the physical interaction between MDM2 and Rb is required for the action of MDM2 in stimulating E2F.

Rb is best known as a transcriptional repressor, which is tethered to promoters through its interaction with members of the E2F family proteins. Rb can directly bind to and block the activation domain of E2F proteins. Perhaps, more importantly, when Rb binds E2F, it recruits chromatin-remodeling factors such as histone deacetylases, SWI/SNF factors, Polycythe group proteins, or methyltransferases to nearby surrounding nucleosome structures, thereby actively repressing transcription of promoters that contain E2F sites (4, 30). Thus, the assembly of such Rb-E2F repressor complexes on promoters is
critical for Rb-mediated growth suppression (30). Our data indicate that the overexpression of MDM2 inhibits the formation of the Rb-E2F-DNA complex, suggesting that MDM2 stimulates E2F activity probably through interacting with Rb and modulating the Rb-E2F-1 repressor complexes on DNA. Interestingly, although the overexpression of MDM2 can markedly reduce Rb-E2F-DNA complexes, no significant changes in free E2F-1 complexes were observed in the same cells as assessed by EMSA. These data suggest that MDM2 may function in inhibiting Rb association with E2F-DNA complexes, thus blocking the assembly of transcription repressor complexes rather than the dissociation of existing Rb-E2F complexes. Consistent with this notion, the ectopic expression of wild type MDM2, but not Rb-binding mutant MDM2, inhibits Rb-mediated suppression of E2F.

In addition, we have shown that the 11-amino acid module (amino acid residues 254–264) located in central acidic domain of MDM2 is critical for Rb binding and for MDM2-mediated E2F activation. Interestingly, the acidic domain of MDM2 (amino acids 200–300) has been shown as an interaction module for proteins such as alternative reading frame protein (31–33), p300 (34), and ribosomal protein L11 (35). Moreover, this region also appears to be critical for MDM2 function toward p53 protein ubiquitination and degradation (36–38). Since it has been reported that MDM2-p53-Rb can form a trimeric complex resulting in Rb-mediated p53 protein stabilization (12), it raises an interesting possibility that Rb binds MDM2, thereby inhibiting MDM2-mediated p53 ubiquitination and degradation.

One primary function of MDM2 is to negatively regulate p53 by facilitating p53 ubiquitination and degradation. Thus, amplification and overexpression of MDM2 has been viewed as an alternative way of inactivating p53. However, a number of sarcomas, leukemia, and carcinomas have been documented that
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harbor p53 mutations and overproduce MDM2 (39–41). Clinically, it has been shown that sarcoma patients with both mutant p53 and high levels of MDM2 had a much lower survival rate than those patients who had only one of these alterations (39), underscoring the importance of MDM2-mediated p53-independent tumor-promoting function. Here, we demonstrate that MDM2 binds Rb, interferes with Rb-E2F complexes, and inhibits Rb growth suppression function. These findings are consistent with the notion that MDM2 plays an important role in promoting cell cycle progression and human tumorigenesis.

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