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The MDM2 RING Domain and Central Acidic Domain Play Distinct Roles in MDM2 Protein Homodimerization and MDM2-MDMX Protein Heterodimerization*

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Background: MDM2 dimerization is required for p53 ubiquitination and degradation.
Results: MDM2-MDM2 and MDM2-MDMX interactions occur through different mechanisms.
Conclusion: MDM2-MDMX interaction requires the proper MDM2 RING domain structure, whereas MDM2-MDM2 interaction does not require the proper RING structure but requires the MDM2 acidic domain.
Significance: Our findings offer mechanistic details of MDM2 dimerization, which are important for understanding p53 regulation as it relates to cancer.

The oncoprotein murine double minute 2 (MDM2) is an E3 ligase that plays a prominent role in p53 suppression by promoting its polyubiquitination and proteasomal degradation. In its active form, MDM2 forms homodimers as well as heterodimers with the homologous protein murine double minute 4 (MDMX), both of which are thought to occur through their respective C-terminal RING (really interesting new gene) domains. In this study, using multiple MDM2 mutants, we show evidence suggesting that MDM2 homo- and heterodimerization occur through distinct mechanisms because MDM2 RING domain mutations that inhibit MDM2 interaction with MDMX do not affect MDM2 interaction with WT MDM2. Intriguingly, deletion of a portion of the MDM2 central acidic domain selectively inhibits interaction with MDM2 while leaving intact the ability of MDM2 to interact with MDMX and to ubiquitinate p53. Further analysis of an MDM2 C-terminal deletion mutant reveals that the C-terminal residues of MDM2 are required for both MDM2 and MDMX interaction. Collectively, our results suggest a model in which MDM2-MDMX heterodimerization requires the extreme C terminus and proper RING domain structure of MDM2, whereas MDM2 homodimerization requires the extreme C terminus and the central acidic domain of MDM2, suggesting that MDM2 homo- and heterodimers utilize distinct MDM2 domains. Our study is the first to report mutations capable of separating MDM2 homo- and heterodimerization.

True to its title of "guardian of the genome," the tumor suppressor p53 is a transcription factor that promotes the transcription of an array of genes responsible for preventing cells from proliferating in the presence of DNA damage. Various stresses, such as DNA damage, activate p53, which, in turn, up-regulates several genes involved in cell cycle arrest and apoptosis (1–4). As a major factor involved in the inhibition of cell growth and division, the *TP53* gene is mutated in many different types of cancer (5). Interestingly, many cancers that do not harbor a mutation directly affecting p53 often harbor mutations in genes that regulate p53, resulting in p53 inactivation (6–8). The oncoprotein murine double minute 2 (MDM2)[2] is a particularly important p53 regulator that is overexpressed in nearly one-third of sarcomas that harbor wild-type p53, consistent with the idea that the overexpression of MDM2 could substitute for p53 mutation (9).

MDM2 is best known for its role as an E3 ubiquitin ligase that ubiquitinates p53, thereby targeting p53 for proteasomal degradation (10–12). Knockout of *Mdm2* results in embryonic lethality in mice that can be rescued through the concomitant deletion of p53, emphasizing the importance of MDM2 in p53 regulation (13). In its active form, MDM2 forms homodimers and/or heterodimers with its homologous partner MDMX, presumably through their C-terminal really interesting new gene (RING) domains (14). In addition to its role in dimerization, the RING domain of MDM2 is also necessary for catalyzing the transfer of ubiquitin to p53 (15). Although MDMX contains a RING domain that is very similar to that of MDM2, MDMX lacks appreciable E3 ligase activity toward p53. Nonetheless, the MDM2-MDMX heterodimer appears to be required for p53 degradation, at least during embryonic development, because mice harboring MDMX deletion or MDMX

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[2] The abbreviations used are: MDM2, murine double minute 2; MDMX, murine double minute 4; RING, really interesting new gene; AD, acidic domain; IP, immunoprecipitation.

mutations that prevent heterodimerization with MDM2 show an embryonic lethal phenotype that can be rescued by concomitant p53 deletion (16, 17).

Many of the studies that have contributed to our understanding of MDM2 dimerization have used isolated domains of MDM2, such as the RING domain (human MDM2 residues 384–491 (18, 19)). Although the study of isolated protein domains can offer insights into the function of individual protein domains, these studies fail to account for the behavior of these domains in the context of the full-length protein. Therefore, a comprehensive understanding of MDM2 behavior and function can only be obtained by studying the full-length protein. In this study, we use several known and novel MDM2 mutant constructs in the context of the full-length protein to show that MDM2-MDM2 interaction requires the central acidic domain and the extreme C-terminal residues of MDM2, whereas MDM2-MDMX interaction requires the proper RING domain structure and the extreme C-terminal residues of MDM2.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—U2OS and H1299 cells were obtained from the ATCC and were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma or Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cells were grown at 37 °C in a humidified incubator in the presence of 5% CO_2. MG132 was purchased from Calbiochem (catalog no. 474790). Cycloheximide was purchased from Sigma (catalog no. C7698). Phusion polymerase (catalog no. M0530S) and all restriction enzymes were purchased from New England Biolabs. XtremeGene HP transfection reagent was purchased from Roche through the University of North Carolina Tissue Culture Facility, and transient transfections were performed according to the instructions of the manufacturer. Mouse anti-actin (MAB1501, Chemicon), mouse anti-p53 (catalog no. DO-1, Labvision, AB-6, catalog no. MS-187P), mouse anti-FLAG (M2, Sigma, catalog no. F3165), and mouse anti-HA (16B12, Covance, catalog no. MMS101P) antibodies were purchased. Mouse anti-MDM2 (4B11 and 2A10) and rabbit anti-myc (9E10) antibody supernatants were obtained from hybridoma cell lines.

Cloning and Plasmids—MDM2 mutants were generated by using the QuikChange II XL site-directed mutagenesis protocol (Agilent Technologies, catalog no. 200521). Briefly, wild-type human MDM2 encoded in the pCMV mammalian expression vector was used as a template for all site-directed mutagenesis reactions. PCR reactions (50 μl total volume) contained 100 μg of template plasmid DNA, 20 pmol of forward primer, 20 pmol of reverse primer, 1× Phusion polymerase buffer, 50 μM dNTPs (Thermo Scientific, catalog no. R0182), 2 units of Phusion polymerase, and distilled water. The primers used were as follows: N447A (T_m 55 °C), gtgatttgtcaaggtcgacctaaagccggctgcattgtccatggcaaaac (forward) and gtttgccatggacaatgcagccggctttaggtcgaccttgacaaatcac (reverse); N447D (T_m 55 °C), gtgatttgtcaaggtcgacctaaagacggctgcattgtccatggcaaaac (forward) and gttttgccatggacaatgcagccgtctttaggtcgaccttgacaaatcac (reverse); C-terminal deletion (T_m 55 °C), ccctgcccagtatgtagatagttgacctgtctataagaggaatt (forward) and aattctcttatagacaggtcaactatctacatactgggcaggg (reverse); RING deletion (T_m 55 °C), cccttaatgccattgaacaaccaattcaaatgattg (forward) and caatcatttgaattggttgttcaatggcattaaggg (reverse); RING and C-terminal deletion (T_m 55 °C), cccttaatgccattgaatagttgacctgtctataagagaatt (forward) and aattctcttatagacaggtcaactattcaatggcattaaggg (reverse); and acidic domain deletion (T_m 60 °C), atcaggattcagtttcagatcaggaaatttccttagctgactattgg (forward) and ccaatagtcagctaaggaaatttcctgatctgaaactgaatcctgat (reverse). Mutagenesis primers were designed to harbor a novel restriction endonuclease site that did not alter the protein coding sequence and were used to amplify the intended product using a thermocycler (Applied Biosystems, model 2720) with the following program: 98 °C for 4 min, then 30 cycles of 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 5 min. A final extension step at 72 °C for 10 min was performed after the final cycle. Reactions were digested with DpnI (New England Biolabs) for 2 h, and then 10 μl of each reaction was transformed into chemically competent XL-1 blue *Escherichia coli* cells. All clones were submitted to the University of North Carolina Genome Analysis Facility for sequence verification.

Transfections—Cells were plated in 6-well plates overnight, and transfections were performed using XtremeGene HP transfection reagent (Roche) according to the instructions of the manufacturer. Briefly, cells were transfected at ~90% confluence with a mixture of 2 μg of total DNA and 6 μl of transfection reagent. All transfections included a GFP plasmid to visually confirm transfection efficiency (in all transfections, at least 50% of cells were GFP-positive). Twenty-four hours after the transfection, the indicated treatment was applied, or the cells were collected and lysed for Western blotting.

Immunoprecipitation—Cells were transfected with the appropriate plasmids for 24 h, after which the cells were lysed in 0.1% Nonidet P-40 buffer containing 1× protease inhibitor mixture (leupeptin, catalog no. L2884; aprotinin, catalog no. A1155; benzamidine, catalog no. B6506; and trypsin inhibitor, catalog no. T9003; all from Sigma), 1 mM PMSF (Sigma, catalog no. P7626), 1 mM $NaVO_3$ (Fisher Scientific, catalog no. S454-50), and 1 mM DTT (Roche, catalog no. 03117014001) for 2 h. Lysates (500 μg of each) were probed with 5 μl of EZ-view anti-FLAG M2 affinity gel (Sigma, catalog no. F2426) by rotating the samples at 4 °C for 2 h to overnight. Beads were washed three times in 0.1% Nonidet P-40 lysis buffer, after which the beads and inputs were analyzed by Western blotting.

Half-life Assay—U2OS cells were transfected with the indicated vectors for 24 h, after which cycloheximide (100 μg/ml) was added to all samples. Cells were collected in 0.5% Nonidet P-40 lysis buffer containing 1× protease inhibitor mixture, 1 mM PMSF, 1 mM $NaVO_3$, and 1 mM DTT at the indicated time point after cycloheximide addition. Cells were lysed for 2 h by rotation at 4 °C, after which the samples were centrifuged, and the supernatants were collected. Lysates were separated by SDS-PAGE and subjected to Western blotting for the indicated proteins. Bands were quantified after normalization to actin and plotted as the amount of protein remaining relative to time 0. Bands were compared quantitatively by using ImageJ software version 1.48.

Western Blotting—Cell lysates (100 μg/sample) were separated on 10% or 12.5% SDS-PAGE gels, after which the proteins were transferred to nitrocellulose membranes (Bio-Rad). Transfers were assessed by staining the membranes with Pon-

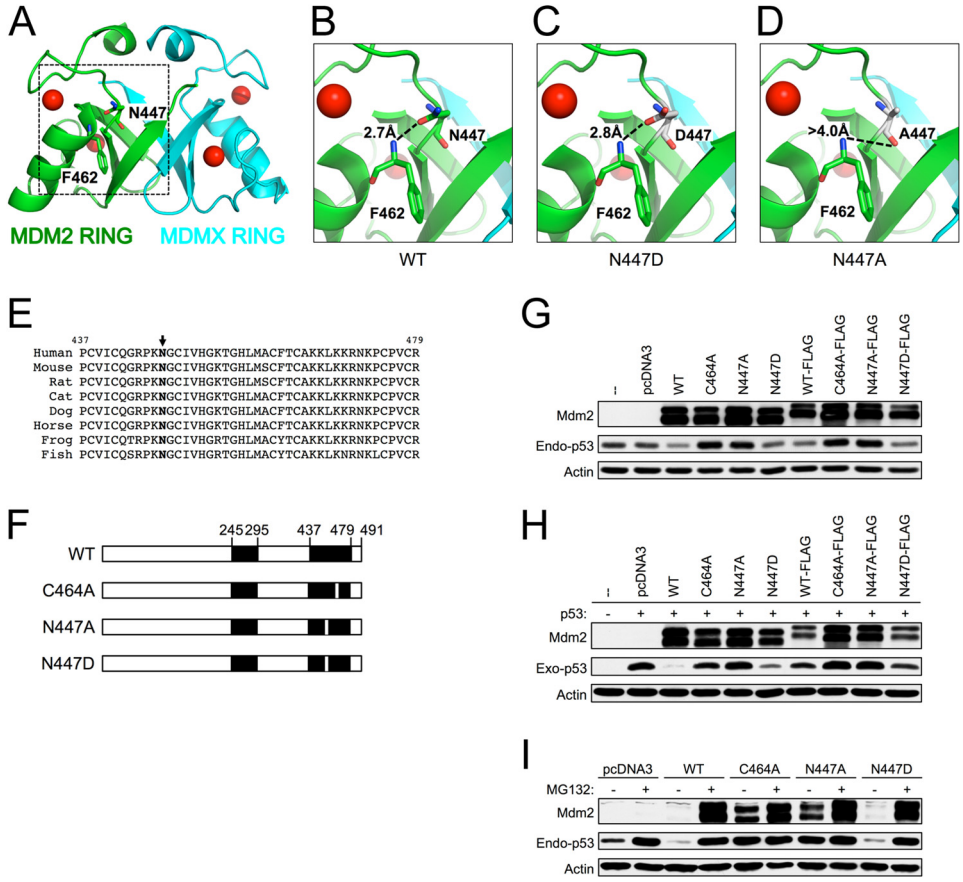

FIGURE 1. *A*, PyMOL image on the basis of the published crystal structure of the human MDM2 (*green*)-MDMX (*blue*) RING domain heterodimer. Residues Phe-462 and Asn-447 are shown as *stick figures*. *B*, close-up image of *A* depicting the intramolecular bond between the Asn-447 side chain and the amino group of Phe-462. *C*, PyMOL image simulating the conservative N447D mutation. *D*, PyMOL image simulating the non-conservative N447A mutation. *E*, protein sequence alignment for the MDM2 RING domain (human residues 437–479) in several species. *F*, diagram of MDM2 with amino acid positions of the AD (245–295), RING (437–479), and extreme C terminus (479–491) indicated. *G*, U2OS cells (WT p53) transfected with empty vector or untagged or FLAG-tagged MDM2 constructs and then blotted for MDM2 (4B11) and p53 (DO-1). *H*, H1299 cells (p53-null) cotransfected with WT p53 and empty vector or untagged or FLAG-tagged MDM2 constructs and then blotted for MDM2 (4B11) and p53 (DO-1). *I*, U2OS cells were transfected with empty vector or FLAG-MDM2 constructs for 24 h and then treated for 2.5 h with 10 μM MG132 and 100 μg/ml cycloheximide. Membranes were blotted for MDM2 (2A10) and p53 (DO-1).

ceau S (Sigma, catalog no. P3504) for 5 min, followed by several brief washes with double-distilled H_2O. Membranes were blocked for at least 1 h in phosphate-buffered saline containing 5% nonfat milk and 0.1% Tween 20. Membranes were then incubated with the appropriate primary antibody diluted in blocking buffer for 2 h to overnight. Membranes were washed three times in phosphate-buffered saline containing 0.1% Tween 20 and then incubated with the appropriate HRP-conjugated secondary antibody diluted in blocking buffer for 1 h. Membranes were washed four times in PBS-T and then developed with Supersignal West Pico chemiluminescent substrate according to the instructions of the manufacturer (Pierce, catalog no. 34080).

In Vivo Ubiquitination Assay—Equal amounts of U2OS or H1299 cells were transfected with the indicated plasmid constructs for 24 h, and then the cells were trypsinized and collected. One-fifth of each sample was lysed with 0.1% Nonidet P-40 lysis buffer as input. The remaining cells were boiled in SDS lysis buffer containing 1× protease inhibitor, 1 mM PMSF, 1 mM $NaVO_4$, and 1 mM DTT for 10 min. Then the lysates were diluted into 0.1% Nonidet P-40 lysis buffer containing protease inhibitors and subjected to immunoprecipitation using anti-FLAG beads. Western blotting was performed to probe for the indicated proteins.

RESULTS

MDM2 Residue Asn-447 Is Important for p53 Degradation—Kostic *et al.* (20) performed a detailed investigation of MDM2-MDMX RING domain binding by NMR (20). In this study, radiolabeled purified MDM2 RING domains were titrated with unlabeled purified MDMX RING domains, and differences in the heteronuclear single quantum coherence spectra were obtained for each MDM2 residue within the RING domain upon MDMX binding. The greatest chemical shift occurred at asparagine residue 447 in MDM2, suggesting that Asn-447 may be important for MDM2-MDMX binding. Initial analysis of the MDM2 sequence indicated that many of the residues within the C-terminal MDM2 RING domain show a high degree of identity among different species. Asn-447 is conserved in many species from humans to zebrafish, consistent with the idea that this residue serves an important role in MDM2 function (Fig. 1*E*). Additionally, upon analyzing the MDM2-MDMX RING domain heterodimer crystal structure (21), Asn-447 appears to form an intramolecular hydrogen bond with the backbone

amino group of Phe residue 462 (Fig. 1, *A* and *B*). To determine whether the Asn-447 side chain is indeed important for MDM2 function, we used site-directed mutagenesis to convert the Asn-447 residue to aspartic acid or alanine (Fig. 1*F*). We predicted that aspartic acid, which contains a side chain of comparable size and polarity to asparagine, should maintain the hydrogen bond and the proper three-dimensional structure and function of MDM2 (Fig. 1*C*). However, the alanine substitution mutant, which lacks a side chain oxygen residue and is considerably smaller than the asparagine side chain, should lose the ability to bond with Phe-462, thereby preventing the proper structure and function of MDM2 (Fig. 1*D*).

To test the importance of Asn-447 in MDM2 function, MDM2 point mutation constructs were assembled in untagged and FLAG-tagged expression plasmids. Consistent with our modeling predictions, endogenous p53 levels decreased in cells transfected with either WT MDM2 or the N447D mutant compared with the empty vector control. Likewise, p53 levels increased in cells transfected with the E3-dead C464A mutant or the N447A mutant (Fig. 1*G*). These results suggest that although the N447A mutation inhibits the ability of MDM2 to degrade p53, the N447D mutation does not appreciably affect MDM2-mediated p53 degradation. We observed similar results when we tested these mutants on exogenously overexpressed p53 in H1299 cells (p53-null, Fig. 1*H*). Although cells transfected with the N447D mutant showed significant p53 degradation, a noticeable increase in p53 levels was observed with the N447D mutant compared with WT MDM2, which reflects decreased E3 ligase activity, likely because of differences between the Asp side chain and the naturally occurring Asn side chain. Treatment of the transfected cells with MG132 prevented degradation of p53 and MDM2 by WT MDM2 and the N447D mutant, suggesting that degradation occurs predominantly through the proteasome (Fig. 1*I*).

The N447A Half-life Increases because of Lack of Ubiquitination Activity—To further determine the effect of the Asn-447 mutations on p53 and MDM2 stability, half-life assays were conducted for the N447A and N447D mutants in U2OS cells. Expression of the E3-active WT or N447D mutant constructs shortened the half-life of p53 (58 and 69 min, respectively, compared with 116 min for the empty vector), which is consistent with intact MDM2 E3 activity (Fig. 2*A*). Conversely, the p53 half-life was extended dramatically in the presence of the C464A or N447A MDM2 mutants (over 210 min for both compared with 116 min for the empty vector), consistent with diminished MDM2 E3 activity (Fig. 2*A*). Analysis of the half-life of the overexpressed MDM2 revealed a similar trend where WT and N447D mutants showed relatively short half-lives (69 and 77 min, respectively) compared with the C464A and N447A mutants (>210 min for both), consistent with the ability of ectopically overexpressed WT and N447D MDM2 proteins to promote their own degradation (Fig. 2*A*).

Next we conducted *in vivo* ubiquitination assays to determine whether the MDM2 mutants show differences in p53 and/or MDM2 ubiquitination. To assess p53 ubiquitination, H1299 cells (p53-negative) were transfected with FLAG-p53, HA-ubiquitin, and untagged MDM2 constructs for 24 h. As in previous results (Fig. 2*A*), WT MDM2 and the N447D mutant polyubiquitinated p53, whereas the C464A and N447A mutants were unable to ubiquitinate p53 (Fig. 2*B*). To assess MDM2 autoubiquitination, the FLAG-MDM2 constructs were individually transfected into H1299 cells along with HA-ubiquitin. Consistent with the p53 ubiquitination assay results, the WT and the N447D mutant showed autoubiquitination laddering, whereas the C464A and N447A mutants displayed significantly less laddering (Fig. 2*C*).

RING-disruptive Mutations Inhibit MDM2 Binding to MDMX but Not to MDM2—Because MDM2 is thought to require homo- and/or heterodimerization with MDMX to function as an effective E3 ligase (11, 14), we sought to determine how these mutations affect MDM2 homo- and heterodimerization. To this end, U2OS cells were cotransfected with myc-tagged WT MDMX and each of the FLAG-MDM2 mutant constructs and were then subjected to coimmunoprecipitation (co-IP). As expected, the MDM2 constructs capable of degrading p53 (WT and N447D) formed robust heterodimers, whereas MDM2 constructs incapable of degrading p53 (C464A and N447A) failed to heterodimerize (Fig. 3*A*). We noticed a consistent decrease in heterodimerization between MDMX and the N447D mutant relative to WT MDM2, suggesting that the N447D mutation marginally affects MDM2-MDMX binding.

To determine whether the MDM2 mutant constructs could interact with MDM2, we conducted a similar co-IP experiment with WT myc-MDM2 instead of myc-MDMX. Surprisingly, all constructs were able to pull down WT MDM2 to similar extents (Fig. 3*B*). Because the RING domain of MDM2 has been thought to be responsible for MDM2 homodimerization, we expected that the C464A and N447A mutants, which were unable to heterodimerize with MDMX, would also fail to interact with MDM2. However, despite harboring point mutations that prevent binding with MDMX, C464A and N447A mutant MDM2 maintained robust binding with WT MDM2 to an extent similar to WT MDM2 itself, suggesting that MDM2-MDMX and MDM2-MDM2 interactions require different sets of residues for binding.

Deletion of the Extreme C-terminal Residues Prevents MDM2 Interaction with MDM2 and MDMX—Previous studies have shown that the MDM2 sequence at the extreme C terminus is necessary for homo- and heterodimer formation (18, 22). Because our co-IP results suggested that MDM2 homo- and heterodimers may require different residues, we sought to determine the effect of an extreme C terminus deletion of MDM2 on its ability to interact with full-length MDM2. To this end, we constructed MDM2 C-terminal deletion mutants lacking the RING domain (amino acids 437–479, Δ*RING*), the extreme C-terminal 12 residues (480–491, Δ*C-term*), or both the RING domain and the C-terminal residues (437–491, Δ*RING*/Δ*C-term*) (Fig. 4*A*). Consistent with our expectations, all of these constructs lacked the ability to degrade endogenous p53 (Fig. 4*B*). Furthermore, co-IP experiments conducted to determine their ability to bind MDMX or MDM2 revealed that all three deletion constructs failed to interact with either MDMX or MDM2 full-length constructs (Fig. 4, *C* and *D*). These data suggest that although the proper structure of the RING domain may not be required for MDM2-

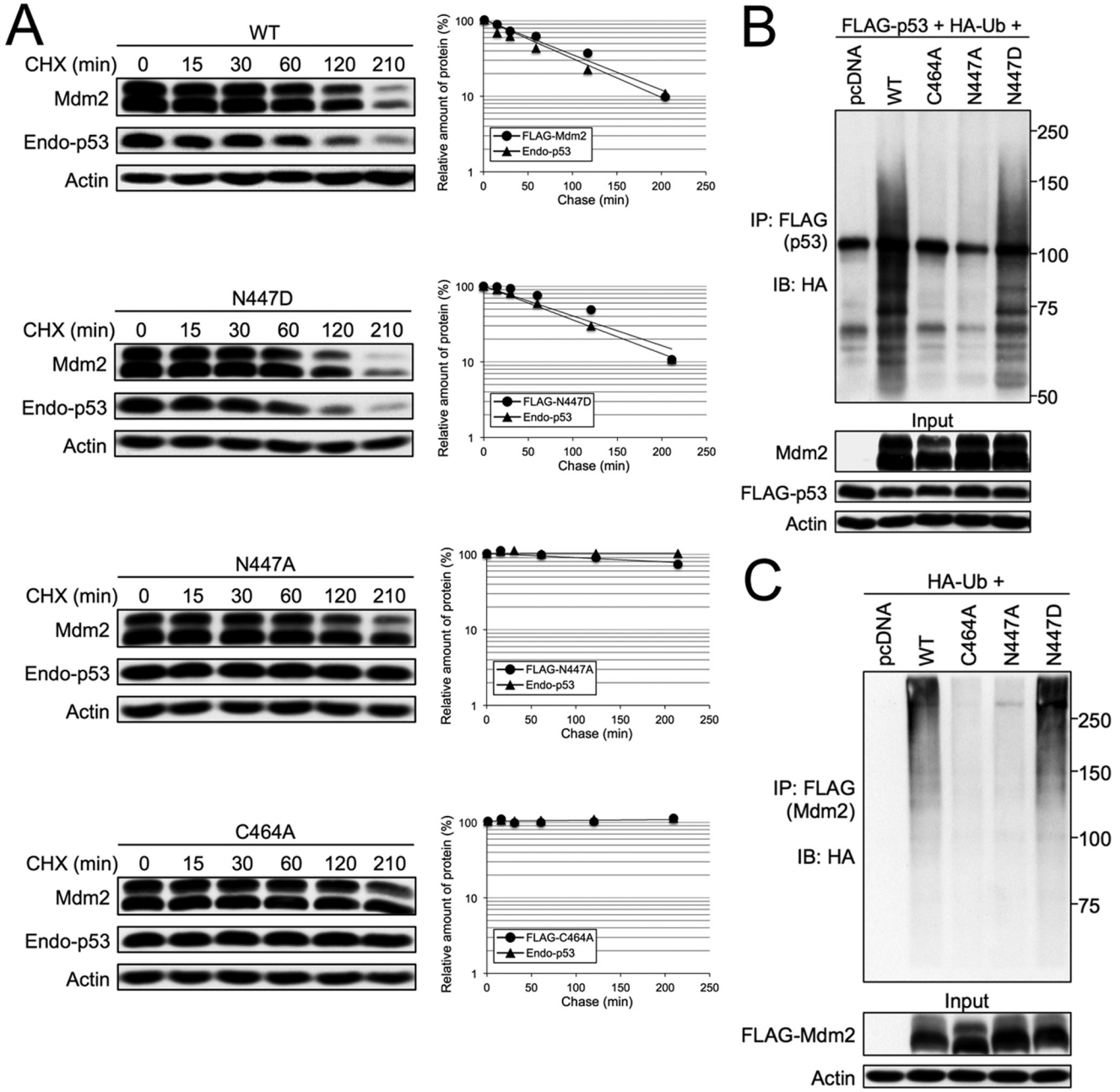

FIGURE 2. *A*, U2OS cells were transfected with constructs encoding MDM2 for 24 h and then chased with 100 μg/ml cycloheximide (*CHX*) for various amounts of time. Lysates were resolved by SDS-PAGE and then probed by Western blot analysis for p53 (DO-1) and MDM2 (4B11). *B* and *C*, H1299 cells were cotransfected with FLAG-p53, HA-ubiquitin, and one of the untagged MDM2 constructs (*B*) or with HA-ubiquitin and FLAG-MDM2 (*C*) for 24 h. Cells were subjected to a ubiquitination assay, probing with anti-HA antibody to detect ubiquitinated p53 or MDM2. Inputs representing 20% of the immunoprecipitated protein are included. *IB*, immunoblot.

MDM2 interaction, the presence of the RING domain and the C-terminal residues that lie downstream of the RING domain of MDM2 are necessary for interaction with both MDMX and MDM2.

The MDM2 Acidic Domain Is Necessary for Interaction with MDM2 but Not with MDMX—MDM2 has been reported to dimerize through RING-acidic domain (AD) interactions (23). However, a more recent study has suggested that AD-RING interactions occur intramolecularly, which may actually be more physiologically relevant than potential intermolecular AD-RING domain interactions. Consistent with the idea that intermolecular RING-AD interactions may not significantly affect MDM2-MDM2 binding in the cellular milieu, the ΔRING MDM2 mutant, which harbors an intact AD, is severely impaired in its ability to bind to WT MDM2, which contains both the AD and the RING domain (Fig. 4*D*). Nonetheless, to investigate the possible effects of the AD on the observed interactions of our mutant constructs, we deleted a portion of the AD (Δ245–295) in the WT, C464A, N447A, and N447D constructs to determine the effect of AD deletion on MDM2 dimerization and p53 degradation (Fig. 5*A*). Consistent with previous reports, AD deletion inhibited the degra-

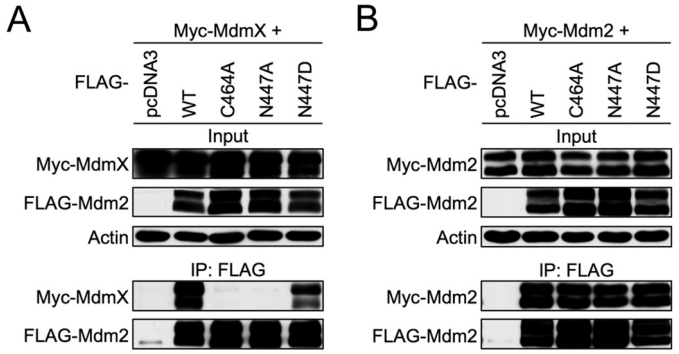

FIGURE 3. *A* and *B*, U2OS cells were cotransfected with constructs encoding myc-MDMX (*A*) or myc-MDM2 (*B*) and each of the FLAG-MDM2 constructs for 24 h. Cell lysates were subjected to immunoprecipitation, resolved by SDS-PAGE, probed with anti-myc antibody, and reprobed with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included.

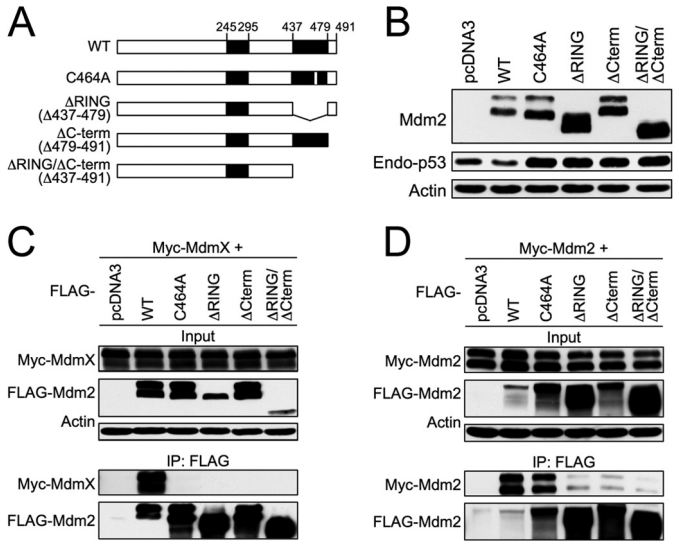

FIGURE 4. *A*, WT and mutant MDM2 constructs. *B*, U2OS cells were transfected with empty vector or FLAG-MDM2 constructs for 24 h, and then the cell lysates were resolved by SDS-PAGE and subjected to Western blot probing for FLAG and p53. *C* and *D*, U2OS cells were cotransfected with constructs encoding myc-MDMX (*C*) or myc-MDM2 (*D*) and each of the indicated FLAG-MDM2 constructs for 24 h. Cell lysates were immunoprecipitated with anti-FLAG beads, resolved by SDS-PAGE, and then subjected to Western blot probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included.

dation of endogenous p53 (Fig. 5*B*) and ectopically expressed p53 (Fig. 5*C*).

Co-IP analysis for dimerization with MDMX revealed that AD deletion does not affect heterodimer formation of WT or N447D MDM2, consistent with the idea that heterodimerization is necessary for ubiquitination activity (Figs. 3*A* and 6*A*). Furthermore, these results suggest that the AD is dispensable for heterodimerization, which likely occurs exclusively through RING domain/C-terminal interactions. However, when analyzed for their ability to interact with WT myc-MDM2, the AD deletion mutants displayed an inverse trend. When the AD was deleted in the E3-dead C464A or N447A constructs, the ability to interact with WT MDM2 was largely unaffected. However, when the AD was deleted in the E3-active WT or N447D constructs, their ability to bind WT MDM2 was abrogated, suggesting that MDM2-MDM2 binding depends on the AD (Fig. 6*B*).

To further investigate the E3-dead (C464A and N447A) AD deletion mutants, the extreme C-terminal residues were deleted to determine whether these dimers require the C terminus. When the extreme C terminus was deleted, the C464A and N447A AD deletion mutants failed to interact with WT MDM2 (Fig. 6*C*), suggesting that the C-terminal tail is absolutely required for MDM2 dimerization.

When subjected to p53 and MDM2 *in vivo* ubiquitination assays, AD deletion did not affect ubiquitination of p53 or MDM2 (Fig. 6, *D* and *E*). These results are consistent with some early reports analyzing various AD deletions and may suggest that the AD is necessary for p53 and MDM2 degradation downstream of ubiquitination (24, 25).

To address whether coexpression of an AD-binding protein might affect MDM2 dimer formation, we cotransfected U2OS cells with constructs encoding FLAG-MDM2, myc-p14ARF (0, 0.1, or 0.5 μg), and either myc-MDMX or myc-MDM2. After protein extraction, the lysates were probed with anti-FLAG beads and then examined by Western blot analysis to determine how much myc-MDMX or myc-MDM2 was pulled down. Our results show that p14ARF coexpression decreased MDMX levels, resulting in less MDMX co-IP while increasing MDM2 levels and, correspondingly, increasing the amount of MDM2 pulled down by co-IP (Fig. 6, *F* and *G*, respectively). Although it is difficult to determine whether there is a change in binding affinity between FLAG-MDM2 and myc-MDMX or myc-MDM2, it appears that p14ARF at least increases the overall amount of MDM2 homodimers and decreases the overall amount of MDM2-MDMX heterodimers, which is consistent with previous findings (26–29).

DISCUSSION

MDM2 has long been known to form homodimers and heterodimers with the structurally related MDMX protein. Although data have convincingly shown the importance and necessity of MDM2-MDMX binding for p53 regulation, the physiological significance of MDM2 homodimers remains unknown. *In vitro* studies have shown that purified MDM2 in the absence of MDMX is sufficient to ubiquitinate and, presumably, elicit the degradation of p53 (11, 22). Furthermore, several independent mouse studies have shown that, when MDM2 is present but unable to bind MDMX, embryonic development progresses to a later stage than *Mdm2* knockout mice (~embryonic day 7.5 or greater *versus* embryonic day 5.5), suggesting that MDM2 alone may offer a primitive mechanism of p53 control (13, 16, 17, 30–32). MDM2 homodimers are indeed stable enough to be detected when overexpressed *in vitro* and in cell-based assays, suggesting that MDM2 homodimers could also exist *in vivo* (20, 26).

To better understand MDM2 homodimers and MDM2-MDMX heterodimers, detailed crystal structures of the two dimers could be particularly valuable. However, complete crystal structures of MDM2 dimers remain elusive. Therefore, many structure- and mechanism-based MDM2 studies have focused on isolated MDM2 domains such as the RING domain. Although these studies have provided valuable insights into the

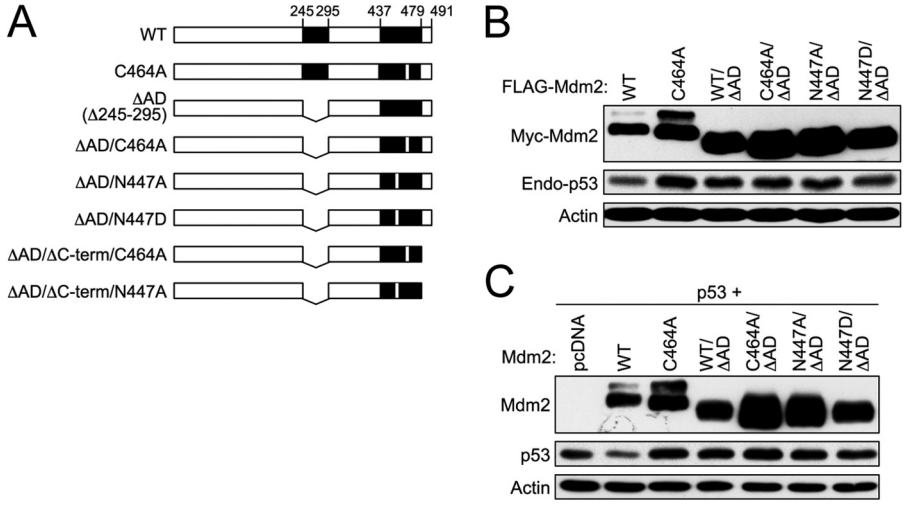

FIGURE 5. *A*, WT and mutant MDM2 constructs. *B*, U2OS cells were transfected with empty vector or FLAG-MDM2 constructs for 24 h, and then cell lysates were subjected to Western blot probing for FLAG and p53. *C*, H1299 cells were cotransfected with WT p53 and empty vector or FLAG-MDM2 constructs for 24 h, and then cell lysates were subjected to Western blotting for FLAG and p53.

binding mechanisms of the individual domains of MDM2, they are unable to assess MDM2 binding in the context of the complete protein. Although protein binding studies such as ours require verification using purified proteins in an *in vitro* binding assay, our experience using purified recombinant full-length MDM2 and MDMX protein suggests that they behave differently under *in vitro* conditions compared with their behavior in the cytoplasmic milieu (data not shown). This could indicate that other factors, such as small proteins that bind MDM2 or the intracellular environment itself, are required for proper function. However, using full-length proteins overexpressed in cells, our study provides mechanistic insight with respect to the domains of MDM2 that are necessary for homo- and heterodimerization within the cell.

Using point mutations in the highly conserved Cys-464 and Asn-447 residues, which reside within the RING domain but interact with different residues, we show that the proper MDM2 RING domain structure may not be necessary for interaction with WT MDM2 but is critical for heterodimerization with MDMX. Previous studies investigating the conserved zinc-coordinating residues within the RING domain have shown the importance of these residues for heterodimerization and p53 degradation (31, 33). Our study expounds these observations by showing that RING domain mutants (C464A and N447A) retain the capacity to form stable homodimers with WT MDM2 comparable with WT MDM2 itself (Fig. 3*B*). This observation was unexpected because previous studies using isolated MDM2 and MDMX RING domains have suggested that although some residues may differ, MDM2 homodimers and heterodimers form in a similar fashion (20, 21).

The extreme C-terminal residues have been implicated in MDM2 RING domain homo-oligomer formation (18, 22). By deleting a small portion of the C terminus of full-length MDM2, our data confirm that the C-terminal residues are required for MDM2 binding to WT MDM2 and MDMX. Furthermore, deletion of the C-terminal 12 residues (ΔC-term) abrogated MDM2 interaction in all constructs tested (WTΔAD, C464AΔAD, and N447AΔAD, Figs. 4*D* and 6*C*). Although the presence of the C terminus appears to be necessary for MDM2 homo- and heterodimerization, the mechanisms that dictate the formation of homo- and/or heterodimers appear to involve the MDM2 AD.

Previous studies have suggested that the AD plays a role in MDM2 regulation and function (24, 25, 34, 35). The AD could also play a role in regulating MDM2 protein binding, such as dimerization preference. Deletion of the MDM2 AD can affect binding between MDM2 and other proteins, such as p300 and various proteasome components (36, 37). It is possible that our AD deletion mutants lack a motif required for their interaction with MDM2 because AD deletion from WT MDM2 selectively inhibits binding with WT MDM2. Furthermore, we observed an increase in MDM2-MDMX heterodimerization upon AD deletion, suggesting that the AD may also be inhibitory toward MDM2-MDMX heterodimerization (Fig. 6*A* compare *lanes 1* and *3*). These data collectively suggest that the AD may control the balance between MDM2 homo- and heterodimerization. Chen and co-workers (26–28) and others (29) have shown that manipulation of the MDM2 AD in response to various p53-activating stimuli can affect MDM2 dimerization, thereby stabilizing p53. We suspect that modification of the MDM2 AD or binding by p14ARF and/or ribosomal proteins in response to stress could also affect MDM2 homo- and heterodimerization. Consistent with this idea, our data show that overexpression of the MDM2-binding protein p14ARF decreases the overall amount of MDM2-MDMX heterodimers while increasing the amount of MDM2-MDM2 homodimers in the cell, likely by affecting MDM2 and MDMX protein stability (Fig. 6, *F* and *G*).

Interestingly, when the AD is deleted from E3-inactive MDM2 constructs (C464A and N447A), the ability of these mutants to interact with WT MDM2 persists (Fig. 6*B*). Although we find this observation intriguing, we are currently unable to fully explain this E3-associated correlation. One possible explanation that will require further investigation is that the AD functions as a trigger that, when activated, allows homodimerization to occur through the C-terminal residues. The unstructured state of the C464A and N447A RING

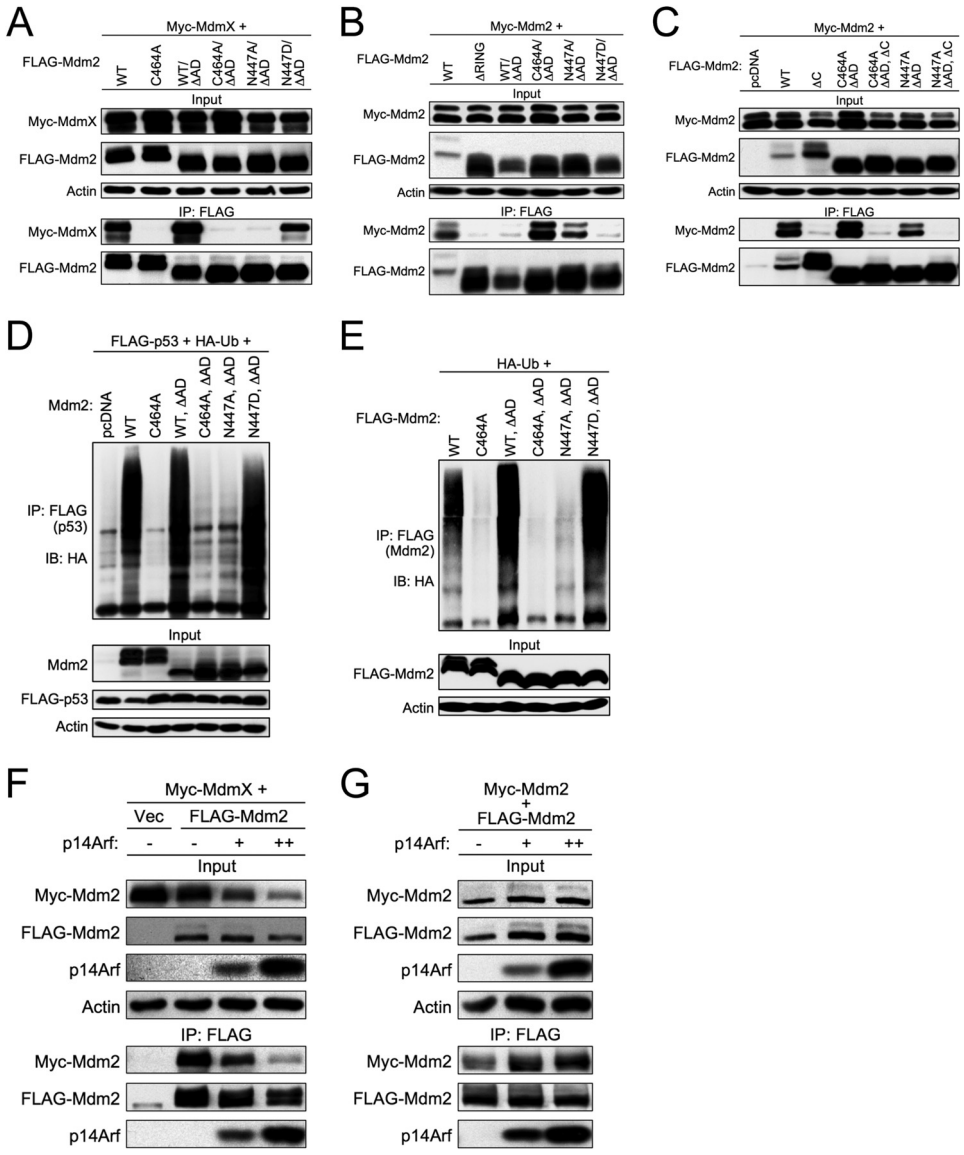

FIGURE 6. *A* and *B*, U2OS cells were cotransfected with constructs encoding myc-MDMX (*A*) or myc-MDM2 (*B*) and each of the indicated FLAG-MDM2 constructs for 24 h. *A*, cells were treated for 3 h with 20 μM MG132 prior to lysis. Cell lysates were precipitated with anti-FLAG beads overnight and then subjected to Western blot probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included. *C*, U2OS cells were cotransfected with myc-MDM2 and each of the indicated FLAG-MDM2 constructs for 24 h. Cell lysates were immunoprecipitated with anti-FLAG beads and then subjected to Western blot probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included. *D* and *E*, U2OS cells were transfected with HA-ubiquitin, FLAG-p53, and one of the untagged constructs as indicated (*D*) or with HA-ubiquitin and one of the FLAG-tagged constructs as indicated (*E*) for 24 h. Cell lysates were immunoprecipitated with anti-FLAG beads and then subjected to Western blot probing with anti-HA antibody. Inputs representing 20% of the total protein immunoprecipitated are included. *IB*, immunoblot. *F* and *G*, U2OS cells were cotransfected with constructs encoding myc-MDMX (*F*) or myc-MDM2 (*G*) and each of the indicated FLAG-MDM2 and p14ARF constructs for 24 h. Cell lysates were precipitated with anti-FLAG beads overnight and then subjected to Western blot probing with anti-myc antibody and reprobing with anti-FLAG antibody. Inputs representing 10% of the immunoprecipitated protein are included.

domains could constitutively expose the C terminus for homodimerization, whereas the WT and N447D RING domains maintain the C terminus in a state that allows homo- and heterodimer formation depending on the state of the AD. When the AD is removed from WT MDM2, then only heterodimers can form. Future studies investigating possible RING structure-dependent binding mechanisms could shed light on the binding mechanics of MDM2 and other dimeric E3 ligases.

Altogether, our results are the first to definitively show evidence for differences in the mechanism of dimerization for MDM2 homodimers and heterodimers. Our data show that although MDM2-MDMX heterodimers form through RING domain and extreme C-terminal contacts, MDM2 homodimers form in an AD- and extreme C-terminal residue-dependent manner (Fig. 7). To our knowledge, this AD deletion mutant represents the first reported MDM2 mutation that can selectively inhibit MDM2-MDM2 interaction while leaving heterodimerization and E3 activity intact. We believe that further investigation into the MDM2 AD will prove critical for a more comprehensive understanding of MDM2 regulation.

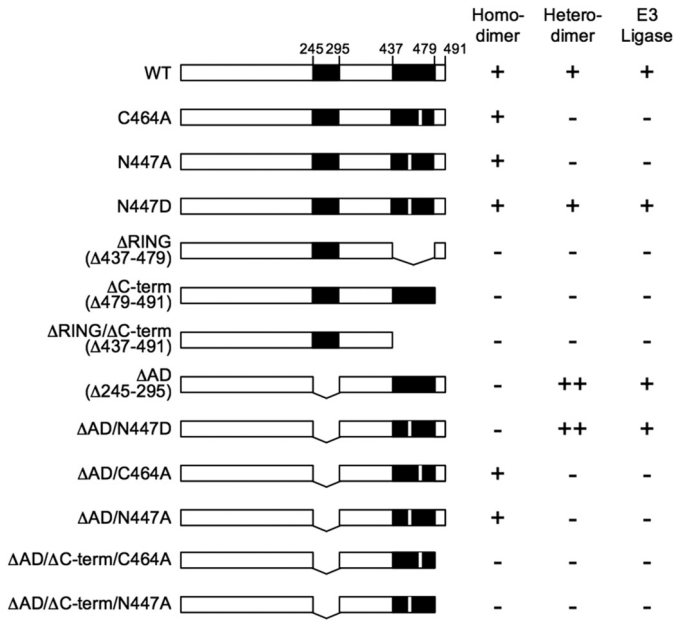

FIGURE 7. **MDM2 requires the AD and C-terminal residues to interact with MDM2, whereas MDM2 requires the properly structured RING domain and C-terminal residues to interact with MDMX.** *R*, RING domain; *C*, extreme C-terminal residues.

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