RING Domain–Mediated Interaction Is a Requirement for MDM2's E3 Ligase Activity

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Abstract

The RING domain of MDM2 that is essential for its E3 ligase activity mediates binding to itself and its structural homologue MDMX. Whereas it has been reported that RING domain interactions are critical, it is not well understood how they affect the E3 ligase activity of MDM2. We report that the E3 ligase activity requires the RING domain–dependent complex formation. *In vivo*, MDM2 and MDMX hetero-RING complexes are the predominant form versus the MDM2 homo-RING complex. Importantly, the MDM2/MDMX hetero-RING complexes exhibit a greater E3 ligase activity than the MDM2 homo-RING complexes. Disruption of the binding between MDM2 and MDMX resulted in a marked increase in both abundance and activity of p53, emphasizing the functional importance of this heterocomplex in p53 control. [Cancer Res 2007;67(13):6026–30]

Introduction

The p53 tumor suppressor is a critical regulator of cell fate. Due to its growth inhibitory functions, p53 must be maintained at low levels under physiologic conditions to ensure cell survival and proper development (1, 2). Regulation of p53 is largely achieved at the protein level by the RING domain E3 ligase MDM2, which targets itself and p53 for proteasome-mediated degradation and mediates p53 nucleocytoplasmic shuttling (3). Moreover, p53 and MDM2 exist in a negative regulatory feedback loop in which p53 transcriptionally activates MDM2 expression (3).

MDMX, a homologue of MDM2, has emerged as another critical negative regulator of p53. MDM2 and MDMX share two important conserved domains: an N-terminal p53 binding domain and a C-terminal RING domain (4). These proteins interact through their RING domains; and although similar, MDMX does not have an intrinsic E3 ligase activity *in vivo* (5, 6). Genetic studies show that deletion of *mdmx* leads to p53-dependent lethality (7, 8). These mouse genetic studies have further characterized the role of MDM2 and MDMX in controlling p53, concluding that the two proteins have distinct roles: MDM2 mediates the degradation of p53; MDMX inhibits p53 transcriptional activity (9).

However, on a biochemical and cellular level, there is overwhelming evidence that there is mutual dependence between MDM2 and MDMX in the inhibition of p53 (10). It has been shown that DNA damage mediates MDM2-dependent degradation of MDMX, allowing for maximal p53 activation (11, 12). Furthermore, MDMX enhances the E3 ligase activity of MDM2 *in vitro* and can restore the E3 ligase activity of MDM2 mutants that have lost intrinsic E3 ligase activity (13, 14). These studies implicate MDMX in facilitating ubiquitylation by MDM2.

The RING domain typically mediates protein/protein interactions. In the case of E3 ligase function, RING E3s seem to form scaffolds to bring substrates in close proximity to cognate E2s (15). Some RING E3 ligases have increased activity with RING cooperativity. For example, the E3 ligase BRCA1 displays low activity alone but functions at full capacity when it is paired with one or more components of BARD1, a RING containing protein that itself has no intrinsic E3 ligase activity (16). One study has elegantly shown that BRCA1 and BARD1 form supramolecular structures that contribute to the observed increased E3 ligase activity (17). Recently, it has been reported that MDM2 is also capable of forming supramolecular structures *in vitro* (18).

In this report, we show compelling evidence that RING-mediated complex formation between MDM2 and MDMX, which seems to be the predominant form present in cells, is essential for its E3 ligase activity toward p53, making it a better E3 ligase than MDM2 alone.

Materials and Methods

Cell culture and transfections. All cells were maintained in minimal essential medium (Life Technologies-Bethesda Research Laboratories) containing 10% fetal bovine serum (Sigma), 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Transfections were done by the calcium phosphate method for 293T cells and the LipofectAMINE method for U2OS and MEFs. Luciferase activities were assayed 24 h posttransfection with a dual luciferase assay kit (Promega).

Plasmids. The p53 fusion proteins were prepared by a two-step PCR using primers carrying a 12-nucleotide tail of the p53 or MDM2 RING finger domain (RFD) or MDMXRFD to be fused. DNA sequencing confirmed the identity of each construct.

Immunoprecipitation and immunoblot analysis. Immunoprecipitations were done as described elsewhere (10). Cell lysates were prepared in 1% Triton X-100 lysis buffer or radioimmunoprecipitation assay buffer (RIPA) and incubated with anti-MDM2 (Ab-1–coated agarose beads; EMD Biosciences) or Flag antibody (M2-coated sepharose beads; Sigma) for 4 to 6 h. Immune complexes and whole lysates were separated by SDS-PAGE and transferred to nitrocellulose filters. Filters were incubated with anti-p53 (Ab-6; EMD Biosciences), anti-Mdm2 (Ab-1; EMD Biosciences), anti-MDMX (Bethyl Laboratories), anti-WAF1 (Ab-3; EMD Bioscience), antigreen fluorescent protein (Clontech), and anti-Flag (M5; Sigma) antibodies. Proteins were detected with an enhanced chemiluminescence system (NEN).

Cross-link assay. Cells were transfected with the indicated expression plasmid and lysed as described above 36 h posttransfection. Each lysate was

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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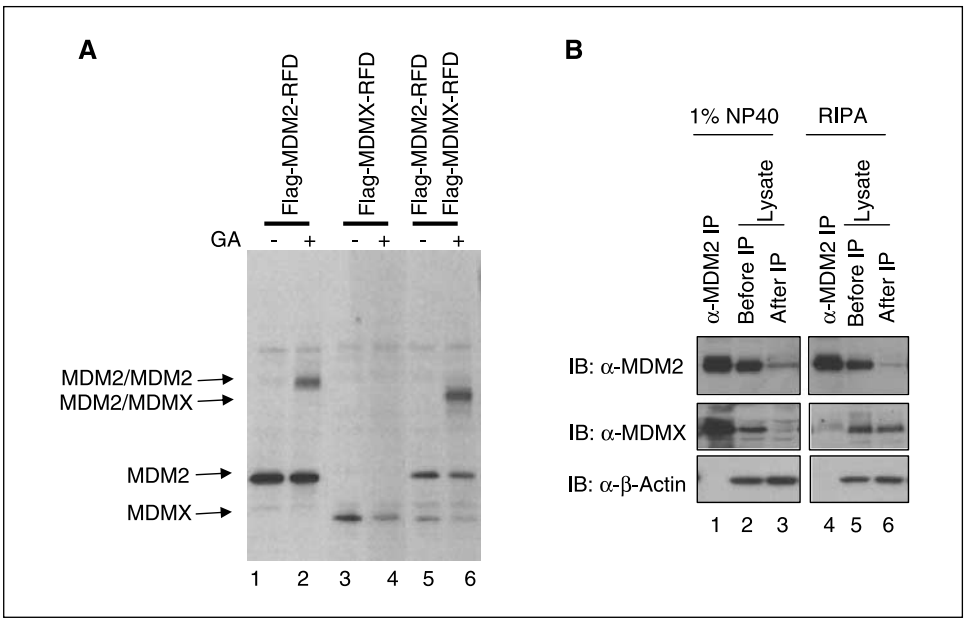

Figure 1. MDM2 and MDMX exist predominantly as the MDM2/MDMX hetero-RING complex in cells. *A,* plasmids encoding the RFD of MDM2 or MDMX were either expressed separately (*lanes 1 and 2,* MDM2 alone; *lanes 3 and 4,* MDMX alone) or together (*lanes 5 and 6,* 1 μg each DNA) in *mdm2*$^{-/-}$/*p53*$^{-/-}$ MEFs. The cells were harvested 24 h after transfection and lysed. The cross-linking reaction was done by incubation of cell lysates on ice in the presence (+) or absence (−) of 0.01% glutaraldehyde. The products were resolved on 10% SDS-PAGE and visualized by Western blotting. *B,* MCF-7 cells were treated with MG132 for 6 h before harvesting. Cells were lysed in either 1% NP40 or a RIPA lysis buffer and subjected to immunoprecipitation (*IP*) with an anti-MDM2 antibody.

divided into three tubes, and glutaraldehyde was added to the final concentration of 0, 0.01%, or 0.1%. After incubation on ice for 15 min, equal volumes of 2× loading buffer were added and the samples were boiled and analyzed as described above.

Subcellular distribution assay. Cells were grown on 25 mm × 25 mm glass coverslips (VWR) and transfected with indicated plasmids. Cells were washed with cold PBS 36 h posttransfection and fixed with 4% paraformaldehyde (Sigma) for 30 min at 4°C. After washing with PBS, the cells were permeabilized with 0.2% Triton X-100 PBS. The slides were washed, incubated with 4′,6-diamidino-2-phenylindole (DAPI; 10 μg/mL; Sigma), and mounted with Fluoromount-G (Southern Biotechnology Associates) containing 2.5 mg/mL of *n*-propyl gallate (Sigma). Specimens were examined under a fluorescence microscope (Nikon).

Results

The MDM2/MDMX heterocomplex is the predominant form present in cells. To test the RING-mediated interactions *in vivo* in mammalian cells, we used a protein cross-linking assay. When expressed alone, the predominant species of MDM2RFD captured by the cross-linking reaction is a dimer, whereas no self-association could be detected for MDMXRFD under the same condition (Fig. 1*A*, *lane 2 versus lane 4*). Interestingly, when MDM2RFD and MDMXRFD were coexpressed, the heterodimer becomes the only detectable species. The formation of dimers seemed to depend on the RING domain–mediated binding as MDM2 (C464A) and MDMX

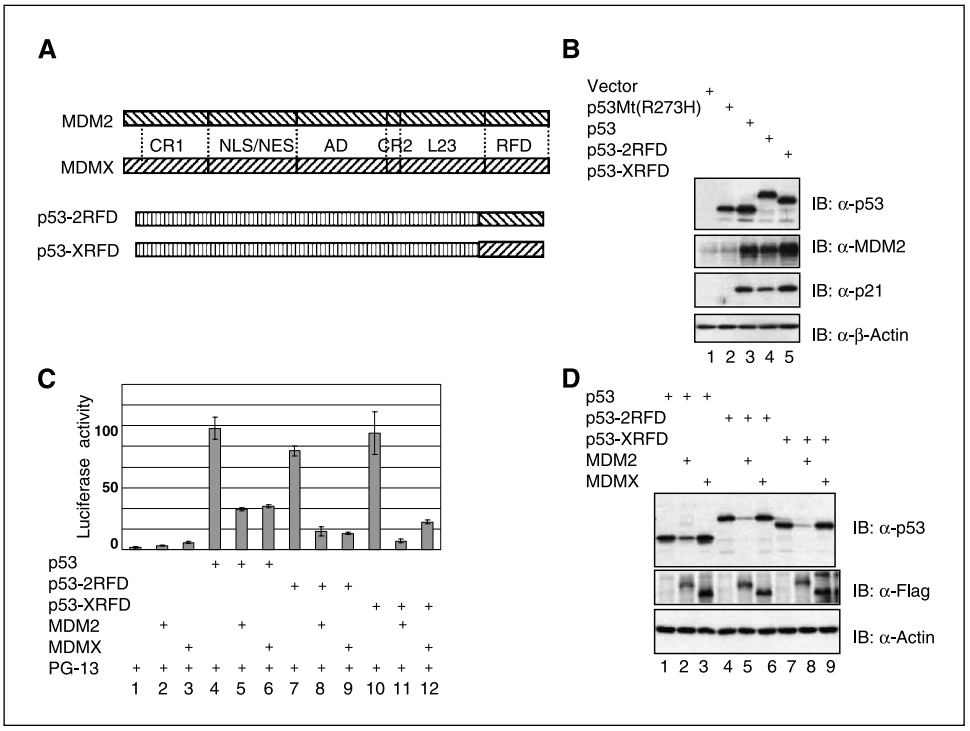

Figure 2. The p53-2RFD and p53-XRFD fusion proteins behave as wild-type p53. *A,* cartoon diagrams show the structure of MDM2, MDMX, and the p53 fusion proteins. *B,* plasmid encoding p53 fusion proteins was transfected into *mdm2*$^{-/-}$/*p53*$^{-/-}$ MEFs, which were harvested for Western analysis 24 h after transfection with the indicated antibodies. Wild-type (*lane 3*) and transcription-deficient mutant of p53 (R273H, *lane 2*) were included as controls. *C,* a similar transfection was done with a luciferase gene containing p53-responsive promoter (PG-13) for luciferase assay. *D,* p53 or the fusion proteins were coexpressed with either MDM2 (*lanes 2, 5, or 8*) or MDMX (*lanes 3, 6, or 9*) in *mdm2*$^{-/-}$/*mdmx*$^{-/-}$/*p53*$^{-/-}$ MEFs. The cells were harvested 24 h posttransfection for Western analysis.

(C463A) RFD mutants defective in RING binding (13) failed to produce such complexes (Supplementary data).

To verify that full-length endogenous MDM2/MDMX interactions behaved similarly, we did anti-MDM2 immunoprecipitation with MCF-7 cell lysates and compared the MDMX protein levels before and after immunoprecipitation. MDM2 immunoprecipitation resulted in almost complete depletion of MDMX from the cell lysates, suggesting that most, if not all, of MDMX is in a complex with MDM2 (Fig. 1*B*). The existence of an MDM2/MDMX heterocomplex as the predominant form is consistent with the finding that the binding affinity between the hetero-RING domains is stronger than the MDM2 homo-RING complex (19).

The RING domain–mediated complex formation is essential for E3 ligase activity. Having confirmed that MDM2 and MDMX exist predominantly as a heterocomplex, we wanted to know if this interaction affects MDM2's E3 ligase activity. As an initial line of experiments, we created p53 fusion proteins in which the RFD of either MDM2 or MDMX was fused to the COOH terminus of p53 (Fig. 2*A*). The addition of the RFD to p53 did not seem to alter its transcriptional activity or stability as evidenced by comparable expression of both p53-2RFD and p53-XRFD to that of wild-type p53, exhibition of transcriptional activities close to that of wild-type p53, and similar inhibition by MDM2 and MDMX (Fig. 2*B* and *C*). Analogous to wild-type p53, both p53-2RFD and p53-XRFD were found to be refractory to MDMX and susceptible to MDM2-mediated degradation (Fig. 2*D*). Collectively, the data indicate that the p53 fusion proteins behave closely to wild-type p53.

We next asked how the RING-mediated interaction could affect the E3 ligase activity of MDM2 by examining whether the heterocomplex could function similarly to the MDM2 homocomplex in p53 ubiquitylation and if its E3 ligase activity would differ. To ensure that the interaction of MDM2 with the fusion proteins was mediated through the RING domain only, we deleted the p53-binding motif (Δ100MDM2). As expected, the N-terminal deletion mutants alone were unable to induce detectable ubiquitylation of wild-type p53. Coexpression of Δ100MDM2 with p53-2RFD was associated with detectable but relatively weak ubiquitylation of the fusion protein (Fig. 3*A, lane 5*). This poor activity was not due to its low intrinsic E3 ligase activity, as the very same deletion mutant induced marked ubiquitylation of the p53-XRFD fusion protein implicating the hetero-RING complex as a better E3 ligase (Fig. 3*A, lane 8*). Consistently, Δ100MDM2/XRFD induced robust ubiquitylation of p53-2RFD (Fig. 3*A, lane 6*). A requirement of the RING-mediated binding for p53 ubiquitylation was also evidenced in a p53-2RFD (C464A) mutant, which was resistant to both Δ100MDM2 and Δ100MDM2/XRFD (Fig. 3*B*). Together, these results support a requirement for a RING-mediated interaction in eliciting E3 ligase activity. Furthermore, the heterocomplex of MDM2 and MDMX seems to be a better E3 ligase than an MDM2 homocomplex.

Because MDM2-mediated p53 ubiquitylation is associated with nuclear export of p53, we made use of a subcellular distribution assay to examine the importance of the RING-mediated interaction in p53 ubiquitylation. Consistent with published results, p53 was primarily nuclear in the absence of MDM2 and substantially redistributed to the cytoplasm upon MDM2 expression (Fig. 3*C*). Importantly, coexpression of MDM2 with MDMX resulted in a much greater induction of p53 cytoplasmic distribution.

Disruption of the MDM2/MDMX heterocomplex results in marked p53 activation. If MDM2 and MDMX function as a heterocomplex in p53 inactivation, disruption of the complex with MDMXRFD that would competitively bind to MDM2 should result in p53 activation. Using a mammalian two-hybrid assay in which MDMX and MDM2 were fused in frame to either Gal4 DNA binding domain or VP16 transactivation domain, along with the luciferase reporter under the control of Gal4-responsive elements, we show that ectopically expressed MDMXRFD inhibited the binding of MDM2 with MDMX, whereas MDMX(C463A)RFD failed to show such an effect (Fig. 4*A*).

To examine the effect of MDMXRFD expression on endogenous p53, we generated a U2OS cell line, with wild-type p53, that stably

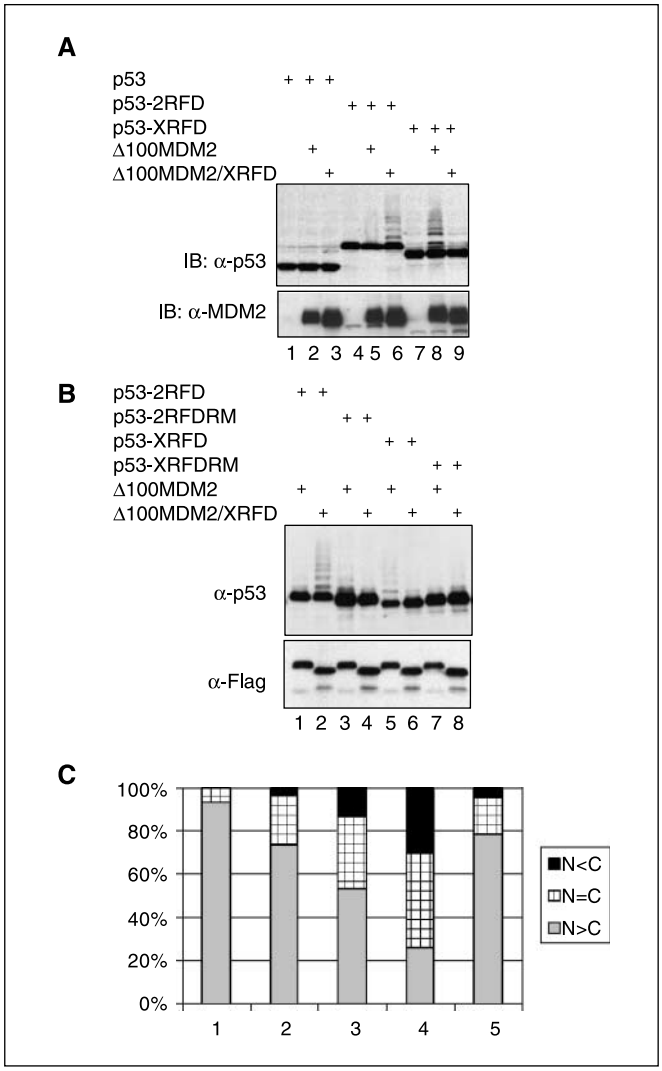

Figure 3. The RING domain–mediated association is essential for the activity of MDM2 E3 ligase. *A,* The N-terminal deletion mutant of MDM2 (Δ100MDM2) or the Δ100MDM2/XRFD chimera was coexpressed with p53 or the p53 fusion proteins in *mdm2*$^{-/-}$/*mdmx*$^{-/-}$/*p53*$^{-/-}$ MEFs. The cells were harvested 24 h posttransfection for Western analysis. *B,* Δ100MDM2 or Δ100MDM2/XRFD chimera was coexpressed with the p53 fusion proteins or the RING domain mutant fusion proteins in *mdm2*$^{-/-}$/*mdmx*$^{-/-}$/*p53*$^{-/-}$ MEFs. The cells were harvested 24 h posttransfection for Western analysis. *C,* EGFP-p53 vector was cotransfected with an empty vector (*column 1*), MDMX (*column 2*), MDM2 (*column 3*), MDMX and MDM2 (*column 4*), or MDMX(C463A) and MDM2 (*column 5*) into *mdm2*$^{-/-}$/*mdmx*$^{-/-}$/*p53*$^{-/-}$ MEFs. MG132 was added to the culture 6 h before fixing. Cellular nuclei were identified by DAPI staining. Direct green fluorescence and DAPI staining were visualized under a fluorescence microscope. Cells were scored as having GFP-tagged proteins distributed in the following manner: strong nuclear (N > C), equally distributed in two compartments (N = C), or strong cytoplasmic (N < C). For each condition, 200 cells from random fields were scored.

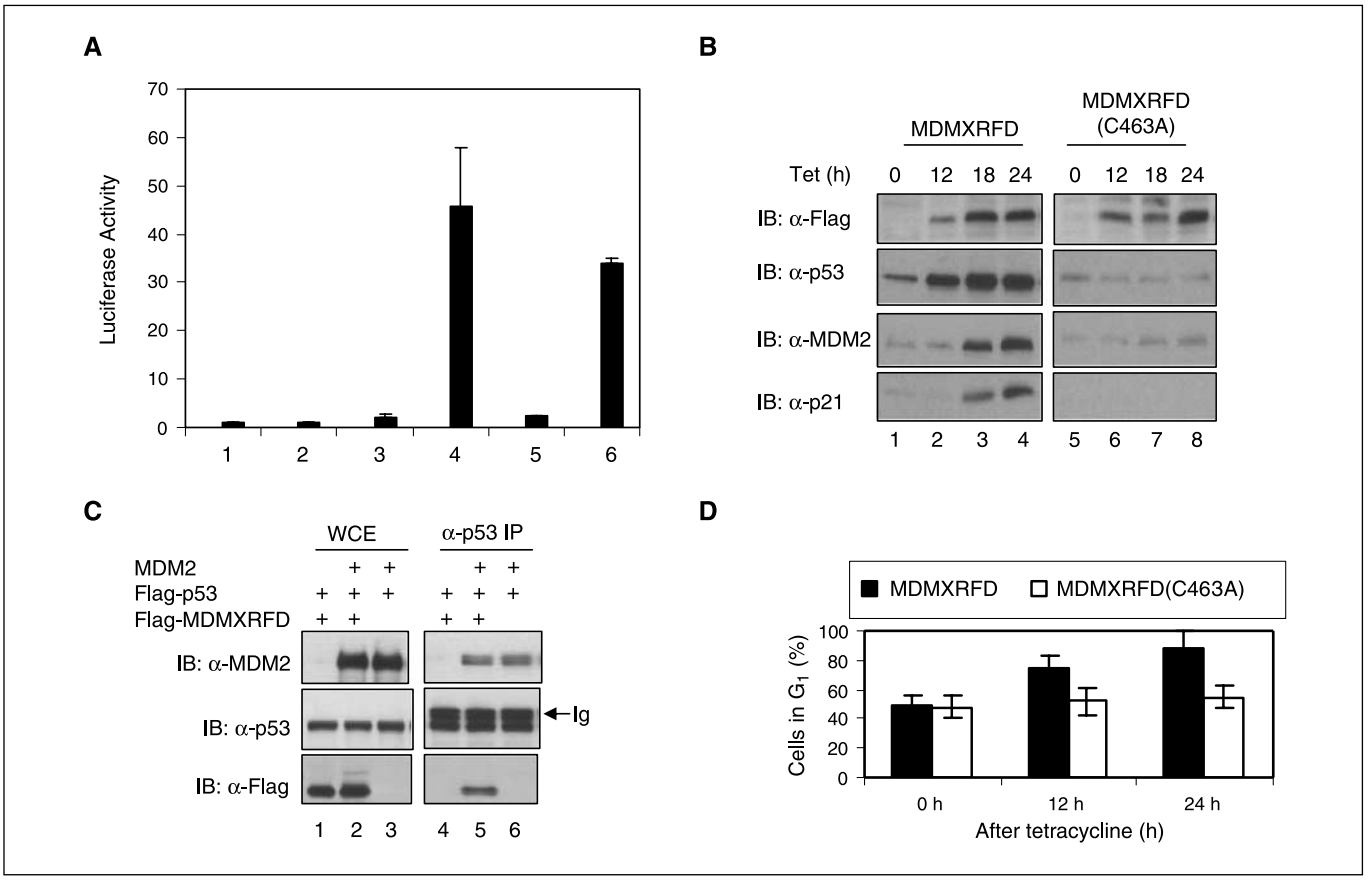

Figure 4. Disruption of the binding of MDMX to MDM2 results in marked p53 activation. *A, mdm2*$^{-/-}$*/p53*$^{-/-}$ MEFs were transfected, along with pGL3-G5SV, with vector (*column 1*), Gal4 fusions of MDMX (*column 2*), VP16 constructs of MDM2 (*column 3*), or with combination of Gal4MDMX and VP16MDM2 (*column 4*) in the presence of Flag-MDMXRFD (*column 5*) or Flag-MDMXRFD(C463A) (*column 6*). *Columns,* values are mean luciferase activity after being adjusted for transfection efficiency of three separate experiments, each done in duplicate; *bars,* SD. *B,* U2OS cells stably expressing MDMXRFD (*lanes 1–4*) or MDMXRFD(C463A) (*lanes 5–8*) under the control of tetracycline were harvested at the indicated time. The cells were studied using Western analysis using the indicated antibodies. *C,* MDMXRFD-expressing cells were induced by tetracycline for 24 h (*lanes 1 and 2*) or control U2OS cells were treated with MG132 for 6 h. The cells were subjected to anti-p53 immunoprecipitation, and the p53 immunocomplexes were analyzed via Western analysis. *D,* U2OS cells stably expressing MDMXRFD or MDMXRFD(C463A) under the control of tetracycline were harvested at 12 h (*lane 2*) or 24 h (*lane 3*) after incubation with tetracycline. The cells were harvested, fixed, and subjected to FACS analysis. Results represent the percentage of G_1-phase cells. *Columns,* mean of two experiments done in triplicate; *bars,* SD.

expressed MDMXRFD under the control of a tetracycline-inducible promoter. MDMXRFD expression was accompanied by marked p53 activation, as evidenced by significant increase of p53 levels and induction of p21 and MDM2 expression. Inability of the MDMX(C463A)RFD to exhibit such activity supports that p53 activation is a result of disruption of the MDM2/MDMX heterocomplex (Fig. 4*B*). Immunoblot analysis of anti-p53 immunocomplexes revealed no significant difference in MDM2 binding in the presence or absence of MDMXRFD expression, ruling out that its expression was interfering with MDM2's ability to directly bind with p53 (Fig. 4*C, lane 5 versus lane 6*).

The elevated p21 levels in the MDMXRFD-expressing cells would predict a delay in cell cycle progression. Fluorescence-activated cell sorting (FACS) analysis of cellular DNA content proved that this was indeed the case (Fig. 4*D*). The increase in G_1 population highly correlated with the induced levels of p21 in response to MDMXRFD expression.

Discussion

Genetic studies have firmly established both MDM2 and MDMX as essential negative regulators of p53 (9). By demonstrating that MDM2 and MDMX exist predominantly in cells as an MDM2/MDMX heterocomplex, it is hard to deny their mutual dependence on each other. Endogenous MDMX was virtually depleted from cell lysates by anti-MDM2 immunoprecipitation. Such a strong physical interaction between the two proteins is reflected at the level of their functional regulation of p53, as highlighted by the finding that disruption of the MDM2/MDMX heterocomplex results in marked p53 activation.

Hetero-RING domain-mediated interaction has been shown to be essential for the activity of a number of RING-containing E3 ligases. Our p53 fusion proteins allowed us to focus and obtain clear evidence in their requirement for the RING-mediated interaction in p53 ubiquitylation. They enabled us to compare the homo-RING with hetero-RING complexes and to show that the MDM2/MDMX heterocomplex is a much better E3 ligase than the MDM2 homocomplex. Whereas it has been reported that *in vitro* MDMX stimulates the E3 ligase activity of MDM2 and that the extreme COOH terminus of MDMX restores E3 ligase activity when replacing the corresponding region of MDM2 (13, 14, 20), this is the first study to specifically compare the MDM2 homo-RING complex with the MDM2/MDMX hetero-RING complex in its ability to ubiquitylate p53.

Given our observation that the MDM2/MDMX heterocomplex is the predominant form present in cells, the higher E3 ligase activity of the hetero-RING complex is functionally significant. Disruption of MDM2/MDMX resulted in a robust increase of p53 abundance, leaving us with the intriguing notion that this complex may be a novel target for therapeutics in cancers carrying wild-type p53.

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