Hdmx Protein Stability Is Regulated by the Ubiquitin Ligase Activity of Mdm2

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Petrade Graaf, Natalie A. Little, Yolande F. M. Ramos, Erik Meulmeester, Stef J. F. Letteboer‡, and Aart G. Jochemsen§

From the Leiden University Medical Center, Department of Molecular and Cell Biology and Center for Biomedical Genetics, P. O. Box 9503, 2300 RA Leiden, The Netherlands

The stability of the p53 tumor suppressor protein is critically regulated by the Hdm2 and Hdmx proteins. Hdm2 protein levels are auto-regulated by the self-ubiquitination activity of Hdm2 and on the transcriptional level by p53-activated transcription of the hdm2 gene. Little is known about the regulation of Hdmx expression levels, apart from the observation that the Mdmx protein can be cleaved by caspase-3 in a p53-inducible manner. In the functional analysis of two mutant Hdmx proteins, products of two alternatively spliced mRNAs, it was found that Hdmx proteins are targets for ubiquitination by Mdm2. The stability of the Hdmx protein is partly dependent on the presence of its internal acidic domain. Mdm2 appears only to require an intact RING domain to be able to ubiquitinate Hdmx and target it for proteasomal degradation. These findings highlight the intricate functional relationships between p53, Mdm2, and Hdmx.

The p53 tumor suppressor protein plays a major role in maintaining the integrity of the genome. In response to various forms of cellular stress p53 is stabilized and activated, which leads to induction of cell cycle arrest, DNA repair, or apoptosis, dependent on the type of cells and stress (1, 2). With such a pivotal role in numerous cellular processes, the frequency of functional p53 inactivation in human cancer is expected to be higher than estimated from the p53 mutation alone (for review see Ref. 2). The major regulator of p53 activity is the Mdm2 protein (reviewed in Refs. 3 and 4). Mdm2 inhibits p53 activity in two ways: by blocking the transcription regulation by p53, and also through targeting p53 for proteasomal degradation by acting as a RING-type E3 (5–8).

The Mdm2-related protein Mdmx binds p53 with similar requirements as Mdm2 and can inhibit p53-induced transcription (9, 10). However, transfection studies indicated that Mdmx could not ubiquitinate or degrade, but instead stabilized p53 and Mdm2 (11–14). This activity requires an interaction between the RING finger domains of Mdmx and Mdm2 (15). Although these studies suggest that Mdmx and Mdm2 have opposite effects on p53 stability, it is very possible that under certain conditions Mdm2 and Mdm2 cooperate in the inactivation of p53 (16). The critical importance of both Mdmx and Mdm2 in the regulation of p53 is best illustrated by the observations that both mdm2-null and mdmx-null mice are embryonic lethal. In both cases, lethality can be rescued in a p53-null background (17–20).

It has been generally accepted that the mdm2 gene functions as an oncogene, as shown by transformation experiments in cell culture and by analysis of human tumors (reviewed in Ref. 21). More recent data suggest that Hdmx might also perform a similar function. It was found that the hdmx gene is amplified in a subset of human gliomas without mutations in p53 or amplification of hdm2 (22, 23). Moreover, we found the levels of Hdmx protein to be increased in more than 30% of human tumor cell lines tested (24). Aberrant expression of Hdmx in these tumor cells appeared to correlate with the presence of wild type p53. However, whether Hdmx actually contributes to transformation and tumor formation remains to be determined.

Because both Hdmx and Mdm2 are important in regulation of p53 activity, Hdmx expression levels, like Mdm2 expression, might be strictly regulated. However, not much is known about the ways that Hdmx/Mdmx expression levels or activities are controlled. We found that after certain types of DNA damage the mdmx mRNA level was not increased (9). Another report (25) suggests that Mdmx levels are constant during cell growth, differentiation, and after DNA damage. On the other hand, it has been shown that Mdmx protein can be cleaved by caspase-3 and that this cleavage can be stimulated by activation of wild type p53 (26). A completely different level of Hdmx regulation is the control of its subcellular localization. It has been shown that localization of Hdmx proteins is distinct between different cell lines, with most cell lines showing a predominantly cytoplasmic localization (16, 27). Upon co-expression of Mdm2 or p53, the majority of Hdmx protein is translocated into the nucleus (27). In addition, following DNA damage, a p53- and Mdm2-independent nuclear translocation has been described (28).

We report here the identification of two new splicing variants of Hdmx, which we have called Hdmx-A and Hdmx-G. Hdmx-A lacks exon 9 sequences encoding the acidic domain, whereas Hdmx-G lacks the p53-binding domain. These proteins were characterized for their ability to associate with and subsequently inhibit p53-induced transcription activation and for their ability to stabilize Mdm2. Surprisingly, the Hdmx-A pro-
tein could not significantly stabilize Mdm2 and was particularly susceptible to Mdm2-triggered degradation. Importantly, both full-length Hdmx, Hdmx-A, and Hdmx-G were found to be targets for Mdm2-induced ubiquitination and degradation. These data provide new insights into the delicate regulation of the balance between the Hdmx, Mdm2, and p53 proteins, which might have implications for the control of Hdmx protein level upon stress-induced activation of p53 and subsequent increase in Mdm2 levels.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**Anti-Mdm2 antibody was mouse monoclonal antibody 4B2 (29). Anti-Hdmx antibody was the rabbit polyclonal serum p55 (24). The p53 monoclonal antibody DO-1, the DO-1 coupled to horseradish peroxidase (HRP), the anti-p53 rabbit polyclonal FL393, and the 9E10 anti-Myc monoclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-HA antibody H11 was obtained from Babco, and the mouse monoclonal anti-LacZ antibody D19–2F2-2 was obtained from Roche Applied Science. Secondary antibodies goat anti-mouse-HRP, goat anti-rabbit-HRP, goat anti-mouse-Rhodamine, and goat anti-rabbit fluorescein isothiocyanate were obtained from Jackson ImmunoResearch.

**Cell Lines and Transfections—**H1299 cells were grown in RPMI supplemented with 10% FBS and antibiotics. p53−/−, p53−/−/mdm2−/−, p53−/−/mdm2−/−/hdmx−/−, U2OS, and C33A cells were maintained in DMEM supplemented with 10% FBS and antibiotics. Cells were seeded 6–16 h before transfection in DMEM and transfected by the calcium phosphate method as described (30) or with FuGENE 6 transfection reagent as described by the manufacturer (Roche Applied Science).

**Western Blot Analysis—**Cell lysates were made in IPB 0.7 (20 m M triethanolamine, pH 7.4, 0.7 % NaCl, 0.5 % Nonidet P-40, 0.2 % sodium deoxycholic acid supplemented with protease inhibitors) and analyzed by Western blot as described previously (13). Protein bands were detected by enhanced chemiluminescence and visualized by autoradiography or with the use of a Lumi-Image (Roche Applied Science).

**RNA Isolation and RT-PCR—**Total cytoplasmic RNA was isolated from C33A cells with the Nonidet P-40 method (31) and treated with DNase I (Promega). Single-stranded cDNA was made with reverse transcriptase from C33A cells with the Nonidet P-40 method (31) and treated with DNase I (Promega). Single-stranded cDNA was made with reverse transcriptase (Invitrogen) from 3 μg of RNA using oligo(dT) as primer. Subsequently, PCR was carried out to detect Hdmx cDNAs. Primers at the 5′-end contained sequences from exon 2 (5′-CGCGTCGAGGCCGGGATCCACATCATTTTCCACCTGCTC-3′), and two different primers located in exon 11 (A, up to nucleotide 1172 within the coding sequence 5′-CCATCTCTTCAGAGGCC-3′, or B, up to nucleotide 1894 within the 3′-end untranslated region 5′-GGTGCACCTAATCTGTA-3′). The exon numbers are based upon the published genomic organization of the mdm2 gene (32). PCR products were carried out for 35 cycles with an annealing temperature of 55 °C. PCR products were analyzed by electrophoresis on agarose gels. PCR products were cloned into pCR2.1-TOPO (Invitrogen) and analyzed by DNA sequencing.

**Plasmid Constructs—**Full-length hdmx and the alternative splicing variants were cloned into the XhoI and BamHI sites of pCDNA3.1Myc-His (Invitrogen). Sequences encoding the hemagglutinin epitope (HA) tag were cloned at the 5′-end of hdmx and mdm2-G by PCR (5′ primer, 5′-GATCAGGTGTCGAGGCCCGACATCATTTTCCAAGTGTCCTC-3′; 3′ primer, 5′-ACAGTATTCCCCCTGCCTTC-3′) and XhoI and BamHI sites. The expression vectors for Hdm2, Hdm2ΔRING, Hdm2 S395A, and Hdm2 S395D were kindly gifted from M. Oren. pcMV Hdm2 was constructed by B. Vogelstein and described with Hdm2 S395A and Hdm2 S395D (39). Hdm2ΔRING is generated from the pcMV Hdm2 vector by deletion of a HindIII-HincII fragment followed by religation, removing amino acids 442–491. pcDNA3.1 Mdm2αacidic domain was generated by removing sequences encoding amino acids 202–303 using the Quick-Change method (Strategene) and using pcDNA3.1 1 Mdm2 as a template. pcDNA3.1 1 Mdm2+Myc-Mdm2-RING, expressing the C-terminal 94 amino acids of Mdm2, was made by PCR amplification on wild type mouse mdm2 cDNA with the use of 5′ primers containing a BamHI endonuclease recognition sequence, a translation initiation site, and the 9E10 Myc epitope coding sequence and with 3′ primers containing a stop codon and an EcoRI endonuclease recognition sequence. PCR fragments were cloned in pcDNA3.1 (Invitrogen). Expression vectors for Mv-Mdm2 and Mv-Mdm2Ap53 (lacking the N-terminal 111 amino acids of Mdm2) were the gift of J. C. Marine and were made by PCR amplification on wild type mouse mdm2 cDNA with the use of 5′ primers containing an EcoRI endonuclease recognition sequence, a translation initiation site, and the 9E10 Myc epitope coding sequence and with 3′ primers containing a stop codon and an EcoRI endonuclease recognition sequence. PCR fragments were cloned in pcDNA3.1 (Invitrogen). To generate the mouse mdmx/mdm2 chimeric constructs pcDNA3.1 2XZ and pcDNA3.1 2X expressed the 5′-untranslated region of mouse mdmx (Strategene): Sgpl at position 106 (Mdm2) or 105 (Mdm2) and BorGI at position 303 (Mdm2) or 304 (Mdmx). pcDNA3.1 2XZ consists of Mdm2 (aa 1–106)–Mdmx (aa 107–304)–Mdm2 (aa 304–489), pcDNA3.1 2XZ consists of Mdm2 (aa 1–303)–Mdmx (aa 305–489). The mdmx/mdm2 chimeras are described in more detail in Meulmeester et al. (40). All plasmids produced by PCR were checked with sequencing and restriction fragment analysis.

**Luciferase Assays—**Transfections were performed in triplicate into H1299 cells grown on 6-well plates and transfected with 1.5 μg of luciferase reporter construct, 60 ng of p53, 1.2 μg of effector plasmid (Mdm2 and Hdmx plasmids) and 1 μg of CMV-LacZ in 300 μl of calcium phosphate precipitate. Luciferase assay was performed with a commercial kit (Promega), and luciferase activity was measured in a luminometer (Berthold, Germany). Luciferase values were corrected for β-galactosidase activity.

**Immunoprecipitations—**Cells were washed with PBS and subsequently lysed in Giordano buffer (250 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100) with 10% glycerol, supplemented with protease inhibitors. Protein concentrations of the cell lysates were determined with Bradford Reagent (Bio-Rad). For direct Western blot analysis samples of 25 μg of total lysate were separated by SDS-PAGE. Anti-Hdmx immunoprecipitations were performed on 200 μg of total protein from H1299 cells and full-length antibodies (Babco). Precipitated complexes were analyzed by Western blot.

**Immunofluorescence—**H1299 and U2OS cells were seeded on coverslips and transiently transfected as described in luciferase assays (H1299 cells) or with HA-Hdmx, HA-Hdmx-A, or HA-Hdmx-G expression vectors, either alone or in combination with plasmid encoding Hdm2 or the Hdm2ΔRING mutant (U2OS cells). 24 h after transfection, cells were washed twice in PBS, fixed for 20 min in 4% formaldehyde, washed twice with PBS, and permeabilized by a 10-min incubation with 0.2% Triton X-100 in PBS. Detection of expressed proteins was performed as described (13). Mdm2 was detected with mouse monoclonal 4B2, the HA-tagged Hdmx, and mutant Hdmx proteins with p55 or with monoclonal H11 (anti-HA). P53 was detected by rabbit polyclonal FL393. Nuclei were visualized by DAPI staining.

**Cycloheximide Chase—**H1299 cells were transiently transfected using the calcium phosphate method. 24 h after transfection, culture medium was replaced by RPMI supplemented with 10% FBS, antibiotics, and cycloheximide (50 μg/ml). Cells were lysed in IPB 0.7 at 0, 1, 2, 4, 6, or 8 h after addition of cycloheximide. Lysates were analyzed by Western blotting.

**In Vivo Ubiquitination Assay—**Purification of His-tagged ubiquiti- nated conjugates was performed essentially as described previously (14). 40 h after transfection, cells were either mock-treated or treated with 20 μg MG132 for 4 h. Cells were then washed twice with PBS and scraped in PBS. 20% of the cell suspension was lysed in IPB 0.7 and analyzed by Western blot. Lysis of the remaining 80% of the cells and the subsequent isolation of Htagged (ubiquitinated) proteins was performed exactly as described previously (14). Eluates were analyzed by Western blotting.
RESULTS
Identification of Alternatively Spliced Hdmx mRNAs—In order to isolate alternative splicing variants of the hdmx gene in C33A cells, we performed RT-PCR. In this study, we characterized two of the splicing variants that still contained the RING, and which we called Hdmx-A and Hdmx-G. The hdmx-A mRNA lacks exon 9 encoded sequences, resulting in the in-frame deletion of 50 amino acids (225–274) in the predicted Hdmx-A protein (Fig. 1). These amino acids fall within the “acidic region” of Hdmx. The hdmx-G mRNA lacks sequences from exons 3–5 and part of exon 6, resulting in an in-frame deletion of amino acids 27–124, encompassing the p53-binding domain (Fig. 1). Within exon 6 a cryptic splice acceptor site is present. As an indication of the accuracy of our method, we also isolated an hdmx mRNA lacking exon 6 sequences. This splicing variant is the human homologue of the mouse mdmx cDNA missing a stretch of 68 nucleotides described by Rallapalli et al. (41). Like in mdmx, the hdmx exon 6 contains 68 nucleotides, which means that skipping exon 6 results in a frameshift leading to translation termination after incorporation of 26 alternative amino acids. Confirming the results from Rallapalli et al. (41), we found that the exon 6 splicing variant very efficiently inhibited p53-induced transcription activation.

To study the functions of the alternative proteins Hdmx-A and Hdmx-G, the parts of the cDNAs containing the alternative splicing were recloned into pcDNA3.1 expression vectors into a full hdmx background. To detect the different proteins with a similar affinity, the cDNAs were provided with sequences encoding the hemagglutinin (HA) tag at their 5′-end.

Hdmx-A, but Not Hdmx-G, Can Bind p53 and Inhibit p53-mediated Transcription (in a Promoter-dependent Fashion)—To investigate the ability of Hdmx-A and Hdmx-G to associate with p53, we transfected C33A cells, containing high levels of endogenous p53, with the different HA-tagged Hdmx constructs. Expression levels of the Hdmx proteins and p53 in the lysates are shown in the left panels of Fig. 2A. Immunoprecipitations were performed using anti-HA antibodies, and the ability to co-precipitate p53 was tested by Western blotting. As expected, p53 co-immunoprecipitated with Hdmx and with Hdmx-A, but not with Hdmx-G, which lacks the p53-binding domain (Fig. 2A, right bottom panel). Reprobing the same blot after stripping with anti-HA antibody showed a similar efficiency of immunoprecipitation of the HA-tagged Hdmx proteins (Fig. 2A, right upper panel).

The Mdmx/Hdmx protein has been reported to inhibit p53-mediated transcription activation (9, 11, 13, 42). We determined whether alternative splicing of Hdmx would affect this function. To address this question, H1299 cells were co-transfected with Mdm2, the different Hdmx constructs, wild type p53 expression vector, and the bax-luciferase reporter. Luciferase activity was determined, and values were corrected for transfection efficiency by measuring β-galactosidase activity. All luciferase transfections were performed at least twice in triplicate. Mdm2 co-expression reduces p53-induced transcription back to basal levels. Hdmx and Hdmx-A also significantly inhibit p53-induced transcription activation (Fig. 2B), whereas Hdmx-G partly inhibits p53-mediated transcription activation. Although possibly unexpected because the Hdmx-G protein lacks the N-terminal p53-binding domain, it fits with our earlier report (13) that the RING of Hdmx alone can partly inhibit the transcription activation by p53. This effect might be mediated through stabilization of endogenous Hdm2. Co-transfection of Hdmx, Hdmx-A, or Hdmx-G with Mdm2, p53, and bax-luciferase did not affect the Mdm2-mediated inhibition of transcription activation by p53, as reported before (13). It is important to note here that exogenous expressed Hdmx, Hdmx-A, and Hdmx-G proteins are mainly located in the nucleus of H1299 cells, both when expressed alone or together with p53 (Fig. 2C), in contrast to what is found in other cells, e.g. U2OS (16, 27). Also, the observation that Hdmx can inhibit p53-activated transcription in H1299 cells fits with earlier reports (11, 43).

In addition to the effect on the bax promoter, we also studied the effect of the Hdmx proteins on p53-dependent transcription of the p21 promoter. Again, Mdm2 inhibited p53-mediated transcription activation back to basal levels (Fig. 2D). Co-expression of p53 with the Hdmx or Hdmx-A proteins, which significantly inhibited p53-mediated transcription activation of the bax-luc reporter, only partly inhibited the transcription activation of the p21-luc reporter (Fig. 2D). Similar experiments with the use of the mdm2-luc reporter showed results comparable with those obtained with the bax-luc reporter (results not shown). The observed promoter dependence is in agreement with our results (42) obtained in Saos-2 cells. Because the Hdmx proteins hardly affected the activation of the p21-luc reporter by p53, we investigated whether in this case co-transfection of Hdmx proteins would affect the Mdm2-mediated inhibition of transcription from the p21 promoter. It was found that, as with the bax promoter, the Hdmx proteins could not prevent the strong inhibition of p53-mediated transcription by Mdm2 (Fig. 2D). Expression levels of transfected constructs were checked by Western blot analysis of the luciferase lysates. A representative example of p53, Hdmx, Mdm2/Hdm2, and LacZ expression is shown in Fig. 2E. The lysates analyzed were pooled triplicates of the bax-luc assay shown in Fig. 2B. All transfected plasmids are clearly expressed, and it can be seen that Hdmx and Hdmx-G clearly stabilize Mdm2, whereas Hdmx-A had not much of an effect. Although the importance was not realized fully at that time, the protein levels of the Hdmx proteins are clearly reduced when co-expressed with Mdm2, an observation that is explained below.

Association of the Alternative Hdmx Proteins with Hdm2/Mdm2—It has been shown previously (16, 27) that in U2OS cells ectopically expressed Hdmx is almost exclusively located in the cytoplasm but that co-expression of Hdm2 leads to a translocation into the nucleus. This effect of Hdm2 was shown to be completely dependent on the interaction between the RING domains of Hdm2 and Hdmx. Therefore, to study whether the Hdmx-A and Hdmx-G proteins could associate with Hdm2 via the RING, we expressed the different HA-Hdmx proteins in U2OS cells, either alone or together with Hdm2. As a control, HA-Hdmx was also co-expressed with Hdm2/RING. Cells were fixed 24 h after transfection and stained for Hdmx and Hdm2 expression. The subcellular localization was investigated by immunofluorescence. As reported previously (16, 27), Hdmx is a cytoplasmic protein in U2OS cells (Fig. 3A, a), but is translocated into the nucleus when co-expressed with Hdm2 (Fig. 3A, c–d). As expected, the Hdm2/RING mutant is incapable of translocating Hdmx into the nucleus (Fig. 3A, f and g). Like Hdmx, both Hdmx-A and Hdmx-G are also cytoplasmic proteins (Fig. 3A, i and n) and are translocated into the nucleus when co-expressed with Hdm2 (compare Fig. 3A, f and l, p and q), indicating binding of Hdmx-A and Hdmx-G to the RING of Hdm2. However, it was observed that expression of Hdm2 leads to a reduction in the number of cells detectably expressing Hdmx-A. This raised the question whether Hdm2 could somehow reduce the expression levels of Hdmx-A. Analysis of lysates made from U2OS cells transfected with the same precipitate on duplicate dishes supported this possibility (Fig. 3B). The original goal of this experiment was to test the effect of the different Hdmx proteins on the stability of Hdm2, because it has been reported by us and others (11–14, 16) that
Hdmx stabilizes Hdm2 in co-transfection experiments. Cell extracts were made 40 h after transfection and analyzed by Western blot for Hdmx and Hdm2 expression. In the absence of Hdm2 co-transfection, Hdmx and Hdmx-A were expressed to comparable levels (Fig. 3B), but Hdmx-G expression was somewhat lower in this particular experiment. Even so, co-expression of both Hdmx-G and full-length Hdmx strongly increased the levels of transfected Hdm2 (Fig. 3B, compare the lane containing Hdm2 only with the lanes Hdmx and Hdmx-G in panel Hdm2). This result was as expected, because Hdmx-G contains the Hdmx RING finger structure, which is sufficient for binding to the RING finger of Mdm2 (12, 15). Unexpectedly, Hdmx-A, although also containing the Hdmx RING finger, affected the level of Hdm2 protein much less. The explanation for this surprising result might be the different expression levels of the Hdmx proteins when co-expressed with Hdm2. The levels of Hdmx-A are strongly reduced, whereas Hdmx and Hdmx-G expression levels are not much affected upon co-expression of Hdm2. These results reflect our observation in the immunofluorescence study mentioned above and the results of the Western analysis of luciferase lysates presented in Fig. 2E, and again suggest that Hdm2 can strongly reduce expression levels of Hdmx-A.

The hypothesis that co-expression of Hdm2 with Hdmx-A strongly decreases Hdmx-A levels was strengthened by the results of the co-immunoprecipitation experiment aimed to investigate the association of the Hdmx proteins with Mdm2 in a more direct manner. We expressed HA-Hdmx, HA-Hdmx-A, or HA-Hdmx-G together with Mdm2 in H1299 cells and as control HA-Hdmx alone. Cell extracts were made 40 h after transfection, and immunoprecipitation was performed with an anti-HA antibody. Subsequently, 80% of the immunoprecipitate was separated on a gel to detect co-immunoprecipitated Mdm2, whereas 20% was used to analyze the immunoprecipitated Hdmx proteins. Immunoblotting analysis showed that Hdmx and Hdmx-G are well expressed and immunoprecipitated and that both proteins associate with Mdm2 (Fig. 3C). However, only a low amount of Hdmx-A is expressed/immunoprecipitated, and a small quantity of co-precipitated Mdm2 is detected (Fig. 3C). Taken together, the results of the experiments presented in Fig. 3 strongly indicate that Hdmx-A and Hdm2/Mdm2 can associate but also that Mdm2 strongly reduces the expression level of Hdmx-A.

Hdmx and Mdm2 Have Opposite Effects on Each Other’s Stability—To study the effect of Mdm2 on the expression levels of Hdmx, Hdmx-A, and Hdmx-G in more detail, we transiently transfected a constant amount of HA-Hdmx, HA-Hdmx-A, and HA-Hdmx-G expression vectors into H1299 cells, with increasing amounts of Mdm2. As shown in Fig. 4A, Hdmx and Hdmx-G are reduced upon Mdm2 expression at relatively high ratios Mdm2:Hdmx(-G) (1:1 or higher). This explains why we had not noted this effect before, because in most previous transfections an excess of Hdmx/Mdmx relative to Mdm2 had been used (13, 14). Strikingly, a significant decrease of Hdmx-A level is already observed at a ratio of 1:4 (Mdm2:Hdmx-A), and Hdmx-A is almost absent at higher ratios, confirming our earlier obser-
To exclude a possible interfering effect of endogenously expressed Mdm2 and Mdmx proteins, similar experiments were performed in cells lacking both Mdm2/p53 or Mdmx/p53, or only lacking p53, established from mouse embryo fibroblast cultures of the respective knock-out mice (18, 19, 44). Fig. 4B shows the result of such a transfection into the p53-/- /mdm2-/- cells. Like in H1299 cells, the expression levels of Hdmx, Hdmx-A, and Hdmx-G proteins are declining upon co-expression of Mdm2, with again a particular susceptibility of the Hdmx-A protein. Similar results were obtained after transfection into p53-/- and p53-/- /mdmx-/- cells (results not shown).

To find out whether the levels of Hdmx and Hdmx-A are decreased upon co-expression of Mdm2 as a result of a shorter half-life, a cycloheximide chase experiment was performed. HA-Hdmx and HA-Hdmx-A were expressed in H1299 cells either alone or together with Mdm2. A ratio of Hdmx:Mdm2 of 2:1 was chosen, because otherwise no Hdmx-A could be detected even at the zero time point. Mdm2 was also expressed alone, to be able to investigate again the effect of Hdmx and Hdmx-A on the stability of Mdm2. Cycloheximide was added 24 h after transfection, and cells were harvested at the indicated time points. Extracts were analyzed by Western blotting. Although at a ratio of Hdmx:Mdm2 of 2:1 the levels of Hdmx are not very strongly decreased in a steady state situation (see Fig. 4A), a clear effect on the stability of Hdmx by Mdm2 can be observed.
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Fig. 3. Hdmx-A and Hdmx-G both associate with Hdm2/Mdm2, and Hdmx-A expression is dramatically reduced by Hdm2/Mdm2. A, U2OS cells were transfected with 4 μg of HA-Hdmx constructs and 2 μg of Hdm2 constructs and analyzed by immunofluorescence 24 h after transfection. Hdm2 and Hdm2ARING were detected with 4B2 monoclonal anti-Mdm2 antibody (a, c, f, i, k, n, and p) and Hdmx, Hdmx-A, and Hdmx-G with the p55 polyclonal anti-Hdmx antibody (d, g, l, and q). Nuclei were stained with DAPI (b, e, h, j, m, o, and r). B, U2OS cells were transiently transfected with 4 μg of HA-Hdmx, HA-Hdmx-A, or HA-Hdmx-G expression vector in combination with empty vector or 2 μg of Hdm2 expression vector, all in the presence of 1 μg of CMV-LacZ. Cell lysates were prepared 40 h after transfection, and equal protein amounts were analyzed by Western blotting. The blot was incubated with anti-LacZ antibody as a transfection control (IP: Hdmx; WB: Hdmx). C, H1299 cells were transiently transfected with 4 μg of HA-Hdmx, HA-Hdmx-A, or HA-Hdmx-G together with empty vector or 2 μg of Mdm2 as indicated. Cell lysates were prepared 40 h after transfection. Immunoprecipitations (IP) were performed with anti-HA antibody, and precipitated proteins were analyzed by Western blotting (WB). Precipitated Hdmx, Hdmx-A, or Hdmx-G were detected with anti-HA antibodies; co-precipitated Mdm2 was detected with 4B2 anti-Mdm2 antibody.

The results of the cycloheximide chase with Hdmx-A protein are somewhat complicated. The Hdmx-A protein almost always resolves into two distinct bands, with the lower band more intense. However, during the cycloheximide chase experiment a shift in abundance is observed, i.e., the upper band actually increases in intensity relative to the lower band, starting at 4 h after addition of cycloheximide, whereas the lower band decreases. This effect is independent of the co-expression of Mdm2. Even so, in the presence of Mdm2 the level of Hdmx-A proteins (mainly the lower band) strongly decreases in the first 2 h of cycloheximide chase (Fig. 4C), whereas this effect is not seen in the absence of Mdm2 and is also not observed in the cycloheximide chase of Hdmx with Mdm2. At later time points the upper Hdmx-A band appears and is not decreasing significantly anymore. These results indicate that indeed the half-life of Hdmx-A protein is decreased very strongly by Mdm2 co-expression, more so than the decrease of Hdmx stability by Mdm2. An additional effect of the cycloheximide chase is the accumulation of the upper Hdmx-A protein, which appears to be very stable. The nature of the different protein forms is unknown at present, but most likely reflects a post-translational modification. Investigating the Mdm2 levels from the same transfection clearly showed that Hdmx strongly increases the half-life of Mdm2, whereas Hdmx-A has a minor effect, as was suggested already from the results presented in Fig. 3B.

By having shown that the stability of human Hdmx proteins are decreased when co-expressed with Mdm2, we were interested in the pathway leading to the destabilization. It had been shown that Mdmx proteins can be cleaved by caspase-3 in a p53-inducible manner (26). Because the experiments shown in Fig. 4, A–C, were performed in H1299 cells, lacking p53, it seemed unlikely that this pathway would play a role. However, to exclude this possibility, two types of Hdmx and Hdmx-A mutants were generated. We either replaced the second aspartic acid in the caspase-3 cleavage recognition site (DVPD) by an alanine (D361A) or mutated the cysteine following the caspase-3 site into a stop codon (C362Stop). The D361A mutants can no longer be cleaved by caspase-3 as has been shown for Mdmx (26), whereas the C362Stop mutant mimics the N-terminal caspase-cleaved product. In a cycloheximide chase assay as described above, we tested the effect of the D361A and the C362Stop mutation in Hdmx and Hdmx-A on the Mdm2-induced degradation. As shown in Fig. 4D, HA-HdmxD361A behaves comparably with wild type HA-Hdmx, indicating that caspase-3 cleavage is not necessary for the degradation of Hdmx by Mdm2. The stability of HA-HdmxC362Stop was unaffected by co-expression with Mdm2, most likely because this mutant protein lacks the RING finger and can no longer interact with Mdm2. The same effect was observed on the mutants of Hdmx-A. Hdmx-A C362Stop protein levels are decreased in time when co-transfected with Mdm2, whereas Hdmx-A C362Stop protein levels are unaffected by co-transfection with
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**Hdmx Proteins Are Ubiquitinated When Co-expressed with Mdm2**—We have shown above that Hdmx-A and Hdmx can interact with Mdm2 and are degraded when co-expressed with Mdm2. Because Mdm2 is a member of the RING family of ubiquitin E3 ligases, we investigated whether Mdm2 can ubiquitinate Hdmx and Hdmx-A. Therefore, we performed an *in vivo* ubiquitination experiment as described previously (14, 34). H1299 cells were co-transfected with the indicated expression constructs (Fig. 5), together with CMV-LacZ and the His6-tagged ubiquitin expression vector. Transfected cells were mock-treated or treated with the proteasome inhibitor MG132 for 4 h prior to harvesting. Ubiquitinated proteins were isolated using nickel beads, separated by SDS-PAGE, and subsequently analyzed by Western blotting using an anti-HA antibody to detect specifically ubiquitinated Hdmx proteins. The results clearly indicated that both Hdmx and Hdmx-A are ubiquitinated upon co-expression of Mdm2 (Fig. 5, *upper panels*). The amount of ubiquitinated proteins is not detectably increased upon treatment with MG132. In addition, only a slight increase in total levels of both Hdmx and Hdmx-A is observed after treatment with MG132. This suggests that a 4-h incubation with MG132 is too short to observe a significant rescue of the degradation of Hdmx/Hdmx-A. We do observe an increase in Mdm2 levels in the total lysate blots, indicating the proteasome inhibitor worked. These results indicate that both Hdmx and Hdmx-A are substrates for the ubiquitin E3 ligase activity of Mdm2.

**The RING Domain of Mdm2 Is Sufficient for Degradation of Hdmx**—In order to investigate in more detail whether indeed the ubiquitin ligase activity of Mdm2 is required and sufficient for the degradation of Hdmx, we tested a series of Mdm2 mutants for their ability to degrade Hdmx-A. Simultaneously, these mutants were also tested for their ability to degrade p53.

H1299 cells were transiently transfected with Hdmx-A or p53, either alone or in combination with Mdm2 and Mdm2 mutant constructs. Both Hdmx-A and p53 are degraded by Mdm2, with or without N-terminal Myc tag (Fig. 6A, *lanes 2 and 3*). Degradation of Hdmx-A and p53 requires an intact Mdm2 RING domain, because both Mdm2-(C464A) containing the Zinc/RING domain of Mdmx (22X) were unable to degrade Hdmx-A or p53 (Fig. 6A, *lanes 3 and 5*). The p53-binding domain is required for p53 degradation but not for Hdmx-A degradation (Fig. 6A, *lane 7*). This is as expected, because Hdmx and Mdm2 interact via their RING domains. Both the Mdm2/Mdmx chimera 2X2 and the deletion mutant Mdm2AD (lacking amino acids 202–303) efficiently degrade Hdmx-A, whereas the levels of p53 are not affected. The 2X2 protein is difficult to detect because it has a very short half-life (40) and is only seen after a long exposure of the blot (Fig. 6A, *Mdm2 (longer exposure)*). The critical role for the central domain of Mdm2 in the degradation of p53 is in agreement with previous publications (40, 45–47). In addition to the Mdm2 mutants shown here, we tested Mdm2 NLS-mutant, Mdm2 NES-mutant, Hdm2, Hdm2ARING, Hdm2-S395D, and Hdm2-S395A mutants. With the exception of Hdm2ARING, all these proteins were able to degrade Hdmx-A (data not shown). These analysis of the lysates as in C. E. H1299 cells were transiently transfected with 5 μg of HA-Hdmx-A D361A and HA-Hdmx-A C362Stop vectors alone and in combination with 2.5 μg of Mdm2 expression vector, in the presence of CMV-LacZ. Treatment with cycloheximide and Western analysis of the lysates as in C.
results strongly suggest that the RING domain of Mdm2 alone is sufficient to stimulate degradation of Hdmx-A. To test whether indeed expression of the RING of Mdm2 would be sufficient to trigger not only the degradation of Hdmx-A but also of full-length Hdmx, we generated a construct directing the expression of the C-terminal 94 amino acids of Mdm2, encompassing the Mdm2 RING domain, fused to an N-terminal Myc tag. Co-expression of Myc-Mdm2-RING together with Hdmx or Hdmx-A in H1299 cells or in p53−/−/mdm2−/− cells resulted in degradation of both proteins, similar to the effect of co-expression of full-length Myc-Mdm2 (Fig. 6B, upper panels). The lower panels show the expression of Myc-Mdm2 and Myc-Mdm2-RING in the same cell lysates. As expected, p53 levels are unaffected by co-expression of Myc-Mdm2-RING (Fig. 6C). These results indicate that the RING domain of Mdm2 is both necessary and sufficient for the ubiquitination and degradation of Hdmx proteins. In addition, the data show that the requirements for degradation of p53 and Hdmx are distinct, raising the possibility that the regulation of p53 and Hdmx levels by Mdm2 can be controlled, at least partly, independently.

DISCUSSION

In this study we show that Hdmx is not only affecting the stability and activity of p53 and Mdm2 but that in turn Mdm2 can ubiquitinate and target Hdmx for degradation. These results add another level to the intricate relationship between p53 and the Mdm family members. It is known from previous work (19, 20) that Mdmx is a critical inhibitor of p53 function in vivo. This inhibition appears to be more on the regulation of activity rather than on the stability of p53, because the levels of p53 in mdm2−/− mouse embryo fibroblasts are only slightly increased, but p21WAF1 levels are strongly elevated (19). On the other hand, most transfection studies indicated that Hdmx overexpression can stabilize both p53 and Mdm2 (11–14). However, a study in which low amounts of Mdm2 were co-expressed with p53 and Mdm2 suggested that Mdmx could actually cooperate with Mdm2 in the degradation and functional inhibition of p53 (16). These results implicate that the effects of Mdmx on the function of Mdm2 and p53 strongly depend on the relative expression levels of Mdm2 and Mdmx. The perception that Mdm2 can trigger the degradation of Hdmx proteins was initiated through our studies on the activity of two Hdmx deletion mutants that are encoded by alternatively spliced hdmx mRNAs isolated from C33A cells. Hdmx-A lacks exon 9, resulting in a protein lacking the acidic domain, whereas Hdmx-G lacks exons 3 to part of exon 6, resulting in an Hdmx mutant lacking the p53 binding domain. As expected Hdmx-A can interact with p53, whereas Hdmx-G could not. Luciferase reporter assays showed that Hdmx and Hdmx-A affected the transcription activation by p53 to a similar extent, whereas Hdmx-G had a minor effect. However, the ability of Hdmx and Hdmx-A to inhibit transcription activation by p53 appears to be promoter-dependent. Activation of the bax luciferase and the mdm2 luciferase construct by p53 was rather efficiently inhibited by Hdmx, as we reported before (13, 42), and similarly by Hdmx-A. However, the p53-mediated activation of the p21 luciferase was hardly affected by Hdmx/Hdmx-A. In contrast, Mdm2 fully inhibited the transcription activation by p53. A recent report (48) shows that different p53-responsive elements (REs) need varying levels of p53 to be fully activated. It was found that the p21 RE is already strongly activated by low levels of p53, whereas the mdm2 REs need much higher levels. Because Hdmx, in contrast to Mdm2, cannot degrade p53, a fraction of p53 will always be available for transcription activation. Apparently, our p53 + Hdmx co-transfection conditions result in a level of active p53 that is still sufficient to activate efficiently the p21-luc reporter but is reduced sufficiently to see a significant reduction in the activation of the bax-luc and mdm2-luc reporter. This presumption is backed up by preliminary results from analyses of p53 target genes in embryos from the mdm2−/− mice. In these embryos the expression level of p53 is only slightly increased. However, quantitative PCR showed the level of p21WAF1 mRNA to be strongly elevated (about 10–15-fold), whereas expression of bax and mdm2 mRNAs were only slightly increased (2.5-fold). These data again suggest that a significant induction of transcription from the bax and mdm2 promoters needs more active p53 than activation of the p21 promoter.

The Hdmx-A and Hdmx-G mutant proteins still contain the RING domain, suggesting that both would be capable of interacting with and stabilizing Mdm2. Indeed, both Hdmx-A and
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Fig. 6. The RING domain of Mdm2 is sufficient for degradation of Hdmx. A, H1299 cells were transiently transfected with the 250 ng of CMV-LacZ and 500 ng of Mdm2 (or mutant Mdm2) expression vector together with either 25 ng of p53 or 1 μg of HA-Hdmx-A expression vector. Cell lysates were prepared 40 h after transfection, and equal amounts of protein were separated on a 10% SDS-polyacrylamide gel and blotted on polyvinylidene difluoride membrane. Blots were incubated with anti-LacZ (transfection control), with anti-Myc, and with anti-p53 to detect Mdm2 and most Mdm2 mutants. To detect Myc-Mdm2-RING the blots were incubated with the 9E10 anti-Myc antibody. HA-Hdmx-A (upper panel) or wild type p53 (middle panel) in combination with pcDNA3.1-Mdm2 (lane 1), pcDNA3.1-Mdm2 (lane 2), pcDNA3.1-Mdm2-(C464A) (lane 3), pcDNA3.1-Mdm2ΔAD (lane 4), pcDNA3.1-22X (lane 5), pcDNA3.1-2X2 (lane 6), pcDNA3.1-Myc-Mdm2Δp53 (lane 7), and pcDNA3.1-Myc-Mdm2 (lane 8). Expression of Mdm2 and Mdm2 mutants are shown in the bottom panels. B, p53−/−/mdm2−/− cells and H1299 were transiently transfected with expression vectors encoding HA-Hdmx or HA-Hdmx-A (1 μg for H1299, 2 μg for p53−/−/mdm2−/− cells) in combination with pcDNA3.1 (lane 1), Myc-Mdm2−/− (lane 2), or Myc-Mdm2-RING (lane 3) (2 μg for H1299 and 4 μg for p53−/−/mdm2−/− cells), together with CMV-LacZ (200 ng for H1299 and 400 ng for p53−/−/mdm2−/− cells). Cell lysates were prepared 40 h after transfection. Equal protein amounts were analyzed by Western blotting with anti-LacZ (transfection control), with the anti-HA antibody to detect the HA-tagged Hdmx proteins and with anti-Mdm2. Lysate samples containing myc-Mdm2-RING were separately analyzed on a 15% SDS-PAGE gel and detected with anti-Myc. C, H1299 cells were transiently transfected with wild type p53 in combination with pcDNA3.1 (lane 1), pcDNA3.1-Myc-Mdm2 (lane 2), or with pcDNA3.1-Myc-Mdm2-RING (lane 3), together with CMV-LacZ. Cell lysates were prepared 40 h after transfection. Equal protein amounts were analyzed by Western blotting with anti-LacZ (transfection control), with anti-Myc, and with anti-p53 antibody (DO-1). Expression of Myc-Mdm2 and Myc-Mdm2RING is shown in bottom panel.
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likely. It will be very important to identify physiological conditions that affect the post-translational modification and to determine the functional effects of this modification.

Whereas these splicing variants identified from C33A cells provide excellent tools to study Hdmx regulation and activity, a physiological function of these alternative Hdmx proteins is unclear. We have not been able to detect the Hdmx-A and Hdmx-G proteins in C33A cells, although we tried to increase the expression level by treatment of the cells with the proteasome inhibitor MG132. Full-length Hdmx was readily detected in these cells, as reported before (24).

A physiological relevance for the degradation of Hdmx by Mdm2 has not been shown yet. It is tempting to speculate that it has a function in the cellular stress response. When cells are stressed, e.g., by DNA damage, p53 levels increase, and p53 target genes, like the hdm2 gene, are activated. Consequently, Hdm2 protein levels rise, resulting in a higher Hdm2:Hdmx ratio, possibly resulting in degradation of Hdmx. Indeed, we have preliminary evidence that upon various types of stress the Hdmx protein levels strongly decrease. This down-regulation might be important for the functional activation of p53 and, if indeed under certain conditions Hdmx can stimulate the degradation of p53 by Hdm2, for the stabilization of p53. We are indeed under certain conditions Hdmx can stimulate the degradation of p53 by Hdm2, for the stabilization of p53. We are therefore under certain conditions Hdmx can stimulate the degradation of p53 by Hdm2, for the stabilization of p53. We are indeed under certain conditions Hdmx can stimulate the degradation of p53 by Hdm2, for the stabilization of p53. We are indeed under certain conditions Hdmx can stimulate the degradation of p53 by Hdm2, for the stabilization of p53.

In conclusion, we show in this paper that the closely related proteins Mdm2 (Hdm2) and Mdmx (Hdmx) strictly regulate each others' half-life. Hdmx stabilizes Hdm2, but when the ratio Hdm2:Hdmx increases above a certain threshold, Hdmx is degraded via the proteasome pathway.

Acknowledgments—We thank M. Oren, S. Mittnacht, D. Xirodimas, and J. C. Marine for expression plasmids. We thank T. Jacks for the hdm2 gene, are activated. Consequently, the expression level by treatment of the cells with the proteasome inhibitor MG132. Full-length Hdmx was readily detected in these cells, as reported before (24).

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