Hdmx Stabilizes Mdm2 and p53*

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The Mdm2 protein is a key regulator of p53 activity and stability. Upon binding, Mdm2 inhibits the transcription regulatory activity of p53 and promotes its rapid degradation. In this study we investigated the effect of the human Mdm2 homologue Hdmx on p53 stability. We found that Hdmx does not target p53 for degradation, although, like Mdm2, it inhibits p53-mediated transcription activation. On the contrary, Hdmx was found to counteract the degradation of p53 by Mdm2, and to stabilize both p53 and Mdm2. The RING finger of Hdmx was found to be necessary and sufficient for this stabilization, and it probably involves hetero-oligomerization with the RING finger of Mdm2, which may lead to inhibition of Mdm2’s ubiquitin ligase activity. However, Hdmx does not relieve the inhibition by Mdm2 of transcription activation by p53, probably due to the formation of a trimeric complex consisting of Hdmx, Mdm2, and p53. We propose a model in which Hdmx secures a pool of largely inactive p53, which, upon the induction of stress, can be quickly activated.

The tumor suppressor protein p53 is a key regulator in maintaining the integrity of the cell. Signaling pathways activated by DNA damage, ribonucleotide depletion, oxidative stress, and incomplete mitotic stimuli lead to the activation of p53. This activation is mainly due to posttranslational modifications, which lead to both increased DNA binding and accumulation of p53 protein levels. Downstream responses of p53 are growth arrest or apoptosis, which are mediated by its target genes including p21 (cip1) and bax. The activities and stability of p53 are tightly regulated. A key regulator of p53 half-life is the Mdm2 protein.

The mdm2 oncogene was discovered as an amplified gene on a murine double minute chromosome in the spontaneously transformed 3T3DM cell line (3). The Mdm2 protein associates with the transactivation domain of p53, thereby inhibiting the transcription regulatory functions of p53 and the p53-induced apoptosis and growth arrest responses (6). In addition, it has recently been shown that upon binding of Mdm2, the ubiquitin-dependent degradation of p53 is promoted by enhancing the ubiquitination of p53 (7, 8), most likely by an intrinsic E3-like ubiquitin-ligase activity of Mdm2 (9). For the latter activity of Mdm2 not only the p53-binding domain is necessary, but also its nuclear localization signal and nuclear export signal to allow nuclear-cytoplasmic shuttling (10) and the RING finger domain which contains the putative ubiquitin-binding site (9).

We previously reported the identification of a mouse and human Mdm2 homologue, Mdmx and Hdmx, respectively (11, 12). Several structural domains are conserved between Mdm2 and Mdmx: most notably, the p53-binding domain, the zinc finger, and the RING finger. In a recent study, the similar requirements of Mdm2 and Hdmx to bind to p53 have been clearly demonstrated (13). Like Mdm2, Mdmx can inhibit p53-mediated transcription activation via binding of its N terminus to the transcriptional activation domain of p53 (11).

In this study we describe the effect of Hdmx on the regulation of p53 stability. It was found that Hdmx does not target p53 for proteasome-mediated degradation but, on the contrary, enhances levels of p53 and Mdm2. This effect is mainly mediated by the RING finger of Hdmx, which, through the interaction with the RING finger of Mdm2, probably inhibits Mdm2’s ubiquitin ligase activity. Binding of the RING finger of Hdmx to Mdm2 does not release p53 from Mdm2; hence, a trimeric complex can be formed that is not able to activate transcription.

Experimental Procedures

Plasmids—Full-length human HA1-tagged hdmx was cloned into pCDNA3.1myc-his (Invitrogen). The hdmx-ΔR and hdmx-R constructs, coding for amino acids 1–392 and 393–490, respectively, were made by PCR with the 3 primer encoding the HA tag and 5 primer: 5′-TAAGCTTCTAGAGGCTAGC-3′ (1) and bax (2). The activities and stability of p53 are tightly regulated. A key regulator of p53 half-life is the Mdm2 protein.

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2 The abbreviations used are: HA, hemagglutinin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; CMV, cytomegalovirus; DAPI, 4,6-diamidino-2-phenylindole; MEF, mouse embryonic fibroblast; IP, immunoprecipitation.

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**RESULTS**

Hdmx Does Not Target p53 for Degradation—Binding of Mdm2 to p53 results in p53 being targeted for degradation. Both the p53-binding domain and the RING finger of Mdm2 are required for this degradation (20), and since these domains are conserved in Hdmx, we investigated whether Hdmx can also target p53 for degradation. C33A cells, which contain high levels of endogenous mutant p53 that can be degraded upon overexpression of Mdm2 (20), were transfected with either hdmx or mdm2. Immunofluorescence was used to analyze the expression of Hdmx, Mdm2 and p53. As shown in Fig. 1 (A–C), the cells expressing Hdmx showed no decreased p53 levels, indicating that Hdmx does not target p53 for degradation, whereas overexpression of Mdm2 clearly leads to a reduction of p53 signal (Fig. 1, D–F).

Hdmx Prevents Mdm2-induced p53 Degradation—Since both Hdmx and Mdm2 bind to the same N-terminal region of p53 (13), we questioned whether Hdmx could interfere with Mdm2-directed p53 degradation, for instance by competing with Mdm2 for p53 binding. Therefore, C33A cells, which contain high levels of endogenous mutant p53, were co-transfected with mdm2 and hdmx. Immunofluorescence indicated that now the Mdm2-positive cells did not show decreased levels of p53 (Fig. 1, G–I). Double staining for Mdm2 and Hdmx on coverslips from the same transfection confirmed co-expression of Hdmx and Mdm2 (data not shown). This result indicates that Hdmx can prevent Mdm2-directed p53 degradation.

In order to assess the effect of Hdmx on p53 stability more quantitatively, transfections were performed into p53−/− MEFs. To prevent induction of endogenous Mdm2 by transfected p53, a DNA binding mutant p53 (Ala-143) was used. In these experiments, 100 ng of p53 was transfected alone or in the presence of 2 μg of mdm2 with either none or increasing amounts of hdmx. As a control for transfection efficiency, 1.5 μg of CMV-lacZ plasmid was co-transfected in all cases. As shown previously, co-transfection of p53 and mdm2 results in a reduction of p53 protein levels (Fig. 2A, compare lanes 1 and 2). Co-transfection of 2, 4, or 7 μg of hdmx results in restoration of p53 levels (lanes 3, 4, and 5). Besides increased levels of p53, also increased levels of Mdm2 were observed (Fig. 2A, lanes 3, 4, and 5). This stabilization of Mdm2 was independent of the presence of p53 (data not shown). The same effect was observed in other p53-deficient cells (H1299 and Saos2 cells; data not shown).
shown). In general, after co-transfection of high amounts of hdmx, p53 levels exceeded the levels observed after transfection of p53 alone (compare lane 1 with lanes 4 and 5). This could be due to the effect of transfected hdmx on the endogenous Mdm2. Fig. 2B shows that hdmx can also stabilize p53 in the absence of transfected mdm2, probably due to the inhibition of the endogenous Mdm2 protein. In all cases, LacZ protein levels were comparable, indicating that Hdmx has no effect on the transcription levels of the transfected plasmids.

Hdmx Stabilizes Both p53 and Mdm2 through Its RING Finger—One possible explanation for this inhibition of Mdm2-mediated p53 degradation was that Hdmx competes with Mdm2 for p53 binding. However, during the course of this study, it was reported that the RING fingers of Mdm2 and Hdmx could interact with each other, leading to stabilization of the Mdm2 protein (21). To investigate the mechanism of p53 and Mdm2 stabilization, we constructed two deletion mutants of Hdmx, one encoding the RING finger (hdmx-R) and another containing all but the RING finger (hdmx-ΔR) and tested their effects on p53 and Mdm2 stability. Fig. 3A shows that only full-length Hdmx (lane 3) and Hdmx-R (lane 5) are able to stabilize both p53 and Mdm2, while Hdmx-ΔR is not (lane 4). These results favor the model, in which the RING finger of Hdmx, by binding to the RING finger of Mdm2, inhibits Mdm2’s ubiquitin ligase activity, resulting in stabilization of both p53 and Mdm2.

Transfection of G401 cells, which contain endogenous wild type p53, showed that full-length Hdmx and the Hdmx RING finger could also stabilize endogenous wild type p53 (Fig. 3B, panels A–C and G–I, respectively). Under these conditions the Hdmx mutant lacking the RING finger was also able to stabilize p53 (panels D–F). In this setting, however, the ratio between Hdmx-ΔR and the endogenous Hdm2 is strongly increased so that competition for p53 binding probably also results in stabilization of p53.

Stabilized p53 Is Not Active as a Transcription Activator—Since Hdmx expression leads to increased levels of p53 due to inhibition of Mdm2-mediated degradation, we wondered whether the ability of p53 to activate transcription would also be modulated. Hep3B cells, lacking endogenous p53, were transfected with the Bax-luc reporter. Co-transfection of 20 ng of wild type p53 results in a 9-fold induction of the luciferase activity (see Fig. 4A, lane 2). Mdm2 completely inhibits this p53 activity (lane 3), whereas full-length Hdmx partially inhibits p53 (lane 4). Co-expression of Hdmx-ΔR leads to a stronger inhibition of p53, probably due to higher expression levels of Hdmx-ΔR compared with full-length Hdmx (compare lane 4 and 5). To our surprise, Hdmx-R, which lacks the N-terminally located p53-binding site,
also partially inhibits p53 activity (lane 6). Co-transfection of the different hdmx constructs with mdm2 did not reverse the inhibition of p53 activity (lanes 7–9), indicating that the stabilized p53 protein is not able to activate transcription.

Fig. 4. Hdmx does not relieve the inhibition of p53-mediated transcription activation by Mdm2. Hep3B cells were transfected with 20 ng of wild type p53, 2 μg of mdm2, 4 μg of hdmx-fl, hdmx-ΔR, or hdmx-R, and 2 μg of the Bax-luciferase reporter as outlined in transfection scheme. A, luciferase activity is shown as -fold induction compared with Bax-Luc alone, with the standard deviation of the triplicates. B, expression levels of Mdm2, Hdmx-fl, Hdmx-ΔR, Hdmx-R, and p53, with the use of the antibodies mentioned in Fig. 3A; p53 was detected with a mixture of monoclonal antibodies DO-1, PAb421, and PAb1801.

Fig. 5. Association of different Hdmx forms with p53 and Mdm2 and formation of a trimeric complex. A, H1299 cells were transfected with 1 μg of mutant p53, 7 μg of mdm2, and 7 μg of HA-tagged hdmx-fl, hdmx-ΔR, and hdmx-R as outlined in the transfection scheme. Cell lysates were immunoprecipitated with a rabbit polyclonal anti-HA antibody (α-Hdmx) or with the anti-Mdm2 monoclonal antibody 2A10 (α-Mdm2). p53 was detected with rabbit polyclonal antibody 28042 in the anti-Mdm2 IP and with mouse monoclonal antibody DO-1 in the anti-HA IP. Mdm2 and the different Hdmx forms were detected with same antibodies as mentioned in Fig. 2. The asterisk marks the presence of cross-reactivity with Ig heavy chain. B, H1299 cells were transfected with 5 μg of hdmx and mdm2-2XM and 2 μg of mutant p53 according to transfection scheme. p53 was immunoprecipitated with a mixture of PAb421 and PAb1801 and Mdm2 with 2A10. Hdmx and p53 were detected with same antibodies as mentioned in Fig. 2; Mdm2-ΔN was detected with a mixture of monoclonal antibodies 2A10 and 3G5.

Identification of Endogenous Protein Complexes—To investigate whether endogenously expressed p53, Hdmx, and Hdm2 proteins interact, immunoprecipitations were performed on G401 cell lysates, which contain detectable levels of endogenous Hdmx, Hdm2, and wild type p53. Clear binding of Hdmx to Hdm2 could be detected by immunoprecipitation of Hdmx with either the rabbit polyclonal antisera p56 or with a mixture of antibodies 11F4D and 12G11G and by immunoprecipitation of Hdm2 with antibody 2A10, with non-immune rabbit serum and anti-large T antibody serving as controls (Fig. 6A). Binding of p53 to both Hdmx and Hdm2 was determined by immunoprecipitation with polyclonal antibody p56 and monoclonal antibody 2A10, respectively (Fig. 6B) and by immunoprecipitation of p53 with a mixture of PAb421 and PAb1801 (Fig. 6C). The presence of an endogenous trimeric complex cannot be determined in this way, since all three proteins can also form heterodimers.

Interestingly, p56 and 11F/12G precipitate equal amounts of Hdmx, but the amount of co-precipitated Hdm2 is much less with the use of 11F/12G. This difference is most likely caused by the fact that the epitopes of 11F4D and 12G11G map within
Hdm2 proteins were detected with the same antibodies as mentioned in and a mouse monoclonal anti-SV40 large-T antibody (KT3 precipitations were performed with non-immune rabbit serum). Two anti-p53 antibodies PAb421 and PAb1801. As a control, immunoprecipitations were performed on G401 cell lysates, with the anti-Hdmx rabbit polyclonal antibody p56, with a mixture of anti-Hdmx mouse monoclonal antibodies 11F4D and 12G11G, with anti-Mdm2 antibody 2A10 and with a mixture of two anti-p53 antibodies PAAb21 and PAAb101. As a control, immunoprecipitations were performed with non-immune rabbit serum (N.I.) and a mouse monoclonal anti-SV40 large-T antibody (KT3). Hdmx and Hdm2 proteins were detected with the same antibodies as mentioned in Fig. 2. p53 protein was detected with mouse monoclonal antibody DO-1.

**FIG. 6. Identification of endogenous Hdmx-Hdm2 and Hdmx-p53 complexes.** Immunoprecipitations were performed on G401 cell lysates, with the anti-Hdmx rabbit polyclonal antibody p56, with a mixture of anti-Hdmx mouse monoclonal antibodies 11F4D (11F) and 12G11G (12G), with anti-Mdm2 antibody 2A10 and with a mixture of two anti-p53 antibodies PAAb21 and PAAb101. As a control, immunoprecipitations were performed with non-immune rabbit serum (N.I.) and a mouse monoclonal anti-SV40 large-T antibody (KT3). Hdmx and Hdm2 proteins were detected with the same antibodies as mentioned in Fig. 2. p53 protein was detected with mouse monoclonal antibody DO-1.

the Hdmx RING finger, and thus apparently disturb the interaction between Hdmx and Hdm2.

**DISCUSSION**

In this study we show that the Hdmx protein does not target p53 for degradation, but even prevents Mdm2-triggered degradation. Thus, although the domain structures of Hdmx and Mdm2 are very similar, their biological activities differ considerably. The inability of Hdmx to destabilize p53 could be due to several reasons. First, analysis of the Hdmx amino acid sequence does not reveal a good consensus for a NES, which is present in Mdm2, and is essential for degradation of p53 (10). Second, although the RING finger structure is conserved between Mdm2 and Hdmx, the amino acid identity is only 44%, leaving the possibility that residues essential for the ubiquitin ligase activity of Mdm2 are absent in Hdmx. The differences in structure of the RING fingers of Hdmx and Mdm2 is underscored by the observation that hetero-oligomerization is much more efficient than homo-oligomerization (21). We have started to swap domains between Mdmx and Mdm2, to investigate which motifs are responsible for the difference in function between the two proteins.

The inhibition of the Mdm2-mediated p53 degradation by Hdmx is mainly caused by the Hdmx RING finger and most likely involves the hetero-oligomerization with the RING finger of Mdm2, which possibly prevents the ubiquitin ligase activity of Mdm2. Alternatively, through binding of Hdmx, the nucleo-cytoplasmic shuttle of Mdm2 might be impaired, as has been shown for the p19ARF protein (23). In addition, in the case of high overexpression of Hdmx compared with endogenous Mdm2, the effect might also be caused by competition with Mdm2 for p53 binding.

The interaction of the RING fingers of Mdm2 and Hdmx has been reported by Tanimura et al. (21). Like us, they find that the interaction leads to stabilization of Mdm2. They suggest, however, that the increased levels of Mdm2 would lead to an even more rapid degradation of p53. Our results suggest a model in which the interaction of the RING fingers prevents both the ubiquitination of p53 and the auto-ubiquitination of Mdm2. These findings are in line with the observation that the inhibition of Mdm2-mediated ubiquitination by p14ARF also leads to increased levels of Mdm2 (24).

The biological function of the effect of Hdmx on p53 half-life remains to be elucidated. Stabilized p53 protein seems not to be able to activate transcription, possibly due to the formation of a trimeric complex. We propose the model that Hdmx secures the presence of a pool of inactive p53 that can be instantly activated upon certain cellular stresses. It has recently been shown that the interaction of Hdmx and p53 is disrupted after in vitro phosphorylation of p53 (25). Thus, the kinetics of the p53 response after cellular stress might be partially dependent upon the levels of Hdmx. It is of interest to note that G401 cells, in which we can detect an endogenous Hdm2/Hdmx complex, contain significant levels of relatively stable wild type p53 (half-life of about 4 h; Ref. 26). It would be interesting to see what the effect on p53 levels would be by decreasing the Hdmx levels, for instance by an antisense approach, or by disrupting the Hdmx-Hdm2 interaction by microinjection of the 11F4D or 12G11G antibodies, which recognize an epitope in the RING finger of Hdmx. We are currently testing this hypothesis. Relatively high levels of Hdmx might also explain the observation that a high percentage of certain tumor types contain high levels of wild type p53 and Mdm2, e.g. germ-cell tumors (27, 28), which show a very good response to radiation and chemotherapy. This would mean that the efficacy of tumors showing overexpression of Hdmx and containing wild type p53 will be very high.

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Addendum—During revision of this manuscript, two publications have appeared that together largely confirm the findings described in this manuscript (29, 30).

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