Stabilization of the MDM2 Oncoprotein by Interaction with the Structurally Related MDMX Protein*

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The MDM2 oncprotein has transforming potential that can be activated by overexpression, and it represents a critical regulator of the p53 tumor suppressor protein. To identify other factors with a potential role in influencing the expression and/or function of MDM2, we utilized a yeast two-hybrid screening protocol. Here we report that MDM2 physically interacts with a structurally related protein termed MDMX. The results obtained in these studies provide evidence that C-terminal RING finger domains, contained within both of these proteins, play an important role in mediating the association between MDM2 and MDMX. The interaction of these proteins interferes with MDM2 degradation, leading to an increase in the steady-state levels of MDM2. MDMX also inhibits MDM2-mediated p53 degradation, with subsequent accumulation of p53. Taken together, these data indicate that MDMX has the potential to regulate the expression and function of the MDM2 oncprotein.

An accumulating number of observations have implicated aberrant expression of the MDM2 oncogene in the pathogenesis of human neoplasias. This mammalian gene has transforming potential that can be activated by overexpression (1, 2). Originally identified as a gene amplified and overexpressed in a spontaneously transformed mouse 3T3 cell line (3), MDM2 is now known to be amplified in a variety of human tumors, particularly soft tissue sarcomas (4–7). Additionally, there are several cases reported of tumor cells having an elevated expression of MDM2 that results from mechanisms other than gene amplification, including enhanced translation of MDM2 transcripts (8–10).

The MDM2 gene encodes a key negative regulator of the p53 tumor suppressor protein, and the role of MDM2 overexpression in cell transformation has been attributed, at least in part, to its disruption of the biological activities of p53 (11, 12). MDM2 tightly associates with the N-terminal region of the p53 protein, inhibiting the trans-activation and G1 growth arrest functions of p53 (13–16). Moreover, binding of MDM2 targets p53 for rapid degradation via the ubiquitin-proteasome pathway (17, 18). In recent reports, evidence has been obtained suggesting that MDM2 can function as an E3 ubiquitin ligase and is responsible for targeting both itself, and p53, for degradation (19, 20). Interestingly, the MDM2 gene itself is a transcriptional target of p53. When activated as a transcription factor, p53 binds to a promoter region within the first intron of the MDM2 gene and up-regulates its expression (21–24). Thus, there is evidence for an autoregulatory feedback loop involving the expression and function of MDM2 and p53 (25). Although the best characterized activities of MDM2 concern its functional interactions with p53, MDM2 also associates with other proteins. Some of these include E2F1 (25), pRb (26), and p300 (27). Such interactions could contribute to the transforming potential of MDM2 or may be concerned with modulating MDM2 function. Recently, a link between MDM2 and yet another tumor suppressor protein, p14ARF (p19ARF in mouse) was identified. An important consequence of complex formation with p14ARF is an abrogation of the ability of MDM2 to mediate p53 degradation and to inhibit the trans-activation function of p53 (28–30).

As illustrated by such examples, the identification of novel MDM2-interacting proteins would be expected to offer new clues to understand better the biological activities and/or regulation of the MDM2 oncprotein. Toward that goal, we sought to identify cellular proteins that physically associate with MDM2. In initiating these studies, we noted that MDM2 contains several conserved structural motifs that likely are important for its biological activities (1). In addition to the N-terminal p53-binding domain, MDM2 has a central acidic domain, a putative nuclear localization signal, and a nuclear export signal. The C-terminal region of the MDM2 protein contains two cysteine-rich elements, classified as a C4 zinc finger domain and a C3HC4 RING finger (31). Recent investigations directly implicate certain zinc finger and RING finger domains in mediating protein-protein interactions or the formation of multi-protein complexes (32, 33). To test whether the C-terminal segment of the MDM2 protein, comprising the zinc finger and RING finger motifs, also might participate in complex formation with other proteins, we carried out a yeast two-hybrid screening assay.

As described in this report, we have identified an interaction between MDM2 and a structurally related protein, MDMX (34, 35). Our data indicate that the RING finger domains contained within both of these proteins are necessary to mediate this interaction. Notably, complex formation between MDM2 and MDMX leads to a stabilization of MDM2, resulting in an elevation of steady-state levels of MDM2 protein. MDMX also interferes with MDM2-mediated degradation of p53, resulting in an increase in p53 protein levels. The data presented here suggest a role for MDMX in the regulation of MDM2 expression and have implications for understanding the cellular functions of the MDM2 oncprotein.

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The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; aa, amino acids; IP, immunoprecipitation; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; AD, activation domain; BD, binding domain.

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EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—A DNA fragment encoding the C-terminal 215 amino acid (aa) residues of human MDM2 was fused in-frame with the yeast GAL4 DNA binding domain in the pBd-M2 GAL4 Cam phagemid vector (Stratagene) to create the hybrid bait protein. A randomly primed cDNA library was constructed in the pAD-GAL4 phagemid (Stratagene) using poly(A)\(^+\) RNA isolated from human thymus (Clontech). The bait and target plasmids were co-transformed into YRG-2 yeast cells. Colonies with plasmid DNA encoding target proteins that interact with the bait protein are identified by transcription of the HIS3 and lacZ reporter genes integrated into the genome of the yeast host. Approximately 3 \times 10^9 transformants were screened for colonies that would grow on media lacking tryptophan (Trp), leucine (Leu), and histidine (His). To confirm the presence of interacting proteins, positive transformants were assayed for expression of the lacZ reporter gene using an X-gal filter assay for detection of \(\beta\)-galactosidase activity. Typically, blue coloration of colonies appeared within 1–3 h of incubation of the lacZ-gal-gal buffer at 30 °C. To verify further protein-protein interactions, plasmid DNA was rescued from positive colonies and re-transformed into YRG-2 yeast cells with the MDM2 bait plasmid. Nucleotide sequence analysis was carried out to characterize the target DNA of positive colonies.

Plasmid Constructs and Antibodies—A full-length human MDMX cDNA fragment encoding aa 1–490 was generated by a random hexamer-primed reverse transcription reaction using human kidney RNA (Clontech) followed by PCR using forward and reverse primers containing the first and last coding triplets of the human MDMX cDNA. All nucleotide and aa sequence numbers for MDMX primer design related to the first ATG coding triplet as number 1 (35). The 5' primer included an XhoI site, and the 3' primer included a BamHI site for subsequent cloning reactions. This PCR product was cloned into the TOPO PCR 2.1 vector (Invitrogen) to generate the MDMX plasmid termed pCK4; the DNA sequence was verified, and this clone was used as a template to generate additional MDMX constructs, as described below. DNA fragments encoding full-length MDMX and MDMX lacking the C-terminal 153 aa of MDMX without RING were both cloned into the yeast phagemid vector pAD-GAL4 by including into the oligonucleotide primers 5' SstI and 3' SfuI restriction sites. The 5' and 3' primers for the full-length MDMX construct included sequences representing the first ATG codon and last (stop) codon, respectively. For the MDMX without RING construct, the 5' primer included sequences for the first ATG codon, and the 3' primer included MDMX coding sequence up to base pair 1290. For the MDMX-(1–490)-Myc construct, the 5' primer included the sequences for an internal ATG at aa residue 101, and the 3' primer included the last (stop) codon of the MDMX cDNA. To generate the MDMX mammalian expression plasmid MDMX-Myc, a C-terminal Myc epitope tag was added to the MDMX coding sequence by using a BamHI-XhoI restriction digest to release the insert from plasmid pCK4, the released insert was subcloned into the pcDNA 3.1+ Myc His A mammalian expression vector (Invitrogen). An N-terminal His A tag was added to the C-terminal Myc His B epitope of MDMX as a template for MDMX-(1–490)-Myc, MDMX-(1–300)-Myc, and MDMX-(1–392)-Myc constructs. The PCR product was then self-ligated following gel purification. Point mutants were generated by Karpal Singh.

RESULTS

Cell Culture, DNA Transfections, and RNA Blot Analysis—H1299 lung adenosquamous carcinoma cells (p53 null) and JEG-3 choriocarcinoma cells (wild-type p53) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. H1299 cell transfections were carried out using the Lipotaxi reagent according to guidelines of the manufacturer (Stratagene) or by a standard calcium phosphate precipitation protocol. In all transfections the total amount of each vector was equalized, as was the total amount of DNA. Approximately 24–48 h post-transfection, the cells were harvested and lysed (1% Nonidet P-40, 250 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 7.4, 0.1 mM sodium deoxycholate) to generate a cell lysate containing target proteins. DNA was transfected into JEG-3 cells using the calcium phosphate precipitation protocol. At 48 h post-transfection, the cells were harvested and lysed. For Northern blot analysis, total cellular RNA from transfected H1299 cells was isolated using RNA Isolator (Genosys). Samples (10 μg) were resolved on formaldehyde-agarose gels and transferred to nitrocellulose as described previously (36). Probes were radiolabeled with \(^32\)P using random primers (Prime-It-II, Stratagene).

Immunoprecipitation and Western Blot Analysis—For immunoprecipitation assays, cell lysates (approximately 1–3 mg of protein) were incubated with 1–3 μg of the appropriate antibody and lysis buffer (final volume, 500 μl) for 1 h at 4 °C with mixing. Following the addition of 40 μl of a protein A/protein G-agarose bead mix, the reactions were incubated for 30 min at 4 °C with mixing. The beads were washed twice in RIPA buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 0.1% SDS, 1% sodium deoxycholate), resuspended in 50 μl of SDS sample loading buffer and boiled for 5 min. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). For immunoblotting experiments, 50–75-μg samples of total cellular lysate in SDS loading buffer were separated by 7.5–10% SDS-PAGE. Western blot analysis was performed using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

RESULTS

Yeast Two-hybrid Analysis Identifies MDMX as an MDM2-binding Protein—To identify cellular proteins that interact

The yeast two-hybrid assays were extended to test further the importance of the C-terminal protein domains in the MDMX-MDM2 interaction. The results (Fig. 2) demonstrated that full-length versions of the MDM2 and MDMX proteins can interact in yeast cells. In addition, deletion of the N-terminal p53-binding region, or the central acidic domain, of MDM2 did not interfere with MDM2-MDMX interactions. However, a truncated MDM2 protein, lacking the C-terminal 49 aa residues, failed to interact with MDMX (Fig. 2A). Deletion of the C-terminal 59 aa residues of MDMX also resulted in a loss of interaction of the two proteins.

Evidence suggests that the C-terminal region of MDM2 binds two molecules of zinc in an interleaved fashion (37). To examine more specifically the role of the RING domain in MDM2-MDMX interaction, we therefore introduced mutations at conserved cysteine residues implicated in this metal ligation. MDM2 proteins containing a single mutation (C441G or C478R) or a double mutation (C438G,C441G or C475Y,C478R) within the RING domain failed to interact with MDMX (Fig. 2A). Mutation of a threonine residue (T455A) that is located within the RING domain, but does not seem to be involved in metal ligation (37), did not disrupt the ability of MDM2 and MDMX to associate (Fig. 2A). The introduction of either a single mutation (C437G) or a double mutation (C437G,C440G) within the RING domain of MDMX also disrupted interaction with MDM2 (Fig. 2B). It should be noted that both MDM2 and MDMX proteins containing such point mutations, and those with the C-terminal deletions noted above, still retained the ability to associate with p53 protein in this yeast two-hybrid system (data not shown). Taken together, these results indicate that the RING finger domain of MDM2 is necessary for interaction with MDMX; in contrast, the RING domain of MDMX likely is both necessary and sufficient for binding to MDM2. Interestingly, we have found no evidence for homo-oligomerization of either the MDMX or MDM2 proteins (data not shown). This result lends additional support to the specificity of the RING-RING interaction between MDM2 and MDMX.

MDMX and MDM2 Associate in Vitro and in Vivo—The MDM2-MDMX interaction also was detected in a cell-free system. Full-length MDMX, containing a c-Myc epitope tag (MDMX-Myc), and full-length MDM2 were individually produced by translation in a rabbit reticulocyte lysate system. The MDMX and MDM2 proteins are very similar in size (85–95 kDa; Fig. 3, lanes 1 and 2). Thus, to ensure accurate identification of the proteins, co-immunoprecipitation reactions were carried out following the 35S labeling of only one of the proteins per reaction. 35S-Labeled MDMX-Myc protein was incubated with unlabeled MDM2 to allow complex formation, and the reactions were immunoprecipitated with anti-MDM2 antibody. The 35S-labeled MDMX-Myc protein is clearly detectable in these immunocomplexes (Fig. 3, lane 3). Consistent results were obtained when 35S-labeled MDM2 protein was incubated with unlabeled MDMX-Myc followed by immunoprecipitation with anti-Myc antibody; the presence of 35S-labeled MDM2 in the immunoprecipitates is illustrated in Fig. 3, lane 4. In contrast, there was no evidence for cross-reaction between the anti-MDM2 antibody and MDMX-Myc (Fig. 3, lane 5) or between the anti-Myc antibody and MDM2 (Fig. 3, lane 6). These results suggest that MDM2 and MDMX interact directly.

MDM2-MDMX binding was assessed in mammalian cells following transient transfection analysis. Mammalian expression plasmids encoding either a Myc-tagged MDMX protein or MDM2 protein were transfected into H1299 human lung carcinoma cells, either separately or together. Cell extracts were analyzed by IP-Western blot analysis. For the co-transfected cells, immunoprecipitation with anti-MDM2 antibodies, fol-
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During the course of these studies, we noted that co-transfection of the MDM2 and MDMX-myc expression plasmids resulted in a reproducible and significant increase in the steady-state level of MDM2 protein. An example of this can be seen in Fig. 4A (compare lanes 8 and 9). This result suggested that MDMX may be modulating MDM2 expression. To assess further this possibility, H1299 cells were co-transfected with a constant amount of MDM2 plasmid and increasing amounts of MDMX-myc plasmid. Steady-state MDM2 protein levels in the transfected cells were examined by Western blotting. The results demonstrate that increased MDMX-Myc protein expression is accompanied by a dose-dependent increase in the steady-state levels of MDM2 protein (Fig. 5A). Expression of the transfected MDMX-myc construct in these cells was confirmed by Western blotting (data not shown).

Interestingly, MDMX protein levels were not obviously affected by co-expression of MDM2 (Fig. 4A, compare lanes 4 and 5). To confirm this, H1299 cells were co-transfected with a constant amount of plasmid for FLAG-tagged MDMX and with increasing amounts of MDM2 plasmid. We carried out these transfections at higher MDM2:MDMX plasmid ratios (1:1 and 5:1) than previously utilized, to ensure that adequate levels of MDM2 would be present to test any effects it might have on MDMX levels. Cell lysates were analyzed for steady-state MDM2 protein levels in the transfected cells by Western blotting. The results obtained from this experiment were consistent with previous data, indicating that that co-expression of MDM2 has no discernible effect on the steady-state levels of MDMX-Myc protein. Northern blot assays demonstrated that MDM2 mRNA levels were not different in the presence of MDMX (Fig. 5C). It is likely, therefore, that the increase in MDM2 is mediated by a change in the half-life of the protein. To test this, we examined the relative stability of MDM2 in the presence or absence of MDMX. For these assays, cellular protein synthesis was inhibited by the addition of cycloheximide, and the abundance of MDM2 protein was examined by Western blotting. We determined that under these experimental conditions, the initial half-life of transfected MDM2 in the H1299 cells is approximately 30 min (Fig. 5D, lanes 1–4); this value is consistent with previous estimates of the half-life of endogenous MDM2 (15–30 min).
MDM2 and MDMX-Myc were produced in separate absence of 35S-labeled methionine. Samples were resolved by SDS-PAGE and visualized by x-ray film exposure following fluorography. Molecular masses of protein size markers are indicated. Aliquots of the individually translated, 35S-labeled MDMX-Myc (lane 1) and 35S-labeled MDM2 (lane 2) proteins are shown. 35S-Labeled Myc-MDMX was incubated in the presence (lane 3) or absence (lane 5) of unlabeled MDM2, followed by precipitation with anti-MDM2 antibody. Similarly, 35S-labeled MDM2 was incubated in the presence (lane 4) or absence (lane 6) of unlabeled MDMX-Myc, followed by precipitation with anti-Myc antibody. Rxns, reactions.

Stabilization of MDM2 by MDMX Is Dependent on Protein-Protein Interaction—Experiments were carried out to test whether the increase in MDM2 protein by MDMX depends on a physical interaction between these proteins. Data obtained in our yeast two-hybrid analyses indicated that the C terminus of MDMX is important for its interaction with MDM2. Therefore, for these studies we utilized MDMX constructs encoding modified MDMX proteins, as follows: MDMX(C437G,C440G) protein contains mutations of two conserved cysteine residues at positions 437 and 440 within the RING domain; MDMX(1–392) is missing the C-terminal 98 aa residues of the full-length MDMX protein, including the RING domain (432–490); MDMX-(1–153) encodes a more severely truncated MDMX protein (392) is missing the C-terminal 98 aa residues of the full-length protein (data not shown). The importance of the MDMX-MDM2 interaction for stabilization of MDM2 is again illustrated by comparing the steady-state MDM2 protein levels present in the input lysates for these IP-Western analyses (Fig. 7, lanes 5–7). Cell lysates were immunoprecipitated with anti-Myc antibody, anti-FLAG antibody, or with a nonspecific antiserum (rabbit IgG), as indicated, followed by Western blot analysis for MDM2 protein (IF2 antibody).

In summary, these experiments demonstrate that stabilization of MDM2 by MDMX requires an interaction between these proteins.

Interaction of MDMX with MDM2 and p53—The MDMX protein contains the MDM2-binding domain at its C terminus and the p53-binding domain at its N terminus. This raises the possibility that MDMX may be able to form ternary complexes with MDM2 and p53. However, testing this idea is complicated by the ability of both MDMX and MDM2 to independently bind to p53. To approach this question, therefore, we constructed an N-terminally truncated MDMX mutant that is missing the first 100 amino acid residues. This mutant retains the C-terminal MDM2-binding region but lacks the p53-binding domain. We then asked whether a ternary complex could form, under conditions where MDM2 would serve as a “bridging” partner for p53 and the MDMX-(101–490) protein. Control IP-Western blot analysis of transfected cells confirmed that the N-terminally truncated MDMX-(101–490) protein interacts well with MDM2 (lane 2) but is impaired in its ability to bind to p53 (lane 3). For cells transfected with expression constructs for all three proteins, IP-Western blot analysis revealed the presence of MDMX-(101–490), as well as MDM2, in the p53 immunocomplexes (Fig. 8, lane 5). These results indicate that MDMX,

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null H1299 lung carcinoma cells, and steady-state p53 protein levels were examined by Western blotting. Co-expression of MDM2 with p53 led to a reduction in p53 levels (Fig. 9, lanes 1 and 2) as previously observed (17, 18). However, when MDMX was co-expressed with p53 and MDM2, the p53 protein level was restored to a level comparable to that seen in the absence of the transfected MDM2 (Fig. 9, lane 4). Notably, the levels of p53 were restored in these triple-transfected cells, even in the presence of quite high levels of MDM2 protein resulting from MDMX binding (Fig. 9, lane 4). The data obtained in these experiments, therefore, demonstrate that MDMX blocks MDM2-mediated degradation of p53.

The transfection of cells with a construct expressing MDMX together with p53 reproducibly resulted in a p53 protein level that was actually higher than that detected in the absence of MDMX (Fig. 9, compare lanes 1 and 3). This finding indicates that, unlike MDM2, MDMX does not target p53 for degradation; it is also consistent with the idea that MDMX is inhibiting p53 degradation mediated by the endogenous MDM2 protein.

To investigate further the effects of MDMX on this critical MDM2 function, transfection assays were also carried out using the N-terminally truncated MDMX-(101–490) mutant. Since this protein is impaired in its ability to bind p53 directly, any influence it might have on p53 stability would result from any influence it might have on p53 stability would result from the ability of MDMX to promote p53 degradation. Transient transfections were carried out using the p53-MDMX Binds and Stabilizes MDM2

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Fig. 9. MDMX inhibits MDM2-mediated degradation of p53. H1299 cells were transiently transfected with combinations of expression plasmids encoding MDM2 (1 μg), p53 (0.025 μg), MDMX (5 μg), or Myc-tagged MDMX (101–490) (5 μg), as indicated. To determine steady-state levels of p53 and MDM2 protein, total cell lysates were subjected to Western blot analysis using anti-p53 monoclonal antibody (DO-1) or anti-MDM2 monoclonal antibody (IF2). Equal loading of cell lysates was confirmed by probing the blot with an anti-β-actin antibody.

DISCUSSION

This investigation was initiated with the goal of identifying interactions between MDM2 and proteins that may be important in influencing its cellular function or expression. The studies described here provide evidence for hetero-oligomerization between MDM2 and MDMX. The interaction of these proteins inhibits the degradation of MDM2, as well as the MDM2-mediated turnover of p53. Thus, MDMX can modulate MDM2 expression and function.

Both MDM2 and MDMX contain C-terminal RING finger motifs. Such domains, which have been defined as a spatially conserved set of cysteine-histidine residues of the type C3HC4, are present in a large and structurally diverse group of proteins (32, 33). Some RING domains seem to provide a surface for protein-protein interactions (32, 33, 40–43). In agreement with this idea, our results indicate that the RING finger domains of MDM2 and MDMX mediate their interaction; an intact RING finger motif within both MDM2 and MDMX is necessary for their association. However, for MDM2 as opposed to MDMX, this domain may not be sufficient; for MDM2 it seems likely that the zinc finger domain also may be required for hetero-oligomerization. Nonetheless, the RING-RING interaction between MDM2 and MDMX provides further evidence that RING domains mediate protein-protein interactions.

Whereas MDM2 and MDMX are structurally related proteins, little is known about the normal cellular functions of the MDMX gene. A major advantage of this scenario, however, MDMX has the potential to modulate the expression and biological function of both MDM2 and p53.

An association between MDM2 and MDMX proteins could influence MDM2/p53 expression in a variety of tissues, perhaps affecting cell growth or differentiation pathways. Northern blot analyses indicate that MDMX transcripts are expressed at very low levels in most tissues, with relatively higher levels in the thymus (34, 35). Reagents, particularly high quality antibodies, needed to examine MDMX protein expression and function in different cells are just beginning to be developed. Nevertheless, we have been able to demonstrate an interaction of epitope-tagged MDMX with endogenous MDM2 in mammalian cells. Interestingly, our yeast two-hybrid screen for MDM2-interacting proteins was carried out using a human thymus cDNA library, and it led to the isolation of several MDMX-derived clones. As suggested by this study, high levels of MDMX protein in a tissue or cell would be expected to interfere with MDM2 turnover, resulting in elevated expression of MDM2 and p53. In a preliminary Western blot analysis to test this possibility, we found that MDM2 and p53 protein levels in the thymus are, in fact, higher than in a number of other tissues examined.2 Clearly, however, additional studies are needed to test rigorously this hypothesis. A clarification of the interplay among MDM2, MDMX, and p53 should provide a better understanding of the function of these proteins in normal and neoplastic cells.

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