MdmX Is a RING Finger Ubiquitin Ligase Capable of Synergistically Enhancing Mdm2 Ubiquitination*

Received for publication, August 22, 2002, and in revised form, October 15, 2002 Published, JBC Papers in Press, October 21, 2002, DOI 10.1074/jbc.M208593200

James C. Badciong‡ and Arthur L. Haas§
From the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

It has been well documented that Mdm2 and its homologue MdmX not only are critical negative regulators of the tumor suppressor p53 but that both Mdm2 and MdmX interact to affect the function of the other. The mechanisms through which these effects are manifested, however, remain unclear. Although Mdm2 has been established as a RING finger ubiquitin ligase, MdmX has not been shown to possess this activity despite the extensive sequence homology between their respective RING finger domains. Here we demonstrate that MdmX acts as a ubiquitin ligase in vitro, being capable of autoubiquitination, as well as mediating the ubiquitination of p53. The addition of Mdm2 to in vitro ubiquitination assays containing MdmX results in a synergistic increase of ubiquitin conjugation. Analysis of the resulting ubiquitin conjugates reveals that this observed synergy reflects an increase in Mdm2 ubiquitination. This study also suggests that ubiquitination of Mdm2 and MdmX may not serve as a signal for degradation, as we show that each are capable of synthesizing non-lysine 48 polyubiquitin chains and, in fact, utilize multiple lysine linkages. Taken together, these findings suggest a more active role for MdmX in the Mdm2-MdmX-p53 regulatory network than has been proposed previously.

The covalent attachment of ubiquitin to proteins plays a fundamental role in the regulation of cellular function and is achieved through three enzymatic steps (1–4). In an ATP-dependent process, the ubiquitin activating enzyme (E1) catalyzes the formation of a reactive thiolester bond with ubiquitin, followed by its subsequent transfer to the active site cysteine of a ubiquitin carrier protein (E2). The specificity of ubiquitin ligation arises from the association of the E2-ubiquitin thiolester with ubiquitin-protein isopeptide ligase (E3), which facilitates the formation of the isopeptide linkage between ubiquitin and its target protein. Eukaryotes possess a single ubiquitin carrier protein (E2). The specificity of ubiquitin ligases is achieved through three enzymatic steps (1–4). In an ATP-dependent process, the ubiquitin activating enzyme (E1) catalyzes the formation of a reactive thiolester bond with ubiquitin, followed by its subsequent transfer to the active site cysteine of a ubiquitin carrier protein (E2). The specificity of ubiquitin ligation arises from the association of the E2-ubiquitin thiolester with ubiquitin-protein isopeptide ligase (E3), which facilitates the formation of the isopeptide linkage between ubiquitin and its target protein. Eukaryotes possess a single ubiquitin carrier protein (E2) and several families of E2 isoforms that are easily identified by conservation of a bipartite E2 consensus motif (1). The recent discovery that RING finger proteins often exhibit ubiquitin ligase activity has led to the rapid identification of numerous novel ligases (5–7). One of the first of these RING finger ubiquitin ligases to be characterized was the oncoprotein Mdm2 (8). The Mdm2 gene is amplified in a broad range of tumors including breast carcinomas, lung cancers, and osteosarcomas with the highest frequency (20%) in soft tissue tumors (9). It is well established that Mdm2 regulates the p53 tumor suppressor protein by inhibiting p33-mediated transactivation and by promoting its degradation (10, 11). For both of these mechanisms, elevated levels of Mdm2 can inhibit the protective functions of p53 and lead to oncogenesis. The ability of Mdm2 to negatively regulate p53 has sparked much interest in understanding how Mdm2 levels within the cell are controlled, demonstrated by numerous studies aimed at developing therapies designed to attenuate Mdm2 activity (12–15). One obvious mechanism to control Mdm2 function is regulation of protein stability. Like several other RING finger ligases, Mdm2 is capable of catalyzing its own ubiquitination thus providing a possible mechanism for autoregulation (8).

The Mdm2-related protein MdmX shares several regions of homology with Mdm2 including the p53 binding domain, a zinc finger motif, and a C-terminal RING finger domain (16). Although the RING finger domains of MdmX and Mdm2 are very similar (46% identity over 52 residues), with the residues implicated in zinc chelation being absolutely conserved, there has been no reported ubiquitin ligase activity by MdmX. This apparent lack of ligase activity has contributed to the ambiguity surrounding the biological role of MdmX. Although structurally similar, genetic evidence demonstrates that MdmX cannot compensate for the loss of Mdm2 expression during embryonic development in mouse (17, 18) nor can Mdm2 compensate for the loss of MdmX expression (19, 20), suggesting that the two proteins have non-overlapping functions. The fact that the embryonic lethal phenotype displayed by MdmX null mice can be rescued in a p53 null genetic background has further demonstrated the importance of MdmX as a negative regulator of p53 function (19, 20). The suggestion from these genetic studies that elevated MdmX levels may contribute to oncogenesis correlates with the recent reports of increased MdmX expression in tumor cell lines and malignant gliomas (21, 22).

Previous studies have demonstrated that Mdm2 and MdmX form both homo- and hetero-oligomers in vitro and in vivo, with hetero-oligomers exhibiting a more stable association; in addition, deletions that include the RING finger domain of either protein abrogate this interaction (23). Recent studies have shown that the half-life of Mdm2 is increased when co-transfected with MdmX and that this stabilization is dependent on the RING finger of MdmX, suggesting a requirement for hetero-oligomer formation (16). One current model proposes that this observed stabilization of Mdm2 is the result of the Mdm2-RING finger ligase activity of MdmX.
MdmX interaction inhibiting the E3 ligase activity of Mdm2, thereby preventing its efficient ubiquitination and stabilizing Mdm2 protein levels.

Here we demonstrate that MdmX possesses ubiquitin ligase activity requiring the Ubc4/5 family of E2 isoforms and that the RING finger domain of MdmX is necessary for this activity. We further show that in addition to its capacity for autoubiquitination, MdmX catalyzes the ubiquitination of p53 in vitro. Our investigation into the proposed ability of MdmX to inhibit Mdm2 ubiquitination has revealed that the interaction between Mdm2 and MdmX results in a synergistic increase in Mdm2 ubiquitination. That MdmX autoubiquitination, as well as the MdmX-mediated increase in Mdm2 ubiquitination, may not target these proteins for degradation is suggested by our demonstration that both Mdm2 and MdmX are capable of forming non-lysine 48-linked polyubiquitin chains. These observations challenge the current hypothesis that the MdmX-mediated stability of Mdm2 reflects a decrease in Mdm2 ubiquitination and support the classification of MdmX as a new member of the RING finger family of ligases.

MATERIALS AND METHODS

Bovine ubiquitin, creatine phosphokinase, and yeast inorganic pyrophosphatase were purchased from Sigma. Thrombin, purified from bovine plasma, was purchased from Amersham Biosciences. The ubiquitin was purified to apparent homogeneity (24) and then radiolabeled by the chloramine T method (25). Alternatively, some of the purified ubiquitin was modified by reductive methylation (26). Carrier-free Na125I and [2,8-3H]ATP were purchased from Amersham Biosciences. Human E1 from outdated human erythrocytes was purified to apparent homogeneity by modifying affinity chromatography and FPLC methods (27) and then quantitated by [35S]-ubiquitin thiolester assays and confirmed by the stoichiometric formation of ubiquitin [35S]H adenylate (28, 29). Plasmids—The entire coding sequence of human MdmX was amplified from a full-length cDNA (expressed sequence tag clone 177586) by polymerase chain reaction using Pfu polymerase (Stratagene). Appropriate primers were used during this amplification to allow for the in-frame insertion of the MdmX cDNA into the pGEX 4T-1 vector at the BamHI and XhoI sites. The construction of pGEX-MdmX394 was achieved by digesting pGEX-MdmX with EcoR I and XhoI and blunting the 5’-overhangs with Klenow fragment, followed by re-ligation. This resulted in the deletion of the 96 carboxyl-terminal amino acids of MdmX. As was done with MdmX, the entire open reading frame of human p53 was amplified from a full-length p53 cDNA (expressed sequence tag clone 610916) utilizing appropriate primers to allow for the in-frame insertion of the p53 cDNA into the pGEX 4T-1 vector at the BamHI and XhoI sites. A full-length human Mdm2 cDNA was obtained by polymerase chain reaction using a pGEX-Mdm2 cDNA construct kindly provided by Dr. Allan Weissman. Primers used in this amplification facilitated the insertion of this Mdm2 cDNA into the EcoRI and XhoI sites of a modified pGEX-T4-1 vector containing a FLAG epitope fused to the C terminus of the GST coding sequence.

Recombinant Protein Production—The production and purification of human recombinant E2 proteins was performed as described previously (30). The concentration of active E2 protein in each preparation was determined by quantitation of the E1-catalyzed stoichiometric formation of the corresponding [35S]-ubiquitin thiolester. Ubiquitin lysine to arginine point mutants were produced and purified as described previously (24, 31). DNA sequencing of the open reading frame of each plasmid, as well as mass spectrometry of each of the purified polypeptides, were performed to confirm that the expressed protein contained the appropriate lysine to arginine mutation. The human MdmX, MdmX394, and FLAG-Mdm2 were expressed as N-terminal GST fusions in logarithmic phase Escherichia coli (Rosetta™Novagen, Madison, WI) grown at 30 °C. Expression at 30 °C for 2 h was induced with 0.1 mM isopropyl-1-thio-galactopyranoside. Following purification by nickel affinity chromatography, the fusion proteins were further purified by FPLC. The fusion proteins were adsorbed to a Mono Q HR5/5 anion exchange FPLC column equilibrated with 50 mM Tris-HCl (pH 7.6) at a flow rate of 1 ml/min and then eluted with a linear 0–0.5 M NaCl gradient (12.5 ml/min). Fractions containing the fusion protein, as determined by immunoblotting, were pooled and concentrated using an Ultrafree-4 centrifugal filtration device with a 5-kDa molecular mass exclusion (Millipore). The GST-FLAG-Mdm2 fusion protein was digested with thrombin (30 units/ml) for 3 h at room temperature to cleave the GST moiety, which was subsequently removed by gluthathione affinity chromatography. Quantitation of GST-MdmX, GST-MdmX394, and FLAG-Mdm2 was performed by silver staining using known concentrations of bovine serum albumin as standards.

Ubiquitin Conjugation Assays—Conjugation reactions were adapted from earlier kinetic methods (30). Reaction volumes of 25 μl contained 50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM dithiothreitol, 2 mM ATP, 10 mM creatine phosphate, 20 IU/ml creatine phosphokinase, 5 mM human ubiquitin activating enzyme, 400 nM of the indicated ubiquitin conjugating enzyme, and the indicated concentration of Mdm2 or MdmX. Reactions were initiated by addition of either [35S]-ubiquitin (typically ~104 cpm/μmol) or unlabeled ubiquitin and allowed to proceed for 15 min at 37 °C. Reactions were quenched with 25 μl of Laemmli sample buffer containing 2% (v/v) β-mercaptoethanol and boiled for 3 min. The conditions for conjugation reactions containing GST-p53 were similar to those stated above with the following modifications. Reaction volumes of 50 μl containing 40 nM human ubiquitin activating enzyme, 150 nM HuUbuc5A, 400 nM GST-p53, 4 μg FLAG-Mdm2, and 400 nM GST-MdmX were incubated for 2 h at 37 °C either in the absence or presence of 10 μM unlabeled ubiquitin. Reactions were quenched with 50 μl of Laemmli sample buffer containing 2% (v/v) β-mercaptoethanol and boiled for 3 min.

Avidity of selected recombinant human E2 isoforms to support in vitro ubiquitination has revealed that the interaction between Mdm2 and MdmX results in a synergistic increase in Mdm2 ubiquitination. That MdmX autoubiquitination, as well as the MdmX-mediated increase in Mdm2 ubiquitination, may not target these proteins for degradation is suggested by our demonstration that both Mdm2 and MdmX are capable of forming non-lysine 48-linked polyubiquitin chains. These observations challenge the current hypothesis that the MdmX-mediated stability of Mdm2 reflects a decrease in Mdm2 ubiquitination and support the classification of MdmX as a new member of the RING finger family of ligases.
MdmX Ubiquitin Ligase Activity Is Dependent on Its RING Finger Domain—After we established that MdmX possessed E3 activity, we sought to confirm that this property was associated with its RING finger. Toward this end, we generated a truncated MdmX construct (MdmXΔ394) in which translation was terminated at codon 394, deleting the 96 C-terminal residues of the wild-type protein in which the RING finger is located (Fig. 2A). MdmXΔ394 was expressed as an N-terminal GST fusion and compared with the full-length GST-MdmX to confirm the deletion of 11 kDa (Fig. 2B, lane 2 versus lane 3). MdmX has been reported to migrate at ~80 kDa contrary to its theoretical mass of 54 kDa (36), which is similar to the aberrant migration of Mdm2 (~90-kDa apparent versus 54-kDa theoretical) (37). The apparent relative molecular masses of full-length GST-MdmX and of GST-MdmXΔ394 corresponding to 110 and 97 kDa, respectively, are consistent with the pre-

Fig. 1. GST-MdmX interacts specifically with HsUbc5A to catalyze the formation of GST-MdmX-Ub conjugates. A, in vitro conjugation assays containing 5 nM human E1, 5 μM 125I-ubiquitin, and 400 nM of the indicated E2 paralog were incubated in the absence or presence of 800 nM GST-MdmX as described under “Materials and Methods.” The 15-min incubation time was empirically shown to be within the initial velocity region for the assay. The reactions were resolved by SDS-PAGE followed by autoradiography. B, GST-MdmX was immunoprecipitated with an anti-MdmX polyclonal antibody from a parallel ubiquitin conjugation reaction identical to lane 6). Immuno-precipitated GST-MdmX (IP) was resolved by SDS-PAGE followed by autoradiography. Samples obtained in the absence (−; lane 2) or presence of anti-MdmX (++; lane 3) were volume normalized to equal an aliquot of the original reaction (Input; lane 1). The mobilities of molecular weight standards are shown to the left.

lane 5 versus lane 6). As has been documented with other RING finger ligases, these ubiquitin conjugates were polyubiquitinated and migrated at the top of the gel (6). That the conjugates contained polyubiquitin chains was demonstrated by the complete abrogation of the high molecular weight adducts when 125I-reductively methylated ubiquitin was substituted for wild-type radiolabeled ubiquitin (data not shown).

We next addressed the nature of the ubiquitin conjugates by determining whether they represented MdmX autoubiquitination or the ubiquitination of trace contaminating proteins present in the enzyme preparations. After repeating the conjugation reaction of Fig. 1A (lane 6), we isolated GST-MdmX by immunoprecipitation with an anti-MdmX polyclonal antibody and found that GST-MdmX-Ub conjugates represented the majority (~70%) of the total ubiquitin conjugates in the reaction (Fig. 1B). Because antibody concentrations were empirically optimized in all instances to ensure quantitative immunoprecipitation, the inability quantitatively to immunoprecipitate the ubiquitin adducts suggests MdmX either is capable of catalyzing the formation of free polyubiquitin chains, as found for E2γ3K (35), or mediates conjugation to contaminating proteins present in the assay.

MdmX Ubiquitin Ligase Activity Is Dependent on Its RING Finger Domain
dicted anomalous migration for deletion of the RING finger domain from GST-MdmX.

The ligase activity of MdmXΔ394 relative to that of full-length MdmX was assayed using biochemically defined in vitro ubiquitin conjugation reactions. Although full-length MdmX displayed ligase activity, we observed no ubiquitin conjugates formed in the presence of an equal concentration of MdmXΔ394 or in the presence of MdmXΔ394 at a level three times greater than the full-length protein (Fig. 2C, lane 2 versus lanes 3 and 4). In an effort to determine whether less drastic alterations to MdmX would also abrogate its E3 activity, we attempted to selectively disrupt its RING finger. Because Zn$^{2+}$ stabilizes RING finger domains (38, 39), we sequestered Zn$^{2+}$ from our MdmX protein preparations using the specific Zn$^{2+}$ chelator TPEN (40), which abrogates the E3 activity of Mdm2 (8). When MdmX was treated with 2 mM TPEN, we observed a biphasic decrease in ligase activity over time, presumably reflecting the time-dependent removal of Zn$^{2+}$ from the RING finger domain (Fig. 2D). Disruption of either Zn$^{2+}$ within the RING finger results in loss of ubiquitin ligase activity (8). Therefore, the biphasic kinetics for loss of MdmX activity most likely reflects different affinities for the two positions, the loss of either of which abrogates activity. Unlike Mdm2, whose activity is quantitatively restored after TPEN treatment by addition of ZnCl$_2$ (8), similar reconstitution of MdmX activity resulted in only a 4-fold increase in ubiquitin ligase activity (11% of the initial activity) after an overnight incubation in the presence of 3 mM ZnCl$_2$. data not shown. These observations are consistent with an intact RING finger motif being required for MdmX-mediated ubiquitin ligase activity.

**MdmX and Mdm2 Catalyze the Formation of Polyubiquitin Chains through Multiple Lysine Linkages**—The ability of GST-MdmX to catalyze autoubiquitination suggests a mechanism for autoregulation. Because polyubiquitin chains linked via lysine 48 are the principle signal for targeting proteins for degradation (41) we sought to determine the linkage utilized by MdmX for chain formation. For this determination, we utilized our collection of ubiquitin lysine point mutants (24), each of which had one of the seven lysine residues contained within human ubiquitin mutated to arginine. These Lys$\rightarrow$Arg point mutants have been used successfully in the past to demonstrate formation of unique chain linkages by ScUbc2/Rad6 and HsE2epf (24). Using a ubiquitin-specific rabbit polyclonal antibody to detect the formation of polyubiquitin conjugates, we reconfirmed the ability of MdmX to form large molecular weight ubiquitin conjugates (Fig. 3A, lane 2). That these conjugates reflected the formation of polyubiquitin chains was confirmed by their elimination when reductively methylated ubiquitin, which is unable to form chains (24), was used (Fig. 3A, lane 3 versus lane 2). Substitution of ubiquitin Lys$\rightarrow$Arg point mutants in these conjugation reactions revealed that GST-MdmX was capable of using each mutant to catalyze polyubiquitin chain formation (Fig. 3A, lanes 4–10). This experiment demonstrates not only that MdmX is capable of synthesizing non-Lys$^{48}$ chains but that MdmX does not use one lysine exclusively for chain formation.

Given the homology between Mdm2 and MdmX, we asked whether Mdm2 could also utilize multiple lysine residues for chain formation. Using both wild-type and reductively methylated ubiquitin we were able to show that FLAG-Mdm2 catalyzed the formation of large molecular weight ubiquitin conjugates that were the result of polyubiquitin chain formation (Fig. 3B, lanes 2 and 3). Just as was seen with MdmX, when the ubiquitin Lys$\rightarrow$Arg point mutants were substituted, Mdm2 was able to catalyze polyubiquitin chain formation (Fig. 3B, lanes 4–10). Recent reports have shown that HsUbc5A has been associated with the catalysis of Lys$^{29}$- and Lys$^{48}$-linked chains (42, 43); however, the signaling function of Lys$^{29}$ ubiquitin chains remains unclear. It is worthwhile to note that other members of the RING finger family of ligases have been shown to generate non-Lys$^{48}$ ubiquitin chains, although their ability to utilize multiple lysine linkages have not been examined (44). The generation of ubiquitin Lys$\rightarrow$Arg double mutants will be used in future experiments to determine whether both Lys$^{29}$ and Lys$^{48}$ are required for MdmX and Mdm2-mediated chain formation.

**The Interaction Between Mdm2 and MdmX Enhances Ubiquitin Ligase Activity**—Several studies have demonstrated through transfection assays that MdmX overexpression stabilizes Mdm2 in vivo (16, 45). This has led to the suggestion that Mdm2-MdmX association results in the inhibition of Mdm2 autoubiquitination and a concomitant decrease in the rate of 26 S proteasome-mediated Mdm2 degradation (45, 46). To test this directly, we examined the ability of MdmX to inhibit Mdm2-mediated autoubiquitination in vitro. That FLAG-Mdm2 and GST-MdmX associated with each other in in vitro assays was confirmed by co-immunoprecipitation of GST-MdmX with FLAG-Mdm2 in the presence, but not in the absence, of an anti-Mdm2 monoclonal antibody (data not shown).

To ensure that the FLAG-Mdm2 in the conjugation reaction was associated with GST-MdmX and not with other FLAG-Mdm2 molecules, GST-MdmX was added at a 200-fold molar excess. This large ratio of MdmX:Mdm2, in addition to the

**Fig. 3.** GST-MdmX and FLAG-Mdm2 utilize multiple lysine linkages for polyubiquitin chain formation. **A**, in vitro ubiquitin conjugation reactions identical to those in Fig. 1, lane 6, contained either no GST-MdmX (lane 1) or 900 nM GST-MdmX (lanes 2–10) and 10 μM unlabeled wild-type (wt), reductively methylated (rm), or mutant ubiquitin. The MdmX-ubiquitin conjugates were resolved by SDS-PAGE (7.5% gel) and then visualized by Western blot analysis using an anti-ubiquitin polyclonal antibody. Migration of molecular weight standards is shown to the left. **B**, in vitro ubiquitin conjugation reactions containing either no FLAG-Mdm2 (lane 1) or 4 nM FLAG-Mdm2 (lanes 2–10) as the source of ligase activity and the indicated unlabeled ubiquitin were assembled as described in A, with resulting Mdm2-ubiquitin conjugates being visualized by Western blot analysis using an anti-ubiquitin polyclonal antibody.
The mobility of poly-[125I]-ubiquitin conjugates following SDS-PAGE resolution. The mobility of molecular weight standards is shown in the left panel. In vitro ubiquitin conjugation reactions contained 5 nM human Mdm2, 800 nM GST-MdmX, or 800 nM GST-MdmXΔ394. The resulting ligase activity was visualized by autoradiography of [125I]-ubiquitin conjugates following SDS-PAGE resolution. The mobility of molecular weight standards is shown to the left. In vitro ubiquitin conjugation reactions contained 5 nM human Mdm2, 800 nM GST-MdmX, or 800 nM GST-MdmXΔ394. The resulting ligase activity was visualized by autoradiography of [125I]-ubiquitin conjugates following SDS-PAGE resolution. The mobility of molecular weight standards is shown in the left panel. 

We examined whether this increased activity required the interaction of Mdm2 and MdmX by substituting MdmXΔ394 for full-length MdmX in these assays, because similar MdmX truncations fail to interact with Mdm2 both in vitro and in vivo (23, 45). The failure of MdmXΔ394 to induce an increase in ubiquitin conjugation (Fig. 4A, lane 2 versus lane 4) suggests that the association of Mdm2 and MdmX is necessary for the increased conjugation and that this increase does not result from conjugation to contaminating bacterial proteins acting as non-specific substrates. Quantitation of the initial rates of ligase activity revealed that the increase was not simply an additive effect of Mdm2 and MdmX activity but instead reflected an approximate doubling of total ligase activity (Fig. 4B). These results demonstrate that the Mdm2-MdmX association does not inhibit E3 activity as proposed in the literature; rather, it enhances the rate of ubiquitin ligation.

If the association between Mdm2 and MdmX induces this synergistic increase in ubiquitin ligation, one would expect to see this same effect at lower MdmX:Mdm2 ratios than were used in the previous assay. In the experiment of Fig. 4A, the concentration of GST-MdmX was twice that of HsUbe5A, leading to the potential that such a large excess could actually reduce the rate of conjugation in the reaction by competing with Mdm2-MdmX complexes for HsUbe5A binding. This was addressed by performing in vitro conjugation reactions in which the concentration of FLAG-Mdm2 was held constant at 2.5 nM and [GST-MdmX], varied from 2.5 to 320 nM at a HsUbe5A concentration of 400 nM. Parallel in vitro conjugation reactions, which did not include FLAG-Mdm2, were performed to measure the initial rate of ubiquitin conjugation attributed to GST-MdmX ligase activity at the various concentrations. These values were then used to correct the initial rate of ubiquitin ligation in the reactions containing both FLAG-Mdm2 and GST-MdmX so that Fig. 4C represents the net initial rate of ubiquitin conjugation. These data are plotted as the net increase in ubiquitin conjugate formation above that observed for either Mdm2 or MdmX alone. The line through the data represents a hyperbolic regression fit of the data.
transient Mdm2₂-MdmX₃ heterotetramer; therefore, it is not possible to interpret the physical meaning of the Kᵢ₂ value. However, the data demonstrate that a significant MdmX-mediated synergistic increase in ubiquitin conjugation occurs at a modest, presumably physiological, molar excess of MdmX (panel C) and that sequestering of HsUbc5A is a potential problem in interpreting the effects of MdmX when in excess over HsUbc5A (panel B).

**MdmX Induces a Synergistic Increase in Mdm2 Ubiquitination**—Although the experiments described in Fig. 4 showed that MdmX had a positive effect on ubiquitin ligation, they did not address the identity of the substrate for the enhanced ubiquitin conjugation. The tendency of Mdm2 and MdmX to form heterocomplexes raised the question of whether this complex, when associated with p53, would enhance the ubiquitination of the substrate. Because we had demonstrated that MdmX is a RING finger ubiquitin ligase with similar catalytic properties to Mdm2, we next addressed whether MdmX could ubiquinate p53, a known Mdm2 substrate (5, 8, 47). Conjugation reactions containing GST-MdmX as the only source of ligase activity and GST-p53 revealed that MdmX was capable of ubiquitinating p53 in vitro, as detected by immunostaining of GST-p53 (Fig. 6). The pattern and level of p53 ubiquitination by MdmX was similar to that of FLAG-Mdm2, except that MdmX-derived conjugates exhibited a greater level of p53 ubiquitination and cross-linking, as detected by immunoprecipitation using an anti-MdmX polyclonal antibody. In contrast, detection with anti-Mdm2 revealed a dramatic increase in the formation of polyubiquitinated Mdm2 on addition of MdmX that correlated with a decrease in non-ubiquitinated Mdm2 (Fig. 5). From this observation, we conclude that the Mdm2-MdmX heterocomplex has an enhanced ability to ubiquitinate Mdm2 over that of the Mdm2 homocomplex. Taken together with the earlier observation that MdmX increases the overall rate of ubiquitin ligation (Fig. 4B), these results indicate that contrary to models based on in vivo overexpression of MdmX, the interaction between MdmX and Mdm2 results in a synergistic increase in the ubiquitination of Mdm2.

**MdmX Catalyzes the Ubiquitination of p53 but Does Not Affect Mdm2-mediated Ubiquitination of p53**—Because we had demonstrated that MdmX is a RING finger ubiquitin ligase with similar catalytic properties to Mdm2, we next addressed whether MdmX could ubiquinate p53, a known Mdm2 substrate (5, 8, 47). Conjugation reactions containing GST-MdmX as the only source of ligase activity and GST-p53 revealed that MdmX was capable of ubiquitinating p53 in vitro, as detected by immunostaining of GST-p53 (Fig. 6, lane 4). The pattern and level of p53 ubiquitination by MdmX was similar to that of FLAG-Mdm2-mediated ubiquitination of p53 (Fig. 6, lane 4 versus lane 2); however, it must be noted that MdmX levels were 100-fold higher than Mdm2 in these assays, because MdmX consistently displayed lower ligase activity when compared with Mdm2.

**In vivo**, Mdm2, MdmX and p53 form a heterotrimeric complex (45). Our finding that the formation of the Mdm2-MdmX heterocomplex was associated with increased ligase activity raised the question of whether this complex, when associated with p53, would enhance the ubiquitination of the substrate.
When both GST-MdmX and FLAG-Mdm2 were included in the p53 conjugation assay, we found no increase in the levels of p53-Ub conjugate formation when compared with levels catalyzed by either MdmX or Mdm2 alone (Fig. 6, lane 6 versus lanes 2 and 4). These results not only provide the first evidence that MdmX is capable of ubiquitinating p53 in vitro but also suggest that the increased ligase activity associated with the MdmX-Mdm2 heterocomplex involves its auto-ubiquitination and not the ubiquitination of other substrates.

**DISCUSSION**

Genetic studies investigating the physiological consequences of the loss of either Mdm2 or MdmX function have demonstrated the requirement of both proteins for normal development and that each has non-overlapping roles (17–20). Although one clear finding from these genetic studies is that each protein is involved in the negative regulation of the tumor suppressor p53, how these two related proteins affect the function of the other remains ambiguous. Recent studies have demonstrated that overexpression of MdmX stabilizes Mdm2 protein levels in vivo (16, 45, 46); however, the mechanism by which this occurs remains unclear. One proposed model asserts that the MdmX-Mdm2 interaction abrogates the E3 ligase activity of Mdm2, thereby reducing the ability of Mdm2 to target itself for 26 S proteasome-dependent degradation (45, 46). Central to this model are the assumptions that MdmX does not possess intrinsic ubiquitin ligase activity and that the oligomerization of these proteins, presumably via their RING finger domains, inhibits Mdm2 ligase activity. Contrary to these assumptions, we have directly demonstrated in biochemically defined assays that MdmX is a RING finger ubiquitin ligase (see Figs. 1 and 2) and that the association of MdmX with Mdm2 results in the increased polyubiquitination of Mdm2 (see Figs. 4 and 5). At present, we cannot unambiguously distinguish whether MdmX directly conjugates Mdm2 or whether hetero-oligomer formation exerts a positive allosteric effect on the ligase activity of Mdm2. However, observation that heterodimer formation increases initial rates for Mdm2, as well as Mdm2 conjugation (Fig. 5), tends to favor a mechanism of mutual allosteric activation.

The fact that the ligase activity of MdmX has been overlooked until now may be a reflection of its relatively low activity in autoubiquitination, ranging from 0.1 to 1% of that observed with Mdm2. The observed increase in ligase activity of the Mdm2-MdmX complex correlates with growing evidence that the dimerization of RING finger ligases does not interfere with their intrinsic E3 activity a priori. Recent studies with the breast and ovarian cancer tumor suppressor BRCA1 and one of its interacting proteins, BARD1, both of which are RING finger proteins possessing E3 activity, demonstrate that ubiquitin ligation is dramatically enhanced for the heterodimer (44, 48). In fact, the structure of the BRCA1-BARD1 RING-RING complex reveals that the RING finger domains of the heterodimer make relatively few contacts (49).

Our finding that MdmX enhances the ubiquitination of Mdm2, as well as our demonstration of MdmX-mediated ubiquitination of p53, appears to be in conflict with studies demonstrating MdmX-mediated stabilization of both Mdm2 and p53, respectively. In the case of Mdm2, this discrepancy can be resolved by invoking a mechanism other than the inhibition of Mdm2 ubiquitination to explain the observed increase in the half-life of Mdm2. One possible mechanism is that MdmX acts to sequester Mdm2 to a subcellular compartment having a lower rate of Mdm2 degradation. Mdm2 contains a leucine-rich nuclear export sequence that has been shown to be required for its exit from the nucleus (50, 51). In contrast, MdmX contains a nuclear export sequence similar to Mdm2 with the exception of a four-amino acid insertion (52) that could prevent MdmX from interacting with nuclear export machinery and may account for MdmX predominantly localizing within the nucleus (52). As a result, the association of MdmX with Mdm2 could impede nuclear export, thereby increasing the half-life of Mdm2.

Stad et al. (46) have invoked a similar nuclear sequestration scenario to explain the observed stabilizing effect of MdmX on p53. In this way, the interaction between MdmX and p53 could stabilize p53 levels even if MdmX ubiquitimates p53.

Alternatively, resolution of these apparent conflicting observations may rest in the expression levels of MdmX during transfection assays. Evidence for the stabilization of Mdm2 by MdmX is predicated on overexpression of the latter (45, 46); however, MdmX levels within cells are normally relatively constant (53). Quantitative biochemically defined rate assays in the present study demonstrate that, like Mdm2, MdmX is a RING finger ligase requiring ubiquitin conjugating enzymes of the Ubč4/5 family for activity (Fig. 1). Therefore, stabilization of Mdm2 by MdmX overexpression may simply represent the sequestration of Ubč4/5 isoforms by abnormally elevated levels of ectopic MdmX, thus interfering with the ability of Mdm2 to catalyze Ubč4/5-mediated auto-ubiquitination. The potential problems associated with overexpression of components of the ubiquitin ligation pathways for cellular dynamics have been discussed previously (1).

It is also important to note we have shown for the first time that both Mdm2 and MdmX can catalyze the formation of non-lysine 48 polyubiquitin chains. This strongly suggests that the increase in Mdm2 ubiquitination in the presence of MdmX also involves noncanonical chains, although this has not been experimentally established. If the Mdm2-MdmX complex does synthesize noncanonical chains, it raises the possibility that the increase in Mdm2 ubiquitination may not, in fact, serve as a signal for degradation as there is evidence that ubiquitin chains that utilize noncanonical lysine linkages are not appropriate signals for degradation (54). Again, a parallel can be drawn between the Mdm2-MdmX complex and the BRCA1-BARD1 complex, because auto-ubiquitination of the latter involves the formation of non-lysine 48 chains resulting in the mutual stabilization of BRCA1 and BARD1 (55).

Although it has clearly been shown in the literature that MdmX interacts with both p53 and Mdm2, the assignment of a specific biological role for MdmX has yet to be established. Although the fact of MdmX remains elusive, its amplification in several tumor cell lines (22), and its requirement during development (20) leave little doubt of its biological significance. Indeed, the present observation that MdmX enhances Mdm2 ubiquitination redefines the functional relationships within this fundamentally important regulatory network. Our finding that MdmX is a new member of the RING finger family of ubiquitin ligases also offers new insights into possible function(s) of MdmX unrelated to Mdm2 and p53 interactions and suggests MdmX mediates the ubiquitination of novel substrates yet to be identified.

Acknowledgments—We thank Dr. A. M. Weissman for the generous gift of the human Mdm2 cDNA clone and members of our laboratory for helpful discussions.

**REFERENCES**

1. Haas, A. L., and Siepmann, T. J. (1997) FEBS Lett. 411, 1257–1268
2. Herskine, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
3. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–533
4. Stroob, G. J., and Govers, R. (1999) J. Cell Sci. 112, 1417–1423
5. Honda, R., Tanaka, H., and Yasuda, H. (1997) FEBS Lett. 398, 25–27
6. Lebeck, R. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11364–11369
7. Joaeno, C. A., Wing, S. S., Huang, H., Leveryson, J. D., Hunter, T., and Liu, Y. C. (1999) Science 286, 309–312
8. Fang, S., Jensen, J. P., Ludwig, R. L., Vouwen, K. H., and Weissman, A. M. (2000) J. Biol. Chem. 275, 8845–8851
