The Mdm-2 Amino Terminus Is Required for Mdm2 Binding and SUMO-1 Conjugation by the E2 SUMO-1 Conjugating Enzyme Ubc9*

Received for publication, April 27, 2001
Published, JBC Papers in Press, May 30, 2001, DOI 10.1074/jbc.M103786200

Thomas Buschmann, Dimitri Lerner, Chee-Gun Lee‡, and Ze’ev Ronai§

From the Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York 10029, the ‡Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07013

Covalent attachment of SUMO-1 to Mdm2 requires the activation of a heterodimeric Aos1-Uba2 enzyme (ubiquitin-activating enzyme (E1)) followed by the conjugation of Sumo-1 to Mdm2 by Ubc9, a protein with a strong sequence similarity to ubiquitin carrier proteins (E2s). Upon SUMO-1 conjugation, Mdm2 is protected from self-ubiquitination and elicits greater ubiquitin-protein isopeptide ligase (E3) activity toward p53, thereby increasing its oncogenic potential. Because of the biological implication of Mdm2 sumoylation, we mapped Ubc9 binding on Mdm2. Here we demonstrate that Ubc9 can associate with Mdm2 only if amino acids 40–59 within the N terminus of Mdm2 are present. Mdm2 from which amino acids 40–59 have been deleted can no longer be sumoylated. Furthermore, addition of a peptide that corresponds to amino acids 40–59 on Mdm2 to a sumoylation reaction efficiently inhibits Mdm2 sumoylation in vitro and in vivo. In UV-treated cells Mdm2 exhibits reduced association with Ubc9, which coincides with decreased Mdm2 sumoylation. Our findings regarding the association of Ubc9 with Mdm2, and the effect of UV-irradiation on Ubc9 binding, point to an additional level in the regulation of Mdm2 sumoylation under normal growth conditions as well as in response to stress conditions.

Regulation of protein stability is important in cellular proliferation, differentiation, and response to stress. A delicate balance between protection of a protein from, or targeting it for, degradation underlies the regulation of proteolysis and determines the duration and magnitude of activities elicited by key regulatory proteins. Conjugation of ubiquitin or ubiquitin-like proteins is mediated by multiple enzymatic reactions catalyzed by a single ubiquitin-activating enzyme (E1), a few ubiquitin carrier proteins (E2s), and when applicable, by a large variety of ubiquitin-protein isopeptidase ligases (E3s) (reviewed in Refs. 1–3). The side chains of lysyl residues serve as a conjugation site for ubiquitin, as well as for the small ubiquitin-like modifier protein SUMO-1 (4–6). SUMO-1, which is 18% identical to ubiquitin, requires a heterodimeric Aos1-Uba2-activating enzyme (E1) and E2-Ubc9 for conjugation to its respective sub-strates (7–11). Ubc9 is the first Sumo conjugating enzyme, which does not conjugate ubiquitin. Human Ubc9, which is composed of 158 amino acids, consists of conserved cysteines within the ubiquitin-accepting domain. Structure analysis of Ubc9 revealed important differences when compared with other ubiquitin-conjugating enzymes. Within the amino-termin al helix both structural and sequence alignments do not match to ubiquitin-conjugating enzymes due to one mismatched amino acid, which confers a different recognition surface on Ubc9 (12, 13). This together with a profound change in the electrostatic surface likely are critical to the recognition of selective substrates in the SUMO-1 conjugation pathways (12, 13). Covalent attachment of SUMO-1 (Sentrin, Ubl1, or Smt3) to lysines was demonstrated for a growing number of proteins, including RanGAP1 (Ran GTPase-activating protein 1) (14), IeBo (15), PML (16), p53 (17, 18), and Mdm2 (19).

Ubc9-mediated sumoylation has different physiological consequences, depending on the substrate. Whereas, sumoylation of RanGAP1 and RanBP2 affect their nucleoeytoplasmic trafficking (14, 20, 21), the Ubc9-mediated sumoylation of PML, sp100, TEL, and HIPK results in their localization within subnuclear structures (22–24). Ubc9-mediated SUMO-1 conjugation to IeB and Mdm2 was shown to take place at the same aa residue required for ubiquitination (15, 19). Sumoylation of androgen receptor, Ets, p53, and p73α was implicated in altering their transcriptional activities (17, 18, 25, 26); Ubc9-mediated SUMO-1 conjugation to topoiso merase 1, RAD51, and RAD52 was associated with better recognition of damaged DNA and DNA repair activities (27–29).

SUMO-1 conjugation is also implicated in regulating septin ring dynamics during the cell cycle in budding yeast (30) and in neural differentiation in Drosophila (31). Although SUMO-1 conjugation is implicated in various physiological processes (32), the function of SUMO-1 modification is not always understood. A growing list of proteins was shown to exhibit Ubc9 binding followed by SUMO-1 conjugation, although the implication of this modification to the function of the proteins is not yet clear. Among such proteins are Fas (Apo-1/CD95; Ref. 33), Werner syndrome protein (34) ATF2 (35), c-Jun (36), poly(ADP-ribose) polymerase (37), and viral proteins including bovine papillomavirus E1 (38), the cytomegalovirus 1E2-p86 (39), and the adenovirus E1A (40).

As an oncogene, Mdm2’s transforming potential is largely attributed to its ability to down-regulate the functions of the p53 tumor suppressor protein. Mdm2 has been implicated in the proteosome-dependent degradation of p53 (41, 42) via targeting p53 ubiquitination in vitro and in vivo (43, 44). Mdm2 mediates p53 ubiquitination through the carboxyl-terminal RING finger domain, which serves as a bona fide RING finger E3 (45, 46). Mdm2 belongs to a growing number of RING finger proteins, which directly bind their substrates and exhibit ubiquitin ligase activity, including mammalian homologues of seven...
in absentea, A07, cChl/Sll-1, and BRCA1 (47–50).

E3 activity of Mdm2 is directed toward self- and targeted ubiquitination of the tumor suppressor protein p53; thus, it maintains low levels of p53 under normal nonstressed conditions. Central to Mdm2 E3 activities is its Sumo modification, which attenuates self-ubiquitination and increases Mdm2-targeted ubiquitination of p53 (19). Sumoylation of Mdm2 takes place on a lysine residue at position 446, which also serves as the primary site for ubiquitin conjugation to Mdm2. Thus, upon its Sumo modification, Mdm2 is no longer subject to ubiquitination, gaining stability and greater ubiquitin-ligase activity toward p53. In response to radiation the degree of Mdm2 sumoylation decreases in a manner that coincides with the increased level of p53 (19).

Here we identify a 19-aa region on Mdm2 that is required for Ubc9 association and demonstrate the ability to alter the degree of Mdm2 sumoylation with a peptide corresponding to this region. We further illustrate the change in Ubc9 binding to Mdm2 after exposure to UV treatment, which coincides with altered Mdm2 sumoylation.

MATERIALS AND METHODS

Expression Vectors—Wild type Mdm2 cDNA was amplified by polymere chain reaction (HiFi Taq polymerase, Life Technologies, Inc.) and unidirectionally cloned, with the addition of two NH₂-terminal FLAG tags, between the EcoRI and BamHI sites of pcDNA3.1 (19). The sequence of the Ubc9 binding domain on Mdm2 (aa 40–59, mdm2 Ubc9 binding site; mu9bs) in wt or scrambled orientation (mu9sb) was cloned in frame between EcoRI and Xhol restriction sites into pcDNA3 that carries the penetratin sequence, followed by the sequence encoding the EcoRI tag. The bacterial expression vector of GST-Mdm2 (in pGEX-4T-1) carries the penetratin sequence, followed by the sequence encoding the XhoI tag. The bacterial expression vector of GST-Ubc9 (in pGEX-KG-1) was kindly provided by Dr. A. Weissman. p53³⁵ expression vector was described previously (45). pcDNA3-HA-Sumo-1 expression vector was kindly provided by Dr. R. Hay (10). The PGMV-HA-Ub expression construct was a gift from Dr. D. Bohmann (51). All Mdm2 mutants were cloned in pBlueprint KS⁺. All Mdm2 mutants in pBlueprint KS⁺ and kindly provided by Dr. J. Chen. Mutant Ubc9 (mut9b) was a gift from Olli Jänne.

Cells, Nondenaturing Immunoprecipitation, and Luciferase Assay—293T, adenovirus-transformed human embryonic kidney cells (a kind gift of Dr. E. Spanopoulou), p53/-/Mdm2 double null cells (kindly provided by Dr. S. Aaronson) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% heat-inactivated fetal bovine serum and antibiotics in 5% CO₂. Transfections were performed by lipofection (LipoFectAMINE Plus, Life Technologies, Inc.). Medium was changed 5 h after transfection, and cells were harvested 36 h later. To monitor Ubc9 protein complexes, lysates were lysed as described previously (19). Western blot analyses were carried out as described previously (44), except peptide analysis. To assure proper separation of small peptides Tricine gels were used according to standard protocol.

Antibodies—Monoclonal antibodies against HA epitopes (HA11) were purchased from Babco. Antibodies (monoclonal) against SUMO-1 (GMP-1) were purchased from Zymed Laboratories Inc. Polyclonal antibodies against Sumo-1 were produced in rabbits and affinity-purified on His-Sumo columns. 2A10 monoclonal antibodies (Oncogene) were used for Mdm2 analysis. Monoclonal antibodies against ubc9 were purchased (Transduction Laboratories, Lexington, KY).

In Vitro Conjugation of Sumo-1—Glutathione beads (Sigma) were used to purify GST-Mdm2 as described previously (45). Bacterially expressed purified Mdm2 (1–2 μg) proteins were incubated with in vitro translated [35S]-SUMO-1 (NTT, Promega), purified Aos1/Uba2 (15 ng), and Ubc9 (0.5 μg) for 30 min at 37 °C in conjunction buffer (20 mM Hapes, pH 7.4, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 1 unit of creatine phosphokinase). Bead-bound complexes were washed (3 × TBS, 0.5% Triton X-100) before denaturing for 5 min at 95 °C in 3 × sample buffer. Proteins were separated on 8% SDS-PAGE. Gel was stained with Coomassie Blue, dried, and exposed to x-ray film (X-Omat, Eastman Kodak Co.). For competition with in vitro sumoylation of Mdm2, a peptide corresponding to the Ubc9 binding region on Mdm2 (aa 40–59) or a non-specific peptide corresponding to was added as indicated in results.

Peptide Conjugation to Amino Link Column—Immunobilization of peptides to the Amino Link column was carried out exactly according to Pierce protocol. In brief, the column was equilibrated with coupling buffer, and 1 column volume of solution containing 1 mg of peptide was incubated with gel for 6 h. Later the column was drained and washed with coupling buffer. Coupling buffer was replaced by quenching buffer containing a reducing solution to block remaining active sites for 30 min. Final washing steps were carried out with washing buffer. The column was stored in storage buffer at 4 °C.

In Vivo Sumoylation/Ubiquitination—Cells were transfected with the indicated plasmids to analyze sumoylation or ubiquitination, respectively. Harvested cell pellets were lysed by incubation with 1 vol of 2% SDS in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) at 95 °C for 10 min. Nine volumes of 1% Triton X-100 in TBS were added, and lysates were sonicated for 2 min. The solution was incubated for 30 min at 4 °C with protein A/G beads (Life Technologies, Inc.) and clarified by 30-min centrifugation (14,000 rpm) at 4 °C. The protein concentration was determined using the Bradford assay. For immunoprecipitation 500 μg of protein was incubated with the appropriate antibodies at 4 °C overnight before protein A/G beads (25 μl) were added for 2 h. Beads were washed with 0.5 M LiCl in TBS followed by two additional washes with TBS. Proteins were loaded onto 8% SDS-PAGE, followed by immunoblot analysis with the indicated antibodies and ECL detection (Amersham Pharmacia Biotech).

UV Treatment—UV irradiation was administered as described previously (19). Briefly, cells were subjected to UV-C irradiation (254 nm) in calibrated areas within the tissue culture hood. The medium that was removed prior to irradiation was returned to minimize serum-induced changes.

RESULTS

Ubc9 Binding Is Mapped to the Mdm2 Amino-Terminal Domain—As noted, binding of Ubc9, which serves as an E2-conjugating enzyme for SUMO-1 (19), is prerequisite to Mdm2 sumoylation. To map the Ubc9 binding site on Mdm2, we incubated a series of [35S]-labeled Mdm2 deletion mutants with GST-Ubc9. These GST pull-down assays enabled us to determine which of the Mdm2 forms lost the ability to associate with Ubc9. As shown in Fig. 1A, Mdm2 from which aa 40–59 had been deleted was the only construct that was no longer capable of associating with Ubc9. This finding suggests that Ubc9 binding may require aa 40–59 of the amino-terminal domain of Mdm2. Forced expression of Mdm2 that lacks aa 40–59 confirmed that this protein is no longer associated with Ubc9 (Fig. 1B). Furthermore Mdm2⁴⁰–⁵⁹ migrates as a 75-kDa protein, which represents the nonsumoylated form of Mdm2 (Fig. 1B). These data confirm that aa 40–59 of Mdm2 are required for Ubc9 association.

Ubc9 Binds to a Peptide Corresponding to aa 40–59 from Mdm2—To further assess the role of the Ubc9 binding domain in Mdm2 sumoylation, we synthesized a peptide that corresponds to aa 40–59 of the amino-terminal domain of Mdm2. Forced expression of Mdm2 that lacks aa 40–59 confirmed that this protein is no longer associated with Ubc9 (Fig. 1B). Furthermore Mdm2⁴⁰–⁵⁹ migrates as a 75-kDa protein, which represents the nonsumoylated form of Mdm2 (Fig. 1B). These data confirm that aa 40–59 of Mdm2 are required for Ubc9 association.

Ubc9 Binds to a Peptide Corresponding to aa 40–59 from Mdm2—To further assess the role of the Ubc9 binding domain in Mdm2 sumoylation, we synthesized a peptide that corresponds to aa 40–59 of the amino-terminal domain of Mdm2. Forced expression of Mdm2 that lacks aa 40–59 confirmed that this protein is no longer associated with Ubc9 (Fig. 1B). Furthermore Mdm2⁴⁰–⁵⁹ migrates as a 75-kDa protein, which represents the nonsumoylated form of Mdm2 (Fig. 1B). These data confirm that aa 40–59 of Mdm2 are required for Ubc9 association.

To further confirm the ability of the peptide corresponding to mu9bs to associate with Ubc9, we carried out in vivo reactions. We synthesized the mu9bs peptide into an HA-tagged mamma- llian expression vector that also carried the penetratin peptide in frame, ensuring its localization into the nucleus. This expression vector was used for the expression of short peptides in mammalian cells (52, 44). Immunoprecipitation of the peptide from cells (with antibodies to HA tag) followed by immunoblot analysis with antibodies to Ubc9 revealed that the mu9bs peptide, but not its scrambled counterpart, associated with Ubc9 (Fig. 2B). These data establish that the region corresponding to aa 40–59 of Mdm2 serves as a Ubc9 binding site.

A Peptide Corresponding to the Ubc9 Binding Site on Mdm2 Attenuates Mdm2 Sumoylation in Vitro and in Vivo—To test the effect of the mu9bs peptide on Mdm2 sumoylation, we first
determined the kinetics of this reaction. Efficient sumoylation was observed as early as 30 min after initiation of the in vitro sumoylation reaction, and it reached maximal levels within 50–60 min (Fig. 3A). Addition of the wt mu9bs peptide for different periods of time during the in vitro sumoylation reaction was sufficient to attenuate Mdm2 sumoylation in a manner that coincided with the length of the incubation (Fig. 3B). 0' reflects no addition of peptide and thus maximal degree of sumoylation; 10' reflects the degree of sumoylation when the peptide was added for 10 min of the reaction; and 50' represents the reaction in which the peptide was added for 50 (out of 60') of the reaction. Similarly, addition of increasing concentrations of mu9bs to the in vitro sumoylation reaction led to a dose-dependent decrease of Mdm2 sumoylation (Fig. 3C). These observations suggest that the presence of the peptide that corresponds to the Ubc9 association site on Mdm2 is sufficient to attenuate Mdm2 sumoylation in vitro.

Important confirmation of the effect of the Mdm2-driven peptide on Mdm2 sumoylation comes from in vivo studies. Co-expression of the mu9bs peptide with Mdm2 in p53/Mdm2 double null cells led to a dose-dependent decrease in the degree of Mdm2 sumoylation in vivo, whereas the mu9sb peptide had no effect (Fig. 3D). Together, these observations identify a Mdm2 domain that is required for Ubc9 binding and consequent sumoylation of Mdm2.

Expression of the Corresponding Ubc9 Binding Site Peptide Does Not Affect the Association of Mdm2 with p53—Given the proximity of p53 association on the Mdm2 and Ub9 binding domains, we tested whether inhibition of Mdm2 sumoylation by overexpression of the mu9bs peptide would also affect degree of Mdm2 association with p53. To do so, we transfected human fibroblasts with mu9bs or mu9sb peptides. Immunoprecipitation of p53 was followed by Western blot analysis to determine the type and level of Mdm2 bound to p53 under each of these conditions. As revealed in Fig. 4, both the 75- and the 90-kDa forms of Mdm2, reflecting the non- and the sumoylated forms, respectively, were found in complex with p53. Whereas expression of the control peptide mu9sb did not affect the association with either form of Mdm2, cells expressing the mu9bs peptide exhibit p53 association only with the nonsumoylated form of Mdm2 (Fig. 4). Forced expression of wt Mdm2 resulted in an association of p90 with p53, whereas expression of the K1 form (a mutant form of Mdm2 on aa 446, which cannot be ubiquitinated or sumoylated; Ref. 19) revealed an association of the nonsumoylated form with p53 (Fig. 4). Effect of mu9bs expression on the form of Mdm2 associated with p53 is expected to also affect the level of p53 in the cell. Indeed, overall levels of p53 expression coincided with the type of peptide expressed; mu9bs expression led to an increased level of p53, in accord with decreased sumoylation of Mdm2, which is the po-

Fig. 1. A, identification of the Ubc9 binding region on Mdm2. Purified GST-Ubc9 was incubated with the indicated in vitro translated 35S-labeled Mdm2 forms for 1 h on ice. Glutathione beads were added for 30 min. Beads were extensively washed (three times with TBS, 0.5% Triton X-100), and the proteins remaining on the washed beads were eluted in 3x Laemmli buffer. Proteins were separated by 10% SDS-PAGE and analyzed by autoradiography. The upper part shows the input of in vitro translated Mdm2 forms in a pull-down assay. The lower part shows the association of the Mdm2 forms with Ubc9 after GST pull-down. B, Mdm2 is not sumoylated upon deletion of the Ubc9 binding site. Mdm2/p53 double null cells were transfected with the indicated Mdm2 plasmids and HA-tagged Sumo-1. Cells were harvested 36 h later, and 500 μg of protein was immunoprecipitated using Mdm2 antibodies (2A10). The immunoprecipitates were washed and the proteins loaded onto 10% SDS-PAGE. Immunoprecipitated Mdm2 was detected using monoclonal Mdm2 antibodies (2A10, left). The same membrane was reprobed using monoclonal antibodies against Sumo-1 (Zymed Laboratories Inc., right). The bottom panel shows binding of Ubc9 to wt Mdm2, but not Mdm2 that is missing aa 40–59.

Fig. 2. A, a peptide corresponding to aa 40–59 on Mdm2 binds Ubc9. mu9bs (Mdm2 Ubc9 binding site) corresponding to aa 40–59 of Mdm2 and its control scrambled counterpart peptide mu9sb (were immobilized on an AminoLink column (Pierce)). 50 μl of peptide-bound beads were incubated with 1 mg of cell lysates from EJ mouse fibroblast cells. Beads were washed extensively with 0.5 M LiCl, TBS, and eluates were separated on a 14% gel. B, Ubc9 binds to mu9bs in vivo. NIH3T3 cells were transfected with wt or mutant Ubc9 binding site peptide. 36 h after transfection cells were lysed, and lysates were immunoprecipitated with HA antibodies directed against the HA tag of transfected peptides. The upper panel depicts the co-immunoprecipitation of Ubc9 with the wt, but not the mutant, peptide. The lower panel shows the expression level of transfected peptides.
tent form for mediating p53 degradation. These data suggest that Ubc9 binding does not affect Mdm2 association with p53, but rather, the form of Mdm2 expressed and associated with p53. Mutant Ubc9, Which No Longer Conjugates SUMO-1, Decreases Mdm2 Sumoylation and p53 Ubiquitination—A specific mutation of conserved cysteine (C93S) was previously shown to abrogate Ubc9 ability to conjugate SUMO-1 to the androgen receptor while increasing the androgen receptor’s transcriptional activities (53). To determine whether Ubc9 association with Mdm2 may affect its ability to target p53 ubiquitination, we have tested the effect of this mutant form of Ubc9. Addition of Ubc9C93S to in vitro sumoylation reaction of Mdm2 revealed that it failed to cause Mdm2 sumoylation and was capable of decreasing wt Ubc9-mediated Mdm2 sumoylation (Fig. 5A). Corresponding to decreased Mdm2 sumoylation has been the increase in its self-ubiquitination (Fig. 5A). In vitro analysis was carried out via transfection of wt or mutant Ubc9 together with HA-Ub into normal human fibroblasts. Whereas expression of wt Ubc9 increased the level of Mdm2 sumoylation in

Fig. 3. A, time-dependent Mdm2 sumoylation in vitro. Bacterially expressed and purified GST-Mdm2 were subjected to in vitro sumoylation reaction in the presence of E1, Ubc9, and 35S-Sumo, as indicated under “Material and Methods.” Reaction was terminated after 10, 30, 50, or 60 min. Proteins were separated on 8% gel, Coomassie-stained, dried, and exposed overnight (upper panel). The lower panel depicts Coomassie Blue staining of Mdm2 forms. B, a peptide corresponding to the Ubc9 binding site on Mdm2 decreases Mdm2 in vitro sumoylation of Mdm2 in a time-dependent manner. Upper part, the Mdm2 sumoylation assay was carried out as detailed under “Materials and Methods.” The specific Ubc9 binding site peptide or a nonspecific peptide was added to the reaction for the indicated time periods. The upper panel shows a radiograph of 35S-labeled Sumo-1 conjugation to Mdm2 under the indicated conditions. The lower panel depicts Coomassie staining visualizing the relative amounts of sumoylated and nonsumoylated forms of purified GST-Mdm2. C, a peptide corresponding to the Ubc9 binding site on Mdm2 decreases in vitro sumoylation of Mdm2 in a dose-dependent manner. The Mdm2 sumoylation assay was carried out in the presence of the Ubc9 binding site peptide (mu9bs) or a nonspecific peptide (mu9bs), which was added to the reaction at the indicated concentrations. The upper panel shows a radiograph of 35S-labeled Sumo-1 conjugation to Mdm2 under the indicated condition. The lower panel shows a Coomassie staining visualizing relative amounts of sumoylated and nonsumoylated forms of purified GST-Mdm2. D, a peptide corresponding to the Ubc9 binding site on Mdm2 decreases Mdm2 sumoylation in vivo. Mdm2/p53 double null cells were transfected with FLAG-tagged Mdm2, HA-tagged Sumo-1, and increasing amounts of the specific or nonspecific Ubc9-peptides, respectively. Cells were harvested 36 h later, and 500 μg of protein was immunoprecipitated using Mdm2 antibodies (2A10). Immunoprecipitated Mdm2 levels were detected using 2A10 antibodies (top). The same membrane was stripped and reprobed using monoclonal antibodies against Sumo-1 (Zymed Laboratories Inc., middle) to visualize the level of Sumo-1 conjugation to Mdm2 after Ubc9 peptide expression. The bottom panel shows the expression level of the respective Ubc9 peptides.

Fig. 4. Ubc9 binding affects the form of Mdm2 associated with p53. Mdm2/p53 double null cells were transfected with mu9bs or the wild type form mu9bs together with p53 and Mdm2. 36 h after transfection protein extracts were prepared and used for immunoprecipitation of p53 (using D01 antibodies). Immunoprecipitated material was analyzed via immunoblotting using antibodies to Mdm2, enabling them to reveal the two major forms of Mdm2 indicated. Western blotting with anti-p53 antibodies (top) revealed expression of the peptides, whereas immunoblot with p53 depicts changes in the levels of p53 expression. Wt Mdm2 and K1 (mutant at aa 446) forms were used as controls.
these cells, the expression of mutant Ubc9<sup>C93S</sup> resulted in decreased Mdm2 sumoylation (Fig. 5B). Changes in Mdm2 sumoylation will lead to altered p53 ubiquitination. Dose-dependent increase of transfected mutant Ubc9<sup>C93S</sup> revealed a corresponding decrease in the level of p53 ubiquitination (Fig. 5B). Similarly, co-expression of the K1 mutant (Mdm2 form that is mutated on aa 446, which is the sumoylation and ubiquitination site), which elicits efficient ubiquitination of p53, did not alter its effect on p53 ubiquitination <em>in vivo</em>, suggesting that Ubc9 binding is not required for K1 ability to mediate efficient p53 ubiquitination and degradation (data not shown). It is likely that the conformational change elicited by the Lys<sup>446</sup> mutation confers on Mdm2 better E3 activities, in addition to greater stability. These observations suggest Ubc9 binding to Mdm2 without conjugation of Sumo (<i>i.e.</i> Ubc9 binding <i>per se</i>) are not sufficient to increase Mdm2’s ability to target p53 ubiquitination, but rather increases Mdm2 self-ubiquitination and decreases p53 ubiquitination.

**Ubc9 Binding to Mdm2 Is Decreased after UV Irradiation**—Since UV irradiation has been shown to reduce the degree of Mdm2 sumoylation, we have monitored possible changes in the association of Ubc9 with Mdm2 in UV-treated cells. UV irradiation of NIH3T3 cells that were co-transfected with Mdm2 and Sumo revealed a time-dependent decrease in the binding of Ubc9 to Mdm2, which coincided with decreased Mdm2 sumoylation (Fig. 6). This observation demonstrates that Ubc9 binding to Mdm2 is under a dynamic regulation and affected by changes elicited upon stress and DNA damage, as shown here for UV irradiation. Our finding further establishes the requirement for Ubc9 binding to enable sumoylation of Mdm2.

**DISCUSSION**

Conjugation of Sumo-1 to Mdm2 is a key event underlying Mdm2’s ability to elicit efficient degradation of p53 (19). Increased Mdm2 E3 activity toward p53 is explained in light of attenuated Mdm2 self-ubiquitination, since sumoylation of Mdm2 displaces its primary ubiquitin conjugation site. The biological importance of Mdm2 sumoylation requires a better understanding of the regulation of Mdm2 sumoylation be better understood. Central to the covalent attachment of Sumo-1 to Mdm2 is the conjugating enzyme Ubc9. Here we identify the
Ubch9 binding domain on Mdm2, which is required for Mdm2 sumoylation and concomitant Mdm2 E3 activity toward p53. Mdm2, whose Ubch9 association domain has been deleted, is no longer capable of either association with Ubch9 or of sumoylation. Furthermore, expression of the peptide that corresponds to this region efficiently inhibited Mdm2 sumoylation both in vitro and in vivo. This region, which was mapped in the current study, is sufficient for Mdm2 association as revealed by in vitro and in vivo data.

The proximity of the Ubch9 binding site on Mdm2 to that required for association with p53 led us to explore possible effects on Mdm2 association with p53; we found that the Ubch9 association does not affect the Mdm2-p53 association, but rather the form (sumoylated or not) of Mdm2 that is bound to p53. Importantly, changes in the form of Mdm2 that bound to p53 was also reflected in the relative levels of p53 expression. Inhibition of Ubch9 binding led to increased levels of p53.

Interestingly, a recent study by Matunis and colleagues (55) identified, based on the analysis of 11 proteins that were reported to undergo sumoylation, that the LLKXE motif serves as the target site for Ubc9 binding. A similar sequence (LLKE) is known to undergo sumoylation, that the LLKXE motif serves as the site of SUMO-1 conjugation, suggesting that the regulatory role of Ubc9 is highly dependent on its proper conformation.

Acknowledgments—We thank Dr. R. Hay for SUMO-1 construct, Dr. D. Bohmann for Ub-HA construct, Drs. A. Weissman and J. Chen for Mdm2 expression vectors, Dr. O. Jänne for Ubch9 expression, Dr. S. Jones for p53/Mdm2 double null cells, and Dr. S. Aaronson for 3T3 cells. We also thank the members of the Ronai laboratory for helpful comments and discussions.

Note Added in Proof—Experiments performed in our laboratory over the past few weeks revealed that K446 is not the site for Sumo-1 conjugation to Mdm2. K446 is the primary site for Mdm2 self-ubiquitination, explaining why this mutant is more potent than wild-type Mdm2 in its effect on p53, and the nature of its prolonged half-life. Whereas sumoylation of Mdm2 in vitro decreases its self ubiquitination and increases its ubiquitin ligase activity, this change can no longer be attributed to displacement of ubiquitin with Sumo-1 at the same site, but possibly to conformationally based effects. Accordingly, it is clear that the reactivity of the relationship between Ubch9 association- and Sumo-1 conjugation sites requires additional studies. These new results do not, however, affect the conclusions of this paper.
45. Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000) *J. Biol. Chem.* 275, 8945–8951
46. Honda, R., and Yasuda, H. (2000) *Oncogene* 19, 1473–1476
47. Hu, G., Zhang, S., Vidal, M., Baer, J. L., Xu, T., and Fearon, E. R. (1997) *Genes Dev.* 11, 2701–2714
48. Honda, R., and Yasuda, H. (1999) *EMBO J.* 18, 22–27
49. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Altroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) *Mol. Cell* 6, 1029–1040
50. Lorick, K. 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
51. Treier, M., Stazewski, L. M., and Bohmann, D. (1994) *Cell* 78, 787–798
52. Buschmann, T., Potapova, O., Bar-Shira, A., Ivanov, V. N., Fuchs, S. Y., Henderson, S., Fried, V. A., Minamoto, T., Alarcon-Vargas, D., Fiscus, M. R., Gaarde, W. A., Holbrook, N. J., Shiloh, Y., and Ronai, Z. (2001) *Mol. Cell. Biol.* 21, 2743–2754
53. Poukka, H., Karvonen, U., Janne, O. A., and Palvimo, J. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 14145–14150
54. Maya, R., Balass, M., Seong-Tae, K., Shkedy, D., Juan-Fernando, M. T., Shifman, O., Moas, M., Buschmann, T., Ronai, Z., Shiloh, Y., Kastan, M. B., Katzir, E., and Oren, M. (2001) *Gene (Amst.)* Dev. 15, 1067–1077
55. Sampson, D. A., Wang, M., and Matunis, M. J. (2001) *J. Biol. Chem.* 276, 21664–21669
