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

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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 isopeptide 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 substrates (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-terminal 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 are likely to contribute to the recognition of selective substrates in the SUMO-1 conjugation pathways (12, 13). Covalent attachment of SUMO-1 (Sentrin, Ub11, or Smt3) to lysines was demonstrated for a growing number of proteins, including RanGAP1 (Ran GTTPase-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 nucleocytoplasmic 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 topoisomerase 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 proteasome-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...
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 Ubch 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 polymerase 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 Ubch binding domain on Mdm2 (aa 40–59; Mdm2 Ubch expression vector; mu9bs) in wt or scrambled orientation (mu9bs) was cloned in frame between EcoRI and XhoI restriction sites into pcDNA3 that carries the penetratin sequence, followed by the sequence encoding the XhoI site of pcDNA3 that carries the penetratin sequence, followed by the sequence encoding the primary site for ubiquitin conjugation to Mdm2 (Fig. 1B). Western blot analyses were carried out 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

Ubch Binding Is Mapped to the Mdm2 Amino-terminal Domain—As noted, binding of Ubch, which serves as an E2-conjugating enzyme for SUMO-1 (19), is prerequisite to Mdm2 sumoylation. To map the Ubch 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 Ubch. 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 Ubch. This finding suggests that Ubch 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 Ubch (Fig. 1B). Furthermore Mdm21240–59 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 Ubch association.

Ubc-binding Site Mapping—To further assess the role of the Ubch binding domain in Mdm2 sumoylation, we synthesized a peptide that corresponds to aa 40–59 of Mdm2. The wt, but not mutant (scrambled), peptide captured Ubc9 association. This finding suggests that Ubch 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 Ubch. 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 Ubch. This finding suggests that Ubch binding may require aa 40–59 of the amino-terminal domain of Mdm2. For this reason, we measured the effect of the mu9bs peptide on Mdm2 sumoylation, we first assayed the difference in the capacity of Mdm2 to capture Ubch in vitro. As shown in Fig. 1B, Mdm21240–59 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 Ubch association.

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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.

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 Ubc9 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
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 vivo 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 vitro, expression of mutant Ubc9 failed to cause Mdm2 sumoylation and was capable of decreasing wt Ubc9-mediated Mdm2 sumoylation (Fig. 5A). In vivo 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 vitro, expression of mutant Ubc9 failed to cause Mdm2 sumoylation and was capable of decreasing wt Ubc9-mediated Mdm2 sumoylation (Fig. 5A).
these cells, the expression of mutant Ubc9C93S resulted in decreased Mdm2 sumoylation (Fig. 5B). Changes in Mdm2 sumoylation will lead to altered p53 ubiquitination. Dose-dependent increase of transfected mutant Ubc9C93S 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 in vivo, 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 Lys446 mutation confers on Mdm2 better E3 activities, in addition to greater stability. These observations suggest Ubc9 binding to Mdm2 without conjugation of Sumo (i.e. Ubc9 binding per se) 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 better understood. Central to the covalent attachment of Sumo-1 to Mdm2 is the conjugating enzyme Ubc9. Here we identify the
Ub9 binding domain on Mdm2, which is required for Mdm2 sumoylation and concomitant Mdm2 E3 activity toward p53. Mdm2, whose Ub9 association domain has been deleted, is no longer capable of either association with Ub9 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 Ub9 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 Ub9 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 Ub9 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 site for Ub9 binding. A similar sequence (LLKXE) is found on aa 37–40 of Mdm2, proximal to aa 40–59, which was characterized in the present studies. The deletion mutant of Mdm2 that encompass aa 1–49 revealed a noticeable binding to Ub9, suggesting that the actual domain engaged in binding of Mdm2 is indeed within an aa 40–59.

Of interest, note that association of Ub9 may affect protein function even if sumoylation would not take place. The C93S substitution of Ub9 prevents Sumo-1 conjugation by abrogating the formation of a thiol ester bond between Sumo-1 and Ub9. Expression of the C93S form was found to elicit stimulation of androgen receptor-dependent transactivation similar to wt Ub9. This finding implies that Ub9 association per se may be sufficient for changes in protein conformation and function (53). Along those lines we have tested whether the ability of Mdm2 mutant at aa 446 (K1) to elicit stronger E3 activity toward p53 may be attributed to Ub9 association, since this mutant can no longer be sumoylated. Our data suggest that K1 elicits its efficient targeting of p53 ubiquitination independent of Ub9, since the mutant form of Ub9 did not affect K1 activities. Possible changes in conformation of the Mdm2 RING, which are expected to take place due to the Lys446 mutation, may contribute to its elevated E3 activities, in addition to increased stability of this mutant.

Support for the dynamic regulation of Ub9 association with Mdm2 comes from the observation that UV irradiation decreases binding of Ub9 while increasing the association of p19ARF. It is likely that UV irradiation decreases the association of Ub9 as a result of phosphorylation of Mdm2 by stress kinases, including ATM (54), which are expected to elicit conformational changes in Mdm2, resulting in an altered subset of its associated proteins. The distant location of Ub9 binding (aa 40–59) from that of Mdm2 sumoylation (aa 446) further supports the importance of Mdm2 conformation in relation to its ability to undergo sumoylation.

Overall, the present study adds important new information regarding Mdm2’s association with Ub9, which is a prerequisite for Mdm2’s sumoylation. Our data provide evidence for the existence of dynamic regulation of Ub9 association, which is altered upon UV treatment. We further demonstrate for the first time a case where Ub9 association is distant to the site of SUMO-1 conjugation, suggesting that the regulation of Mdm2 sumoylation is highly dependent on its proper conformation.

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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 relationship between Ub9 association- and Sumo-1 conjugation-sites requires additional studies. These new results do not, however, affect the conclusions of this paper.
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