Sumoylation of Mdm2 by Protein Inhibitor of Activated STAT (PIAS) and RanBP2 Enzymes*

Received for publication, August 14, 2002, and in revised form, October 18, 2002
Published, JBC Papers in Press, October 18, 2002, DOI 10.1074/jbc.M208319200

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Mdm2, a ubiquitin ligase that acts on p53, is regulated by sumoylation. In the current study, we identify the enzymes responsible for the sumoylation of Mdm2. When mammalian cells are co-transfected with cDNAs encoding Mdm2 and PIAS1 or PIASxβ (protein inhibitor of activated STAT) as sumoylation enzymes, Mdm2 is highly sumoylated. Mdm2 is also sumoylated in an in vitro system containing PIASxβ, PIAS1, and RanBP2. When several lysine residues of Mdm2 were sequentially mutated to arginine, the K182R mutant was not sumoylated in intact cells; however, in the in vitro system this mutant was sumoylated by PIAS1, PIASxβ, and RanBP2 as efficiently as the wild-type Mdm2 protein. Lysine residues 182 and 185 map within the nuclear localization signal of Mdm2. A K185R mutant of Mdm2 is sumoylated in intact cells, whereas a K182R protein is not. Only a Mdm2 protein bearing the K182R mutation is localized exclusively in the cytoplasm. Because RanBP2 is a nuclear pore protein and PIAS proteins are localized within the nucleus, our data suggest that Mdm2 is sumoylated during nuclear translocation by RanBP2 and then further sumoylated once in the nucleus by PIASxβ and PIAS1.

The oncoprotein Mdm2 has been shown to be a ubiquitin ligase toward itself and tumor suppressor p53 (1). Its ligase activity is dependent on the RING finger domain in its carboxyl terminus, because a mutation that disrupts this domain diminishes the ubiquitin ligase activity (2, 3). The ubiquitin ligase activity of Mdm2 is important for the regulation of the level of p53 in a cell. When DNA in a cell is damaged by genotoxic stress, p53 is phosphorylated at its amino terminus by ATM kinase and/or chk2 (4–7). Mdm2 cannot bind and ubiquitynate phosphorylated p53, and thus p53 becomes stable. When mammalian cells were transfected with v-ras, myc, or adenovirus E1A, the mouse p14ARF (p14ARF in humans) was induced (8–11), and then ARF bound to and inhibited the Mdm2 activity to stabilize p53 (12).

The ubiquitin ligase activity of Mdm2 is also thought to be regulated by its post-translational modifications, including phosphorylation and sumoylation. When cells were exposed to genotoxic stress, ATM kinase phosphorylated not only p53 but also Mdm2 to inhibit its activity (13, 14). Furthermore, when Akt kinase was activated by inositol 1,4,5-trisphosphate (IP₃) kinase, active Akt kinase phosphorylated Mdm2, resulting in activation of the ubiquitin ligase activity of Mdm2 and consequently the destabilization of p53 (15, 16).

SUMO is a ubiquitin-like protein, and mammals have three types of SUMO protein, i.e. SUMO-1, -2, and -3 (17–20). The sumoylation pathway very much resembles the ubiquitylation pathway. In the latter, three enzymatic steps are necessary to ubiquitylate the target protein (21–23). First, a ubiquitin moiety is activated by E1, the ubiquitin-activating enzyme, in the presence of ATP and binds to a cysteine residue of the E1 enzyme through a thioester bond. The activated ubiquitin is then transferred to E2, the ubiquitin-conjugating enzyme, and then the ubiquitin-E2 complex binds to E3, the ubiquitin ligase, which then transfers the ubiquitin moiety to the target protein, or the ubiquitin moiety of the ubiquitin-E2 complex is transferred to E3, the ubiquitin ligase, and then the ubiquitin moiety is bound to the target protein. The sumoylation pathway in vitro requires the Sua1/Uba2 heterodimer as its E1 enzyme and Ubc9 as its E2 enzyme (24–27). Recently, the protein inhibitor of activated STAT (PIAS) family proteins have been proposed to function as a SUMO ligase or E3 (28–30). PIAS1 and PIASy were shown to catalyze sumoylation of p53 and LEF-1, respectively (28, 32). PIAS1 and PIAS3 were initially cloned as transcriptional coregulators of the JAK-STAT pathway (33, 34). The PIAS family consists of at least the following six members: PIAS1, PIAS3, PIASxα, PIASxβ, PIASγ, and a hypothetical protein (GenBankTM accession number CAB66507). All of them have RING finger-like domain, and their SUMO–E3 ligase activities are dependent on this domain (28, 30, 31). More recently, RanBP2 was shown to be another type of SUMO–E3 ligase toward Sp100 or HDAC4 (35, 36). This protein is thought to act at the cytoplasmic filaments of the nuclear pore complex (NPC) and to serve to modify substrates on their way to the nucleus. The SUMO–E3 ligase activity is found within a 33-kDa domain of RanBP2 that lacks the RING finger-like domain, and this domain does not resemble any domain found in members of the PIAS family (35).

At least four kinds of functions have been proposed for protein sumoylation (37). One is a modulation of protein-protein
interaction. When RanGAP1 is sumoylated, modified RanGAP1 can then bind to the RanBP2 (18, 19, 38). A second function is competition with ubiquitinylation, because sumoylation and ubiquitinylation share the same site (26). In this case, once a protein is sumoylated the ubiquitinylation does not occur any longer, and the protein thus remains stable. A third function is specification of the subcellular localization of a protein. For example, when the promyelocytic leukemia (PML) protein is sumoylated, it becomes localized in PML bodies (39–41). The fourth function is the modification of the activities of other enzymes or factors that have biological functions (42–44). The sumoylation of histone deacetylase 1 (HDAC1) is a case in point (44). When HDAC1 is sumoylated, its enzyme activity is increased.

Concerning the sumoylation of Mdm2, the site and role of this modification have not yet been clarified (45, 46). Also, the SUMO-E3 for Mdm2 remains to be identified. Therefore, in this present study we sought to identify the SUMO-E3 for Mdm2. In addition to identifying E3 ligases for Mdm2, we also found the nuclear localization of Mdm2 to be important for the modification.

**EXPERIMENTAL PROCEDURES**

**Expression and Preparation of Recombinant Proteins**—Human SUMO-1 cDNA and human Ubc9 were obtained by the reverse transcription PCR method and were expressed in *Escherichia coli* strain BL21 (DE3) LysS. *E. coli*-expressing recombinant proteins were purified as described previously (24). Human GST-Mdm2 and GST-tagged mutants, which were prepared by the PCR mutation method described previously (2), were expressed in a baculovirus expression system and purified by use of a glutathione-Sepharose 4B resin as described earlier (12).

**DNA Transfection**—U2OS cells were transfected with pFLAG-Mdm2, pFLAG-SUMO1, pFlag-Flag-SUMO1, pEGFP-Pias, or pEGFP-Mdm2 by using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After a 24-h incubation, the cells were lysed with SDS-PAGE sample buffer and subjected to SDS-PAGE. After the proteins had been transferred to a polyvinylidene difluoride membrane, Western blotting using anti-Mdm2 antibody (Santa Cruz Biotechnology) or anti-GFP antibody (MBL) was performed; horseradish peroxidase-conjugated anti-mouse IgG and ECL reagents (Amer sham Biosciences) were used for detection, as described previously (1).

**Fluorescence Microscopy**—Logarithmically growing U2OS cells were grown on coverslips and transfected with wild-type Mdm2 or the mutants by using FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. At 24 h after transfection, the cells were fixed for 20 min with 3.7% formaldehyde in PBS (–), and washed 3 times with PBS (–). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Microscopic observation was made using a Olympus LSC101.

**In Vitro Sumoylation Assay**—An in vitro sumoylation assay was done as described previously (28). Briefly, GST-Mdm2 or mutants were purified with glutathione-Sepharose 4B, mixed with purified 0.36 μg of E1 (GST-Sua1/His-Uba2), 20 ng of Ubc9, 1.1 μg of SUMO-1, and a specified amount of GST-PIASxβ or RanBP2 and then incubated at 25°C for 30 min in the presence of 50 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol, and 3 mM ATP in a 30-μl volume. Ubc9, SUMO-1, and RanBP2ΔFG were expressed in *E. coli* (BL21) by using the pET vector; E1, Pias, and Mdm2 were expressed in SF9 cells by using a baculovirus protein expression system (Invitrogen). The glutathione-Sepharose 4B resin was washed with 10 mM Tris-HCl (pH 7.4) buffer containing 0.01% Brij. The resin was subjected to SDS-PAGE and Western-blotted with anti-SUMO-1 antibody (Zymed Laboratories Inc.) or anti-Mdm2 antibody.

**RESULTS**

**Sumoylated Form of Mdm2 in a Cell**—The most prominent form of Mdm2 in a cell migrates as a protein of ~92 kDa in an SDS-polyacrylamide gel, although the molecular mass calculated from its cDNA sequence is 55.2 kDa. This ~92-kDa Mdm2 in a cell has been proposed to be its sumoylated form, and a smaller prominent Mdm2 with a molecular mass of about 75 kDa has been considered to be its non-modified form (45). However, another report indicated a 75-kDa protein to be translated from the second methionine of Mdm2 (47). Therefore, when we started our study on the sumoylation of Mdm2, we addressed the issue as to whether this ~92-kDa Mdm2 is a SUMO-modified form or not. When the Mdm2 in USO2 cells was immunoblotted with anti-Mdm2 antibody, the prominent Mdm2 protein showed a molecular mass of about 92 kDa (Fig. 1, lane 2). When FLAG-tagged Mdm2 was expressed in U2OS cells, the FLAG-Mdm2 showed a molecular mass of ~93 kDa, which was bigger by 1 kDa than the Mdm2 in U2OS cells (Fig. 1, lane 3). This difference corresponds to the molecular mass of the FLAG tag (DYKDDDDK). To know whether this 92-kDa Mdm2 was sumoylated Mdm2, we expressed non-tagged Mdm2 in *E. coli*, which does not have a SUMO modification system. The prominently expressed Mdm2 that reacted with the anti-Mdm2 antibody migrated to the same place in the SDS gel as did the Mdm2 in U2OS cells (Fig. 1, lanes 1 and 2). Furthermore, two minor anti-Mdm2-reacting proteins, whose molecular masses were ~75 and 76 kDa, were detected in both *E. coli* and U2OS cells (Fig. 1, lanes 1 and 3). The faster migrating protein could be Mdm2 translated from the second ATG at codon 50 as described previously (47), although the nature of the slower migrating one is unknown. These data indicate that the most prominent cellular form of Mdm2, having a molecular mass of 92 kDa, is not its sumoylated form. The molecular mass of its sumoylated form on an SDS-PAGE gel would be expected to be ~110 kDa.

Both PIAS Family Proteins, PIASxβ and PIAS1, as Well as RanBP2, Function as SUMO-E3 toward Mdm2—PIAS1 and PIASy have been shown to have SUMO-E3 activities (28, 32). Therefore, we explored the possibility that PIAS family members can function as an E3 toward Mdm2. U2OS cells were co-transfected with FLAG-tagged Mdm2, FLAG-tagged SUMO-1, and a given GFP-PIAS protein; the sumoylation of Mdm2 was then checked by immunoblotting with the anti-Mdm2 antibody. In the case of no expression of PIAS proteins (Fig. 2A, lane 10), two proteins were detected by the anti-Mdm2 antibody; the faster migrating band was thought to be an endogenous Mdm2, and the slower migrating one was thought to be ectopically expressed FLAG-Mdm2 (as was shown in Fig. 1). In contrast, when PIAS1 or PIASxβ was co-expressed with the Mdm2 plasmid and the FLAG-SUMO-plasmid, a band migrating more slowly than the Mdm2 bands appeared (Fig. 2A, lanes 2 and 8) and was suspected to be sumoylated Mdm2. To confirm that this anti-Mdm2 antibody-reactive band was indeed sumoylated Mdm2, we transfected the cells with FLAG-FLAG (2×FLAG)-tagged SUMO-1 in place of FLAG-tagged
SUMO-1. The slower migrating band of Mdm2 was shifted to a higher molecular mass when 2×FLAG-tagged SUMO-1 was used than when FLAG-tagged SUMO-1 had been used (Fig. 2A, lanes 3 and 9). These results indicate that the slower migrating band was sumoylated Mdm2. In contrast, the expression of PIASα did not enhance the sumoylation of Mdm2 (Fig. 2A, lanes 4–6). Furthermore, when cells were transfected with the PIASβ RING finger mutant (C362S or C362A), the sumoylation of Mdm2 was not enhanced (Fig. 2B). In Fig. 2C, the expression level of each PIAS protein is shown. Although the cells were transfected with the same amount of each PIAS protein, the expression levels of PIASα and wild-type PIASβ were lower than those of PIAS1, PIASβ-CS and PIASβ-CA. These data thus indicate that PIAS1 and PIASβ enhanced the sumoylation of Mdm2 in intact cells in a RING finger-like domain-dependent manner.

Next, we conducted in vitro sumoylation by using purified recombinant Mdm2 proteins as the substrate (Fig. 3, A and B). To confirm that PIASβ has indeed SUMO-E3 ligase activity toward the Mdm2, we prepared and purified the recombinant protein and checked its activity. In the absence of PIASβ, no sumoylated Mdm2 bands were detected (Fig. 3A, lane 1). In contrast, the addition of PIASβ to the assay system enhanced the sumoylation of the Mdm2 in a dose-dependent manner (Fig. 3A, lanes 2–4). When we used PIAS1 in place of PIASβ, similar results were obtained (data not shown). More recently, RanBP2 was shown to be another type of SUMO-E3 toward Sp100 or HDAC4 (35, 36). Therefore, we examined whether RanBP2 might also function as a SUMO-E3 toward Mdm2. We
performed an in vitro sumoylation assay in the presence or absence of GST-RanBP2ΔFG, which is the minimal fragment containing the SUMO-E3 ligase activity (35). In the absence of RanBP2ΔFG, no sumoylated Mdm2 bands were detected by anti-SUMO-1 antibody (Fig. 3B, lane 1). The addition of RanBP2ΔFG to the assay system enhanced the sumoylation of Mdm2 in a dose-dependent manner (Fig. 3B, lanes 2–4). Thus, RanBP2 also functioned as a SUMO-E3 toward Mdm2. When a 10-fold greater amount of Ubc9 than the usual amount was added to this assay, the sumoylated band was detected even in the absence of RanBP2 (Fig. 3B, lane 5).

RanBP2 has been shown to be localized at nuclear pores. So next we examined the subcellular location of PIAS1 and PIASxβ. As shown in Fig. 4, both PIAS1 and PIASxβ, appearing as dot-like structures, were localized in nuclei. These data suggest that Mdm2 is sumoylated during nuclear translocation by RanBP2 and then further sumoylated in nuclei by PIASxβ or PIAS1.

K182R Mutant Is Not Sumoylated in Cells but Is Sumoylated in Vitro—Next, to determine the sumoylation site of Mdm2, we mutated eight lysine residues of Mdm2 and then transfected U2OS cells with this mutant in the presence of GFP-PIASxβ and FLAG-tagged SUMO-1. This mutant, which we call 8KR (K182R/K185R/K334R/K336R/K338R/K344R/K346R/K422R), was not sumoylated in the U2OS cells (Fig. 5A, lanes 1 and 2). Therefore, we tried to identify the responsible lysine residue(s) for the sumoylation. The K182R mutant was proven not to be sumoylated in the cells (Fig. 5A, lanes 1–7). This lysine 182 residue is in the nuclear localization signal (NLS), and the GFP-tagged K182R mutant was directly observed by fluorescence microscopy to be localized exclusively in the cytoplasm (Fig. 6). The lysine 185 is also localized in the NLS, but K185R was sumoylated just like the wild-type Mdm2 (Fig. 6, lanes 9–12). The wild-type Mdm2 and K185R mutant were localized in nuclei and not in the cytoplasm, but K182R/K185R was localized in the cytoplasm (Fig. 6). Furthermore, 2RTL (R181T/R183L), a mutant that has mutations in its NLS, was hardly sumoylated (Fig. 5A, lanes 3 and 4); this mutant was localized mainly in the cytoplasm (data not shown).

As described above, the mutant K182R was not sumoylated in intact cells, so we next we examined whether the mutant could be sumoylated in vitro. The purified wild-type Mdm2 was sumoylated in the presence of PIASxβ in vitro, as was the K182R mutant (Figs. 3A and 5, B and C). RanBP2 also functioned as a SUMO-E3 toward both wild-type Mdm2 and the mutant in vitro (Figs. 3B and 5, B and C). Therefore, these data suggest that nuclear import is necessary for Mdm2 to be sumoylated.

DISCUSSION

Here we showed that the sumoylation of Mdm2, a ubiquitin ligase toward p53, was enhanced in the presence of PIAS1, xβ, or RanBP2 acting as a SUMO-E3. We also showed that the K182R mutant, which was localized exclusively in the cytoplasm, was not sumoylated in intact cells.

Although sumoylation of the proteins has been shown to occur in the presence of SUMO-E1, E2, Ubc9, and SUMO in the absence of a SUMO-E3, recently the extent of the sumoylation was shown to increase markedly in the presence of the PIAS family or RanBP2 serving as a SUMO-E3 (28–31, 35). PIAS family members, but not RanBP2, have been shown to function as a SUMO-E3 toward p53 (28, 35, 36). On the other hand,
RanBP2, but not PIAS family members, functions as a SUMO-E3 toward HDAC4 (36). Mdm2 can be sumoylated in vitro without E3, but SUMO-E3 may function in vivo. To assess the possibility that PIAS family members function as a SUMO-E3, we transfected U2OS cells with Mdm2 and SUMO along with a PIAS family member and found that Mdm2 was clearly sumoylated in the presence of PIASx or PIAS1. Furthermore, PIASx\textsubscript{K182R} mutants with a mutation in the RING finger like domain did not have SUMO-E3 activity. In the in vitro SUMO-conjugating system, both PIASx\textsubscript{K182R} and RanBP2 functioned as a SUMO-E3 toward Mdm2, but in the case of p53 sumoylation, PIAS family members functioned as a SUMO-E3, but RanBP2 did not (28, 35). On the contrary, it has been shown that RanBP2 functions as a SUMO-E3 toward HDAC4 and Sp100 but that members of the PIAS family do not. Mdm2 is the first protein whose sumoylation is enhanced by both the PIAS family and RanBP2. This suggests that there are three kinds of sumoylated protein: 1) the protein sumoylated only by RanBP2 at the nuclear pore during import into the nucleus; 2) the protein sumoylated only in the nucleus by a PIAS; and 3) the protein sumoylated by both RanBP2 and a PIAS at the nuclear pore during import into the nucleus and in the nucleus, respectively.

The lysine residues of Mdm2 were sequentially mutated to arginine, and the sumoylation of the mutants was checked in the cells transfected with a PIAS or in vitro in the presence of a PIAS or RanBP2 as SUMO-E3. The K182R mutant was not sumoylated in intact cells. However, this mutant was sumoylated in vitro in the presence of the PIAS protein or RanBP2 as a SUMO-E3. K182R is located in the NLS of Mdm2, and the K182R mutant was exclusively localized in the cytoplasm. Possibly, nuclear import is necessary for Mdm2 to be sumoylated (48), because PIAS1 and PIASx\textsubscript{K182R} are localized in nuclei, and RanBP2 is in the nuclear pore. A recent paper (36) indicated that HDAC4 was sumoylated in the presence of RanBP2 but not in the presence of PIAS and that the molecule with its NLS mutated was not sumoylated. We propose that Mdm2 is sumoylated when it enters the nucleus through the nuclear pore, where RanBP2 is and that Mdm2 is further sumoylated in the nucleus by PIAS protein. Furthermore, Lys-182 is not a lysine residue conforming to the consensus site YKXE for protein sumoylation, where Y is a large hydrophobic residue and X is any amino acid residue.

To elucidate the meaning of the sumoylation of Mdm2, it is important to know what kinds of signal can induce the sumoylation or de-sumoylation of Mdm2. The level of the sumoylation is a balance between the activities of sumoylation enzyme (E1, or Sua1/ub2, E2, or Ubc9, and maybe SUMO-ligase, the PIAS protein, or RanBP2) and SUMO-isopeptidase. Of the sumoylation enzymes, E1, Ubc9, and/or SUMO-ligase are possible candidates to be regulated in the course of signal exposure. There are several SUMO-isopeptidases reported to date (37). Possibly one or more of them are responsible for removing the SUMO moiety from Mdm2, but the responsible enzymes remain to be identified.

To further discuss the function of sumoylation of Mdm2, it is necessary to identify the sumoylated lysine residue(s) in Mdm2 without affecting the nuclear localization signal. However, because both RanBP2 and PIAS protein seem to function as a SUMO-E3 for Mdm2 and may sumoylate it at a different lysine residue(s), it may not be easy to identify which E3 sumoylates which residue. Because the sumoylation site of p53 sumoylated by E1 and E2 is the same as the site using E1, E2, and SUMO-E3 or PIAS1,\textsuperscript{2} the sumoylated site in Mdm2 could possibly be the same site detected by using E1 and E2 in vitro (49, 50).

Acknowledgments—We are grateful to Dr. Y. Nakao and Dr. H. Saitoh, Kumamoto University, for kindly giving us U2OS cells and RanBP2\textsubscript{ΔFG}, respectively.

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