Binding of p53 to the Central Domain of Mdm2 Is Regulated by Phosphorylation*

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The Mdm2 protein is the major regulator of the tumor suppressor protein p53. We show that the p53 protein associates both with the N-terminal and with the central domain of Mdm2. The central p53-binding site of Mdm2 encompasses amino acids 235–300. Binding of p53 to the central domain is significantly enhanced after phosphorylation of the central domain of Mdm2. The N-terminal and central domains of Mdm2 act synergistically in binding to p53. p53 mutants that have mutations in the tetramerization domain and that fail to oligomerize do not show such an enhancement of binding in the presence of the other binding site.

One of the major functions of the Mdm2 protein is to control abundance and activity of the tumor suppressor protein p53. This activity requires direct interaction between p53 and Mdm2 (1) that is brought about by the N-terminal domains of both proteins (2). This interaction prevents p53 from activating its target genes and allows Mdm2-mediated polyubiquitylation and degradation of p53 (reviewed in Ref. 3). The importance of this interaction has been supported convincingly by in vivo experiments. Mice lacking Mdm2 die before implantation. They will, however, survive by concomitant deletion of p53 (4, 5).

The p53-binding pocket of Mdm2 comprises the first 100 amino acids of Mdm2. Other motifs of Mdm2 include a Ring finger (aa 450–480),2 a nuclear localization signal (aa 160–180), a nuclear export signal (aa 180–195), a nucleolar localization signal (aa 464–471), and a central acidic domain (aa 200–300) (6–8). The central acidic domain of Mdm2 is necessary for the interaction with a number of proteins including p300, L11, and p19ARF (reviewed in Ref. 3). Recently, this domain was found to contribute to p53 turnover. Mdm2 mutants with point mutations or deletions in the central domain may allow p53 ubiquitylation but prevent p53 degradation (9–11).

p53 and Mdm2 receive and integrate signals that monitor genome stability, replication state, and growth conditions. In response to these signals, the p53/Mdm2 system regulates transcription of genes whose products prevent cell cycle progression or induce programmed cell death (reviewed in Ref. 28). Transcriptional activity as well as degradation of p53 requires its assembly into tetramers, which is brought about by an oligomerization domain (aa 324–355) (Refs. 12 and 13; reviewed in Ref. 14). Oligomerization-deficient mutants of p53 display low binding affinity for Mdm2 and are relatively poor substrates for ubiquitylation (15, 16).

Although the N-terminal domain of p53 is the major Mdm2-binding site, more recent studies suggest that p53 may possess a second site for Mdm2 interaction (17, 18). However, if p53 has a second binding site for Mdm2, Mdm2 may also bear a second binding site for p53.

The purpose of this study was to determine whether such a second binding site for p53 exists on the Mdm2 protein and how binding to this site is regulated. By using different deletion mutants of Mdm2 and p53, we mapped a second p53-binding site within the central domain of Mdm2 and the core domain of p53. Binding of p53 to the second binding site was significantly enhanced after phosphorylation of the central domain of Mdm2. In the presence of both binding sites, binding of p53 to Mdm2 was stronger than the two individual bindings. p53 mutants with substitutions in the oligomerization domain failed to show this assistance in binding, suggesting that two different p53 molecules of one tetramer may bind to the two p53-binding sites on one Mdm2 molecule.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmids pDWM 659 (Myc-Mdm2), pGEX4T-2-Hdm2 (aa 200–300), and pcDNA3-p53 have been described previously (10, 19). The p53 mutants R337C and L344P (22), the GST–Hdm2 mutants pGEX-4T2-Hdm2-(aa200–300)-S246D/S253D/S256D/S260D/S262D and pGEX-4T2-Hdm2-(aa200–300)-S253/S256A were gene-rated by site-directed mutagenesis. The p53 deletion mutants pcDNA3-V5-p53-aa150–300 and pcDNA3-V5-p53-aa300–393 and pcDNA3-V5-p53-aa150–393 were generated by PCR amplification of the central and C-terminal domain using pcDNA3-p53 as a template and primers encoding the V5 tag. The PCR products were cloned into pcDNA3. The plasmids pcDNA3-myc-mdm2-aa1–199 and pcDNA3-3-flag-mdm2aa200-300 were generated by PCR amplification of the N-terminal and central domains of Mdm2 using primers encoding the corresponding tags. The PCR products were cloned into pcDNA3. The pcDNA3-myc-mdm2-S244A, pcDNA3-myc-mdm2-S251A, pcDNA3-myc-mdm2-S254A, and pcDNA3-myc-mdm2-S244/ S251A/S254A were generated by site-directed mutagenesis. To

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2 The abbreviations used are: aa, amino acid(s); PBS, phosphate-buffered saline; wt, wild type; GST, glutathione S-transferase; PCNA, proliferating nuclear cell antigen; HRP, horseradish peroxidase.

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create Mdm2 deletion mutants pcDNA3-myc-mdm2-Δaa200-300, pcDNA3-myc-mdm2-Δaa200–235, pcDNA3-myc-mdm2-Δaa235–260, pcDNA3-myc-mdm2-Δaa260–300, pcDNA3-myc-mdm2-Δaa300–400, and pcDNA3-myc-mdm2-Δaa400–460. Two PCRs, each containing only one of the primers, were set up, and five cycles of PCR were performed using pDWM659 as a template. The two PCRs were combined, and another 30 PCR cycles were carried out. The DNA template was digested with Dpn1 before Escherichia coli were transformed with the PCR product. Primer sequences are available on request.

Antibodies—The following antibodies were used: the anti-Myc antibody 9E10 (Santa Cruz) as well as cell culture supernatant from an 9E10 hybridoma cell line, the anti-proliferating nuclear cell antigen (PCNA) monoclonal antibody PC10 (Santa Cruz), the anti-FLAG antibody M2 (Sigma), the anti-p53 antibody DO-1 (Santa Cruz), the anti-Mdm2 antibody 4B2 (obtained from David Lane), an HRP-coupled anti-V5 antibody (Invitrogen), an anti-acetyl-p53 antibody directed against lysine 373 and lysine 382 (Upstate), an HRP-coupled TrueBlot anti-mouse antibody (eBioscience), and a HRP-coupled anti-mouse antibody (DAKO).

Cell Lines and Their Treatments—293T cells were obtained from M. Litfin. H1299 cells were purchased from ATCC. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 units/ml penicillin/streptomycin at 37 °C and 5% CO2 in a humidified atmosphere. The cells were transiently transfected with calcium-phosphate or jet-PEI (PolyPlus). Nutilin-3 (Calbiochem) was added to a final concentration of 100 μM; curcumin (Calbiochem) was added to a final concentration of 100 μM, and MG132 (Calbiochem) was added to a final concentration of 5 μM.

Immuno precipitation—The cells were washed twice with ice-cold PBS and lysed in Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The protein extract was cleared by centrifugation at 13,000 × g at 4 °C for 15 min, and the protein concentration was determined by the method of Bradford. 50 μg of protein extract were loaded onto a 10% SDS-PAGE gel and transferred onto Immobilon P blotting membrane (Millipore). Immunodetection was performed as described (10). For the analysis of trimeric complexes, the membrane was incubated over night at 4 °C with cell culture supernatant of an anti-Myc hybridoma cell line diluted 1:5 in 5% bovine serum albumin, 0.05% Tween 20 in PBS. For visualization of p53 oligomers, the lysate was incubated for 15 min on ice with a 0.03% final concentration of glutaraldehyde.

Far Western Blotting—Mdm2 was immunoprecipitated as described above. The Mdm2-antibody complexes were separated by SDS-PAGE and blotted onto Immobilon P blotting membrane. After transfer, the membrane was blocked with 5% dry milk in PBS, 0.1% Tween 20 for 30 min. The blocking solution was discarded, and the membrane was incubated for 1 h with p53 protein that has been transcribed and translated in vitro (TN-T-T7; Promega). The membrane was washed three times with PBS, 0.1% Tween 20. Subsequently, standard Western blotting was performed as described above using the DO-1 antibody.

GST Pull-down—GST-Mdm2 fragments were expressed in bacteria, coupled to glutathione agarose, and phosphorylated with CKIδ in the presence of 1 mM ATP as described (19). p53 was transcribed and translated in vitro (TN-T-T7; Promega), phosphorylated by CKIδ where indicated, and added to GST-Mdm2. The mixture was incubated for 1.5 h with end-over-end rotation, washed three times in PBS, separated by a 10% SDS gel, and blotted. For phosphatase treatment, 50 units of λ-phosphatase (Upstate) were added, and the mixture was incubated for 10 min at 37 °C.

Ubiquitylation Assay—Ubiquitylation assays were performed as described (10).

Two-dimensional Peptide Mapping—Two dimensional peptide mapping was performed as described (19).

RESULTS

p53 Binds to Amino Acids 235–300 of the Central Domain of Mdm2—Previous investigations of the p53 and Mdm2 interaction indicated the presence of a second binding site for the Mdm2 protein on p53 (17, 18). To investigate whether, correspondingly, a second binding site for p53 exists on the Mdm2 protein, we generated a series of Myc-tagged Mdm2 deletion mutants (Fig. 1B) and transfected them into 293T cells. We precipitated Mdm2 with an anti-Myc antibody, separated the protein-antibody complexes by SDS-PAGE, and determined binding of p53 to the different Mdm2 fragments by far Western blotting. As expected, p53 associated with wild type Mdm2 (Fig. 1A). Surprisingly, deletion of the first 200 amino acids of Mdm2, which contain the classical p53-binding site, did not interfere with the binding of p53 to Mdm2 when the interaction was monitored by far Western blotting. This result strongly suggested that binding to the classical p53-binding site cannot be monitored by this technique. Because we still observed binding of p53 to an Mdm2 fragment lacking the N-terminal p53-binding site, we concluded that this interaction is due to binding to a “novel” second binding site on the Mdm2 protein. This idea was further supported by data that we obtained upon deletion of the central domain of Mdm2. Deletion of amino acids 200–300 of Mdm2 completely abolished binding of p53 to Mdm2. Because the “first” p53-binding site of the Mdm2 protein was still present in this construct, this result can only be explained with the removal of the novel second p53-binding site.

To determine the location of the second p53-binding site more precisely, we employed additional deletion mutants of
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Prior binding experiments of bacterially expressed Mdm2 and p53 with the central domain of Mdm2 were performed by GST pull-down experiments of bacterially expressed Mdm2 and p53 transcribed and translated in vitro. The association of p53 with a bacterially expressed Mdm2 fragment of the central domain was, however, barely detectable (Fig. 2, A and B). Because Mdm2 is constitutively phosphorylated at several sites in the central domain (10), we reasoned that the difference between the binding of p53 to the central domain of Mdm2 in far Western assays and the weak binding in GST pull-down experiments might be due to the lack of phosphorylation of bacterially expressed proteins. To test this idea, we incubated bacterially expressed Mdm2 with CKI\(\beta\) prior to its employment in the binding assay. As we show in Fig. 2 (A and B), binding of p53 to the central domain of Mdm2 was significantly enhanced when the central domain of Mdm2 was phosphorylated by recombinant CKI\(\beta\). Incubation of phosphorylated Mdm2 with \(\lambda\)-phosphatase prior to its employment in the binding assay prevented the enhancement in binding (Fig. 2B). Phosphorylation of p53 instead of Mdm2 did not influence the association of p53 and Mdm2 (Fig. 2B).

We next determined the phosphorylation sites that regulate the binding of p53 to the central domain of Mdm2. When we employed an Mdm2 mutant where serine 246, serine 253, serine 256, serine 260, and serine 262 of human Hdm2 were replaced with an aspartic acid, in the GST pull-down assay, binding of p53 to Hdm2 was enhanced, although not to a great extent. More importantly, prior incubation of this Hdm2 mutant with CKI\(\beta\) no longer enhanced the binding to p53 (Fig. 2C). The major CKI\(\beta\) phosphorylation sites of Mdm2 are serine 240, serine 242, and serine 246 of human Hdm2 (serine 238, 240, 242, 246, 253, 256, 260, 262, replaced with an aspartic acid).

Monitor Mdm2 levels and with an anti-V5 antibody to monitor p53 levels, followed by incubation with HRP-coupled anti-mouse antibody. To monitor expression levels, 50 \(\mu\)g of total cell lysate was separated by SDS-PAGE and blotted onto Immobilon P. The membrane was incubated with an anti-V5 antibody and with an anti-PCNA antibody, for loading control. IP, immunoprecipitation; WB, Western blot.

FIGURE 1. p53 associates with aa 235–aa 300 of the central domain of Mdm2. A, 293T cells were transfected with a cDNA encoding Myc-tagged wild type Mdm2 or the indicated Mdm2 deletion mutants. 24 h after transfection, the cells were harvested, and Mdm2 was precipitated using the anti-Myc antibody to Mdm2. Binding of p53 to Mdm2 was slightly reduced when amino acids 200–235 were removed (Fig. 1A). Deletion of amino acids 235–260 caused an even stronger decrease in binding, whereas removal of amino acids 260–300 completely eliminated the association of p53 and Mdm2 (Fig. 1A). In contrast, deletion of amino acids 300–400 of Mdm2 did not influence the association of p53 and Mdm2 (Fig. 1A).

We next investigated which part of p53 associated with the central region of Mdm2. We transfected three V5-tagged fragments of p53, encompassing the N terminus (aa 1–150), the p53 core (aa 150–300), and the C terminus (aa 300–393), into H1299 cells together with FLAG-tagged Mdm2 (aa 200–300) and determined the amount of p53 that was associated with Mdm2 by immunoprecipitation/Western. As shown in Fig. 1C, the core domain of p53 associated with the central part of Mdm2, whereas no interaction with the C-terminal domain of p53 was detectable. Because of rapid degradation, the N-terminus of p53 was hardly detectable in total cell lysate and gave no result in the immunoprecipitation/Western (data not shown).

Binding of p53 to the Central Domain of p53 Is Regulated by Phosphorylation—To further investigate the interaction of p53 with the central domain of Mdm2, we performed GST pull-down experiments of bacterially expressed Mdm2 and p53 transcribed and translated in vitro. The association of p53 with a bacterially expressed Mdm2 fragment of the central domain was, however, barely detectable (Fig. 2, A and B). Because Mdm2 is constitutively phosphorylated at several sites in the central domain (10), we reasoned that the difference between the binding of p53 to the central domain of Mdm2 in far Western assays and the weak binding in GST pull-down experiments might be due to the lack of phosphorylation of bacterially expressed proteins. To test this idea, we incubated bacterially expressed Mdm2 with CKI\(\beta\) prior to its employment in the binding assay. As we show in Fig. 2 (A and B), binding of p53 to the central domain of Mdm2 was significantly enhanced when the central domain of Mdm2 was phosphorylated by recombinant CKI\(\beta\). Incubation of phosphorylated Mdm2 with \(\lambda\)-phosphatase prior to its employment in the binding assay prevented the enhancement in binding (Fig. 2B). Phosphorylation of p53 instead of Mdm2 did not influence the association of p53 and Mdm2 (Fig. 2B).
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serine 240, and serine 244 of mouse Mdm2) (19), all of which are outside of the interaction site (Fig. 1A). However, by two-dimensional peptide mapping, we also identified serine 253 and serine 256 (these sites correspond to serine 251 and serine 254 of mouse Mdm2) as sites that are phosphorylated by CKIβ, at least in vitro (Fig. 2D). Correspondingly, overexpression of CKIβ also enhanced the binding of a p53 deletion mutant (aa 150–393) lacking the N-terminal domain to Mdm2 in vivo (Fig. 2E) and substitution of serine 251 or serine 254 with an alanine significantly reduced the increase in binding after overexpression of CKIβ. Likewise, substitution of serine 244, serine 251, and serine 254 with an alanine also reduced the CKIβ-mediated increase in binding. It should be noted that in several experiments, we observed an enhancement of p53 expression levels after overexpression of CKIβ (Fig. 2E). Nevertheless, the increase in binding in the presence of wild type Mdm2 after overexpression of CKIβ was always higher than the CKIβ-mediated up-regulation of p53 levels.

Both the N-terminal and the Central p53-binding Site Contribute to Total p53 Binding—We next determined the contribution of the two individual binding sites to the total binding of p53 to Mdm2. We transfected H1299 with Myc-tagged Mdm2 lacking an N-terminal domain (Δ1–199) or with Myc-tagged Mdm2 lacking the central domain (Δ200–300). To prevent the interaction of the N-terminal domain of Mdm2 with the N-terminal domain of p53, we added nutlin, an inhibitor of the N-terminal interaction of p53 and Mdm2 (20), to some of the culture dishes. We precipitated Mdm2 with the anti-Myc antibody and determined both with an anti-Myc and with an anti-Flag antibody, and the relative amount of co-precipitating p53 for each immunoprecipitation was determined by Western blotting. In these experiments, p53 co-precipitated both with the Myc-tagged N-terminal domain of Mdm2 and with the FLAG-tagged central domain of Mdm2. The Mdm2 protein fragments were precipitated both with an anti-Myc and with an anti-Flag antibody, and the relative amount of co-precipitating p53 for each immunoprecipitation was determined by Western blotting. In these experiments, p53 co-precipitated both with the Myc-tagged N-terminal domain of Mdm2 and with the FLAG-tagged central domain of Mdm2. The Mdm2 protein fragments were precipitated both with an anti-Myc and with an anti-Flag antibody, and the relative amount of co-precipitating p53 for each immunoprecipitation was determined by Western blotting.

Under physiologic conditions, the p53 protein forms dimers and tetramers (21). This property of p53 raised the intriguing question of whether the enhanced binding in the presence of
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FIGURE 3. The N-terminal domain and the acidic domain of Mdm2 and p53 form a trimeric complex. A, H1299 cells were transfected with cDNAs encoding p53 and Myc-tagged wild type Mdm2 or the indicated deletion mutants. 24 h after transfection, the cells were treated with 100 μM nutlin-3 for 4 h. The cells were harvested, and Mdm2 was precipitated using the anti-Myc antibody coupled to protein A-agarose. The complexes were separated by SDS-PAGE and blotted. The blotting membrane was divided into two parts. The upper part was incubated with an anti-Myc antibody to monitor Mdm2 levels, and the lower part was incubated with an DO-1 antibody to monitor p53 levels, followed by incubation with HRP-coupled anti-mouse antibody. To monitor expression levels, 50 μg of total cell lysate were separated by SDS-PAGE and blotted onto Immobilon P. The membrane was divided into two parts. The upper part was incubated with an antibody directed against p53, and the lower part was incubated with an anti-PCNA antibody for loading control. B, 293 cells were transfected with cDNAs encoding a Myc-tagged N-terminal fragment (aa 1–199) and a FLAG-tagged central domain (aa 200–300) of Mdm2. 19 h after transfection, MG132 was added for 5 h. The central domain of Mdm2 was precipitated with an anti-FLAG antibody coupled to protein A-Sepharose. The complexes were washed twice with ice-cold PBS and loaded onto a 12% SDS-PAGE gel. The FLAG-tagged central domain and the co-precipitating Myc-tagged N-terminal domain were detected by Western blotting using anti-Myc and anti-FLAG antibodies, respectively. To monitor expression levels, 50 μg of total cell lysate were separated by SDS-PAGE and blotted onto Immobilon P. The membrane was first hybridized with an anti-Myc antibody and developed before it was divided into two parts. The upper part was incubated with an antibody directed against p53, and the lower part was incubated with an anti-PCNA antibody for loading control. IP, immunoprecipitation; WB, Western blot; TCL, total cell lysate.

FIGURE 4. The N-terminal and central domains of Mdm2 act synergistically in binding to p53. A, H1299 cells were transfected with plasmids encoding wild type p53, the mutants R337C and L344P or vector DNA for control together with plasmids encoding a Myc-tagged N-terminal fragment of Mdm2 (aa 1–199) and/or a FLAG-tagged fragment of the central domain of Mdm2 (aa 200–300). 24 h after transfection, the cells were lysed and divided into two aliquots from which Mdm2/p53 complexes were precipitated both with the anti-Myc antibody and with the anti-FLAG antibody. The complexes were separated by SDS-PAGE and blotted. The membranes were divided into two parts. The upper part was incubated with an anti-FLAG or anti-Myc antibody, respectively, and the upper part was incubated with an anti-p53 antibody followed by incubation with HRP-coupled TrueBlot anti-mouse antibody. To monitor expression levels, 50 μg of total cell lysate were separated by SDS-PAGE and blotted onto Immobilon P. The membrane was divided into two parts. The upper part was incubated with an anti-p53 antibody and the lower part was incubated with an anti-PCNA antibody for loading control. B, 8, H1299 cells were transfected with wt p53, the R337C, or L344P mutant and lysed after 24 h. The lysate was incubated with 0.03% glutaraldehyde (final concentration) for 15 min before 50 μg of cellular lysate were separated by SDS-PAGE. After transfer onto Immobilon P, the membrane was divided into two parts. The upper part was incubated with an antibody for p53, and the lower part was incubated with an antibody for PCNA and subsequently with an HRP-linked anti-mouse antibody. Western blots were developed by ECL. IP, immunoprecipitation; WB, Western blot; TCL, total cell lysate.

Binding to Both p53-binding Sites on the Mdm2 Protein Correlates with p53 Degradation—Previous reports on p53 degradation have shown that the central domain of Mdm2 assists in both binding sites is due to the binding of one p53 molecule to both binding sites on the Mdm2 protein or to the binding of different p53 proteins of an oligomer. To test this objective, two p53 mutants (R337C and L344P) from Li-Fraumeni patients with mutations in the oligomerization domain (22) were employed. The ability of these mutants to oligomerize was assayed by incubating cell lysates with glutaraldehyde followed by SDS-PAGE and Western blotting. Although p53 dimers, trimers, and oligomers were present in the lysate of cells transfected with wild type p53, no p53 dimers or multimers were present in lysates from cells transfected with the p53 oligomerization mutants R337C or L344P (Fig. 4B). As shown in Fig. 4A, p53 proteins with mutations in the oligomerization domain still associated with the N-terminal first p53-binding site and also with the central second p53-binding site of Mdm2. However, binding in the presence of both the N-terminal fragment and the central fragment was much reduced in comparison with wild type p53, most likely because of the lack of assistance in binding of p53 oligomerization mutants.
ubiquitylated proteins were purified by adsorption to Ni\(^{2+}\) agarose. In consistency with previous reports (10, 25), p53 was efficiently polyubiquitylated and degraded by wild type Mdm2, whereas deletion of the central domain prevented p53 polyubiquitylation and degradation (Fig. 5). Deletion of amino acids 200–235 slightly reduced p53 degradation, although p53 was still polyubiquitylated. In contrast, when we deleted amino acids 235–260 or amino acids 260–300, p53 was still mono- and bi-ubiquitylated (or bi-mono-ubiquitylated), but polyubiquitylation was largely impaired, and the protein accumulated to high levels (Fig. 5). These results show that sequences within the Mdm2 protein that are required for binding of p53 to the second binding site are identical to sequences of Mdm2 that mediate polyubiquitylation of p53.

The observation that the second p53-binding site on the Mdm2 protein overlapped with the domain of Mdm2 that is required for ubiquitylation suggested that binding of p53 to the second p53-binding site may be an obligatory step for polyubiquitylation. To further investigate this thought, we investigated Mdm2-mediated degradation of the p53 oligomerization mutants R337C and L344P. We transfected H1299 cells with cDNAs encoding either wild type p53 or the p53 oligomerization mutants R337C and L344P together with cDNAs encoding Mdm2 or vector DNA for control. Twenty-four hours after transfection, we monitored total p53 levels by straight Western blotting and the level of ubiquitylated p53 by purification of ubiquitylated proteins and subsequent Western blotting. Although co-transfection of Mdm2 reduced the amount of wild type p53, it failed to reduce the levels of the two p53 oligomerization mutants R337C and L344P (Fig. 6). Interestingly, similar to the deletion of the central domain of Mdm2, mutation of the oligomerization domain of p53 allowed mono- and bi-ubiquitylation of p53, whereas polyubiquitylation of p53 oligomerization mutants was significantly reduced.

A prerequisite for p53 ubiquitylation is prior deacetylation (26), which is mediated by Mdm2 (27). We therefore wondered whether the reduction in p53 polyubiquitylation in the absence of synergistic binding might result from a failure in deacetylation. To address this question, we determined p53 acetylation status of the oligomerization mutants R377C and L344P and of wild type p53 in the presence of an Mdm2 deletion mutant lacking the second p53-binding site (Δ260–300) by Western blotting using an acetyl-p53-specific antibody. As also shown by others (26, 27), a significant part of cellular p53 was constitutively acetylated (Fig. 7A). In the presence of wild type Mdm2, p53 was deacetylated, and p53 was degraded (Fig. 7A). The Mdm2 deletion mutant (Δ260–300), however, failed to mediate p53 deacetylation and p53 degradation. Interestingly, the two Li-Fraumeni mutants were efficiently deacetylated by wild type Mdm2 (Fig. 7A) despite the failure in polyubiquitylation and degradation (Figs. 6 and 7A). We therefore speculated that mediating p53 deacetylation might not be the only function of the second p53-binding site. To test this idea, we transfected H1299 cells with p53 in the presence of wild type Mdm2 or the Mdm2 mutant lacking the second p53-binding site (Δ260–300). Twenty-four hours prior to lysis, we incubated cells with the p300 acetyl-transferase inhibitor curcumin and determined total p53 levels and acetylation status of p53 by Western blotting. As shown in Fig. 7B, acetylation of p53 was completely prevented in the presence of curcumin. However, despite the absence of p53 acetylation, the Mdm2 mutant lacking the second p53-binding site failed to target p53 for degradation.

**DISCUSSION**

To better understand the regulation of p53, we investigated the association of p53 with its negative regulator Mdm2. Inspired by the recently described second Mdm2-binding site on the p53 protein (17, 18), we searched for a potential second p53-binding site on Mdm2. By applying the far Western blotting technique, we identified a second p53-binding site in the central domain of Mdm2 spanning from amino acids 235–300. Interestingly, by the far Western blotting technique, we only detected binding of p53 to the central domain and not to the classical N-terminal first p53-binding site. Most likely, the first p53-binding site lost some structural determinants during gel electrophoresis. Although the far Western technique involves a renaturation step, renaturation may be insufficient for complete refolding of the N-terminal domain of Mdm2, and in consequence, binding of p53 to the N-terminal p53-binding site is lost. Because binding of p53 to the central domain was easily detected by this technique, we assume that the folding of the
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FIGURE 6. Failure in p53 oligomerization prevents p53 polyubiquitylation and degradation. H1299 cells were co-transfected with cDNAs encoding His-tagged ubiquitin, wt p53, C337R, or L344P together with wild type Mdm2. 24 h after transfection, the cells were lysed, and an aliquot of the lysate was separated by SDS-PAGE to monitor total p53 and Mdm2 expression. From the remaining lysate, ubiquitylated proteins were purified by adsorption to Ni²⁺-agarose and separated by SDS-PAGE. After transfer, the blotting membrane was incubated with the anti-p53 antibody DO-1 followed by incubation with an HRP-coupled anti-mouse antibody. The Western blots were developed by ECL. TCL, total cell lysate.

A

B

FIGURE 7. The central domain of Mdm2 mediates p53 deacetylation. A, H1299 cells were transfected with cDNAs encoding wt p53 in the absence or presence of a cDNA encoding wt Mdm2 or mutant Mdm2 lacking amino acids 260 to 300 (Δ260–300) or with cDNAs encoding mutant p53 (R337C and L344P) in the presence of absence of a cDNA encoding wild type Mdm2. 24 h after transfection, the cells were lysed. 50 μg of cellular extract were separated by SDS-PAGE and blotted onto Immobilon blotting membrane. The membrane was divided into three parts. The upper part was hybridized with the anti-Mdm2 antibody 4B2, and the lower part was hybridized with an antibody directed against PCNA for loading control. The third part was first hybridized with an antibody directed against acetylated p53 and rehybridized with the anti-p53 antibody DO-1. B, H1299 cells were transfected with cDNAs encoding wt p53 in the absence or presence of a cDNA encoding wt Mdm2 or mutant Mdm2 lacking amino acids 260–300 (Δ260–300). Twenty-four hours prior to lysis, curcumin was added to a final concentration of 100 μM. Forty-eight hours after transfection, the levels of acetylated and total p53 were determined as described in the legend to A.

central domain of Mdm2 is less complex or that the central domain of Mdm2 is less structured.

The second binding site on Mdm2 interacted with the core domain of p53, as also shown by others (11, 18). In contrast to cell-derived Mdm2, p53 bound only weakly to the central domain of Mdm2 when Mdm2 was expressed in bacteria. However, binding was significantly enhanced when the bacterially expressed central domain of Mdm2 was phosphorylated by CKIδ prior to GST pull-down. Overexpression of CKIδ also enhanced binding of p53 to the central domain of Mdm2 in vivo. Serine 251 and serine 254 (of mouse Mdm2) turned out to be particularly important for this enhanced interaction after CKIδ overexpression. Interestingly, these phosphorylation sites do not encompass known CKIδ phosphorylation sites (19).

While this manuscript was under review, Yu et al. (30) reported about binding of p53 to the central domain of Mdm2. In their hands, binding of p53 to the central domain was insensitive to phosphorylation. However, they missed investigating the most critical residues serine 251 and serine 254.

Most importantly, p53 not only binds to two different sites on one Mdm2 molecule; in the presence of both binding sites, binding of Mdm2 to p53 was significantly enhanced. Because p53 mutants that fail to oligomerize are incapable in showing this enhanced binding, we speculate that two different p53 molecules of one tetramer may occupy the two binding sites. This second p53 molecule of one tetramer that binds to the same Mdm2 molecule may strengthen the interaction between p53 and Mdm2, thus leading to the observed enhanced binding of tetramerization-competent p53 molecules. Interestingly, this enhanced binding was even observed when the central domain and the N-terminal domain of Mdm2 were located on two different molecules. Thus it is possible that binding to one domain of Mdm2 induces a conformational change in p53 that facilitates binding to the other p53-binding site. Accordingly, p53 oligomerization mutants such as C337R or L344P did not show such an enhanced binding.

The p53-binding site in the central domain of Mdm2 overlaps with a region of the Mdm2 protein that is important for p53 degradation. Replacement of phosphorylation sites in this domain with an alanine resulted in accumulation of ubiquitylated p53 (10), whereas removal of larger parts of the central domain of Mdm2 interfered (23, 24) with p53 ubiquitylation. Interestingly, deletion of amino acids 235–260 or deletion of amino acids 260–300 prevented p53 polyubiquitylation, whereas p53 was still mono- or bi-ubiquitylated. (Whether bi-ubiquitylation occurs at a single lysine or at two different lysines could not be determined by this experimental setting). In consequence, p53 was not degraded. In contrast, deletion of amino
acids 200–235 resulted in a marginal increase in the 53-kDa form of p53, and the p53 protein was fully ubiquitylated. p53 sequences that are required for polyubiquitylation thus completely overlap with sequences that are involved in p53 binding to the central domain of Mdm2. Our data would therefore be consistent with a requirement for binding of p53 to the central domain for p53 polyubiquitylation. It is, however, rather unlikely that Mdm2 itself has an enzymatic activity in the central domain that is required for efficient polyubiquitylation. Instead, it is much more conceivable that binding of p53 to the central domain may bring the tumor suppressor protein into a position that allows other proteins that also associate with the central domain of Mdm2 to exert their activity on p53. In this context it is of particular interest that p300, a “chain assembly factor” or “E4” for p53, associates with Mdm2 within this domain (29). p53 mutants that are deficient for oligomerization (e.g. C337R or L344P) are most likely not properly presented to these factors. In consequence they are not efficiently polyubiquitylated and hence not degraded.

A prerequisite for p53 ubiquitylation is prior removal of acetyl groups in the C-terminal domain of p53 because both modifications compete with the e-amino group of C-terminal lysines (26, 27). Moreover, Mdm2 has been shown to mediate p53 deacetylation, p53 bringing about deacetylase activity. However, although the central domain of Mdm2 is required for p53 deacetylation, p53 oligomerization mutants that fail to show enhanced binding are efficiently deacetylated by Mdm2 but nevertheless only inefficiently polyubiquitylated and not degraded. Likewise, treatment of cells with curcumin prevented p53 acetylation but did not restore p53 degradation by a Mdm2 mutant lacking the second p53-binding site.

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