A Role for the Polyproline Domain of p53 in Its Regulation by Mdm2

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The p53 protein plays a key role in the cellular response to stress by inducing cell growth arrest or apoptosis. The polyproline region of p53 has been shown to be important for its growth suppression activity. p53 protein lacking the polyproline region has impaired apoptotic activity and altered specificity for certain apoptotic target genes. Here we describe the role of this region in the regulation of p53 by its inhibitor Mdm2. p53 lacking the polyproline region was identified to be more susceptible to inhibition by Mdm2. Furthermore, the absence of this region renders p53 more accessible to ubiquitination, nuclear export, and Mdm2-mediated degradation. This increased sensitivity to Mdm2 results from an enhanced affinity of Mdm2 toward p53 lacking the polyproline region. Our results provide a new explanation for the impaired growth suppression activity of p53 lacking this region. The polyproline region is proposed to be important in the modulation of the inhibitory effects of Mdm2 on p53 activities and stability.

The p53 tumor suppressor has been implicated in the prevention of many types of cancer (1). In response to various stress signals, such as DNA damage, wild type (wt) p53 promotes cell cycle arrest (2). However, in response to certain oncogenic changes, or when the stress is excessive, p53 can induce apoptotic cell death (3). The apoptotic activity of p53 is mediated by both transcriptional-dependent and -independent pathways that cooperate to mediate a full apoptotic response (3, 4). Several p53 apoptotic target genes have been identified, including bax, Fas/Apo-1, KILLER/DR5, IGF-BP3, PAG-608, PIG3, PERP, and MCG10 (Ref. 3 and references therein; Refs. 5 and 6). However, each of these target genes contribute only partially to the overall apoptotic response mediated by p53 (for reviews, see Refs. 3 and 4). Hence, multiple apoptotic pathways presumably operate in parallel.

Little is known about the transactivation-independent apoptotic activity of p53. New insights have been provided from studies defining functional domains responsible for growth suppression. Initially, Walker and Levine (8) revealed a conserved PXXP motifs that are important for growth suppression. Deletion of these motifs impairs the ability of p53 to induce apoptosis, without affecting its ability to induce growth arrest (7–9). Intriguingly, p53 lacking the polyproline region has altered specificity for endogenous target promoters. There is a decrease in the induction of several apoptotic genes: PIG3, PIG6, PIG11, p85, and BTG2 (9), many of which were implicated in the cellular apoptotic response to oxidative stress (10, 11). However, the induction of other apoptotic genes, such as bax and KILLER/DR5, is unaffected (9). This proline-rich region was also shown to be required for p53-dependent cell growth arrest through Gas-1 (12), a plasma membrane protein highly expressed during G0. The identifications of a germ line mutation within the polyproline region (proline 82) in cancer patients with Li-Fraumeni syndrome (13) and of somatic mutations in bladder tumors (proline 85 and 89; Ref. 14) are consistent with this region playing an important role in regulating p53 activity. Moreover, the phenotype described for p53 lacking the polyproline region is similar to that observed in tumor-derived p53 mutants (15).

The p53 protein is subject to tight regulation at multiple levels, including protein stability, post-translational modifications, and subcellular localization (for reviews, see Refs. 16 and 17). The key player in p53 regulation is the proto-oncogene mdm2, which is amplified in a variety of tumors (18). Mdm2 blocks the transcriptional activity of p53 and its ability to induce growth arrest and apoptosis (for reviews, see Refs. 18–20). In addition, Mdm2 promotes the degradation of p53 through the ubiquitin-proteasome pathway (21, 22) by acting as an E3-ligase (23). Negative regulation of p53 by Mdm2 is modulated by specific modifications, such as phosphorylation (20), or through the action of partner proteins, such as p19ARF and c-Abl (for reviews, see Refs. 3 and 24).

In this study we examined the role of the polyproline-rich region of p53 in the regulation of p53 by Mdm2. We found that the apoptotic and transcriptional activities of a p53 protein lacking the polyproline region were more susceptible to negative regulation by Mdm2 than its wt counterpart. In the absence of this region, p53 is more accessible than wt p53 to ubiquitination and nuclear export, and consequently more susceptible to Mdm2-mediated degradation. These effects result from an enhanced affinity of Mdm2 for p53 that lacks the polyproline region. Our results support a role for the polyproline region of p53 in modulating p53 regulation by Mdm2.

EXPERIMENTAL PROCEDURES

Cells and Transfection Assays—Mouse embryo fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium, and H1299 and Saso-2 cells were grown in Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum at 37 °C. The Saso-2 cell line is derived from an osteosarcoma, and the H1299 cell line is derived from a lung carcinoma; both lines are devoid of any p53 expression. MEFs were derived from p53−/− knockout mice (KO) or from the p53−/−/
mdm2−/− double knockout mice (2KO) (25). Transfections were carried out as outlined previously (26). The amounts of expression plasmids used in each experiment are indicated in the corresponding figure legends. To maintain a constant amount of plasmid DNA in each sample, an empty vector was added.

The luciferase assay and Western blot analysis were carried out as previously described (27). The apoptotic assay was carried out essentially as previously described (26). Samples were analyzed in a cell sorter (FACSCalibur) using the CellQuest software (Becton Dickinson).

The apoptotic fraction was determined by measuring the number of cells possessing a sub-G1 DNA content (27).

For pulse-chase analysis, H1299 cells were transfected as indicated. Twenty-four h post-transfection, cells were metabolically labeled with 150 μCi of [35S]Met plus Cys for 30 min. Cells were then washed and chased in nonradioactive medium for the indicated periods. Samples containing the same amount of radioactivity were subjected to immunoprecipitation with the anti-p53 antibody PAb421 as previously described (27). Immunocomplexes were resolved by SDS-polyacrylamide gel electrophoresis and exposed to x-ray film. The half-life of p53 proteins was quantified by scanning the autoradiogram using a densitometer (Aida).

For immunofluorescent staining, H1299 cells were plated on glass coverslips. Twenty-four h post-transfection, cells were treated for 4 h with the proteasome inhibitor ALLN (150 μm; Calbiochem) to prevent the degradation of p53 in the cytoplasm. Cells were fixed in 4% methanol and stained with anti-p53 antibodies (PAb1801 and DO1) followed by Cy3-conjugated goat anti-mouse secondary antibody. Cells were stained simultaneously for DNA using 4',6-diamidino-2-phenylindole. Stained cells were observed under the confocal microscope (Zeiss).

The antibodies used were as follows: anti-human p53 monoclonal antibodies PAb1801, PAb421, and DO1, anti-Hdm2 SM14 (28), and an anti-α-tubulin antibody (DM1A, Sigma).

Plasmids—Expression plasmids were as follows: human wt p53 (pRC/CMV wt-p53), human mutant p53 lacking the proline-rich region (pRC/CMV p53ΔproAE; Ref. 8), mouse wt mdm2 (pCOV-mdm2 x2; Ref. 27), and human Hdm2 (PCMV-Neo-Bam-Hdm2). The reporter plasmid used was the βgal luciferase (29).

Ubiquitination Assay in Vivo—The ubiquitination of p53 in vivo was detected by transfecting H1299 cells with 1.5 μg of hp53Δpro or hp53 expression plasmids alone or together with the indicated amounts of expression plasmid for mdm2. Twenty-two h post-transfection, cells were treated with 150 μm ALLN for 4 h. Following treatment, cells were subjected to nuclear cytoplasmic fractionation. To prepare the cytoplasmic fraction, the cell pellets were resuspended in cytoplasmic buffer (10 mM Tris·HCl, pH 8.0, 10 mM KCl). Cells were allowed to swell for 2 min, and then Nonidet P-40 was added to 0.6% followed by centrifugation. The supernatant contained the soluble cytoplasmic fraction. The pellets were washed once more with the cytoplasmic buffer before proceeding to nuclear fractionation. Preparation of the nuclear fraction from the remaining cell pellet was undertaken by resuspending in high salt radioimmune precipitation buffer (50 mM Tris, pH 8.0, 5 mM NaCl, 400 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, and 0.025% SDS). The purity of the cytoplasmic fraction was verified by probing with anti-α-tubulin antibody, whereas that of the nuclear fraction was verified with anti-histone H2B antibody. Nuclear and cytoplasmic extracts were subjected to Western blot analysis using the indicated antibodies.

RESULTS

The Inhibition of the Apoptotic Activity of p53Δpro by Mdm2

Is Greater than That of wt p53—Deletions of the polyproline region of p53 has been shown to impair the apoptotic activity of p53 (30). Because Mdm2 inhibits the apoptotic activity of p53 (27, 31), we argued that one possible explanation for this impairment is that the deletion of the polyproline region of p53 renders it more sensitive to inhibition by Mdm2. This conjecture was tested by a transient apoptosis assay in Saos-2 cells. Cells were transfected with expression plasmids for wt human p53 or mutant p53 lacking all five proline motifs (p53Δpro; Ref. 8). Seventy-two h post-transfection, cells were harvested, stained for p53, and subjected to flow-cytometric analysis (27). Cells with background levels of fluorescence represent the non-transfected subpopulation (NT in Fig. 1A), whereas cells with high fluorescent intensity represent the successfully transfected subpopulation (T in Fig. 1A). The cell cycle distribution of each subpopulation was analyzed separately, and the proportion of cells with sub-G1 DNA content was determined (Fig. 1B). Expression of wt p53 in these cells was shown to induce apoptosis in 26% of the transfected subpopulation, whereas expression of p53Δpro induced only 14% apoptosis (Fig. 1, C–E), which is consistent with previous reports (7, 30). It should be noted that the expression level of p53Δpro was similar, or even slightly higher, than that of wt p53, as measured by the fluorescent intensity of the two transfected subpopulations (Fig. 1F). This result demonstrates that under these experimental conditions the impaired apoptotic activity of p53Δpro is more sensitive than wt p53 to inhibition by Mdm2. Saos-2 cells were transfected with 1 μg of either wt p53 or p53Δpro expression plasmid alone or together with expression plasmid for mdm2. Seventy-two h post-transfection, cells were harvested, fixed, and stained for p53 using anti-p53 antibodies, DO1 and PAb1801, followed by fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody. Labeled cells were subjected to flow-cytometric analysis. Even numbers of cells from each population were collected and analyzed separately. A, fluorescent intensity of the nontransfected (NT) subpopulation and of the p53-transfected subpopulation (T). B, the cell cycle distribution of the nontransfected subpopulation as determined by DNA content. The area of the apoptotic cells is marked by Sub-G1. Also shown is the DNA content of cells transfected with wt p53 (C) or with p53Δpro (D). The percentage of apoptotic subpopulation is shown, E, a summary of triplicates from one of five independent experiments. F, histogram showing the fluorescent intensity of wt p53 (dark line) and p53Δpro (light line). Also shown is the DNA content of cells transfected with wt p53 and mdm2 expression plasmids (G) or p53Δpro and mdm2 (H). I, relative apoptotic activity of wt p53 and p53Δpro alone or together with mdm2. The extent of apoptosis obtained for each p53 plasmid alone was taken as 100% relative apoptosis (black bars), and the residual activity in the presence of 2 μg (gray bars) or 4 μg (white bars) of Mdm2 was calculated relative to this value. Standard errors from three independent experiments are indicated. J, histogram showing the fluorescent intensity of wt p53 + Mdm2 (dark line) or p53Δpro + Mdm2 (light line).
p53Δpro is not due to reduced levels of expression, consistent with previous findings (7, 30).

At the next step, we tested whether the impaired apoptotic activity of p53Δpro results from an increased sensitivity to Mdm2-mediated inhibition. For this purpose, the apoptotic activities of wt p53 and p53Δpro were compared in the presence of increasing amounts of mdm2 expression plasmid. Saos-2 cells were transfected with 1 μg of expression plasmid for wt p53 or p53Δpro, together with an expression plasmid for mdm2. In the presence of 2 and 4 μg of mdm2 expression plasmid, the apoptotic activity of wt p53 was reduced by 25 and 50%, respectively (Fig. 1, A and B), whereas that of p53Δpro was reduced by 50 and 80%, respectively (Fig. 1, C and D). Here too the p53 fluorescent intensities of cells transfected with wt p53 or p53Δpro were identical, even in the presence of Mdm2 (Fig. 1E). Taken together, these results strongly implicate the involvement of Mdm2 in the impaired apoptotic activity of p53 lacking the polyproline region.

Mdm2 Inhibition of Transcriptional Activity Is Greater for p53Δpro than for wt p53—The inhibition of p53-mediated apoptosis by Mdm2 was shown to be largely due to inhibition of the transcriptional activity of p53 (27, 31). Based on the findings above, we predicted that the impaired apoptotic activity of p53Δpro results from enhanced sensitivity of its transcriptional activity to inhibition by Mdm2. To test this prediction, the transcriptional activity of wt p53 and of p53Δpro was compared in fibroblasts that lack p53 and either express or lack endogenous Mdm2. MEFs derived from p53Δ−/− KO or from p53Δ−/− /mdm2Δ−/− 2KO were used (25; a generous gift from Dr. G. Lozano). Transcriptional activity was measured using the luciferase reporter plasmid driven under the bax promoter. The induction of the bax reporter by low levels of p53Δpro was previously shown to be impaired (7). The KO and 2KO MEFs were transfected with the bax luciferase reporter plasmid together with expression plasmids for wt p53 or p53Δpro. In MEFs expressing endogenous mdm2, the transcriptional activity of p53Δpro was 50% lower than that induced by wt p53 (Fig. 2A), consistent with previous reports using similar conditions (7). However, this difference in activity was diminished in MEFs lacking mdm2 expression, where the transcriptional activity of p53Δpro was even slightly higher than that of wt p53 (Fig. 2A). These results support the notion that the impaired transcriptional activity of p53Δpro, at least for some promoters such as bax, is due to increased sensitivity to inhibition by Mdm2. The complete absence of transcriptional activity for certain genes, such as PIG3, involve a loss of DNA binding ability to the corresponding promoters (7, 9, 15).

Next we determined whether the differences in the transcriptional activities between wt p53 and p53Δpro in the 2KO line were due to altered expression of p53Δpro. The steady state level of each form of p53 was measured by Western blot analysis using anti-p53 antibodies. For this purpose a sample was taken from one of multiple dishes that were used in the luciferase assay. This analysis revealed that within each cell line p53Δpro was expressed at a similar level, or even slightly higher than wt p53 (Fig. 2B). The expression levels of both proteins were reduced in the KO cells due to endogenous mdm2 expression (Fig. 2B). These results support the notion that the impaired transcriptional activity of p53Δpro results from an increased sensitivity to inhibition by Mdm2 as compared with wt p53.

Absence of the Polyproline Region Renders p53 More Susceptible to Mdm2-mediated Degradation—Because Mdm2 promotes p53 for degradation, it was predicted from the above findings that p53Δpro would be more sensitive than wt p53 to degradation by Mdm2. In the assays described above the steady state levels of wt p53 and p53Δpro were found to be equivalent (Figs. 1, A and B, and 2B). To examine the possibility that p53Δpro is more susceptible to Mdm2-mediated degradation, we used two assays. First, we compared the steady state levels of wt p53 and p53Δpro by using very low amounts of expression plasmids for each p53 form in the presence or absence of Mdm2. For this purpose lung adenocarcinoma cells, H1299, lacking p53 expression were transfected with 50 ng of each p53 expression plasmid alone or together with increasing amounts of mdm2 expression plasmids (100, 200, or 300 ng). Twenty-four h post-transfection, cells were harvested, and the luciferase activity was determined. The luciferase activity is shown in arbitrary units; the average and S.D. from three independent experiments are shown. B, one dish from each type (as indicated) was used to visualize the p53 protein by Western blot analysis using a mixture of anti-p53 antibodies, DO1 and PA1B1801.

In the second assay, the half-life of p53Δpro and wt p53 was compared. H1299 cells were transfected with expression plasmids for wt p53 or p53Δpro. Twenty-four h post-transfection, cells were metabolically labeled with [35S]Met/Cys and then chased in nonradioactive medium for 0, 1, 3, or 6 h. The p53 proteins were precipitated from each cell extract and subjected to SDS-polyacrylamide gel electrophoresis analysis. At 0 h chase time, the amount of p53Δpro was similar to, or even higher than, that of wt p53, hence excluding the possibility that...
The Polyproline Region Modulates p53 Regulation by Mdm2

FIG. 3. p53Δpro is more sensitive than wt p53 to Mdm2-mediated degradation. H1299 cells were transfected with 50 ng of either wt p53 (lane 1) or p53Δpro (lane 5) expression plasmid alone or together with 100 ng (lanes 2 and 6), 200 ng (lanes 3 and 7), or 300 ng (lanes 4 and 8) of expression plasmid for mdm2. Twenty-four h post-transfection, cells were harvested, and cell extracts were subjected to Western blot analysis as in Fig. 2. The amount of p53 in each lane was quantified using a densitometer (Aida). The amount of protein loaded was monitored by reactivity of the same blot with anti-α-tubulin. The positions of wt p53, p53Δpro, and α-tubulin are marked by arrows.

The Lack of the Polyproline of p53 Enhances Its Nuclear Export—The nuclear export of p53 is essential for its degradation (data not shown). Even in the presence of ALLN, as observed above (e.g. Figs. 3 and 6), the nuclear export of p53 continues to be mediated by Mdm2. However, with increasing chase time, the amount of radioactively labeled wt p53 was elevated (Fig. 4, lanes 3 and 5), consistent with previous findings (21), suggesting that the half-life of exogenous wt p53 was longer than 6 h. By contrast, the amount of radioactively labeled p53Δpro increased after a 1-hour chase, and thereafter the levels decreased. Thus, the half-life of p53Δpro is significantly shorter than that of wt p53 (Fig. 4). Taken together, these two assays demonstrate that p53Δpro is less stable than wt p53 and is more sensitive than wt p53 to degradation by Mdm2.

p53Δpro Is More Susceptible to Ubiquitination than Is wt p53—The findings that p53Δpro is less stable than wt p53 (Fig. 4) raised the possibility that p53Δpro is more susceptible to ubiquitination than is wt p53. This possibility was tested in an in vivo ubiquitination assay. It has recently been reported that p53 undergoes ubiquitination in the nucleus (33). Therefore, the extent of ubiquitination of wt p53 and p53Δpro was examined in the nuclear and cytoplasmic fractions. To test the effect in vivo, H1299 cells were transfected with expression plasmids for wt p53 or p53Δpro. Twenty-four h post transfection, cells were treated with ALLN for 4 h, to prevent p53 degradation, prior to harvest. Extracts from nuclear and cytoplasmic fractions were subjected to Western blot analysis using anti-p53 antibody (PAb421). p53 bands of molecular weight larger than p53 and p53Δpro represent p53-ubiquitin conjugates (Fig. 5A, lane 3). These bands do not appear in the absence of ALLN and were previously shown to contain ubiquitin molecules by coimmunoprecipitation assay using ubiquitin-hemagglutinin tag (data not shown). Effective ubiquitination of p53Δpro but not of wt p53 was observed (Fig. 5A, lanes 1 and 3). This ubiquitination is believed to be mediated by Mdm2. Attempts to measure the effect of exogenous Mdm2 on the ubiquitination of p53Δpro failed because of its high sensitivity to Mdm2-mediated degradation (data not shown) even in the presence of ALLN, as observed above (e.g. Figs. 3 and 6). Interestingly, the ubiquitination was confined almost exclusively to the nuclear fraction. This result supports the notion that p53Δpro is more susceptible to ubiquitination than wt p53 and that the ubiquitination of p53Δpro occurs largely in the nucleus.

The overall increased sensitivity of p53Δpro to Mdm2 suggested that p53 lacking the polyproline region may be more accessible to binding by Mdm2. This notion was tested by comparing the binding of human Mdm2 (Hdm2) to wt p53 or p53Δpro using a coimmunoprecipitation assay. H1299 cells were transfected with each expression plasmid alone or with Hdm2 together with each p53 expression plasmid. Twenty-two h post-transfection, cells were incubated with ALLN (150 μM) for 2 h to protect p53 from degradation, before cell extracts were prepared. Immunocomplexes were identified by immunoprecipitation of p53 using anti-p53 antibody (PAb421), followed by Western blot analysis using anti-Hdm2 antibody (SMP14). As shown in Fig. 6A, Hdm2 bound stronger to p53Δpro than to wt p53 despite the higher expression of the latter. When the binding was normalized to the amount of p53 proteins expressed, Hdm2 bound p53Δpro 10-fold stronger than wt p53. These results suggest that the absence of the proline-rich region of p53 enhances the binding affinity of p53 to Hdm2.

A p53 protein lacking the polyproline region of p53 has impaired apoptotic activity (7, 9, 30). This impairment has been correlated with altered specificity of p53Δpro for certain promoters (7, 9). Notably, Zhu et al. (9) have shown that p53Δpro has reduced ability to induce certain endogenous apoptotic target genes, including BTP2, p58, PIG6, PIG11, and BTG2 (10, 13, 35). However, several lines of evidence argue for an additional explanation for the impaired apoptotic activity of
p53Δpro. First, a direct contribution of these apoptotic genes to the apoptotic response of p53 has not yet been demonstrated. Second, the induction of several other p53 apoptotic target genes, such as KILLER/DR5, BAX, PIG2, and PIG7, is not affected by the deletion of the proline-rich region (9). Third, deletion of the corresponding region in p73b did not affect the induction of PIG3 (15). Fourth, despite the phenotypic similarity between tumor-derived p53 mutants and p53Δpro, very few mutants have been identified in this region. Therefore, it appears that the differential regulation of certain p53 apoptotic target genes provides only a partial explanation for the impaired apoptotic activity of p53Δpro.

In this study we searched for an alternative explanation for the impaired apoptotic activity of p53Δpro. The high sensitivity of p53 to its negative regulator Mdm2 prompted us to ask whether the polyproline region of p53 affects its regulation by Mdm2. Indeed, the Mdm2 inhibition of the apoptotic activity is greater for p53Δpro than for wt p53 (Fig. 1). Similarly, Mdm2 inhibition of the transcriptional activity is greater for p53Δpro than for wt p53 (Fig. 2). These differences are likely to have been underestimated because the induction of endogenous Mdm2 by p53Δpro is ~30% that of wt p53 (9). This differential induction of Mdm2 is consistent with the findings that the steady state level of the p53Δpro protein appears to be higher than that of the wt p53 protein (Figs. 2 and 3). These findings provide an additional explanation for the impaired growth suppression activity of p53Δpro observed in previous studies (7–9, 30). Furthermore, our findings help to explain the low frequency of mutations within the polyproline region of p53 in tumors. Such mutations are expected to have increased sensitivity to Mdm2-mediated degradation, and consequently are unlikely to be selected during tumor development.

The exact contribution of Mdm2-mediated degradation of p53 to the overall inhibition of p53 by Mdm2 is difficult to assess. It appears that low ratios of Mdm2 to p53 may be sufficient for inhibiting p53 activities, without promoting its degradation. This notion is consistent with our findings that the mere presence of endogenous Mdm2 was sufficient to render p53Δpro less active than wt p53 (Fig. 2) and more susceptible to nuclear export and ubiquitination than wt p53 (Fig. 5), although its presence was insufficient for promoting p53Δpro for degradation (Figs. 1–4). Interestingly, almost all the detected ubiquitination of p53 was observed in the nucleus. The requirement for the ubiquitination of p53 in the nucleus for its nuclear export (33, 34) is consistent with the enhanced
shuttle of p53Δpro to the cytoplasm (Fig. 6), providing further support for the link between the ubiquitination and nuclear export of p53 (32, 33). On the other hand, in the presence of high ratios of Mdm2 to p53, as achieved by the addition of exogenous Mdm2, p53Δpro was more sensitive than wt p53 to Mdm2-mediated degradation (Fig. 4). This increased sensitivity to Mdm2 inhibitory effects is explained by the enhanced binding of p53Δpro to Mdm2, as compared with wt p53 (Fig. 7).

It is not clear at this stage why p53Δpro binds Mdm2 with higher affinity than does wt p53. The polyproline region may be involved directly or indirectly in Mdm2 binding. This region may serve as an anchor for a third partner, presumably an SH3-containing protein, which may modulate the interaction between p53 and Mdm2. In the absence of the polyproline region the putative protein would be unable to bind p53, and consequently would be unable to modulate Mdm2 binding to p53. Further studies aimed at identifying proteins interacting with the polyproline region of p53 are required to test this conjecture.

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