Activation of an MDM2-specific Caspase by p53 in the Absence of Apoptosis*

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Cells undergoing p53-mediated apoptosis activate caspase 3-like activities, resulting in the cleavage of the MDM2 oncoprotein and other apoptotic substrates such as poly(ADP-ribose) polymerase. To investigate the mechanism of p53-mediated apoptosis and to determine whether cleavage of MDM2 has a potential role in regulating p53, we examined caspase activation and cleavage of MDM2 in a cell line undergoing p53-mediated growth arrest and delayed apoptosis. We found that in H1299 cells expressing a temperature-sensitive human p53, a distinct caspase activity specific for the MDM2 cleavage site DVDP is induced by p53 prior to the onset of apoptosis and loss of viability. This is accompanied by the cleavage of MDM2 but not the apoptotic substrate poly(ADP-ribose) polymerase. The cleaved MDM2 loses the ability to promote p53 degradation and may potentially function in a dominant-negative fashion to stabilize p53. These results suggest that p53 activation may induce a positive feedback effect by cleavage of MDM2 through a unique caspase.

The p53 tumor suppressor can be activated by DNA damage (1), deregulated oncogenes (2), and hypoxia (3). Activation of p53 results in growth arrest or apoptosis by induction of downstream target genes (4, 5) and possibly transcription-independent mechanisms (6, 7). The activity of p53 is regulated by the MDM2 oncoprotein, which binds to p53 and inhibits the transcription activation function (8). MDM2 binding also promotes p53 degradation through the ubiquitin-dependent proteasome pathway (9, 10). MDM2 expression is induced by p53 (11, 12), thus forming a negative feedback loop that maintains p53 at low levels in the absence of stress. Loss of MDM2 results in embryonic lethality due to activated p53 (13, 14), and overexpression of MDM2 in tumors results in suppression of p53 transcription function and apoptosis (15). Increasing evidence suggests that regulation of the interaction between p53 and MDM2 by phosphorylation (16–18), and inhibition of MDM2 by the tumor suppressor p19ARF are important mechanisms of p53 activation (19, 20).

Activation of p53 can lead to growth arrest or apoptosis, depending on cell types (21–23). The cell cycle-arrest activity of p53 is largely mediated by induction of the p21WAF1 gene (24). Despite the identification of several p53-inducible genes that can regulate apoptosis, such as Bax (25) and KILLER/DR5 (26), the apoptotic mechanism of p53 is not completely understood. Furthermore, it is unclear why different tumor cells or different cell lines of the same tumor type often exhibit either a growth arrest or apoptosis response to p53, which may be clinically important. Recent studies using cell fusion revealed that the apoptotic response phenotype is dominant over growth arrest (23), suggesting that expression of a specific factor may confer an apoptotic response to p53.

Apoptosis typically involves the activation of proteolytic cascades of caspases and cleavage of vital cellular proteins (27). Fas/CD95- and tumor necrosis factor-α-induced apoptosis involves activation of caspase 8 through receptor-mediated oligomerization (28). Caspase 8 can directly activate effector caspase 3 or induce mitochondria cytochrome c release and activation of the Apaf1-caspase 9 complex (29), which then activates effector caspases. Because p53-induced apoptosis also involves activation of effector caspases (23, 30), it is conceivable that certain upstream signaling events may be activated by p53. The ability of p53 to induce KILLER/DR5 expression and to promote Fas/CD95 export to the cell surface (31) provides a possible apoptotic pathway. Whether this is a general mechanism of p53-mediated apoptosis is still not clear.

During p53-induced apoptosis, the MDM2 oncoprotein is also cleaved by a caspase after the aspartic acid residue 361 (30, 32). Proteolytic cleavage of MDM2 has been proposed to play a role in eliminating its p53-inhibitory effect and possibly other cell survival functions of MDM2. However, due to the rapid and non-synchronous nature of p53-induced apoptosis, whether MDM2 cleavage is an early event that can contribute to p53 regulation has not been determined. Furthermore, the caspase cleavage fragment of MDM2 can still bind to p53 and inhibit its transcription function when overexpressed (30). Thus the functional significance of the cleavage is still unclear.

We recently observed that certain tumor cell lines express high levels of a 60-kDa isoform of MDM2 protein (p60) similar to the caspase cleavage fragment of MDM2 (33). The expression of p60 can be inhibited by culturing cells in caspase inhibitor ZVAD-FMK, can be produced from a transfected full-length MDM2 cDNA, and is blocked by point mutation of residue 361 (33). Cells can produce high levels of p60 in normal culture conditions, with no detectable apoptosis and cleavage of another apoptosis substrate PARP. These results suggest that caspase-mediated processing of MDM2 can occur in the absence of apoptosis; MDM2 is unique compared with other known apoptotic substrates in being targeted by a caspase in non-apoptotic cells. The variable levels of p60 in different cell lines suggest that this caspase may be regulated by unknown mechanisms.

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In this report, we describe the characterization of MDM2 cleavage using a cell line that undergoes p53-mediated growth arrest followed by delayed, partial apoptosis. This system enabled us to examine p53-induced events before the onset of apoptosis. We found that p53-induced growth arrest results in the activation of a unique caspase that is specific to MDM2, resulting in the production of p60 after p53 activation. Activation of the MDM2-specific caspase occurs before the activation of apoptotic caspases. p60 is deficient in promoting p53 degradation and can stabilize p53 by a dominant-negative mechanism.

**MATERIALS AND METHODS**

**Cell Lines and Plasmids—**JAR, SJSA, and HT1080 cells were obtained from the American Type Cell Collection and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Both cell lines contain MDM2 gene amplifications (34, 35). Human p53-null cell line H1299 was provided by Arnold J. Levine. A Bluescript vector containing human p53 cDNA with an 138 alanine-to-valine mutation was kindly provided by Ute Moll of the State University of New York at Stony Brook. The p53 valine 138 cDNA was cloned into the pCMV-neo-BamH I cloning vector driven by the CMV promoter (36). The CMV promoter-driven Flag-MDM2 cDNA was kindly provided by Douglas Tchakou of the Seattle Veterans Medical Center. Escherichia coli expression vectors of caspase 6 and 8 were kindly provided by Dr. Emad Alnemri of Thomas Jefferson University.

**Transfection—**In a stable transfection, 2 × 10⁵ cells were seeded into 10-cm dishes for 24 h and transfected with 15 μg of plasmid using the standard calcium phosphate precipitate protocol. Two days after transfection, cells were cultured in medium containing 750 μg/ml G418 for 2 weeks, and G418-resistant colonies were cloned or pooled. For secondary stable transfections, H1299-Val-138 cells were transfected with a mixture of 10 μg of pBPl00-luciferase or pSG-HDM1B plasmid (encoding Flag-MDM2) with 5 μg of pEG-hygro plasmid. Cells were selected with a medium containing 750 μg/ml G418 and 200 units/ml hygromycin. Double drug-resistant colonies were pooled for analysis.

**Western Blot—**Cells were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), centrifuged for 5 min at 10,000 × g, and the insoluble debris discarded. Cell lysate (100–200 μg of protein) was fractionated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P filters. The filter was blocked for 5 min with phosphate-buffered saline (PBS) containing 5% non-fat dry milk, 0.1% Tween 20, and then incubated for 1 h with 1/10 dilution of anti-MDM2 monoclonal antibody 3G9 or 4B11 (37) in PBS containing 5% non-fat dry milk. For detection of p21WAF1, the filter was incubated with 1/500 dilution of anti-p21 antibody (Onogene Research Product) as recommended by the supplier. p53 was detected by incubating with monoclonal antibody DO-1. The filter was then washed four times (5 min each) with PBS containing 0.1% Tween 20. Bound primary antibody was detected by incubating for 1 h with protein A-peroxidase or horseradish peroxidase goat anti-mouse IgG (for p21) diluted in PBS containing 5% non-fat dry milk. The filter was washed with PBS containing 0.1% Tween 20 and developed using the ECL-plus reagent (Amersham Pharmacia Biotech). For detection of PARP, 10⁵ cells were lysed in sample buffer (50 mM Tris-Cl, pH 6.8, 6 mM urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, 5% freshly added 2-mercaptoethanol), sonicated, boiled, and loaded onto 10% SDS-polyacrylamide gel electrophoresis. Fractionated proteins were transferred onto Immobilon P filters. The filter was incubated with an anti-PARP monoclonal antibody (PharMingen), washed, incubated with horseradish peroxidase goat anti-mouse IgG, and developed using the ECL-plus system.

**Protease Cleavage Assay—**Peptide substrates Ac-DEVD-AMC, Ac-WEHD-AMC, and Z-VAD-AMC were purchased from Bachem. The MDM2-specific substrate Ac-DYDV-AMC was synthesized by Dr. Chi Yang (Synpep Corp., Dublin, CA) by solution phase chemistry. To measure caspase activities, cytosol was prepared using a procedure described in Nicholson et al. (38). The cell pellet (~10⁶ cells) was suspended in 0.15 ml of cytosol buffer (10 mM Hepes/KOH, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, 50 μM leupeptin, and 10 μg/ml aprotinin). The mixture was homogenized by 30 strokes in a Dounce homogenizer with a type B pestle and centrifuged at 15,000 × g for 5 min at 5 °C, and the pellet was discarded. Fluorogenic assays for caspase activities contained 100 μl of cytosol buffer, 20 μg of cytosolic protein, 20 μM tetrapeptide-AMC substrate. After incubating for 2 h at 37 °C, free AMC concentration was measured using a fluorometer with a 380 nm excitation filter and 460 nm emission filter.

**Viability Assay—**H1299-Val-138 cells were cultured to 60% confluency per 10-cm dish and incubated for 10 days at 39 °C. The plates were stained, and visible colonies were counted. The experiment was performed three times, each with duplicate plates for each time point.

**Cytochrome c Release Assay—**Cytochrome c release from mitochondria was determined as described by Yang et al. (39). Cells were washed once with ice-cold phosphate-buffered saline and resuspended in 5 volumes of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose). Cells were disrupted by 10 strokes in a Dounce homogenizer using a type B pestle and centrifuged twice at 1,000 × g for 6 min each at 4 °C, and the supernatant was centrifuged at 10,000 × g for 15 min at 4 °C. The pellet was saved as the mitochondrial fraction. The supernatant was centrifuged at 100,000 × g for 1 h, and the supernatant was saved as cytoplasmic soluble fraction. Cytochrome c was detected by Western blot using an antibody from PharMingen.

**RESULTS**

**The Human p53 Val-138 Mutant Is Temperature-sensitive—**A CMV promoter-driven human p53 cDNA construct containing an amino acid 138 alanine-to-valine point mutation (Val-138) was stably transfected into the p53-null human lung carcinoma cell line H1299 (40). The Val-138 mutation is equivalent to the murine p53 codon 135 alanine-to-valine mutation (Val-153) that renders the protein temperature-sensitive (41). H1299 cells were transfected at 39 °C, and a stable cell line (H1299-Val-138) expressing the p53 protein was selected and maintained at 39 °C. Incubation of H1299-Val-138 cells at the permissive temperature of 32 °C resulted in growth arrest at the first 48 h, very few cells displayed apoptotic morphology (Fig. 1). About 84 h after temperature shift, a small subset (~10%) of cells displayed membrane blebbing and detached from the plate. The appearance of cells with apoptotic morphologies was associated with a strong increase in a caspase activity specific to the DEVD sequence (see below). The remaining
cell population was stable for up to 10 days, when revertant colonies of proliferating cells began to appear. Therefore, the phenotype of H1299-Val-138 cells at 32 °C is growth arrest, followed by delayed and incomplete apoptosis.

Western blot analysis of two p53-inducible products, p21\textsuperscript{WAF1} and MDM2, showed that both were strongly induced after incubation at 32 °C (Fig. 2A), without significant change in the level of p53. This is consistent with the Val-138 mutant protein being activated at 32 °C by a conformational change. When the H1299-Val-138 cells were further stably transfected with the p53-responsive reporter BP100-luciferase, up to 270-fold activation of luciferase expression was detected after 24 h at 32 °C and persisted through the 96-h observation period (Fig. 2B). These results confirm that the human p53 Val-138 mutant is temperature-sensitive in its transcription activation function, similar to its murine p53 counterpart.

**MDM2 Is Cleaved into a p60-kDa Product after p53 Activation**—Previous studies showed that MDM2 can be cleaved into a 60-kDa fragment (p60) by caspase in cells undergoing apoptosis (30, 32). We have also found that MDM2 is cleaved in certain tumor cell lines (e.g. JAR) in the absence of apoptosis (33), suggesting that significant cleavage of MDM2 can also be carried out by a caspase that is active in non-apoptotic cells. This appears to be a property unique to MDM2. To determine whether cleavage of MDM2 can be regulated by p53 before the onset of apoptosis, H1299-Val-138 cells were incubated at 32 °C for up to 72 h, and MDM2 protein was detected by Western blot using monoclonal antibody 3G9 (detects both full-length MDM2 and the N-terminal cleavage product p60) or 4B11 (detects the C-terminal fragment) (37). At early time points up to 24 h at 32 °C, only high levels of full-length MDM2 were detected. Both 60- and 30-kDa cleavage fragments were detectable 30 h after p53 activation (Fig. 3A). The production of p60 was partially inhibited by treatment with Z-VAD-FMK, a cell-permeable broad spectrum caspase inhibitor. The timing of p60 production correlates with the timing of p53 transcription activation reaching maximum (Fig. 2B).

The delayed production of p60 could be caused by the slow accumulation of p60 generated by a protease that is constitutively active or due to the activation of a caspase by p53. To distinguish between these possibilities, the cleavage status of basal MDM2 was examined. H1299 cells express very low levels of MDM2 at 39 °C, when p53 is not active. To facilitate the detection of MDM2 cleaving activity, H1299-Val-138 cells were stably transfected with a CMV-driven Flag-tagged MDM2 cDNA. Flag-MDM2 was detected by anti-Flag immunoprecipitation and 3G9 anti-MDM2 Western blot. JAR cells stably transfected with Flag-MDM2 was used as positive control for Flag-p60 production.

**Detection of Caspase Activity after p53 Activation**—To test directly the activation of caspase by p53, cytosol was prepared from H1299-Val-138 cells cultured at 32 °C at different time points and incubated with commercially available caspase flu-
The p53-inducible Caspase Activity in H1299 Is Distinct from Caspase 8—Since activation of p53 may activate the KILLER/DR5 or Fas signaling pathways (31, 42), both capable of activating caspase 8 (FLICE), we tested whether caspase 8 may be activated in H1299-Val-138 cells. Recombinant caspase 3 and 8, tagged by polyhistidine, were expressed in E. coli. The reaction mixtures were fractionated by SDS-polyacrylamide gel electrophoresis, and MDM2 fragments were detected by autoradiography. 

A. In vitro translated, 35S-labeled MDM2 and the non-cleavable 361 aspartic acid to alanine point mutant were incubated with purified caspases produced in E. coli. The reaction mixtures were fractionated by SDS-polyacrylamide gel electrophoresis, and MDM2 fragments were detected by autoradiography. 

B. Relative efficiencies of recombinant caspases in the cleavage of peptide-AMC substrates. Identical amounts of each purified recombinant enzyme were incubated with substrate, and the release of free AMC was determined by fluorometry. The amounts of each enzyme were adjusted such that Ac-DEVD-AMC was cleaved at similar rates.

orogenic substrates Z-VAD-AMC, Ac-DEVD-AMC (detects caspase 3 and 8), and Ac-WEHD-AMC (caspase 1). Use of these substrates failed to detect a significant increase in cleavage activity in growth-arrested H1299-Val-138 cells (incubated at 32 °C for up to 48 h) (Fig. 4A). An activity that rises strongly at 84 h was detected using Ac-DEVD-AMC (representing the apoptotic substrate PARP), correlating with the appearance of apoptosis in a subset of cells.

Since the cleavage of Flag-MDM2 after temperature shift gave a strong indication that an MDM2-cleaving enzyme was activated before 30 h, a MDM2-specific substrate Ac-DVPD-AMC was synthesized based on the caspase cleavage site of MDM2 codon 361. Incubation of Ac-DVPD-AMC with H1299-Val-138 cytosol revealed an activity that is present at a very low basal level in 39 °C cells but is induced over 3-fold after 32 °C (Fig. 4D). This is consistent with the interpretation that the caspase-cleaving DVPD at early time points is different from the DEVD-cleaving protease.

The p60 MDM2 fragment was found to be produced at high levels in JAR cells but not in SJSA cells, both overexpressing full-length MDM2 (33–35). When directly compared for their caspase activities, both cell lines showed similar activities with three commercial substrates, whereas JAR cytosol cleaved Ac-DVPD-AMC with 10-fold higher efficiency than SJSA (Fig. 4D). The level of DVPD-specific caspase activity in JAR cells is comparable with H1299-Val-138 cells incubated for 28 h at 32 °C (Fig. 4C), consistent with the relative ratio of full-length MDM2 and p60 in these cells. These results confirm the presence of a MDM2-specific caspase activity at various levels in different cell lines, correlating with the levels of p60.

The p53-inducible Caspase Activity in H1299 Is Distinct from Caspase 8—Since activation of p53 may activate the KILLER/DR5 or Fas signaling pathways (31, 42), both capable of activating caspase 8 (FLICE), we tested whether caspase 8 may be activated in H1299-Val-138 cells. Recombinant caspases 3 and 8, tagged by polyhistidine, were expressed in E. coli and purified using Ni²⁺-chelating chromatography. When incubated with in vitro translated MDM2, caspase 8 was able to cleave wild type MDM2 but not the 361 aspartic acid to alanine point
p53 Activates an MDM2-specific Caspase

p53 activation. A, cleavage status of PARP in H1299-Val-138 cells incubated at 32 °C was determined by Western blot. H1299-Val-138 cells treated with 10 μM camptothecin or Adriamycin for 24 h at 32 °C were used as positive controls. B, viability of H1299-Val-138 cells cultured at 32 °C. Cells incubated for indicated time points at 32 °C were trypsinized and seeded into duplicate plates at low density. Colony formation efficiencies were determined after incubation at 39 °C for 10 days. Data are averages of three experiments. C, absence of cytochrome c release after p53 activation. Soluble cytochrome c in H1299-Val-138 cells were determined after incubation at 32 °C for 30 h by Western blot. Apoptotic JAR cells induced by staurosporine were used as a positive control. P, pelleted mitochondrial fraction; S, soluble cytoplasmic fraction.

mutant (Fig. 5A), suggesting that MDM2 can be a substrate of caspase 8, and the cleavage is dependent on the DVPD sequence at residue 361. Caspase 6 only has weak activity against in vitro translated MDM2 (data not shown), whereas caspase 3 was highly effective in cleaving MDM2 (Fig. 5A), as reported previously (30).

When assayed using the peptide fluorogenic substrates, recombinant caspases 3, 6, and 8 cleaved Ac-DEVD-AMC more efficiently than Ac-DVPD-AMC (Fig. 5B). Caspase 6 was nearly inactive against Ac-DVPD-AMC. These substrate specificities are opposite that of the H1299-Val-138 32 °C cytosol, which showed a preference for Ac-DVPD-AMC. Therefore, the protease that is initially activated by p53 appears to be distinct from caspases 3, 6, and 8. Western blot of H1299-Val-138 cells at 32 °C did not reveal a change in the level of caspase 8 proenzyme (data not shown), consistent with a lack of caspase 8 activation in this system. A previous study also ruled out caspase 1, which cannot cleave MDM2 (32).

p53 Induces Caspase Activation before Commitment to Apoptosis—H1299-Val-138 cells cultured for 30–48 h at 32 °C did not show obvious signs of apoptosis, despite significant cleavage of MDM2. To determine whether the cells were indeed non-apoptotic, the cleavage status of an apoptotic caspase substrate PARP (43) was examined. Western blot analysis of PARP using a monoclonal antibody capable of detecting full-length 117-kDa and the large 87-kDa fragment of PARP showed that in adherent H1299-Val-138 cells incubated at 32 °C for up to 72 h, PARP was not cleaved (Fig. 6A). As a positive control, H1299-Val-138 cells were treated with DNA-damaging agents camptothecin or Adriamycin at 32 °C for 24 h. This resulted in apoptosis accompanied by the cleavage of PARP. Therefore, the H1299-Val-138 cells with significant cleavage of MDM2 are not at the end stage of apoptosis characterized by PARP cleavage. This result also excluded the possibility that the p60 we detected at early 32 °C time points was contributed by a small number of apoptotic cells.

Next, the viability of the growth-arrested cells after returning to 39 °C was determined. After incubation at 32 °C, adherent H1299-Val-138 cells were trypsinized; 200 cells were plated in each 10-cm dish and incubated for 10 days at 39 °C before visible colonies were counted. The results showed that after 30 h at 32 °C, there was no decrease in cell viability compared with cells maintained at 39 °C (Fig. 6B). Trypan blue exclusion tests also did not reveal the appearance of dead cells from the adherent population (not shown). Therefore, the majority of H1299-Val-138 cells that have undergone p53 activation, caspase activation, and MDM2 cleavage have not permanently lost viability.

Cytochrome c release from mitochondria plays an important role in the amplification of apoptosis signals by activation of the Apaf1-caspase 9 complex and is regulated by the apoptosis suppressor Bcl-2 (29, 39, 44, 45). Cytochrome c release was determined in H1299-Val-138 cells at 32 °C. As a positive control, apoptosis was induced in JAR cells by treating with the broad spectrum protein kinase inhibitor staurosporine for 5 h (33). This resulted in a significant increase of soluble cytochrome c in the cell extract devoid of mitochondria (Fig. 6C). In contrast, H1299-Val-138 cells incubated at 32 °C for 30 h did not show an increase in soluble cytochrome c. Therefore, activation of the MDM2-cleaving protease occurs without the release of cytochrome c from mitochondria.

Caspase Cleavage of MDM2 Inactivates Its p53 Degradation Function—Previous experiments showed that the p60 fragment of MDM2 was still capable of binding to p53 (30), consistent with its intact p53 binding domain. A recent report suggested that the C terminus of MDM2 may be important for its ability to direct ubiquitination of p53 (46). Therefore, we tested the ability of p60 to promote p53 degradation. A plasmid expressing p60 was cotransfected with a plasmid encoding the inactive p53 mutant 175R-H, which eliminated the interference from an apoptotic response. Transient cotransfection of p53 with full-length MDM2 resulted in a significant reduction of p53 level; however, this effect was not apparent with the p60 MDM2 fragment (Fig. 7A), suggesting that p60 is not capable of promoting p53 degradation.

Since p60 is still capable of binding to p53 with similar efficiency as full-length MDM2, it is possible that it can function in a dominant-negative fashion to prevent p53 degradation by full-length MDM2. To test this possibility, a cDNA expression plasmid encoding p60 was stably transfected into a human fibrosarcoma cell line HT1080, which contains wild type p53. p53 protein levels in a pool of stable transfectants was analyzed by Western blot. Overexpression of p60 induced strong accumulation of p53 (Fig. 7B). A similar result was also observed when p60 is transiently expressed in HT1080 cells (not shown). Immunofluorescence staining of p60-overexpressing cells revealed an increase in nuclear p53 (not shown). This result suggests that p60 binding to p53 can protect p53 from degradation by full-length MDM2. Therefore, the p60 fragment has
HT1080 cells were stably transfected with MDM2 expression plasmids. The levels of p53 bound to MDM2 were determined by anti-MDM2 immunoprecipitation with 2A9 antibody and anti-p53 Western blot. The ability of p53 to regulate p53 turnover is a dominant-negative mechanism.

Because p60 was overproduced in transfection experiments, the stabilized p53 was not transcriptionally active. Thus, there was no obvious alteration in cell proliferation and the level of p21WAF1 expression (Fig. 7B). High levels of p53 were coprecipitated with p60 (Fig. 7B), indicating that most p53 molecules were sequestered in a complex with p60. Further experiments are needed to determine whether producing physiological levels of p60 can result in increase of p53 level and activity.

**DISCUSSION**

The data presented here suggest that p53 can regulate a proteolytic activity specific for the caspase cleavage site of MDM2, before the onset of apoptosis and activation of other apoptotic caspases. The p53-induced protease activity is less efficient against peptide substrates for caspases 1, 3, and 8, suggesting the presence of a distinct MDM2-specific protease. The ability of this protease to cleave after the aspartic acid residue in the caspase cleavage site of MDM2 (DVPD361), the partial sensitivity to caspase inhibitor Z-VAD-FMK, and resistance to conventional serine and cysteine protease inhibitors suggest that it is a caspase.

By using the MDM2 cleavage site substrate Ac-DVPD-AMC, we also detected an activity that is present in JAR cells, which constitutively produce cleaved MDM2 (p60) (33). Furthermore, this activity is nearly absent from SJSA cells, which lack significant p60 (33). In contrast, use of other fluorogenic peptide substrates revealed similar levels of cleavage activities in both cell lines. This further supports the presence of a protease that preferentially targets MDM2. The variability of the MDM2-specific protease activity suggests that it may be a regulatory rather than an effector protease in apoptosis. Thus, JAR cells with a significant level of MDM2 cleavage proliferate normally without committing to apoptosis (33).

Cleavage after DVPD361 results in the separation of the highly conserved MDM2 C-terminal Ring finger domain from the p53 binding domain. It has been shown that cysteine 464 is important for the ubiquitin ligase activity of MDM2 in vitro (46); p60 does not promote p53 degradation; furthermore, it can function in a dominant-negative fashion to protect p53 from degradation by full-length MDM2. These results suggest that the 362–491 region of MDM2 is an important functional domain for promoting p53 degradation. The C-terminal cleavage fragment also contains the RNA binding activity of MDM2 (30, 47), which may also be associated with the ability of MDM2 to promote p53 degradation.

The ability of p53 to activate an MDM2-specific caspase suggests a signaling pathway for p53-mediated apoptosis. A threshold level of p53 may induce cleavage of MDM2 before commitment to cell death, resulting in a positive feedback effect on p53 level and further activation of the protease. In addition to cleavage of MDM2, the protease may be responsible for subsequently activating the DEVD-reactive caspase, forming signal transduction and amplification cascades that lead to commitment to apoptosis. This model is consistent with the dosage-dependent apoptotic effect of p53 (48). Certain tumor cell lines undergo growth arrest after p53 activation; it will be interesting to determine whether these cells fail to activate the MDM2-cleaving caspase.

During apoptosis induced by survival factor withdrawal in the mouse myeloid cell line M1, p53 activity is required for caspase cleavage of pRb (49). The pRb-cleaving caspase can also be activated during the preparation of cytosol (49). However, pRb is not cleaved in H1299-Val-138 cells producing p60, or by cytosol prepared from 32 °C H1299-Val-138 cells does not cleave in vitro translated pRb,2 suggesting important differences between the caspases involved. The activation of MDM2 and pRb-cleaving caspases may be related events in a p53-regulated pathway; the pRb-cleaving caspase may be activated in a step more proximal to the commitment to apoptosis.

In addition to caspase 3, MDM2 is also efficiently cleaved by recombinant caspase 8, suggesting that signaling pathways involving caspase 8 may be able to regulate p53 stability by modification of MDM2. Two cell death-promoting factors that act by activation of caspase 8, i.e. tumor necrosis factor-α and FasL, are known to induce p53 accumulation (50, 51). It is possible that caspase 8 cleavage of MDM2 contributes to the stabilization of p53 by these factors. Proteolytic modification of MDM2 may provide a mechanism for other signaling pathways to interact with the p53 pathway.

Activation of DEVD-specific caspases in the absence of apoptosis have been observed in mitogen-activated lymphocytes undergoing active proliferation, before the appearance of apoptosis (52). Our results provide further evidence for the existence of a novel caspase active in proliferating or growth-arrested cells, prior to commitment to cell death.

In summary, this study revealed the presence of a unique p53-regulated caspase that mediates the pre-apoptotic cleavage and inactivation of MDM2. This caspase activity may function in a positive feedback mechanism of p53 and may also have a role in the apoptotic signaling by p53. The protease is constitutively active in certain tumor cell lines, suggesting that it may also have a function in non-apoptotic conditions. Identification of the MDM2-specific caspase may shed light on the regulation of MDM2 and the apoptotic mechanism of p53.

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